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34	08 Jul 2014	Response after Final Action
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I hereby certify that the attached **Non-Provisional Application** and all marked attachments are being deposited by Electronic Filing on **November 14, 2013** by using the EFS Web patent filing system and addressed to Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

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METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS

BACKGROUND OF THE INVENTION

[0001] Phenylephrine is a selective α1-adrenergic receptor agonist used primarily as a decongestant, as an agent to dilate the pupil, and to increase blood pressure. Phenylephrine is marketed as a substitute for the decongestant pseudoephedrine, though clinical studies differ regarding phenylephrine's effectiveness in this role.

SUMMARY OF THE INVENTION

[0002] In accordance with the present invention, the present invention provide a composition comprising at least 95% *R*-phenylephrine hydrochloride and an aqueous buffer, wherein the composition substantially maintains an initial chiral purity of *R*-phenylephrine hydrochloride for at least 6 months stored between -10 to 10 degree Celsius.

[0003] In another aspect, provided herein are methods of stabilizing a phenylephrine hydrochloride composition comprising storing a solution of aqueous *R*-phenylephrine hydrochloride at less than 10 degree Celsius, wherein the composition substantially maintains the initial chiral purity of *R*-phenylephrine hydrochloride for at least 6 months.

[0004] In one aspect, provided herein are methods of assaying chiral purity of *R*-phenylephrine hydrochloride, wherein the chiral purity is determined by chiral column chromatography, optical rotation, capillary electrophoresis, circular dichroism, or Nuclear Magnetic Resonance.

[0005] In another aspect provides compositions comprising *R*-phenylephrine hydrochloride, wherein the composition substantially maintains the initial chiral purity of *R*-phenylephrine hydrochloride for at least 6 months.

[0006] In another aspect provides methods of dilating the pupil comprising administering a composition comprising *R*-phenylephrine hydrochloride topically to a mammal, wherein the composition substantially maintains the initial chiral purity of *R*-phenylephrine hydrochloride for at least 6 months.

[0007] In another aspect provides methods of treating Uveitis in a subject comprising administering a composition comprising *R*-phenylephrine hydrochloride to said subject, wherein the composition substantially maintains the initial chiral purity of *R*-phenylephrine hydrochloride for at least 6 months

[0008] In another aspect provides methods of performing certain ocular testing such as ultrasonography, provocative closed angle glaucoma test, Retinoscopy, compromised circulation (i.e., blanching test), Refraction, fundus examination comprising administering a composition

comprising *R*-phenylephrine hydrochloride, wherein the composition substantially maintains the initial chiral purity of *R*-phenylephrine hydrochloride for at least 6 months.

[0009] In another aspect provides methods of aiding surgical procedures requiring visualization of the posterior chamber comprising administering a composition comprising *R*-phenylephrine hydrochloride to a subject, wherein the composition substantially maintains the initial chiral purity of *R*-phenylephrine hydrochloride for at least 6 months.

INCORPORATION BY REFERENCE

[0010] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[0011] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[0012] Figure 1 shows a HPLC chromatogram of racemic *R*-phenylephrine hydrochloride by a chiral column purification (OJ-RH (150×4.6) mm). Two peaks at the retention time 5.225 minutes and 6.444 minutes are shown.

[0013] Figure 2 shows a HPLC chromatogram of the exemplary *R*-Phenylephrine Hydrochloride Opthalmic Solution (10%) before storage. The chiral purity was determined to be 99.3% ee based on the peaks at 5.184 minutes (area: 9931.84) and at 6.425 minutes (area: 32.5748).

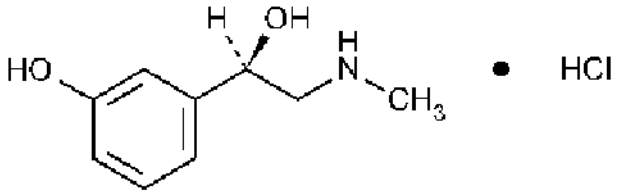
[0014] Figure 3 shows a HPLC chromatogram of the exemplary *R*-Phenylephrine Hydrochloride Opthalmic Solution (10%) stored at 2 to 8 °C after 6 months. The chiral purity was determined to be 99.3% ee based on the peaks at 5.089 minutes (area: 8454.34) and at 6.363 minutes (area: 30.7874).

[0015] Figure 4 shows a HPLC chromatogram of the purified "impurity" which is a *S*-Phenylephrine Hydrochloride. The chiral purity was determined to be 82.4% ee based on the peaks at 5.183 minutes (area: 255.971) and at 6.347 minutes (area: 2851.08).

DETAILED DESCRIPTION OF THE INVENTION

[0016] Phenylephrine differs chemically from epinephrine only in lacking one hydroxyl group (OH) in the four position on the benzene ring. It is a bitter-tasting crystalline material soluble in water and alcohols, with a melting point of 140°–145° C. Chemically it is Benzenemethanol, 3- hydroxy-α-

[(methylamino)methyl]-, hydrochloride or (R)- (-)-m-hydroxy-α-[methylamino)methyl]benzyl alcohol hydrochloride with the following chemical structure.



[0017] It is known in the art that a Phenylephrine Hydrochloride solution should be stored protected from light. The benzylic hydrogen is acidic and can be deprotonated easily. The hydroxyl group may be oxidized to form a carbonyl moiety conjugated with phenyl group, especially with help of the adjacent basic amino group. Thus, it is known in the art that a Phenylephrine Hydrochloride solution should be stored protected from light. For example, an insert from a commercially available Phenylephrine Hydrochloride Ophthalmic Solution provides that the solution should be stored at 20° to 25 °C (USP controlled room temperature) and keep container tightly closed. Do not use if solution is brown or contains precipitate. (AKORN Package Insert)

[0018] However, a solution under such condition often turns brown over time despite of carefully keeping container tightly closed at 20° to 25 °C (USP controlled room temperature). Those packages containing the brown solution cannot be used and thus create waste.

[0019] The present invention provides the improvement to overcome such instability problem.

[0020] In some embodiments, there are provided a composition comprising at least 95% *R*-phenylephrine hydrochloride and an aqueous buffer for substantially maintaining chiral purity of *R*-phenylephrine hydrochloride for at least 6 months, the improvement comprising storing the composition between -10 to 10 degree Celsius. In certain embodiments, the composition is stored between 2 to 8 degree Celsius. In certain embodiments, the composition comprises at least 99% or 99.3%, *R*-phenylephrine hydrochloride. In certain embodiments, the chiral purity of *R*-phenylephrine hydrochloride is at least 95%, 97%, 99%, or 99.5% of the initial chiral purity after 6 months. In certain embodiments, the composition comprises 2.5% w/v or 10% w/v *R*-phenylephrine hydrochloride by weight. In certain embodiments, the composition further comprises a preservative such as benzalkonium chloride, stearalkonium chloride, polyaminopropyl biguanide, or the like. In some embodiments, the composition is in a 1-15 ml plastic or glass bottle. In some embodiments, the composition is in a glass or plastic bottle of about 2 ml, about 3 ml, about 5 ml, about 10 ml or about 15 ml. In certain embodiments, the plastic or glass bottle is opaque.

[0021] In some embodiments provide methods of stabilizing a phenylephrine hydrochloride composition such as a solution of aqueous *R*-phenylephrine hydrochloride at less than 10 degree Celsius wherein the composition substantially maintains the initial chiral purity of *R*-phenylephrine hydrochloride for at least 6 months.

[0022] In some embodiments provide herein compositions comprising *R*-phenylephrine hydrochloride, wherein the composition substantially maintains the initial chiral purity of *R*-phenylephrine hydrochloride for at least 6 months.

[0023] In some embodiments, the composition is stored at -10 to 10 degree Celsius. In certain embodiments, the composition is stored at -5 to 10 degree Celsius. In certain embodiments, the composition is stored at 0 to 10 degree Celsius. In certain embodiments, the composition is stored at 2 to 8 degree Celsius.

[0024] The term "substantial" or "substantially maintains" described herein refers to not more than 15% deviation of the initial purity. In some embodiments, the chiral purity of the composition is at least 85%, 90%, 95%, 97%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8% or 99.9% of the initial chiral purity.

[0025] In some embodiments provide herein methods of assaying chiral purity of *R*-phenylephrine hydrochloride, wherein the chiral purity is determined by chiral column chromatography, optical rotation, capillary electrophoresis, circular dichroism, or Nuclear Magnetic Resonance.

[0026] In certain embodiments, the chiral purity is determined by chiral column chromatography.

Chiral Column Chromatography

[0027] Chiral column chromatography is a variant of column chromatography in which the stationary phase contains a single enantiomer of a chiral compound rather than being achiral. The two enantiomers of the same analyte compound differ in affinity to the single-enantiomer stationary phase and therefore they exit the column at different times.

[0028] The chiral stationary phase can be prepared by attaching a suitable chiral compound to the surface of an achiral support such as silica gel, which creates a Chiral Stationary Phase (CSP). Many common chiral stationary phases are based on oligosaccharides such as cellulose or cyclodextrin (in particular with β-cyclodextrin, a seven sugar ring molecule). As with all chromatographic methods, various stationary phases are particularly suited to specific types of analytes.

[0029] The packing material of the chiral column may be amylose tris(3,5-dimethylphenylcarbamate), β-cyclodextrin, cellobiohydrolase, selector R-(−)—N-(3,5-dinitrobenzoyl)-phenylglycine, cellulose tris(3,5-dimethylphenylcarbamate), cellulose tris(3,5-dichlorophenylcarbamate), or combinations thereof. In some embodiments, the chiral column for

analytical purpose is packed with amylose tris(3,5- dichlorophenylcarbamate). The column may have a packing particle of a size of about 3 μm to about 50 μm. In some embodiments, the column has a packing particle a size of about 3 μm, 5 μm, 10 μm, 20 μm, 30 μm, 40 μm, or 50 μm. In certain embodiments, the column has a packing particle a size of about 3 μm. In some embodiments, when using a chiral column system, the first mobile phase is non-polar solvent such as n-hexane, n-pentane, and the like, and the second mobile phase is polar solvent such as isopropanol, ethanol, methanol, or the like. In certainly embodiments, the mobile phase comprises small amount of amine such as ethylenediamine. The first mobile phase may be present in an amount of about 75% to about 95% by volume and the second mobile phase is present in an amount of about 5% to about 25% by volume. In some embodiments, the first mobile phase is present in an amount of about 85% by volume and the second mobile phase is present in an amount of about 15% by volume with or without ethylenediamine.

Other Chiral Compound Analysis Methods

[0030] There are several chiral compound purification and analysis methods available besides chiral column chromatography. For example, it is known in the art chiral purity can be determined by optical rotation. In some embodiments, the chiral purity of *R*-phenylephrine hydrochloride in the stabilized compositions and methods thereof can be determined by comparison of optical rotation of pure *R*-phenylephrine hydrochloride.

Optical Purity Measured by Optical Rotation

[0031] Molecules with chrial centers cause the rotation of plane polarised light and are said to be "optically active" (hence the term optical isomers). Enantiomeric molecules rotate the plane in opposite directions but with the same magnitude. This provides a means of measuring the "optical purity" or "enantiomeric excess (ee)" of a sample of a mixture of enantiomers.

[0032] Specific rotation is a physical property like boiling point and can be looked up in references. It is defined according to the following equation based on the experimental measurements: Specific rotation $[\alpha]D = \alpha_{obs}/cl$ where "α_{obs}" is the experimentally observed rotation, "c" is the concentration in g/ml and "l" is the path length of the cell used expressed in dm (10 cm).

[0033] A non-racemic mixture of two enantiomers will have a net optical rotation. It is possible to determine the specific rotation of the mixture and, with knowledge of the specific rotation of the pure enantiomer, the optical purity can be determined.

% Optical purity of sample = 100 * (specific rotation of sample) / (specific rotation of a pure enantiomer)

[0034] In some embodiments, there are provided methods of assaying chiral purity of *R*-phenylephrine hydrochloride, wherein the chiral purity is determined by optical rotation. In certain embodiments, the optical rotation is determined by comparison of optical rotation of pure *R*-phenylephrine hydrochloride.

Capillary electrophoresis

[0035] Capillary electrophoresis (CE), also known as capillary zone electrophoresis (CZE), can be used to separate ionic species by their charge and frictional forces and hydrodynamic radius.

[0036] Capillary electrophoresis (CE) in general offers highly efficient separations. To achieve chiral separation, the capillary is filled with a separation buffer containing a chiral additive. Although many chiral selectors have been used successfully, the most comprehensive separation strategies have been achieved with highly sulfated cyclodextrins. In some embodiments, the chiral purity of the compositions provided herein is determined by capillary electrophoresis. In certain embodiments, the capillary electrophoresis uses cyclodextrin or its derivatives (such as sulfated cyclodextrins).

Chiral Purity Measured by Circular Dichroism

[0037] Circular dichroism (CD) refers to the differential absorption of left and right circularly polarized light. This phenomenon is exhibited in the absorption bands of optically active chiral molecules. CD spectroscopy has a wide range of applications in many different fields. For example, vibrational circular dichroism, which uses light from the infrared energy region, is used for structural studies of small organic molecules, and most recently proteins and DNA. In general, this phenomenon will be exhibited in absorption bands of any optically active molecule. As a consequence, circular dichroism is exhibited by biological molecules, because of their dextrorotary and levorotary components. Even more important is that a secondary structure will also impart a distinct CD to its respective molecules.

[0038] Optical rotation and circular dichroism stem from the same quantum mechanical phenomena and one can be derived mathematically from the other if all spectral information is provided. In some embodiments, the chiral purity is determined by circular dichroism. In certain embodiments, the chiral purity is determined by Fourier transform infrared vibrational circular dichroism (FTIR-VCD). A skilled person in the art can readily apply the general knowledge and procedure to determine chirality of the compositions provided herein.

NMR spectroscopy of stereoisomers

[0039] It is known in the art that NMR spectroscopy techniques can determine the absolute configuration of stereoisomers such as cis or trans alkenes, R or S enantiomers, and R,R or R,S

diastereomers. In a mixture of enantiomers, these methods can help quantify the optical purity by integrating the area under the NMR peak corresponding to each stereoisomer. Accuracy of integration can be improved by inserting a chiral derivatizing agent with a nucleus other than hydrogen or carbon, then reading the heteronuclear NMR spectrum: for example fluorine-19 NMR or phosphorus-31 NMR. Mosher's acid contains a -CF3 group, so if the adduct has no other fluorine atoms, the 19F NMR of a racemic mixture shows just two peaks, one for each stereoisomer. In some embodiments, the chiral purity of the compositions provided herein is determined by Nuclear Magnetic Resonance Spectroscopy (NMR). In certain embodiments, a chirally pure complexing reagent (i.e., a chiral derivatizing agent) is used in measuring NMR. A skilled person in the art can readily utilize NMR and any suitable chiral complexing agent to determine the chirality of the compositions provided herein.

DOSAGE FORMS AND STRENGTHS

[0040] In some embodiments, the stabilized compositions provided herein comprise a solution of 2.5% w/v or 10% w/v *R*-phenylephrine hydrochloride by weight. In certain embodiments, the compositions further comprise sodium phosphate monobasic, sodium phosphate dibasic, boric acid and benzalkonium chloride. The followings are non-limited exemplary compositions:

[0041] Phenylephrine Hydrochloride Ophthalmic Solution, 2.5% is a clear, colorless to yellowish, sterile topical ophthalmic solution containing phenylephrine hydrochloride 2.5%.

[0042] Phenylephrine Hydrochloride Ophthalmic Solution, 10% is a clear, colorless to yellowish, sterile topical ophthalmic solution containing phenylephrine hydrochloride 10%.

Application of the Stabilized Compositions Comprising *R*-Phenylephrine hydrochloride

[0043] It has been established that Phenylephrine Hydrochloride Ophthalmic Solution is recommended as a vasoconstrictor, decongestant, and mydriatic in a variety of ophthalmic conditions and procedures. Some of its uses are for pupillary dilation in uveitis (to prevent or aid in the disruption of posterior synechia formation), for many ophthalmic surgical procedures and for refraction without cycloplegia. Phenylephrine Hydrochloride Ophthalmic Solution may also be used for funduscopy and other diagnostic procedures.

[0044] For example, *R*-Phenylephrine is used to dilate the iris through α-adrenergic stimulation of the iris dilator muscle. Sympathetic stimulation of the ciliary muscle is believed to be inhibitory, decreasing accommodative amplitude. *R*-Phenylephrine is formulated in an eye drop to dilate the pupil in order to facilitate visualization of the retina. It is often used in combination with tropicamide as a synergist when tropicamide alone is not sufficient. Surprisingly it was found that *S*-Phenylephrine dilated the eye only slightly more than that was untreated. Thus it is important that an

eye drop containing Phenylephrine Hydrochloride used for dilation of the pupil contains predominantly the *R*-isomer in order to maintain maximum efficacy of the ophthalmic solution.

[0045] Sympathetic innervation leads to pupillary dilation. It is innervated by the sympathetic system, which acts by releasing noradrenaline, which acts on α1-receptors causing dilation.

[0046] The alpha-1 (α_1) adrenergic receptor is a G protein-coupled receptor (GPCR) associated with the G_q heterotrimeric G-protein. It consists of three highly homologous subtypes, including α_{1A}-, α_{1B}-, and α_{1D}-adrenergic. Catecholamines like norepinephrine (noradrenaline) and epinephrine (adrenaline) signal through the α_1-adrenergic receptor in the central and peripheral nervous systems.

[0047] Phenylephrine is a selective α_1-adrenergic receptor agonist used primarily as a decongestant, as an agent to dilate the pupil, and to increase blood pressure. Dilation is controlled by the dilator pupillae, a group of muscles in the peripheral 2/3 of the iris. Sympathetic innervation begins at the cortex with the first synapse at the cilliospinal center (also known as Budge's center after German physiologist Julius Ludwig Budge). Post synaptic neurons travel down all the way through the brain stem and finally exit through the cervical sympathetic chain and the superior cervical ganglion. They synapse at the superior cervical ganglion where third-order neurons travel through the carotid plexus and enter into the orbit through the first division of the trigeminal nerve.

[0048] In the anesthetized rats, infusion of large amount of (+)-epinephrine, (+)-norepinephrine, epinine, and (-)-or (+)-phenylephrine induces tachyphylaxis to vasopressor effect of (-)-epinephrine, (-)-norepinephrine, and tetraethylammonium. The tachyphylactic potency of the amines was (-)-phenylephrine (*R*-phenylephrine) > epinine > (+)-norepinephrine = (+)-epinephrine > (+)-phenylephrine.

[0049] Two ophthalmic formulations, formulated 10% Phenylephrine hydrochloride (*S*-isomer) and the exemplary invention composition, 10% Phenylephrine hydrochloride (*R*-isomer) were tested for their ocular activity in NZW rabbits. It was observed that formulated *S*-isomer showed minimal dilation, responded to light exposure and constricted slightly more slowly than the untreated eye, where as the exemplary invention composition, 10% Phenylephrine hydrochloride showed maximal dilation with in 15 min of dosing and the pupil did not respond to light and remained dilated for 4 hrs.

[0050] According to the above study it could be postulated that, when an ophthalmic solution of phenylephrine hydrochloride, (R-isomer) containing S-isomer as an impurity is used for dilation of pupil, the s-isomer may cause the saturation of the a-adrenergic receptors resulting in the decrease in

the response of the drug after its administration (tachyphylasis). Furthermore, the presence of *S*-isomer in the ophthalmic solution may lead to poor/ delayed dilation of the pupil.

[0051] In some embodiments provide methods of dilating the pupil comprising administering a composition comprising *R*-phenylephrine hydrochloride topically to a mammal, wherein the composition substantially maintains the initial chiral purity of *R*-phenylephrine hydrochloride for at least 6 months. It is evident from the literature that the pharmacological evaluation of both R & S-Phenylephrine hydrochloride is not same. *R*-Phenylephrine is referenced as useful synthetic adrenergic drug.

Uveitis

[0052] Uveitis is, broadly, inflammation of the uvea. The uvea consists of the middle, pigmented, vascular structures of the eye and includes the iris, ciliary body, and choroid. Uveitis requires an urgent referral and thorough examination by an ophthalmologist or Optometrist and urgent treatment to control the inflammation. Anterior uveitis (iritis) affects the front portion of the eye, intermediate uveitis (cyclitis) affects the ciliary body, and posterior uveitis (choroiditis) affects the back portion of the uvea. Diffuse uveitis affects all portions of the uvea. Anterior uveitis commonly occurs in conjunction with juvenile rheumatoid arthritis, but does not manifest in all juvenile arthritis patients. Uveitis is most likely to be present in juvenile arthritis patients with pauciarticular disease (fewer than five joints involved), a positive anti-nuclear antibody test, and a negative rheumatoid factor test. It has been demonstrated that after phenylephrine hydrochloride ophthalmic solution instillation, flare intensity and pain were significantly decreased only in eyes with iridocyclitis and without fibrinoid reaction (FR). The decreasing level of flare intensity, and paralysis of the pupil after phenylephrine instillation seem to alleviate pain in those eyes. *See* e.g., Zaczek, et. al., Acta Ophthalmol Scand. 2000 Oct;78(5):516-8.

[0053] In some embodiments provide methods of treating Uveitis in a subject comprising administering a composition comprising *R*-phenylephrine hydrochloride to said subject, wherein the composition substantially maintains the initial chiral purity of *R*-phenylephrine hydrochloride for at least 6 months.

[0054] In some embodiments provide methods of performing certain ocular testing such as ultrasonography, provocative closed angle glaucoma test, Retinoscopy, compromised circulation (i.e., blanching test), Refraction, fundus examination comprising administering a composition comprising *R*-phenylephrine hydrochloride, wherein the composition substantially maintains the initial chiral purity of *R*-phenylephrine hydrochloride for at least 6 months.

[0055] In some embodiments provide methods of aiding surgical procedures requiring visualization of the posterior chamber comprising administering a composition comprising *R*-phenylephrine hydrochloride, wherein the composition substantially maintains the initial chiral purity of *R*-phenylephrine hydrochloride for at least 6 months.

[0056] After presentation of *R*-phenylephrine hydrochloride ophthalmic solution 2.5% or 10% to the ocular surface, a broad variation in the delay of onset of dilation is widely reported, varying between 20-to-30 minutes and as much as up to 60 minutes. While a number of contributors to this delay of onset have been theorized, the absence of phenylephrine hydrochloride's pharmacologic activity in the eye due to the presence of *S*-phenylephrine may in fact be the explanation for such delay.

Dropper Bottle or Storage Bottle

[0057] Conventional dropper bottles for administering ophthalmic fluid are well known in the prior art. The basic commercial design of such dropper bottles has remained fairly unchanged over the last several decades: a squeezable container is provided with a tapered dispenser that terminates in a discharge aperture. To administer ophthalmic fluid, the discharge aperture is aligned above a target eye and the bottle is squeezed to urge out a drop or dose of the fluid.

[0058] Alternatively, liquid dispensers have been developed in which the formulation is supplied from a storage bottle through a dropper, for example (dropper bottles or EDO-Ophthiols). The aqueous formulation usually flows out of the dropper opening as a result of manual pressure being applied to the compressible storage bottle.

[0059] In some embodiments, the composition described herein is stored in a plastic or glass bottle. In certain embodiments, the plastic bottle is a low-density polyethylene bottle. In certain embodiments, the composition described herein is stored in a glass bottle with or without a liquid dispenser. In certain embodiments, the plastic or glass bottle is opaque.

[0060] Additionally, the compositions described herein are either packaged for single use or for multiple uses with or without a preservative.

Certain Pharmaceutical and Medical Terminology

[0061] The term "acceptable" with respect to a formulation, composition or ingredient, as used herein, means having no persistent detrimental effect on the general health of the subject being treated.

[0062] The term "carrier," as used herein, refers to relatively nontoxic chemical compounds or agents that facilitate the incorporation of a compound into cells or tissues.

[0063] The terms "co-administration" or the like, as used herein, are meant to encompass administration of the selected therapeutic agents to a single patient, and are intended to include

treatment regimens in which the agents are administered by the same or different route of administration or at the same or different time.

[0064] The term "diluent" refers to chemical compounds that are used to dilute the compound of interest prior to delivery. Diluents can also be used to stabilize compounds because they can provide a more stable environment. Salts dissolved in buffered solutions (which also can provide pH control or maintenance) are utilized as diluents in the art, including, but not limited to a phosphate buffered saline solution.

[0065] The terms "effective amount" or "therapeutically effective amount," as used herein, refer to a sufficient amount of an agent or a compound being administered which will relieve to some extent one or more of the symptoms of the disease or condition being treated. The result can be reduction and/or alleviation of the signs, symptoms, or causes of a disease, or any other desired alteration of a biological system. For example, an "effective amount" for therapeutic uses is the amount of the composition comprising a compound as disclosed herein required to provide a clinically significant decrease in disease symptoms. An appropriate "effective" amount in any individual case may be determined using techniques, such as a dose escalation study.

[0066] The terms "enhance" or "enhancing," as used herein, means to increase or prolong either in potency or duration a desired effect. Thus, in regard to enhancing the effect of therapeutic agents, the term "enhancing" refers to the ability to increase or prolong, either in potency or duration, the effect of other therapeutic agents on a system. An "enhancing-effective amount," as used herein, refers to an amount adequate to enhance the effect of another therapeutic agent in a desired system.

[0067] The term "subject" or "patient" encompasses mammals. Examples of mammals include, but are not limited to, any member of the Mammalian class: humans, non-human primates such as chimpanzees, and other apes and monkey species; farm animals such as cattle, horses, sheep, goats, swine; domestic animals such as rabbits, dogs, and cats; laboratory animals including rodents, such as rats, mice and guinea pigs, and the like. In one embodiment, the mammal is a human.

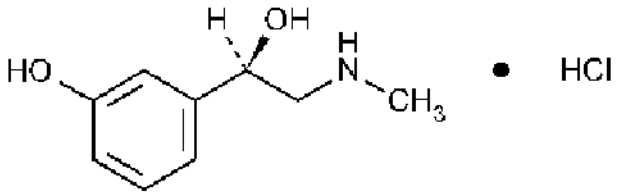
[0068] The terms "treat," "treating" or "treatment," as used herein, include alleviating, abating or ameliorating at least one symptom of a disease or condition, preventing additional symptoms, inhibiting the disease or condition, e.g., arresting the development of the disease or condition, relieving the disease or condition, causing regression of the disease or condition, relieving a condition caused by the disease or condition, or stopping the symptoms of the disease or condition either prophylactically and/or therapeutically.

[0069] All of the various embodiments or options described herein can be combined in any and all variations. The following Examples serve only to illustrate the invention and are not to be construed in anyway to limit the invention.

Examples

Example 1. Exemplary Phenylephrine HCl Ophthalmic Formulation

[0070] *R*-Phenylephrine Hydrochloride Ophthalmic Solution, USP 2.5% or 10%, is a sterile, clear, colorless to light yellow, topical mydriatic agent for ophthalmic use. The chemical name is (R)-3-hydroxy-α-[(methylamino)methyl]benzenemethanol hydrochloride. *R*-Phenylephrine hydrochloride is represented by the following structural formula:



[0071] Phenylephrine hydrochloride has a molecular weight of 203.67 and an empirical formula of $C_9H_{13}NO_2$-HCl.

[0072] Each mL of *R*-Phenylephrine Hydrochloride Ophthalmic Solution, 2.5% contains: ACTIVE: phenylephrine hydrochloride 25 mg (2.5%); INACTIVES: sodium phosphate monobasic, sodium phosphate dibasic; boric acid, water for injection. Hydrochloric acid and/or sodium hydroxide may be added to adjust pH (6.0 to 6.4). The solution has a tonicity of 500 mOsm/kg; PRESERVATIVE: benzalkonium chloride 0.01%.

[0073] Each mL of *R*-Phenylephrine Hydrochloride Ophthalmic Solution, 10% contains: ACTIVE: *R*-phenylephrine hydrochloride 100 mg (10%); INACTIVES: sodium phosphate monobasic, sodium phosphate dibasic; water for injection. Hydrochloric acid and/or sodium hydroxide may be added to adjust pH (6.3 to 6.7). The solution has a tonicity of 1000 mOsm/kg; PRESERVATIVE: benzalkonium chloride 0.01%.

[0074] The composition of Phenylephrine HCl Ophthalmic Solution, 2.5% and 10% is listed in Table 1.

[0075] Table 1:Phenylephrine HCl Ophthalmic Solution, 2.5% and 10% Quantitative Composition

Component	**2.5% Formulation Quantity (%w/v)**	**10% Formulation Quantity (%w/v)**	**Function**	**Quality Standard**
R-Phenylephrine HCl	2.5%	10%	Active	USP
Sodium Phosphate Monobasic,	0.5%	0.5%	Buffer	USP

Sodium Phosphate Dibasic, Anhydrous	0.3%	0.3%	Buffer	USP
Boric Acid	1.0%		Buffer	USP
Benzalkonium Chloride	0.01%	0.01%	Antimicrobial preservative	USP
Sodium Hydroxide	As needed	As needed	pH adjustment	USP
Hydrochloric Acid	As needed	As needed	pH adjustment	USP

Example 2. Stability (impurity) Test and Results

[0076] Stability studies of 2.5% and 10% Phenylephrine HCL solutions prepared as in Example 2 were conducted at 2 to 8 °C for 12 months.

[0077] While the testing performed during the historical stability analysis is limited, those parameters evaluated show excellent results. For the 3 batches of 2.5% formulation evaluated, the initial assay averaged 101.2% of label claim (range 99.8% - 102.9%), and after 12 months of storage at the labeled storage condition (2-8°C) the average potency was 99.7% of label claim (range 97.0%– 103.4%). All other parameters evaluated (appearance, preservative effectiveness, sterility) conformed to specifications.

[0078] For the 3 batches of 10% formulation evaluated, the initial assay averaged 100.4% of label claim (range 99.8% - 101.6%), and after 12 months of storage at the labeled storage condition (2-8°C) the average potency was 99.8 % of label claim (range 98.8% – 101.0%). All other parameters evaluated (appearance, preservative effectiveness, sterility) conformed to specifications.

Example 3. Chiral HPLC Analysis

[0079] The following are non-limited exemplary chiral columns and relevant mobile phases in the methods for analyzing chiral purity of *R*-phenylephrine.

[0080] Column-OJ-RH (150×4.6) mm, 5 µm, Flow: 1 mL min−1, Mobile Phase: Methanol, Column Temp: 25° C., Detection wavelength: 270 nm.

[0081] Column-OJ-RH (150×4.6) mm, 5 µm, Flow: 0.8 mL min−1, Mobile Phase: 0.05% Ethylenediamine in Methanol, Column Temp: 25° C., Detection wavelength: 270 nm.

[0082] Column-OJ-RH (150×4.6) mm, Flow: 1 ml min−1, Mobile Phase: 0.05% Ethylenediamine in Methanol, Column Temp: Ambient, Detection wavelength: 270 nm.

[0083] Column-OJ-RH (150×4.6) mm, 5 µm, Flow: 1 ml min−1, Mobile Phase: 0.05% Ethylenediamine in Methanol, Column Temp: 25° C., Detection wavelength: 270 nm.

[0084] Column-OJ-RH (150×4.6) mm, 5 µm, Flow: 1 ml min−1, Mobile Phase: 0.05 Ethylenediamine in Methanol, Column Temp: 25° C., Detection wavelength: 270 nm.

[0085] Column-OJ-RH (150×4.6) mm, 5 µm, Flow: 1 ml min−1, Mobile Phase: 0.05% Ethylenediamine in Water (05%):Methanol (95), Column Temp: 25° C., Detection wavelength: 270 nm.

[0086] Column-OJ-RH (150×4.6) mm, 5 µm, Flow: 1 ml min−1, Mobile Phase: 0.05% Ethylenediamine in Methanol, Column Temp: 25° C., Detection wavelength: 270 nm.

[0087] Column-OJ-RH (150×4.6) mm, 5 µm, Flow: 1 ml min−1, Mobile Phase: 0.05% Ethylenediamine in Methanol, Column Temp: 25° C., Detection wavelength: 270 nm.

[0088] Column-OJ-RH (150×4.6) mm, 5 µm, Flow: 0.5 ml min−1, Mobile Phase: Acetonitrile: 0.05% Ethylenediamine in water (30:70) Column Temp.: 25°C, Detection wavelength: 270 nm.

[0089] Column-OJ-RH (150×4.6) mm, 5 µm, Flow: 0.5 ml min−1; Mobile Phase: Acetonitrile: 0.05% Ethylenediamine in water (40:60) Column Temp.: 25, Detection wavelength: 270 nm.

[0090] Column-Chiralpak IC-3 (150×4.6) mm, 3 µm, Flow: 1.0 ml min−1, Mobile Phase: 0.1% Ethylenediamine in n-Hexane (85%):Ethanol (15%), Column Temp: 25° C., Detection wavelength: 270 nm; ref 600 nm.

[0091] Column-Chiralpak IC-3 (150×4.6) mm, 3 µm, Flow: 1.2 ml min−1, Mobile Phase: 0.1% Ethylenediamine in n-Hexane (50%):IPA (50%), Column Temp: 25° C., Detection wavelength: 270 nm.

[0092] Column-OJ-RH (150×4.6) mm, Flow: 0.6 ml min−1, Mobile Phase: 0.05% Ethylenediamine in Methanol, Column Temp: 25° C.; Detection wavelength: 270 nm. 4.0 mg sample in 1 mL ethanol was analyzed. The injection volume to HPLC is 3.0 µL. The HPLC chromatogram is shown in Figure 1.

[0093] The HPLC chromatogram clearly show separation of racemic sample. Chiral HPLC method was thus established to analyze Phenylephrine.

Example 4. Determination of Chiral Purity after 6 Months Storage at Low Temperature

[0094] *R*-Phenylephrine Hydrochloride Opthalmic Solution, 2.5% and 10% prepared as in Example 1 were stored at 2 to 8 °C. The chiral purity of Sample 1 (10% solution) was assessed before low temperature stability test. The HPLC chromatogram is shown in Figure 2.

[0095] The chiral purity of *R*-Phenylephrine Hydrochloride was determined by the method and conditions as shown in Example 3. The result showed 99.3% ee.

[0096] After 6 months of low temperature storage (i.e., 2 to 8 °C), the chiral purity of *R*-Phenylephrine Hydrochloride in the solution was determined to be 99.3% ee. The HPLC chromatogram is shown in Figure 3.

[0097] To confirm the "impurity" shown in the chromatogram, the "impurity" was purified and determined by the same method. The "impurity" (i.e., S- Phenylephrine Hydrochloride) was determined to possess 82.4% ee of *S*-form. The HPLC chromatogram is shown in Figure 4.

[0098] Thus, it is clearly shown that the solution remain substantially maintains the initial chiral purity of *R*-phenylephrine hydrochloride for at least 6 months.

Example 5. Dilation Assay of *S* Form Phenylephrine Solution

[0099] Both *R* and *S* form solutions (10% solution prepared as in Example 1) were test for dilation on rabbits. The first test rabbit received 3 drops of the *S* form formulation and the second test rabbit received 3 drops of the *R* form solution.

[00100] The results were as follows:

[00101] Test Rabbit No. 1: Minimal Dilation, within 15 minutes of dilation the pupil was only slightly more dilated than the untreated eye. The treated eye responded to light exposure and constricted slowly. The control eye constricted rapidly as was expected.

[00102] Test Rabbit No 2: Maximal dilation within 15 minutes of dosing. The pupil did not respond to light exposure and remained fully dilated for 4 hours then regressed.

[00103] These results clearly show that an ophthalmic solution of phenylephrine containing *S*-isomer does not dilate the rabbit pupil as it is achieved with an ophthalmic solution of phenylephrine containing *R* isomer. Thus it is evident that maintaining the chiral purity of the ophthalmic solution is crucial to keep drug potency.

[00104] While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

WHAT IS CLAIMED IS:

1. A composition comprising at least 95% *R*-phenylephrine hydrochloride and an aqueous buffer for substantially maintaining chiral purity of *R*-phenylephrine hydrochloride for at least 6 months, the improvement comprising storing the composition between -10 to 10 degree Celsius.
2. The composition of claim 1, wherein the composition is stored between 2 to 8 degree Celsius.
3. The composition of claim 1, wherein the composition comprises at least 99% *R*-phenylephrine hydrochloride.
4. The composition of claim 1, wherein the composition comprises at least 99.3% *R*-phenylephrine hydrochloride
5. The composition of claim 1, wherein the chiral purity of *R*-phenylephrine hydrochloride is at least 95% of the initial chiral purity after 6 months.
6. The composition of claim 1, wherein the chiral purity of *R*-phenylephrine hydrochloride is at least 97% of the initial chiral purity after 6 months.
7. The composition of claim 1, wherein the chiral purity of *R*-phenylephrine hydrochloride is at least 99% of the initial chiral purity after 6 months.
8. The composition of claim 1, wherein the chiral purity of *R*-phenylephrine hydrochloride is at least 99.5% of the initial chiral purity after 6 months.
9. The composition of claim 1, wherein the composition comprises 2.5% w/v or 10% w/v *R*-phenylephrine hydrochloride by weight.
10. A packaged composition comprising the composition of claim 1, in a 1-15 ml plastic or glass bottle.
11. The packaged composition of claim 10, wherein the package identifies storing the composition at a temperature between -10 to 10 C.
12. The packaged composition of claim 11, wherein the package identifies storing the composition at a temperature between 2 to 8 C.
13. The packaged composition of claim 10, wherein the composition is in a plastic or glass bottle of about 2 ml, about 3 ml, about 5 ml, about 10 ml or about 15 ml.
14. The packaged composition of claim 10, wherein the plastic or glass bottle is opaque.

METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS

ABSTRACT

The invention is directed to methods and compositions of stabilizing phenylephrine formations. The composition has good time-dependent stability at low temperature and has no change in its outward appearance even after having been stored at least 6 months.

Figure 1

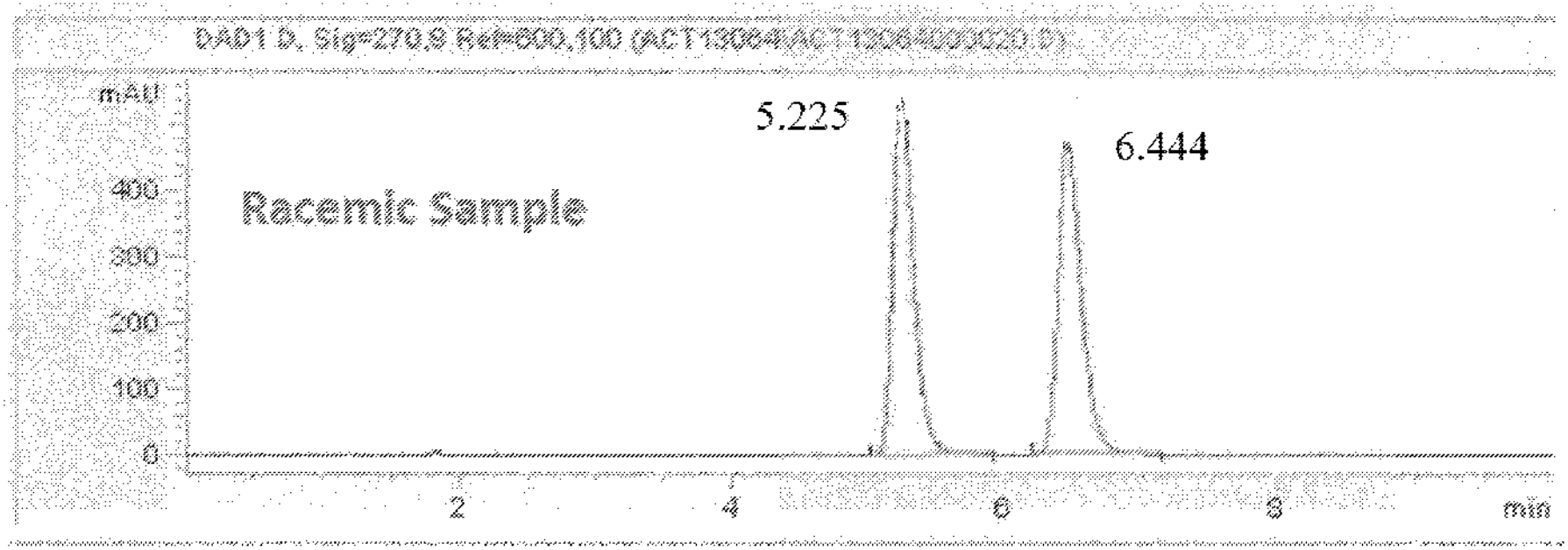


Figure 2

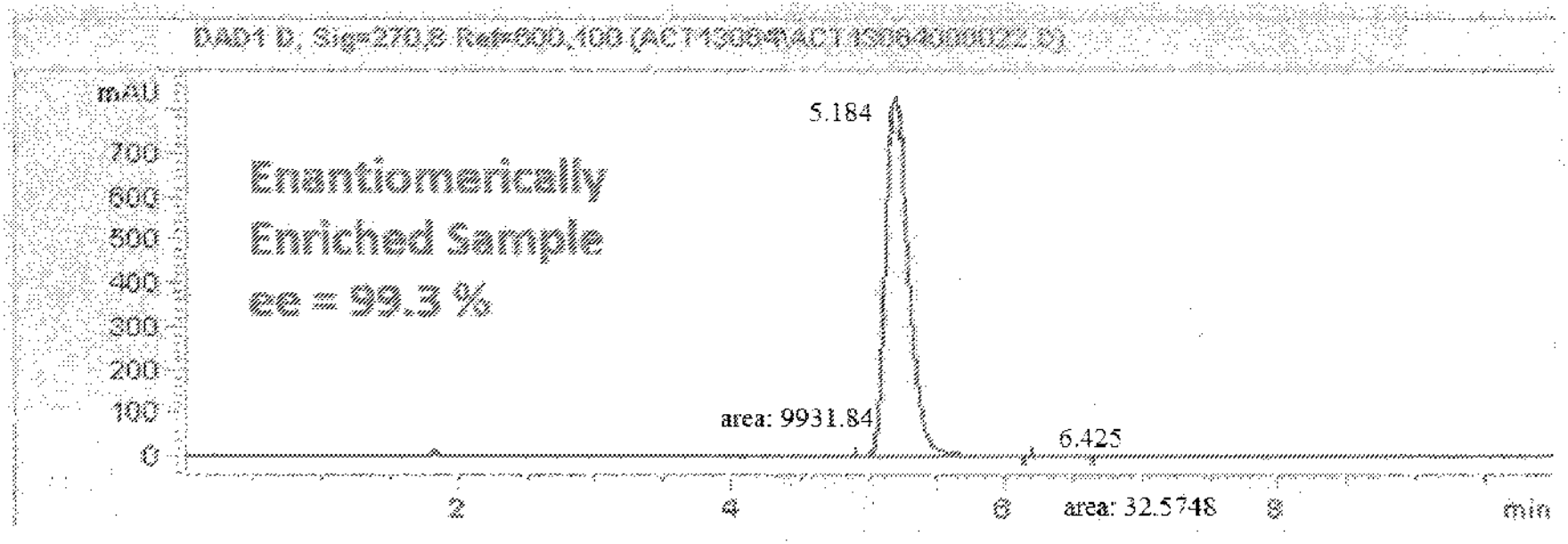


Figure 3

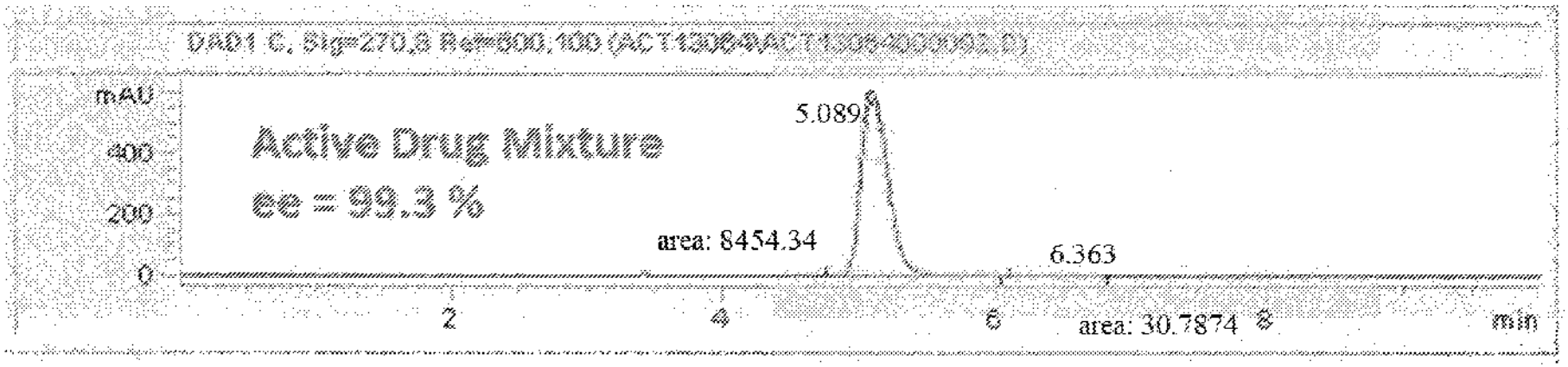
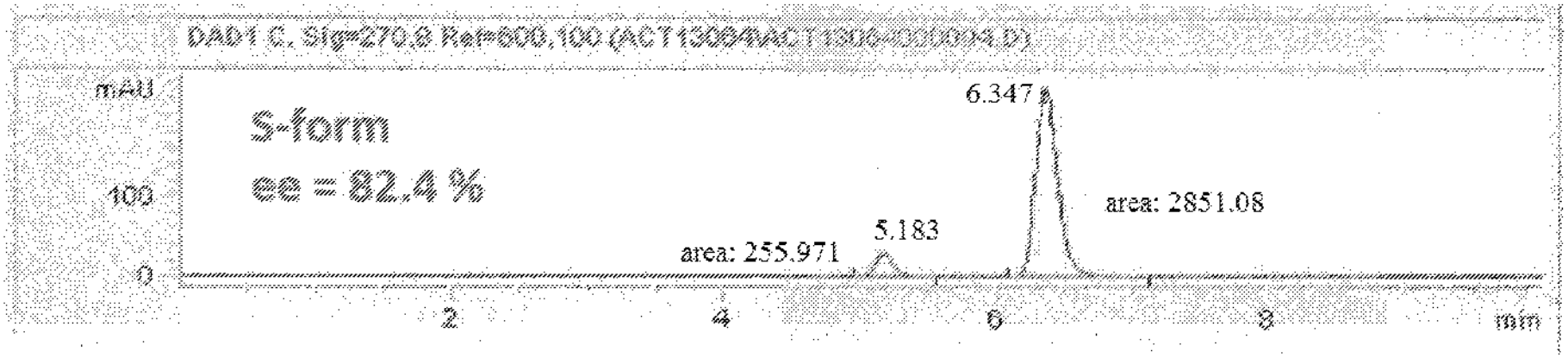


Figure 4



PTO/AIA/08 (06-12)
Approved for use through 01/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63)

☒ Declaration Submitted With Initial Filing OR ☐ Declaration Submitted After Initial Filing (surcharge (37 CFR 1.16(f)) required)

Attorney Docket Number	44630-701.201
First Named Inventor	Patrick H. Witham et al.
COMPLETE IF KNOWN	
Application Number	Not yet assigned
Filing Date	Not yet assigned
Art Unit	Not yet assigned
Examiner Name	Not yet assigned

METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS

(Title of the Invention)

As a below named inventor, I hereby declare that:

This declaration is directed to:

☒ The attached application,

OR

☐ United States Application Number or PCT International application number _____

filed on _____.

The above-identified application was made or authorized to be made by me.

I believe I am the original inventor or an original joint inventor of a claimed invention in the application.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

Authorization To Permit Access To Application by Participating Office

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In accordance with 37 CFR 1.14(h)(3), access will be provided to a copy of the above-identified patent application with respect to: 1) the above-identified patent application-as-filed; 2) any foreign application to which the above-identified patent application claims priority under 35 U.S.C. 119(a)-(d) if a copy of the foreign application that satisfies the certified copy requirement of 37 CFR 1.55 has been filed in the above-identified patent application; and 3) any U.S. application-as-filed from which benefit is sought in the above-identified patent application.

In accordance with 37 CFR 1.14(c), access may be provided to information concerning the date of filing the Authorization to Permit Access to Application by Participating Offices.

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DECLARATION — Utility or Design Patent Application

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LEGAL NAME OF SOLE OR FIRST INVENTOR:

(*E.g.*, Given Name (first and middle (if any)) and Family Name or Surname)

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■ Additional inventors are being named on the _____ supplemental sheet(s) PTO/AIA/10 attached hereto

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SUPPLEMENTAL SHEET FOR DECLARATION | **ADDITIONAL INVENTOR(S)**
Supplemental Sheet (for PTO/AIA/08,09) Page 3 of 3

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Inventor's Signature | Date (Optional)

Residence: City | State | Country

Mailing Address

City | State | Zip | Country

Doc Code: TRACK1.REQ
Document Description: TrackOne Request

PTO/SB/424 (12-11)

CERTIFICATION AND REQUEST FOR PRIORITIZED EXAMINATION UNDER 37 CFR 1.102(e) (Page 1 of 1)

First Named Inventor:	Patrick H. Witham	Nonprovisional Application Number (if known):	
Title of Invention:	METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS		

APPLICANT HEREBY CERTIFIES THE FOLLOWING AND REQUESTS PRIORITIZED EXAMINATION FOR THE ABOVE-IDENTIFIED APPLICATION.

1. The processing fee set forth in 37 CFR 1.17(i), the prioritized examination fee set forth in 37 CFR 1.17(c), and if not already paid, the publication fee set forth in 37 CFR 1.18(d) have been filed with the request. The basic filing fee, search fee, examination fee, and any required excess claims and application size fees are filed with the request or have been already been paid.

2. The application contains or is amended to contain no more than four independent claims and no more than thirty total claims, and no multiple dependent claims.

3. The applicable box is checked below:

I. ☑ Original Application (Track One) - Prioritized Examination under § 1.102(e)(1)

i. (a) The application is an original nonprovisional utility application filed under 35 U.S.C. 111(a). This certification and request is being filed with the utility application via EFS-Web.

---OR---

(b) The application is an original nonprovisional plant application filed under 35 U.S.C. 111(a). This certification and request is being filed with the plant application in paper.

ii. An executed oath or declaration under 37 CFR 1.63 is filed with the application.

II. ☐ Request for Continued Examination - Prioritized Examination under § 1.102(e)(2)

i. A request for continued examination has been filed with, or prior to, this form.
ii. If the application is a utility application, this certification and request is being filed via EFS-Web.
iii. The application is an original nonprovisional utility application filed under 35 U.S.C. 111(a), or is a national stage entry under 35 U.S.C. 371.
iv. This certification and request is being filed prior to the mailing of a first Office action responsive to the request for continued examination.
v. No prior request for continued examination has been granted prioritized examination status under 37 CFR 1.102(e)(2).

Signature /Michael Hostetler/	Date 2013-11-14
Name (Print/Typed) Michael Hostetler	Practitioner Registration Number 47664

Note: *Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required in accordance with 37 CFR 1.33 and 11.18. Please see 37 CFR 1.4(d) for the form of the signature. If necessary, submit multiple forms for more than one signature, see below*.*

☑ *Total of 1 forms are submitted.

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6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

PTO/AIA/14 (03-13)
Approved for use through 01/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	44630-701.201
	Application Number	
Title of Invention	METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS	

The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.
This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.

Secrecy Order 37 CFR 5.2

☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

Inventor Information:

Inventor 1 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Patrick	H.	Witham	

Residence Information (Select One) ◉ US Residency ○ Non US Residency ○ Active US Military Service

City	Eugene	State/Province	OR	Country of Residence i	US

Mailing Address of Inventor:

Address 1	5563 Jeffrey Way		
Address 2			
City	Eugene	State/Province	OR
Postal Code	97402	Country i	US

Inventor 2 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Sailaja		Machiraju	

Residence Information (Select One) ◉ US Residency ○ Non US Residency ○ Active US Military Service

City	Beaverton	State/Province	OR	Country of Residence i	US

Mailing Address of Inventor:

Address 1	16275 NW Schendel Avenue		
Address 2	#16D		
City	Beaverton	State/Province	OR
Postal Code	97006	Country i	US

Inventor 3 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Lauren	Mackensie-Clark	Bluett	

Residence Information (Select One) ◉ US Residency ○ Non US Residency ○ Active US Military Service

PTO/AIA/14 (03-13)
Approved for use through 01/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	44630-701.201
	Application Number	
Title of Invention	METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS	

City	Milwaukie	State/Province	OR	Country of Residence i	US

Mailing Address of Inventor:

Address 1	2585 SE Martha CT		
Address 2			
City	Milwaukie	State/Province	OR
Postal Code	97222	Country i	US

All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the **Add** button. Add

Correspondence Information:

Enter either Customer Number or complete the Correspondence Information section below.
For further information see 37 CFR 1.33(a).

☐ **An Address is being provided for the correspondence Information of this application.**

Customer Number	21971		
Email Address	patentdocket@wsgr.com	Add Email	Remove Email

Application Information:

Title of the Invention	METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS		
Attorney Docket Number	44630-701.201	Small Entity Status Claimed	☒
Application Type	Nonprovisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	4	Suggested Figure for Publication (if any)	

Publication Information:

☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)

☐ **Request Not to Publish.** I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application **has not and will not** be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32).
Either enter Customer Number or complete the Representative Name section below. If both sections are completed the customer Number will be used for the Representative Information during processing.

Please Select One:	◉ Customer Number	○ US Patent Practitioner	○ Limited Recognition (37 CFR 11.9)

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	44630-701.201
	Application Number	
Title of Invention	METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS	
Customer Number	21971	

Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, or 365(c) or indicate National Stage entry from a PCT application. Providing this information in the application data sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78.

Prior Application Status			Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)

Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the **Add** button. Add

Foreign Priority Information:

This section allows for the applicant to claim priority to a foreign application. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55(d). When priority is claimed to a foreign application that is eligible for retrieval under the priority document exchange program (PDX) [i]the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(h)(1) and (2). Under the PDX program, applicant bears the ultimate responsibility for ensuring that a copy of the foreign application is received by the Office from the participating foreign intellectual property office, or a certified copy of the foreign priority application is filed, within the time period specified in 37 CFR 1.55(g)(1).

			Remove
Application Number	Country [i]	Filing Date (YYYY-MM-DD)	Access Code[j] (if applicable)

Additional Foreign Priority Data may be generated within this form by selecting the **Add** button. Add

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

☐ This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.
NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.

PTO/AIA/14 (03-13)
Approved for use through 01/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	44630-701.201
	Application Number	
Title of Invention	METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS	

Authorization to Permit Access:

☒ Authorization to Permit Access to the Instant Application by the Participating Offices

If checked, the undersigned hereby grants the USPTO authority to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the World Intellectual Property Office (WIPO), and any other intellectual property offices in which a foreign application claiming priority to the instant patent application is filed access to the instant patent application. See 37 CFR 1.14(c) and (h). This box should not be checked if the applicant does not wish the EPO, JPO, KIPO, WIPO, or other intellectual property office in which a foreign application claiming priority to the instant patent application is filed to have access to the instant patent application.

In accordance with 37 CFR 1.14(h)(3), access will be provided to a copy of the instant patent application with respect to: 1) the instant patent application-as-filed; 2) any foreign application to which the instant patent application claims priority under 35 U.S.C. 119(a)-(d) if a copy of the foreign application that satisfies the certified copy requirement of 37 CFR 1.55 has been filed in the instant patent application; and 3) any U.S. application-as-filed from which benefit is sought in the instant patent application.

In accordance with 37 CFR 1.14(c), access may be provided to information concerning the date of filing this Authorization.

Applicant Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Applicant 1 Remove

If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.

Clear

◉ Assignee	○ Legal Representative under 35 U.S.C. 117	○ Joint Inventor
○ Person to whom the inventor is obligated to assign.	○ Person who shows sufficient proprietary interest	

If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:

Name of the Deceased or Legally Incapacitated Inventor :

If the Applicant is an Organization check here. ☒

Organization Name	Paragon BioTeck, Inc.

PTO/AIA/14 (03-13)
Approved for use through 01/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	44630-701.201
	Application Number	
Title of Invention	METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS	

Mailing Address Information:			
Address 1	4640 SW Macadam Avenue		
Address 2	Suite 80		
City	Portland	**State/Province**	OR
Country i	US	Postal Code	97239
Phone Number		Fax Number	
Email Address			

Additional Applicant Data may be generated within this form by selecting the Add button. Add

Assignee Information including Non-Applicant Assignee Information:

Providing assignment information in this section does not subsitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Assignee 1

Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication . An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.

Remove

If the Assignee is an Organization check here. ☐

Prefix	**Given Name**	Middle Name	**Family Name**	Suffix

Mailing Address Information:			
Address 1			
Address 2			
City		**State/Province**	
Country i		Postal Code	
Phone Number		Fax Number	
Email Address			

Additional Assignee Data may be generated within this form by selecting the Add button. Add

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	44630-701.201
	Application Number	
Title of Invention	METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS	

Signature:

Remove

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications

Signature	/Michael Hostetler/			Date (YYYY-MM-DD)	2013-11-14
First Name	Michael	Last Name	Hostetler	Registration Number	47664

Additional Signature may be generated within this form by selecting the Add button. Add

This collection of information is required by 37 CFR 1.76. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 23 minutes to complete, including gathering, preparing, and submitting the completed application data sheet form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor: Patrick H. Witham	Group Art Unit: To Be Assigned
Serial Number: To Be Assigned	Examiner: To Be Assigned
Filing Date: Herewith	**CONFIRMATION NO:** To Be Assigned
Title: Methods and Compositions of Stable Phenylephrine Formulations	

FILED ELECTRONICALLY ON: November 14, 2013

Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

INFORMATION DISCLOSURE STATEMENT UNDER 37 CFR §1.97

Madam:

An Information Disclosure Statement along with attached PTO/SB/08 is hereby submitted. A copy of each listed publication is submitted, if required, pursuant to 37 CFR §§1.97-1.98, as indicated below.

The Examiner is requested to review the information provided and to make the information of record in the above-identified application. The Examiner is further requested to initial and return the attached PTO/SB/08 in accordance with MPEP §609.

The right to establish the patentability of the claimed invention over any of the information provided herewith, and/or to prove that this information may not be prior art, and/or to prove that this information may not be enabling for the teachings purportedly offered, is hereby reserved.

This statement is not intended to represent that a search has been made or that the information cited in the statement is, or is considered to be, prior art or material to patentability as defined in §1.56.

A. ☒ *37 CFR §1.97(b).* This Information Disclosure Statement should be considered by the Office because:

☐ (1) It is being filed within 3 months of the filing date of a national application and is other than a continued prosecution application under §1.53(d);

-- OR --

☐ (2) It is being filed within 3 months of entry of the national stage as set forth in §1.491 in an international application;

-- OR --

☒ (3) It is being filed before the mailing of a first Office action on the merits;

-- OR --

☐ (4) It is being filed before the mailing of a first Office action after the filing of a request for continued examination under §1.114.

B. ☐ *37 CFR §1.97(c).* Although this Information Disclosure Statement is being filed after the period specified in *37 CFR §1.97(b),* above, it is filed before the mailing date of the earlier of (1) a final office action under §1.113, (2) a notice of allowance under §1.311, or (3) an action that otherwise closes prosecution on the merits, this Information Disclosure Statement should be considered because it is accompanied by one of:

☐ a statement as specified in §1.97(c) provided concurrently herewith;

-- OR --

☐ a fee of $180.00 as set forth in §1.17(p) authorized below, enclosed, or included with the payment of other papers filed together with this statement.

C. ☐ *37 CFR §1.97(d).* Although this Information Disclosure Statement is being filed after the mailing date of the earlier of (1) a final office action under §1.113 or (2) a notice of allowance under §1.311, it is being filed before payment of the issue fee and should be considered because it is accompanied by:

i. a statement as specified in §1.97(e);

-- AND --

ii. a fee of $180.00 as set forth in §1.17(p) is authorized below, enclosed, or included with the payment of other papers filed together with this Statement.

D. ☐ *37 CFR §1.97(e).* Statement.

☐ A statement is provided herewith to satisfy the requirement under 37 CFR §§1.97(c);

-- AND/OR --

☐ A statement is provided herewith to satisfy the requirement under 37 CFR §§1.97(d);

-- AND/OR --

☐ A copy of a dated communication from a foreign patent office clearly showing that the information disclosure statement is being submitted within 3 months of the filing date on the communication is provided in lieu of a statement under 37 C.F.R. § 1.97(e)(1) as provided for under MPEP 609.04(b) V.

E. ☐ *Statement Under 37 C.F.R. §1.704(d).* Each item of information contained in the information disclosure statement was first cited in a communication from a foreign patent office in a counterpart application that was received by an individual designated in § 1.56(c) not more than thirty (30) days prior to the filing of this information disclosure statement. This statement is made pursuant to the requirements of 37 C.F.R. §1.704(d) to avoid reduction of the period of adjustment of the patent term for Applicant(s) delay.

F. ☒ *37 CFR §1.98(a)(2).* The content of the Information Disclosure Statement is as follows:

☐ Copies of each of the references listed on the attached Form PTO/SB/08 are enclosed herewith.

-- OR --

☒ Copies of U.S. Patent Documents (issued patents and patent publications) listed on the attached Form PTO/SB/08 are NOT enclosed.

-- AND/OR --

☒ Copies of Foreign Patent Documents and/or Non Patent Literature Documents listed on the attached Form PTO/SB/08 are enclosed in accordance with 37 CFR §1.98 (a)(2).

-- AND/OR --

☐ Copies of pending unpublished U.S. patent applications are enclosed in accordance with 37 CFR §1.98(a)(2)(iii).

G. ☐ *37 CFR §1.98(a)(3).* The Information Disclosure Statement includes non-English patents and/or references.

☐ Pursuant to 37 CFR §1.98(a)(3)(i), a concise explanation of the relevance of each patent, publication or other information provided that is not in English is provided herewith.

☐ Pursuant to MPEP 609(B), an English language copy of a foreign search report is submitted herewith to satisfy the requirement for a concise explanation where non-English language information is cited in the search report.

-- OR --

☐ A concise explanation of the relevance of each patent, publication or other information provided that is not in English is as follows: _____

☐ Pursuant to 37 CFR §1.98(a)(3)(ii), a copy of a translation, or a portion thereof, of the non-English language reference(s) is provided herewith.

H. ☐ *37 CFR §1.98(d).* Copies of patents, publications and pending U.S. patent applications, or other information specified in 37 C.F.R. § 1.98(a) are not provided herewith because:

☐ Pursuant to 37 CFR §1.98(d)(1) the information was previously submitted in an Information Disclosure Statement, or cited by examiner, for another application under which this application claims priority for an earlier effective filing date under 35 U.S.C. 120.

Application in which the information was submitted: _____

Information Disclosure Statement(s) filed on: _____

AND

☐ The information disclosure statement submitted in the earlier application complied with paragraphs (a) through (c) of 37 CFR §1.98.

I. ☒ *Fee Authorization.* The Commissioner is hereby authorized to charge the above-referenced fees of $0.00 and charge any additional fees or credit any overpayment associated with this communication to Deposit Account No. 23-2415 (Docket No. 44630-701.201).

Respectfully submitted,

WILSON SONSINI GOODRICH & ROSATI

Dated: November 14, 2013

By: /Michael Hostetler/
Michael J. Hostetler
Reg. No. 47,664

650 Page Mill Road
Palo Alto, CA 94304-1050
858/350-2306
Customer No. 021971

Substitute for form 1449/PTO **INFORMATION DISCLOSURE STATEMENT BY APPLICANT** *(Use as many sheets as necessary)*				***Complete if Known***	
				Application Number	To Be Assigned
				Filing Date	Herewith
				First Named Inventor	Patrick H. Witham
				Art Unit	To Be Assigned
				Examiner Name	To Be Assigned
Sheet	1	of	1	Attorney Docket Number	44630-701.201

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] *(if known)*	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3] – Number[4] – Kind Code[5] *(if known)*	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
	1.	Brown, et al., "Activities of octopamine and synephrine stereoisomers on α-adrenoceptors." Br. J. Pharmacol. (1988), 93, 417-429	
	2.	El-Shibini, et al. "The Stability of Phenylephrine- Part 1: The Rate of Degradation of the Amino Group."Arzneimittelforschung. 1969 Apr;19(4):676-8.	
	3.	El-Shibini, et al. "The Stability of Phenylephrine- Part 2: The discolouration reaction and the influence of some ions on the rate of degradation of the drug." Arzneimittelforschung. 1969 May;19(5):828-31.	
	4.	El-Shibini, et al. "The Stability of Phenylephrine- Part 3: The racemisation reaction." Arzneimittelforschung. 1969 Sep;19(9):1613-4.	
	5.	Millard, et al., "The Stability of Aqueous Solutions of Phenylephrine at Elevated Temperatures: Identification of the Decomposition Products." J. Pharm. Pharmac., 1973, 25, Suppl., 24P-31P	
	6.	"Report of the International Workshop on In Vitro Methods for Assessing Acut Systemic Toxicity." Results of an International Workshop Organized by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM); pages 1-370.	
	7.	Zaczek, et. al., "The effect of phenylephrine on pain and flare intensity in eyes with uveitis."Acta Ophthalmol Scand. 2000 Oct;78(5):516-8	

Examiner Signature		Date Considered	

**EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.* [1]Applicant's unique citation designation number (optional). [2]See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3]Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4]For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5]Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6]Applicant is to place a check mark here if English language Translation is attached.

PTO/AIA/15 (03-13)
Approved for use through 01/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No.	44630-701.201
First Named Inventor	Patrick H. Witham
Title	METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS
Express Mail Label No.	

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

1. ☐ **Fee Transmittal Form** (PTO/SB/17 or equivalent)
2. ☑ **Applicant asserts small entity status.** See 37 CFR 1.27
3. ☐ **Applicant certifies micro entity status.** See 37 CFR 1.29. Applicant must attach form PTO/SB/15A or B or equivalent.
4. ☑ **Specification** [*Total Pages* 18] Both the claims and abstract must start on a new page. *(See MPEP § 608.01(a) for information on the preferred arrangement)*
5. ☑ **Drawing(s)** (35 U.S.C. 113) [*Total Sheets* 4]
6. **Inventor's Oath or Declaration** [*Total Pages* 3] *(including substitute statements under 37 CFR 1.64 and assignments serving as an oath or declaration under 37 CFR 1.63(e))*
   a. ☑ Newly executed (original or copy)
   b. ☐ A copy from a prior application (37 CFR 1.63(d))
7. ☑ **Application Data Sheet** ** See note below.* See 37 CFR 1.76 (PTO/AIA/14 or equivalent)
8. **CD-ROM or CD-R** in duplicate, large table, or Computer Program (*Appendix*)
   ☐ Landscape Table on CD
9. **Nucleotide and/or Amino Acid Sequence Submission** (*if applicable, items a. – c. are required*)
   a. ☐ Computer Readable Form (CRF)
   b. ☐ Specification Sequence Listing on:
      i. ☐ CD-ROM or CD-R (2 copies); or
      ii. ☐ Paper
   c. ☐ Statements verifying identity of above copies

ADDRESS TO:

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

ACCOMPANYING APPLICATION PAPERS

10. ☐ **Assignment Papers** (cover sheet & document(s))
    Name of Assignee _____
11. ☐ **37 CFR 3.73(c) Statement** ☐ **Power of Attorney** (*when there is an assignee*)
12. ☐ **English Translation Document** (*if applicable*)
13. ☑ **Information Disclosure Statement** (PTO/SB/08 or PTO-1449)
    ☑ Copies of citations attached
14. ☐ **Preliminary Amendment**
15. ☐ **Return Receipt Postcard** (*MPEP § 503*) (*Should be specifically itemized*)
16. ☐ **Certified Copy of Priority Document(s)** (*if foreign priority is claimed*)
17. ☐ **Nonpublication Request** Under 35 U.S.C. 122(b)(2)(B)(i). Applicant must attach form PTO/SB/35 or equivalent.
18. ☑ **Other:** Request for Prioritized Examination

***Note**: (1) Benefit claims under 37 CFR 1.78 and foreign priority claims under 1.55 **must** be included in an Application Data Sheet (ADS).
(2) For applications filed under 35 U.S.C. 111, the application must contain an ADS specifying the applicant if the applicant is an assignee, person to whom the inventor is under an obligation to assign, or person who otherwise shows sufficient proprietary interest in the matter. See 37 CFR 1.46(b).

19. CORRESPONDENCE ADDRESS

☑ The address associated with Customer Number: 21971 **OR** ☐ Correspondence address below

Name					
Address					
City		State		Zip Code	
Country		Telephone		Email	

Signature	/Michael Hostetler/	Date	2013-11-14
Name (Print/Type)	Michael Hostetler	Registration No. (Attorney/Agent)	47664

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Patent Application Fee Transmittal

Application Number:	
Filing Date:	
Title of Invention:	METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS
First Named Inventor/Applicant Name:	Patrick H. Witham
Filer:	Michael J. Hostetler/Jason Liang/Adriana Serrano
Attorney Docket Number:	44630-701.201

Filed as Small Entity

Track I Prioritized Examination - Nonprovisional Application under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Utility filing Fee (Electronic filing)	4011	1	70	70
Utility Search Fee	2111	1	300	300
Utility Examination Fee	2311	1	360	360
Request for Prioritized Examination	2817	1	2000	2000
Pages:				
Claims:				
Miscellaneous-Filing:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Publ. Fee- Early, Voluntary, or Normal	1504	1	300	300
OTHER PUBLICATION PROCESSING FEE	1808	1	130	130
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	**3160**

Electronic Acknowledgement Receipt

EFS ID:	17409389
Application Number:	14080771
International Application Number:	
Confirmation Number:	6889
Title of Invention:	METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS
First Named Inventor/Applicant Name:	Patrick H. Witham
Customer Number:	21971
Filer:	Michael J. Hostetler/Jason Liang/Adriana Serrano
Filer Authorized By:	Michael J. Hostetler
Attorney Docket Number:	44630-701.201
Receipt Date:	14-NOV-2013
Filing Date:	
Time Stamp:	22:47:44
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$3160
RAM confirmation Number	7416
Deposit Account	232415
Authorized User	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /zip	Pages (if appl.)

1	Transmittal of New Application	44630_701_201_Transmittal_Form.pdf	278146 1cb6336ccd6e933b8e0dfce9413a52ddd1f5f2b5	no	2
Warnings:					
Information:					
2	TrackOne Request	44630_701_201_Track_One_Request.pdf	140995 91047fae183f13a69b397a2b053077ac54a9c31b	no	2
Warnings:					
Information:					
3	Application Data Sheet	44630_701_201_Application_Data_Sheet.pdf	1505454 04d9b5c45a76d286160d5940051212d4b7123fee7	no	7
Warnings:					
Information:					
4	Oath or Declaration filed	44630_701_201_Signed_Declaration.pdf	149406 cda78d6d5e02a71ac488d09d695b3c814af84b6b	no	3
Warnings:					
Information:					
5		44630_701_201_IDS_Nov_14_2013.pdf	207628 909bc0ac7c926fcaf220b5c7a219ff1b541f99b0	yes	5
	Multipart Description/PDF files in .zip description				
	Document Description		**Start**	**End**	
	Transmittal Letter		1	4	
	Information Disclosure Statement (IDS) Form (SB08)		5	5	
Warnings:					
Information:					
6	Non Patent Literature	BRWON.pdf	829033 10068bbd31d616bbbcddaa5a7cd8b7e69585b11	no	13
Warnings:					
Information:					
7	Non Patent Literature	ElShabini_Part1.pdf	5823332 a0bf543b3c2fb9df12fb920bcc2475909f1c0ec3c	no	3
Warnings:					
Information:					
8	Non Patent Literature	ElShabini_Part2.pdf	8026192 1acd49f3fc302dd9b2f1949863cbcd94ec88f3e	no	4

Warnings:					
Information:					
9	Non Patent Literature	ElShabini_Part3.pdf	4085582 43587873bfc317cdee6b22fd1a168aa0f3f98cfa	no	2
Warnings:					
Information:					
10	Non Patent Literature	MILLARD.pdf	8985495 c3217d2aee17fa439c51e5f5087bba79dda53c5d	no	8
Warnings:					
Information:					
11	Non Patent Literature	Report_AcuteSystemicToxicity.pdf	4502900 11a8c2332JJ18/99d2f1b92fbe4J8acacJe9b634	no	370
Warnings:					
Information:					
12		44630_701_201_Application_Methods_and_Compositions_of_Stable_Phenylephrine_Formulations.pdf	1010668 0739acf251ab203Jee2ec906005bcb9b98c8b511	yes	22

Multipart Description/PDF files in .zip description

Document Description	Start	End
Specification	1	16
Claims	17	17
Abstract	18	18
Drawings-only black and white line drawings	19	22

Warnings:					
Information:					
13	Non Patent Literature	ZACZEK.pdf	88913 3b199bb27fd77bf4c58169315efb13e145d0J90d	no	3
Warnings:					
Information:					
14	Fee Worksheet (SB06)	fee-info.pdf	40308 99e8dfca8d3209054491fGda131555b0472aa001	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	35674052		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

SCORE Placeholder Sheet for IFW Content

Application Number: 14080771 Document Date: 11/14/2013

The presence of this form in the IFW record indicates that the following document type was received in electronic format on the date identified above. This content is stored in the SCORE database.

- Drawings – Other than Black and White Line Drawings

Since this was an electronic submission, there is no physical artifact folder, no artifact folder is recorded in PALM, and no paper documents or physical media exist. The TIFF images in the IFW record were created from the original documents that are stored in SCORE.

To access the documents in the SCORE database, refer to instructions below.

At the time of document entry (noted above):

- Examiners may access SCORE content via the eDAN interface.
- Other USPTO employees can bookmark the current SCORE URL (http://Score.uspto.gov/ScoreAccessWeb/).
- External customers may access SCORE content via the Public and Private PAIR interfaces.

Form Revision Date: September 30, 2013

Document code: WFEE

United States Patent and Trademark Office
Sales Receipt for Accounting Date: 12/02/2013

VVAN11 SALE #00000007 Mailroom Dt: 11/14/2013 232415 14080771
01 FC : 2830 70.00 DA

Document code: WFEE

United States Patent and Trademark Office
Sales Receipt for Accounting Date: 12/02/2013

VVAN11 ADJ #00000002 Mailroom Dt: 11/14/2013
Seq No: 7416 Sales Acctg Dt: 11/15/2013 232415 14080771
06 FC : 1808 130.00 CR

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 14/080,771

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	70		N/A	
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A	300		N/A	
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	360		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	14 minus 20 =	*	x 40 =	0.00	OR		
INDEPENDENT CLAIMS (37 CFR 1.16(h))	1 minus 3 =	*	x 210 =	0.00			
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			0.00			
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				0.00			
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	730		TOTAL	

APPLICATION AS AMENDED - PART II

AMENDMENT A

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE(S)	OTHER THAN SMALL ENTITY ADDITIONAL FEE(S)
Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
Application Size Fee (37 CFR 1.16(s))									
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

AMENDMENT B

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE($)	ADDITIONAL FEE($)		RATE(S)	ADDITIONAL FEE(S)
Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
Application Size Fee (37 CFR 1.16(s))									
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
14/080,771	11/14/2013	1629	1030	44630-701.201	14	1

CONFIRMATION NO. 6889

21971
WILSON, SONSINI, GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050

FILING RECEIPT

Date Mailed: 12/05/2013

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

Patrick H. Witham, Eugene, OR;
Sailaja Machiraju, Beaverton, OR;
Lauren Mackensie-Clark Bluett, Milwaukie, OR;

Applicant(s)

Paragon BioTech, Inc., Portland, OR

Assignment For Published Patent Application

Paragon BioTech, Inc., Portland, OR

Power of Attorney: None

Domestic Applications for which benefit is claimed - None.
A proper domestic benefit claim must be provided in an Application Data Sheet in order to constitute a claim for domestic benefit. See 37 CFR 1.76 and 1.78.

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access - A proper **Authorization to Permit Access to Application by Participating Offices** (PTO/SB/39 or its equivalent) has been received by the USPTO.

If Required, Foreign Filing License Granted: 12/02/2013
The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 14/080,771**

Projected Publication Date: 05/14/2015

Non-Publication Request: No

Early Publication Request: No
** **SMALL ENTITY** **
Title

METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS

Preliminary Class

514

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

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This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

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UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents
United States Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450
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650 PAGE MILL ROAD
PALO ALTO CA 94304-1050

MAILED
DEC 13 2013
OFFICE OF PETITIONS

Doc Code: TRACK1.GRANT

Decision Granting Request for Prioritized Examination (Track I or After RCE)	Application No.: 14/080,771

1. THE REQUEST FILED November 14, 2013 IS **GRANTED.**

The above-identified application has met the requirements for prioritized examination

A. ☒ for an original nonprovisional application (Track I).
B. ☐ for an application undergoing continued examination (RCE).

2. **The above-identified application will undergo prioritized examination.** The application will be accorded special status throughout its entire course of prosecution until one of the following occurs:

A. filing a **petition for extension of time** to extend the time period for filing a reply;
B. filing an **amendment to amend the application to contain more than four independent claims, more than thirty total claims**, or a multiple dependent claim;
C. filing a **request for continued examination**;
D. filing a notice of appeal;
E. filing a request for suspension of action;
F. mailing of a notice of allowance;
G. mailing of a final Office action;
H. completion of examination as defined in 37 CFR 41.102; or
I. abandonment of the application.

Telephone inquiries with regard to this decision should be directed to **Michelle R. Eason** at (571) 272-4231. In his/her absence, calls may be directed to Brian W. Brown at (571) 272-5338.

/Michelle R. Eason/
(*Signature*)

Paralegal Specialist, Office of Petitions
(Title)

PTO/AIA/80 (07-12)
Approved for use through 11/30/2014. OMB 0651-0035
U.S. Patent and Trademark Office; U.S DEPARTMENT OF COMMERCE

POWER OF ATTORNEY TO PROSECUTE APPLICATIONS BEFORE THE USPTO

I hereby revoke all previous powers of attorney given in the application identified in the attached statement under 37 CFR 3.73(c).

I hereby appoint:

■ Practitioners associated with Customer Number: 21971

OR

☐ Practitioner(s) named below (if more than ten patent practitioners are to be named, then a customer number must be used):

Name	Registration Number	Name	Registration Number

As attorney(s) or agent(s) to represent the undersigned before the United States Patent and Trademark Office (USPTO) in connection with any and all patent applications assigned only to the undersigned according to the USPTO assignment records or assignments documents attached to this form in accordance with 37 CFR 3.73(c).

Please change the correspondence address for the application identified in the attached statement under 37 CFR 3.73(c) to:

■ The address associated with Customer Number: 21971

OR

☐

Firm or Individual Name			
Address			
City		State	Zip
Country			
Telephone		Email	

Assignee Name and Address: Paragon BioTeck, Inc.
4640 SW Macadam Avenue, Suite 80
Portland, Oregon 97239

A copy of this form, together with a statement under 37 CFR 3.73(c) (Form PTO/AIA/96 or equivalent) is required to be Filed in each application in which this form is used. The statement under 37 CFR 3.73(c) may be completed by one of The practitioners appointed in this form, and must identify the application in which this Power of Attorney is to be filed.

SIGNATURE of Assignee of Record

The individual whose signature and title is supplied below is authorized to act on behalf of the assignee

Signature	Patrick H. Witham	Date	Nov 25, 2013
Name	Patrick H. Witham	Telephone	
Title	CEO of Paragon BioTeck, Inc.		

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

PTO/AIA/96 (08-12)
Approved for use through 01/31/2013. OMB 0651-0031
U.S. Patent and Trademark Office:U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of1995, no persons arerequired to respond to a collection of information unless it displays a valid OMB control number.

STATEMENT UNDER 37 CFR 3.73(c)

Applicant/Patent Owner: Patrick H. Witham, et al.

Application No./Patent No.: 14/080,771 Filed/Issue Date: 11-14-2013

Titled: METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS

Paragon BioTeck, Inc., a Corporation

(Name of Assignee) (Type of Assignee. e.g., corporation, partnership. university. government agency. etc.)

states that, for the patent application/patent identified above, it is (choose **one** of options 1, 2, 3 or 4 below):

1. ☑ The assignee of the entire right, title, and interest.

2. ☐ An assignee of less than the entire right, title, and interest (check applicable box):

   ☐ The extent (by percentage) of its ownership interest is _____%. Additional Statement(s) by the owners holding the balance of the interest must be submitted to account for 100% of the ownership interest.

   ☐ There are unspecified percentages of ownership. The other parties, including inventors, who together own the entire right, title and interest are:

   Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

3. ☐ The assignee of an undivided interest in the entirety (a complete assignment from one of the joint inventors was made). The other parties, including inventors, who together own the entire right, title, and interest are:

   Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

4. ☐ The recipient, via a court proceeding or the like (*e.g.*, bankruptcy, probate), of an undivided interest in the entirety (a complete transfer of ownership interest was made). The certified document(s) showing the transfer is attached.

The interest identified in option 1, 2 or 3 above (not option 4) is evidenced by either (choose **one** of options A or B below):

A. ☑ An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the United States Patent and Trademark Office at Reel _____, Frame _____, or for which a copy thereof is attached.

B. ☐ A chain of title from the inventor(s), of the patent application/patent identified above, to the current assignee as follows:

   1. From: _____ To: _____

      The document was recorded in the United States Patent and Trademark Office at

      Reel _____, Frame _____, or for which a copy thereof is attached.

   2. From: _____ To: _____

      The document was recorded in the United States Patent and Trademark Office at

      Reel _____, Frame _____, or for which a copy thereof is attached.

STATEMENT UNDER 37 CFR 3.73(c)

3. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

4. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

5. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

6. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

☐ Additional documents in the chain of title are listed on a supplemental sheet(s).

☑ As required by 37 CFR 3.73(c)(1)(i), the documentary evidence of the chain of title from the original owner to the assignee was, or concurrently is being, submitted for recordation pursuant to 37 CFR 3.11.

[NOTE: A separate copy (i.e., a true copy of the original assignment document(s)) must be submitted to Assignment Division in accordance with 37 CFR Part 3, to record the assignment in the records of the USPTO. See MPEP 302.08]

The undersigned (whose title is supplied below) is authorized to act on behalf of the assignee.

/Michael Hostetler/	December 18, 2013
Signature	Date
Michael J. Hostetler, Ph.D., Esq.	47,664
Printed or Typed Name	Title or Registration Number

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that yoube given certain informationin connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, pleasebe advised that: (1) the general authority forthe collection of thisinformation is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and(3) the principal purpose forwhich the information isused by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent applicationor patent. If you do not furnish the requested information,the U.S. Patent and Trademark Office may not be able to process and/or examineyour submission,which may result in termination of proceedings or abandonment of the applicationor expiration of the patent.

The informationprovided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the informationin order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
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6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, arecord may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from thissystem of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

PATENT ASSIGNMENT	Docket Number 44630-701.201

WHEREAS, the undersigned:

1. Patrick H. Witham,	2. Sailaja Machiraju	3. Lauren Mackensie-Clark Bluett
Citizen of U.S.A., Residing at	Citizen of India, Residing at	Citizen of U.S.A., Residing at
5563 Jeffrey Way	16275 NW Schendel Ave, #16D	2585 SE Martha Ct
Eugene, OR 97402	Beaverton, OR 97006	Milwaukie, OR 97222

(hereinafter "Inventor(s)," have invented certain new and useful improvements in

METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS

- ☐ for which a United States patent application is executed on even date herewith;
- ☒ for which Application No. 14/080,771 was filed on 11-14-2013 in the United States Patent Office;
- ☐ for which Application No. __ was filed on __ in the U.S. Receiving Office of the Patent Cooperation Treaty;
- ☐ for which Application No. __ was filed on __ in the __ Patent Office; and/or
- ☐ for which an application was filed upon which a United States Patent issued on __, as U.S. Patent No.

(hereinafter "Application(s)").

WHEREAS, Paragon BioTeck, Inc., a corporation of the State of Nevada, having a place of business at 4640 SW Macadam Avenue, Suite 80, Portland, Oregon 97239, (hereinafter "Assignee"), is desirous of acquiring the entire right, title and interest in and to said Application(s) and the inventions disclosed therein, and in and to all embodiments of the inventions, heretofore conceived, made or discovered, whether jointly or severally, by said Inventor(s) (hereinafter collectively referred to as "Inventions"), and in and to any and all patents, inventor's certificates and other forms of protection (hereinafter "Patent(s)") thereon granted in the United States, foreign countries, or under any international convention, agreement, protocol, or treaty.

NOW, THEREFORE, in consideration of good and valuable consideration acknowledged by said Inventor(s) to have been received in full from said Assignee:

1. Said Inventor(s) do hereby sell, assign, transfer and convey unto said Assignee the entire right, title and interest (a) in and to said Inventions, including the right to claim priority to said Inventions; (b) in and to all rights to all United States and corresponding non-United States patent applications and Patent(s), including those filed under the Paris Convention for the Protection of Industrial Property, The Patent Cooperation Treaty or otherwise; (c) in and to any and all applications filed and any and all Patent(s) granted on said Inventions in the United States, in any foreign country, or under any international convention, agreement, protocol, or treaty, including each and every application filed and any and all Patent(s) granted on any application which is a divisional, substitution, continuation, or continuation-in-part of any of said Application(s); and (d) in and to each and every reissue, reexamination, or extensions of any of said Patent(s).

2. Said Inventor(s) hereby covenant and agree to cooperate with said Assignee to enable said Assignee to enjoy to the fullest extent the right, title and interest herein conveyed in the United States, foreign countries, or under any international convention, agreement, protocol, or treaty. Such cooperation by said Inventor(s) shall include prompt production of pertinent facts and documents, giving of testimony, execution of petitions, oaths, specifications, declarations or other papers, and other assistance all to the extent deemed necessary or desirable by said Assignee (a) for perfecting in said Assignee the right, title and interest herein conveyed; (b) for prosecuting any applications covering said Inventions; (c) for filing and prosecuting substitute, divisional, continuing or additional applications covering said Inventions; (d) for filing and prosecuting applications for reissuance of any said Patent(s); (e) for interference or other priority proceedings involving said Inventions; and (f) for legal proceedings involving said Inventions and any applications therefore and any Patent(s) granted thereon, including without limitation reissues and reexaminations, opposition proceedings, cancellation proceedings, priority contests, public use proceedings, infringement actions and court actions; provided, however, that the expense incurred by said Inventor(s) in providing such cooperation shall be paid for by said Assignee.

3. The terms and covenants of this assignment shall inure to the benefit of said Assignee, its successors, assigns and other legal representatives, and shall be binding upon said Inventor(s), their respective heirs, legal representatives and assigns.

4. Said Inventor(s) hereby warrant and represent that they have not entered and will not enter into any assignment, contract, or understanding in conflict herewith.

5. Said Inventor(s) hereby request that any Patent(s) issuing in the United States, foreign countries, or under any international convention, agreement, protocol, or treaty, be issued in the name of the Assignee, or its successors and assigns, for the sole use of said Assignee, its successors, legal representatives and assigns.

6. Said Inventor(s) hereby authorize and request the attorneys appointed in said application to hereafter complete this assignment by inserting above the **filing date** and **serial number** of said application when known;

PATENT ASSIGNMENT	Docket Number 44630-701.201

IN WITNESS WHEREOF, said Inventor(s) have executed and delivered this instrument to said Assignee as of the dates written below:

Date: Nov 25, 2013 Patrick H. Witham

Patrick H. Witham

State/Commonwealth of _____)
County of _____)

On _____ before me, _____ (Name/Title of Notary) personally appeared **Patrick H. Witham** (Name of Signer) who proved to me on the basis of satisfactory evidence to be the person whose name is subscribed to the within instrument and acknowledged to me that s/he executed the same in his/her authorized capacity, and that by his/her signature on the instrument the person, or entity upon behalf of which the person acted, executed the instrument.

I certify under PENALTY OF PERJURY under the laws of the State/Commonwealth of _____ that the foregoing paragraph is true and correct.

WITNESS my hand and official seal.

Signature: _____ (Notary Seal)

Date: 14 NOV 2013 M. Sailaja

Sailaja Machiraju

State/Commonwealth of Oregon)
County of Multnomah)

On Nov. 13, 2013 before me, Pamela A Malone (Name/Title of Notary) personally appeared **Sailaja Machiraju** (Name of Signer) who proved to me on the basis of satisfactory evidence to be the person whose name is subscribed to the within instrument and acknowledged to me that s/he executed the same in his/her authorized capacity, and that by his/her signature on the instrument the person, or entity upon behalf of which the person acted, executed the instrument.

I certify under PENALTY OF PERJURY under the laws of the State/Commonwealth of Oregon that the foregoing paragraph is true and correct.

WITNESS my hand and official seal.

Signature: Pamela Anne Malone (Notary Seal)

OFFICIAL SEAL
PAMELA ANNE MALONE
NOTARY PUBLIC-OREGON
COMMISSION NO. 451986
MY COMMISSION EXPIRES SEPTEMBER 02, 2014

Date: 14 NOV 2013

Lauren Mackensie-Clark Bluett

State/Commonwealth of Oregon)
County of Multnomah)

On Nov. 13, 2013 before me, Pamela A. Malone (Name/Title of Notary) personally appeared **Lauren Mackensie-Clark Bluett** (Name of Signer) who proved to me on the basis of satisfactory evidence to be the person whose name is subscribed to the within instrument and acknowledged to me that s/he executed the same in his/her authorized capacity, and that by his/her signature on the instrument the person, or entity upon behalf of which the person acted, executed the instrument.

I certify under PENALTY OF PERJURY under the laws of the State/Commonwealth of Oregon that the foregoing paragraph is true and correct.

WITNESS my hand and official seal.

Signature: Pamela Anne Malone (Notary Seal)

OFFICIAL SEAL
PAMELA ANNE MALONE
NOTARY PUBLIC-OREGON
COMMISSION NO. 451986
MY COMMISSION EXPIRES SEPTEMBER 02, 2014

PATENT
Attorney Docket No. 44630-701.201

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application:

Inventor: *Patrick H. Witham, et al.*

Application No.: 14/080,771

Filed: 11-14-2013

Title: **METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS**

Confirmation No.: ***6889***

Customer No. **021971**

FILED ELECTRONICALLY ON: December 18, 2013

FILING FORMALITY DOCUMENTS

Mail Stop Missing Parts
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Applicant submits the following:

1. ☐ Copy of Notice to File Missing Parts is provided
2. ☐ **Filing Fees are authorized herewith**
3. ☒ **Applicant claims small entity status.** See 37 CFR 1.27.
4. ☐ **Replacement Specification is provided** *[Total Pages]* Both the claims and abstract must start on a new page. *(For information on the preferred arrangement, see MPEP 608.01(a))*
5. ☐ **Replacement Drawing(s)** (35 U.S.C. 113) is provided *[Total Pages]*
6. ☐ **Oath or Declaration is provided** *[Total Pages]*
   a. ☐ Newly executed (original or copy)
   b. ☐ Copy from a prior application (37 CFR 1.63(d))
7. ☐ **Information Disclosure** (PTO/SB/08 or PTO-1449) is provided
   ☐ Copies of citations attached
8. ☐ ***Preliminary Amendment* is provided**
9. ☐ **CD-Rom or CD-R** in duplicate, large table or Computer *Program (Appendix)* is provided
   ☐ Landscape table on CD
10. ***Nucleotide and/or Amino Acid Sequence Submission* is provided** *(if applicable, items a.-c. are required)*
    a. ☐ *Computer Readable Form (CRF)*
    b. ☐ *Specification Sequence Listing* on:
       i. ☐ *CD ROM* or CD-R (2 copies); or
       ii. ☐ *Paper*
    c. ☐ Statement verifying identity of above copies
11. ☒ **Assignment Papers** (cover sheet & document(s) are provided
12. ☒ **Power of Attorney is provided**
    ☒ **37 CFR 3.73(b) *Statement*** *(when there is an assignee)*
13. ☐ **English Translation Document** *(if applicable)* is provided
14. ☐ **Certified Copy of Priority Document(s)** *(if foreign priority is claimed)* is provided
15. ☐ Applicants request an Extension of Time under 37 C.F.R. §1.136 of:
    ☐ 1 Month ☐ 2 Months ☐ 3 Months
    ☐ 4 Months ☐ 5 Months
16. ☐ Other: **REPLACEMENT SHEET**

FEE AUTHORIZATION

The Commissioner is authorized to charge any additional fees which may be required, including petition fees and extension of time fees, to Deposit Account No. **23-2415** (Docket No. 44630-701.201).

Respectfully submitted,

Date: December 18, 2013

By: /Michael Hostetler/
Michael J. Hostetler, Ph.D., Esq.
Registration No. 47,664

WILSON SONSINI GOODRICH & ROSATI
650 Page Mill Road
Palo Alto, CA 94304-1050
Direct Dial: (650) 493-9300

5979079_1.doc

Electronic Acknowledgement Receipt

EFS ID:	17693942
Application Number:	14080771
International Application Number:	
Confirmation Number:	6889
Title of Invention:	METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS
First Named Inventor/Applicant Name:	Patrick H. Witham
Customer Number:	21971
Filer:	Michael J. Hostetler/Ashley Garcia
Filer Authorized By:	Michael J. Hostetler
Attorney Docket Number:	44630-701.201
Receipt Date:	18-DEC-2013
Filing Date:	14-NOV-2013
Time Stamp:	18:53:38
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal Letter	44630_701_201_Formalities_Transmittal.pdf	31456 c74660f37fb0352dacd8ccd40b4dbf1a0fdcbe1	no	1

Warnings:	
Information:	

2		44630_701_201_Formal_Documents.pdf	250276 50e1e689ff0872bb5dbade5de99a1fc1aa8dc1	yes	7

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Power of Attorney	1	2
	Assignee showing of ownership per 37 CFR 3.73.	3	7

Warnings:

The page size in the PDF is too large. The pages should be 8.5 x 11 or A4. If this PDF is submitted, the pages will be resized upon entry into the Image File Wrapper and may affect subsequent processing

Information:

Total Files Size (in bytes):	281732

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
14/080,771	11/14/2013	Patrick H. Witham	44630-701.201

CONFIRMATION NO. 6889

21971
WILSON, SONSINI, GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050

POA ACCEPTANCE LETTER

OC000000065653934

Date Mailed: 12/27/2013

NOTICE OF ACCEPTANCE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 12/18/2013.

The Power of Attorney in this application is accepted. Correspondence in this application will be mailed to the above address as provided by 37 CFR 1.33.

/nhassani/

Office of Data Management, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor: Patrick H. Witham, et al.	**CONFIRMATION NO: 6889**
Serial Number: 14/080,771	Group Art Unit: 1629
Filing Date: 11/14/2013	Examiner: Unassigned
Title: METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS	

FILED ELECTRONICALLY ON JANUARY 14, 2014

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

REQUEST FOR CORRECTED FILING RECEIPT

Sir:

There are errors with respect to the following, which are omitted, or misspelled:

Error in	***Correct data***
Applicant(s) names	***Paragon Bioteck, Inc.***
Applicant(s) addresses	
Priority Information	
Entity Status	

A copy of the Filing Receipt with corrections noted thereon is enclosed.

Issuance of a corrected Filing Receipt is respectfully requested.

Respectfully submitted,

Dated: January 14, 2014 By: /Sabrina Diane Poulos/
Sabrina D. Poulos, Reg. No. 62,387

WILSON SONSINI GOODRICH & ROSATI
650 Page Mill Road
Palo Alto, CA 94304-1505
(650)493-9300

Paragon / 44630-701.201
MHOS / JLIA / SPS / SILC

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY DOCKET NO	TOT CLAIMS	IND CLAIMS
14/080,771	11/14/2013	1629	1030	44630-701.201	14	1

CONFIRMATION NO. 6889

21971
WILSON, SONSINI, GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050

FILING RECEIPT

OC000000065298100

Date Mailed: 12/05/2013

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

Patrick H. Witham, Eugene, OR;
Sailaja Machiraju, Beaverton, OR;
Lauren Mackensie-Clark Bluett, Milwaukie, OR;

Applicant(s)

~~Paragon BioTech,~~ Inc., Portland, OR Correct spelling of Applicant is:

Assignment For Published Patent Application

~~Paragon BioTech~~, Inc., Portland, OR Paragon BioTeck, Inc.

Power of Attorney: None

Domestic Applications for which benefit is claimed - None.
A proper domestic benefit claim must be provided in an Application Data Sheet in order to constitute a claim for domestic benefit. See 37 CFR 1.76 and 1.78.

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access - A proper **Authorization to Permit Access to Application by Participating Offices** (PTO/SB/39 or its equivalent) has been received by the USPTO.

If Required, Foreign Filing License Granted: 12/02/2013
The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 14/080,771**

Projected Publication Date: 05/14/2015

Non-Publication Request: No

Early Publication Request: No
** **SMALL ENTITY** **
Title

METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS

Preliminary Class

514

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

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The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

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Electronic Acknowledgement Receipt

EFS ID:	17914066
Application Number:	14080771
International Application Number:	
Confirmation Number:	6889
Title of Invention:	METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS
First Named Inventor/Applicant Name:	Patrick H. Witham
Customer Number:	21971
Filer:	Sabrina D. Poulos/Linda Anders
Filer Authorized By:	Sabrina D. Poulos
Attorney Docket Number:	44630-701.201
Receipt Date:	14-JAN-2014
Filing Date:	14-NOV-2013
Time Stamp:	20:03:43
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Request for Corrected Filing Receipt	44630-701-201-RequestCorrectFR.pdf	212142 235639ff6bdc8262f68b444/46f8c/cc8/5e d33a	no	4

Warnings:	
Information:	

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New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
14/080,771	11/14/2013	1611	1030	44630-701.201	14	1

CONFIRMATION NO. 6889

CORRECTED FILING RECEIPT

21971
WILSON, SONSINI, GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050

OC000000066214245

Date Mailed: 01/24/2014

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

Patrick H. Witham, Eugene, OR;
Sailaja Machiraju, Beaverton, OR;
Lauren Mackensie-Clark Bluett, Milwaukie, OR;

Applicant(s)

Paragon BioTeck, Inc., Portland, OR

Assignment For Published Patent Application

Paragon BioTeck, Inc., Portland, OR

Power of Attorney: The patent practitioners associated with Customer Number 21971

Domestic Applications for which benefit is claimed - None.
A proper domestic benefit claim must be provided in an Application Data Sheet in order to constitute a claim for domestic benefit. See 37 CFR 1.76 and 1.78.

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/080,771	11/14/2013	Patrick H. Witham	44630-701.201	6889

21971 7590 02/04/2014

WILSON, SONSINI, GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050

EXAMINER	
FRAZIER, BARBARA S	

ART UNIT	PAPER NUMBER
1611	

MAIL DATE	DELIVERY MODE
02/04/2014	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No. 14/080,771	Applicant(s) WITHAM ET AL.	
	Examiner BARBARA FRAZIER	Art Unit 1611	AIA (First Inventor to File) Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *14 November 2013*.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5)☒ Claim(s) *1-14* is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *1-14* is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)

2) ☒ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date *11/14/13*.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .

4) ☐ Other: _____.

DETAILED ACTION

1. The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

2. Claims 1-14 are pending in this application.

Claim Rejections - 35 USC § 103

3. The following is a quotation of 35 U.S.C. 103 which forms the basis for all obviousness rejections set forth in this Office action:

> A patent for a claimed invention may not be obtained, notwithstanding that the claimed invention is not identically disclosed as set forth in section 102 of this title, if the differences between the claimed invention and the prior art are such that the claimed invention as a whole would have been obvious before the effective filing date of the claimed invention to a person having ordinary skill in the art to which the claimed invention pertains. Patentability shall not be negated by the manner in which the invention was made.

4. The factual inquiries set forth in *Graham v. John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under 35 U.S.C. 103 are summarized as follows:

1. Determining the scope and contents of the prior art.

2. Ascertaining the differences between the prior art and the claims at issue.

3. Resolving the level of ordinary skill in the pertinent art.

4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

5. This application currently names joint inventors. In considering patentability of the claims the examiner presumes that the subject matter of the various claims was commonly owned as of the effective filing date of the claimed invention(s) absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and effective filing dates of each claim that was not commonly owned as of the effective filing date of the later invention in order for the examiner to consider the applicability of 35 U.S.C. 102(b)(2)(C) for any potential 35 U.S.C. 102(a)(2) prior art against the later invention.

6. Claims 1-14 are rejected under 35 U.S.C. 103 as being unpatentable over Shibini et al. ("Shibini", Arzneimettelforschung, 19(9), pp. 1613-1614, 1969, cited by Applicants in IDS filed 14 November 2013) as evidenced by Valle (US Patent 4,260,600).

Shibini teaches an aqueous buffered composition of L-phenylephrine (e.g., see page 1614). While Shibini does not specifically teach the R-isomer, Valle teaches that the therapeutic form of phenylephrine hydrochloride is (R)-3-hydroxy-α-[(methylamino)methyl]benzenemethanol hydrochloride (col. 1, lines 27-30), and thus the skilled artisan would envisage R-phenylephrine from the teachings of Shibini. Shibini teaches that racemization and oxidation of phenylephrine is prevented through the use of an acidic medium (pH above 2 and below 7) and addition of EDTA (page 1614, column 2). The phenylephrine is present in an aqueous buffer (page 1614, column 1). Regarding the chiral purity at least 95% (claim1), 99% (claim3), or 99.3% (claim 4), since Shibini teaches

prevention of racemization, the skilled artisan would envisage a chiral purity of 100%, absent evidence to the contrary.

While Shibinia does not expressly teach storing the composition between -10 to 10 degrees Celsius (claim 1) or 2 to 8 degrees Celsius (claim 2), it is noted that said limitation is a process limitation, which may be interpreted either as 1) a product-by-process limitation (in order to arrive at the claimed composition), or 2) an intended use of the composition (i.e., the intended use of the composition is to store it between -10 to 10 degrees Celsius). Regarding 1), it is noted that product-by-process claims are not limited to the manipulations of the recited steps, only the structure implied by the steps. See MPEP 2113. Since Shibini already teaches a composition comprising phenylephrine and aqueous buffer, the limitations of the claims are met. Regarding 2), it is noted that the composition of Shibini is capable of the intended use of storing the composition between -10 to 10 degrees Celsius, absent evidence to the contrary, and thus the limitations of the claims are met.

Regarding claims 5-8, Shibini teaches prevention of racemization of phenylephrine, and that the solution can be safely sterilized by autoclaving and will not undergo appreciable decomposition during storage (page 1614). Therefore, the skilled artisan would envisage a chiral purity of at least 95%, 97%, 99%, or 99.3% of the initial chiral purity after 6 months, absent evidence to the contrary.

Regarding claim 9, Shibini teaches using 6.25 g in 200 ml, or approximately 3.12% w/v (page 1614, column 1). This amount is comparable to the amounts taught in claim 9, and/or the skilled artisan would be motivated to manipulate the amounts to within comparable ranges by routine experimentation, in order to optimize the therapeutic efficacy of the resultant composition.

Regarding the size and color of the bottle used (claims 10, 13, and 14), said limitations would be within the purview of the skilled artisan and would be a matter of design choice, absent evidence to the contrary.

Regarding the packaging identifying storage directions (claims 11 and 12), it is noted that said limitation amounts to printed material, and does not affect the structural limitations of the composition itself, and therefore does not impart patentability to the claims, absent evidence to the contrary.

Conclusion

No claims are allowed at this time.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to BARBARA FRAZIER whose telephone number is (571)270-3496. The examiner can normally be reached on Monday-Friday 9am-2:30pm EST.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Daniel Sullivan can be reached on (571)272-0779. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

BSF

/DANIEL SULLIVAN/

Supervisory Patent Examiner, Art Unit 1611

Notice of References Cited	Application/Control No. 14/080,771	Applicant(s)/Patent Under Reexamination WITHAM ET AL.	
	Examiner BARBARA FRAZIER	Art Unit 1611	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
*	A	US-4,260,600	04-1981	Valle, Ronald	424/720
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

PTO/SB/08a (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons required to respond to a collection of information unless it contains a valid OMB control number.

Substitute for form 1449/PTO **INFORMATION DISCLOSURE STATEMENT BY APPLICANT** *(Use as many sheets as necessary)*				***Complete if Known***	
				Application Number	To Be Assigned
				Filing Date	Herewith
				First Named Inventor	Patrick H. Witham
				Art Unit	To Be Assigned
				Examiner Name	To Be Assigned
Sheet	1	of	1	Attorney Docket Number	44630-701.201

U.S. PATENT DOCUMENTS					
Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] *(if known)*	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear

FOREIGN PATENT DOCUMENTS						
Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3] – Number[4] – Kind Code[5] *(if known)*	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]

NON PATENT LITERATURE DOCUMENTS			
Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
	1.	Brown, et al., "Activities of octopamine and synephrine stereoisomers on α-adrenoceptors." Br. J. Pharmacol. (1988), 93, 417-429	
	2.	El-Shibini, et al. "The Stability of Phenylephrine- Part 1; The Rate of Degradation of the Amino Group."Arzneimittelforschung. 1969 Apr;19(4);676-8.	
	3.	El-Shibini, et al. "The Stability of Phenylephrine- Part 2; The discolouration reaction and the influence of some ions on the rate of degradation of the drug." Arzneimittelforschung. 1969 May;19(5);828-31.	
	4.	El-Shibini, et al. "The Stability of Phenylephrine- Part 3; The racemisation reaction." Arzneimittelforschung. 1969 Sep;19(9);1613-4.	
	5.	Millard, et al., "The Stability of Aqueous Solutions of Phenylephrine at Elevated Temperatures: Identification of the Decomposition Products." J. Pharm. Pharmac., 1973, 25. Suppl., 24P-31P	
	6.	"Report of the International Workshop on In Vitro Methods for Assessing Acut Systemic Toxicity." Results of an International Workshop Organized by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM); pages 1-370.	
	7.	Zaczek, et. al., "The effect of phenylephrine on pain and flare intensity in eyes with uveitis."Acta Ophthalmol Scand. 2000 Oct;78(5);516-8	

Examiner Signature	/Barbara Frazier/	Date Considered	01/27/2014

**EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.* [1]Applicant's unique citation designation number (optional). [2]See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3]Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4]For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5]Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6]Applicant is to place a check mark here if English language Translation is attached.

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 (1-800-786-9199) and select option 2.

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /B.F./

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BIB DATA SHEET

CONFIRMATION NO. 6889

SERIAL NUMBER	FILING or 371(c) DATE RULE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
14/080,771	11/14/2013	514	1611	44630-701.201

APPLICANTS

Paragon BioTeck, Inc., Portland, OR, Assignee (with 37 CFR 1.172 Interest);

INVENTORS

Patrick H. Witham, Eugene, OR;
Sailaja Machiraju, Beaverton, OR;
Lauren Mackensie-Clark Bluett, Milwaukie, OR;

** **CONTINUING DATA** **********

** **FOREIGN APPLICATIONS** **********

** **IF REQUIRED, FOREIGN FILING LICENSE GRANTED** ** ** SMALL ENTITY **
12/02/2013

Foreign Priority claimed	35 USC 119(a-d) conditions met	Verified and Acknowledged	Met after Allowance	STATE OR COUNTRY	SHEETS DRAWINGS	TOTAL CLAIMS	INDEPENDENT CLAIMS
☐ Yes ☑ No	☐ Yes ☑ No	/BARBARA S FRAZIER/ Examiner's Signature	☐ Initials	OR	4	14	1

ADDRESS

WILSON, SONSINI, GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050
UNITED STATES

TITLE

METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS

FILING FEE RECEIVED	
1030	FEES: Authority has been given in Paper No._____ to charge/credit DEPOSIT ACCOUNT No._____ for following:

☐ All Fees
☐ 1.16 Fees (Filing)
☐ 1.17 Fees (Processing Ext. of time)
☐ 1.18 Fees (Issue)
☐ Other _____
☐ Credit

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14080771	WITHAM ET AL.
	Examiner	Art Unit
	BARBARA FRAZIER	1611

CPC- SEARCHED		
Symbol	Date	Examiner

CPC COMBINATION SETS - SEARCHED		
Symbol	Date	Examiner

US CLASSIFICATION SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
Inventor name search	1/27/14	BSF
EAST search	1/27/14	BSF
Google scholar search: phenylephrine, cold storage/stored/storing	1/27/14	BSF

INTERFERENCE SEARCH			
US Class/ CPC Symbol	US Subclass / CPC Group	Date	Examiner

/B.F./ Examiner.Art Unit 1611	

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/080,771	11/14/2013	Patrick H. Witham	44630-701.201	6889

21971 7590 03/04/2014

WILSON, SONSINI, GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050

EXAMINER	
FRAZIER, BARBARA S	
ART UNIT	PAPER NUMBER
1611	

NOTIFICATION DATE	DELIVERY MODE
03/04/2014	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

patentdocket@wsgr.com

Applicant-Initiated Interview Summary	Application No.	Applicant(s)	
	14/080,771	WITHAM ET AL.	
	Examiner	Art Unit	
	BARBARA FRAZIER	1611	

All participants (applicant, applicant's representative, PTO personnel):

(1) *BARBARA FRAZIER*. (3)_____.

(2) *Xiaofan Yang*. (4)_____.

Date of Interview: *27 February 2014*.

Type: ☒ Telephonic ☐ Video Conference
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☐ Yes ☒ No.
If Yes, brief description: _____.

Issues Discussed ☐101 ☐112 ☐102 ☐103 ☒Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: *1-14*.

Identification of prior art discussed: *Shibini*.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

See Continuation Sheet.

Applicant recordation instructions: The formal written reply to the last Office action must include the substance of the interview. (See MPEP section 713.04). If a reply to the last Office action has already been filed, applicant is given a non-extendable period of the longer of one month or thirty days from this interview date, or the mailing date of this interview summary form, whichever is later, to file a statement of the substance of the interview

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

☐ Attachment

/BARBARA FRAZIER/ Examiner, Art Unit 1611	

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews
Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiner's responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicant's correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:
1) A brief description of the nature of any exhibit shown or any demonstration conducted,
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
   (The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicant's record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiner's version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, "Interview Record OK" on the paper recording the substance of the interview along with the date and the examiner's initials.

Continuation of Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments: Applicant's representative Mr. Yang ("Applicant") discussed proposed limitations to the instant claims to include adding the term "ophthalmic" to describe the claimed composition. Applicant discussed the limitations of 1) starting with a composition with an initially high chiral purity, and 2) storing the composition at -10 to 10 degrees C to maintain chiral purity. Applicant presented the position that the applied reference Shibini is older art, and does not quantify the level of chiral purity achieved. Applicant also discussed recognizing the importance of the R-isomer of phenylephrine, as well as negative effects of the S-isomer on pupil dilating effect. Applicant also presented the position that the current prior art teaches away from low-temperature storage, citing an insert from a commercially available phenylephrine solution and Shibini's teachings regarding high temperature treatment; Examiner suggesting submitting the insert as prior art. Examiner also suggested Applicants present any data available demonstrating that the product obtained by storing the composition at -10 to 10 degrees C exhibits unexpectedly improved (or unexpectedly maintains) chiral purity compared to compositions stored at room temperature; said data should be presented in the form of a Declaration. If appropriate, Applicants may also present supporting data regarding importance of the R-isomer vs. S-isomer (briefly discussed in specification) in the form of a Declaration. An agreement was not reached with respect to the claims.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of:	Group Art Unit: 1611
Inventors: Patrick H. Witham et al.	Confirmation No.: 6889
Serial No.: 14/080,771	Examiner: Barbara S. Frazier
Filed: November 14, 2013	Customer No. 21971
Title: **Methods and Composition of Stable Phenylephrine Formulations**	Certificate of Electronic Filing I hereby certify that the attached Response and all marked attachments are being deposited by Electronic Filing on March 19, 2014 by using the EFS Web patent filing system and addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450. By: /Linda Anders/ Linda Anders

Mail Stop Amendment
Commissioner For Patents
P.O. Box 1450
Alexandria, VA 22313-1450

RESPONSE TO NON-FINAL OFFICE ACTION DATED FEBRUARY 4, 2014

Dear Madam:

This paper responds to the Office Action dated February 4, 2014, setting an initial due date of May 4, 2014. Accordingly, this response is timely filed. In the event any additional fee is due, the Commissioner is authorized to charge it to Deposit Account No. 23-2415, referencing Docket No. 44630-701.201.

Amendments to the Claims begin on page **2** of this paper

Remarks begin on page **4** of this paper.

Conclusion is on page **10** of this paper.

Amendments to the Claims

The following listing of claims will replace all prior versions, and listings, of claims in this application.

1. (Currently Amended) An ophthalmic composition comprising at least 95% *R*-phenylephrine hydrochloride and an aqueous buffer for substantially maintaining chiral purity of *R*-phenylephrine hydrochloride for at least 6 months in the ophthalmic composition, the improvement comprising storing the ophthalmic composition between -10 to 10 degree Celsius.

2. (Currently Amended) The ophthalmic composition of claim 1, wherein the ophthalmic composition is stored between 2 to 8 degree Celsius.

3. (Currently Amended) The ophthalmic composition of claim 1, wherein the ophthalmic composition comprises at least 99% *R*-phenylephrine hydrochloride.

4. (Currently Amended) The ophthalmic composition of claim 1, wherein the ophthalmic composition comprises at least 99.3% *R*-phenylephrine hydrochloride.

5. (Currently Amended) The ophthalmic composition of claim 1, wherein the chiral purity of *R*-phenylephrine hydrochloride is at least 95% of the initial chiral purity after 6 months.

6. (Currently Amended) The ophthalmic composition of claim 1, wherein the chiral purity of *R*-phenylephrine hydrochloride is at least 97% of the initial chiral purity after 6 months.

7. (Currently Amended) The ophthalmic composition of claim 1, wherein the chiral purity of *R*-phenylephrine hydrochloride is at least 99% of the initial chiral purity after 6 months.

8. (Currently Amended) The ophthalmic composition of claim 1, wherein the chiral purity of *R*-phenylephrine hydrochloride is at least 99.5% of the initial chiral purity after 6 months.

9. (Currently Amended) The ophthalmic composition of claim 1, wherein the composition comprises 2.5% w/v or 10% w/v *R*-phenylephrine hydrochloride by weight.

10. (Currently Amended) A packaged ophthalmic composition comprising the ophthalmic composition of claim 1, in a 1-15 ml plastic or glass bottle.

11. (Currently Amended) The packaged ophthalmic composition of claim 10, wherein the package identifies storing the ophthalmic composition at a temperature between -10 to 10 C.

12. (Currently Amended) The packaged ophthalmic composition of claim 11, wherein the package identifies storing the ophthalmic composition at a temperature between 2 to 8 C.

13. (Currently Amended) The packaged ophthalmic composition of claim 10, wherein the ophthalmic composition is in a plastic or glass bottle of about 2 ml, about 3 ml, about 5 ml, about 10 ml or about 15 ml.

14. (Currently Amended) The packaged ophthalmic composition of claim 10, wherein the plastic or glass bottle is opaque.

REMARKS

This paper is filed in response to the Office Action dated February 4, 2014. In the Office Action, claims 1-14 are rejected as allegedly obvious over Shibini et al., Arzneimettelforschung, 19(9), pp. 1613-14, 1969 ("Shibini") and USPN4,260,600 ("Valle"). In light of an Examiner Interview conducted on February 27, 2014, and for the sole purpose of expediting the prosecution of the present application without acceding to the Office's obviousness conclusion, Applicant has amended the claims to recite an ophthalmic composition, which is supported by at least paragraphs [0040]-[0042] of the present application. No new matter is added. In addition, Applicant submits an Inventor Declaration under 37 C.F.R. 1.132, as suggested by the Office. In view of the claim amendments, Declaration, and remarks submitted herewith, reconsideration and withdrawal of the obviousness rejections asserted in the Office action are respectfully requested.

To support an obviousness rejection, MPEP §2143.03 requires "all words of a claim to be considered" and MPEP § 2141.02 requires consideration of the "[claimed] invention and prior art as a whole." Further, the Board of Patent Appeal and Interferences recently confirmed that a proper, post-KSR obviousness determination still requires the Office make "a searching comparison of the claimed invention – including all its limitations – with the teaching of the prior art." See, *In re Wada and Murphy*, Appeal 2007-3733, citing *In re Ochiai*, 71 F.3d 1565, 1572 (Fed. Cir. 1995) (emphasis in original). In sum, it remains well-settled law that an obviousness rejection requires at least a suggestion of all of the claim elements.

Here, all amended claims recite the features of (1) an ophthalmic composition comprising at least 95% *R*-phenylephrine hydrochloride and an aqueous buffer; and (2) the ophthalmic composition being stored at between -10 to 10 degree Celsius for substantially maintaining chiral purity of *R*-phenylephrine hydrochloride. Neither of those technical feature is taught or suggest in the cited references, whether taken alone or in combination, as discussed in greater detail below.

"Ophthalmic Composition" and "≥95% *R*-Phenylephrine Hydrochloride"

Applicant first notes that Shibini does not disclose any ophthalmic composition containing *R*-phenylephrine hydrochloride of high chiral purity. Instead, Shibini starts with a brief summary the previous two stability studies of L-m-Hydroxy-α-(methylaminomethyl)-benzyl alcohol, and states that "[i]t now remained to study the racemization of L-phenylephrin in order to obtain full information about the stability of the drug." See, Shibini's "Introduction" Section. Following this general statement, Shibini then discloses purported racemization studies of L-phenylephrin solutions at pHs of close to zero (5% and 10% hydrochloric acid) and pHs of 2 and 6.5. See, Shibini's "Experimental Part" and "Results and Discussion" Sections. As such, Shibini is nothing more than a mere academic or theoretical study of racemization of L-phenylephrin in general. In other words, nothing in Shibini teaches or even remotely suggests any therapeutically negative effect of L-phenylephrin's enantiomer, or any therapeutically positive effect of the purported racemization prevention, much less in an ophthalmic composition. Thus, Shibini does not teach or suggest an ophthalmic composition comprising R-phenylephrin of high chiral purity, as recited in the pending claims.

Applicant next notes that Shibini does not disclose any composition comprising ≥95% *R*-phenylephrine hydrochloride. In making the rejection, the Office states that "since Shibini teaches prevention of racemization, the skilled artisan would envisage a chiral purity of 100%, absent evidence to the contrary." See, the sentence bridging pages 3-4 of Office Action. It appears that the Office has taken the position that the ≥95% chiral purity of *R*-phenylephrine hydrochloride is inherently disclosed by Shibini's disclosure of the purported "prevention of racemization."

Applicant disagrees. Under MPEP 2112 IV,

> The fact that a certain result or characteristic may occur or be present in the prior art is not sufficient to establish the inherency of that result or characteristic. *In re Rijckaert*, 9 F.3d 1531, 1534, 28 USPQ2d 1955, 1957 (Fed. Cir. 1993) (reversed rejection because inherency was based on what would result due to optimization of conditions, not what was necessarily present in the prior art); *In re Oelrich*, 666 F.2d 578, 581-82, 212 USPQ 323, 326 (CCPA 1981). "To establish inherency, the extrinsic evidence 'must make clear that the missing descriptive matter is necessarily present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill. Inherency, however, may not be established by probabilities or possibilities. The mere fact that a certain thing may result from a given set of circumstances is not sufficient.' "

Thus, the proper standard for reliance on inherency is not whether one of ordinary skill in the art would "envisage" a claimed feature from the disclosure of prior art, but rather, whether one of ordinary skill in the art would recognize that the claimed feature is "necessarily present" in the things described in the cited reference.

Here, Shibini does not include any disclosure as to the chiral purity of the L-m-Hydroxy-α-(methylaminomethyl)-benzyl alcohol used in its studies. As such, one of ordinary skill in the art would understand that Shibini's purported disclosure of "prevention of racemization" at most indicates that the chiral purity of L-m-Hydroxy-α-(methylaminomethyl)-benzyl alcohol could be maintained, rather than indicating what the chiral purity of L-m-Hydroxy-α-(methylaminomethyl)-benzyl alcohol is. In other words, one of ordinary skill in the art would not recognize that the ≥95% chiral purity of *R*-phenylephrine hydrochloride is necessarily present in Shibini's alleged disclose of "prevention of racemization." Thus, Shibini does not disclose any composition comprising ≥95% *R*-phenylephrine hydrochloride, as recited in the pending claims.

Finally, Applicant notes that the deficiencies of Shibini discussed above cannot be cured by citing to the secondary reference Valle, which is merely cited for allegedly disclosing therapeutic use of (R)-3-hydroxy-α-[(methylamino)methyl]benzenemethanol hydrochloride. See, Office Action on page 3. Specifically, Valle allegedly teaches a "method of treating **depression** by administering several active compounds throughout the day to a patient in need of such treatment" (Abstract of Valle), in which the active compounds, including (R)-3-hydroxy-α-[(methylamino)methyl]benzenemethanol hydrochloride, are orally administered as **tablets** or **capsules** (col. 1, lines 62-66 of Valle). Thus, like Shibani, Valle does not teach or suggest an ophthalmic composition containing ≥95% chiral purity of *R*-phenylephrine hydrochloride.

"Storing at Between -10 to 10 Degree Celsius"

Turning to the feature of the ophthalmic composition being stored at between -10 to 10 degree Celsius for substantially maintaining chiral purity of *R*-phenylephrine hydrochloride, Applicant notes that it is not disclosed or even remotely suggested in Shibani. In making the obviousness rejection, the Office admits that Shibini does not teach "storing the composition between -10 to 10 degree Celsius (claim 1) or 2 to 8 degree Celsius (claim 2)," but appears to have taken the position that the "structure implied by the steps" are disclosed because "Shibini already

teaches a composition comprising phenylephrin and aqueous buffer." See first full paragraph on page 4 of Office Action.

Applicant disagrees. As recited in the pending claims, the ophthalmic composition comprises ≥95% *R*-phenylephrine hydrochloride, and the storing step substantially maintains chiral purity of *R*-phenylephrine hydrochloride. See, definition of "substantially maintains" in paragraph [0024] of the present application. As discussed above, Shibini does not teach or suggest, either expressly or inherently, any ophthalmic composition comprising ≥95% *R*-phenylephrine hydrochloride. Therefore, the "structure implied by the steps," *i.e.* the "substantially maintained" chiral purity of *R*-phenylephrine hydrochloride (based on the initial ≥95% *R*-phenylephrine hydrochloride), is not expressly or inherently taught or suggest by Shibini.

Again, Applicant notes that the deficiencies of Shibini discussed above cannot be cured by citing to the secondary reference Valle. Specifically, Valle is completely irrelevant to the storage of its composition and therefore does not teach or even remotely suggest the feature of an ophthalmic composition being stored at between -10 to 10 degree Celsius for substantially maintaining chiral purity of *R*-phenylephrine hydrochloride.

Technical Feature of the Present Application

While the aforementioned technical features and lack of their disclosure in the cited references should be sufficient to overcome the obviousness rejection, Applicant further submits that the missing technical features are not mere matters of design available to one of ordinary skill in the art without sufficient inventiveness because (1) prior art teaches away from low temperature storage; (2) it is Applicant who identified the need to maintain high optical purity in ophthalmic phenylephrine products; and (3) it is Applicant who discovered the unexpected results of the technical features of the claimed invention, as discussed in greater detail below.

Teaching Away

Applicant first submits that Shibini in fact actively teaches away from the storing step recited in the pending claims. See MPEP 2145, stating that "familiar lines of argument still apply, including teaching away from the claimed invention by the prior art, lack of a reasonable expectation of success, and unexpected results. Indeed, they may have even taken on added importance in view of the recognition in *KSR* of a variety of possible rationales" (emphasis added by Applicant).

Specifically, Shibini's "Results and Discussion" Section (first paragraph) states that "[n]o noticeable changes in the optical rotation of such solutions could be detected after heating at 97°C for several days." Accordingly, in its "Conclusion" Section, Shibini states that "the following conditions for the proper preparation and storage of phenylephrine solutions are deduced: . . . the solution can be safely sterilized by autoclaving and will not undergo appreciable decomposition during storage." Shibini's observation and recommendation are consistent with existing understanding and practice of the storage of phenylnephrine. See, *e.g.* paragraph [[0017] of the present application, stating that "an insert from a commercially available Phenylephrine Hydrochloride Ophthalmic Solution provides that the solution should be stored at 20° to 25 °C (USP controlled room temperature) and keep container tightly closed. Do not use if solution is brown or contains precipitate. (AKORN Package Insert)". To make it clearly, a copy of the insert is provided in the 1.132 Declaration (Exhibit 2 and Paragraph 9), as suggested by the Office during the Examiner Interview.

Identification of New Problem

Applicant next submits that one of ordinary skill in the art, in light of Shibini and knowledge in the art, would not store phenylephrine solutions at the temperatures recited in the pending claims because doing so would amount to "extra work and greater expense for no apparent reason." See MPEP 2143A (Example 3), where the Office discusses *In re Omeprazole Patent Litigation*, 536 F.3d 1361 (Fed. Cir. 2008), and states:

> Office personnel should note that in this case the modification of the prior art that had been presented as an argument for obviousness was an extra process step that added an additional component to a known, successfully marketed formulation. The proposed modification thus amounted to extra work and greater expense for no apparent reason. This is not the same as combining known prior art elements A and B when each would have been expected to contribute its own known properties to the final product. In the *Omeprazole* case, in view of the expectations of those of ordinary skill in the art, adding the subcoating would not have been expected to confer any particular desirable property on the final product. Rather, the final product obtained according to the proposed modifications would merely have been expected to have the same functional properties as the prior art product.

Furthermore, under MPEP 2143A (Example 3),

> The *Omeprazole* case can also be analyzed in view of the discovery of a previously unknown problem by the patentee. If the adverse interaction between active agent and

> coating had been known, it might well have been obvious to use a subcoating. However, since the problem had not been previously known, there would have been no reason to incur additional time and expense to add another layer, even though the addition would have been technologically possible.

Here, it is Applicant who discovered that "[s]urprisingly it was found that *S*-Phenylephrine dilated the eye only slightly more than that was untreated. Thus it is important that an eye drop containing Phenylephrine Hydrochloride used for dilation of the pupil contains predominantly the *R*-isomer in order to maintain maximum efficacy of the ophthalmic solution" (paragraph [0044]), an insight heretofore unknown. The negative effect of the S-enantiomer is further discussed in paragraphs [0049]-[0050], stating that "when an ophthalmic solution of phenylephrine hydrochloride, (R-isomer) containing S-isomer as an impurity is used for dilation of pupil, the s-isomer may cause the saturation of the a-adrenergic receptors resulting in the decrease in the response of the drug after its administration (tachyphylasis). Furthermore, the presence of *S*-isomer in the ophthalmic solution may lead to poor/ delayed dilation of the pupil." To make it clear, the summary page of a final report regarding Applicant's discovery is provided in the 1.132 Declaration (Exhibit 1 and Paragraph 8), as suggested by the Office during the Examiner Interview.

As discussed above, neither Shibini nor Valle contemplates the negative effect of the optical isomer in an ophthalmic composition. Therefore, one of ordinary skill in the art in view of Shibini and Valle would not recognize the technical significance of (1) an ophthalmic composition comprising at least 95% *R*-phenylephrine hydrochloride and an aqueous buffer; and (2) the ophthalmic composition being stored at between -10 to 10 degree Celsius for substantially maintaining chiral purity of *R*-phenylephrine hydrochloride, as provided in the pending claims. Accordingly, there would have been no reason to incur additional time and expense to provide an ophthalmic composition comprising ≥95% *R*-phenylephrine hydrochloride and storing the composition at between -10 to 10 degree Celsius, even if technologically possible.

Unexpected Result

Finally, it is Applicant who discovered the unexpected results of the technical features of the claimed invention, a significant contribution heretofore unknown. As shown in the 1.132 Declaration (Exhibit 3 and Paragraph 10), while a commercial phenylephrine formulation (stored at "room temperature") and a formulation of the present application (stored at "low temperature") both

exhibit no or little chemical degradation, the chiral chromatograms of the two formulations indicate significant difference in term of chiral stability.

For reasons stated above, the obviousness rejections raised in the Office Action should be withdrawn.

CONCLUSION

Applicants submit that this response fully addresses the Office Action mailed February 4, 2014 and respectfully request consideration and allowance of the claims.

Should the Examiner have any questions, the Examiner is encouraged to contact the undersigned attorney at (858) 350-2306. If additional fees are believed to be required, the Commissioner is authorized to charge any additional fees to Deposit Account No. 23-2415 (Attorney Docket No. 44630-701.201).

Respectfully submitted,

WILSON SONSINI GOODRICH & ROSATI
Professional Corporation

Date: March 19, 2014

/Michael Hostetler/
Michael J. Hostetler, Reg. No. 47,664
Attorney for Applicants

650 Page Mill Road
Palo Alto, CA 94304
Direct Dial: (858) 350-2306
Customer No. 021971

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of:	Group Art Unit: 1611
Inventors: Patrick H. Witham et al.	Confirmation No.: 6889
Serial No.: 14/080,771	Examiner: Barbara S. Frazier
Filed: November 14, 2013	Customer No. 21971
Title: **Methods and Composition of Stable Phenylephrine Formulations**	Certificate of Electronic Filing I hereby certify that the attached Response and all marked attachments are being deposited by Electronic Filing on February , 2014 by using the EFS – Web patent filing system and addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450. By: Linda Anders Linda Anders

Mail Stop Amendment
Commissioner For Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION PURSUANT TO 37 CFR §1.132

Madam:

I, Patrick H. Witham, do hereby declare as follows:

1. I am the inventor of U.S. Application No. 14/080,771. I am currently the President and CEO of Paragon BioTeck, Inc. (Assignee of U.S. Application No. 14/080,771). I own stock in Paragon BioTeck, Inc.

2. Paragon BioTeck, Inc. has focused on development of ophthalmic products.

3. I am familiar with the pending claims in U.S. Application No. 14/080,771. I am aware of the rejection of the pending claims under 35 U.S.C. §103 (a) as being allegedly unpatentable on grounds of obviousness. I have reviewed the references cited by the Examiner in the Office Action dated February 4, 2014.

4. I am submitting this declaration and the attached Exhibits to comment on the surprising results we obtained at Paragon BioTeck, Inc. (hereinafter "Paragon").

5. It is my understanding that the currently pending claims of U.S. Application No. 14/080,771 all recite the features of (1) an ophthalmic composition comprising at least 95% *R*-phenylephrine hydrochloride and an aqueous buffer; and (2) the ophthalmic composition being stored at between -10 to 10 degree Celsius for substantially maintaining chiral purity of *R*-phenylephrine hydrochloride (hereinafter "low temperature storage").

6. It is my understanding that Shibini et al., Arzneimettelforschung, 19(9), pp. 1613-14, 1969 ("Shibini") is one of the references cited in the Office action dated February 4, 2014. It is my understanding that Shibini does not disclose (1) an ophthalmic composition comprising at least 95% *R*-phenylephrine hydrochloride and an aqueous buffer; and (2) the ophthalmic composition being stored at between -10 to 10 degree Celsius for substantially maintaining chiral purity of *R*-phenylephrine hydrochloride.

7. I am submitting this declaration to show (1) Paragon surprisingly discovered that *S*-Phenylephrine dilated the eye only slightly more than that was untreated, and it is important that an eye drop containing Phenylephrine hydrochloride used for dilation of the pupil contains predominantly the *R*-isomer in order to maintain maximum efficacy of the ophthalmic solution; (2) current R-Phenyephrine hydrochloride eye products explicitly specify storage at 20 to 25 degree Celsius (hereinafter "room temperature storage"); and (3) chiral chromatogram of *R*-Phenylephrine hydrochloride product of high chiral purity and subject to low temperature storage shows surprisingly better *R*-Phenylephrine preservation, when compared to chiral chromatogram of a commercially available *R*-Phenylephrine hydrochloride product and subject to room temperature storage.

8. **Exhibit 1** below is a summary of an animal study, in which Paragon surprisingly discovered that that *S*-Phenylephrine dilated the eye only slightly more than that was untreated, and it is important that an eye drop containing Phenylephrine hydrochloride used for dilation of the pupil contains predominantly the *R*-isomer in order to maintain maximum efficacy of the ophthalmic solution.

9. **Exhibit 2** below is an insert of a commercially available R-Phenylephrine hydrochloride ophthalmic product (by Akorn Pharmaceuticals), which shows a revision date of July, 2011 and explicitly specifies storage at 20 to 25 degree Celsius.

10. **Exhibit 3** below is a comparison between chiral chromatogram of *R*-Phenylephrine

hydrochloride product of high chiral purity ((*e.g.* ≥99%) and subject to low temperature storage, and chiral chromatogram of a commercially available *R*-Phenylephrine product and subject to room temperature storage. There are several points I would like to make to place the data shown in Exhibit 3 in context.

(i). The figure at the top is a chiral HPLC chromatogram of a commercially available *R*-Phenylephrine hydrochloride product and stored at 20 to 25 °C after 6 months.

(ii). The figure at the bottom is a reproduction of Fig. 3 in U.S. 14/080,771, which shows a chiral HPLC chromatogram of the exemplary *R*-Phenylephrine hydrochloride formulation with high chiral purity (*e.g.* ≥99%) and stored at 2 to 8 °C after 6 months.

(iii). Non-chiral reverse phase column chromatograms (currently published USP HPLC method) show no or little chemical degradation of *R*-Phenylephrine hydrochloride in both formulations **(i)** and **(ii)**.

11. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that making of willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful statements may jeopardize the validity of the applications or any patent issuing thereon.

Respectfully submitted.

Dated: March 12, 2014

Patrick H. Witham

Exhibit 1

SUMMARY

Paragon Bio Teck Inc. test substances identified as Ophthalmic Solutions Containing Phenylephrine Hydrochloride USP 10% R enantiomer (NDA 203510) and S enantiomer (S=LP19) were both tested in order to examine and evaluate their individual abilities to elicit ocular responses. Both test articles were administered, in separate studies conducted 7 days apart, to a test group of adult NZA rabbits (2 males/3 females). The same animals were used for both trials, with a seven day interval between the studies, in order to lessen any possibility of test animal variation. Measurements of Mydriasis (dilation) of the pupil and response to light stimulus were made at 15-30 minute intervals after treatment of the right eye comparing responses to ophthalmic solution containing the R enantiomer with responses to ophthalmic solution containing the S enantiomer.

Both enantiomers S and R exhibited a measurable physiological response when administered at a dose of 3 drops/ 150µL into the right eyes of male and female NZA rabbits. Left eyes remained untreated.

A summary of the measured parameters, in a comparison format, is as follows:

Increase in pupil diameter (mm):
R enantiomer elicited a mean dilation of 3.2 mm
S enantiomer (LP19) elicited a mean dilation of 2.6 mm

Time to maximal dilation in minutes:
R enantiomer elicited a mean time for maximal dilation of 36 minutes
S enantiomer (LP19) elicited a mean time for maximal dilation of 45 minutes

Duration of maximal dilation in minutes:
R enantiomer elicited a mean time for duration of maximal dilation of 156 minutes
S enantiomer (LP19) elicited a mean time for duration of maximal dilation of 93 minutes

Time required for first regression of dilation (post dosing):
R enantiomer elicited a mean time required for first regression of dilation of 195 minutes
S enantiomer (LP19) elicited a mean time required for first regression of dilation of 117 minutes

Complete reversal of dilation (post dosing):
R enantiomer required a mean time of 294 minutes from onset to complete reversal of dilation
S enantiomer (LP19) required a mean time of 210 minutes from onset to complete reversal of dilation

R enantiomer did not elicit the level of constriction in test subjects when exposed to light at 15-30 minute intervals when compared with S enantiomer. Constriction during light exposure in test subjects treated with the R enantiomer was none to mild while subjects treated with S enantiomer exhibited moderate to normal constriction.

Phenylephrine Hydrochloride Ophthalmic Solution, USP 2.5% — Sterile

Storage: Store at 20° to 25°C (68° to 77°F) [see USP Controlled Room Temperature]. Keep container tightly closed. Protect from light and excessive heat.
Do not use if solution is brown or contains precipitate

REFERENCES:

1. Fraunfelder, F.T., and Meyer, S.M.: Possible Cardiovascular Effects Secondary to Topical Ophthalmic 2.5% Phenylephrine. Am. J. Oph. 99:3:362, 1985.
2. Ibid.

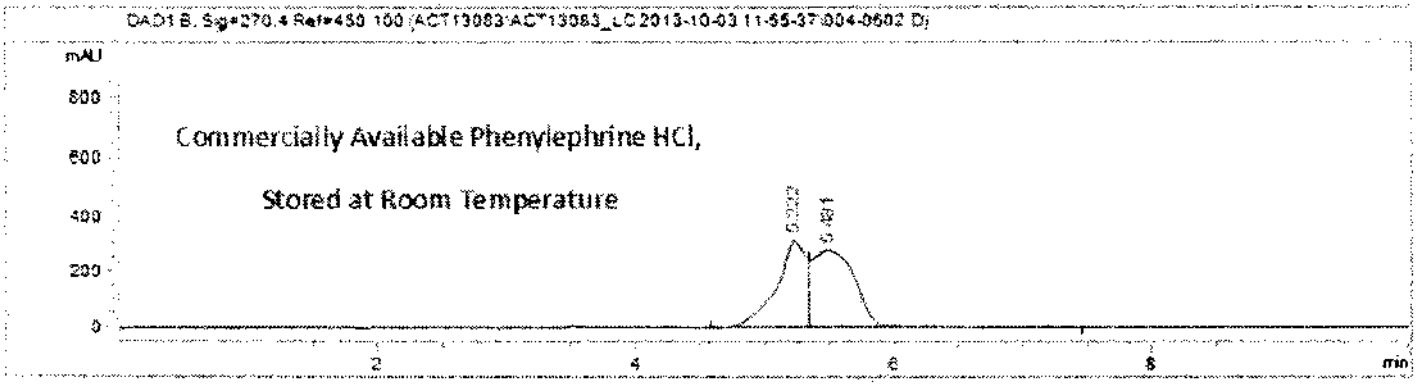
Akorn

Manufactured by: Akorn, Inc.
Lake Forest, IL 60045

DLA0N

Rev. 07/11

Exhibit 3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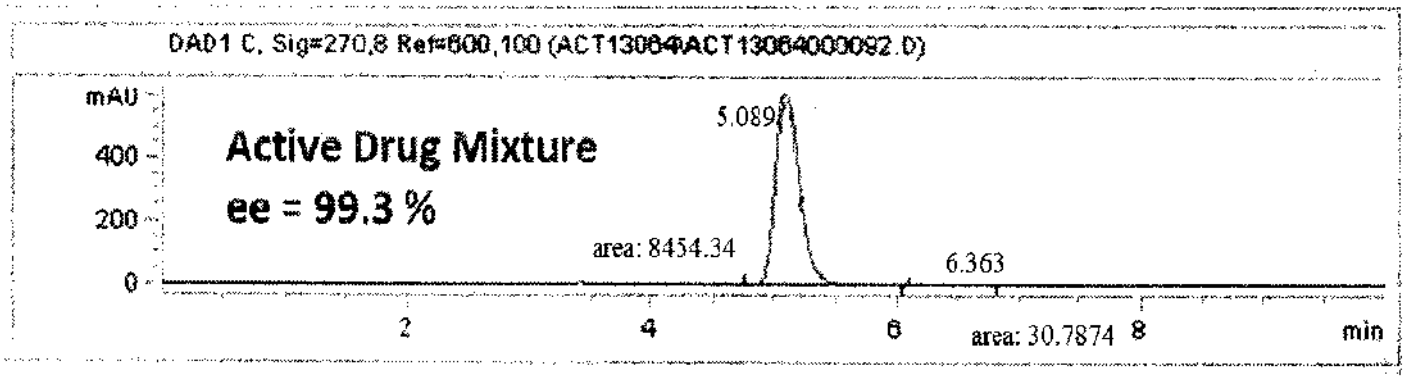
DAD1 C, Sig=270,8 Ref=600,100 (ACT13064\ACT13064000092.D)
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Active Drug Mixture
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area: 8454.34
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Electronic Acknowledgement Receipt

EFS ID:	18528717
Application Number:	14080771
International Application Number:	
Confirmation Number:	6889
Title of Invention:	METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS
First Named Inventor/Applicant Name:	Patrick H. Witham
Customer Number:	21971
Filer:	Michael J. Hostetler/Linda Anders
Filer Authorized By:	Michael J. Hostetler
Attorney Docket Number:	44630-701.201
Receipt Date:	19-MAR-2014
Filing Date:	14-NOV-2013
Time Stamp:	18:28:28
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		44630-701-201-ResponseOA.pdf	241194 bf211649c9d4c11f7c2bcb6fdf4428907ba57d9e	yes	11

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Amendment/Req. Reconsideration-After Non-Final Reject	1	1
	Claims	2	3
	Applicant Arguments/Remarks Made in an Amendment	4	11

Warnings:					
Information:					
2	Affidavit-traversing rejectns or objectns rule 132	44630-701-201-Declaration.pdf	533900 7034319cffd46ab390cbf5d11c24f124fd1ed0 /dc	no	6
Warnings:					
Information:					
		Total Files Size (in bytes):	775094		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/06 (09-11)
Approved for use through 1/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 14/080,771	Filing Date 11/14/2013	☐ To be Mailed

ENTITY: ☐ LARGE ☒ SMALL ☐ MICRO

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	x $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	x $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT 03/19/2014

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	* 14	Minus	** 20	= 0	x $40 =	0
Independent (37 CFR 1.16(h))	* 1	Minus	*** 3	= 0	x $210 =	0
☐ Application Size Fee (37 CFR 1.16(s))						
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
					TOTAL ADD'L FEE	0

AMENDMENT

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	x $ =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	x $ =	
☐ Application Size Fee (37 CFR 1.16(s))						
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
					TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

LIE
/CORALIA BETANCOURT/

UNITED STATES PATENT AND TRADEMARK OFFICE

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Alexandria, Virginia 22313-1450
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/080,771	11/14/2013	Patrick H. Witham	44630-701.201	6889

21971 7590 06/12/2014

WILSON, SONSINI, GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050

EXAMINER	
FRAZIER, BARBARA S	
ART UNIT	PAPER NUMBER
1611	

NOTIFICATION DATE	DELIVERY MODE
06/12/2014	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

patentdocket@wsgr.com

Office Action Summary	Application No. 14/080,771	Applicant(s) WITHAM ET AL.	
	Examiner BARBARA FRAZIER	Art Unit 1611	AIA (First Inventor to File) Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *19 March 2014*.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5)☒ Claim(s) *1-14* is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *1-14* is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☐ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .

4) ☐ Other: _____.

DETAILED ACTION

1. The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

2. Claims 1-14 are pending in this application.

Claim Rejections - 35 USC § 103

3. The following is a quotation of 35 U.S.C. 103 which forms the basis for all obviousness rejections set forth in this Office action:

> A patent for a claimed invention may not be obtained, notwithstanding that the claimed invention is not identically disclosed as set forth in section 102 of this title, if the differences between the claimed invention and the prior art are such that the claimed invention as a whole would have been obvious before the effective filing date of the claimed invention to a person having ordinary skill in the art to which the claimed invention pertains. Patentability shall not be negated by the manner in which the invention was made.

4. Claims 1-14 are rejected under 35 U.S.C. 103 as being unpatentable over Shibini et al. ("Shibini", Arzneimettelforschung, 19(9), pp. 1613-1614, 1969, cited by Applicants in IDS filed 14 November 2013) as evidenced by Valle (US Patent 4,260,600).

Shibini teaches an aqueous buffered composition of L-phenylephrine (e.g., see page 1614). While Shibini does not specifically teach the R-isomer, Valle teaches that the therapeutic form of phenylephrine hydrochloride is (R)-3-hydroxy-α-[(methylamino)methyl]benzenemethanol hydrochloride (col. 1, lines

27-30), and thus the skilled artisan would envisage R-phenylephrine from the teachings of Shibini, and would equate L-phenylephrine with R-phenylephrine (i.e., a recitation of "phenylephrine" would be understood to mean R-phenylephrine), absent evidence to the contrary. Shibini teaches that racemization and oxidation of phenylephrine is prevented through the use of an acidic medium (pH above 2 and below 7) and addition of EDTA (page 1614, column 2). The phenylephrine is present in an aqueous buffer (page 1614, column 1). Regarding the chiral purity at least 95% (claim1), 99% (claim3), or 99.3% (claim 4), since Shibini teaches prevention of racemization, the skilled artisan would envisage a chiral purity of 100%, absent evidence to the contrary.

While Shibini does not expressly teach storing the composition between -10 to 10 degrees Celsius (claim 1) or 2 to 8 degrees Celsius (claim 2), it is noted that said limitation is a process limitation, which may be interpreted either as 1) a product-by-process limitation (in order to arrive at the claimed composition), or 2) an intended use of the composition (i.e., the intended use of the composition is to store it between -10 to 10 degrees Celsius). Regarding 1), it is noted that product-by-process claims are not limited to the manipulations of the recited steps, only the structure implied by the steps. See MPEP 2113. Since Shibini already teaches a composition comprising phenylephrine and aqueous buffer, the limitations of the claims are met. Regarding 2), it is noted that the composition of Shibini is capable of the intended use of storing the composition

between -10 to 10 degrees Celsius, absent evidence to the contrary, and thus the limitations of the claims are met.

Regarding the limitation that the composition is "ophthalmic", said limitation describes an intended use of the composition, and does not distinguish the composition apart from what is already claimed. Since Shibini teaches a composition comprising the same components as the claimed invention (i.e., R-phenylephrine in an aqueous buffer), said composition would be capable of the intended use, absent evidence to the contrary.

Regarding claims 5-8, Shibini teaches prevention of racemization of phenylephrine, and that the solution can be safely sterilized by autoclaving and will not undergo appreciable decomposition during storage (page 1614). Therefore, the skilled artisan would envisage a chiral purity of at least 95%, 97%, 99%, or 99.3% of the initial chiral purity after 6 months, absent evidence to the contrary.

Regarding claim 9, Shibini teaches using 6.25 g in 200 ml, or approximately 3.12% w/v (page 1614, column 1). This amount is comparable to the amounts taught in claim 9, and/or the skilled artisan would be motivated to manipulate the amounts to within comparable ranges by routine experimentation, in order to optimize the therapeutic efficacy of the resultant composition.

Regarding the size and color of the bottle used (claims 10, 13, and 14), said limitations would be within the purview of the skilled artisan and would be a matter of design choice, absent evidence to the contrary.

Regarding the packaging identifying storage directions (claims 11 and 12), it is noted that said limitation amounts to printed material, and does not affect the structural limitations of the composition itself, and therefore does not impart patentability to the claims, absent evidence to the contrary.

Response to Arguments and Declaration

5. Applicant's arguments filed 19 March 2014 have been fully considered but they are not persuasive.

Applicants first argue that Shibini is nothing more than a mere academic or theoretical study of racemization of L-phenylephrine in general, and the does not teach or suggest an ophthalmic composition comprising R-phenylephrine of high chiral purity as recited in the pending claims. This argument is not persuasive because Shibini teaches a composition comprising the same components as that of the claimed invention, i.e., chirally pure phenylephrine in an aqueous buffer (noting that one skilled in the art would recognize L-phenylephrine to be equivalent to R-phenylephrine, i.e., a recitation of "phenylephrine" would be understood to mean R-phenylephrine, as evidenced by Valle), and therefore reads on the claimed invention.

Applicants then argue that Shibini does not include any disclosure as to the chiral purity of L-phenylephrine used in its studies, and that one of ordinary skill in the art would not recognize that the ≥95% chiral purity of R-phenylephrine is necessarily present in Shibini's disclosure of "prevention of racemization". This argument is not persuasive because “prevention of racemization” would indicate to one skilled in the art

that no racemization takes place, and therefore the compound is chirally pure, absent evidence to the contrary. Applicants have not presented objective evidence demonstrating that the initial compound of Shibini is less than 95% chirally pure, and therefore the rejection is maintained.

Applicants further argue that the deficiencies of Shibini cannot be cured by Valle, which does not teach or suggest an ophthalmic composition containing ≥95% chiral purity R-phenylephrine hydrochloride. This argument is not persuasive because the teachings of Valle are relied upon merely as evidence to demonstrate that one skilled in the art would equate L-phenylephrine with R-phenylephrine (i.e., a recitation of "phenylephrine" would be understood to mean R-phenylephrine), absent evidence to the contrary. That Valle teaches other methods or dosage forms of R-phenylephrine is not persuasive because the claims are drawn to a composition, and Shibini, the reference upon which the rejection is based, already teaches a composition comprising the same components as the composition of the claimed invention (i.e., phenylephrine in an aqueous buffer).

Applicants then argue that the storage step substantially maintains chiral purity of R-phenylephrine hydrochloride, and the "structure implied by the steps", i.e., the "substantially maintained" chiral purity of R-phenylephrine hydrochloride is not expressly or inherently taught or suggested by Shibini. This argument is not persuasive because the composition having the "substantially maintained" chiral purity of R-phenylephrine hydrochloride amounts to the same composition as that taught by Shibini, i.e., the compositions still comprise the same components. Since the composition of the claimed

invention after cold storage comprises the same components of the composition of Shibini before storage, the composition of Shibini still reads on the composition of the claimed invention. The Examiner points out that the claims as currently written are drawn to a composition, not a method of preparing or storing. Applicants have not presented objective evidence demonstrating how the composition of the claimed invention itself or components within is distinguished from the composition of Shibini, and therefore the rejection is maintained.

Applicants continue, arguing that the prior art teaches away from low temperature storage; it is Applicant who identified the need to maintain high optical purity in ophthalmic phenylephrine products; and it is Applicant who discovered the unexpected results of the technical features of the claimed invention (pages 7 to 10 of Remarks filed 19 March 2014 and Declaration filed 19 March 2014).

Applicant's arguments and Declaration have been fully considered, but are not persuasive for overcoming the rejection. While Applicants demonstrate a higher chiral purity of R-phenylephrine after the step of cold storage vs. room temperature (or higher) storage, it is noted that the composition of the claimed invention after cold storage is the same as the composition of Shibini before storage. Therefore, while it appears Applicant's inventive feature is the method of cold storing chirally pure R-phenylephrine, this method does not distinguish the composition from known compositions of the prior art, and thus the rejection is maintained. Additionally, while Applicants argue the preferred use of the R-enantiomer is the identification of a new problem, it is noted that Valle already generally teaches that the therapeutic form of phenylephrine hydrochloride

is (R)-3-hydroxy-α-[(methylamino)methyl]benzenemethanol hydrochloride (col. 1, lines 27-30), and thus this identification does not appear to be inventive.

Therefore, it is the Examiner's position that the claims are rendered obvious.

Conclusion

No claims are allowed at this time.

THIS ACTION IS MADE FINAL. Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to BARBARA FRAZIER whose telephone number is (571)270-3496. The examiner can normally be reached on Monday-Friday 9am-2:30pm EST.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Daniel Sullivan can be reached on (571)272-0779. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/B. F./
Examiner, Art Unit 1611

/DANIEL SULLIVAN/

Supervisory Patent Examiner, Art Unit 1611

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14080771	WITHAM ET AL.
	Examiner	Art Unit
	BARBARA FRAZIER	1611

CPC- SEARCHED		
Symbol	Date	Examiner
A61K 31/137 (w/search terms - see history)	6/4/14	BSF

CPC COMBINATION SETS - SEARCHED		
Symbol	Date	Examiner

US CLASSIFICATION SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
Inventor name search	1/27/14	BSF
EAST search	1/27/14	BSF
Google scholar search: phenylephrine, cold storage/stored/storing	1/27/14	BSF
EAST search updated	6/4/14	BSF
Inventor search updated	6/4/14	BSF

INTERFERENCE SEARCH			
US Class/ CPC Symbol	US Subclass / CPC Group	Date	Examiner

/B.F./ Examiner.Art Unit 1611	

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
S1	12560	phenylephrine	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 09:58
S2	69	R near2 phenylephrine	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 09:58
S3	1066	synephrine or neosynephrine	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 09:59
S4	259	(m near3 synephrine) or neosynephrine or (meta near3 synephrine)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 10:08
S5	9048560	stored or storage or storing	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 10:09
S6	6056	S1 and S5	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 10:09
S7	148	S6 and (S5 near20 loss)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 10:09
S9	183	S6 and (S5 near20 (loss or losing or lose))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 10:10
S10	3	S4 and (S5 near20 (loss or losing or lose))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 10:12
S11	103392	S5 near10 degree	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 10:15
S12	341	S1 and S11	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 10:15
S13	1680832	pure or purity	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 10:16

S14	280	S12 and S13	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 10:16
S15	7063	S11 near20 S13	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 10:16
S16	233	S14 and S15	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 10:16
S17	2377	S1.ti,ab,clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 10:16
S18	0	S16 and S17	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 10:16
S19	12703	S1 or S4	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 10:17
S20	0	S19 near5 S5 near5 degree	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 10:18
S21	1	S1 near30 S11	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 10:18
S22	64395	S5 near10 (refrigerator or freezer)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 10:20
S23	280	S1 and S22	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 10:20
S24	0	S1 near10 S22	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 10:20
S25	0	S1 near20 S22	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 10:21
S26	6409295	S5.ti,ab,clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 10:21
S27	0	S23 and S24	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 10:21
S28	17	S23 and S26	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT;	ADJ	ON	2014/01/27 10:21

			IBM_TDB			
S29	58918	cold near5 S5	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 11:12
S30	115	S1 and S29	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 11:12
S31	1	S1 near20 S29	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 11:13
S32	197	stored near5 ambient near5 cold	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 11:20
S33	0	S1 and S32	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 11:20
S34	2	"20050153946".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 11:34
S35	717113	purity	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 11:38
S36	2724	S1 and S35	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 11:38
S37	13	maintain$3 near5 chiral near5 purity	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 11:38
S38	0	S1 and S37	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 11:39
S39	4918	chiral near5 purity	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 11:39
S40	118	S1 and S39	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 11:39
S41	127302	S5 near10 (cold or refrigerator or freezer)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 11:41
S42	13	S40 and S41	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 11:41
S43	2	"20110237616".pn.	US-PGPUB; USPAT;	ADJ	ON	2014/01/27

			USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB			13:35
S44	130	S1 near5 (liquid or aqueous)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 13:40
S45	0	S44 and (by weight)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 13:41
S46	17	S44 and "w/v"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/01/27 13:42
S47	0	14/080771.app.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/05/28 11:42
S48	69	R near2 phenylephrine	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/06/04 11:13
S49	2673472	pure or purified or purity or purification	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/06/04 11:13
S50	43	S48 and S49	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/06/04 11:13
S51	5	S48 near20 S49	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/06/04 11:13
S52	2	"20120149073".pn. and phenylephrine	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/06/04 11:20
S53	2	"20120149073".pn. and (water or aqueous or buffer or buffered)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/06/04 11:20
S54	65	S48 and (water or aqueous or buffer or buffered)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/06/04 11:22
S55	46	S48 and (water or aqueous) and (buffer or buffered)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/06/04 11:22
S56	25	S48 and ((water or aqueous) near10 (buffer or buffered))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/06/04 11:22
S57	16765	A61K31/137.cpc.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/06/04 11:52

S58	1143683	ophthalmic or eye or pupil	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/06/04 11:52
S59	1182	S57 and S58	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/06/04 11:52
S60	7	S48 and S59	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/06/04 11:52
S61	46	S48 and "19"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/06/04 11:54
S62	10	S48 and S57	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/06/04 11:54
S63	19973	(pure or purified or purity or purification) near7 (chiral or chirally or chirality)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/06/04 11:55
S64	143	S57 and S63	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/06/04 11:55
S65	192131	(cold or freeze or freezing or frozen) near20 (stored or storage or storing)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/06/04 11:56
S66	4	S64 and S65	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/06/04 11:57

6/ 4/ 2014 12:01:20 PM
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/080,771	11/14/2013	Patrick H. Witham	44630-701.201	6889

21971 7590 07/03/2014

WILSON, SONSINI, GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050

EXAMINER	
FRAZIER, BARBARA S	
ART UNIT	PAPER NUMBER
1611	

NOTIFICATION DATE	DELIVERY MODE
07/03/2014	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

patentdocket@wsgr.com

Applicant-Initiated Interview Summary	Application No.	Applicant(s)	
	14/080,771	WITHAM ET AL.	
	Examiner	Art Unit	
	BARBARA FRAZIER	1611	

All participants (applicant, applicant's representative, PTO personnel):

(1) *BARBARA FRAZIER*. (3)_____.

(2) *Xiaofan Yang*. (4)_____.

Date of Interview: *25 June 2014*.

Type: ☒ Telephonic ☐ Video Conference
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☐ Yes ☒ No.
If Yes, brief description: _____.

Issues Discussed ☐101 ☐112 ☐102 ☒103 ☐Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: *1-14*.

Identification of prior art discussed: *Shibini*.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

The teachings of Shibini were discussed. Applicant discussed possible ways to demonstrate their position that the composition of Shibini does not possess the required optical purity as recited in the claims. Examiner noted any evidence presented should include why a person having ordinary skill in the art would not expect the composition of the prior art to have an optical purity of at least 95% R-phenylephrine hydrochloride as required by the claims as currently written. Examiner also noted that, because the claims are directed to a composition, evidence demonstrating optical purity after 6 months of storage would not be persuasive for distinguishing over prior art compositions having the same purity prior to storage. An agreement was not reached with respect to the claims.

Applicant recordation instructions: The formal written reply to the last Office action must include the substance of the interview. (See MPEP section 713.04). If a reply to the last Office action has already been filed, applicant is given a non-extendable period of the longer of one month or thirty days from this interview date, or the mailing date of this interview summary form, whichever is later, to file a statement of the substance of the interview

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

☐ Attachment

/BARBARA FRAZIER/ Examiner, Art Unit 1611	

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews
Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiner's responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicant's correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:
1) A brief description of the nature of any exhibit shown or any demonstration conducted,
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
   (The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicant's record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiner's version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, "Interview Record OK" on the paper recording the substance of the interview along with the date and the examiner's initials.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of:	Group Art Unit: 1611
Inventors: Patrick H. Witham et al.	Confirmation No.: 6889
Serial No.: 14/080,771	Examiner: Barbara S. Frazier
Filed: November 14, 2013	Customer No. 21971
Title: **Methods and Composition of Stable Phenylephrine Formulations**	Certificate of Electronic Filing I hereby certify that the attached Response and all marked attachments are being deposited by Electronic Filing on July 8, 2014 by using the EFS Web patent filing system and addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450. By: /Linda Anders/

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Commissioner For Patents
P.O. Box 1450
Alexandria, VA 22313-1450

RESPONSE TO FINAL OFFICE ACTION WITH REQUEST FOR CONSIDERATION UNDER THE AFTER FINAL CONSIDERATION PILOT PROGRAM 2.0

Dear Madam:

This paper responds to the final Office Action dated June 12, 2014, and is filed with an After Final Consideration Pilot 2.0 request. In the event any additional fee is due, the Commissioner is authorized to charge it to Deposit Account No. 23-2415, referencing Docket No. 44630-701.201.

Amendments to the Claims begin on page **2** of this paper
Remarks begin on page **4** of this paper.
Conclusion is on page **9** of this paper.

Amendments to the Claims

The following listing of claims will replace all prior versions, and listings, of claims in this application.

1. (Currently Amended) A[[n]] method of using an ophthalmic composition for pupil dilation, the composition comprising at least 95% *R*-phenylephrine hydrochloride and an aqueous buffer for substantially maintaining chiral purity of *R*-phenylephrine hydrochloride for at least 6 months, the ~~improvement~~ method comprising:

   ~~storing~~ allowing the composition to be stored between -10 to 10 degree Celsius; and

   administering the composition into an eye of an individual in need thereof, wherein the composition comprises at least 95% *R*-phenylephrine hydrochloride when administered after storage.

2. (Currently Amended) The ~~composition~~ method of claim 1, wherein the composition is allowed to be stored between 2 to 8 degree Celsius.

3. (Currently Amended) The ~~composition~~ method of claim 1, wherein the composition comprises at least 99% *R*-phenylephrine hydrochloride.

4. (Currently Amended) The ~~composition~~ method of claim 1, wherein the composition comprises at least 99.3% *R*-phenylephrine hydrochloride.

5. (Currently Amended) The ~~composition~~ method of claim 1, wherein the chiral purity of *R*-phenylephrine hydrochloride is at least 95% of the initial chiral purity after 6 months.

6. (Currently Amended) The ~~composition~~ method of claim 1, wherein the chiral purity of *R*-phenylephrine hydrochloride is at least 97% of the initial chiral purity after 6 months.

7. (Currently Amended) The ~~composition~~ method of claim 1, wherein the chiral purity of *R*-phenylephrine hydrochloride is at least 99% of the initial chiral purity after 6 months.

8. (Currently Amended) The ~~composition~~ method of claim 1, wherein the chiral purity of *R*-phenylephrine hydrochloride is at least 99.5% of the initial chiral purity after 6 months.

9. (Currently Amended) The ~~composition~~ method of claim 1, wherein the composition comprises 2.5% w/v or 10% w/v *R*-phenylephrine hydrochloride by weight.

10. (Currently Amended) ~~A packaged composition comprising the composition~~ The method of claim 1, wherein the composition is packaged in a 1-15 ml plastic or glass bottle.

11. (Currently Amended) The ~~packaged composition~~ method of claim 10, wherein the package identifies storing the composition at a temperature between -10 to 10 C.

12. (Currently Amended) The ~~packaged composition~~ method of claim 11, wherein the package identifies storing the composition at a temperature between 2 to 8 C.

13. (Currently Amended) The ~~packaged composition~~ method of claim 10, wherein the composition is in a plastic or glass bottle of about 2 ml, about 3 ml, about 5 ml, about 10 ml or about 15 ml.

14. (Currently Amended) The ~~packaged composition~~ method of claim 10, wherein the plastic or glass bottle is opaque.

REMARKS

This paper is filed in response to the final Office Action dated June 12, 2014; and with a Certification and Request for Consideration Under the After Final Consideration Pilot Program 2.0 ("AFCP"). Accordingly, Applicant is willing and available to participate in any interview initiated by the Examiner concerning the AFCP submission and response filed herewith.

In the final Office Action, claims 1-14 remain rejected as allegedly obvious over Shibini et al., Arzneimettelforschung, 19(9), pp. 1613-14, 1969 ("Shibini") and USPN4,260,600 ("Valle"). In light of a telephonic discussion with the Examiner on July 3, 2014, and for the sole purpose of expediting the prosecution of the present application, Applicant has amended claim 1 to recite a method of using an ophthalmic composition for pupil dilation, wherein the method comprises allowing the composition to be stored between -10 to 10 degree Celsius and administering the composition into an eye of an individual in need thereof, wherein the composition comprises at least 95% *R*-phenylephrine hydrochloride when administered after storage. Those technical features are properly supported by the specification (*e.g.* paragraphs [0006], [0007], and [0020]). In view of the claim amendments and remarks submitted herewith, reconsideration and withdrawal of the obviousness rejections asserted in the Office action are respectfully requested.

To support an obviousness rejection, MPEP §2143.03 requires "all words of a claim to be considered" and MPEP § 2141.02 requires consideration of the "[claimed] invention and prior art as a whole." Further, the Board of Patent Appeal and Interferences recently confirmed that a proper, post-KSR obviousness determination still requires the Office make "a searching comparison of the claimed invention – including all its limitations – with the teaching of the prior art." See, *In re Wada and Murphy*, Appeal 2007-3733, citing *In re Ochiai*, 71 F.3d 1565, 1572 (Fed. Cir. 1995) (emphasis in original). In sum, it remains well-settled law that an obviousness rejection requires at least a suggestion of all of the claim elements.

Here, all amended claims recite the features of (1) allowing an ophthalmic composition comprising at least 95% *R*-phenylephrine hydrochloride to be stored at between -10 to 10 degree Celsius for substantially maintaining chiral purity of *R*-phenylephrine hydrochloride, and (2) administering the composition to an eye wherein the composition comprises at least 95% *R*-phenylephrine hydrochloride when administered after storage. Neither of those technical feature is

taught or suggest in the cited references, whether taken alone or in combination, as discussed in greater detail below.

I. "Stored at Between -10 to 10 Degree Celsius"

Regarding the feature of the ophthalmic composition being stored at between -10 to 10 degree Celsius for substantially maintaining chiral purity of *R*-phenylephrine hydrochloride, Applicant notes that it is not disclosed or even remotely suggested in Shibani. In making the obviousness rejection, the Office admits, and Applicant agrees, that Shibini does not teach "storing the composition between -10 to 10 degree Celsius (claim 1) or 2 to 8 degree Celsius (claim 2)" (page 3, lines 11-12 of the Office Action). Applicant further submits that Shibini actively teaches away from the storing step recited in the pending claims. See MPEP 2145, stating that "familiar lines of argument still apply, including teaching away from the claimed invention by the prior art, lack of a reasonable expectation of success, and unexpected results. Indeed, they may have even taken on added importance in view of the recognition in *KSR* of a variety of possible rationales" (emphasis added by Applicant).

Specifically, Shibini's "Results and Discussion" Section (first paragraph) states that "[n]o noticeable changes in the optical rotation of such solutions could be detected after heating at 97°C for several days." Accordingly, in its "Conclusion" Section, Shibini states that "the following conditions for the proper preparation and storage of phenylephrine solutions are deduced: . . . the solution can be safely sterilized by autoclaving and will not undergo appreciable decomposition during storage." Shibini's observation and recommendation are consistent with existing understanding and practice of the storage of phenylnephrine. See, *e.g.* paragraph [[0017] of the present application, stating that "an insert from a commercially available Phenylephrine Hydrochloride Ophthalmic Solution provides that the solution should be stored at 20° to 25 °C (USP controlled room temperature) and keep container tightly closed. Do not use if solution is brown or contains precipitate. (AKORN Package Insert)". See also, a copy of the insert provided in the 1.132 Declaration (Exhibit 2 and Paragraph 9) submitted to the Patent Office on March 19, 2014.

Applicant next submits that one of ordinary skill in the art, in light of Shibini and knowledge in the art, would not store phenylephrine solutions at the temperatures recited in the pending claims because doing so would amount to "extra work and greater expense for no apparent reason." See MPEP 2143A (Example 3), where the Office discusses *In re Omeprazole Patent Litigation*, 536 F.3d

1361 (Fed. Cir. 2008), and states:

> Office personnel should note that in this case the modification of the prior art that had been presented as an argument for obviousness was an extra process step that added an additional component to a known, successfully marketed formulation. The proposed modification thus amounted to extra work and greater expense for no apparent reason. This is not the same as combining known prior art elements A and B when each would have been expected to contribute its own known properties to the final product. In the *Omeprazole* case, in view of the expectations of those of ordinary skill in the art, adding the subcoating would not have been expected to confer any particular desirable property on the final product. Rather, the final product obtained according to the proposed modifications would merely have been expected to have the same functional properties as the prior art product.

Furthermore, under MPEP 2143A (Example 3),

> The *Omeprazole* case can also be analyzed in view of the discovery of a previously unknown problem by the patentee. If the adverse interaction between active agent and coating had been known, it might well have been obvious to use a subcoating. However, since the problem had not been previously known, there would have been no reason to incur additional time and expense to add another layer, even though the addition would have been technologically possible.

Notwithstanding Shibini's declaration that chiral purity of L-m-Hydroxy-α-(methylaminomethyl)-benzyl alcohol would not be affected even after "heating at 97°C for several days", Applicant unexpectedly discovered that chiral purity of *R*-phenylephrine hydrochloride cannot be maintained at storage temperatures known in the art. As shown in the 1.132 Declaration (Exhibit 3 and Paragraph 10) submitted to the Patent office on March 19, 2014, while a commercial phenylephrien formulation (stored at "room temperature") and a formulation of the present application (stored at "low temperature") both exhibit no or little chemical degradation, the chiral chromatograms of the two formulations indicate significant difference in term of chiral stability. Therefore, one of ordinary skill in the art in view of Shibini would not recognize the technical significance of allowing an ophthalmic composition comprising at least 95% *R*-phenylephrine hydrochloride to be stored at between -10 to 10 degree Celsius for substantially maintaining chiral purity of *R*-phenylephrine hydrochloride, as provided in the pending claims. Accordingly, there would have been no reason to incur extra work and additional expense to store the composition at between -10 to 10 degree Celsius, even if technologically possible.

Finally, Applicant notes that the deficiencies of Shibini discussed above cannot be cured by citing to the secondary reference Valle. Specifically, Valle is completely irrelevant to the storage of its composition and therefore does not teach or even remotely suggest the feature of an ophthalmic composition being stored at between -10 to 10 degree Celsius for substantially maintaining chiral purity of *R*-phenylephrine hydrochloride.

II. Administration of Composition with Substantially Maintained Chiral Purity After Storage

Turning to the feature of administration of the ophthalmic composition with substantially maintained chiral purity of *R*-phenylephrine hydrochloride after storage, Applicant notes that it is not disclosed or suggested in Shibini. Instead, Shibini starts with a brief summary the previous two stability studies of L-m-Hydroxy-α-(methylaminomethyl)-benzyl alcohol, and states that "[i]t now remained to study the racemization of L-phenylephrin in order to obtain full information about the stability of the drug."

As such, Shibini merely investigates racemization of L-phenylephrin as an academic study of chiral chemistry. In other words, nothing in Shibini teaches or even remotely suggests that its compositions, formulated for the sole purpose of chiral chemistry investigation, is suitable for therapeutic application, much less for administration into an eye of a patient. To that end, Applicant directs the Patent Office's attention to Shibini's "Experimental Part" and "Results and Discussion" Sections, where Shibini discloses two L-phenylephrin solutions at pHs of close to zero (5% and 10% hydrochloric acid) and pHs of 2 and 6.5.

The secondary reference Valle also fails to teach or suggest administration of the ophthalmic composition with substantially maintained chiral purity of *R*-phenylephrine hydrochloride after storage, as recited in the amended claims. Instead, Valle purportedly teaches a "method of treating **depression** by administering several active compounds throughout the day to a patient in need of such treatment" (Abstract of Valle), in which the active compounds, including (R)-3-hydroxy-α-[(methylamino)methyl]benzenemethanol hydrochloride, are orally administered as **tablets** or **capsules** (col. 1, lines 62-66 of Valle).

Furthermore, it is Applicant who discovered the negative effect of the S-enantiomer on pupil dilation, and thus the importance of maintaining the high chiral optical purity of R-phenylephrin ophthalmic compositions after storage. See Section I above regarding the *Omeprazole* case and the

discovery of a previously unknown problem. As discussed in paragraphs [0049]-[0050] of the present application,

> when an ophthalmic solution of phenylephrine hydrochloride, (R-isomer) containing S-isomer as an impurity is used for dilation of pupil, the s-isomer may cause the saturation of the a-adrenergic receptors resulting in the decrease in the response of the drug after its administration (tachyphylasis). Furthermore, the presence of *S*-isomer in the ophthalmic solution may lead to poor/ delayed dilation of the pupil.

See also, the summary page of a final report regarding Applicant's discovery is provided in the 1.132 Declaration (Exhibit 1 and Paragraph 8), submitted to the Patent Office on March 19, 2014.

Neither Shibini nor Valle recognizes the importance of maintaining high chiral purity of R-phenylephrin hydrochloride in ophthalmic composition for pupil dilation. The Office Action cites to Valle for allegedly disclosing that "the therapeutic form of phenylephrin hydrochloride is (R)-3-hydroxy-α-[(methylamino)methyl]benzenemethanol hydrochloride," as Valle states that:

> (l) Phenylephrine hydrochloride which is (R)-3-hydroxy-α-[(methylamino)methyl]benzenemethanol hydrochloride. This compound alone has a therapeutic use as an adrenergic.

However, Valle's disclosure above is silence with respect to the therapeutic effect of the (S)-isomer, much less any negative effect of the (S)-isomer on pupil dilation as unexpectedly discovered by Applicant. Therefore, one of ordinary skill in the art in view of Shibini would not recognize the technical significance of administration of the ophthalmic composition with substantially maintained chiral purity of *R*-phenylephrine hydrochloride after storage.

For reasons stated above, the obviousness rejections raised in the Office Action should be withdrawn.

CONCLUSION

Applicants submit that this response fully addresses the Office Action dated June 12, 2014 and respectfully request consideration and allowance of the claims.

Should the Examiner have any questions, the Examiner is encouraged to contact the undersigned attorney at (858) 350-2306. If additional fees are believed to be required, the Commissioner is authorized to charge any additional fees to Deposit Account No. 23-2415 (Attorney Docket No. 44630-701.201).

Respectfully submitted,

WILSON SONSINI GOODRICH & ROSATI
Professional Corporation

Date: July 8, 2014

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Doc Code: A.NE.AFCP
Document Description: After Final Consideration Pilot Program Request

PTO/SB/434 (05-13)

CERTIFICATION AND REQUEST FOR CONSIDERATION UNDER THE AFTER FINAL CONSIDERATION PILOT PROGRAM 2.0

Practitioner Docket No.:	Application No.:	Filing Date:
44630-701.201	14/080,771	November 14, 2013
First Named Inventor:	**Title:**	
Patrick H. Witham	METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS	

APPLICANT HEREBY CERTIFIES THE FOLLOWING AND REQUESTS CONSIDERATION UNDER THE AFTER FINAL CONSIDERATION PILOT PROGRAM 2.0 (AFCP 2.0) OF THE ACCOMPANYING RESPONSE UNDER 37 CFR 1.116.

1. The above-identified application is (i) an original utility, plant, or design nonprovisional application filed under 35 U.S.C. 111(a) [a continuing application (*e.g.*, a continuation or divisional application) is filed under 35 U.S.C. 111(a) and is eligible under (i)], or (ii) an international application that has entered the national stage in compliance with 35 U.S.C. 371(c).
2. The above-identified application contains an outstanding final rejection.
3. Submitted herewith is a response under 37 CFR 1.116 to the outstanding final rejection. The response includes an amendment to at least one independent claim, and the amendment does not broaden the scope of the independent claim in any aspect.
4. This certification and request for consideration under AFCP 2.0 is the only AFCP 2.0 certification and request filed in response to the outstanding final rejection.
5. Applicant is willing and available to participate in any interview requested by the examiner concerning the present response.
6. This certification and request is being filed electronically using the Office's electronic filing system (EFS-Web).
7. Any fees that would be necessary consistent with current practice concerning responses after final rejection under 37 CFR 1.116, *e.g.*, extension of time fees, are being concurrently filed herewith. [There is no additional fee required to request consideration under AFCP 2.0.]
8. By filing this certification and request, applicant acknowledges the following:
   - Reissue applications and reexamination proceedings are not eligible to participate in AFCP 2.0.
   - The examiner will verify that the AFCP 2.0 submission is compliant, *i.e.*, that the requirements of the program have been met (see items 1 to 7 above). For compliant submissions:
     - The examiner will review the response under 37 CFR 1.116 to determine if additional search and/or consideration (i) is necessitated by the amendment and (ii) could be completed within the time allotted under AFCP 2.0. If additional search and/or consideration is required but cannot be completed within the allotted time, the examiner will process the submission consistent with current practice concerning responses after final rejection under 37 CFR 1.116, *e.g.*, by mailing an advisory action.
     - If the examiner determines that the amendment does not necessitate additional search and/or consideration, or if the examiner determines that additional search and/or consideration is required and could be completed within the allotted time, then the examiner will consider whether the amendment places the application in condition for allowance (after completing the additional search and/or consideration, if required). If the examiner determines that the amendment does not place the application in condition for allowance, then the examiner will contact the applicant and request an interview.
       - The interview will be conducted by the examiner, and if the examiner does not have negotiation authority, a primary examiner and/or supervisory patent examiner will also participate.
       - If the applicant declines the interview, or if the interview cannot be scheduled within ten (10) calendar days from the date that the examiner first contacts the applicant, then the examiner will proceed consistent with current practice concerning responses after final rejection under 37 CFR 1.116.

Signature	Date
/Michael Hostetler/	2014-07-08
Name (Print/Typed) Michael J. Hostetler	**Practitioner Registration No.** 47,664

Note: *This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4(d) for signature requirements and certifications. Submit multiple forms if more than one signature is required, see below*.*

[✓] * Total of 1 forms are submitted.

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Acknowledgement Receipt

EFS ID:	19524164
Application Number:	14080771
International Application Number:	
Confirmation Number:	6889
Title of Invention:	METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS
First Named Inventor/Applicant Name:	Patrick H. Witham
Customer Number:	21971
Filer:	Michael J. Hostetler/Linda Anders
Filer Authorized By:	Michael J. Hostetler
Attorney Docket Number:	44630-701.201
Receipt Date:	08-JUL-2014
Filing Date:	14-NOV-2013
Time Stamp:	20:11:28
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	After Final Consideration Program Request	44630-701-201-AFCP2-0Request.pdf	227053 b5/e9ca24/ca63d45204f9bfc6ac8524bf32e00	no	2

Warnings:	
Information:	

2		44630-701-201-responsefinal.pdf	211582 8e1eef1ff023f9cc35838a5c3e99559 1ad3b0ddf0	yes	9

Multipart Description/PDF files in .zip description		
Document Description	**Start**	**End**
Response After Final Action	1	1
Claims	2	3
Applicant Arguments/Remarks Made in an Amendment	4	9

Warnings:	
Information:	
Total Files Size (in bytes):	438635

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/06 (09-11)
Approved for use through 1/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 14/080,771	Filing Date 11/14/2013	☐ To be Mailed

ENTITY: ☐ LARGE ☒ SMALL ☐ MICRO

APPLICATION AS FILED – PART I

	(Column 1)	(Column 2)		
FOR	NUMBER FILED	NUMBER EXTRA	RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	x $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	x $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT	07/08/2014	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 14	Minus	** 20	= 0	x $40 =	0
	Independent (37 CFR 1.16(h))	* 1	Minus	*** 3	= 0	x $210 =	0
	☐ Application Size Fee (37 CFR 1.16(s))						
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
						TOTAL ADD'L FEE	0

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=	x $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x $ =	
	☐ Application Size Fee (37 CFR 1.16(s))						
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
						TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

LIE
/MOLIKI MAY/

PTO/SB/30 (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Request for Continued Examination (RCE) Transmittal

Address to:
Mail Stop RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Application Number	14/080,771
Filing Date	11/14/2013
First Named Inventor	Patrick H. Witham
Art Unit	1611
Examiner Name	Barbara S. Frazier
Attorney Docket Number	44630-701.201

This is a Request for Continued Examination (RCE) under 37 CFR 1.114 of the above-identified application.
Request for Continued Examination (RCE) practice under 37 CFR 1.114 does not apply to any utility or plant application filed prior to June 8, 1995, or to any design application. See Instruction Sheet for RCEs (not to be submitted to the USPTO) on page 2.

1. Submission required under 37 CFR 1.114 Note: If the RCE is proper, any previously filed unentered amendments and amendments enclosed with the RCE will be entered in the order in which they were filed unless applicant instructs otherwise. If applicant does not wish to have any previously filed unentered amendment(s) entered, applicant must request non-entry of such amendment(s).

   a. [✓] Previously submitted. If a final Office action is outstanding, any amendments filed after the final Office action may be considered as a submission even if this box is not checked.

      i. [ ] Consider the arguments in the Appeal Brief or Reply Brief previously filed on _____

      ii. [✓] Other Response to Final Office Action filed 07/08/2014

   b. [ ] Enclosed

      i. [ ] Amendment/Reply

      ii. [ ] Affidavit(s)/ Declaration(s)

      iii. [ ] Information Disclosure Statement (IDS)

      iv. [ ] Other _____

2. Miscellaneous

   a. [ ] Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a period of _____ months. (Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)

   b. [ ] Other _____

3. Fees The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.

   a. [✓] The Director is hereby authorized to charge the following fees, any underpayment of fees, or credit any overpayments, to Deposit Account No. 232415.

      i. [✓] RCE fee required under 37 CFR 1.17(e)

      ii. [ ] Extension of time fee (37 CFR 1.136 and 1.17)

      iii. [ ] Other _____

   b. [ ] Check in the amount of $ _____ enclosed

   c. [ ] Payment by credit card (Form PTO-2038 enclosed)

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

Signature	/Michael Hostetler/	Date	07/25/2014
Name (Print/Type)	Michael J. Hostetler	Registration No.	47,664

CERTIFICATE OF MAILING OR TRANSMISSION

I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Mail Stop RCE, Commissioner for Patents, P. O. Box 1450, Alexandria, VA 22313-1450 or facsimile transmitted to the U.S. Patent and Trademark Office on the date shown below.

Signature	/Linda Anders/		
Name (Print/Type)	Linda Anders	Date	07/25/2014

Doc Code: TRACK1.REQ
Document Description: TrackOne Request

PTO/SB/424 (12-11)

CERTIFICATION AND REQUEST FOR PRIORITIZED EXAMINATION UNDER 37 CFR 1.102(e) (Page 1 of 1)

First Named Inventor:	Patrick H. Witham	Nonprovisional Application Number (if known):	14/080,771
Title of Invention:	METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS		

APPLICANT HEREBY CERTIFIES THE FOLLOWING AND REQUESTS PRIORITIZED EXAMINATION FOR THE ABOVE-IDENTIFIED APPLICATION.

1. The processing fee set forth in 37 CFR 1.17(i), the prioritized examination fee set forth in 37 CFR 1.17(c), and if not already paid, the publication fee set forth in 37 CFR 1.18(d) have been filed with the request. The basic filing fee, search fee, examination fee, and any required excess claims and application size fees are filed with the request or have been already been paid.

2. The application contains or is amended to contain no more than four independent claims and no more than thirty total claims, and no multiple dependent claims.

3. The applicable box is checked below:

I. ☐ Original Application (Track One) - Prioritized Examination under § 1.102(e)(1)

i. (a) The application is an original nonprovisional utility application filed under 35 U.S.C. 111(a). This certification and request is being filed with the utility application via EFS-Web.

---OR---

(b) The application is an original nonprovisional plant application filed under 35 U.S.C. 111(a). This certification and request is being filed with the plant application in paper.

ii. An executed oath or declaration under 37 CFR 1.63 is filed with the application.

II. ☑ Request for Continued Examination - Prioritized Examination under § 1.102(e)(2)

i. A request for continued examination has been filed with, or prior to, this form.
ii. If the application is a utility application, this certification and request is being filed via EFS-Web.
iii. The application is an original nonprovisional utility application filed under 35 U.S.C. 111(a), or is a national stage entry under 35 U.S.C. 371.
iv. This certification and request is being filed prior to the mailing of a first Office action responsive to the request for continued examination.
v. No prior request for continued examination has been granted prioritized examination status under 37 CFR 1.102(e)(2).

Signature /Michael Hostetler/	Date 07/25/2014
Name (Print/Typed) Michael J. Hostetler	Practitioner Registration Number 47,664

Note: *Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required in accordance with 37 CFR 1.33 and 11.18. Please see 37 CFR 1.4(d) for the form of the signature. If necessary, submit multiple forms for more than one signature, see below*.*

☑ *Total of 1 forms are submitted.

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Patent Application Fee Transmittal

Application Number:	14080771
Filing Date:	14-Nov-2013
Title of Invention:	METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS
First Named Inventor/Applicant Name:	Patrick H. Witham
Filer:	Michael J. Hostetler/Linda Anders
Attorney Docket Number:	44630-701.201

Filed as Small Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Request for Prioritized Examination	2817	1	2000	2000
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
Request for Continued Examination	2801	1	600	600
		Total in USD ($)		**2600**

Electronic Acknowledgement Receipt

EFS ID:	19691665
Application Number:	14080771
International Application Number:	
Confirmation Number:	6889
Title of Invention:	METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS
First Named Inventor/Applicant Name:	Patrick H. Witham
Customer Number:	21971
Filer:	Michael J. Hostetler/Linda Anders
Filer Authorized By:	Michael J. Hostetler
Attorney Docket Number:	44630-701.201
Receipt Date:	25-JUL-2014
Filing Date:	14-NOV-2013
Time Stamp:	18:41:38
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$2600
RAM confirmation Number	5029
Deposit Account	232415
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: Charge any Additional Fees required under 37 C.F.R. Section 1.21 (Miscellaneous fees and charges)	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	TrackOne Request	44630-701-201-trackonerequest.pdf	141265 211acc1eb05c6b4696b61b2b5680441433b8543	no	2
Warnings:					
Information:					
2	Request for Continued Examination (RCE)	44630-701-201-RCE.pdf	55753 b54a868d19f6e9e7f3185f2ff2910c5175554eb1f	no	1
Warnings:					
This is not a USPTO supplied RCE SB30 form.					
Information:					
3	Fee Worksheet (SB06)	fee-info.pdf	32091 6a052992b6b127f386fd0e4b28896bf24fa85094	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	229109		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Document code: WFEE

United States Patent and Trademark Office
Sales Receipt for Accounting Date: 07/31/2014

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01 FC : 2830 70.00 DA

PTO/SB/06 (09-11)
Approved for use through 1/31/2014. OMB 0651-0032
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 14/080,771	Filing Date 11/14/2013	☐ To be Mailed

ENTITY: ☐ LARGE ☒ SMALL ☐ MICRO

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	x $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	x $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT 07/25/2014

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	* 14	Minus	** 20	= 0	x $40 =	0
Independent (37 CFR 1.16(h))	* 1	Minus	*** 3	= 0	x $210 =	0
☐ Application Size Fee (37 CFR 1.16(s))						
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
					TOTAL ADD'L FEE	0

AMENDMENT

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	x $ =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	x $ =	
☐ Application Size Fee (37 CFR 1.16(s))						
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
					TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

LIE
/GLORIA ANTHONY/

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/080,771	11/14/2013	Patrick H. Witham	44630-701.201	6889

21971 7590 07/31/2014

WILSON, SONSINI, GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050

EXAMINER	
FRAZIER, BARBARA S	
ART UNIT	PAPER NUMBER
1611	

NOTIFICATION DATE	DELIVERY MODE
07/31/2014	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

patentdocket@wsgr.com

Advisory Action Before the Filing of an Appeal Brief	**Application No.** 14/080,771	**Applicant(s)** WITHAM ET AL.	
	Examiner BARBARA FRAZIER	**Art Unit** 1611	**AIA (First Inventor to File) Status** Yes

--The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

THE REPLY FILED 08 July 2014 FAILS TO PLACE THIS APPLICATION IN CONDITION FOR ALLOWANCE.

NO NOTICE OF APPEAL FILED

1. ☒ The reply was filed after a final rejection. No Notice of Appeal has been filed. To avoid abandonment of this application, applicant must timely file one of the following replies: (1) an amendment, affidavit, or other evidence, which places the application in condition for allowance; (2) a Notice of Appeal (with appeal fee) in compliance with 37 CFR 41.31; or (3) a Request for Continued Examination (RCE) in compliance with 37 CFR 1.114 if this is a utility or plant application. Note that RCEs are not permitted in design applications. The reply must be filed within one of the following time periods:
   a) ☐ The period for reply expires _____ months from the mailing date of the final rejection.
   b) ☒ The period for reply expires on: (1) the mailing date of this Advisory Action; or (2) the date set forth in the final rejection, whichever is later. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of the final rejection.
   c) ☐ A prior Advisory Action was mailed more than 3 months after the mailing date of the final rejection in response to a first after-final reply filed within 2 months of the mailing date of the final rejection. The current period for reply expires months from the mailing date of the prior Advisory Action or SIX MONTHS from the mailing date of the final rejection, whichever is earlier.

   Examiner Note: If box 1 is checked, check either box (a), (b) or (c). ONLY CHECK BOX (b) WHEN THIS ADVISORY ACTION IS THE FIRST RESPONSE TO APPLICANT'S FIRST AFTER-FINAL REPLY WHICH WAS FILED WITHIN TWO MONTHS OF THE FINAL REJECTION. ONLY CHECK BOX (c) IN THE LIMITED SITUATION SET FORTH UNDER BOX (c). See MPEP 706.07(f).

Extensions of time may be obtained under 37 CFR 1.136(a). The date on which the petition under 37 CFR 1.136(a) and the appropriate extension fee have been filed is the date for purposes of determining the period of extension and the corresponding amount of the fee. The appropriate extension fee under 37 CFR 1.17(a) is calculated from: (1) the expiration date of the shortened statutory period for reply originally set in the final Office action; or (2) as set forth in (b) or (c) above, if checked. Any reply received by the Office later than three months after the mailing date of the final rejection, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

NOTICE OF APPEAL

2. ☐ The Notice of Appeal was filed on _____. A brief in compliance with 37 CFR 41.37 must be filed within two months of the date of filing the Notice of Appeal (37 CFR 41.37(a)), or any extension thereof (37 CFR 41.37(e)), to avoid dismissal of the appeal. Since a Notice of Appeal has been filed, any reply must be filed within the time period set forth in 37 CFR 41.37(a).

AMENDMENTS

3. ☒ The proposed amendments filed after a final rejection, but prior to the date of filing a brief, will not be entered because
   a) ☒ They raise new issues that would require further consideration and/or search (see NOTE below);
   b) ☐ They raise the issue of new matter (see NOTE below);
   c) ☐ They are not deemed to place the application in better form for appeal by materially reducing or simplifying the issues for appeal; and/or
   d) ☐ They present additional claims without canceling a corresponding number of finally rejected claims.

   NOTE: *See Continuation Sheet*. (See 37 CFR 1.116 and 41.33(a)).
4. ☐ The amendments are not in compliance with 37 CFR 1.121. See attached Notice of Non-Compliant Amendment (PTOL-324).
5. ☐ Applicant's reply has overcome the following rejection(s): _____.
6. ☐ Newly proposed or amended claim(s) _____ would be allowable if submitted in a separate, timely filed amendment canceling the non-allowable claim(s).
7. ☒ For purposes of appeal, the proposed amendment(s): (a) ☒ will not be entered, or (b) ☐ will be entered, and an explanation of how the new or amended claims would be rejected is provided below or appended.

AFFIDAVIT OR OTHER EVIDENCE

8. ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.
9. ☐ The affidavit or other evidence filed after final action, but before or on the date of filing a Notice of Appeal will not be entered because applicant failed to provide a showing of good and sufficient reasons why the affidavit or other evidence is necessary and was not earlier presented. See 37 CFR 1.116(e).
10. ☐ The affidavit or other evidence filed after the date of filing the Notice of Appeal, but prior to the date of filing a brief, will not be entered because the affidavit or other evidence failed to overcome all rejections under appeal and/or appellant fails to provide a showing of good and sufficient reasons why it is necessary and was not earlier presented. See 37 CFR 41.33(d)(1).
11. ☐ The affidavit or other evidence is entered. An explanation of the status of the claims after entry is below or attached.

REQUEST FOR RECONSIDERATION/OTHER

12. ☐ The request for reconsideration has been considered but does NOT place the application in condition for allowance because: _____.
13. ☐ Note the attached Information *Disclosure Statement*(s). (PTO/SB/08) Paper No(s). _____
14. ☐ Other: _____.

STATUS OF CLAIMS

15. The status of the claim(s) is (or will be) as follows:
    Claim(s) allowed: .
    Claim(s) objected to: .
    Claim(s) rejected: 1-14.
    Claim(s) withdrawn from consideration: .

/DAVID J BLANCHARD/ Supervisory Patent Examiner, Art Unit 1619	/B. F./ Examiner, Art Unit 1611

Continuation of 3. NOTE: Applicant's request for entry into AFCP 2.0 is acknowledged, but is denied because the response cannot be reviewed and a search conducted in the limited amount of time authorized for this pilot program. Therefore, the response is being reviewed under pre-pilot practice. Applicant's amendments changing the claims from an ophthalmic composition to a method of using an ophthalmic composition for pupil dilation includes new steps which have not previously been a part of the claim set, and require further consideration and/or search.

DO NOT ENTER: /B.F./ 07/25/2014

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of:	Group Art Unit: 1611
Inventors: Patrick H. Witham et al.	Confirmation No.: 6889
Serial No.: 14/080,771	Examiner: Barbara S. Frazier
Filed: November 14, 2013	Customer No. 21971
Title: **Methods and Composition of Stable Phenylephrine Formulations**	Certificate of Electronic Filing I hereby certify that the attached Response and all marked attachments are being deposited by Electronic Filing on July 8, 2014 by using the EFS Web patent filing system and addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450. By: /Linda Anders/

Mail Stop AF
Commissioner For Patents
P.O. Box 1450
Alexandria, VA 22313-1450

RESPONSE TO FINAL OFFICE ACTION WITH REQUEST FOR CONSIDERATION UNDER THE AFTER FINAL CONSIDERATION PILOT PROGRAM 2.0

Dear Madam:

This paper responds to the final Office Action dated June 12, 2014, and is filed with an After Final Consideration Pilot 2.0 request. In the event any additional fee is due, the Commissioner is authorized to charge it to Deposit Account No. 23-2415, referencing Docket No. 44630-701.201.

Amendments to the Claims begin on page **2** of this paper
Remarks begin on page **4** of this paper.
Conclusion is on page **9** of this paper.

UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents
United States Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450
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AUG 01 2014
OFFICE OF PETITIONS

WILSON SONSINI GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO CA 94304-1050

Doc Code: TRACK1.GRANT

Decision Granting Request for Prioritized Examination (Track I or After RCE)	Application No.: 14/080,771

1. THE REQUEST FILED July 25, 2014 IS **GRANTED**.

The above-identified application has met the requirements for prioritized examination

A. ☐ for an original nonprovisional application (Track I).
B. ☒ for an application undergoing continued examination (RCE).

2. **The above-identified application will undergo prioritized examination.** The application will be accorded special status throughout its entire course of prosecution until one of the following occurs:

A. filing a **petition for extension of time** to extend the time period for filing a reply;

B. filing an **amendment to amend the application to contain more than four independent claims, more than thirty total claims**, or a multiple dependent claim;

C. filing a **request for continued examination**;

D. filing a notice of appeal;

E. filing a request for suspension of action;

F. mailing of a notice of allowance;

G. mailing of a final Office action;

H. completion of examination as defined in 37 CFR 41.102; or

I. abandonment of the application.

Telephone inquiries with regard to this decision should be directed to Irvin Dingle at (571)272-3210, Office of Petitions.

Irvin Dingle
/Irvin Dingle/
[*Signature*]

Paralegal Specialist
(Title)

Notice of Allowability	Application No. 14/080,771	Applicant(s) WITHAM ET AL.	
	Examiner BARBARA FRAZIER	Art Unit 1611	AIA (First Inventor to File) Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to *RCE filed 25 July 2014*.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on_____.
2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.
3. ☒ The allowed claim(s) is/are *1-4 and 6-14*. As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.
4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

   Certified copies:

   a) ☐ All b) ☐ Some *c) ☐ None of the:
   1. ☐ Certified copies of the priority documents have been received.
   2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
   3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____.

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).
6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☒ Notice of References Cited (PTO-892)
2. ☐ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date _____
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material
4. ☒ Interview Summary (PTO-413), Paper No./Mail Date *8/19/14* .
5. ☒ Examiner's Amendment/Comment
6. ☒ Examiner's Statement of Reasons for Allowance
7. ☐ Other _____.

/B. F./	/DAVID J BLANCHARD/
Examiner, Art Unit 1611	Supervisory Patent Examiner, Art Unit 1619

DETAILED ACTION

The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

Previous Rejection Withdrawn

The rejection of claims 1-14 under 35 U.S.C. 103 as being unpatentable over Shibini as evidenced by Valle is withdrawn in view of Applicant's amendment to claim 1.

Examiner's Amendment

An examiner's amendment to the record appears below. Should the changes and/or additions be unacceptable to applicant, an amendment may be filed as provided by 37 CFR 1.312. To ensure consideration of such an amendment, it MUST be submitted no later than the payment of the issue fee.

Authorization for this examiner's amendment was given in a telephone interview with Mr. Xiaofan Yang on 19 August 2014.

The application has been amended as follows:

IN THE CLAIMS:

Please amend claim 1 as follows:

1. (Amended) A method of using an ophthalmic composition for pupil dilation, the composition comprising ~~at least 95%~~ *R*-phenylephrine hydrochloride having an initial chiral purity of at least 95% and an aqueous buffer ~~for substantially maintaining chiral purity of *R*-phenylephrine hydrochloride for at least 6 months~~, wherein the chiral purity

of *R*-phenylephrine hydrochloride is at least 95% of the initial chiral purity after 6 months, the method comprising:

~~allowing the composition to be stored between -10 to 10 degree Celsius; and~~

administering the composition into an eye of an individual in need thereof, wherein the composition is stored between -10 to 10 degree Celsius prior to administration, and wherein the composition comprises ~~at least 95%~~ *R*-phenylephrine hydrochloride having a chiral purity of at least 95% when administered after storage.

Claim 2, line 2, please delete “allowed to be”.

Please amend claims 3 and 4 as follows:

3. (Amended) The method of claim 1, wherein the composition comprises ~~at least 99%~~ *R*-phenylephrine hydrochloride having an initial chiral purity of at least 99%.

4. (Amended) The method of claim 1, wherein the composition comprises ~~at least 99.3%~~ *R*-phenylephrine hydrochloride having an initial chiral purity of at least 99.3%.

Please cancel claim 5.

Reasons for Allowance

The following is an examiner’s statement of reasons for allowance: in light of Applicant's amendments, the closest prior art is Akorn, Inc. (package insert for phenylephrine hydrochloride solution/drops, at

http://dailymed.nlm.nih.gov/dailymed/lookup.cfm?setid=c5c51d8b-b50b-4c77-9d55-f64c14b0d0e5, revised 09/2011, included with this Office action). Akorn teaches an ophthalmic solution of

(-)phenylephrine hydrochloride, 2.5%, which may be used as a mydriatic (page 1), wherein the composition is to be stored at 20 to 25 degree Celsius (page 3). Akorn does not teach the composition is stored at -10 to 10 degree Celsius, and also does not specify the chiral purity of the solution before or after storage. Applicant's Declaration filed 19 March 2014 provides evidence that the chiral purity of R-phenylephrine is maintained after 6 months of storage at -10 to 10 degree Celsius, but is not maintained when the composition is stored at room temperature (20 to 25 degree Celsius). Therefore, Applicant's Declaration is sufficient for demonstrating nonobviousness of the claimed invention.

Any comments considered necessary by applicant must be submitted no later than the payment of the issue fee and, to avoid processing delays, should preferably accompany the issue fee. Such submissions should be clearly labeled "Comments on Statement of Reasons for Allowance."

Correspondence

Any inquiry concerning this communication or earlier communications from the examiner should be directed to BARBARA FRAZIER whose telephone number is

(571)270-3496. The examiner can normally be reached on Monday-Friday 9am-2:30pm EST.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, John Mabry can be reached on (571)270-1967. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/B. F./
Examiner, Art Unit 1611

/DAVID J BLANCHARD/
Supervisory Patent Examiner, Art Unit 1619

Notice of References Cited	Application/Control No. 14/080,771	Applicant(s)/Patent Under Reexamination WITHAM ET AL.	
	Examiner BARBARA FRAZIER	Art Unit 1611	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Akorn, Inc., package insert for phenylephrine hydrochloride solution/drops, at http://dailymed.nlm.nih.gov/dailymed/lookup.cfm?setid=c5c51d8b-b50b-4c77-9d55-f64c14b0d0e5, revised 09/2011.
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Issue Classification

Application/Control No.	Applicant(s)/Patent Under Reexamination
14080771	WITHAM ET AL.
Examiner	**Art Unit**
BARBARA FRAZIER	1611

CPC

Symbol			Type	Version
A61K	31 /	137	F	2013-01-01
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CPC Combination Sets

Symbol			Type	Set	Ranking	Version
	/					
	/					

/BARBARA FRAZIER/ Examiner.Art Unit 1611 (Assistant Examiner)	8/19/14 (Date)	**Total Claims Allowed:** 13	
/DAVID J BLANCHARD/ Supervisory Patent Examiner.Art Unit 1619 (Primary Examiner)	08/20/2014 (Date)	O.G. Print Claim(s) 1	O.G. Print Figure None

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14080771	WITHAM ET AL.
	Examiner	**Art Unit**
	BARBARA FRAZIER	1611

US ORIGINAL CLASSIFICATION		INTERNATIONAL CLASSIFICATION										
CLASS	SUBCLASS	CLAIMED					NON-CLAIMED					
514	653	A	6	1	K	31 / 137 (2006.01.01)						

CROSS REFERENCE(S)						
CLASS	SUBCLASS (ONE SUBCLASS PER BLOCK)					

/BARBARA FRAZIER/ Examiner.Art Unit 1611	8/19/14	Total Claims Allowed: 13	
(Assistant Examiner)	(Date)		
/DAVID J BLANCHARD/ Supervisory Patent Examiner.Art Unit 1619	08/20/2014	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	None

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14080771	WITHAM ET AL.
	Examiner	**Art Unit**
	BARBARA FRAZIER	1611

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original
1	1														
2	2														
3	3														
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5	6														
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9	10														
10	11														
11	12														
12	13														
13	14														

/BARBARA FRAZIER/ Examiner.Art Unit 1611	8/19/14	**Total Claims Allowed:**	
(Assistant Examiner)	(Date)	13	
/DAVID J BLANCHARD/ Supervisory Patent Examiner.Art Unit 1619	08/20/2014	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	None

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P.O. Box 1450
Alexandria, Virginia 22313-1450
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BIB DATA SHEET

CONFIRMATION NO. 6889

SERIAL NUMBER	FILING or 371(c) DATE RULE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
14/080,771	11/14/2013	514	1611	44630-701.201

APPLICANTS

Paragon BioTeck, Inc., Portland, OR, Assignee (with 37 CFR 1.172 Interest);

INVENTORS

Patrick H. Witham, Eugene, OR;
Sailaja Machiraju, Beaverton, OR;
Lauren Mackensie-Clark Bluett, Milwaukie, OR;

** **CONTINUING DATA** **********

** **FOREIGN APPLICATIONS** **********

** **IF REQUIRED, FOREIGN FILING LICENSE GRANTED** ** SMALL ENTITY **
12/02/2013

Foreign Priority claimed	35 USC 119(a-d) conditions met	Verified and Acknowledged	Met after Allowance	STATE OR COUNTRY	SHEETS DRAWINGS	TOTAL CLAIMS	INDEPENDENT CLAIMS
☐ Yes ☑ No	☐ Yes ☑ No	/BARBARA S FRAZIER/ Examiner's Signature	☐ Initials	OR	4	~~14~~ 13	1

ADDRESS

WILSON, SONSINI, GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050
UNITED STATES

TITLE

METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS

FILING FEE RECEIVED	FEES: Authority has been given in Paper
1030	No._____ to charge/credit DEPOSIT ACCOUNT No._____ for following:

☐ All Fees
☐ 1.16 Fees (Filing)
☐ 1.17 Fees (Processing Ext. of time)
☐ 1.18 Fees (Issue)
☐ Other _____
☐ Credit

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14080771	WITHAM ET AL.
	Examiner	Art Unit
	BARBARA FRAZIER	1611

CPC- SEARCHED		
Symbol	Date	Examiner
A61K 31/137 (w/search terms - see history)	6/4/14	BSF
updated	8/19/14	BSF

CPC COMBINATION SETS - SEARCHED		
Symbol	Date	Examiner

US CLASSIFICATION SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
Inventor name search	1/27/14	BSF
EAST search	1/27/14	BSF
Google scholar search: phenylephrine, cold storage/stored/storing	1/27/14	BSF
EAST search updated	6/4/14	BSF
Inventor search updated	6/4/14	BSF
EAST search updated	8/19/14	BSF
Inventor search updated	8/19/14	BSF

INTERFERENCE SEARCH			
US Class/ CPC Symbol	US Subclass / CPC Group	Date	Examiner
A61K	31/137 (w/search terms - see history)	8/19/14	BSF

/B.F./ Examiner.Art Unit 1611	

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	17073	A61K31/137.cpc.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/08/19 21:34
L2	1160733	ophthalmic or eye or pupil	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/08/19 21:34
L3	1236	L1 and L2	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/08/19 21:34
L4	195497	(cold or freeze or freezing or frozen) near20 (stored or storage or storing)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/08/19 21:40
L5	80	3 and 4	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/08/19 21:40
L6	13280	phenylephrine or Rphenylephrine	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/08/19 21:43
L7	787	1 and 6	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/08/19 21:43
L8	265	2 and 7	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/08/19 21:43
L9	154950	chiral or chirality	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/08/19 21:43
L10	48	8 and 9	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/08/19 21:44
L11	46	10 and (cold or freeze or freezing or frozen or store or storing or stored)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/08/19 22:02
L12	23	10 and (freeze or freezing or frozen or store or storing or stored)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/08/19 22:03

L13	1213030	eye or retina or retinal or ophthalmic or ophthamology or pupil	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/08/19 22:04
L14	46	11 and 13	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/08/19 22:05

EAST Search History (Interference)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L15	3586	A61K31/137.cpc.	US-PGPUB; USPAT; UPAD	ADJ	ON	2014/08/19 22:09
L16	685637	ophthalmic or eye or retina or retinal or pupil or ophthamology	US-PGPUB; USPAT; UPAD	ADJ	ON	2014/08/19 22:09
L17	989	15 and 16	US-PGPUB; USPAT; UPAD	ADJ	ON	2014/08/19 22:10
L18	545	17 and (freeze or freezing or frozen or store or storing or stored)	US-PGPUB; USPAT; UPAD	ADJ	ON	2014/08/19 22:10
L19	6668	(dilat$3 or enlarg$3) near3 pupil	US-PGPUB; USPAT; UPAD	ADJ	ON	2014/08/19 22:12
L20	50	18 and 19	US-PGPUB; USPAT; UPAD	ADJ	ON	2014/08/19 22:12
L21	11590	phenylephrine	US-PGPUB; USPAT; UPAD	ADJ	ON	2014/08/19 22:18
L22	23	20 and 21	US-PGPUB; USPAT; UPAD	ADJ	ON	2014/08/19 22:18

8/19/2014 10:19:45 PM
C:\Users\bfrazier\Documents\EAST\Workspaces\14080771.wsp

Examiner-Initiated Interview Summary	Application No.	Applicant(s)	
	14/080,771	WITHAM ET AL.	
	Examiner	Art Unit	
	BARBARA FRAZIER	1611	

All participants (applicant, applicant's representative, PTO personnel):

(1) *BARBARA FRAZIER*. (3)_____.

(2) *Xiaofan Yang*. (4)_____.

Date of Interview: *19 August 2014*.

Type: ☒ Telephonic ☐ Video Conference
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☐ Yes ☒ No.
If Yes, brief description: _____.

Issues Discussed ☐101 ☐112 ☐102 ☒103 ☐Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: *1-14*.

Identification of prior art discussed: *Shibini*.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

Examiner contacted Applicant's representative Mr. Yang ("Applicant") to discuss allowable subject matter. Examiner suggested amending the claims to include limitations regarding chiral purity and storing the composition between -10 and 10 degree Celsius. Applicant agreed to amending the claims as outlined in the Examiner's Amendment of this Office action.

Applicant recordation instructions: It is not necessary for applicant to provide a separate record of the substance of interview.

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

☐ Attachment

/BARBARA FRAZIER/ Examiner, Art Unit 1611	

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NOTICE OF ALLOWANCE AND FEE(S) DUE

21971 7590 09/03/2014
WILSON, SONSINI, GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050

EXAMINER	
FRAZIER, BARBARA S	
ART UNIT	PAPER NUMBER
1611	

DATE MAILED: 09/03/2014

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/080,771	11/14/2013	Patrick H. Witham	44630-701.201	6889

TITLE OF INVENTION: METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	SMALL	$480	$0	$0	$480	12/03/2014

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 1/2 the amount of undiscounted fees, and micro entity fees are 1/2 the amount of small entity fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: Mail **Mail Stop ISSUE FEE**
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or Fax (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

21971 7590 09/03/2014
WILSON, SONSINI, GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission
I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/080,771	11/14/2013	Patrick H. Witham	44630-701.201	6889

TITLE OF INVENTION: METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	SMALL	$480	$0	$0	$480	12/03/2014

EXAMINER	ART UNIT	CLASS-SUBCLASS
FRAZIER, BARBARA S	1611	514-653000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).
- ☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.
- ☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.
1 _ _ _ _ _ _ _ _ _ _
2 _ _ _ _ _ _ _ _ _ _
3 _ _ _ _ _ _ _ _ _ _

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent): ☐ Individual ☐ Corporation or other private group entity ☐ Government

4a. The following fee(s) are submitted:
- ☐ Issue Fee
- ☐ Publication Fee (No small entity discount permitted)
- ☐ Advance Order - # of Copies _ _ _ _ _ _

4b. Payment of Fee(s): (**Please first reapply any previously paid issue fee shown above**)
- ☐ A check is enclosed.
- ☐ Payment by credit card. Form PTO-2038 is attached.
- ☐ The Director is hereby authorized to charge the required fee(s), any deficiency, or credits any overpayment, to Deposit Account Number _ _ _ _ (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)
- ☐ Applicant certifying micro entity status. See 37 CFR 1.29 — NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.
- ☐ Applicant asserting small entity status. See 37 CFR 1.27 — NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.
- ☐ Applicant changing to regular undiscounted fee status. — NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ Date _ _ _ _ _ _ _ _ _ _ _ _ _ _ _

Typed or printed name _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ Registration No. _ _ _ _ _ _ _ _ _ _ _ _ _

PTOL-85 Part B (10-13) Approved for use through 10/31/2013. OMB 0651-0033 U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/080,771	11/14/2013	Patrick H. Witham	44630-701.201	6889

21971 7590 09/03/2014
WILSON, SONSINI, GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050

EXAMINER	
FRAZIER, BARBARA S	

ART UNIT	PAPER NUMBER
1611	

DATE MAILED: 09/03/2014

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450. Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of:	Group Art Unit: 1611
Inventor(s): Patrick H. Witham, et al.	Examiner: Frazier, Barbara S.
Serial No.: 14/080,771	Confirmation No.: 6889
Filed: November 14, 2013	Customer No.: 21971
Title: METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

NOTIFICATION OF CHANGE IN ENTITY STATUS

Sir:

Please be advised that it was discovered that the small entity status was established in error. In accordance with the requirements of 37 C.F.R. §1.27(g)(2), Applicants hereby notify the U.S. Patent and Trademark Office that the above-identified application is no longer entitled to small entity status. Please change this application status from small entity to large entity status.

Applicants provide an itemization of fees and the deficiency payment as outlined on the following page.

Deficiency Payment

Description	Date Paid	Small Entity Fee Paid	Large Entity Fee	Deficiency Owed
Prioritized Exam	07/28/2014	$2,000	$4000	S2000
RCE	07/28/2014	$600	$1200	S600
Utility Filing Fee	11/15/2013	$70	$280	S210
Utility Search Fee	11/15/2013	$300	$600	S300
Utility Exam Fee	11/15/2013	$360	$720	S360
Track One Request Fee	11/15/2013	$2000	$4000	S2000
			TOTAL AMOUNT OWED:	**$5,470**

The Commissioner is authorized to charge the above fees, and any additional fees which may be required, including petition fees, or credit any overpayment to Deposit Account No. 23-2415 (Docket No. 44630-701.201).

Respectfully submitted,

WILSON SONSINI GOODRICH & ROSATI

Dated: September 9, 2014

By: /Michael Hostetler/

Michael J. Hostetler, Registration No.: 47,664

650 Page Mill Road
Palo Alto, California 94304
(650) 493-9300
Customer No.: 21971

Electronic Acknowledgement Receipt

EFS ID:	20086544
Application Number:	14080771
International Application Number:	
Confirmation Number:	6889
Title of Invention:	METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS
First Named Inventor/Applicant Name:	Patrick H. Witham
Customer Number:	21971
Filer:	Michael J. Hostetler/Linda Anders
Filer Authorized By:	Michael J. Hostetler
Attorney Docket Number:	44630-701.201
Receipt Date:	09-SEP-2014
Filing Date:	14-NOV-2013
Time Stamp:	14:29:39
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Miscellaneous Incoming Letter	44630-701-201-entitystatuschange.pdf	94632 947a9938f6b5e80c0fc972fbe9508ffb22bd01dc9	no	2

Warnings:	
Information:	

Total Files Size (in bytes):	94632

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: Mail **Mail Stop ISSUE FEE**
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or Fax (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

21971 7590 09/03/2014
WILSON, SONSINI, GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050

Filed via EFS on 9/10/2014

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission
I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/080,771	11/14/2013	Patrick H. Witham	44630-701.201	6889

TITLE OF INVENTION: METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	undiscounted	$960	$0	$0	$960	12/03/2014

EXAMINER	ART UNIT	CLASS-SUBCLASS
FRAZIER, BARBARA S	1611	514-653000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).
☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.
☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 Wilson Sonsini
2 Goodrich & Rosati
3

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE: PARAGON BIOTECK, INC.

(B) RESIDENCE: (CITY and STATE OR COUNTRY): PORTLAND, OREGON

Please check the appropriate assignee category or categories (will not be printed on the patent): ☐ Individual ☑ Corporation or other private group entity ☐ Government

4a. The following fee(s) are submitted:
☑ Issue Fee
☐ Publication Fee (No small entity discount permitted)
☐ Advance Order - # of Copies

4b. Payment of Fee(s): (**Please first reapply any previously paid issue fee shown above**)
☐ A check is enclosed.
☐ Payment by credit card. Form PTO-2038 is attached.
☑ The Director is hereby authorized to charge the required fee(s), any deficiency, or credits any overpayment, to Deposit Account Number 23-2415 (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)
☐ Applicant certifying micro entity status. See 37 CFR 1.29
NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.
☐ Applicant asserting small entity status. See 37 CFR 1.27
NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.
☑ Applicant changing to regular undiscounted fee status.
NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature /Michael Hostetler/ Date September 10, 2014
Typed or printed name Michael Hostetler Registration No. 47,664

PTOL-85 Part B (10-13) Approved for use through 10/31/2013. OMB 0651-0033 U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Electronic Patent Application Fee Transmittal

Application Number:	14080771
Filing Date:	14-Nov-2013
Title of Invention:	METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS
First Named Inventor/Applicant Name:	Patrick H. Witham
Filer:	Michael J. Hostetler/Jennifer Vail
Attorney Docket Number:	44630-701.201

Filed as Large Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Utility Appl Issue Fee	1501	1	960	960
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
			Total in USD ($)	**960**

Electronic Acknowledgement Receipt

EFS ID:	20097512
Application Number:	14080771
International Application Number:	
Confirmation Number:	6889
Title of Invention:	METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS
First Named Inventor/Applicant Name:	Patrick H. Witham
Customer Number:	21971
Filer:	Michael J. Hostetler/Jennifer Vail
Filer Authorized By:	Michael J. Hostetler
Attorney Docket Number:	44630-701.201
Receipt Date:	10-SEP-2014
Filing Date:	14-NOV-2013
Time Stamp:	13:24:27
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$960
RAM confirmation Number	7204
Deposit Account	232415
Authorized User	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /zip	Pages (if appl.)

1	Issue Fee Payment (PTO-85B)	44630-701-201_IssueFee.pdf	159485 3d5aaef0aeaac8faeba9856f01edfcd537fe7e3d8f	no	1
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	30771 07f06b6b79b2816fddfc13beb955f412d75999fb916	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	190256		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of:	Group Art Unit: 1611
Inventor(s): Patrick H. Witham, et al.	Examiner: Frazier, Barbara S.
Serial No.: 14/080,771	Confirmation No.: 6889
Filed: November 14, 2013	Customer No.: 21971
Title: METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

SUPPLEMENTAL NOTIFICATION OF CHANGE IN ENTITY STATUS

Sir:

Applicants provide a supplemental itemization of fees in furtherance of the Notification of Change of Entity Status filed September 9, 2014. The additional deficiency payments are outlined on the following page.

Deficiency Payment

Description	Date Paid	Small Entity Fee Paid	Large Entity Fee	Deficiency Owed
Processing Fee	11/14/2013	$70	$140	$70
Processing Fee	07/25/2014	$70	$140	$70
			TOTAL AMOUNT OWED:	**$140**

The Commissioner is authorized to charge the above fees, and any additional fees which may be required, including petition fees, or credit any overpayment to Deposit Account No. 23-2415 (Docket No. 44630-701.201).

Respectfully submitted,

WILSON SONSINI GOODRICH & ROSATI

Dated: September 10, 2014

By: /Michael Hostetler/

Michael J. Hostetler, Registration No.: 47,664

650 Page Mill Road
Palo Alto, California 94304
(650) 493-9300
Customer No.: 21971

Electronic Acknowledgement Receipt

EFS ID:	20102993
Application Number:	14080771
International Application Number:	
Confirmation Number:	6889
Title of Invention:	METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS
First Named Inventor/Applicant Name:	Patrick H. Witham
Customer Number:	21971
Filer:	Michael J. Hostetler/Linda Anders
Filer Authorized By:	Michael J. Hostetler
Attorney Docket Number:	44630-701.201
Receipt Date:	10-SEP-2014
Filing Date:	14-NOV-2013
Time Stamp:	17:06:59
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Miscellaneous Incoming Letter	44630-701-201-entitystatuschange2.pdf	92772 298e1bd3c0f8900daab5e57e7a5281741 1c9576de	no	2

Warnings:	
Information:	

Total Files Size (in bytes):	92772

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/080,771	10/14/2014	8859623	44630-701.201	6889

21971 7590 09/24/2014

WILSON, SONSINI, GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050

ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment is 0 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Data Management (ODM) at (571)-272-4200.

APPLICANT(s) (Please see PAIR WEB site http://pair.uspto.gov for additional applicants):

Paragon BioTeck, Inc., Portland, OR, Assignee (with 37 CFR 1.172 Interest);
Patrick H. Witham, Eugene, OR;
Sailaja Machiraju, Beaverton, OR;
Lauren Mackenzie-Clark Bluett, Milwaukie, OR;

PHENYLEPHRINE HYDROCHLORIDE solution/ drops
[Akorn, Inc.]

Category	DEA Schedule	Marketing Status
HUMAN PRESCRIPTION DRUG LABEL		unapproved drug other

NOTE: THIS DRUG HAS NOT BEEN FOUND BY FDA TO BE SAFE AND EFFECTIVE, AND THIS LABELING HAS NOT BEEN APPROVED BY FDA. For further information about unapproved drugs, click here.

Drug Label Sections
Description | Clinical Pharmacology | Indications & Usage | Contraindications | Warnings | Precautions | Adverse Reactions
Overdosage | Dosage & Administration | How Supplied | Patient Counseling Information | Supplemental Patient Materials
Boxed Warning | Patient Package Insert | Highlights | Full Table of Contents | Medication Guide

2.5% — Sterile

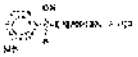
WARNING: PHYSICIANS SHOULD COMPLETELY FAMILIARIZE THEMSELVES WITH THE COMPLETE CONTENTS OF THIS LEAFLET BEFORE PRESCRIBING PHENYLEPHRINE HYDROCHLORIDE OPHTHALMIC SOLUTION.

Rx only

DESCRIPTION:

Phenylephrine Hydrochloride Ophthalmic Solution, USP is a vasoconstrictor and mydriatic for use in ophthalmology. Phenylephrine is a synthetic sympathomimetic compound structurally similar to epinephrine and ephedrine. Phenylephrine Hydrochloride Ophthalmic Solution is supplied as a sterile topical ophthalmic solution. The active ingredient, phenylephrine hydrochloride is represented by the chemical structure:

$C_9H_{13}NO_2 \cdot HCl$ Molecular weight 203.67



ESTABLISHED NAME: Phenylephrine Hydrochloride

CHEMICAL NAME: (-)-*m*-Hydroxy-α- [(methyl-amino)methyl] benzyl alcohol hydrochloride

Each mL of solution contains:

Active: Phenylephrine Hydrochloride 2.5%. **Inactives:** Sodium Phosphate Dibasic, Sodium Phosphate Monobasic, Sodium Hydroxide and/or Phosphoric Acid may be added to adjust pH (4.0 to 7.5), and Purified Water USP. **Preservative:** Benzalkonium Chloride 0.1 mg (0.01%).

CLINICAL PHARMACOLOGY:

Phenylephrine Hydrochloride Ophthalmic Solution is an alpha receptor sympathetic agonist used in local ocular disorders because of its vasoconstrictor and mydriatic action. It exhibits rapid and moderately prolonged action, and it produces little rebound vasodilation. Systemic side effects are uncommon.

Although rare, systemic absorption of sufficient quantities of phenylephrine may lead to systemic α-adrenergic effects, such as a rise in blood pressure which may be accompanied by a reflex atropine-sensitive bradycardia.

INDICATIONS AND USAGE:

Phenylephrine Hydrochloride Ophthalmic Solution is recommended as a vasoconstrictor, decongestant, and mydriatic in a variety of ophthalmic conditions and procedures. Some of its uses are for pupillary dilation in uveitis (to prevent or aid in the disruption of posterior synechia formation), for many ophthalmic surgical procedures and for refraction without cycloplegia. Phenylephrine Hydrochloride Ophthalmic Solution may also be used for funduscopy and other diagnostic procedures.

CONTRAINDICATIONS:

Ophthalmic solutions of phenylephrine hydrochloride are contraindicated in patients with anatomically narrow angles or narrow angle glaucoma. Phenylephrine hydrochloride may be contraindicated in low birth weight infants and in some elderly adults with severe arteriosclerotic cardiovascular or cerebrovascular disease. Phenylephrine hydrochloride may be contraindicated during intraocular operative procedures when the corneal epithelial barrier has been disturbed. This preparation is also contraindicated in persons with a known sensitivity to phenylephrine hydrochloride or any of its components.

WARNINGS:

Not for intraocular use. As with other adrenergic drugs, when Phenylephrine Hydrochloride Ophthalmic Solution is administered simultaneously with, or up to 21 days after, administration of monoamine oxidase (MAO) inhibitors, careful supervision and adjustment of dosages are required since exaggerated adrenergic effects may result. The pressor response of adrenergic agents may also be potentiated by tricyclic antidepressants. Systemic side effects are more common in patients taking beta adrenergic blocking agents such as propranolol. Concomitant use of phenylephrine and atropine may enhance the pressor effects and induce tachycardia in some patients, especially infants.[1]

There have been rare reports associating the use of phenylephrine hydrochloride 10% ophthalmic solutions with the development of serious cardiovascular reactions, including ventricular arrhythmias and myocardial infarctions. These episodes, some ending fatally, have usually occurred in elderly patients with preexisting cardiovascular diseases.

PRECAUTIONS:

General: Ordinarily, any mydriatic, including phenylephrine hydrochloride, is contraindicated in patients with glaucoma, since it may occasionally raise intraocular pressure. However, when temporary dilation of the pupil may free adhesions, this advantage may temporarily outweigh the danger from coincident dilation of the pupil. Rebound miosis has been reported in older persons one day after receiving phenylephrine hydrochloride ophthalmic solutions, and reinstillation of the drug may produce less mydriasis than previously. This may be of clinical importance in dilating the pupils of older subjects prior to retinal detachment or cataract surgery. The lacrimal sac should be compressed by digital pressure for two to three minutes after instillation to avoid excessive systemic absorption. Due to a strong action of the drug on the dilator muscle, older individuals may also develop transient pigment floaters in the aqueous humor 40 to 45 minutes following the administration of phenylephrine hydrochloride ophthalmic solution. The appearance may be similar to anterior uveitis or to a microscopic hyphema. To prevent pain, a drop of suitable topical anesthetic may be applied before using Phenylephrine Hydrochloride Ophthalmic Solution. Prolonged exposure to air or strong light may cause oxidation and discoloration. Monitor blood pressure in geriatric patients with known cardiac disease. Use caution in infants with known cardiac anomalies. Exceeding recommended dosages or applying Phenylephrine Hydrochloride Ophthalmic Solution to the instrumented, traumatized, diseased or postsurgical eye or adnexa, or to patients with suppressed lacrimation, as during anesthesia, may result in the absorption of sufficient quantities of phenylephrine to produce a systemic vasopressor response.

INFORMATION FOR PATIENTS: DO NOT TOUCH DROPPER TIP TO ANY SURFACE AS THIS MAY CONTAMINATE THE SOLUTION. DO NOT USE IF SOLUTION IS BROWN OR CONTAINS A PRECIPITATE.

Drug Interaction: As with all other adrenergic drugs, when Phenylephrine Hydrochloride Ophthalmic Solution is administered simultaneously with, or up to 21 days after, administration of monoamine oxidase (MAO) inhibitors, careful supervision and adjustment of dosages are required since exaggerated adrenergic effects may occur. The pressor response of adrenergic agents may also be potentiated by tricyclic antidepressants, propranolol, reserpine, guanethidine, methyldopa, and atropine-like drugs. Phenylephrine hydrochloride ophthalmic solutions may potentiate the cardiovascular depressant effects of potent inhalation anesthetic agents.

Carcinogenesis, Mutagenesis, Impairment of Fertility:

Carcinogenicity studies with phenylephrine hydrochloride have been completed in mice at doses up to 2500 ppm in feed and in rats at doses up to 1250 ppm in feed. Phenylephrine hydrochloride demonstrated no carcinogenic effect in male or female mice and rats.

Pregnancy: Pregnancy Category C. Animal reproduction studies have not been conducted with phenylephrine hydrochloride ophthalmic solution. It is also not known whether phenylephrine hydrochloride ophthalmic solution can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Phenylephrine Hydrochloride Ophthalmic Solution should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Phenylephrine Hydrochloride Ophthalmic Solution is administered to a nursing woman.

Pediatric Use: Phenylephrine Hydrochloride Ophthalmic Solution may be contraindicated in low birth weight neonates and infants. For use in older children see DOSAGE AND ADMINISTRATION.

Exceeding recommended dosages or applying Phenylephrine Hydrochloride Ophthalmic Solution to the instrumented, traumatized, diseased or post surgical eye or adnexa, or to patients with suppressed lacrimation, as during anesthesia, may result in the absorption of sufficient quantities of phenylephrine to produce a systemic vasopressor response.

The hypertensive effects of phenylephrine may be treated with an alpha-adrenergic blocking agent such as phentolamine mesylate, 5 mg to 10 mg intravenously, repeated as necessary.

The oral LD_{50} of phenylephrine in the rat: 350 mg/kg, in the mouse: 120 mg/kg.

Concomitant use of phenylephrine and atropine may enhance the pressor effects and induce tachycardia in some patients, especially infants.

Use with caution in infants with known cardiac anomalies.

ADVERSE REACTIONS:

A marked increase in blood pressure has been reported in low-weight premature neonates, infants and adult patients with idiopathic orthostatic hypotension. Cardiovascular reactions which have occurred primarily in elderly patients include marked increase in blood pressure, syncope, myocardial infarction, tachycardia, arrhythmia, and fatal subarachnoid hemorrhage.[2]

Other reactions include bradycardia, headache, and excitability.

DOSAGE AND ADMINISTRATION:

Vasoconstriction and Pupil Dilatation: Phenylephrine Hydrochloride Ophthalmic Solution is especially useful when rapid and powerful dilatation of the pupil without cycloplegia and reduction of congestion in the capillary bed are desired. A drop of a suitable

topical anesthetic may be applied, followed in a few minutes by 1 drop of Phenylephrine Hydrochloride Ophthalmic Solution to the upper limbus. The anesthetic prevents stinging and consequent dilution of the solution by lacrimation. It may occasionally be necessary to repeat the instillation after one hour, again preceded by the use of the topical anesthetic.

Uveitis: Posterior Synechiae: Phenylephrine Hydrochloride Ophthalmic Solution may be used in patients with uveitis when synechiae are present or may develop. The formation of synechiae may be prevented by the use of this solution and atropine or other cycloplegics to produce wide dilation of the pupil. For recently formed posterior synechiae one drop of Phenylephrine Hydrochloride Ophthalmic Solution may be applied to the upper surface of the cornea and be repeated as necessary, not to exceed three times. Treatment may be continued the following day, if necessary. Atropine sulfate and the application of hot compresses should also be used if indicated.

Glaucoma: Phenylephrine Hydrochloride Ophthalmic Solution may be used with miotics in patients with open angle glaucoma. It reduces the difficulties experienced by the patient because of the small field produced by miosis, and still it permits and often supports the effect of the miotic in lowering the intraocular pressure in open angle glaucoma. Hence, there may be marked improvement in visual acuity after using Phenylephrine Hydrochloride Ophthalmic Solution in conjunction with miotic drugs.

Surgery: When a short-acting mydriatic is needed for wide dilation of the pupil before intraocular surgery, Phenylephrine Hydrochloride Ophthalmic Solution may be applied topically from 30 to 60 minutes before the operation.

Refraction: Phenylephrine Hydrochloride Ophthalmic Solution may be used effectively to increase mydriasis with homatropine hydrobromide, cyclopentolate hydrochloride, tropicamide hydrochloride and atropine sulfate.

FOR ADULTS: One drop of the preferred cycloplegic is placed in each eye, followed in 5 minutes by one drop of Phenylephrine Hydrochloride Ophthalmic Solution. Since adequate cycloplegia is achieved at different time intervals after the instillation of the necessary number of drops, different cycloplegics will require different waiting periods to achieve adequate cycloplegia.

FOR CHILDREN: For a "one application method," Phenylephrine Hydrochloride Ophthalmic Solution may be combined with one of the preferred rapid acting cycloplegics to produce adequate cycloplegia.

Ophthalmoscopic Examination: One drop of Phenylephrine Hydrochloride Ophthalmic Solution is placed in each eye. Sufficient mydriasis to permit examination is produced in 15 to 30 minutes. Dilation lasts one to three hours.

Diagnostic Procedures:

Provocative Test for Angle Closure Glaucoma:

Phenylephrine Hydrochloride Ophthalmic Solution may be used cautiously as a provocative test when interval narrow angle closure glaucoma is suspected. Intraocular tension and gonioscopy are performed prior to and after dilation of the pupil with phenylephrine hydrochloride. A "significant" intraocular pressure (IOP) rise combined with gonioscopic evidence of angle closure indicates an anterior segment anatomy capable of angle closure. A negative test does not rule this out. This pharmacologically induced angle closure glaucoma may not simulate real life conditions and other causes for transient elevations of IOP should be excluded.

Retinoscopy (Shadow Test): When dilation of the pupil without cycloplegic action is desired for retinoscopy, Phenylephrine Hydrochloride Ophthalmic Solution may be used.

NOTE: Heavily pigmented irides may require larger doses in all of the above procedures.

Blanching Test: One or two drops of Phenylephrine Hydrochloride Ophthalmic Solution should be applied to the injected eye. After five minutes, examine for perilimbal blanching. If blanching occurs, the congestion is superficial and probably does not indicate iridocyclitis.

HOW SUPPLIED:

Phenylephrine Hydrochloride Ophthalmic Solution, USP is supplied as a sterile solution in plastic dropper bottles in the following sizes:

2 mL-NDC 17478-200-20

15 mL-NDC 17478-200-12

Storage: Store at 20° to 25°C (68° to 77°F) [see USP Controlled Room Temperature]. Keep container tightly closed.

Protect from light and excessive heat.

Do not use if solution is brown or contains precipitate.

REFERENCES:

1. Praunfelder, P.T., and Meyer, S.M.: Possible Cardiovascular Effects Secondary to Topical Ophthalmic 2.5% Phenylephrine, Am. J. Oph. 99:3:362, 1985.
2. Ibid.

Akorn

Manufactured by: Akorn, Inc.
Lake Forest, IL 60045

DLA0N
Rev. 07/11

Principal Display Panel Text for Container Label:

NDC 17478-200-20

Phenylephrine

Hydrochloride

Ophthalmic

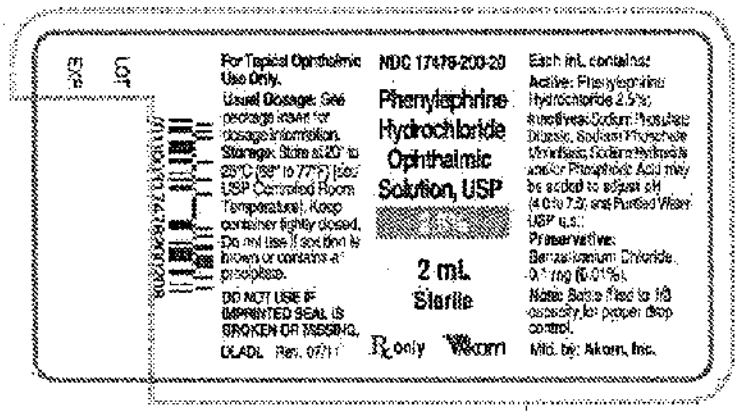
Solution, USP

2.5%

2 mL

Sterile

Rx only [Akorn logo]



Principal Display Panel Text for Carton Label:

NDC 17478-200-20

Phenylephrine

Hydrochloride

Ophthalmic

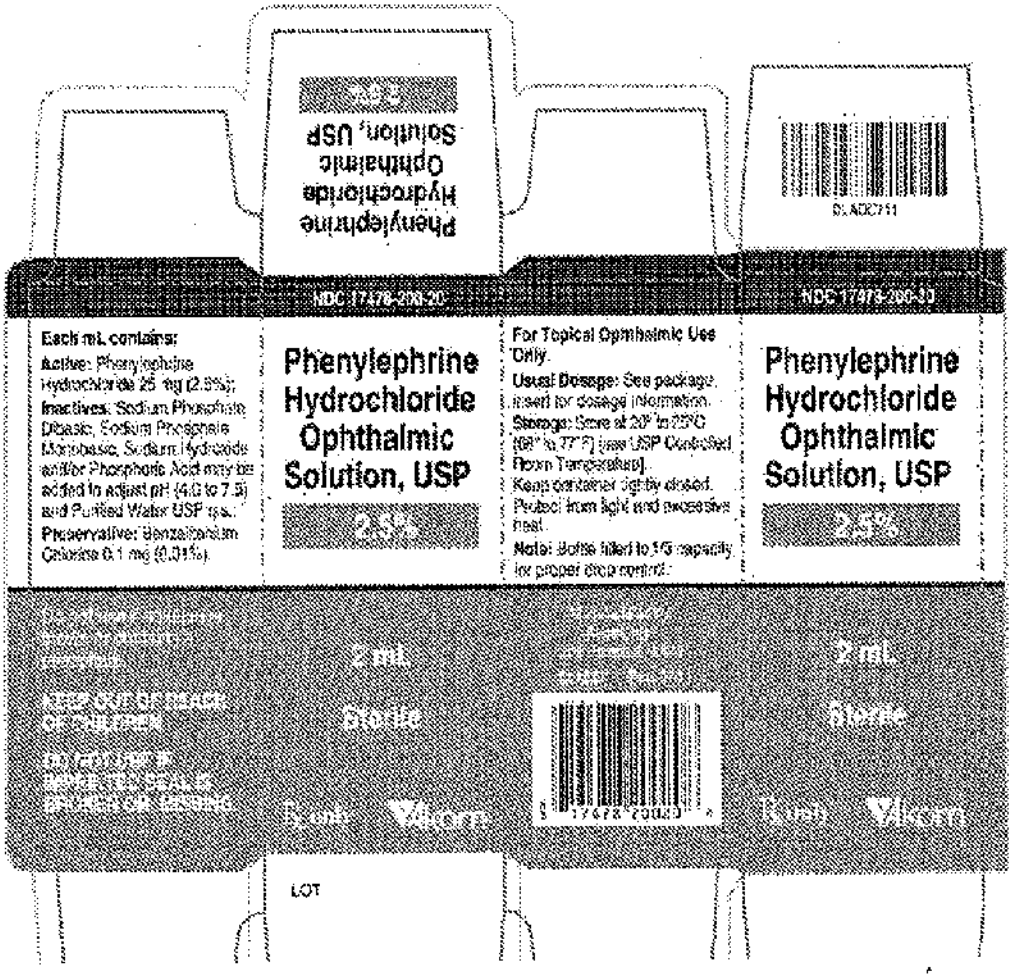
Solution, USP

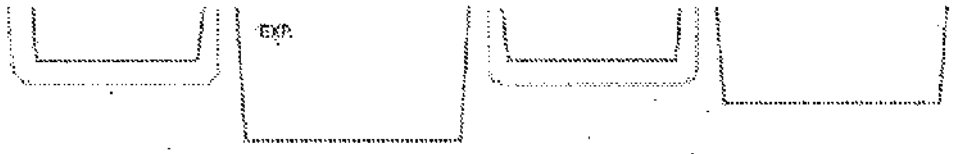
2.5%

2 mL

Sterile

Rx only [Akorn logo]





PHENYLEPHRINE HYDROCHLORIDE

phenylephrine hydrochloride solution/ drops

Product Information

Product Type	HUMAN PRESCRIPTION DRUG LABEL	Item Code (Source)	NDC:17478-200
Route of Administration	OPHTHALMIC	DEA Schedule	

Active Ingredient/Active Moiety

Ingredient Name	Basis of Strength	Strength
Phenylephrine Hydrochloride (Phenylephrine)	Phenylephrine Hydrochloride	25 mg in 1 mL

Inactive Ingredients

Ingredient Name	Strength
sodium phosphate, dibasic	
sodium phosphate, monobasic	
sodium hydroxide	
phosphoric acid	
water	
benzalkonium chloride	

Packaging

#	Item Code	Package Description	Marketing Start Date	Marketing End Date
1	NDC:17478-200-10	1 in 1 CARTON		
1		2 mL in 1 BOTTLE, DROPPER		
2	NDC:17478-200-12	1 in 1 CARTON		
2		15 mL in 1 BOTTLE, DROPPER		

Marketing Information

Marketing Category	Application Number or Monograph Citation	Marketing Start Date	Marketing End Date
unapproved drug other		05/01/1991	

Labeler - Akorn, Inc. (062649876)

Establishment

Name	Address	ID/FEI	Business Operations
Akorn, Inc.		153313783	MANUFACTURE, REPACK, ANALYSIS

Revised: 9/2011 Akorn, Inc.

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Activities of octopamine and synephrine stereoisomers on α-adrenoceptors

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1 The activities of the (−)- and (+)-forms of *m*- and *p*-octopamine and *m*- and *p*-synephrine on α_1-adrenoceptors from rat aorta and anococcygeus and α_2-adrenoceptors from rabbit saphenous vein were compared with those of noradrenaline (NA).

2 The rank order of potency of the (−)- forms on α_1-adrenoceptors from rat aorta and α_2-adrenoceptors was NA > *m*-octopamine = *m*-synephrine > *p*-octopamine = *p*-synephrine. The two *m*-compounds were 6 fold less active than NA on α_1-adrenoceptors from rat aorta and 150 fold less active on α_2-adrenoceptors. The two *p*- compounds were 1,000 fold less active than NA on both α_1-adrenoceptors from rat aorta and α_2-adrenoceptors. The rank order of potency of the (−)- forms on α_1-adrenoceptors from rat anococcygeus was NA = *m*-synephrine > *m*-octopamine > *p*-octopamine = *p*-synephrine. *m*-Octopamine was 4 fold less active than NA and (−)-*m*-synephrine. The two *p*-compounds were 30 fold less active than NA.

3 The rank order of potency of the (+)- forms was NA > *m*-octopamine > *m*-synephrine > *p*-octopamine > *p*-synephrine on both α_1- and α_2-adrenoceptors. The potency of each (+)-form was 1–2 orders of magnitude less than that of the (−) counterpart, the differences being greater for the stereoisomers of synephrine than for those of octopamine on both α_1- and α_2-adrenoceptors.

4 The yohimbine diastereoisomer antagonists, rauwolscine and corynanthine, were tested against (−)-NA and (−)-*m*-octopamine-induced contractions in both preparations. Based upon the known selectivities of these isomers for α-adrenoceptor subtypes, it is concluded that the rat aorta contains only α_1-adrenoceptors while the rabbit saphenous vein possesses predominantly α_2-adrenoceptors.

5 Ligand binding data for the octopamine and synephrine stereoisomers at α_1- and α_2-binding sites from rat cerebral cortex was also obtained. (−)-Forms were more active than (+)-forms. The rank order of affinity of the (−)-forms for both α_1- and α_2-binding sites was NA > *m*-octopamine = *m*-synephrine > *p*-synephrine > *p*-octopamine. The relative affinities of the members of the series against α_1-binding sites were very similar to their relative functional activities on rat aorta. However, the affinities of both *m*- and *p*-compounds relative to that of (−)-NA were much greater at the α_2-binding sites than were the relative activities in rabbit saphenous vein, possibly suggesting low intrinsic efficacy. Functional antagonist responses to NA by the (−)-octopamine and synephrines could not, however, be demonstrated on rat aorta or rabbit saphenous vein.

6 The activities of *m*-octopamine and *m*-synephrine were not significantly different from each other on either α_1-adrenoceptors from rat aorta or α_2-adrenoceptors; however, *m*-synephrine is more active than *m*-octopamine on α_1-adrenoceptors from rat anococcygeus. Both *m*-octopamine and *m*-synephrine can be considered to be naturally occurring α_1-selective amines. However, if *m*- and *p*-octopamine are co-released with NA in amounts proportional to their concentration, it is concluded that their activities on α_1- and α_2-adrenoceptors are too low to be physiologically significant.

Introduction

meta-Octopamine together with its positional isomer *para*-octopamine and their two N-methyl derivatives, *meta*-synephrine (phenylephrine) and *para*-synephrine are now known to occur naturally in mammalian tissues (Ibrahim *et al.*, 1985). However, *m*- and *p*-synephrine are found only in adrenal gland, whilst *m*-

and *p*-octopamine are found in several sympathetically innervated organs (heart, spleen, vas deferens, intestine, kidney, liver, lung, brain) and in adrenal medulla. Treatments which increase (monamine oxidase inhibition) or decrease (6-hydroxydopamine) tissue noradrenaline (NA) levels also affect *m*- and *p*-octopamine concentrations in identical fashion. Radioactive *m*- and *p*-octopamine are both taken up in noradrenergic nerve terminals, accumulated in storage vesicles, and released together with NA (Kopin *et al.*, 1964; Reimann, 1984). It is therefore probable that both *m*- and *p*-octopamine coexist with NA in mammalian sympathetic nerves and are released with NA as co-transmitters by adrenergic nerve stimulation, in the manner first proposed for *p*-octopamine by Axelrod & Saavedra (1977). Although co-transmission is now known to occur throughout the central and peripheral nervous system and it is probable that all nerves contain two or more co-transmitters (O'Donohue *et al.*, 1985), little is known about the mechanisms of neuromodulation produced by the release of multiple co-transmitters. The actions of structurally dissimilar co-transmitters such as adenosine triphosphate (ATP) and NA are mediated by purinoceptors and adrenoceptors respectively (Burnstock & Sneddon, 1985). However, *m*- and *p*-octopamine are structurally so similar to NA that it is reasonable to suppose that their actions might be mediated by one or more of the well-characterized adrenoceptors. The physiological effects of *p*-octopamine and *m*-octopamine were first determined with racemates (Lands & Grant, 1952; Lands, 1952). Later investigations were performed with the (−) enantiomers of *p*-octopamine (Korol *et al.* (1968) and *m*-octopamine (Della Bella & Galli, 1955) but they were carried out on selected *in vivo* responses before the different subtypes of adrenoceptors were recognized. As a result, the activities of the pure (−)- and (+)-forms of *m*- and *p*-octopamine on α_1- and α_2-adrenoceptors have not been determined.

Here we describe the activities of the stereoisomers of *m*- and *p*-octopamine and *m*- and *p*-synephrine on postjunctional α_1- and α_2-adrenoceptors. We used the rat aorta and anococcygeus for postjunctional α_1-adrenoceptors and the rabbit saphenous vein for postjunctional α_2-adrenoceptors. Rat aorta was chosen because it is now generally accepted that only α_1-adrenoceptors are located there (Downing *et al.*, 1983; Digges & Summers, 1983a; Hamed *et al.*, 1983; Ruffolo, 1985). Rat anococcygeus is also believed to contain only α_1-adrenoceptors (McGrath, 1984). Smooth muscle contraction of the rabbit saphenous vein is mediated mainly, if not entirely, by adrenoceptors of the α_2-subtype (Alabaster *et al.*, 1985). This conclusion is based on the evidence that phenylephrine is 250 fold less potent than NA and that the responses to NA and phenylephrine are insensitive to the selective α_1-antagonist prazosin, but sensitive to the selective α_2-antagonist rauwolscine. Availability of these compounds also provided an opportunity for further study of the structural requirements for affinity and activity of α-adrenoceptor subtypes.

Methods

Male Wistar rats (250–300 g) were stunned and killed by cervical dislocation. The thoracic aorta were removed and sectioned into 2 mm rings from which the endothelium was removed by mechanical rubbing. This procedure shifts the concentration-response curves of NA and phenylephrine to the left and increases maximum attainable tension (Godfraind *et al.*, 1985). The rings were then suspended in a Krebs bicarbonate solution at 37°C under 1.5 g tension for 1 hour and allowed to equilibrate before examining the contractile response. Functional disruption of the endothelium-derived relaxant factor was demonstrated on each tissue by raising its tone with NA (10^{-7} M) and showing that acetylcholine (3×10^{-6} M), which always produced relaxation in unrubbed controls, was then ineffective.

The whole of each anococcygeus (excluding the ventral bar) was suspended in Krebs solution at 37°C under 0.5 g tension for 1 hour. Then cocaine (3×10^{-6} M) was added to block neuronal uptake of catecholamines 10 min before each concentration-response curve was determined.

Male rabbits were stunned and exsanguinated. The saphenous vein was removed and sectioned into 2–3 mm long rings. The rings were then mounted in an organ bath containing Krebs solution at 37°C, given an initial resting tension of 2 g and allowed to equilibrate for 1 hour. Cocaine (3×10^{-6} M) to block neuronal uptake of catecholamines was added to the baths 10 min before each concentration-response curve was determined; it shifted the concentration-response curve to the left. Cocaine was not present in the aorta experiments nor was a β-blocker added with either tissue since these treatments did not affect control concentration-response curves to NA. pD_2 values were determined in the absence of β-adrenoceptor antagonists because, as demonstrated by Jordan *et al.* (1987), these compounds are not active at β-adrenoceptors. However, when pA_2 or $-\log K_B$ values for antagonists were determined propranolol (1 μM) was included to eliminate completely the possibility of concomitant stimulation of β-adrenoceptors.

Krebs bicarbonate-saline composition in mmol l^{-1} was: NaCl 119, KCl 4.7, $MgSO_4$ 1.0, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.0 and glucose 11.1. It was gassed with 95% O_2 plus 5% CO_2. Contractile responses were recorded by Grass isometric transducers by means of either a Grass polygraph or Linseis recorder.

Protocol for concentration-response curves

Cumulative concentration-response curves to NA and to the octopamine and synephrine stereoisomers were constructed by adding them to the baths in steps of 0.5 log units. Curves to NA and to other test agonists were alternated. After obtaining initial concentration-response curve to NA the preparations were washed 3 times with Krebs solution over a 15 min period. A concentration-response curve to NA was then obtained before the subsequent addition of each test substance. In each experiment four rings were cut from one aorta and four different agonists were tested on each ring. The concentration-response curves to NA obtained after that of each test substance did not change significantly; neither the EC_{50} nor the maximum tension achieved were altered significantly. An exception to this was the rat anococcygeus; the first NA curve lay to the left of subsequent ones and was discarded. Agonist potency was measured as the concentration required to produce 50% of the maximum contraction to NA (EC_{50} NA) because, for some agonists, maxima could not be obtained, due to an insufficient supply of test substances to complete the curves.

Calculation of potency was made by graphical interpolation of the curve for log (agonist concentrations) versus response to find the pD_2NA ($-\log$ agonist concentration) which gave 50% of the maximum contraction to NA. For individual tissues EC_{50} = antilog ($-pD_2$). The average potency for a given compound is expressed as the mean of the pD_2NA values ± s.e.mean.

Following construction of a concentration-response curve to either NA or *m*-octopamine, preparations of the rat aorta or rabbit saphenous vein were exposed to an antagonist for a minimum of 40 min and the concentration-response curve repeated. The agonist concentration-ratio (i.e., EC_{50} of the agonist in the presence of the antagonist divided by the control EC_{50} value) produced by the antagonist was determined at different concentrations spanning a range of 50 fold. According to Arunlakshana & Schild (1959) if antagonism is competitive, a plot of the log of (concentration-ratio − 1) against the log of the molar concentration of the antagonist yields a straight line whose slope is 1 and the intercept along the abscissa scale is the pA_2 which is equal to the K_B (equilibrium conditions). In all experiments, one preparation was run in parallel with the experimental tissue, but received no antagonist, and was used to correct for time-dependent changes in agonist sensitivity (Furchgott, 1972).

In addition, $-\log K_B$ values for the antagonists were also determined in each tissue at each concentration of the antagonist, by the concentration-ratio method of Furchgott (1972) for those agents that did not display competitive antagonism.

Ligand binding assays

Male Sprague-Dawley rats (150–200 g) were killed by cervical dislocation, the brains rapidly removed and dissected on ice. Cerebral cortices were homogenized in 20 volumes of Tris buffer (50 mmol l^{-1} Tris. HCl, 5 mmol l^{-1} EDTA; pH 7.4 at 25°C) using a Polytron PT 10 tissue disruptor (setting 10; 2 × 10 s bursts). The homogenate was filtered through a single layer of cheesecloth and the filtrate centrifuged at 38,000 g_{av} for 15 min. The pellet obtained was washed 3 times by resuspension and centrifugation in Tris assay buffer (50 mmol l^{-1} Tris HCl, 0.5 mmol l^{-1} EDTA; pH 7.4 at 25°C). The final pellet was resuspended in assay buffer for direct use in binding studies.

Competition α_1-adrenoceptor binding assays were performed by incubating washed rat cerebrocortical membranes (0.5 mg ml^{-1} membrane protein) with [^{3}H]-prazosin 1.0 nmol l^{-1} in the presence or absence of a range of 13 concentrations of the competing ligands in a total volume of 0.25 ml of Tris assay buffer. Non-specific binding was defined as the concentration of bound ligand in the presence of 1×10^{-5} mmol l^{-1} phentolamine. Following equilibrium (30 min at 25°C) bound ligand was separated from free by vacuum filtration over Whatman GF/B glass fibre filters, which were then rinsed with 3 × 5 ml ice-cold buffer. Radioactivity bound to the glass fibre filters was determined by liquid scintillation spectrophotometry.

α_2-Adrenoceptor binding assays were performed in a similar manner by incubation of washed rat cerebrocortical membranes (1.0 mg ml^{-1} membrane protein) with [^{3}H]-yohimbine (2.0 nmol l^{-1}). Membrane protein was determined by the method of Lowry *et al.* (1951).

The inhibition of specific binding of the radioligands by competing ligands was analysed graphically to estimate the IC_{50} (concentration of competitor displacing 50% of specifically bound radioligand), using a non-linear least squares programme which is specially designed for the interpretation of sigmoidal concentration-response curves in terms of total and non-specific binding as well as inhibition constants and curve steepness.

Drugs used

The following drugs were used: (−)-noradrenaline bitartrate; corynanthine · HCl (Sigma); (+)-noradrenaline bitartrate; prazosin HCl (Pfizer); rauwolscine · HCl (Carl Roth); (−)-*m*-synephrine · HCl (m.p. 141–142°C, $[\alpha]_D^{22}$ −43°C, c 0.1 (H_2O)), B.D.H. Ltd.; (+)-*m*-synephrine · HCl (m.p. 142°C, $[\alpha]_D^{22}$ + 50.3°C, c 0.1 (H_2O), Ganes Chemicals Inc. Racemic *m*- and *p*-octopamine and *p*-synephrine were

resolved with appropriate (+)- and/or (−)-organic acids, followed by fractional crystallization of the diastereoisomeric salts and ion-exchange to afford the corresponding optically active hydrochloride salt. Full experimental details of these procedures and determinations of the absolute configurations of these compounds will be published elsewhere. (+)-*m*-Octopamine · HCl (Aldrich Chem. Co. Ltd; (+)- and (−)-*O,O*-dibenzoyltartaric acid, Aldrich Chem. Co. Ltd.) afforded (−)-*m*-octopamine · HCl (m.p. 127°C, $[\alpha]_D^{22}$ −39°C, c 0.1 (H_2O)) and (+)-*m*-octopamine · HCl (m.p. 125°C, $[\alpha]_D^{22}$ + 37.5°C, c 0.1 (H_2O)). (±)-*p*-Octopamine · HCl (Aldrich Chem. Co. Ltd.; (+)-10-camphorsulphonic acid monohydrate, Aldrich Chem. Co. Ltd.) gave (−)-*p*-octopamine · HCl (m.p. 176°C, $[\alpha]_D^{22}$ −50°C, c 0.1 (H_2O)) and (+)-*p*-octopamine · HCl (m.p. 177–178°, $[\alpha]_D^{22}$ + 46°, c 0.1 (H_2O)). (+)-*p*-Synephrine · HCl (Sigma; (+)- and (−)-bromocamphorsulphonic acid, ammonium salt, Aldrich Chem. Co. Ltd. and Chemical Dynamic Corp. respectively) yielded (−)-*p*-synephrine · HCl (m.p. 176°C, $[\alpha]_D^{22}$ −39°C, c 0.1 (H_2O)) and (+)-*p*-synephrine · HCl (m.p. 178°C, $[\alpha]_D^{22}$ + 42°C, c 0.2 (H_2O)). Drugs were dissolved in distilled water except for NA which was diluted in distilled water containing 23 μM EDTA. [^{3}H]-prazosin (specific activity 80.9 Ci mmol^{-1}) and [^{3}H]-yohimbine (specific activity 80.9 Ci mmol^{-1}) were obtained from Dupont.

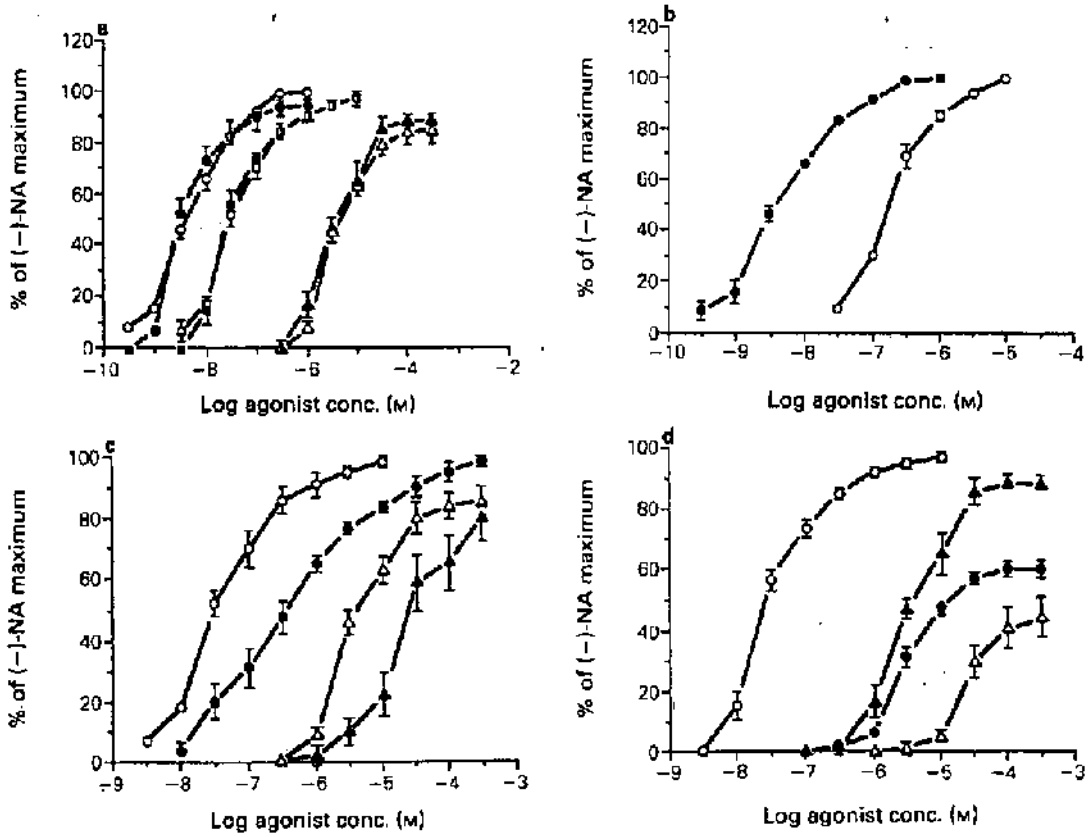

Figure 1 Concentration-response curves produced by (−)- and (+)-noradrenaline (NA), the stereoisomers of octopamine and synephrine and (−)-adrenaline in the rat isolated thoracic aorta. (a) Comparison of the (−)-isomers of noradrenaline (○), adrenaline (●), *m*-octopamine (□), *p*-octopamine (△), *m*-synephrine (■) and *p*-synephrine (▲). (b) Comparison of (−)-noradrenaline (○) and (+)-noradrenaline (●). (c) Comparison of the stereoisomers of octopamine: (−)-*m*-octopamine (○), (+)-*m*-octopamine (●), (−)-*p*-octopamine (△), (+)-*p*-octopamine (▲). (d) Comparison of the stereoisomers of synephrine: (−)-*m*-synephrine (○), (+)-*m*-synephrine (●), (−)-*p*-synephrine (▲), (+)-*p*-synephrine (△). All responses are expressed as a % of the maximum response to (−)-noradrenaline in a minimum of four preparations from different animals and the vertical bars indicate the s.e.mean from these observations.

Results

α_1-Adrenoceptor activity in rat aorta and rat anococcygeus

The α_1-adrenoceptor activities of the (−)- forms of NA, adrenaline, *m*-octopamine, *m*-synephrine (phenylephrine), *p*-octopamine and *p*-synephrine in rat aorta without endothelium are shown in Figure 1a. The concentration-response curves for NA and adrenaline were superimposable as were the pairs of curves for *m*-octopamine and *m*-synephrine and for *p*-octopamine and *p*-synephrine. This resulted in a rank order of potency of NA = adrenaline > *m*-octopamine = *m*-synephrine > *p*-octopamine = *p*-synephrine. The activities of *m*-octopamine and *m*-synephrine were about six fold less than for NA and the activities of *p*-octopamine and *p*-synephrine about 1,000 fold less. These values are in good agreement with an earlier investigation on the rat aorta in which racemic *m*-octopamine and (−)*m*-synephrine were 3–4 fold less active than NA and racemic *p*-octopamine was 1,000 fold less (Ress *et al.*, 1980). In contrast to the (−)-forms, the rank order of potency of the (+)- forms was NA > *m*-octopamine > *m*-synephrine > *p*-octopamine > *p*-synephrine. The pD_2 NA values for these compounds for α_1-adrenoceptors are presented in Table 1 together with isomeric activity ratios. The pD_2 value for rat aorta with endothelium removed of 8.30 for NA is in excellent agreement with the value of 8.17 obtained by Godfraind *et al.* (1985).

It is apparent that α_1-adrenoceptor activity is associated predominantly with the (−)-isomers whereas the (+)- isomers are from 1 to 3 orders of magnitude weaker (Figure 1b,c,d). The (+)- isomers of the *m*-octopamine and *m*-synephrine pair and the *p*-octopamine and *p*-synephrine pair were not equiactive like their (−)-counterparts: the (+)- isomers of *m*- and *p*-octopamine were 8 and 5 fold less active than the (−)- isomers (Figure 1c) while the (+)- isomers of *m*- and *p*-synephrine were 420 and 75 fold less active than the (−)- forms, respectively (Figure 1d).

The properties of the α_1-adrenoceptors in rat anococcygeus were similar but not identical to those in rat aorta. The concentration-response curves (Figure 2) of NA and (−)-*m*-synephrine were superimposable and (−)-*m*-octopamine was about 4 fold less active than either. The two (−)-*p*- compounds gave concentration-response curves which were not significantly different. This resulted in a rank order of potency of NA = (−)-*m*-synephrine > (−)-*m*-octopamine > (−)-*p*-octopamine = (−)-*p*-synephrine. In contrast to the α_1-adrenoceptors in rat aorta the two (−)-*p*-compounds were only 30 fold less active than NA. The rank order of potency of the (+)- forms was identical to that observed with the α_1-adrenoceptors in rat aorta, i.e. *m*-octopamine > *m*-synephrine > *p*-octopamine > *p*-synephrine. The pD_2 NA values for these compounds are presented in Table 2 together with isomeric activity ratios.

α_2-Adrenoceptor activity in rabbit saphenous vein

The α_2-adrenoceptor effects of the (−)- forms of NA, adrenaline, *m*- and *p*-octopamine and *m*- and *p*-synephrine in rabbit saphenous vein are shown in Figure 3a. The rank order of potency was the same as for the α_1-adrenoceptors in rat aorta, i.e. NA = adrenaline

Table 1 The activity of the stereoisomers octopamine and synephrine on rat aorta with endothelium removed

	n	pD_2NA (± s.e.mean)	Relative potency	Fraction of NA maximum	Isomeric activity ratio (−)/(+)	Potency relative to corresponding octopamine
(−)-Adrenaline	3	8.48 (± 0.09)	1.51	0.95	—	
(−)-Noradrenaline	31	8.30 (± 0.04)	1.00	1.00	—	
(−)-*m*-Octopamine	11	7.50 (± 0.08)	0.16	0.98	—	
(−)-*m*-Synephrine	8	7.50 (± 0.09)	0.16	0.97	—	1.0
(−)-*p*-Octopamine	7	5.34 (± 0.09)	0.001	0.85	—	
(−)-*p*-Synephrine	7	5.38 (± 0.13)	0.001	0.89	—	1.0
(+)-Noradrenaline	3	6.78 (± 0.04)	0.03	1.00	33	
(+)-*m*-Octopamine	12	6.61 (± 0.15)	0.02	0.98	8	
(+)-*m*-Synephrine	12	4.88 (± 0.07)	0.0004	0.60	420	0.02
(+)-*p*-Octopamine	8	4.66 (± 0.25)	0.0002	0.80*	5	
(+)-*p*-Synephrine	8	3.50 (± 0.31)	0.00002	0.49*	75	0.1

*Satisfactory maximum not attained (see Figure 1). This is the mean of the responses to the highest concentration tested.

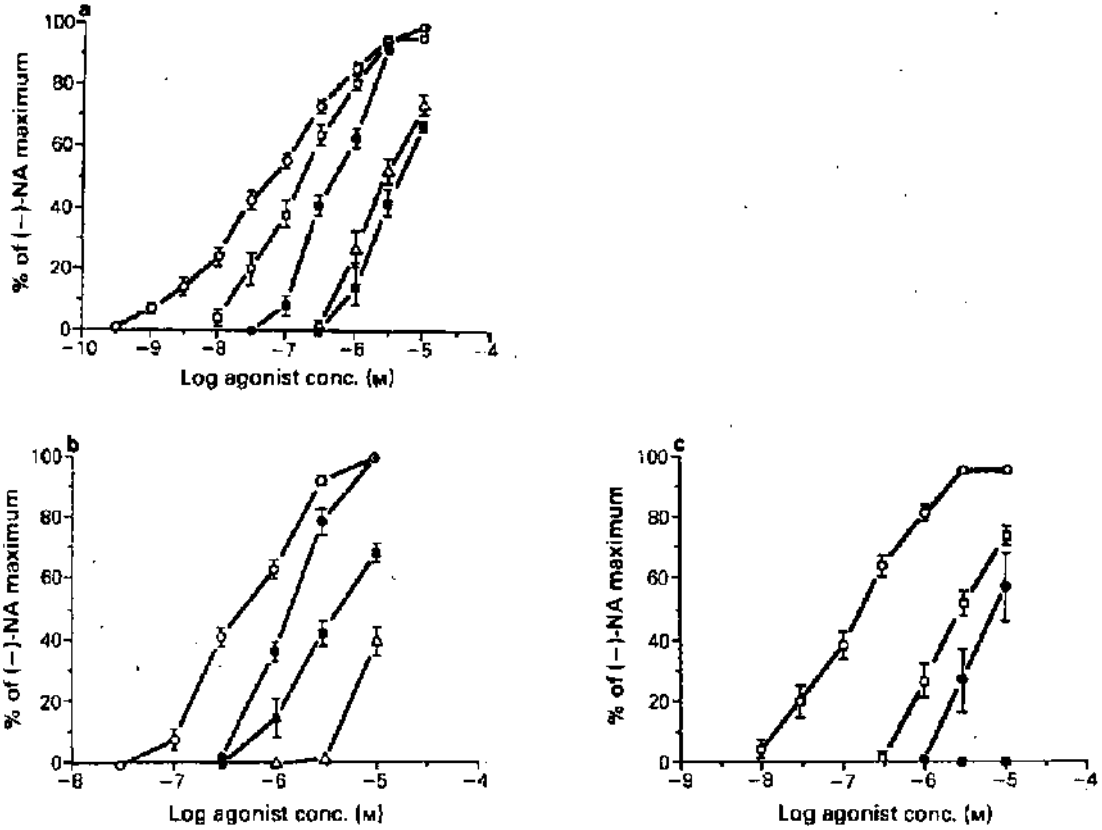

Figure 2 Concentration-response curves produced by (−)-noradrenaline (NA) and the stereoisomers of octopamine and synephrine in the rat isolated anococcygeus muscle. (a) Comparison of the (−) isomers of noradrenaline (○), *m*-octopamine (●), *p*-octopamine (■), *m*-synephrine (□) and *p*-synephrine (△). (b) Comparison of the stereoisomers of octopamine: (−)-*m*-octopamine (○), (+)-*m*-octopamine (●), (−)-*p*-octopamine (■), (+)-*p*-octopamine (△). (c) Comparison of the stereoisomers of synephrine: (−)-*m*-synephrine (○), (+)-*m*-synephrine (●), (−)-*p*-synephrine (□), (+)-*p*-synephrine (■). All responses are expressed as a % of the maximum response to (−)-noradrenaline in a minimum of four preparations from different animals and the vertical bars indicate the s.e.mean from these observations.

> *m*-octopamine = *m*-synephrine > *p*-octopamine = *p*-synephrine. However, the magnitudes of the shifts in the concentration-response curve were not consistently similar to those found in rat aorta (shown in parentheses): the *m*-octopamine-*m*-synephrine pair were about 150 fold (6 fold) less active than NA and the *p*-octopamine-*p*-synephrine pair were about 1,000 fold (1,000 fold) less active than NA. The rank order of potency of the (+)- compounds was NA > *m*-octopamine > *m*-synephrine > *p*-octopamine > *p*-synephrine, i.e. the same sequence as for the α_1-adrenoceptors. The pD_2NA values for these compounds are presented in Table 3 together with the isomeric activity ratios. The pD_2 for NA in the rabbit saphenous vein of 7.60 was in good agreement with the value of 7.20 obtained by Alabaster *et al.* (1985): their value of 0.004 for the potency of (−)-phenylephrine relative to (−)-NA was also in good agreement with our value of 0.007.

As with the α_1-adrenoceptors, the activities of the (−)- forms were 1–3 orders of magnitude greater than for the (+)- forms and the shift to the right by the (+)-forms of the two octopamines was less than the shift to the right by the (+)- forms of the two synephrines (Figure 3b,c,d).

Assessment of antagonist effects of octopamine and synephrine stereoisomers on α_1- and α_2-adrenoceptors

The effects of the (−)-stereoisomers as α-adrenoceptor antagonists were determined by testing them as antagonists to NA at concentrations known from the

Table 2 The activity of the stereoisomers of octopamine and synephrine on the rat anococcygeus

	n	*pD_2NA (± s.e.mean)*	*Relative potency*	*Fraction of NA maximum*	*Isomeric activity ratio (−)/(+)*	*Potency relative to corresponding octopamine*
(−)-Noradrenaline	17	6.95 (± 0.09)	1.00	1.00	—	
(−)-*m*-Synephrine	14	6.75 (± 0.07)	0.63	0.95	—	2.7
(−)-*m*-Octopamine	14	6.31 (± 0.05)	0.23	1.00	—	
(−)-*p*-Synephrine	15	5.54 (± 0.07)	0.04	0.74*	—	1.3
(−)-*p*-Octopamine	15	5.35 (± 0.06)	0.03	0.68*	—	
(+)-*m*-Octopamine	4	5.84 (± 0.04)	0.08	0.99	3	
(+)-*m*-Synephrine	4	5.08 (± 0.19)	0.01	0.57*	45	0.13
(+)-*p*-Octopamine	4	4.82** —	<0.007	0.39*	3	
(+)-*p*-Synephrine	4	<<5 —	<<0.005	—	—	—

* Satisfactory maximum not attained (see legend to Table 1).
**Estimated by extrapolation.

earlier experiments to be at the threshold for contraction. A shift of the NA concentration-response curves could not be demonstrated (1) in rabbit saphenous vein at levels up to 10^{-5} M for *m*-octopamine and *m*-synephrine and for levels as high as 10^{-5} M for *p*-octopamine and *p*-synephrine; nor (2) in rat aorta for levels as high as 10^{-6} M for *p*-octopamine and *p*-synephrine.

Antagonism of noradrenaline and (−)-m-octopamine by corynanthine and rauwolscine

In the rat aorta both corynanthine and rauwolscine produced parallel shifts in the concentration-response curve for (−)-NA and (−)-*m*-octopamine and the slope of the Schild plots did not differ significantly from unity. Based upon the values of pA_2, corynanth-

Table 3 The activity of the stereoisomers of octopamine and synephrine on the rabbit saphenous vein

	n	*pD_2NA (± s.e.mean)*	*Relative potency*	*Fraction of NA maximum*	*Isomeric activity ratio (−)/(+)*	*Potency relative to corresponding octopamine*
(−)-Noradrenaline	56	7.60 (± 0.04)	1.00	1.00	—	
(−)-Adrenaline	8	7.33 (± 0.10)	0.53	1.00	—	
(−)-*m*-Octopamine	12	5.39 (± 0.07)	0.006	0.85	—	
(−)-*m*Synehrine	12	5.45 (± 0.06)	0.007	1.00	—	1.2
(−)-*p*-Octopamine	11	4.66 (± 0.08)	0.001	1.00	—	
(−)-*p*-Synephrine	12	4.36 (± 0.08)	0.0006	0.88*	—	5.9
(+)-Noradrenaline	4	5.92 (± 0.09)	0.02	0.93*	48	
(+)-*m*-Octopamine	4	4.63 (± 0.06)	0.001	0.89	6	
(+)-*m*-Synephrine	4	3.85 (± 0.04)	0.0002	0.81*	40	0.08
(+)-*p*-Octopamine	4	3.51 (± 0.06)	0.00008	0.67*	14	
(+)-*p*-Synephrine	4	<3.00 —	<0.00002	0.13*	>20	<0.3

*Satisfactory maximum not attained (see Figure 3). This is the mean of the responses to the highest concentration tested.

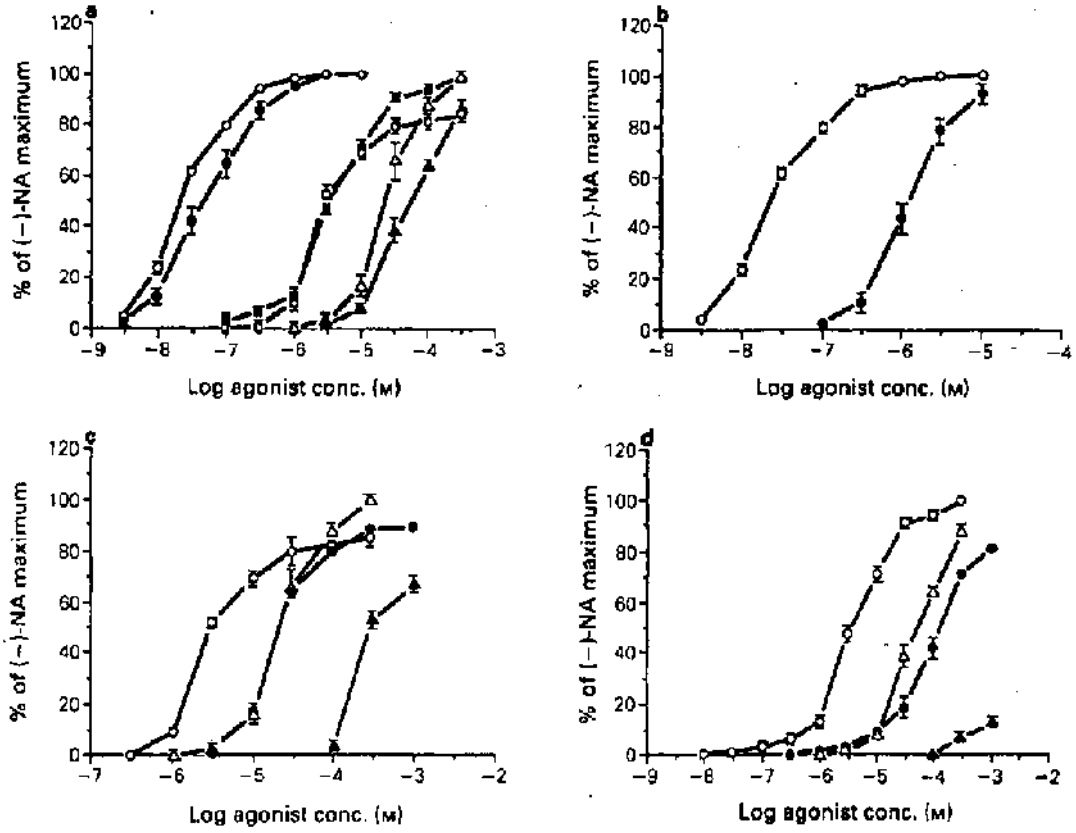

Figure 3 Concentration-response curves produced by (−)- and (+)-noradrenaline (NA), the stereoisomers of octopamine and synephrine and (−)-adrenaline in the rabbit isolated saphenous vein. (a) Comparison of the (−) isomers of noradrenaline (○), adrenaline (●), *m*-octopamine (□), *p*-octopamine (△), *m*-synephrine (■) and *p*-synephrine (▲). (b) Comparison of (−)-noradrenaline (○) and (+)-noradrenaline (●). (c) Comparison of the stereoisomers of octopamine: (−)-*m*-octopamine (○), (+)-*m*-octopamine (●), (−)-*p*-octopamine (△), (+)-*p*-octopamine (▲). (d) Comparison of the stereoisomers of synephrine: (−)-*m*-synephrine (○), (+)-*m*-synephrine (●), (−)-*p*-synephrine (△), (+)-*p*-synephrine (▲). All responses are expressed as a % of the maximum response to (−)-noradrenaline in a minimum of four preparations from different animals and the vertical bars indicate the s.e.mean from these observations.

ine was significantly more potent than rauwolscine against both agonists. In the rabbit saphenous vein rauwolscine, in contrast to corynanthine, produced non-parallel rightward shifts of the concentration-response curves to both agonists. Rauwolscine was more potent than corynanthine, and the antagonism effected was not competitive (slope of Schild plot significantly less than unity). $-\log K_B$ values for the antagonists in the rabbit saphenous vein are shown in Table 4.

Ligand binding data

The relative affinities of the stereoisomers of octopamine and synephrine for the central α_1- and α_2-binding sites was determined by their potency to compete for the binding of the α_1-selective radioligand [^{3}H]-prazosin or the α_2-selective radioligand [^{3}H]-yohimbine. All the compounds acted in a concentration-dependent manner although showing only weak affinity. The affinity of each of the (−)-forms was greater than that of the (+)- counterpart. The data are presented in Tables 5 and 6.

For the (−)-enantiomers the rank order of affinity for the α_1-binding site was NA > *m*-octopamine > *m*-synephrine > *p*-synephrine > *p*-octopamine. The relative affinities of the four compounds correlated well with the pharmacological data for α_1-receptors in the rat aorta, with the two *m*-compounds being about 1/8th as active as NA and the two *p*-compounds being

Table 4 pA_2 values and slopes of Schild plots for corynanthine and rauwolscine in rat aorta and rabbit saphenous vein

Tissue	*Antagonist*	*Agonist*	n	pA_2	*Slope of Schild plot*
Rat aorta[a]	Corynanthine	(−)-NA	5	7.74 (± 0.08)	0.94 (± 0.04)
		(−)-*m*-Octopamine	5	7.49 (± 0.07)	0.96 (± 0.05)
	Rauwolscine	(−)-NA	6	6.73 (± 0.05)[b]	1.01 (± 0.13)
		(−)-*m*-Octopamine	5	6.90 (± 0.10)	0.93 (± 0.1)
Rabbit saphenous vein	Corynanthine	(−)-NA	5	6.56 (± 0.05)[c]	——
		(−)-*m*-Octopamine	5	6.02 (± 0.10)[c]	——
	Rauwolscine	(−)-NA	5	8.19 (± 0.06)[c]	——
		(−)-*m*-Octopamine	5	8.20 (± 0.06)[c]	——

Data shown are means ± s.e.mean.
[a] Endothelium removed.
[b] Value taken from Downing *et al.* (1983).
[c] $-\log K_B$ values determined in the presence of 50 nmol l^{-1} rauwolscine or 2500 nmol l^{-1} corynanthine.

about 1/1000th as active. All the stereoisomers produced Hill slopes close to unity (0.81–1.03).

The rank order of affinities for the (−)-enantiomers for the α_2-binding site was NA > *m*-synephrine > *m*-octopamine > *p*-synephrine > *p*-octopamine. However, in this case the relative potency did not correlate well with the pharmacological potency. Analysis of the nH values for the competitors of [^{3}H]-yohimbine binding revealed that similar to the catecholamines NA and adrenaline (−)-*m*-octopamine and (−)-*m*-synephrine had nH values significantly less than 1.0 (range 0.5–0.8), possibly suggesting agonist activity for these compounds (see Discussion). The nH values for the other compounds were not significantly different from unity.

Discussion

The major finding of this study was that (−)-*m*-octopamine and (−)-*p*-octopamine are less active than NA by factors of 4–6 and 30–1,000, respectively, on α_1-adrenoceptors and by factors of 150 and 1,000, respectively on α_2-adrenoceptors. The potency of the two amines varied depending on the tissue (aorta or anococcygeus) used. Since the concentration of NA is approximately 100 times greater than either *m*-octopamine or *p*-octopamine in most sympathetically innervated organs, it is apparent that if stimulation of adrenergic nerves leads to the co-release of these three amines in amounts proportional to their concentration, it is unlikely that modulation of NA neurotransmission by *m*- or *p*-octopamine can be mediated by α_1- or α_2-adrenoceptors. Both *m*- and *p*-octopamine are less active than (−)-NA by more than 3 orders of magnitude on β_1-adrenoceptors, and by more than 4 orders of magnitude on β_2-adrenoceptors (Jordan *et al.*, 1987). It is concluded that, if *m*- and *p*-octopamine have a physiological function, then this function is not mediated by any of the four adrenoceptor subtypes.

Another important finding of this study is that (−)-*m*-octopamine and (−)-*m*-synephrine do not significantly differ in their selectivity for α-adrenoceptor subtypes. Both are about 10–100 times more potent on α_1- than on α_2-adrenoceptors: *m*-octopamine may therefore be regarded as equivalent to *m*-synephrine (phenylephrine) as a selective α_1-agonist.

Based upon the reported selectivity of the yohimbine diastereoisomers corynanthine and rauwolscine for α_1- and α_2-adrenoceptors, respectively (McGrath, 1982), our observations with these antagonists appear to confirm the view that the rat aorta contains only α_1-adrenoceptors (Digges & Summers, 1983b), while the rabbit saphenous vein contains predominantly α_2-adrenoceptors (Alabaster *et al.*, 1985). The possible contribution of α_1-adrenoceptors to responses in the rabbit saphenous vein, as evidenced by the non-linearity of the Schild plots for rauwolscine, appears to be minimal since the selective α_1-adrenoceptor agonist (−)-phenylephrine/(−)-*m*-synephrine (McGrath, 1982) was markedly less potent than NA. A detailed study of the pharmacological characteristics of α-adrenoceptors on the rabbit saphenous vein will be published separately (McGrath & Wilson, unpublished observations).

In general, the isomeric ratios found in this study were low compared with those published for other tissues and were not greater for α_2- than for α_1-adrenoceptors (Ruffolo *et al.*, 1982). The outstanding feature of the isomeric ratios was the large ratio for each form of synephrine at α_1-adrenoceptors particularly *m*-synephrine with a ratio of 420. This was greater than that for NA or for the equivalent octopamine, suggesting that when the configuration of

Table 5 Ligand binding data for stereoisomers of octopamine and synephrine on α_1-adrenoceptor binding sites in the rat cerebral cortex

	pIC_{50}[1]	nH[1]	Affinity ratios: *Relative to NA*	Affinity ratios: *Relative to corresponding octopamine*	Affinity ratios: *Isomeric ratio (+)/(−)*	$pD_2NA - pIC_{50}$
(−)-Noradrenaline	5.04 ± 0.03	0.71 ± 0.05*	1			3.26**
(−)-Adrenaline	5.35 ± 0.05	0.70 ± 0.03*	2.04			3.13**
(−)-*m*-Octopamine	4.17 ± 0.05	0.97 ± 0.02	0.13			3.33
(−)-*m*-Synephrine	4.05 ± 0.11	0.85 ± 0.02	0.1	0.76		3.45
(−)-*p*-Octopamine	2.45 ± 0.05	0.84 ± 0.10	0.003			2.89
(−)-*p*-Synephrine	3.35 ± 0.10	0.87 ± 0.14	0.02	1.37		2.03
(+)-*m*-Octopamine	2.93 ± 0.09	1.02 ± 0.04	0.008		17.4	3.68
(+)-*m*-Synephrine	3.33 ± 0.07	1.03 ± 0.05	0.019	7.69	5.2	1.55
(+)-*p*-Octopamine	2.00 ± 0.15	0.81 ± 0.10	0.0009		3.5	2.66
(+)-*p*-Synephrine	2.42 ± 0.1	0.94 ± 0.02	0.002	2.63	8.5	1.08

[1] Data shown are means ± s.e.mean.
* Hill coefficient significantly different from 1.
** Value calculated for agonist with a Hill coefficient significantly different from 1.

Table 6 Ligand binding data for stereoisomers of octopamine and synephrine on α_2-adrenoceptor binding sites in the rat cerebral cortex

	pIC_{50}[1]	nH[1]	Affinity ratios: *Relative to NA*	Affinity ratios: *Relative to corresponding octopamine*	Affinity ratios: *Isomeric ratio (+)/(−)*	$pD_2NA - pIC_{50}$
(−)-Noradrenaline	6.07 ± 0.10	0.62 ± 0.05*	1			1.53**
(−)-Adrenaline	6.75 ± 0.07	0.53 ± 0.04*	4.79			0.58**
(−)-*m*-Octopamine	5.29 ± 0.17	0.72 ± 0.02*	0.16			0.1**
(−)-*m*-Synephrine	5.60 ± 0.08	0.72 ± 0.06*	0.34	2.1		−0.15**
(−)-*p*-Octopamine	4.62 ± 0.08	0.97 ± 0.02	0.04			0.04
(−)-*p*-Synephrine	5.01 ± 0.08	0.89 ± 0.09	0.09	2.4		−0.65
(+)-*m*-Octopamine	4.86 ± 0.13	1.02 ± 0.03	0.06		2.7	−0.23
(+)-*m*-Synephrine	4.66 ± 0.10	1.00 ± 0.05	0.04	0.63	8.7	−0.81
(+)-*p*-Octopamine	4.68 ± 0.04	0.81 ± 0.08	0.04		0.87	−1.07
(+)-*p*-Synephrine	4.17 ± 0.05	1.14 ± 0.11	0.01	0.31	6.9	< −1.17

[1] Data shown are means ± s.e.mean.
* Hill coefficient significantly different from 1.
** Value calculated for agonist with a Hill coefficient significantly different from 1.

the molecule is unfavourable for reaction at the receptor, the presence of the methyl substituent on the nitrogen and the loss of the *p*-OH group further diminish the interaction. Neither of these factors applied to the same extent to activity at α_1-adrenoceptors in rabbit saphenous vein, although the ratios were still slightly larger for the stereoisomers of synephrines than for those of octopamine. This appears to confirm that the position of the ring OH plays a less critical role at α_2- (indicated by the smaller difference in potency between all pairs of *p*- and *m*-compounds) than at α_1-adrenoceptors.

Ligand binding studies provide an alternative more direct means of determining the affinity of compounds for α_1- and α_2-adrenoceptors. The ligand binding data from the cerebral cortex showed that all octopamine and synephrine isomers were weak displacers of [^{3}H]-prazosin and [^{3}H]-yohimbine binding.

There is considerable evidence that the cerebral and peripheral subtypes of α-adrenoceptors are similar (Bylund & U'Prichard, 1983). However, caution is necessary in extrapolating too far, particularly as α_2-adrenoceptor isotypes have been proposed to explain the pharmacological differences between rodent and non-rodent species (Cheung *et al.*, 1982; Latifpour *et al.*, 1982; Feller & Bylund, 1984; Alabaster *et al.*, 1986) and there is mounting evidence of a heterogeneity within species (Bylund, 1985). In addition the ligand binding was carried out at a lower temperature (25°C cf 37°C).

Nevertheless if a similarity between receptors is assumed, and given that the ligand binding affords a measure of affinity, some deductions concerning intrinsic efficacy can be made by comparing the relative activities on smooth muscle. A comparison of the binding and functional activities is shown in Tables 5 and 6.

Considering the members of a series of compounds, if the difference between pD_2 and affinity remains constant as the pD_2 falls, then loss of activity can be attributed entirely to loss of affinity. This is essentially the case for α_1-adrenoceptors in rat aorta where, with the exception of (−)-NA and (−)-adrenaline, the nH values are near unity and the pIC_{50} determined at 1 nM [^{3}H]-prazosin should correlate with the pK_i (Table 5) and is true also for α_1-adrenoceptors in rat anococcygeus (not shown), with the exception of the (+)-synephrines whose activities are relatively poorer than is indicated by their affinities. Thus, within this series, all of the compounds appear to be full agonists with similar intrinsic efficacies and their different activities are attributable entirely to varying affinity for the receptor. This confirms a similar conclusion drawn from experiments with adrenaline and synephrines on α_1-adrenoceptors in the guinea-pig aorta (Ruffolo & Waddell, 1983). The (+)-synephrines, which deviate from this rule, are thus partial agonists relative to the other compounds.

For the octopamines the isomeric activity ratio can be accounted for solely by binding. The far larger ratio for the synephrines can be explained by postulating that the N-methyl group does not affect binding but dramatically reduces intrinsic efficacy in the (+) isomers. Partial agonism of the (+)-synephrines is confirmed by the low maximum (Table 1, Figure 3).

In contrast, for the α_2-adrenoceptors it is more difficult to draw comparisons between the binding data and the functional response, not only because of the heterogeneity of α_2-adrenoceptors mentioned above but also because the difference between agonist and antagonist binding must be considered (Hoffman & Lefkowitz, 1980). Analysis of the binding data showed that (−)-*m*-octopamine and (−)-*m*-synephrine had nH values <1.0, as had (−)-NA and (−)-adrenaline. One interpretation of this complex ligand/receptor interaction, particularly in view of the structural relationship between these two compounds and the catecholamines, would be to predict that the compounds have agonist activity, as indeed was seen in the functional studies. Partial agonist activity could be predicted for other compounds with nH values of 0.8−1.00. This might be verified in the binding studies by introducing guanine nucleotides such as GTP or the nonhydrolyzable analogue Gpp (NH)p which reduce the affinity of agonists at sites labelled by tritiated antagonists. However, this was not carried out since the partial agonist activity was clear in the rabbit saphenous vein.

In general, the fall in pIC_{50} (determined at 2 nM [^{3}H]-yohimbine) relative to that in pD_2 increased with diminishing activity, except that for the synephrines the loss was relatively even greater. Thus, as the compounds lose affinity for α_2-adrenoceptors, they also lose intrinsic activity; in contrast to α_1-adrenoceptors at which only affinity changed. This leads to the conclusion that all of the compounds are partial agonists relative to NA at α_2-adrenoceptors.

The isomeric ratio of the octopamines at α_1-adrenoceptors could be accounted for solely by affinity, while the synephrines had an additional element from intrinsic efficacy. For α_2-adrenoceptors the isomeric ratios were consistently higher for activity than for affinity, suggesting that, in this case, both intrinsic efficacy and affinity change in tandem.

One of the objectives of the study was to assess the potency of the series of compounds at α_1-adrenoceptors. The rat aorta was selected for its high sensitivity to agonists since this is necessary for some of the less potent compounds. However, it can be argued that rat aorta α_1-adrenoceptors are not 'typical'. First, their agonist potency series, particularly for non-phenylethanolamine agonists, show several deviations not found over the small group of other tissues where α_1-adrenoceptors have been studied in equivalent detail (Ruf-

folo, 1985; Digges & Summers, 1983a,b). Secondly, the potencies of several antagonists lie at the end of the spectrum of values found for them, including key compounds in α_1-adrenoceptor classification such as yohimbine and prazosin, both of which have relatively high pA_2 values (Randriantsoa *et al.*, 1981; Decker *et al.*, 1984). In order to verify 'α_1 potency', we repeated the assessment of the series on rat anococcygeus which has more typical α_1-adrenoceptors and at which prazosin has its 'normal' pA_2 values of 8.2 to 9.3 (Docherty & Starke, 1981; McGrath, 1984; Drew, 1985). Figure 4 shows that there is a good correlation between the potencies of NA, octopamine and synephrine stereoisomers on rat aorta and anococcygeus. The pA_2 values for the selective α_1-antagonist corynanthine against NA of 7.74 or (−)-*m*-octopamine of 7.49 are similar in separate studies of rat aorta (7.35 for NA, Digges & Summers, 1983b) and anococcygeus (7.3 for NA, McGrath, 1984). This validates the use of the aorta and confirms that for phenylethanolamines its α_1-adrenoceptor need not be considered unusual.

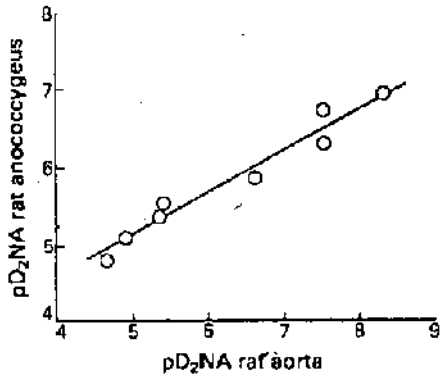

Figure 4 Correlation of activity of (−)-noradrenaline (NA) and the stereoisomers of octopamine and synephrine on α_1-adrenoceptors in rat aorta and anococcygeus.

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nen nicht. Andere Stämme — mit Chloramphenicol 4 und mit Nitrofurantoin 5 — lieferten wiederum wie bei Penicillin im Prinzip gleiche Resultate.

Für Staphylokokken läßt sich damit zusammenfassend sagen, daß Penicillin, Chloramphenicol und Nitrofurantoin zusammen mit Lysozym synergistisch geprägte antibakterielle Effekte erzielen, die Aussichten auf günstige Kombinationsmöglichkeiten eröffnen.

Entsprechende Versuche an *E. coli* mit Chloramphenicol und Nitrofurantoin ergaben dagegen nicht diese günstigen Ergebnisse. Auch hier wurden 100 und 500 γ/ml Lysozym mit steigenden Dosen der beiden antibakteriellen Substanzen kombiniert. In allen Versuchen wurde die Kombinationswirkung jedoch nur von der antibakteriell wirksamen Komponente, d. h. von Chloramphenicol oder Nitrofurantoin geprägt. Lysozym als die zweite Komponente der Kombination ließ keine Wirkungssteigerung eintreten.

Nach den vorliegenden Ergebnissen ist die Aussage erlaubt, daß unter der gegebenen Versuchsanordnung Penicillin, Chloramphenicol und Nitrofurantoin durch Kombination mit Lysozym auf Staphylokokken gesteigerte Wirksamkeit zeigen, während für *E. coli* entsprechende Effekte nicht festzustellen sind. Ob sich daraus Konsequenzen für das therapeutische Vorgehen am Krankenbett ergeben können, muß die intensive Zusammenarbeit mit dem Kliniker erst klären.

Zusammenfassung

Das Polypeptid Lysozym (Muramidase) — ein Komplex von über 100 Aminosäuren, der die Mucopolysaccharidmembran von Bakterien angreift, und der polyvalente Proteinaseinhibitor Trasylol®, wurden mit antibakteriellen Substanzen kombiniert um deren Hemmeffekt zu erhöhen. Während mit Trasylol keine Wirkung beobachtet wurde, zeigte die Kombination mit Lysozym, z. T. gute Beeinflussungsmöglichkeit, wie durch Messung des O_2-Verbrauchs von Mikroorganismen festgestellt wurde. Staphylokokken wurden durch die Kombination von Lysozym mit Penicillin, Chloramphenicol und Nitrofurantoin besser gehemmt als durch die Einzelsubstanzen. *E. coli* wurde in den Kombinationsversuchen dagegen nur durch den Chloramphenicol- bzw. Nitrofurantoin-Anteil beeinflußt.

Summary

Enzymatic Influence on the Effect of Antibiotics

The polypeptide lysozyme (muramidase), a complex of more than 100 amidoacids, which damages the mucopolysaccharide membrane of bacteria, and the polyvalent proteinase inhibitor trasylol were combined with antibacterial substances in order to increase their inhibitory activities. While trasylol showed no effect, in some cases of combination with lysozyme remarkable influence was observed as could be stated by measuring the O_2 consumption of the microorganisms. Staphylococci were more effectively inhibited by the combinations of lysozyme with penicillin, chloramphenicol or nitrofurantoin than by these substances alone. *E. coli*, on the other hand, was inhibited only by the chloramphenicol or nitrofurantoin part of the combinations.

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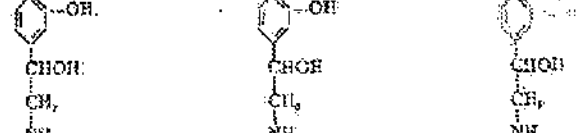
The Stability of Phenylephrine

Part I: The Rate of Degradation of the Amino Group

By H. A. M. El-Shibini, N. A. Daabis, and M. M Motawi

1. Introduction

L-m-Hydroxy-α-(methylaminomethyl)-benzylalcohol (phenylephrine; I) is a synthetic sympathomimetic drug. It is chemically very much related to adrenaline (II) and synephrine (III).



(I) (II) (III)

As could be depicted from its structure, phenylephrine is likely to undergo various changes including oxidation, racemisation, and cyclisation. However, only little work has been done on the stability of the drug. Thus, the presence of unidentified degradation products was reported by Schriftman [9]. The stability of phenylephrine in presence of acetylsalicylic acid in tablet form was investigated by Troup and Mitchner [10] who reported that acetylation of the secondary amino group of the phenylephrine molecule occurred by the acetic acid liberated from the hydrolysis of acetylsalicylic acid. Apart from these two publications, nothing is reported in the literature about the decomposition of phenylephrine. The previous investigations on the discolouration of phenylephrine solutions will be reviewed in the next part of this study.

As regards the proper choice of the method of assay for such a stability study, it is evident that the procedure of Auerbach [1] cannot be applied, since this method is based on the coupling reaction of phenylephrine with diazotised p-nitroaniline; it only indicates the presence of the phenolic group but fails to detect changes in the side chain of the molecule. The method of Troup and Mitchner [10], on the other hand, is based on the formation of a dialkyl dithiocarbamate by the interaction of phenylephrine with carbon disulphide. It is therefore an indication of the intact secondary amino group of the side chain which is an important centre for the pharmacological activity of the drug. It thus appears that the latter procedure is very suitable for the proper evaluation of the rate of degradation of phenylephrine.

2. Experimetal Part

2.1. Analytical procedure

The procedure developed by Troup and Mitchner [10] was applied in this study. A Unicam SP 500 spectrophotometer was used for this purpose. In the concentration range of 5 to 17.5 mg of phenylephrine per 100 ml of solution, the reaction obeyed Beer's law.

2.2. Kinetic studies

50 mg of phenylephrine, accurately weighed, were added to 50 ml of the appropriate buffer solution in a stoppering flask and maintained at the desired temperature in a thermostatically controlled water bath. After complete dissolution of the drug, an aliquot volume was withdrawn, suitably diluted, rapidly cooled in an ice bath

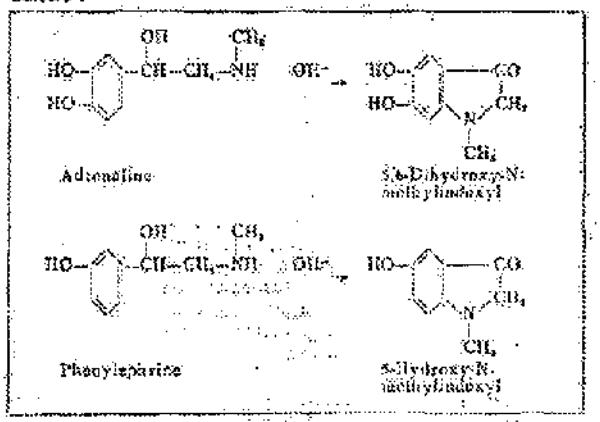
and then assayed by the above method. This was taken as the initial concentration of the solution. Samples were then assayed at suitable time intervals depending on the experimental conditions.

3. Results and discussion

3.1. Nature of the degradation reaction

The results obtained in the course of this investigation have demonstrated the gradual disappearance of the secondary amino group of the phenylephrine molecule. It is reasonable to suppose that the amino group of the side chain thereby participates in a process of cyclisation involving the activated carbon atom in p-

Scheme 1



position to the phenolic group. Although no oxidation products have been isolated, yet phenylephrine, in analogy to adrenaline [2—8], is thus most probably converted into 5-hydroxy-N-methylindoxyl (Scheme 1).

3.2. Reaction order

As is evident from the linear logarithmic plots illustrated in Fig. 1 to 6, the rate of degradation of phenylephrine in various buffered media appears to be first-order with respect to the drug concentration. This relationship was found over a wide range of pH (6.7—11) and temperature (57—97° C).

3.3. Effect of pH

An attempt was made to study the rate of disappearance of phenylephrine over a wide range of pH covering both the acid and alkaline sides of the scale. However, it appeared that the deterioration in acid medium proceeded so slowly that no detectable losses of phenylephrine could be observed after keeping the solution at pH 2 and 97° C for more than 10 days. So, the investigation of the rate of degradation of phenylephrine was carried out in almost neutral and alkaline media (pH 6.7—11).

As is illustrated in Fig. 7, the rate of deterioration increases with rise of pH, particularly above pH 9. In this respect, phenylephrine is similar to adrenaline and noradrenaline, although the respective rates of decomposition of these various drugs differ in their magnitude.

The results could be explained on the basis of the influence of pH on the activation of the benzene nucleus by the phenolic group. In acid medium, the phenolic group is completely undissociated whereas in alka-

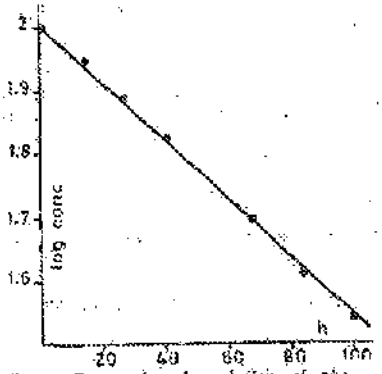


Fig. 1: First-order degradation of phenylephrine at pH 11 and 97° C; $k = 1.08 \times 10^{-1}\ h^{-1}$.

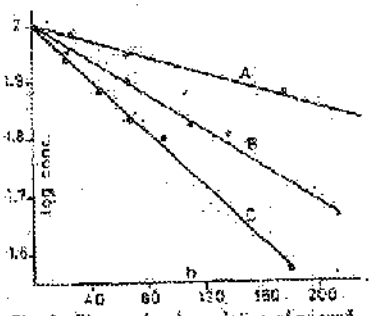


Fig. 2: First-order degradation of phenylephrine at 97° C. A = pH 7.1; B = pH 8.5; C = pH 9; $k = 4.703 \times 10^{-2}\ h^{-1}$; $k = 5.50 \times 10^{-2}\ h^{-1}$; $k = 9.4 \times 10^{-2}\ h^{-1}$.

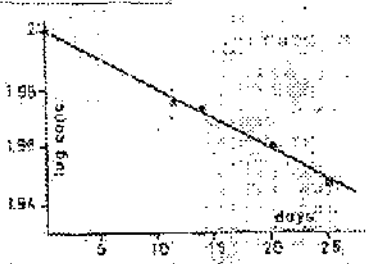


Fig. 3: First-order degradation of phenylephrine at 97° C and pH 8.2; $k = 2.09 \times 10^{-1}\ h^{-1}$.

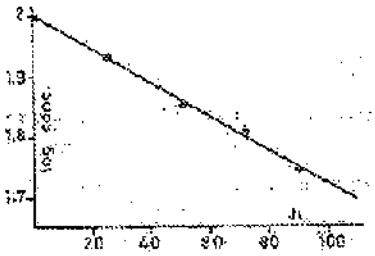


Fig. 4: First-order degradation of phenylephrine at pH 11 and 87° C; $k = 6.4 \times 10^{-2}\ h^{-1}$.

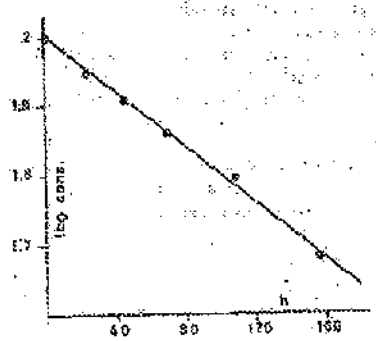


Fig. 5: First-order degradation of phenylephrine at pH 11 and 77° C; $k = 4.6 \times 10^{-2}\ h^{-1}$.

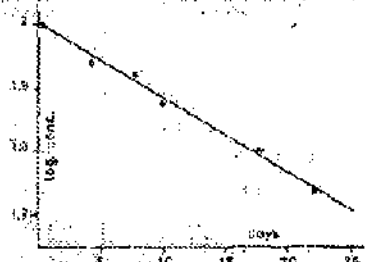


Fig. 6: First-order degradation of phenylephrine at pH 11 and 57° C; $k = 1.08 \times 10^{-2}\ h^{-1}$.

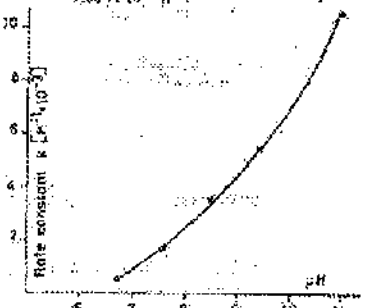


Fig. 7: Rate-pH profile for the degradation reaction of phenylephrine at 97° C.

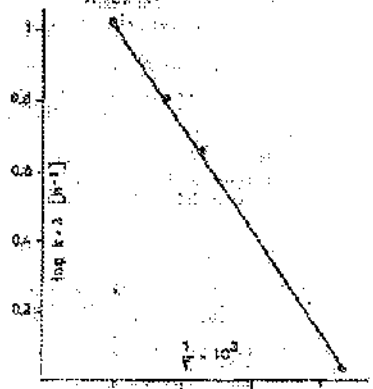


Fig. 8: Arrhenius plot for the degradation reaction of phenylephrine at pH 11.

line medium it would exist as a phenoxide ion. The activating effect of the latter on the p-position is obviously much more pronounced than that of the undissociated phenol; consequently, there should be a much greater possibility for the cyclisation reaction to take place in alkaline medium.

These results are of practical importance, as they clearly indicate that an acid medium is an essential condition for the stabilisation of phenylephrine solutions.

3.4. Effect of temperature

The effect of temperature on the rate of disappearance of the secondary amino group of phenylephrine was investigated with solutions of pH 11 in the range of 57 to 97° C. It appears from the results illustrated in Fig. 8 that the rate of decomposition of the drug is temperature-dependent and that the reaction obeys the Arrhenius equation for the thermal activation of molecules:

$$\log k = \log A - \frac{E_a}{2.303\,R} \cdot \frac{1}{T}$$

where k is the reaction rate constant,
A is the frequency factor,
E_a is the energy of activation,
R is the gas constant, and
T is the absolute temperature.

Now, the postulated oxidative cyclisation of phenylephrine into 5-hydroxy-N-methylindoxyl in buffered aqueous solutions must involve the participation of dissolved atmospheric oxygen. Accordingly, the reaction should not have followed Arrhenius equation, because the concentration of oxygen in solution decreases with rise of temperature. The fact that the rate of degradation of the drug is actually accelerated by raising the temperature indicates that free radicals and accompanying chain reactions are involved in the process; in such instances, the reaction rate is practically independent of the oxygen concentration of the solution.

The energy of activation, as graphically calculated from Fig. 8, was found to be equal to 13.728 kilocal./Mole.

Summary

The rate of degradation of L-m-hydroxy-a-(methylaminomethyl)benzyl alcohol (phenylephrine) in aqueous solutions was investigated by following the disappearance of the secondary amino of the side chain.

The results obtained indicate that the decomposition follows a first-order kinetic equation.

The reaction rate is pH-dependent, being very pronounced in alkaline medium and almost completely undetectable in the strong acid range (pH 2).

The degradation process follows Arrhenius equation in spite of the decrease of the oxygen concentration of the solutions with rise of temperature. At pH 11, the energy of activation for the reaction amounts to 13.728 kilocal./Mole.

Zusammenfassung

Die Stabilität von Phenylephrin.
Teil I: Abbaugeschwindigkeit der Amino-Gruppe

Die Abbaugeschwindigkeit des L-m-Hydroxy-a-(methylaminomethyl)benzylalkohols (Phenylephrin) wurde durch Beobachtung des Verschwindens der sekundären Aminogruppe der Seitenkette untersucht. Die erzielten Ergebnisse lassen erkennen, daß die Zersetzung gemäß einer Reaktion 1. Ordnung verläuft.

Die Reaktionsgeschwindigkeit ist pH-abhängig; sie ist sehr ausgeprägt in alkalischem und kaum nachweisbar in stark saurem Bereich (pH 2).

Der Abbau folgt der Arrhenius-Gleichung trotz der Erniedrigung der Sauerstoffkonzentration der Lösungen durch Erhöhung der Temperatur. Bei pH 11 beträgt die Aktivierungsenergie für die Reaktion 13.728 kilocal./Mol.

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Evaluation Studies on 4% Gelatin Solution Containing 1% Glycine for Intravenous Use

Effect of Gelatin Infusions on the Concentrations of Sodium, Potassium, Calcium and Magnesium in Blood of Rabbits

By Y. Gabr, El S. Amin, and A. El Waseef

With increasing knowledge of the normal clotting mechanism, it was realized that better results could be obtained, in the treatment of haemorrhage, by the application of substances supposed to hasten the clotting of blood *in vitro*. Such things as gelatin and pectin were used, which had the great advantage of being comparatively harmless. Gelatin is an unusual protein from the transfusion point of view, since it is not antigenic. These two advantages of gelatin prompted us to study some effects of gelatin solution on experimental animals.

In this paper, there are given the mean values of the concentrations of sodium, potassium, calcium, and magnesium in blood of experimental animals receiving infusions of a 4% autoclaved gelatin solution containing 1% glycine, as a plasma protein substitute. The molecular weight of this preparation was found to be in the range of 40 000 by the method of specific viscosity [1].

Accelerated aging tests, showed that the physicochemical characteristics of gelatin solution were improved by the addition of glycine [1], which was shown by Knoop and Bullitt [2] to abolish pseudo-agglutination of erythrocytes following intravenous infusion of gelatin.

Morphological changes were not detected in both kidney and liver cells of the gelatin-infused rats. There was only an increase in the amount of sudanophile granules in the liver cells. The activity of phosphatases, especially acid phosphatase, was found to increase one week post infusion of gelatin and began to normalise in the second, third, fourth, fifth, and sixth weeks post infusion of gelatin.

Methods

Sodium and potassium

Determination of sodium and potassium in rabbits' blood plasma was carried out by flame-photometry using a Beckman spectrophotometer, model DU and Beckman flame-photometer attachment (manufactured by National Technical Laboratories, South Pasadena, California), according to the method described by Mosher *et al.* [3]. The accuracy of this method depends on the use of synthetic standards in an effort to approximate the physical and chemical properties of the plasma samples to be tested. The viscosity of the standard solutions was made approximately equal to that of plasma by the addition of gelatin and cholesterol. Glucose, urea and all cations in concentrations approximating those of the samples to be analyzed, were also included in the stock solutions in order to carry out the

die Serumkonzentrationen nach 24 h in dem von Tab. 1 her bekannten Bereich. Die im 24-h-Rhythmus verabfolgte Erhaltungsdosis von 100 mg Doxycyclin hält die Serumspiegel auf diesem Niveau. Lediglich in einem Falle (Patient Tx) war nach 7 Tagen Doxycyclin-Medikation eine geringfügige Kumulationstendenz erkennbar, ohne daß jedoch eine ungewöhnliche Spiegelhöhe überschritten worden wäre. In den anderen Fällen blieb das initial erreichte Niveau während der Behandlungszeit erhalten. Auch bei diesen Ergebnissen fällt im Vergleich zu ähnlichen Untersuchungen bei nierengesunden Personen das gegenüber diesen praktisch unveränderte Verhalten der Doxycyclin-Serumspiegel auf [2].

Dieses fast identische Verhalten der Doxycyclin-Serumspiegel wie auch der Halbwertszeiten bei Nierengesunden und bei Patienten mit schwerer Niereninsuffizienz bzw. bei nierenlosen Kranken führt zwangsläufig zu der Frage nach dem Ausscheidungsmodus des Doxycyclins bei der Niereninsuffizienz.

Tab. 4 zeigt mit den aufgeführten Doxycyclin-Werten im ausgeschiedenen Urin, daß die renale Elimination in den eigenen Versuchsreihen zu vernachlässigen ist. Erschienen hierbei doch lediglich wenige Prozent der verabreichten Dosis innerhalb von 72 h im Urin wieder. Der Nierengesunde scheidet im Vergleich dazu etwa 40% der Doxycyclin-Dosis innerhalb von 72 h im Urin aus [2].

Weiteren Untersuchungen bleibt es vorbehalten, das Schicksal des Medikamentes zu klären. Möglicherweise kommt es zu einer Aktivierung der Ausscheidung über die Galle und den Darm, die beim Nierengesunden etwa 1/3 der Gesamtausscheidung ausmacht. Da mit großer Wahrscheinlichkeit das Medikament im Darm auch als Metabolit erscheint, kann dieser mit dem in den eigenen Untersuchungen angewendeten Testverfahren nicht erfaßt werden.

Zusammenfassung

Bei Patienten mit stark eingeschränkter Nierenfunktion sowie bei Kranken, denen zur Vorbereitung einer Transplantation beide Nieren entfernt worden waren, wurden die Serumkonzentrationen nach einmaliger und wiederholter Verabreichung von α-6-Desoxy-5-hydroxytetracyclinhydrochlorid (Doxycyclin, Vibramycin®) ermittelt. In beiden Versuchsanordnungen lagen die Serumspiegel in dem bei Nierengesunden gefundenen Bereich. Die fast identischen Halbwertszeiten des Doxycyclins bei Nierengesunden und bei Nierenkranken führen zu der Frage nach dem Schicksal des Doxycyclins bei Niereninsuffizienz.

Summary

Doxycycline Levels in the Serum of Patients with Renal Insufficiency

In patients with strongly reduced renal function and in such whose both kidneys had been removed in preparation of a transplantation the drug levels in the serum were determined after single and repeated administration of α-6-desoxy-5-hydroxytetracycline hydrochloride (doxycycline, Vibramycin®). In both test models the serum levels were in the same range as those found in persons with normally functioning kidneys. The nearly identical half-life values of doxycycline in subjects with normal and with defect kidneys suggest the question for the mode of excretion of doxycycline in the case of renal insufficiency.

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The Stability of Phenylephrine

Part II: The discolouration reaction and the influence of some ions on the rate of degradation of the drug)*

By H. A. M. El-Shibini, N. A. Daabis, and M. M. Motawi

1. Introduction

The discolouration of aqueous solutions of L-m-hydroxy-α-(methylaminomethyl)benzyl alcohol (phenylephrine) was first investigated by Schou and Rhodes [6], who showed that 1% injections were stable in this respect if made oxygen-free and kept in strongly acid medium (pH 1). Later, West and Whittet [8] reinvestigated the reaction using a rather highly concentrated solution (10%). With such solutions, the authors [8] found that the colour intensity was no indication of the decrease in activity. They also reported that the presence of heavy metals increased the discolouration of the solution. In the first part of this study [3] we had determined the rate of degradation of the secondary amino group of the phenylephrine molecule and thereby found that the drug was very rapidly deteriorated in alkaline medium and very stable in strong acid solutions. The aim of this work is to carry out detailed investigations in order to attempt to correlate between the rate of disappearance of the amino group and the discolouration of phenylephrine solutions. In addition, the present work includes a study on the effect of iron, copper, lead, calcium and magnesium on the rate of degradation of the drug.

2. Experimental

2.1. Analytical procedures

The determination of the secondary amino group of phenylephrine was carried out by application of the dialkyl dithiocarbamate method according to the procedure of Troup and Mitchner [7].

The rate of discolouration of phenylephrine solutions was followed by measuring the intensity of the yellow colour at 450 mμ. Structural changes in the phenylephrine molecule were detected by tracing the absorption spectrum of the solutions in the ultraviolet region.

In all cases, a UNICAM SP 500 spectrophotometer was used.

2.2. Kinetic studies

Aqueous buffered solutions containing 20 mg of phenylephrine in 100 ml were used and the same procedure adopted in the previous part of this study [3] was applied.

In certain experiments, 10 p.p.m. of calcium, magnesium, iron, copper, and lead were added using calculated amounts of their chlorides.

The effect of ethylenediamine tetraacetic acid (EDTA) was studied using 50 mg of the disodium salt per 100 ml of solution.

3. Results and discussion

3.1. Rate of discolouration

The yellow colour developed in solutions of phenylephrine does not exhibit any definite absorption maximum, which means that it is most probably due to the presence of a mixture of oxidation products. Its intensity was arbitrarily measured at 450 mμ which is usually a suitable wavelength for the determination of yellow coloured materials.

The rate of discolouration of phenylephrine solutions is illustrated in Fig. 1 and 2, from which it appears that the process is both pH- and temperature-dependent.

However, it can be readily deduced by careful analysis of those results that the rate of discolouration is very slow in comparison to that of the disappearance of the secondary amino group. For example, the extinction of the phenylephrine solution of pH 11 does not exceed 0.15 at 450 mμ after keeping at 97°C for 40 h. The solution is thus only pale yellowish coloured. According to our previous results [3], the reaction rate constant k for the degradation of the amino group of phenylephrine at pH 11 and 97°C amounts to 1.06 × 10⁻² h⁻¹. This means that such a solution will have lost about 35% of its phenylephrine content after 40 h at 97°C.

*) Part I s. [3].

Thus, the *discolouration* cannot be taken as a measure for the degree of degradation of phenylephrine, because the yellow colour becomes noticeable only after considerable decomposition of the drug. These results are in contradiction with those obtained by West and Whittet [8]. However, this difference can be explained by the fact that the latter authors [8] used a very concentrated solution (10%) as compared to that present in our experiments (0.02%). It follows that a slight degree of decomposition of phenylephrine in the concentrated solution would be accompanied by a marked discolouration of the solution.

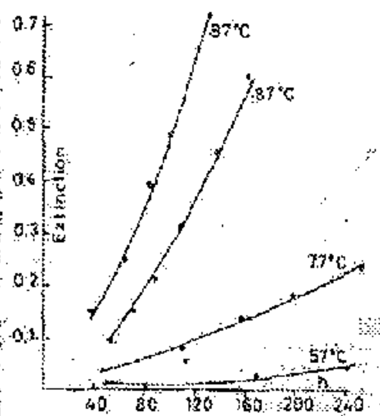

Fig. 1: Rate of discolouration of phenylephrine solutions of pH 11 and various temperatures.

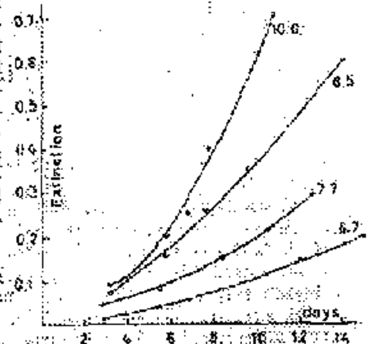

Fig. 2: Rate of discolouration of phenylephrine solutions at 97 °C and various pH values.

Apart from the yellow colour produced in phenylephrine solutions, a definite peak at 370 mμ appeared together with the disappearance of the secondary amino group of the phenylephrine molecule. This maximum is completely absent in the absorption spectrum of phenylephrine (Fig. 3). It increases in intensity with rise of pH (Fig. 4) and temperature (Fig. 5). This new peak corresponds most probably to the formation of 5-hydroxy-N-methylindoxyl by the oxidative cyclisation of phenylephrine.

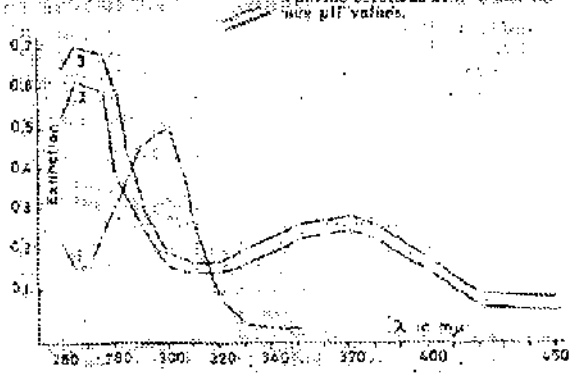

Fig. 3: Ultraviolet absorption spectrum of partially decomposed phenylephrine solutions. 1: Undecomposed phenylephrine; 2: after 4 days at pH 11 and 97° C; 3: after 5 days at pH 11 and 97° C.

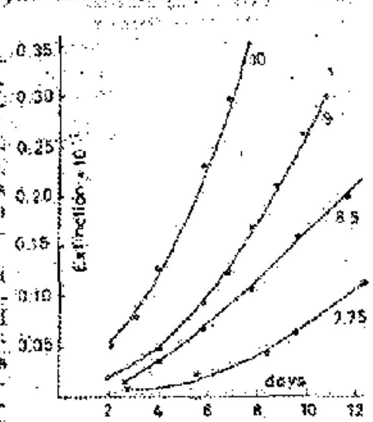

Fig. 4: Rate of formation of the decomposition product with λ_{max} 370 mμ at 97° C and various pH values.

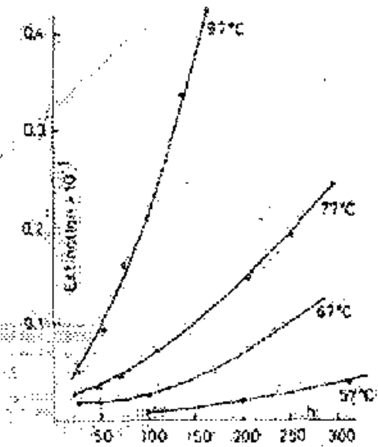

Fig. 5: Rate of formation of the decomposition product with λ_{max} 370 mμ at pH 11 and various temperatures.

It thus appears that the disappearance of the secondary amino group of phenylephrine represents the main reaction responsible for the decomposition of the drug. The yellow colour, on the other hand, results from a subsidiary reaction. The determination of the intensity of absorption of the solution at 370 mμ constitutes therefore, in addition to the estimation of the secondary amino group by the dialkyl dithiocarbamate method, a useful procedure for detecting early signs of decomposition of phenylephrine.

3.2. Effect of some metal ions

This study was performed at pH 11.67 and 97° C. The concentration of the investigated cations was 10 p.p.m.

The cupric ion was found to exert a marked effect on the rate of disappearance of the secondary amino group. Lead and iron showed only a little activity, calcium had no significant influence, while magnesium, on the other hand, proved to act as stabiliser (Fig. 6). Similar results were obtained with the investigation of the rate of formation of the degradation compound with the absorption maximum at 370 mμ (Fig. 7).

The assumption of a catalytic effect of the metals on the rate of oxidative decomposition of phenylephrine is reasonable for the interpretation of the influence of copper, lead and iron, particularly if it is taken

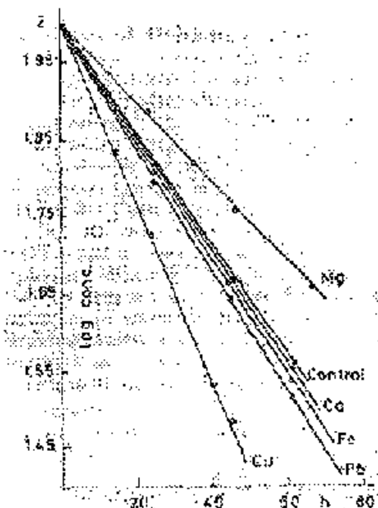

Fig. 6: Effect of metal ions on the rate of degradation of phenylephrine at 97° C and pH 11.67.

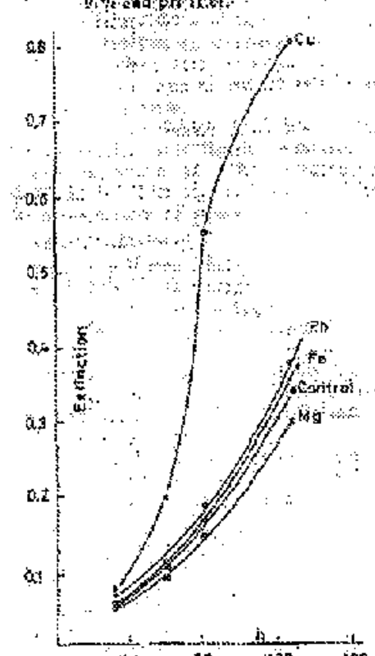

Fig. 7: Effect of metal ions on the rate of formation of the decomposition product with λ_{max} 370 mμ at 97° C and pH 11.67.

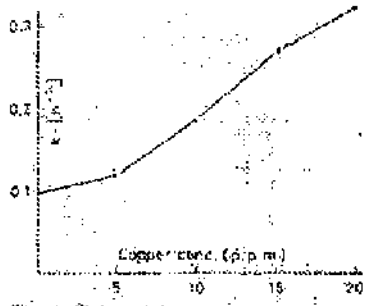

Fig. 8: Effect of the copper concentration on the rate of degradation of phenylephrine at pH 11 and 97°C.

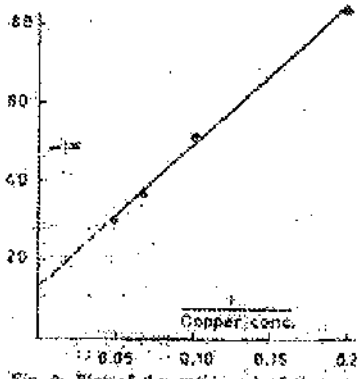

Fig. 9: Plot of the reciprocal of the copper concentration showing a limiting value for the catalytic action of the metal.

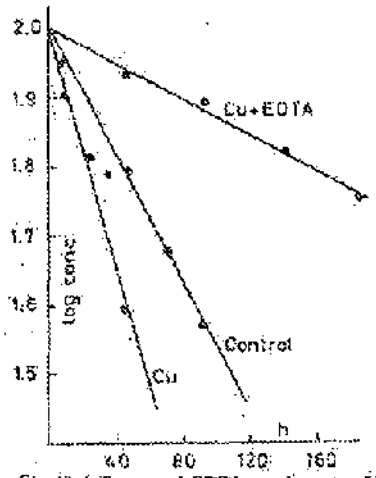

Fig. 10: Influence of EDTA on the rate of degradation of phenylephrine at pH 11 and 97° C.

in consideration that copper has a much higher oxidation reduction potential than lead and iron. However, this assumption does not explain the stabilising effect of magnesium. This shows that the catalytic effect of these elements is not of a simple nature. In addition to the redox potential, other factors must be involved in the process.

In a study on adrenaline [1], the formation of adrenaline-metal complexes of variable stability (depending on the nature of the metal) had been postulated. This opinion could be applied to explain the results obtained with phenylephrine. It could thus be said that copper, lead, and iron form with phenylephrine autoxidisable metal complexes of which the copper complex is the most instable. Also accordingly, calcium is unable to form such a complex; while magnesium gives with phenylephrine a complex which is less susceptible to oxidation than phenylephrine.

Of course, nothing can be decided with certainty about the validity of this hypothesis. The possible formation of such intermediate metal complexes requires further investigations and will be the subject of a later publication.

3.3. Effect of different copper concentrations

Since copper exerts a marked adverse influence on the stability of phenylephrine, it showed necessary to investigate this effect in more details. For this purpose, phenylephrine solutions buffered at pH 11 and containing different concentrations of copper ranging from 5 to 20 p.p.m. were examined at 97°C.

The results plotted in Fig. 8 indicate a non-linear relationship between the specific reaction rate and the copper concentration. This is more clearly illustrated in Fig. 9 where the reciprocal of the reaction rate constant k is plotted against the reciprocal of the copper concentration. Extrapolation of the straight line thereby obtained to infinite copper concentration indicates a limiting rate constant of 7.1×10^{-3} h^{-1}.

The fact that there does exist a copper concentration beyond which any additional amount of the metal does not produce a corresponding increase in the rate of oxidation of the drug could be attributed to one of the following factors:

a) Copper is consumed by phenylephrine to form a drug-metal complex. A degree of saturation exists when all the phenylephrine exists in a state of complex.

b) A state of equilibrium is established between phenylephrine, copper and the phenylephrine-copper complex.

These results represent a confirmation for the previously expressed assumption that the oxidative degradation of phenylephrine in presence of some metals proceeds through the formation of drug-metal intermediates.

Such saturation effects have been previously noted in studies on the cysteine-cystine system [2] and in the oxidation of 2,3-dimercaptopropanol (dimercaprol) [5].

3.4. Effect of chelating agents

From the previous experiments it was established that copper played a decisive catalytic role in the degradation of phenylephrine. So, an attempt was made to investigate the possible beneficial effect of EDTA as chelating agent in this respect.

The results of addition of 50 mg of EDTA to 100 ml of phenylephrine solution on the rate of degradation of the drug are illustrated in Fig. 10. It is evident that, unlike its action in the presence of adrenaline [4], EDTA has a very marked stabilising effect on phenylephrine. When EDTA is added, the rate of degradation is reduced to a value far below that of a normal solution of phenylephrine. This is due to its ability to build chelates with the traces of heavy metals normally present as impurities in distilled water and the components of the buffer solutions.

Summary

The rate of discolouration of L-m-hydroxy-α-(methylaminomethyl)-benzyl alcohol (phenylephrine) solutions cannot be taken as a measure of the decomposition of the drug because it is noticeable only after appreciable degradation of the drug. Early stages of decomposition can be detected by measuring the intensity of light absorption at 370 mμ. This peak is completely absent in the undecomposed phenylephrine.

Heavy metals accelerate the rate of degradation of phenylephrine, copper exerting the most harmful effect. Calcium has no effect while magnesium exerts a stabilising action.

There is evidence that the metals act by building autoxidisable intermediate complexes with the drug.

The addition of ethylenediamine tetraacetic acid (EDTA) inhibits the influence of heavy metals on the decomposition of phenylephrine.

Zusammenfassung

Die Stabilität von Phenylephrin

Teil II: Die Verfärbungsreaktion und der Einfluß einiger Ionen auf die Abbaugeschwindigkeit des Medicaments

Die Verfärbungsgeschwindigkeit von Lösungen des L-m-Hydroxy-α-(methylaminomethyl)benzylalkohols (Phenylephrin) kann nicht als Maß für die Zersetzung des Arzneimittels betrachtet werden, denn die Verfärbung ist erst nach erheblichem Abbau des Phenylephrins bemerkbar.

Frühe Abbaustufen können durch Messung der Intensität der Lichtabsorption bei 370 mμ nachgewiesen werden. Dieses Maximum fehlt beim unzersetzten Phenylephrin völlig.

Schwermetalle beschleunigen die Abbaugeschwindigkeit des Phenylephrins. Kupfer übt den schädlichsten Einfluß aus. Calcium hat keine Wirkung, während Magnesium einen stabilisierenden Einfluß zeigt.

Es gibt Anhaltspunkte dafür, daß die Metalle durch Bildung autooxydierbarer Komplexe mit dem Arzneimittel wirken.

Durch Zusatz von Äthylendiamin-tetraessigsäure ADTA wird der Einfluß der Schwermetalle auf die Zersetzung des Phenylephrins verhindert.

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KURZE MITTEILUNGEN

From the Department of Parasitology, Veterinary Faculty, Ankara (Turkey)

The Efficiency of Bithionol in the Treatment of Dog Tapeworms

By Nevzat Gürelp and Yilmaz Tigin

In Turkey tapeworms in dogs have a wide distribution. At the necropsy Mimioğlu, Gürelp, and Savin [2] found *Dipylidium caninum* in 26%, *Taenia pisiformis* in 18%, *Joyeuxiella pasquali* in 8%, *Taenia hydatigena* and *Echinococcus granulosus* in 4%, *Multiceps serialis* and *Mesocestoides lineatus* in 2% of 50 dogs.

According to Poimaken and Erkiprik [3], 169 out of 627 dogs which came from the city of Ankara and its vicinity were found infested with different parasites: 31.6% of the infected dogs were carrying *D. caninum*, 15.2% *T. hydatigena*, 1.8% *M. lineatus*, 1.2% *T. pisiformis*, *Multiceps multiceps*, and *E. granulosus* and 0.6% *J. pasquali*.

Very limited work has been done on the effect of 2,2'-thiobis(4,6-dichlorophenol) (bithionol) against dog tapeworms. The drug used by Enzie and Colglazier [1] against *Dipylidium* and *Taenia* infestations at the rates of 110—550 mg/kg and 220 mg/kg and higher were found completely effective against *Taenia* in dogs. However, when 110 and 165 mg/kg were used, the efficiency of the drug was found to be 55% and 76% respectively. With the minimal dose level the drug was ineffective against *D. caninum* in one dog.

7 of 15 animals treated with bithionol vomited and 10 showed a moderate to marked softening of the faeces [1].

Materials and Methods

Bithionol was tested in 91 dogs of different ages, weight and breed. Two of the animals were naturally infested with *D. caninum*, 12 of the dogs were artificially infested with *T. hydatigena*, 16 with *M. multiceps* and 6 with *T. ovis*. 7 of the animals were kept as controls, and 21 were treated with 100—300 mg/kg of bithionol at the end of the prepatent period when the eggs or segments were detected in the faeces.

To show the side effects and safety margin of the drug, 2 animals were dosed with 500 mg/kg.

Each of the 12 dogs was infected with 5 *Cysticercus tenuicollis*, 10 with 20 *Coenurus cerebralis* scolices and 6 with 3 *Cysticercus ovis*.

4 h prior to treatment, all dogs were fed with minced meat. No special proprietory preparations were given to these animals. The drug was administered in gelatin capsules as a single oral dose. All the test animals were kept in seperate cages and the time of expulsion of the tapeworms was determined.

7 days after dosing all the treated and control animals were necropsied and their intestines carefully examined for tapeworms.

Results

The efficiency of bithionol against different tapeworms of dogs (Table 1)

In all of the 15 cases when bithionol was administered at 150—200 mg/kg the drug completely eliminated all tapeworms, whereas at the dose level of 125 mg/kg there was 100% efficiency in 5 out of 6 animals against *T. hydatigena* and *M. multiceps* (83%) and at 100 mg/kg it was 70—95% efficient in 4 dogs.

In 74% of the treated dogs all tapeworms were removed between 3—5 h after medication, in 17% of the cases the expulsion occurred after 5—7 h and in the remaining 9% of dogs (receiving 150 mg/kg) tapeworms were discharged over the period of 4.5 to 21.5 h after treatment.

Softening of the faeces was seen in 74% of the dogs 3 to 5 h after treatment and within this period 26% of the animals showed diarrhoea. All dogs regained normal conditions within 24 h. No other side effects were observed in the dogs given normal therapeutic doses. In 2 dogs given the high dose level of 500 mg/kg 1 animal vomited 45 min after administration and the other 90 min and 3 h later. Both dogs passed soft faeces 45 min and 5½ h after medication, but there were no other reactions noted; there were no alterations in their appetites, no changes in the blood pictures or alteration in their weights.

Tab. 1

No. of Dogs	Tapeworms	Dosage (mg/kg)	No. of tapeworms recovered at necropsy	Efficiency (%)
1	*Dipylidium caninum*	200	0	100
1	*Dipylidium caninum*	150	0	100
3	*Taenia hydatigena*	200	0	100
5	*Taenia hydatigena*	150	0	100
2	*Taenia hydatigena*	125	6	100
1	*Taenia hydatigena*	100	1	77
1	*Taenia hydatigena*	100	1	77
1	*Taenia hydatigena*	Control	3	--
1	*Taenia hydatigena*	Control	4	--
1	*Taenia hydatigena*	Control	5	--
2	*Taenia ovis*	200	0	100
3	*Taenia ovis*	150	0	100
1	*Taenia ovis*	Control	4	--
1	*Multiceps multiceps*	150	0	100
5	*Multiceps multiceps*	125	0	100
1	*Multiceps multiceps*	125	2	98
1	*Multiceps multiceps*	100	7	95
1	*Multiceps multiceps*	100	6	70
1	*Multiceps multiceps*	Control	12	--
1	*Multiceps multiceps*	Control	31	--
1	*Multiceps multiceps*	Control	27	--

Discussion

Enzie and Colglazier [1] obtained 100% efficiency against *Taenia* in dogs with bithionol when used at 220 mg/kg, and with 165 mg/kg the activity of this drug was found to be 76%. In our experiments, 150 mg/kg of bithionol was found wholly effective against all tapeworms of dogs. With 125 mg/kg the efficiency of the drug was 90—100% against *T. hydatigena* and *M. multiceps*.

No vomiting occurred in dogs which were given doses as high as 200 mg/kg. Emesis was seen only in 2 dogs which were dosed with 500 mg/kg of bithionol although Enzie and Colglazier [1] have reported vomiting at lower doses. In most treated dogs soft faeces and in a few cases, diarrhoea, was apparent but these side effects only lasted 24 h.

The results obtained with these trials showed that bithionol at 150 mg/kg is completely effective against the tapeworms of dogs. Side effects which lasted less than 24 h were never alarming. Following these findings we recommend the use of bithionol for the control of tapeworms in dogs.

Summary

2,2'-Thiobis(4,6-dichlorophenol) (bithionol) was found completely effective against tapeworms of dogs when given at 150 mg/kg. With 125 mg/kg the drug showed 90—100% efficiency to the same parasites. Within these dose ranges soft faeces or diarrhoea were the only side effects which disappeared in the first 24 h.

Bithionol exhibited a wide safety margin in dogs.

Zusammenfassung

Die Wirksamkeit von Bithionol in der Behandlung des Bandwurmbefalls beim Hund

In einer Dosierung von 150 mg/kg war 2,2'-Thiobis-(4,6-dichlorphenol) (Bithionol) voll wirksam gegen

von therapeutischem Interesse sein, zu untersuchen und zu klären, ob und in welchem Umfang höhere Salicylat-Spiegel im Plasma mit einer galenischen Zubereitung zu erzielen sind, die Acetylsalicylsäure in vitro in wasserlösliche Form bringt. Wir haben diese auch im deutschen Schrifttum offengebliebene Frage aufgegriffen und Salicylat-Bestimmungen im Plasma nach oraler Applikation von Acetylsalicylsäure einerseits und von wasserlöslich gemachter Acetylsalicylsäure andererseits durchgeführt. Hierbei kam es uns darauf an, sowohl die Unterschiede der Salicylat-Werte im Plasma in Relation zur jeweiligen Applikationsform der zu prüfenden Substanz als auch den Verlauf der Salicylatspiegel im Plasma über einen Zeitraum von 2 h zu untersuchen.

Für die Untersuchungen wurde in der ersten Bestimmungsreihe normale Acetylsalicylsäure (ASA) verwendet. Für die zweite Versuchsreihe stand Boxazin®*) zur Verfügung, eine galenische Zubereitung, die Acetylsalicylsäure wasserlöslich macht. Eine Tablette Boxazin enthält 330 mg Acetylsalicylsäure und 200 mg Ascorbinsäure. Boxazin löst sich vollständig in Wasser.

Die quantitative Salicylatbestimmung im Plasma erfolgte nach der Methode von Brodie, Udenfriend u. Coburn (1944). 10 Probanden mit gesundem Magen und normaler Resorption (7 Männer, 3 Frauen) im Alter von 22 bis 54 Jahren erhielten in der ersten Versuchsreihe 660 mg ASA. In einer zweiten Versuchsreihe erhielten dieselben Probanden 2 Tabletten Boxazin. Dieses Vorgehen sollte dazu beitragen, den Störfaktor individuell bedingter Resorptionsschwankungen weitgehend zu eliminieren. Die zu applizierende Menge ASA wurde in Wasser suspendiert, Boxazin im gleichen Volumen Wasser gelöst. Die Einnahme erfolgte nüchtern. 15, 30, 60, 90 und 120 min nach der oralen Gabe von ASA bzw. Boxazin wurde der jeweilige Salicylatspiegel im Plasma bestimmt. Die gewonnenen Ergebnisse sind im einzelnen aus der Abbildung und der Tabelle ersichtlich.

In den wesentlich höheren Salicylat-Werten im Plasma nach Boxazin kommt eine bessere und schnellere Resorption der wasserlöslichen Acetylsalicylsäure der Boxazin-Zubereitung zum Ausdruck. Die Differenz war erwartungsgemäß in den ersten 30 min nach der jeweiligen Applikation am stärksten ausgeprägt. Gegenüber ASA erreichte Boxazin nach 15 min den vierfachen, 30 min nach der Applikation mehr als den dreifachen Salicylatspiegel im Plasma. Es ist hervorzuheben, daß die Meßwerte 2 h nach der Verabreichung von Boxazin noch immer deutlich höher lagen als nach Applikation von ASA. Bei Boxazin-Medikation darf man demnach — über den kräftigen Initialanstieg hinaus — einen mindestens 2 h anhaltenden höheren Salicylatspiegel im Plasma erwarten.

Zusammenfassung

Bei 10 Probanden wurden im Verlauf von jeweils 2 h die Salicylatwerte im Plasma nach oraler Applikation von 660 mg Acetylsalicylsäure (= ASA) bzw. nach 2 Tabletten Boxazin®, entsprechend 660 mg Acetylsalicylsäure in wasserlöslicher Form und 400 mg Ascorbinsäure, bestimmt. Gegenüber ASA erreichte Boxazin einen über 2 h anhaltend höheren Salicylatspiegel im Plasma mit einem kräftigen Initialanstieg auf 3- bis 4fache Werte in den ersten 30 min.

Summary

Determination of Salicylate Level in the Plasma Following Administration of Acetylsalicylic Acid and a Combination of Acetylsalicylic Acid and Ascorbinic Acid

In 10 test persons the salicylate values in the plasma were determined over 2 h following application of 660 mg acetylsalicylic acid (ASA) or 2 tablets of Boxazin® resp. equalling 660 mg of water-soluble acetylsalicylic acid and 400 mg ascorbinic acid. Compared to ASA Boxazin attained a higher salicylate level in the plasma for 2 h, showing a pronounced initial rise to the 3- to 4fold within the first 30 min.

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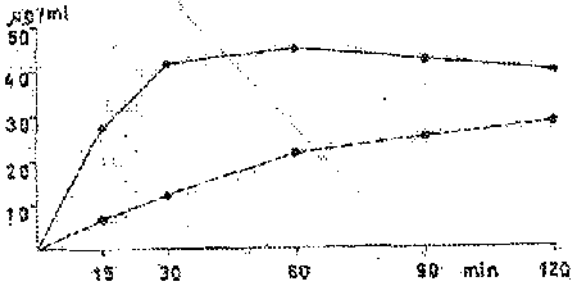


Abb. 1: Salicylatspiegel im Plasma in µg/ml 15 bis 120 min nach oraler Applikation von 2 Tabletten Boxazin (– · –) bzw. 660 mg Acetylsalicylsäure (——).

Tab. 1: Salicylatspiegel im Plasma in γ/ml 15 bis 120 min nach oraler Applikation von ASA bzw. Boxazin.

	660 mg Acetylsalicylsäure					2 Tabletten Boxazin				
Werte nach	15'	30'	60'	90'	120'	15'	30'	60'	90'	120'
Fall 1	5,5	7,5	11,5	14,5	19,5	30	30	38	34	–
Fall 2	12	20	34	36	38	26	49	47	43	37
Fall 3	4	6	18	28	44	31	39	50	98	48
Fall 4	10	18	30	30	28	32	46	50	54	45
Fall 5	8	16	20	22	20	38	61	61	57	55
Fall 6	4	14	24	26	25	31	34	50	65	57
Fall 7	6	6	8	10	16	20	68	60	50	46
Fall 8	3,5	7,5	12,5	16,5	18,5	8,5	17,5	21,5	26,5	–
Fall 9	12	24	34	42	38	34	68	48	44	36
Fall 10	4	6	22	26	30	12	66	40	40	30
Mittelwert	6,7	12,3	21,4	24,9	28,0	27,7	41,8	44,9	42,5	39,2

*) Dr. Karl Thomae GmbH, 795 Biberach an der Riss.

From the Department of Pharmaceutics, Faculty of Pharmacy, University of Alexandria (U.A.R.); Head: Professor Dr. M. M. Motawi

The Stability of Phenylephrine

Part III: The racemisation reaction

By H. A. M. El-Shibini, N. A. Daabis, and M. M. Motawi

Introduction

In a previous paper [1], the rôle of degradation of the secondary amino group of the molecule of L-m-hydroxy-α-(methylaminomethyl)benzyl alcohol (phenylephrine) was investigated. It was shown that the disappearance of this amino group, which is an important center for the pharmacological activity of the drug, was very rapid in alkaline medium and almost negligible in acid medium. In another study [2], it was found that the discolouration of aqueous phenylephrine solutions occurred only after severe decomposition of the drug. Also the influence of heavy metals and alkaline earth metals was investigated. It now remained to study the racemisation of L-phenylephrine in order to obtain full information about the stability of the drug.

No data are available in the literature about the rate of racemisation of L-phenylephrine. However, it is known that the racemisation of the closely related compound adrenaline is catalysed by hydrogen ion [4]. We have therefore investigated the rate of racemisation of L-phenylephrine in acid medium only.

Experimental part

About 0.25 g of phenylephrine, accurately weighed, were placed in a 250 ml measuring flask and immersed in a thermostatically controlled water bath. The drug was then dissolved in 200 ml of the appropriate buffer solution of the same temperature and the mixture was then completed to volume. A 25 ml sample was rapidly withdrawn, immediately cooled in ice and the optical rotation of the solution was then measured by means of a Carl Zeiss polarimeter. The samples were then analysed at suitable time intervals.

The buffer solutions of pH 2 to 6.5 were prepared by suitable mixing of citric acid, phosphoric acid, hydrochloric acid, boric acid and sodium hydroxide [5]. The solutions of pH less than 2 were not buffered, but consisted simply of a solution of the drug in 5 or 10% hydrochloric acid.

Results and Discussion

The results obtained in this series of investigations have shown that L-phenylephrine does not undergo racemisation at pH values as low as 2. No noticeable changes in the optical rotation of such solutions could be detected after heating at 97°C for several days. In this respect, the drug can therefore be considered as very stable.

In the completion of this study, the racemisation of L-phenylephrine was treated, however, as a purely physico-chemical process. For this purpose, the reaction was investigated in the presence of 5 and 10% hydrochloric acid. This is an extremely acidic medium with a pH value close to zero. In such highly acidic solutions there is no possibility of oxidation of the drug to occur [1—2] so that racemisation becomes the only process involved.

The results are plotted in Fig. 1 and 2. It appears that a straight line is obtained when the logarithm of the specific rotation is plotted as a function of time, indicating a first-order reaction with respect to the phenylephrine concentration. Furthermore, it is evident from the results illustrated in Fig. 1 and 2 that the process of racemisation of L-phenylephrine is subject to hydrogen ion catalysis. The racemisation rate constant k amounts to 4.6×10^{-4} h^{-1} and 8.5×10^{-4} h^{-1} in the presence of 5 and 10% hydrochloric acid respectively.

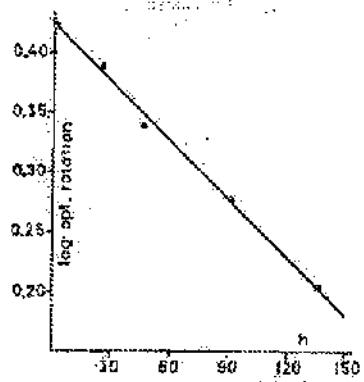


Fig. 1: Rate of racemisation of L-phenylephrine in 5% hydrochloric acid at 97° C.

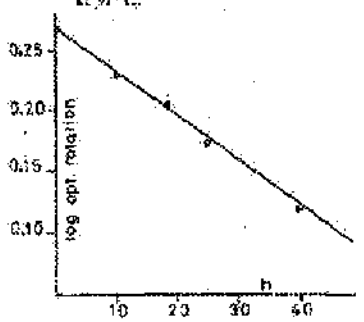
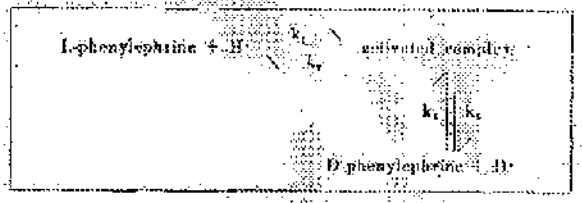


Fig. 2: Rate of racemisation of L-phenylephrine in 10% hydrochloric acid at 97° C.

The mechanism of reaction shown in Scheme 1 is suggested to explain why the rate of racemisation of L-phenylephrine is increased by raising the concentration of hydrochloric acid added.

Scheme 1



Conclusions

From all the previous results obtained in the three parts of this study, the following facts are established:

1. The degradation of the amino group of phenylephrine takes place only in alkaline medium. This holds also for the discolouration of phenylephrine solutions.

2. These reactions are greatly increased by the presence of traces of copper.

3. The addition of ethylenediamine-tetraacetic acid (EDTA) as chelating agent inhibits the catalytic influence of copper on the rate of degradation of the drug.

4. Racemisation of L-phenylephrine takes place in a noticeable way only below pH 2.

Consequently, the following conditions for the proper preparation and storage of phenylephrine solutions are deduced:

1. The reaction of the solution should be adjusted to pH above 2 and below 7, to prevent both racemisation and oxidation.

2. EDTA should be added in a concentration of 50 mg per 100 ml of the solution.

3. Under these conditions, the solution can be safely sterilised by autoclaving and will not undergo appreciable decomposition during storage.

Summary

L-m-Hydroxy-α-(methylaminomethyl)benzyl alcohol (L-Phenylephrine) does not undergo racemisation above pH 2. Below pH 2 the reaction is of first order and is catalysed by hydrogen ion. On the basis of these results and those obtained in previous investigations, the appropriate conditions for the preparation and storage of phenylephrine solutions have been established.

Zusammenfassung

Die Stabilität von Phenylephrin

Teil III: Die Racemisierungsreaktion

Über pH 2 findet keine Racemisierung des L-m-Hydroxy-α-methylaminomethyl)benzyl-alkohols (L-Phenylephrin) statt. Unter pH 2 ist die Reaktion 1. Ordnung in bezug auf die Arzneimittelkonzentration und wird von Wasserstoffionen katalysiert. Auf Grund dieser und anderer in früheren Untersuchungen erzielten Ergebnisse wurden die zur richtigen Herstellung und Aufbewahrung von Phenylephrinlösungen geeigneten Bedingungen festgelegt.

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The stability of aqueous solutions of phenylephrine at elevated temperatures: identification of the decomposition products

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The decomposition of phenylephrine in buffered solution at pH 6·8 was studied using t.l.c. and g.l.c. The major decomposition products were identified by gas chromatography-mass spectrometry following the formation of the trimethylsilyl derivative and are 1,2,3,4-tetrahydro-4,6-dihydroxy-2-methylisoquinoline and the 4,8-dihydroxy analogue. A possible mechanism for the degradation reaction is discussed.

The stability of phenylephrine hydrochloride ((—)-1-(3-hydroxyphenyl)-2-methyl aminoethanol hydrochloride) has been referred to in the literature. The racemization reaction of (—)-phenylephrine hydrochloride, the official stereoisomer was found to be first order (Pratt, 1957; Kisbye, 1967; El-Shibini, Daabis & Motawi, 1969). This route of degradation is only of significance in strongly acidic solutions. Ledbetter, Snyder & Lands (1963) irradiated phenylephrine solutions with ultraviolet light and determined the loss of the drug by biological assay and photofluorometric determination. The authors suggested that adrenaline had been formed from phenylephrine in the irradiated solution and further oxidation of the formed catecholamine resulted in discolouration of the solution. In 1951, Schou & Rhodes showed that injections containing 1% phenylephrine were stable when made strongly acidic and free from oxygen, solutions of higher pH required the addition of 0·1% sodium metabisulphite to prevent discolouration and loss of activity on autoclaving. West & Whittet (1960) found that sodium edetate prevented the discolouration of phenylephrine solutions stored in amber bottles at room temperature whereas sodium metabisulphite did not. The presence of trace quantities of iron and manganese probably accelerated the discolouration. Biological assay of several samples showed that discolouration of solutions did not indicate a significant decrease in biological activity, possibly due to the formation of a biologically active oxidation product. Schriftman (1959) was the first to attempt to isolate and identify the oxidation products. Using paper chromatography techniques, he separated up to five compounds from a degraded sample of phenylephrine. The ultraviolet absorption spectrum of the main breakdown product and of two other products were recorded. The colour reactions of these compounds indicated the presence of phenolic groups and the general absence of the secondary nitrogen group. Recently, El-Shibini & others (1969) suggested, in analogy to adrenaline, that on oxidation phenylephrine was converted to 5-hydroxy-*N*-methyl-indoxyl.

The present work was initiated to determine the nature of the decomposition products formed on heating an aqueous solution of phenylephrine in the presence of oxygen.

METHODS AND RESULTS

Materials

Phenylephrine hydrochloride B.P. was obtained from Hilton Davis Chemicals Ltd. Buffer solutions were prepared using B.P. 1968 formulae, and buffer materials were analar grade.

Decomposition of phenylephrine solutions

0·5% solutions were prepared over the pH range 2·0–9·0 using suitable formulae. These solutions were stored at 85° for 2 weeks in ampoules containing excess air to ensure the availability of sufficient oxygen, and examined by t.l.c. A larger quantity of solution buffered to pH 6·8 was stored similarly at 95° for 6 weeks.

Thin-layer chromatography

Silica Gel G plates (20 × 20 × 0·03 cm) were used. 50 µl quantities of the decomposed solutions and similar quantities of fresh solutions were applied to the plates which were subsequently developed for 10 cm with a solvent system consisting of the organic layer separated from *n*-butanol–acetic acid–water (5:1:4). The spots were located using three detection methods:

(*a*) 0·2% ninhydrin in *n*-butanol, followed by heating at 110° for 10 min for colour development (for primary and secondary amine groups).

(*b*) Millons reagent (for phenolic groups).

(*c*) Ultraviolet light at 366 nm (for fluorescence).

T.l.c. examination showed two major decomposition products at R_F 0·27 and R_F 0·52 with a third product only faintly visible at R_F 0·44 together with the remaining phenylephrine at R_F 0·40, over the pH range 2–9. All the decomposition products were detected with Millons reagent; none gave a positive reaction with the ninhydrin reagent and all fluoresced under ultraviolet light. The amount of degraded material present increased with increasing pH and at a pH below 5 an insoluble pigment was occasionally formed, this coincided with the loss of the fluorescent spots on the t.l.c. plate. When these buffered solutions were stored at 85° under nitrogen, no decomposition products were observed.

The two main decomposition products at R_F 0·27 and 0·52 were isolated by preparative t.l.c. using the decomposed solution in buffer at pH 6·8. The plates were prewashed with an ethanol–methanol (50:50) mixtures. The bands were eluted with methanol and the resultant solution evaporated to dryness under vacuum.

Further storage of these isolated compounds at 95° under air followed by t.l.c. analysis, indicated that the highly coloured material at R_F 0·27 was derived from the material at R_F 0·52. The extraction procedures employing t.l.c. successfully separated the two main oxidation products from each other. However the structures of these compounds could not be determined using infrared, nmr and mass spectrometry, since traces of other impurities were still present and the major components rapidly polymerized, once isolated.

Gas chromatography of the degraded solution

A Pye '104' gas chromatograph with a flame ionization detector was used. The column consisted of 3% OV-17 on chromasorb W, 80–100#, acid washed and

DMCS treated, packed into a 7 ft glass ¼" O.D. column. Operating conditions were as follows: column temperature 190°, inlet temperature 240°, carrier gas flow rate 35 ml min^{-1} and the injection volume within the range 2–5 µl.

The degraded sample (pH 6·8) was freeze-dried and reacted with N,O-bis(trimethylsilyl)-acetamide in pyridine. Some of the phosphate buffer may have been silylated also under these conditions, but would have emerged with the solvent. A sample of the silylated extract was chromatographed (Fig. 1), peak X had the same retention time as a silylated sample of phenylephrine. Peak Y and peak Z were of nearly

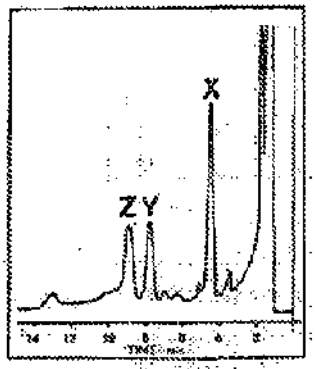


FIG. 1. Gas chromatographic separation of a degraded solution of phenylephrine after silylation; column OV — 17 3%, temp. 190°.

equal area indicating similar quantities of both materials; several other small peaks were also visible.

Gas chromatography-mass spectrometry of the decomposition products

An A.E.I./M.S. 902 mass spectrometer was used. The molecular separator has been described previously (Millard, Priaulx & Shotton, 1971). Helium was used as carrier gas. The source was operated at a temperature of 210° and a beam energy of 70 eV. The resolving power was 1000 (10% valley definition). Accurate mass measurements were obtained at a resolving power of 20,000 using heptacosafluorotributylamine as reference. An amount (5 µl) of a freshly prepared silylated sample of the decomposed solution was injected into the system. Due to the polar nature of the compounds, broadening and tailing of the three major peaks occurred and minor peaks were not detected. The three mass spectra obtained were corrected for background due to column bleed. The mass spectrum of peak X showed it to be that of silylated phenylephrine. The mass spectra of peaks Y and Z were nearly identical suggesting that the constituents were isomers. Peak Y showed two ions at *m/e* 267 and *m/e* 268 while peak Z did not; this was probably due to a memory effect from the silylated phenylephrine in peak X. The spectrum of peak Z is shown in Fig. 2 where the ion of highest *m/e* value occurs at *m/e* 323.

Since this low resolution spectrum was not immediately interpretable as that of any obvious oxidation product of phenylephrine, accurate mass measurements were carried out on the relevant peak emerging from the chromatograph. This was done by the peak matching technique. The accurate masses of the prominent peaks, *m/e* 280, 308, 322 and 323 are given, along with the corresponding atomic compositions, in Table 1.

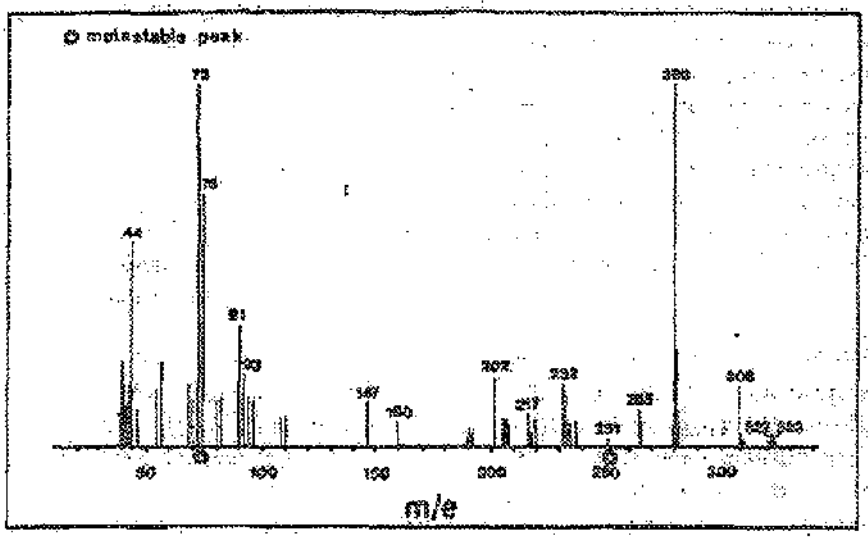
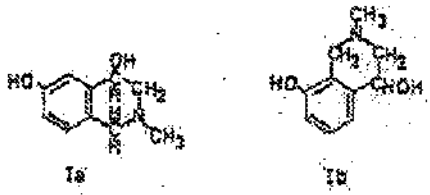


FIG. 2. The Mass spectrum of the main decomposition product after silylation.

Thus the decomposition product might be 1,2,3,4-tetrahydro-4,6-dihydroxy-2-methylisoquinoline (Ia) and 1,2,3,4,-tetrahydro-4,8-dihydroxy-2-methylisoquinoline (Ib). This material was synthesized conveniently from phenylephrine using the Pictet-Spengler reaction.



Ia Ib

Synthesis of 1,2,3,4-tetrahydro-4,6-dihydroxy-2-methylisoquinoline and its 4,8-dihydroxy analogue

Phenylephrine hydrochloride (250 mg) was dissolved in phosphate buffer, adjusted to pH 6·8. Formalin solution AR (0·5 ml) was added and the solution made up to 5 ml. The solution was sealed in ampoules under an atmosphere of nitrogen and stored at 75° for 24 h. It had been shown that under these storage conditions without formaldehyde no oxidation of phenylephrine would occur. The solution was freeze-

Table 1. *Accurate mass measurements obtained from the spectrum of the main silylated decomposition product.*

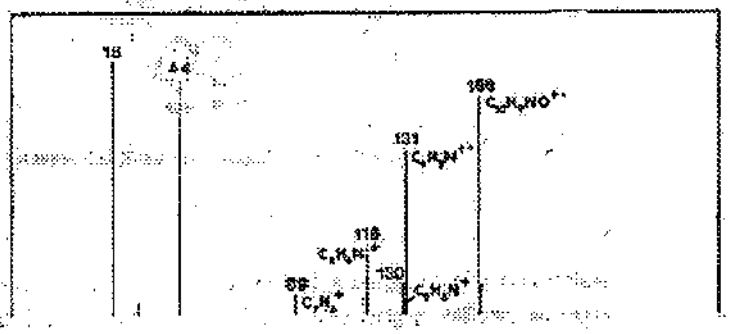
m/e	Empirical formulae	Measured mass value	Calculated mass value	Comment
280	$C_{16}H_{26}O_2Si_2$	280·1315	280·131478	
308	$C_{15}H_{26}O_3NSi_2$	308·1502	308·150201	$(M^+ - 15)$†
322	$C_{16}H_{28}O_3NSi_2$	322·1658	322·165851	$(M^+ - 1)$†
323	$C_{16}H_{29}O_3NSi_2$	323·1736	323·173676	M^+

M^+ . . . is the molecular ion

dried, silylated and chromatographed, no silylated phenylephrine was detected, but two peaks were apparent, having the same retention times as peaks Y and Z produced by the normal oxidation of phenylephrine. The mass spectra of both peaks in the gc–ms system were identical and corresponded to the spectra of the peaks obtained by the molecular oxidation of phenylephrine. This result was evidence for the presence of equivalent amounts of the two isomers 1,2,3,4-tetrahydro-4,6- and 4,8-dihydroxy-2-methylisoquinoline in oxidized solutions. The gc–ms system failed to identify the secondary decomposition product shown by t.l.c. to be produced by oxidation of the tetrahydroisoquinoline. A sample of this further oxidation product was isolated using a cationic exchange resin column.

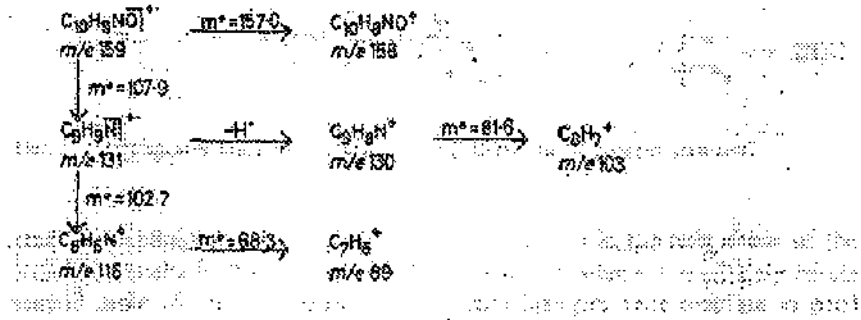
Isolation of the secondary decomposition product by column chromatography

The column consisted of Zeokarb '225' (50–100#) ion exchange resin (Permutit Co. Ltd.) in its hydrogen form, packed into a 60 cm Quickfit chromatography column with sintered glass disc and glass tap. 1 g of phenylephrine hydrochloride was degraded as described earlier and slowly passed through the column followed by thorough washing with distilled water. The ultraviolet adsorption spectrum of the eluent was monitored at intervals using an Optica 4F4R recording spectrophotometer and when the absorption in the range 230–330 nm became negligible, 0·05 M hydrochloric acid AR was passed through the column until the phenylephrine was eluted completely. The secondary oxidation product was next eluted using 0·1M hydrochloric acid and finally the remaining oxidation products including the identified tetrahydroisoquinolines eluted with 3M hydrochloric acid. The various fractions were identified by t.l.c. The solution eluted with 0·1M acid was neutralized with sodium hydroxide and evaporated to dryness at 40° under vacuum. The organic fraction was dissolved in methanol, leaving behind most of the sodium chloride, formed during neutralization; this operation was repeated to remove the last traces of salt. Finally the material was dried in a vacuum oven at 40° for 6 h. Analysis by t.l.c. showed that trace amount of contaminants were present. The mass spectrum obtained on this material via the direct inlet system of the mass spectrometer is shown in Fig. 3. The



atomic compositions of the prominent ions as given in Fig. 3 were confirmed by accurate mass measurements.

The following fragmentation pathways were established by the presence of the appropriate metastable ions (m*):



Since the compound is derived from 1,2,3,4-tetrahydro-4,6-dihydroxy-2-methylisoquinoline and its 4,8-dihydroxy analogue, the most likely structures consistent with the mass spectral data are 2-methyl-isoquinolin-6(2H)-one and -8(2H)-one. The fragmentation pattern of the former is given in Fig. 4.

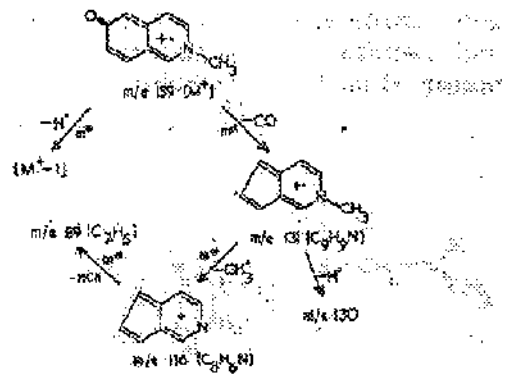


FIG. 4. A fragmentation pattern for 2-methyl-isoquinolin-6(2H)-one.

Ultraviolet spectrum

The λ_{max} in acidic solution was at 246 nm and this solution was colourless. Above pH3 the λ_{max} shifted to 262 nm and the solution was bright yellow, as expected for a quinonoid structure.

These results support the structure of this product as being 2-methyl-6-oxoisoquinoline or 2-methyl-8-oxoisoquinoline.

DISCUSSION

The main oxidation products of phenylephrine were identified as 1,2,3,4-tetrahydro-4, 6-dihydroxy-2-methylisoquinoline and its 4,8-isomer. The mechanism suggested is

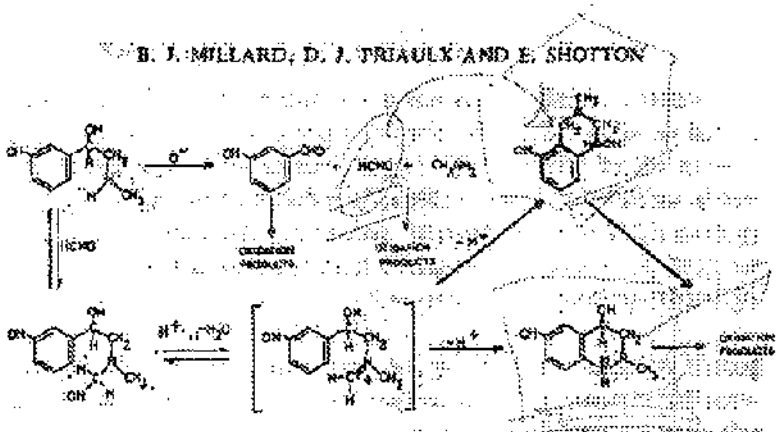
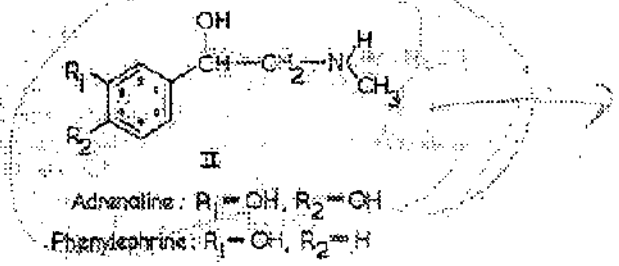


FIG. 5. A proposed oxidation pathway for phenylephrine in aqueous solution.

that the oxidation of the secondary alcoholic group in the side chain of the parent molecule results in the formation of formaldehyde which immediately reacts with a second molecule of phenylephrine in a Pictet-Spengler type reaction to produce the corresponding isoquinoline derivative (Fig. 5).

The first stage in the oxidation is similar to that reported for the degradation of methoxamine (Millard, Priaulx & Shotton, 1971), while the second stage of the reaction has been reported to occur under similar mild conditions for other phenylalkylamines (Buck, 1934; Govindachari & Whaley, 1951). Addition products initially formed after the interaction of a secondary amine and an aldehyde are known to be very unstable and cannot be isolated (Organikum, 1964). Synthesis from phenylephrine and formaldehyde indicated that this second step occurs rapidly, so that free formaldehyde would not be present in an oxidised solution of phenylephrine.



II

Adrenaline: R_1 = OH, R_2 = OH

Phenylephrine: R_1 = OH, R_2 = H

In the phenylephrine molecule (II) the one phenolic group influences the distribution of electrons in the aromatic nucleus resulting in high electron density at positions ortho and para to this phenolic group. After initial oxidation to produce free carbonyl compounds, the electrophilic formaldehyde formed reacts with an intact molecule of phenylephrine to produce an addition product; this new electrophilic species is suitably placed with respect to the nucleophilic carbons C-2, C-6 in the *para*-position in the aromatic ring, resulting in a cyclization reaction to form an isoquinoline. This reaction cannot proceed in the methoxamine oxidation mechanism due to blocking by the methoxy group attached at this position in the aromatic nucleus.

Degradation of adrenaline occurs by a different mechanism. Initial oxidation of this molecule occurs at the two phenolic groups to yield adrenaline quinone having carbon atom six electron deficient. The second stage involves attack by the nucleophilic side chain nitrogen on this electron-deficient position which with either simultaneous or subsequent oxidation yield adrenachrome. It can be seen that this analogy cannot be applied to phenylephrine as a catechol structure is required to form an intermediate *o*-quinone, thus the postulated oxidation product 5-hydroxy-*N*-methylindoxyl cannot be formed.

3-Hydroxybenzaldehyde and methylamine are thought to be produced during initial oxidation of phenylephrine; these have not been detected in decomposed solutions, and are probably further oxidized to a complex mixture of products in this system. Svensson (1972) described the oxidative degradation of terbutaline and showed that oxidation resulted in the formation of formaldehyde which subsequently initiated a Pictet-Spengler reaction to form the corresponding isoquinoline derivative. The author also detected significant quantities of 3,5-dihydroxybenzaldehyde and butylamine giving some evidence for the likely formation of 3-hydroxybenzaldehyde and methylamine during the oxidation of phenylephrine.

Acknowledgements

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Report of the International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity

Results of an International Workshop Organized by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)
and the
National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

National Institute of Environmental Health Sciences
National Institutes of Health
U.S. Public Health Service
Department of Health and Human Services

THE INTERAGENCY COORDINATING COMMITTEE ON THE VALIDATION OF ALTERNATIVE METHODS AND THE NTP INTERAGENCY CENTER FOR THE EVALUATION OF ALTERNATIVE TOXICOLOGICAL METHODS

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) was established in 1997 by the Director of the National Institute of Environmental Health Sciences (NIEHS) to implement NIEHS directives in Public Law 103-43. P.L. 103-43 directed NIEHS to develop and validate new test methods, and to establish criteria and processes for the validation and regulatory acceptance of toxicological testing methods. P. L. 106-545, the ICCVAM Authorization Act of 2000, established ICCVAM as a permanent committee. The Committee is composed of representatives from 15 Federal regulatory and research agencies and programs that generate, use, or provide information from toxicity test methods for risk assessment purposes. The Committee coordinates cross-agency issues relating to development, validation, acceptance, and national/international harmonization of toxicological test methods.

The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (Center) was established in 1998 to provide operational support for the ICCVAM, and to carry out committee-related activities such as peer reviews and workshops for test methods of interest to Federal agencies. The Center and ICCVAM coordinate the scientific review of the validation status of proposed methods and provide recommendations regarding their usefulness to appropriate agencies. The NTP Center and ICCVAM seek to promote the validation and regulatory acceptance of toxicological test methods that will enhance agencies' abilities to assess risks and make decisions, and that will refine, reduce, and replace animal use. The ultimate goal is the validation and regulatory acceptance of new test methods that are more predictive of human and ecological effects than currently available methods.

Additional Information

Additional information can be found at the ICCVAM/Center Website: http://iccvam.niehs.nih.gov and in the publication: *Validation and Regulatory Acceptance of Toxicological Test Methods, a Report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods* (NIH Publication No. 97-3981, or you may contact the Center at telephone 919-541-3398, or by e-mail at iccvam@niehs.nih.gov. Specific questions about ICCVAM and the Center can be directed to the ICCVAM Co-chairs:

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I. ICCVAM Test Method Recommendations

List of Acronyms/Abbreviations

A549	Human alveola Type II epithelia – lung carcinoma
ADAPT	A commercially available system for the evaluation of LD50s and MTDs
ADME	Absorption, distribution, metabolism, elimination
ANOVA	Analysis of Variance
ATC	Acute Toxic Class
ATP	Adenosine triphosphate
ATSDR	Agency for Toxic Substances and Disease Registry/DHHS
BALB/c	Inbred strain of mouse
BBB	Blood-Brain Barrier
BEAS-2B	Human Bronchial-tracheal epithelia/transformed
BgVV	Federal Institute for Health Protection of Consumers and Veterinary Medicine (Germany)
BG1	Breakout Group 1: *In Vitro* Screening Methods for Assessing Acute Toxicity
BG2	Breakout Group 2: *In Vitro* Methods for Assessing Acute Toxicity Biokinetic Determinations
BG3	Breakout Group 3: *In Vitro* Methods for Organ-Specific Toxicity
BG4	Breakout Group 4: Chemical Data Sets for Validation of *In Vitro* Toxicity Tests
BFU-E	Burst-forming unit -- erythrocytes
BMC	Bone marrow cell
BTS	British Transplantation Society
b.w.	Body weight
Caco-2	Human acute leukemia cell line
CASE	QSAR Software
CAS	Chemical Abstract Service
CBC	Cord blood cell
CBER	Center for Biologics Evaluation and Research/FDA
CCL-30	Human nasal septum cells – squamous cell carcinoma
CDC	Centers for Disease Control and Prevention/DHHS
CDER	Center for Drug Evaluation and Research/FDA
CFN	The National Board for Laboratory Animals, Stockholm, Sweden
CFR	Code of Federal Regulations
CFU-GM	Colony-forming unit – granulocyte/macrophage
CFU-MK	Colony-forming unit – megakaryocytes
CFSAN	Center for Food Safety and Nutrition/FDA
CNN	Computational Neural Network
CNS	Central Nervous System
CPH 100	Human neuroblastoma cell line -- differentiated
CPSC	Consumer Product Safety Commission
CTLU	Cytotoxicology Laboratory, Uppsala
DEREK	Deduction of Risk from Existing Knowledge (a commercially available knowledge-based expert system - QSAR);
DHHS	Department of Health and Human Services
DIMDI	The German Institute for Medical Documentation and Information

DIV-BBB	Dynamic *in vitro* blood-brain barrier model
DOD	Department of Defense
DOE	Department of Energy
DOT	Department of Transportation
EC50	Effective concentration of compound that causes 50% of the maximum response
ECITTS	ERGATT/CFN Integrated Toxicity Testing Scheme
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals
EC/HO	European Commission/British Home Office
ECVAM	European Centre for the Validation of Alternative Methods
EDIT	Evaluation-Guided Development on *In Vitro* Tests
ELISA	Enzyme-Linked Immunosorbent Assay
ERGATT	European Research Group for Alternatives in Toxicity Testing
EPA	Environmental Protection Agency
EU	European Union
EUCLID	Electronically Useful Chemistry Laboratory Instructional Database
FACS	Fluorescence activated cell sorting
FDA	Food and Drug Administration/DHHS
FDP	Fixed-Dose Procedure
FOIA	Freedom of Information Act
FRAME	Fund for the Replacement of Animals in Medical Experiments
$GABA_A$	gamma-aminobutyric acid; type A receptor is a ligand-gated ion channel complex
Galileo	Publicly available database of chemicals tested for toxicity
GFAP	Glial Fibrillary Acidic Protein
GHS	Globally Harmonized System
GLP	Good Laboratory Practice
H441	Human pulmonary adenocarcinoma cell line
Hb/g	Blood-air partition
HeLa	Human cervical adenocarcinoma cell line
HepG2	Human hepatocellular carcinoma cell line
HESI	Health and Environmental Science Institute
HL-60	Human acute leukemia cell line
HPV	High Production Volume
IC50	Inhibitory concentration estimated to affect endpoint in question by 50%
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
ICH	International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use;
ILSI	International Life Sciences Institute
IMR32	Human neuroblastoma cell line -- differentiated
INVITTOX	ERGATT FRAME ECVAM Data Bank of In Vitro Techniques in Toxicology (on-line)
IUPAC	The International Union of Pure and Applied Chemistry
JSAAE	Japanese Society for Alternatives to Animal Experiments
Km	Constant that reflects affinity of the enzyme for its substrate

Ko/w	Octanol-water partition; lipophilicity
LC	Lethal blood (or serum) Concentration
LD50	Dose producing lethality in 50% of the animals (median lethal dose)
LDH	Lactate Dehydrogenase
LLC-PK_1	Porcine kidney cell line
LOAEL	Lowest Observable Adverse Effect Level
LR	Likelihood-Ratio
MCASE	A QSAR system for the evaluation of LD50s and MTDs
MDCK	Madin Darby Canine Kidney cells
MEIC	Multicentre Evaluation of *In Vitro* Cytotoxicity
MTD	Maximum Tolerated Dose
MTS	3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MEMO	MEIC Monographs (monographs for 50 MEIC chemicals available from CTLU)
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NCI	National Cancer Institute/NIH
NHK	Normal human keratinocyte
NHNP	Human brain neural progenitor cell line
NICEATM	NTP Interagency Center for the Evaluation of Alternative Toxicological Methods
NIEHS	National Institute of Environmental Health Sciences/NIH
NIH	National Institutes of Health/DHHS
NIOSH	National Institute for Occupational Safety and Health
NLM	National Library of Medicine/NIH
NMDA	N-methyl-D-aspartate; receptor for neurotransmitter glutamate
NOAEL	No Observed Adverse Effect Level
NRU	Neutral Red Uptake
NT2	Human brain neural progenitor cell line; from teratocarcinoma
NTE	Neuropathy Target Esterase
NTP	National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
OPP	Office of Pesticide Programs/EPA
OPPT	Office of Pollution Prevention and Toxics/EPA
OPPTS	Office of Prevention, Pesticides, and Toxic Substances/EPA
PBBK	Physiologically-Based Biokinetics
PCA	Principal Component Analysis
PCC	Poison Control Center
PCNA	Proliferating cell nuclear antigen
PLS	Partial Least Square Analysis
QSAR	Quantitative Structure-Activity Relationship
QSPR	Quantitative Structure-Property Relationship
QPPR	Quantitative Property-Property Relationship
RC	Registry of Cytotoxicity/ZEBET
RITOX	Research Institute of Toxicology – Utrecht University, the Netherlands

ROS	Reactive Oxygen Species
RTECS	Registry of Toxic Effects of Chemical Substances/NIOSH
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SAR	Structure Activity Relationship
SAS	Statistical Analysis System – (SAS Institute, Inc., Cary, NC, USA)
SGOMSEC	Scientific Group on Methodologies for the Safety Evaluation of Chemicals
SH-SY5Y	Human neuroblastoma cell line -- differentiated
SMILES	Simplified Molecular Input Line Entry Specification (chemical nomenclature)
SR-4897	Murine stromal cells
SOP	Standard Operating Procedures
Sw	Water solubility
TD10	Toxic Dose for 10% of the individuals
TG 401	Test Guideline 401 (Acute Oral Toxicity) [OECD]
TG 420	Test Guideline 420(Acute Oral Toxicity - Fixed Dose Method) [OECD]
TG 423	Test Guideline 423 (Acute Oral toxicity - Acute Toxic Class Method) [OECD]
TG 425	Test Guideline 425 (Acute Oral Toxicity: Up-and-Down Procedure) [OECD]
TOPKAT	QSAR Software for the evaluation of LD50s and MTDs
UDP	Up-and-Down Procedure
Vd	Volume of distribution
Vmax	Maximum initial rate of reaction
WEHI-3B	Murine leukemia (myelomonocytic) cells
XTT	sodium 3,3-{1-[(phenylamino)carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate
ZEBET	German Centre for the Documentation and Validation of Alternative Methods (at BgVV)
3Rs	Refinement, Reduction, and Replacement (of Animal Use)
3T3	BALB/c mouse fibroblast cells
9L	Rat glioma cells

Workshop Breakout Groups

The following scientists were invited to serve on the Breakout Group Panels for the International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity, October 17-20, 2000.

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Björn Ekwall, 1940 – 2000

Dr. Björn Ekwall is acknowledged for his extraordinary contributions to alternative test method development and his dedication to the implementation of alternative tests for regulatory purposes. Results from his work in the area of *in vitro* cytotoxicity served as a major impetus for organization of the International Workshop on *In Vitro* Methods for Assessing Acute Toxicity.

Born in Uppsala in 1940, he attended Uppsala University Medical School and obtained his MD in 1969. After a short time as a General Practitioner, he became a lecturer at the Department of Anatomy, Uppsala University, where he earned his Ph.D. in toxicology. He was a postdoctoral fellow for 6 months at Materials Science Toxicology Laboratories, Memphis, TN, 1981-1982, and a Consultant at the Toxicology Laboratory of the Swedish Food Administration, 1982-1983. Between 1989 and 1996 he was an Associate Professor at the Division of Toxicology, Department of Pharmaceutical Biosciences, Uppsala University.

Dr. Ekwall introduced the concept of testing compounds in systems such as cell cultures and extrapolating the results to human toxicity. He felt that one could break down toxicity in complex biological systems to basic elements that could then be analyzed by using *in vitro* methods. To advance these ideas, he founded a small non-profit research institute, the Cytotoxicology Laboratory, Uppsala (CTLU) in 1983. He also established the Scandinavian Society for Cell Toxicology whose mission is to gather scientists for meetings and show that chemical effects on cells should translate to *in vivo* effects. These organizations coordinated a large international evaluation study, the Multicenter Evaluation of In Vitro Cytotoxicity Tests (MEIC), that began in 1989 to scrutinize the relevance of *in vitro* cytotoxicity tests for human acute toxicity of chemicals, and to select batteries of *in vitro* assays for practical testing of chemicals as alternatives to animal acute toxicity tests. Sixty-five different test methods were employed for testing 50 chemicals. Dr. Ekwall continued to work with the MEIC project until his untimely death on August 19, 2000.

Dr. Ekwall published 69 articles/book chapters on *in vitro* toxicology, plus 30 abstracts published in journals and another 30 abstracts published in conference proceedings. He received many international awards and was a member of many scientific societies and associations.

Preface

Acute systemic toxicity testing is conducted to determine the relative health hazard of chemicals and various products. Substances found to cause lethality in animals at or below prescribed doses are labeled to identify their hazard potential. While acute toxicity testing is currently conducted using animals, studies published in recent years have shown a correlation between *in vitro* and *in vivo* acute toxicity. These studies suggest that *in vitro* methods may be helpful in predicting *in vivo* acute toxicity.

An extensive evaluation of *in vitro* methods for acute toxicity, known as the Multicenter Evaluation of *In Vitro* Toxicity (MEIC) Program, was initiated by the Scandinavian Society for Cell Toxicology in 1989 under the direction of Dr. Bjorn Ekwall, Director of the Cytotoxicity Laboratory at the University of Uppsula. Fifty reference chemicals were selected for which there was acute oral toxicity data from animal testing and blood concentrations from fatal human poisonings. Ninety-six laboratories evaluated 30 of the chemicals in 82 different *in vitro* cytotoxicity assays, and all 50 chemicals were evaluated in 61 assays. Detailed analysis of the results identified a battery of three human cell line basal cytotoxicity assays that were highly correlative with peak human lethal blood concentrations.

In 1998, Dr. Willi Halle from Germany published a Register of Cytotoxicity consisting of *in vivo* acute toxicity data and *in vitro* cytotoxicity data for 347 chemicals. These data were used to construct a regression model that could be used to predict estimated LD50 values based on cytotoxicity data. Dr. Horst Spielmann and his colleagues at the German Centre for the Documentation and Evaluation of Alternatives to Testing in Animals subsequently proposed that cytotoxicity methods could be useful for predicting starting doses for *in vivo* acute oral toxicity studies, thereby reducing the number of animals necessary for such determinations.

In 1999, amidst growing awareness of the MEIC and other studies, the National Institute of Environmental Health Sciences (NIEHS) received over 800 letters requesting that the MEIC program results be evaluated by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). Also in 1999, the U.S. Environmental Protection Agency (EPA) Office of Pesticides, Prevention, and Toxic Substances asked ICCVAM to review the validation status of the MEIC proposals.

ICCVAM discussed these requests at its August 1999 meeting and asked the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) to prepare a technical summary of the extensive publications resulting from the MEIC studies. ICCVAM reviewed the MEIC results at its October 1999 meeting and recommended that an expert workshop should be convened to: a) evaluate the current validation status of the proposed MEIC test battery and other available *in vitro* tests that might be useful for predicting acute toxicity; and b) identify research, development, and validation efforts that might further enhance the use of *in vitro* methods to assess acute systemic toxicity.

Names of appropriate scientists to serve on an ICCVAM Workshop Organizing Committee were requested from participating ICCVAM Agencies. The Committee was charged with working with NICEATM to develop the Workshop objectives and program and to identify appropriate expert scientists to participate. The Committee held its first of several meetings in February 2000. Dr. Philip Sayre of the EPA and Dr. John Frazier of the U.S. Air Force co-chaired the Organizing Committee and guided the development of the scope and breadth of the Workshop.

In June of 2000, the International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity was announced in a *Federal Register* notice. Relevant data and nominations of

scientists that should be invited to participate in the Workshop were also requested in the notice. The Organizing Committee invited 33 expert scientists from academia, industry, and Federal agencies to participate in the Workshop. NICEATM assembled relevant background materials for distribution to the invited expert scientists, other workshop participants, and the public. The Organizing Committee also identified knowledgeable agency scientists to participate in the workshop, and developed a series of questions for four breakout groups to address during the three and a half-day meeting. In September 2000, a second *Federal Register* notice announced the availability of the Workshop agenda and background materials, and requested public comments.

Invited scientific experts and ICCVAM agency scientists were assigned to one of the following four Breakout Groups:

- *In Vitro* Screening Methods for Assessing Acute Toxicity;
- *In Vitro* Methods for Toxicokinetic Determinations;
- *In Vitro* Methods for Predicting Organ Specific Toxicity; and
- Chemical Data Sets for Validation of *In Vitro* Acute Toxicity Test Methods.

The Workshop was convened in Arlington, VA on October 17-20, 2000. The NTP, the NIEHS and the EPA sponsored the Workshop, and NICEATM provided logistical, technical, and administrative support. The Workshop was open to the public and was attended by 110 participants from nine countries. In the opening plenary session, speakers provided an overview of *in vitro* acute toxicity methods and described the regulatory use of acute toxicity data. Breakout Groups were then charged with their assigned objectives and asked to develop responses to questions provided by the Organizing Committee.

The Groups reported on their progress each morning of the second and third days and gave a final report on the last day of the meeting. Opportunity for public comment was provided in all plenary and breakout sessions. Following the Workshop, each of the Breakout Groups prepared reports that represented the consensus of the invited scientists assigned to that Group.

The NICEATM subsequently assembled the Breakout Group reports and other relevant information into this Workshop Report. A separate *Guidance Document on Using In Vitro Data to Estimate In Vivo Starting Doses for Acute Toxicity*, based on contributions from Drs. Rodger Curren, Julia Fentem, and Manfred Liebsch, was also prepared after the workshop. The Organizing Committee and ICCVAM reviewed the report and guidance document, and developed test recommendations to forward with these publications to Federal agencies for their consideration in accordance with Public Law 106-545. The ICCVAM recommendations are included in this report as Appendix I. Both publications are available on the Internet at the ICCVAM/NICEATM website (http://iccvam.niehs.nih.gov), and copies may be requested from NICEATM through email at: NICEATM@niehs.nih.gov.

On behalf of the ICCVAM, we gratefully acknowledge the unselfish contributions of all of the Workshop participants. We extend a special thanks to the Breakout Group co-chairs who worked diligently to ensure the timely completion and accuracy of their Group reports. The efforts of the Organizing Committee members and especially the co-chairs, Drs. John Frazier and Philip Sayre, were instrumental in assuring a productive and useful Workshop. The efforts of the NICEATM staff in coordinating local arrangements, providing timely distribution of information, and preparing the final report are acknowledged and appreciated. We especially acknowledge Dr. Ray Tice for preparation of the comprehensive background materials, Brad Blackard for coordinating communications and logistics throughout the entire project, and Michael Paris and Judy Strickland for their efforts in compiling the final workshop report.

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Executive Summary

Toxicity testing is conducted to determine the potential human health hazards of chemicals and products. Acute systemic toxicity testing is used to properly classify and appropriately label materials with regard to their lethality potential in accordance with established regulatory requirements (49 CFR 173; 16 CFR 1500; 29 CFR 1910; 40 CFR 156). Non-lethal parameters may also be evaluated in acute systemic toxicity studies to identify potential target organ toxicity, toxicokinetic parameters, and dose-response relationships. While animals are currently used to evaluate acute toxicity, recent studies suggest that *in vitro* methods may also be helpful in predicting acute toxicity.

To evaluate the validation status and current potential uses of *in vitro* methods as predictors of acute *in vivo* toxicity, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) held a four-day workshop—the International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity, October 17-20, 2000, in Arlington, VA, U.S.A. The Workshop provided a public venue for invited experts and ICCVAM agency participants to review the validation status of available *in vitro* methods for assessing acute systemic toxicity and to develop recommendations for validation efforts necessary to further characterize the usefulness and limitations of these methods. Workshop participants also developed recommendations for future mechanism-based research and development efforts to improve *in vitro* assessments of acute systemic lethal and non-lethal toxicity.

Specific objectives of the Workshop were to:

- Review the status of *in vitro* methods for assessing acute systemic toxicity:
  - — Review the validation status of available *in vitro* screening methods for their usefulness in estimating *in vivo* acute systemic toxicity;
  - — Review *in vitro* methods for predicting toxicokinetic parameters important to acute toxicity (i.e., absorption, distribution, metabolism, and elimination);
  - — Review *in vitro* methods for predicting specific target organ toxicity;
- Recommend candidate methods for further evaluation in prevalidation and validation studies;
- Recommend validation study designs that can be used to adequately characterize the usefulness and limitations of proposed *in vitro* methods;
- Identify reference chemicals that can be used to develop and validate *in vitro* methods for assessing *in vivo* acute toxicity.

Four Breakout Groups were assigned specific objectives and asked to develop responses to questions grouped into general areas of (a) identifying needs, (b) current status, and (c) future directions. Breakout Group 1 (BG1) addressed the use of *in vitro* screening methods to estimate acute *in vivo* toxicity (i.e., median lethal dose [LD50 values]). Breakout Group 2 (BG2) discussed the role of *in vitro* methods for estimating toxicokinetic parameters needed to assess acute *in vivo* toxicity. Breakout Group 3 (BG3) examined *in vitro* methods for assessing target organ toxicity and mechanisms, and Breakout Group 4 (BG4) addressed chemical data sets for validation of acute *in vitro* toxicity tests.

In Vitro Screening Methods for Assessing Acute Toxicity

BG1 was asked to evaluate the validation status of available *in vitro* methods for estimating *in vivo* acute toxicity. The Group identified methods and

appropriate validation studies that might be completed within the next one to two years. The potential uses of quantitative structure-activity relationships (QSAR) as part of an *in vitro* strategy were also considered.

In identifying needs, BG1 noted that the ultimate goal is to be able to predict acute toxicity in humans. To that end, the long-term goal is to develop a battery of *in vitro* tests employing human cells and to integrate the resulting information with that derived from other sources on key physico-chemical parameters (e.g., kinetics, metabolism, and dynamics) to predict human acute toxicity. The Group also recommended investigating ways to reduce and replace animal use in acute oral toxicity tests as detailed and described in the Organisation for Economic Co-operation and Development (OECD) test guidelines 401, 420, 423, and 425. The Group recognized that the use of QSAR (e.g., Barratt et al., 1998) can provide key information in a number of areas, including the selection of test chemicals for validation studies, the interpretation of outliers, and the grouping of chemicals by structure and biological mechanisms of toxicity.

To characterize the current status of the use of *in vitro* cytotoxicity assays to predict acute *in vivo* lethality, BG1 reviewed a number of approaches but focused on the Multicentre Evaluation of *In Vitro* Cytotoxicity (MEIC) and the German Center for the Documentation and Validation of Alternative Methods (ZEBET) approaches. The MEIC program investigated the relevance of *in vitro* test results for predicting acute toxicity in humans by coordinating the generation of *in vitro* cytotoxicity data for 50 chemicals by 96 laboratories using different *in vitro* methods. The MEIC management team correlated the *in vitro* findings with data compiled from human poisoning reports. The ZEBET approach involved using data from the Registry of Cytotoxicity (RC), which contains a regression analysis of *in vitro* cytotoxicity IC50 values and rodent LD50 values for 347 chemicals, to determine starting doses for LD50 tests. BG1 concluded that none of the available *in vitro* methods or proposed testing strategies had been evaluated adequately to replace the use of animals for acute systemic toxicity testing.

In the future, to reduce the use of animals in acute lethality assays, BG1 recommended using *in vitro* cytotoxicity data to predict starting doses for *in vivo* lethality studies as proposed by ZEBET (Spielmann et al., 1999). Data were presented indicating that this approach would reduce and refine animal use for acute toxicity testing. BG1 recommended that test laboratories evaluate and compare the performance of several *in vitro* cytotoxicity tests with the existing RC data. An appropriate *in vitro* cytotoxicity assay for this purpose would be a protocol employing the BALB/c 3T3 mouse fibroblast cell line, a 24-hour exposure time, and neutral red uptake as the measurement endpoint (of cytotoxicity). Other cell lines and cell viability assays could serve the same purpose equally well.

The Group also recommended that to further the goal of replacing the use of animals in acute lethality assays a prevalidation study should be initiated as soon as possible to evaluate various cell types, exposure periods, and endpoint measurements as predictors of acute toxicity. The assay, or battery of assays, determined to be the best predictor of *in vivo* lethality could be optimized further to identify, standardize, and validate simple predictive systems for gut absorption, blood-brain barrier (BBB) passage, kinetics, and metabolism. Such information has been identified as necessary to improve the ability of *in vitro* cytotoxicity data to predict *in vivo* LD50 values (Curren et al., 1998; Seibert et al., 1996; Ekwall et al., 1999). Additionally, other concepts such as TestSmart (CAAT, 1999, 2001), an approach to determine whether "one can measure cellular changes that will predict acute system failure" (A. Goldberg, personal communication) could be incorporated into *in vitro* strategies for predicting acute toxicity *in vivo*.

In the longer-term, preferably as a parallel activity, BG1 recommended focusing on the development and validation of human *in vitro* test systems for predicting human acute toxicity, integrating the approaches suggested by Breakout Groups 2 and 3. BG1 recommended that future

studies identify and evaluate mechanism-based endpoints. . The Group also recognized the potential impact of genomics and proteomics in many areas of toxicology, but noted that acute toxicity testing is not currently an area of high priority for the application of these new technologies.

BG1 made the following recommendations for the prevalidation, validation, and future development of *in vitro* assays for acute lethal toxicity:

- To further reduce the use of animals in acute lethality assays, a guidance document on the application of *in vitro* cytotoxicity data for predicting *in vivo* starting doses, including details of current test protocols and their application should be prepared.
- To support a testing strategy that might eventually replace the use of animals in acute lethality assays, a working group of scientific experts should be established to identify and/or define specific *in vitro* cytotoxicity test protocols for inclusion in a prevalidation study of their use for predicting LD50 values. The working group should design and plan the study in detail and take into account the suggestions made by BG1 (Section 2.7) regarding cell type, exposure period, and endpoint measurement.
- It is anticipated that the use of simple systems that predict gut absorption, BBB passage, key kinetic parameters, and metabolism will improve the ability of *in vitro* cytotoxicity assays to predict rodent LD50 values, or any *in vivo* toxic effects. Continued development and optimization of such systems for this application is encouraged and should receive regulatory support.
- In principle, QSAR approaches, including expert systems and neural networks, could be developed and validated for predicting acute systemic toxicity. Initially, an up-to-date review of current QSAR systems for predicting rodent oral LD50 values should be undertaken. In addition, QSARs for predicting gut absorption, metabolism, and BBB passage should be developed and evaluated and initiatives to increase data sharing should be established.
- The development of simple predictive models for human acute toxicity should be a major focus.
- The evaluation and ultimate acceptance of *in vitro* assays for human acute toxicity will need a larger reference database than is presently available for validation purposes. The MEIC human database should be peer-reviewed, modified if needed, and expanded as soon as possible so that data will be available for future validation studies.

<u>*In Vitro* Methods for Assessing Acute Toxicity: Biokinetic Determinations</u>

The second Breakout Group, BG2, was charged with 1) evaluating the capabilities of *in vitro* methods for providing toxicokinetic information (i.e., absorption, distribution, metabolism, and elimination) that can be used to estimate target organ dosimetry for acute toxicity testing, and 2) providing recommendations for future research to accomplish this goal. BG2 also explored the role of QSAR in toxicokinetic determinations.

In identifying needs, BG2 focused on a short-term goal of improving the prediction of acute lethal effects in rodents and a long-term goal of using *in vitro* techniques to evaluate chemical kinetics and ultimately to predict sublethal acute toxic effects in humans. Needs include the ability to use *in vitro* determinations of metabolic rate and passage of a chemical across membrane barriers to improve kinetic modeling. Such information may be useful for estimating LD50 values from basal cytotoxicity data. BG2 identified the following techniques that need further development to advance *in vitro* determinations of biokinetic parameters:

- *In vitro* determination of partition coefficients, metabolism, protein binding, and stability;
- Characterization of biotransformation enzymology;

- Structural knowledge and its translation into "chemical functionalities," estimation of partition coefficients, metabolism, etc. (i.e., "*in silico*" methods such as QSAR/quantitative structure-property relationships [QSPR]);
- Biokinetic modeling, including the integration of toxicodynamic and biokinetic modeling in predicting systemic toxicity.

Evaluation of the current status of the use of *in vitro* methods to obtain biokinetic information involved a survey of *in vitro* systems for estimating metabolism and passage of membrane barriers. Biotransformation information can currently be obtained using human or animal liver preparations; however, conditions for the preparation and incubation need to be standardized. Several *in vitro* systems for measuring intestinal absorption are also available, but some cell lines lack transporters that are present *in vivo*. Glomerular filtration and reabsorption in the proximal tubule determine the renal excretion of most compounds and can be predicted from a compound's physico-chemical properties and plasma protein binding. Many of the available renal cell lines or primary cultures lack specific transporters implicated in the accumulation of several nephrotoxic compounds.

Future directions for research outlined by BG2 include using a conceptual structure to integrate kinetic information into the estimation of acute oral toxicity. Available *in vitro* data on the absorption, tissue partitioning, metabolism, and excretion of a test material could be used to parameterize a chemical-specific biokinetic model (Clewell, 1993). The model could then be used to relate the concentration at which *in vitro* toxicity occurs to the equivalent dose that would be expected to produce *in vivo* toxicity. Such models could also provide information on the temporal profile for tissue exposure *in vivo*, which can then be used to design the most appropriate *in vitro* experimental protocol (Blaauboer et al., 1999).

BG2 suggested two main testing strategies appropriate for research and development activities. One strategy was a simple method of using chemical-specific partitioning information and the other was a one-compartment model to estimate the oral dose equivalent to the *in vitro* cytotoxicity value. Research and development activities would involve collecting partitioning information for a number of chemicals, making such oral dose estimations, and then comparing the estimations to empirical values to develop a prediction model.

The other testing strategy BG2 recommended for research and development was a tiered approach for using *in vitro* cytotoxicity assays to evaluate the role of metabolism in the production of acute toxicity due to chemical exposure. The first step would be to estimate hepatocyte metabolism at a relatively low concentration (e.g., 10 μM).

If the rate of metabolism (Vmax/Km) is low, then basal cytotoxicity information could be relied upon to predict *in vivo* toxicity. If the metabolism rate is high, then the responsible enzyme system could be identified with *in vitro* studies. If the primary enzyme system is oxidative or reductive, then metabolic activation may be producing toxicity and a hepatocyte cytotoxicity assay should be performed.

If the IC50 value for hepatocytes is much lower than that for basal cytotoxicity, then the concentration-response for metabolism should be characterized to predict the *in vivo* doses that might be associated with toxicity. If the primary metabolism is detoxification (conjugation, sulfation, etc.), then the basal cytotoxicity results could be used with some confidence to predict the LD50 value.

BG2 also recommended identifying the compounds that represent the outliers in the MEIC correlations of *in vitro* basal cytotoxicity assays with LD50 values. By determining the physico-chemical properties of these compounds and their target tissues, it may be possible to identify factors that could improve the correlation between predicted oral LD50 values in rodents and empirical values. Such an exercise would help define a "predictive range" for various chemical properties over which *in vitro* basal cytotoxicity assays might be expected to provide reasonable LD50 estimates, as well as exclusion rules for

identifying compounds for which *in vitro* assays are not reliable.

Other research recommendations made by BG2 include developing validated, stable human hepatocyte systems and *in vitro* systems for key transporters (renal, biliary, etc.). Such data would provide a mechanistic description of barrier functions that could be incorporated into template physiologically-based biokinetic (PBBK) models for various classes of chemicals. Specific QSPR applications need to be developed to provide other information such as metabolic constants, binding, etc., required by PBBK models.

The interaction between kinetics and dynamics also needs to be explored. For example, the effect of toxicity on the metabolism and excretion of a chemical or, conversely, the effect of metabolism or reabsorption on the toxicity of a chemical must be taken into account. The time dimension in the conduct of these assays should be analyzed rigorously to account for duration and frequency of exposure. Other recommendations for research include:

- Understand the relationship between molecular structure, physical-chemical properties, and kinetic behavior of chemicals in biological systems;
- Develop algorithms to determine the optimum kinetic model for a particular chemical;
- Conduct research on modeling of fundamental kinetic mechanisms;
- Develop mathematical modeling techniques to describe complex kinetic systems;
- Develop mathematical modeling techniques for tissue modeling (anatomically correct models);
- Develop an optimal battery of *in vitro* assays to evaluate chemical-specific kinetic parameters;
- Establish a database of chemical-independent parameters (mouse, rat, human);
- Develop a library of generic models that are acceptable for regulatory risk assessments;
- Understand and model the mechanisms regulating the expression of proteins involved in kinetic processes (metabolizing enzymes, transport enzymes, metallothionein, membrane channels, etc.);
- Understand and model effects of changes in physiological processes on kinetics of chemicals;
- Develop mathematical modeling techniques to describe complex dynamic systems and genetic networks at the cellular and at the systemic level;
- Develop mathematical modeling techniques to describe individual variability (genetic background);
- Develop *in vitro* biological models that are equivalent to *in vivo* tissues (i.e., models that maintain specified differentiated functions that are important for the toxicological phenomena under study);
- Establish lines of differentiated human cells (e.g., derived from stem cells);
- Understand and model mechanisms of multi-cellular interactions in development of toxic responses (co-cultures);
- Understand and model relationships between cellular responses and biomarkers of systemic responses;
- Compare genomic differences or species-specific expression differences between species and within species (e.g., polymorphisms in biotransformation enzymes);
- Perform high dose to low dose extrapolation.

In Vitro Methods for Organ-Specific Toxicity

Breakout Group 3 reviewed *in vitro* methods that can be used to predict specific organ toxicity or toxicity associated with alteration of specific cellular or organ functions and developed recommendations for priority research efforts necessary to support the development of methods that can accurately assess target organ toxicity.

In identifying needs, reviewing current status, and suggesting future directions, BG3 focused on the major organ systems most likely to be affected by acute systemic toxicity: liver, central nervous system, kidney, heart, hematopoietic system, and lung.

- Currently it is possible to assess the potential for hepatic metabolism in high throughput screening assay systems when identification of the specific metabolites is not needed. Future work should include development of a system that will be able to recognize the effect of products of hepatic metabolism on other organ systems in a dose responsive manner. A worldwide database is needed to compare human *in vitro* and *in vivo* data for hepatic toxicity.
- Some endpoints, assays, and cell models for the more general endpoints for *in vitro* neurotoxicity have been studied and used extensively and are ready for formal validation. However, most assays and cell models determining effects on special functions still need significant basic research before they can be used as screening systems.
- Several *in vitro* models to assess BBB function are currently being evaluated in a prevalidation study sponsored by the European Centre for the Validation of Alternative Methods (ECVAM). Models being studied include immortalized endothelial cell lines of both human and animal origin, primary bovine endothelial cells co-cultured with glial cells, and barrier-forming continuous cell lines of non-endothelial origin. Preliminary results from the prevalidation study show that the rate of penetration of compounds that pass the BBB by simple diffusion can be estimated by the determination of log P, or by the use of any cell system that forms a barrier. To assess the impairment of the transporter functions of the BBB, an *in vitro* system with a high degree of differentiation is required, including the significant expression of all transporter proteins representing species-specific properties. At present, this can only be achieved in primary cultures of brain endothelial cells co-cultured with brain glial cells.
- To assess kidney function, *in vitro* systems will need to utilize metabolically competent kidney tubular cells and be able to evaluate the barrier function of the kidney. A system to assess this parameter is currently being studied in Europe with support from ECVAM. In addition, *in vitro* systems will need to assess specific transport functions. More research is needed in this area to develop mechanistically based test systems.
- The Group's review of *in vitro* models for cardiovascular toxicity concluded that none have been validated. The likely candidate *in vitro* systems for an acute cardiotoxicity testing scheme could include: (a) short term single-cell suspensions of adult rat myocytes to measure products of oxidation; (b) primary cultures of neonatal myocytes to measure changes in beating rates and plasma membrane potentials; (c) co-culture of smooth muscle cells or endothelial cells with macrophages to examine rate of wound healing (DNA synthesis); and (d) an immortalized cell line (e.g., the human fetal cardiac myocyte line) to measure classical cytotoxic endpoints. It also may be important to include the perfused heart preparation for a comparison with other *in vitro* models since this system is more representative of the *in vivo* situation than cell culture systems.
- Regarding the status of *in vitro* methods for assessing toxicity on the hematopoietic system, ECVAM is supporting a validation study of the use of colony-forming assays to test for the development of neutropenia. Methods to assess effects on thombocytopoiesis and erythropoiesis are also available and can be considered for validation. ECVAM is also supporting a new project to develop and prevalidate *in vitro* assays for the prediction of thrombocytopenia. A preliminary study by ECVAM's laboratories confirmed the usefulness of

the *in vitro* test for screening drug toxicity to megakaryocyte progenitors. The study also showed that cord blood cells (CBC) can be used as a human source, are more suitable for this purpose, and provide a means of avoiding ethical problems connected with the collection of human bone marrow cells (BMC).

- *In vitro* evaluation of acute respiratory toxicity should consider several cell types since the tracheal-bronchial epithelial lining consists of stratified epithelium and diverse populations of other cell types, including ciliated, secretory (e.g., mucous, Clara, serous), and non-secretory cells. BG3 reviewed a number of models that could be used to indicate chemical-induced cell damage or death. The cells of the airways are relatively accessible to brushing, biopsy, and lavage, and therefore lend themselves for harvesting and use as primary cells (Larivee et al., 1990; Werle et al., 1994). The most useful markers are those that relate to the basic mechanisms by which airway epithelia respond to toxic exposure. However, most assays and cell models for determining effects on special functions still need significant basic research before they can be used as screening systems.

BG3 indicated that specific organ toxicity data would not be needed routinely to assess acute systemic toxicity and recommended a tiered approach to assess the acute systemic toxicity potential of xenobiotics. The first step involves physico-chemical characterization and initial biokinetic modeling for the chemical of interest. Such information should be used to compare the test material with chemicals that have a similar structure or properties and for which toxicity data exist that may be useful for predicting organ distribution. The second step is to conduct a basal cytotoxicity assay. The third step is to determine the potential for metabolism-mediated toxicity. The next two steps can be done in either order. Step 4 involves assessing the effect of the test substance on energy metabolism by using a neuronal cell line that expresses good aerobic energy metabolism. Results from this system will help determine if the nervous or cardiovascular systems are likely targets. If there is evidence of metabolism (from Step 3), Step 4 must be done with both the parent compound and the metabolite(s). The fifth step is to assess the ability of the compound to disrupt epithelial cell barrier function using a transepithelial resistance assay across a membrane. The results from such a system will help determine if organs (e.g., brain, and kidney) that depend on barriers for defense against toxic insult are likely to be targets. If the compound causes disruption of barrier function at a concentration lower than the basal cytotoxicity, the endpoint used in determining the effect on the organism might need to be lowered to take this into consideration. If there is evidence of metabolism in Step 3, Step 5 must be done with both the parent compound and the metabolite(s).

Chemical Data Sets for Validation of *In Vitro* Toxicity Tests

Breakout Group 4 defined the chemical data sets required for validation studies, identified existing resources, and recommended approaches for using existing data sets and/or compiling or developing new data sets.

Rather than develop specific lists of chemicals, BG4 developed criteria for establishing a database of chemicals to use to validate individual tests or prediction models. In identifying needs, BG4 noted that chemicals chosen for use in a validation study should be distributed uniformly across a broad range of toxicity. Two sets of chemicals are needed: 1) training sets that can be used for method development and 2) validation sets that can be used to confirm the predictive capacity of the tests. In selecting chemicals for use in validation studies, needs of the user communities must be met. The performance parameters of the *in vivo* tests must be clearly defined prior to chemical selection if the results of these tests are to serve as a baseline for judging success.

To evaluate the current status of chemical data sets for prevalidation and validation activities, a number of databases were discussed. The NTP database would be a useful component of any primary database of chemicals for validation. The

high production volume (HPV) database, containing predominantly industrial chemicals, might not meet the needs of all user communities. The U.S. Environmental Protection Agency pesticides database and the U.S. Food and Drug Administration drugs and food additive databases contain associated LD50 data of good quality, but accessibility of the data may be impeded by confidentiality claims by the sponsors.

For future activities, BG4 recommended convening an expert committee to assemble a reference set of test chemicals from existing databases according to the following criteria:

- Chemicals selected must be consistent with the test protocol and its prediction model, be physically and chemically compatible with the test system, and include the relevant chemical classes.
  - — The definition of chemical class is context-specific.
  - — The developers of the test must specify the parameters that define the class.
  - — The chemicals must be chosen independently.
- The toxicity must cover the range of response with uniform distribution.
- The number of chemicals used in the subset will depend on the nature of the test and the questions being asked, and should be determined with statistical advice.

BG4 also recommended undertaking a study of existing databases to determine the variation in rodent LD50 results introduced by different laboratories and by different protocols used by various regulatory agencies.

To build upon the MEIC foundation, BG4 recommended that an expert panel review the MEIC approach for measuring acute toxicity parameters in humans. The Group agreed that a standard approach for measuring acute toxicity parameters is necessary and that existing sources of information should be searched carefully to ensure that all human data are obtained.

1.0 INTRODUCTION

This report summarizes the proceedings and outcome of the International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity, October 17-20, 2000, in Arlington, VA, U.S. This Workshop, the first convened by ICCVAM and NICEATM, evaluated the status of available *in vitro* methods for assessing acute toxicity. These included screening methods such as those that may be used to predict the starting dose for *in vivo* animal studies, and *in vitro* methods for generating information on toxicokinetics, target organ toxicity, and mechanisms of toxicity. The Workshop also developed recommendations for validation efforts necessary to further characterize the usefulness and limitations of these methods and for research and development efforts that might further improve *in vitro* assessments of acute systemic toxicity. Notice of the Workshop and requests for nomination of scientific experts and submission of information on relevant past, current, or future studies were announced in two Federal Register notices (See **Appendix H**).

This introduction briefly summarizes the purpose and history of acute toxicity testing and the purpose and conduct of the Workshop. The final reports from the Breakout Groups are presented in **Sections 2** through **5**. **Section 6** provides a glossary, while **Section 7** contains the Registry of Cytotoxicity (RC) Data, a database of LD50 values and *in vitro* cytotoxicity IC50 values, and a regression analysis between the two values. **Section 8** contains all references cited in the Breakout Group reports and appendices. The Appendices provide supplementary materials, including the Workshop agenda, a summary of the plenary sessions, guidance for the Breakout Groups, the background document provided to Workshop participants, the NICEATM summary of the Multicenter Evaluation of *In Vitro* Cytotoxicity (MEIC), regulatory requirements for acute toxicity information, a bibliography, the list of Workshop participants, Federal Register notices regarding the Workshop, and ICCVAM test method recommendations forwarded to Federal agencies.

1.1 History and Purpose of Acute Toxicity Testing

Acute oral systemic toxicity testing is conducted to determine the hazard potential of a single oral exposure to various chemicals and products. Four regulatory agencies in the United States, the Department of Transportation (DOT), the Consumer Product Safety Commission (CPSC), the Occupational Safety and Health Administration (OSHA), and the U.S. Environmental Protection Agency (EPA) require industry to label chemicals and products with hazard information based on LD50 estimates. DOT requires oral lethality data to determine the transportation requirements for hazardous substances (49 CFR 173). CPSC requires such information for labeling hazardous substances so as to protect consumers when such products are used in the home, the school, and recreational facilities (16 CFR 1500). OSHA requires the use of acute lethality data to implement labeling requirements for the hazard communication program to protect employees (29 CFR 1910). Certain EPA regulatory programs also require the submission or generation of acute toxicity data for hazard classification purposes (40 CFR 156). During acute toxicity testing, non-lethal endpoints may also be evaluated to identify potential target organ toxicity, toxicokinetic parameters, and/or dose-response relationships.

As shown in Table 1, the international community also uses acute oral toxicity data as the basis for hazard classification and the labeling of chemicals for their manufacture, transport, and use (OECD, 1998a). Other potential uses for acute toxicity testing data include:

- Establishing dosing levels for repeated-dose toxicity studies;
- Generating information on the specific organs affected;
- Providing information related to the mode of toxic action;
- Aiding in the diagnosis and treatment of toxic reactions;
- Providing information for comparison of toxicity and dose response among

- substances in a specific chemical or product class;
- Aiding in the standardization of biological products;
- Aiding in judging the consequences of single, high accidental exposures in the workplace, home, or from accidental release;
- Serving as a standard for evaluating alternatives to animal tests.

Table 1.1 OECD Harmonized Integrated Hazard Classification System for Human Health and Environmental Effects of Chemical Substances-- Oral Toxicity (OECD, 1998a)

Acute Toxicity Route	Toxicity Class 1	Toxicity Class 2	Toxicity Class 3	Toxicity Class 4	Toxicity Class 5
Oral LD50 Values (mg/kg) [approximate]	5	50	300	2000	5000

Historically, lethality has been the primary toxicological endpoint in acute toxicity tests. Trevan (1927) was the first to attempt to standardize a method for assessing the toxicity of potent biological toxicants, the progenitor of the "lethal dose, 50% (LD50) test". The classical LD50 test procedure that evolved from this innovation in the 1970s and early 1980s used from 100 to 200 animals per test substance (Galson, 2000). Although other information, such as the slope of the dose-response curve, confidence interval for the LD50, and toxic signs, could also be obtained from this test, the procedure was severely criticized for both scientific and animal welfare reasons (Zbinden and Flury-Roversi, 1981). These criticisms eventually resulted in the proposal and adoption of a new guideline (OECD TG 401; OECD, 1987) that reduced the required number of animals to 20. This has become the most widely used method for defining the acute toxicity of a chemical and a mandatory-testing requirement for new chemicals. More recently, the acute toxicity test procedure has been modified in various ways to refine and further reduce the number of animals used to a maximum of 16 (OECD, 1992; 1996; 1998b). The Globally Harmonized Scheme for Hazard Classification prompted a re-assessment of all of the OECD *in vivo* test guidelines for acute toxicity (i.e., fixed dose, up and down procedure, acute toxic class method) to ensure that regulatory needs are met while minimizing animal usage and maximizing data quality.

Recent studies suggest that *in vitro* methods may be helpful in predicting acute toxicity and reducing the number of animals necessary to assess acute toxicity. Studies by Spielmann et al. (1999) suggest that *in vitro* cytotoxicity data may be useful in identifying an appropriate starting dose for *in vivo* studies, and thus may potentially reduce the number of animals necessary for such determinations. Other studies (e.g., Ekwall et al., 2000) have indicated an association between chemical concentrations leading to *in vitro* basal cytotoxicity and human lethal blood concentrations. A program to estimate toxicokinetic parameters and target organ toxicity utilizing *in vitro* methods has been proposed that may provide enhanced predictions of toxicity, and potentially reduce or replace animal use for some tests (Ekwall et al., 1999). However, many of the necessary *in vitro* methods for this program have not yet been developed. Other methods have not been evaluated in validation studies to determine their reliability and relevance for generating information to meet regulatory requirements for acute toxicity testing. Development and

validation of *in vitro* methods that can establish accurate dose-response relationships will be necessary before such methods can be considered for the reduction or replacement of animal use for acute toxicity determinations.

1.2 Purpose and Objectives of the Workshop

The International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity examined the status of available *in vitro* methods for predicting acute toxicity, including screening methods for acute toxicity, and other methods that might be suitable to predict the starting dose for *in vivo* animal studies, and methods for generating information on toxicokinetics, target metabolism organ toxicity, and mechanisms of toxicity. The Workshop developed recommendations for validation efforts necessary to further characterize the usefulness and limitations of these methods. Recommendations were also developed for future mechanism-based research and development efforts that might further improve *in vitro* assessments of acute systemic lethal and non-lethal toxicity.

Specific objectives of the Workshop were to:

- Review the status of *in vitro* methods for predicting acute systemic toxicity:
  - — Review the validation status of available *in vitro* screening methods for their usefulness in estimating *in vivo* acute systemic toxicity;
  - — Review *in vitro* methods for predicting toxicokinetic parameters relevant to acute toxicity (i.e., absorption, distribution, metabolism, elimination);
  - — Review *in vitro* methods for predicting specific target organ toxicity;
- Recommend candidate methods for further evaluation in prevalidation and validation studies;
- Recommend validation study designs to adequately characterize the usefulness and limitations of proposed *in vitro* methods;
- Identify reference chemicals for development and validation of *in vitro* methods for assessing *in vivo* acute toxicity;
- Identify priority research efforts necessary to support the development of *in vitro* methods to assess acute systemic toxicity adequately. Such efforts might include incorporation and evaluation of new technologies such as gene microarrays, and development of methods necessary to generate dose response information.

1.3 Conduct of the Workshop

The International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity, which was open to the public, was conducted over three and a half days. The final agenda for the meeting is provided in **Appendix A.** As the agenda shows, the Workshop began with a plenary session to frame the purpose and objectives of the Workshop and formulate the problem of using *in vitro* tests to predict *in vivo* acute toxicity. A summary of the opening plenary session is provided in **Appendix B.** The opening plenary session was followed by Breakout Group discussions for two and a half days. Each of the four Breakout Groups was comprised of 12 to 18 individuals who were invited scientific experts or ICCVAM agency participants. Breakout Groups addressed their assigned objectives for the Workshop by developing responses to questions provided in the background materials for the Workshop (See **Appendix C**). Breakout Groups reported on their progress each morning of the second and third days, and gave a final report on the last day of the meeting. Written reports of each Breakout Group's findings, conclusions and recommendations are provided in **Sections 2** through **5**. Public observers were invited to provide comments in both plenary and breakout sessions of the Workshop. A summary of public comments during plenary sessions is provided in **Appendix B.** After the Workshop, ICCVAM reviewed the Breakout Group reports and developed test method recommendations for Federal agencies (see **Appendix I**).

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2.0 *IN VITRO* SCREENING METHODS FOR ASSESSING ACUTE TOXICITY

2.1 Introduction

Since the early work of Pomerat and Leake (1954), Eagle and Foley (1956), and Smith and colleagues (1963), research over the last 50 years has been conducted to evaluate the potential use of *in vitro* cell systems for predicting acute toxic effects *in vivo*. Significant correlations between cytotoxicity *in vitro* and animal lethality have been demonstrated on numerous occasions (for reviews see Phillips et al., 1990; Garle et al., 1994), as have correlations between cytotoxicity *in vitro* and systemic and topical effects from acute exposures to chemicals. Several newer initiatives directed toward reducing and replacing the use of laboratory animals for acute toxicity testing have emerged (Curren et al., 1998; Ohno et al., 1998; Spielmann et al., 1999; Ekwall et al., 2000); these initiatives were reviewed as part of the charge given to Breakout Group 1 (*In Vitro* Screening Methods) at this Workshop.

2.1.1 *Charge to the Breakout Group*

Breakout Group 1 (BG1) was asked to evaluate the validation status of available *in vitro* methods for estimating *in vivo* acute toxicity and was requested to identify methods and appropriate validation studies that might be completed within the next one to two years. It was also envisaged that the Breakout Group would evaluate potential uses of QSAR as part of an *in vitro* strategy.

2.1.2 *Objectives*

The specific objectives of the Workshop pertinent to the charge given to BG1 were given as follows:

(1) Review the validation status of available *in vitro* screening methods for their usefulness in estimating *in vivo* acute toxicity.

(2) Recommend candidate methods for future evaluation in prevalidation and validation studies.

(3) Recommend validation study designs that can be used to adequately characterize the usefulness and limitations of proposed *in vitro* methods.

(4) Identify priority research efforts necessary to support the development of mechanism-based *in vitro* methods to assess acute systemic toxicity.

In its opening deliberation on these objectives, BG1 members decided to limit the review to methods for reducing or replacing animal use for determining acute lethality with the understanding that Breakout Group 3 would focus on methods for assessing acute systemic toxicity.

2.2 Background

Cytotoxicity has been defined as the adverse effects resulting from interference with structures and/or processes essential for cell survival, proliferation, and/or function (Ekwall, 1983). These effects may involve the integrity of membranes and the cytoskeleton, cellular metabolism, the synthesis and degradation or release of cellular constituents or products, ion regulation, and cell division. Ekwall (1983) described the concept of "basal cell functions" that virtually all cells possess (mitochondria, plasma membrane integrity, etc.) and suggested that, for most chemicals, toxicity is a consequence of non-specific alterations in those cellular functions which may then lead to effects on organ-specific functions and/or death of the organism.

Ekwall drew two important inferences from his early studies: that (a) cell cultures (notably cell lines) can be used to detect basal cytotoxicity; and (b) many chemicals exert cytotoxic effects on these cultures at concentrations which would be lethal in humans. Ekwall recognized that there will be exceptions and ultimately refinements needed in the development of a test battery for predicting human lethality, as, for example, incorporating test strategies for identifying chemicals that produce cell selective (organ specific) toxicity at lower concentrations than "basal" (or general) cytotoxicity.

Others likewise concluded that, since the actions of chemicals that produce injury and death are ultimately exerted at the cellular level, cytotoxicity assays may be useful for the prediction of acute lethal potency (Grisham and Smith, 1984). Based on that premise, a considerable amount of research has been undertaken into the development and evaluation of *in vitro* tests for use as screens and as potential replacements for *in vivo* LD50 tests. Good agreement between cytotoxicity *in vitro* and animal lethality have been reported by numerous groups (see reviews by Phillips et al., 1990; Garle et al., 1994; Guzzie, 1994). However, none of the proposed *in vitro* models have been evaluated in any formal studies for reliability and relevance, and their usefulness and limitations for generating information to meet regulatory requirements for acute toxicity testing have not been assessed.

More recently, Spielmann and colleagues have conducted studies to indicate that, as a first step toward replacement of LD50 tests, *in vitro* cytotoxicity data could be used now to identify the appropriate starting dose for *in vivo* studies, thereby reducing the number of animals necessary for such determinations (Spielmann et al., 1999). Other studies have indicated an association between chemical concentrations inducing cytotoxic effects *in vitro* and human lethal blood concentrations (Ekwall et al., 2000). Several groups have proposed the use of *in vitro* cytotoxicity tests in tiered testing schemes. These tests include proposed strategies for using *in vitro* test data as a basis for classifying and labeling new chemicals, thereby reducing (and possibly replacing) the need for acute toxicity tests in animals (Seibert et al., 1996) and for *in vitro* cytotoxicity data and other information in a tiered approach to replace oral LD50 tests (Curren et al., 1998). Curren and colleagues recognized that the application of their proposal was limited because of insufficient information on the many cellular mechanisms involved in chemical-induced lethality and because the most reliable *in vitro* models for gastrointestinal uptake, blood-brain barrier (BBB) passage, and biotransformation for more precise quantitative *in vivo* toxic dose/exposures were not yet identified.

To summarize, many investigations of the relationship between *in vitro* cytotoxicity and acute toxicity *in vivo* have been reported. Since it was not possible to critically review and discuss all of the published literature in the course of the Workshop, a selection of recent key activities and reports that included the most advanced and extensive efforts to develop alternative methods for lethality was made for consideration by Breakout Group 1 (Appendix D). The most intensive discussions focused on the ZEBET and MEIC approaches, which are outlined below in detail for the reader's reference (Sections 2.2.1-2.2.6 and 2.2.7, respectively).

2.2.1 *Prediction of In Vivo Starting Doses (ZEBET Approach)*

Investigators (Halle et al., 1997; Halle 1998; Spielmann et al., 1999) have proposed a strategy to reduce the number of animals required for acute oral toxicity testing. The strategy is referred to in this document as the ZEBET approach where ZEBET is the acronym for Zentralstelle zur Erfassung und Bewertung von Ersatz- und Ergaenzungsmethoden zum Tierversuch (the National Center for Documentation and Evaluation of Alternative Methods to Animal Experiments). The strategy involves using *in vitro* cytotoxicity data to determine the starting dose for *in vivo* testing. They report the findings of an initial study conducted to assess the feasibility of applying the standard regression between mean IC50 values (i.e., IC50*x*, the mean concentration estimated to affect the endpoint in question by 50%) and acute oral LD50 data included in the Register of Cytotoxicity (RC) to estimate the LD50 value which can then be used to determine the *in vivo* starting dose.

The RC is a database of acute oral LD50 data from rats and mice (taken from the NIOSH Registry of Toxic Effects of Chemical Substances [RTECS]) and IC50*x* values of chemicals and drugs from *in vitro* cytotoxicity assays (Halle and Goeres, 1988; Halle and Spielmann, 1992). It currently contains data on 347 chemicals (Halle, 1998; Spielmann et al., 1999). The main purpose of establishing the RC was to evaluate, with a large amount of non-selected data from various chemicals with different systemic oral toxicities, whether basal

cytotoxicity (averaged over various cells, cell lines, and/or toxicity endpoints) is a sufficient predictor for acute systemic toxicity.

Apart from the fact that basal cytotoxicity was an acceptable predictor (i.e., LD50 values localized in the dose range around the regression line by the empirical factor $F_G \leq \log 5$) of the LD50 for 74% of the RC chemicals (Halle and Spielmann, 1992), the predicted LD50 value can be used as a starting dose in acute oral toxicity testing to reduce the number of animals. This concept was first discussed at an ECVAM workshop (Seibert et al., 1996) as it related to refinements of *in vivo* acute toxicity tests by the use of new sequential dosing methods such as the Acute Toxic Class method ([ATC; OECD TG 423] OECD, 1996) and the Up-and-Down Procedure ([UDP; OECD TG 425] OECD, 1998b). In these tests, the number of animals needed depends upon the correct choice of the starting dose, since the number of consecutive dosing steps would be reduced as the starting dose more closely approximates the true toxicity class (ATC), or the true LD50 (UDP) (i.e., the more precisely the starting dose is predicted, the fewer animals that need to be used).

2.2.2 *Characterization of the RC*

The first registry, RC-I (Halle and Göcres, 1988), contained 117 chemicals and served as a training data set to establish a linear regression model for predicting oral LD50 values. A second data set of 230 chemicals, RC-II, verified the regression obtained with RC-I (Halle, 1998). Currently, a third RC of 150 chemicals that will increase the number of chemicals to almost 500 is in preparation. It is important to note that, in order to keep the registry unbiased, published data that were complete and met the acceptance criteria described below were included in the RC without further restriction. Thus, the RC contains data of nonselected chemicals. However, it has to be noted that selecting only published data may be a slight bias in itself because it identifies chemicals of scientific interest, public concern, etc., so that pharmaceuticals, pesticides, consumer products (e.g., cosmetics, food additives, etc.), and biocides are over-represented compared to industrial chemicals; the majority of the latter are of low toxicity (I. Gerner, BgVV, personal communication, as cited in Spielmann et al., [1999]).

The acceptance criteria for the *in vitro* cytotoxicity data were defined as follows:

- At least two different IC50 values were available, either from different cell types, or from different cell lines, or from different cytotoxicity endpoints.
- Only cytotoxicity data obtained with mammalian cells were accepted.
- Cytotoxicity data obtained with hepatocytes were not acceptable.
- The chemical exposure time in the cytotoxicity tests was at least 16-hr.

Only the following cytotoxicity endpoints were accepted:

- Cell proliferation: cell number, cell protein, DNA content, DNA synthesis, colony formation;
- Cell viability, metabolic indicators: MIT-24, MTT, MTS, XTTC;
- Cell viability, membrane indicators: Neutral Red Uptake (NRU), Trypan blue exclusion, cell attachment, cell detachment;
- Differentiation indicators.

The acceptance criteria for the *in vivo* data were defined as follows:

- Only LD50 values published in RTECS were used.
- If different issues of RTECS reported different LD50 values, then the first LD50 value was used for the RC. This value is also the highest value reported, since NIOSH replaces an LD50 value whenever a smaller value is available in the literature. A continuous change of *in vivo* data in the RC would not have been acceptable because the RC database had to be 'closed' to form a training data set (RC-I) and later a verification data set (RC-II). Therefore, since the beginning of data collection for RC-II, all LD50 values were only taken from

the 1983 RTECS issue, and later issues were not used.

The IC50 values from RC-I and RC-II, for a total of 347 chemicals, were obtained from 157 original publications in the literature. In the regression analysis for 347 chemicals, 1,912 single IC50 values were averaged (geometric means) per chemical to one IC50*x* value and then paired with 347 *in vivo* acute oral LD50 values. Whenever obtainable from RTECS, oral *in vivo* LD50 data from the rat were used (282 values). As a second priority, LD50 data from the mouse were used (65 values). Before data of rats and mice were merged in the RC, regression analyses performed separately with rat and mouse data justified this procedure (Halle, 1998). Although, by pairing 347 *in vitro* IC50*x* data with 347 *in vivo* LD50 data, an equal weight is given to each chemical, it has been criticized by reviewers that the IC50*x* is the geometric mean of a few up to many single data [minimum: n = 2, maximum: n = 32] per chemical. However, if the RC regression is recalculated with the means of only the smallest and the largest IC50 values per chemical, there are no differences in the regression function (Halle, personal communication).

To obtain a prediction model, a linear regression was derived from pairs of the log-transformed IC50*x* values and oral LD50 values (in mmol/kg), where 'a' is the intercept and 'b' is the regression coefficient, to produce the regression model [log (LD50) = b x log (IC50*x*) + a] shown graphically in Figure 2.1:

log (LD50) = 0.435 x log (IC50*x*) + 0.625

To allow comparison of the predictive value of the RC (or parts of the RC) with other similar approaches (prediction of the LD50 from basal cytotoxicity), an empirical linear-shaped prediction interval of a factor (F_Q) of ± log 5 was defined (Figure 2.1). The linear-shaped boundaries should not be confused with the curved boundaries of a probability-based confidence interval. Halle defined this interval empirically as an acceptability measure based on information of the required and expected precision of rodent oral LD50 data (Halle and Spielmann,1992).

To evaluate the validity of the regression model, the key parameters of the regression for RC-I, RC-II, and RC-I+II (Table 2.1) were compared with the regression parameters obtained with single mammalian cell lines. Table 2.1 shows that all regression lines have essentially identical intercepts and regression coefficients (slopes) regardless of whether single parts of the RC or the whole RC were analyzed, or whether data from single studies with only one cell line were used. In addition, the percentage of data within the defined prediction interval (± log 5) is almost constant (73%-77%). In summary, the regression function derived from the RC, and from the RC subsets, seems to be a reliable description of the general relationship between basal cytotoxicity and rodent oral systemic LD50 values. This relationship can consequently be used as a mathematical model for prediction of rodent oral LD50 values from basal cytotoxicity.

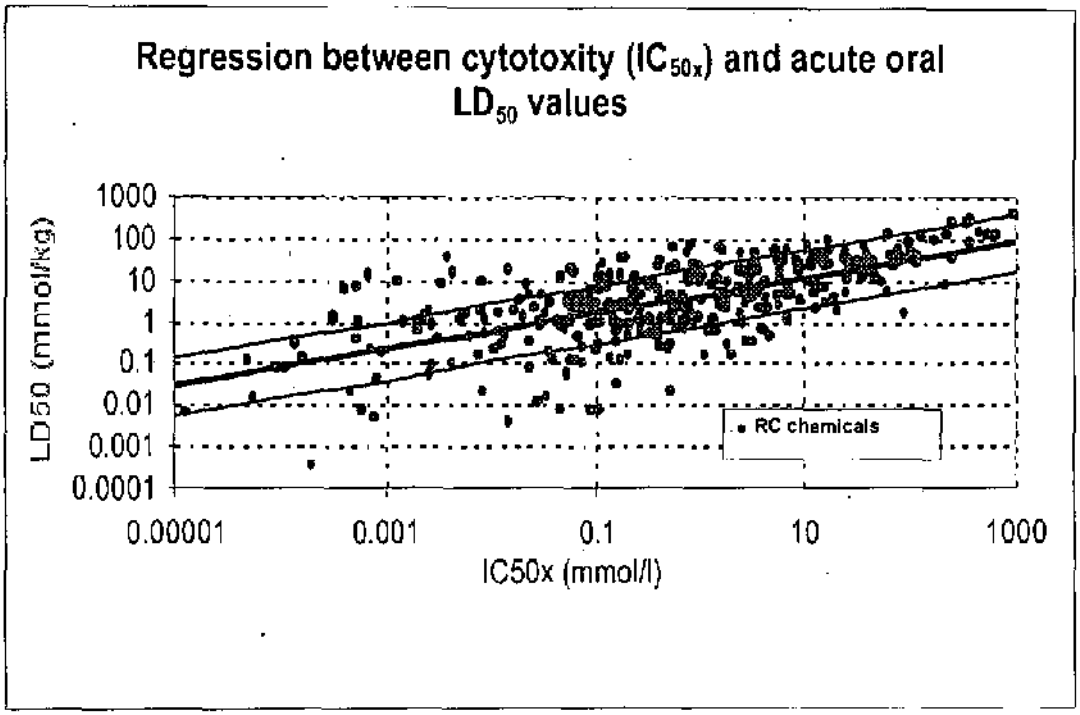

Figure 2.1. Registry of Cytotoxicity regression between cytotoxicity (IC50x) and rodent acute oral LD50 values of 347 chemicals
The heavy line represents the fit of the data to a linear regression model (r=0.67); the two additional lines represent the boundaries of ± log 5, an acceptance interval for this prediction model (Halle and Spielmann, 1992). This factor, F_G = ± log 5, was established based on information of the required and expected precision of LD50 values from rodent studies. The equation of the regression line (prediction model) reads: log (LD50) = 0.435 x log (IC50*x*) + 0.625.

Table 2.1. Linear regression parameters of two RC issues and two single studies using one cell line and one cytotoxicity endpoint

RC or Cell line**	Number of Chemicals (n)	Correlation Coefficient (r)	Intercept (a)	Regression Coefficient (b)	% Chemicals in Prediction Interval[a]	Reference[b]
RC-I *	117	0.667	0.637	0.477	74	1
RC-II *	230	0.666	0.634	0.414	73	2
RC-I+II *	347	0.672	0.625	0.435	73	2, 3, 4
BCL-D1**	22	0.720	0.536	0.633	77	5
3T3-L1 **	91	0.720	0.631	0.427	74	6

[a]Prediction interval for regression line is ± F_G ≤ log 5.
[b]References: 1 = Halle and Göeres, 1988; 2 = Halle, 1998; 3 = Halle et al., 1997; 4 = Spielmann et al., 1999; 5 = Knox et al., 1986; 6 = Clothier et al., 1988.

2.2.3 *Influence of the Starting Dose in the Acute Toxic Class (ATC) Method.*

Introductory note: The current accepted version of the ATC is the version adopted by the OECD in 1996 (OECD TG 423; OECD, 1996). Several updated drafts have been created since the OECD endorsed a new Globally Harmonized System (GHS) for the classification of chemicals in November 1998 (OECD, 1998a). The most recent draft of TG 423 was issued after the ICCVAM Workshop was held (OECD, October, 2000; http://www.oecd.org/ehs/test/health.htm). Consequently, the following analysis focuses on the 1996 OECD version of TG 423, but also attempts to address recent developments.

Following a national and an international experimental validation study of the ATC Method (Schlede et al., 1992, 1994; Diener et al., 1995), the ATC was accepted by the OECD (OECD TG 423; OECD, 1996) as an alternative to the classical LD50 test for acute oral toxicity. In the TG 423 procedure, a substance is tested in a stepwise dosing procedure with each step using three animals of a single sex at the same time. The proportion of survivors dosed at one step determines the next step, which is: (a) no further testing, or (b) dose three additional animals with the same dose, or (c) dose three additional animals at the next higher or the next lower dose. Originally, the method was developed and experimentally validated with two sexes and three different fixed starting doses (25, 200, and 2000 mg/kg body weight [b.w.]) reflecting the European Union (EU) hazard classification system. A thorough biometrical analysis (Diener et al., 1995) showed that the ATC is applicable to all hazard classifications currently in use.

Figure 2.2 shows, for example, that to classify a chemical as "toxic" or "very toxic", 1-2 consecutive steps could be saved if 25 mg/kg b.w. was used as the starting dose instead of the medium dose. With increasing distance between the true toxicity class and the starting dose, the number of dosing steps increases. This effect is shown in more detail in Table 2.2, which shows the expected number of animals used and the number that died in relation to starting dose and true LD50 for a dose-mortality slope of $\beta = 2$. Biometrical calculations with other slopes (from $\beta = 1$ to $\beta = 6$) revealed the dependency in Table 2.2 is only slightly affected by the dose-mortality slope (for details see Diener et al., 1995).

In summary, one to three dosing steps can be avoided if the optimum starting dose can be predicted from a preceding cytotoxicity test. Taking into account that approximately 75% of the LD50 values predicted from basal cytotoxicity tests are expected to fall within the prediction interval of ± log 5 (see Table 2.1), and, moreover, that the space between the three starting doses (25, 200, 2000 mg/kg b.w.) is a factor of about 10, it was anticipated that, for most chemicals, the starting dose predicted from cytotoxicity would have been the dose requiring the fewest consecutive steps to reach a classification.

In November 1998, the GHS for the classification of chemicals, which uses four toxicity classes instead of the three used by the current EU system, was endorsed by the OECD (OECD, 1998a). A fifth toxicity class (> 2000 – 5000 mg/kg b.w.) was additionally introduced for special regulatory purposes. As a consequence, the current updated Draft OECD TG 423 (OECD, October, 2000; http://www.oecd.org/ehs/test/health.htm) now uses four different starting doses (5, 50, 300, and 2000 mg/kg b.w.), but the upper boundary of the fifth class of 5000 mg/kg b.w. is not used as a starting dose. Figure 2.3 shows the proposed revision of the ATC.

For the version of the revised ATC to be consistent with the OECD GHS classification system, biometrical calculations of the expected number of animals used and dead in relation to starting dose, true LD50, and dose-mortality slope, have been published (Diener and Schlede, 1999). While any increase in the number of possible starting doses theoretically increases the potential to save dosing steps when using the optimal starting dose, only a small decrease in animal numbers is expected compared to the current ATC method because (a) the number of starting doses has been increased at the toxic end of the scale, where the prediction of the LD50 by IC50 is less accurate than at the non-toxic end of the scale, and (b) the entire scale is still about the same length.

INTERPRETATION OF RESULTS BASED ON OPTION 1 TESTING FOR COMMONLY USED CLASSIFICATION SYSTEMS

Starting dose: 200 mg/kg body weight

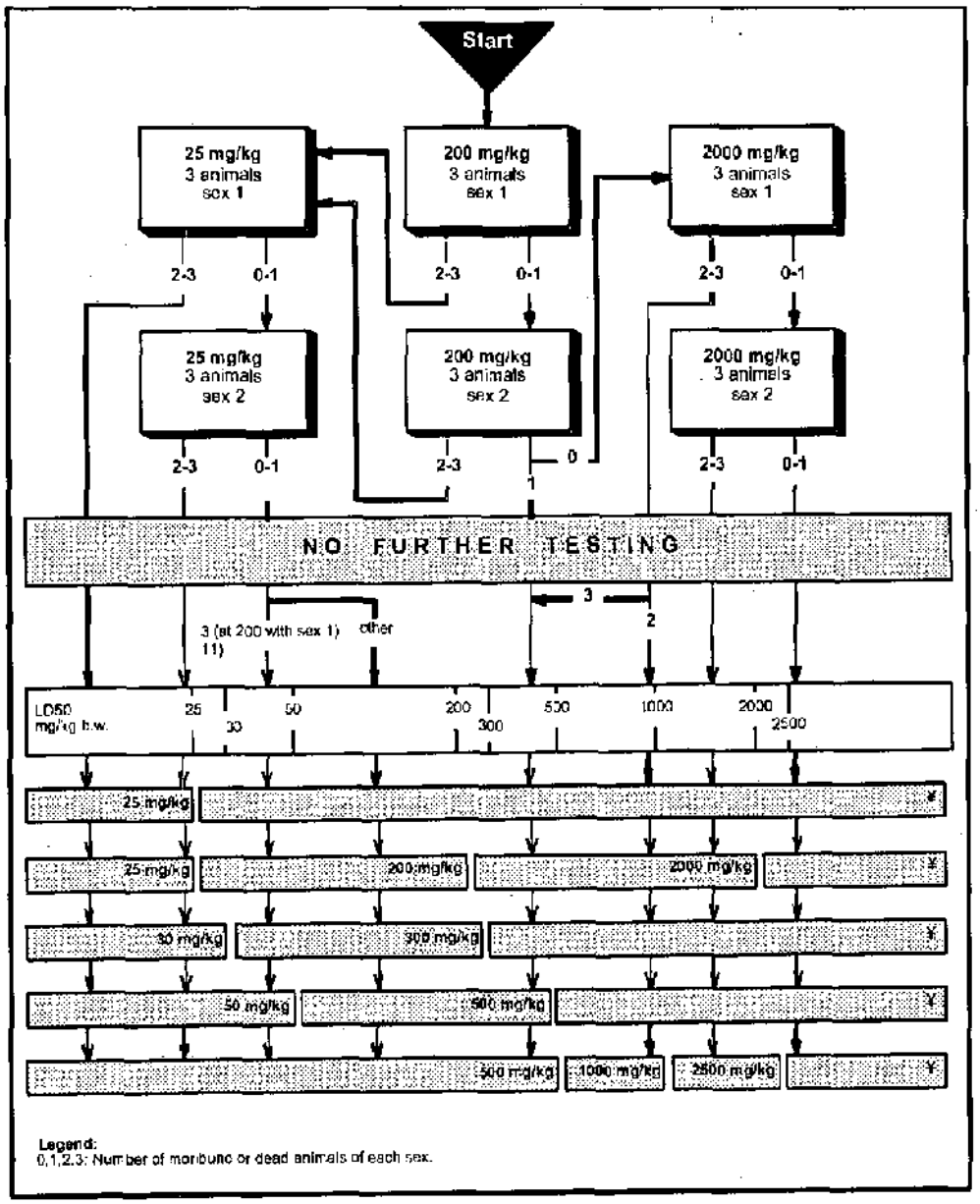

Figure 2.2 **Principle of the Acute Toxic Class (ATC) method: medium starting dose**
Source: OECD TG 423, Annex 3b (OECD, 1996). Example shows the possible dosing steps when 200 mg/kg b.w. is used as the starting dose. Depending on the toxicity of the test substance, 2 to 4 steps may be necessary to reach a classification according to hazard classification systems currently in use.

Table 2.2. Influence of the ATC starting dose on total number of animals (used and dead) in relation to the true LD50 for slope = 2[a]

	Starting dose in mg/kg body weight					
	25		200		2000	
True LD50	Used	Dead	Used	Dead	Used	Dead
1	3.0	3.0	6.0	6.0	9.0	9.0
2	3.0	3.0	6.0	6.0	9.0	9.0
5	3.1	2.8	6.1	5.8	9.1	8.8
10	3.4	2.7	6.4	5.6	9.4	8.6
20	4.6	2.8	7.2	5.3	10.2	8.3
50	7.5	3.3	8.6	4.2	11.6	7.2
100	9.3	3.2	9.3	3.3	12.2	6.2
200	11.2	3.2	9.7	3.1	12.0	5.3
500	14.0	3.3	9.3	3.3	10.0	3.9
1000	14.9	2.6	9.1	2.6	9.2	2.7
2000	15.4	1.8	9.4	1.8	9.3	1.8
5000	16.5	1.0	10.5	1.0	9.0	1.0
10000	17.3	0.4	11.3	0.4	7.7	0.4
20000	17.8	0.1	11.8	0.1	6.6	0.1
50000	18.0	0.0	12.0	0.0	6.1	0.0
100000	18.0	0.0	12.0	0.0	6.0	0.0

[a]Presented by W. Diener at the OECD ad hoc expert meeting on evaluation of the ATC in Berlin, Germany, 1994.

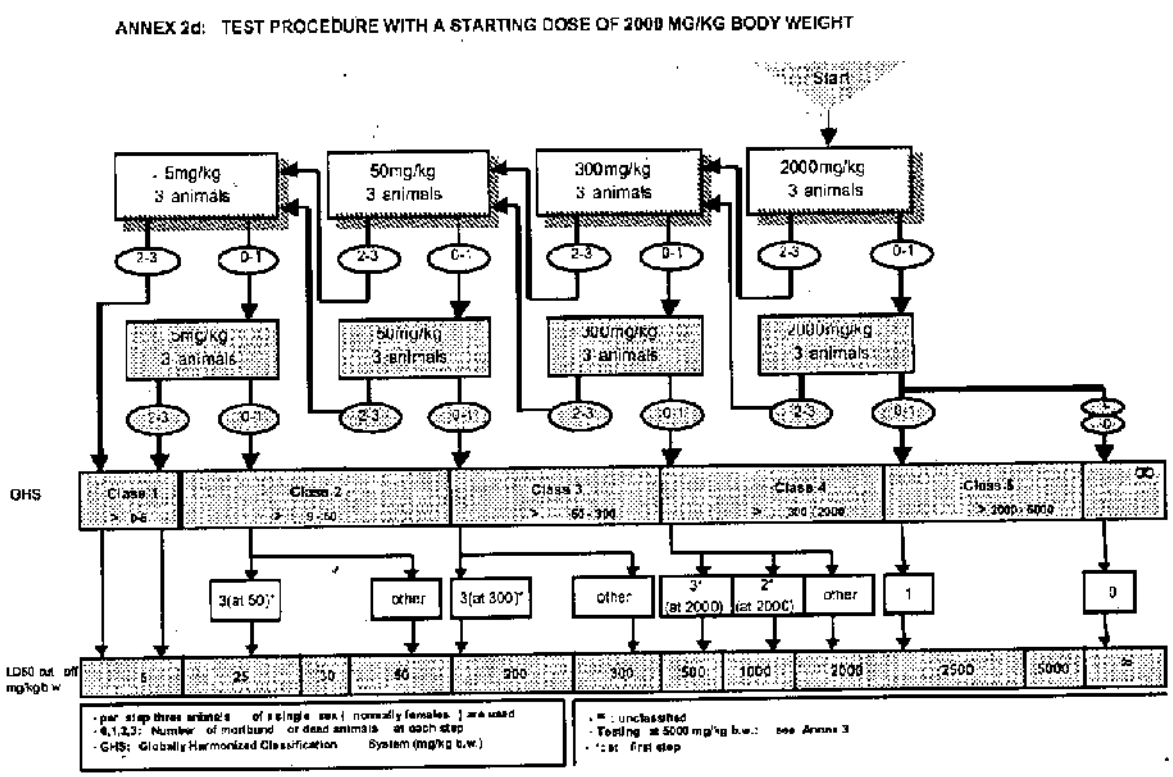

Figure 2.3. Proposed revision of the ATC to meet requirements of the OECD GHS
Source: OECD, Draft TG 423 (OECD, 2000). The number of new starting doses and spaces between have been changed so that the results from this test will allow a substance to be ranked and classified according to the GHS for the classification of chemicals which cause acute toxicity (OECD, 1998a).

2.2.4 *Influence of the Starting Dose in the Up-and-Down-Procedure (UDP)*

Introductory note: The current accepted version of the UDP is the version adopted by the OECD in 1998 (OECD TG 425; OECD, 1998b). Updated drafts of TG 425 have been created to allow for assessment of the confidence interval for the LD50 point estimate, and to include the application of new stopping rules and a larger dose progression factor, both of which tailor the UDP to the most efficient use of animals and improve the point estimate obtained. The most recent draft of TG 425 was issued after the ICCVAM Workshop was held (OECD, October 2000; http://www.oecd.org/ehs/test/health.htm). The analysis of the possible number of animals saved in a tiered approach is therefore based on the currently adopted 1998 OECD version of TG 425, but the significance for both versions can be assumed.

The concept of the up-and-down testing approach was first described by Dixon and Mood (Dixon and Mood, 1948; Dixon, 1965; 1991a, 1991b) and was later proposed to be used for the determination of acute toxicity of chemicals (Bruce, 1985). Apart from many biometrical publications refining the method (not cited here), a key review paper (Lipnick et al., 1995a) compared the results obtained with the UDP, the conventional LD50 test ([TG 401] OECD, 1981) and the Fixed Dose Procedure ([FDP; TG 420] OECD, 1992).

In principle, all versions of the UDP are stepwise procedures that use (as opposed to the ATC) single animals with the first animal receiving a dose at the best estimate of the LD50 (adopted TG 425, OECD 1998b), or one dosing step below the best estimate of the LD50 (most recent draft TG 425). Depending on the outcome for the first animal, the dose for the next is increased or decreased, either by a factor of 1.3 (adopted TG 425), or by a factor of 3.2 (recent draft TG 425). This sequence continues until there is a reversal of the initial outcome (i.e., the point where an increasing dose results in death rather than survival, or decreasing dose results in survival rather than death). After reaching the first reversal of the initial outcome, four additional animals are dosed following the up-down principle according to the adopted TG 425 (OECD, 1998b). In the most recent draft, however, a combination of stopping criteria is used to keep the number of animals to a minimum, while adjusting the dosing pattern to reduce the effect of a poor starting value or low slope. When one of the following criteria is satisfied, dosing is stopped and estimates of the LD50 and confidence interval are calculated according to the maximum likelihood method.

Three stopping criteria are defined in the draft UDP test guideline as follows:

(1) Three consecutive animals survive at the upper bound;
(2) Five reversals occur in any six consecutive animals tested (not just the first six);
(3) At least four animals have followed the first reversal and the specified likelihood-ratios exceed the critical value. (Calculations are made at each dose following the fourth animal after the first reversal.)

Under certain circumstances, which are defined in the draft Guideline, statistical computation will not be possible or will likely give erroneous results. For most applications, testing will be completed with only four to six animals after an or the initial reversal in animal outcome [stopping rule (c)]

Since the UDP test guideline ([TG 425] OECD, 1998b) clearly states that the test performance of the method is optimal if the investigator's best estimate is used as a starting dose, Spielmann et al. (1999) have investigated the quality of LD50 estimates derived from the RC (Halle, 1998) for several chemicals used to validate the UDP (Lipnick et al., 1995a). Of the 35 chemicals used in the UDP validation study (Lipnick et al., 1995a), nine chemicals were also part of the RC (acetonitrile, p-aminophenol, caffeine, coumarin, dimethyl-formamide, mercury (II) chloride, nicotine, phenylthiourea and resorcinol). For four chemicals, the LD50 values predicted by the RC were almost exactly the same as those determined with the UDP *in vivo*, (i.e., the LD50 values determined in the UDP were on the regression line of the RC) (see Figure 1 in Spielmann et al., 1999). For three chemicals,

the predicted LD50 values were within the prediction interval of ± log 5, and for two chemicals (p-aminophenol and caffeine), the predicted LD50 values differed from the *in vivo* LD50 values by one order of magnitude (Spielmann et al., 1999). Thus, even in this small set of data, the 'basic rule' derived from the RC that about 75% of the LD50 values predicted from cytotoxicity (see Section 2.2.2, Table 2.1) are acceptable, was confirmed. This indicates that cytotoxicity assays could be successfully used to determine starting doses, and can reduce the number of animals for *in vivo* studies, particularly the UDP.

To date, no computer simulations have been performed to estimate the possible reduction in animal numbers if the combined *in vitro/in vivo* approach is applied to the UDP. Thus, the Workshop discussions were based on computations taken from the ICCVAM background document for the peer review of a recent revision of the UDP (ICCVAM, 2000) which are shown in a slightly improved way in Figure 2.4a and Figure 2.4b. Figure 2.4a applies to the stopping rule defined in the adopted TG 425 (OECD, 1998b), and Figure 2.4b shows the effect when the likelihood-ratio (LR) stopping-rule (current draft OECD TG 425) applies.

Since the LR rule is only one out of three stopping rules that should be applied in an adaptive way, additional computation will be needed to assess the influence of the starting dose on animal usage. The upper curves of both figures depict the numbers of animals used if the starting dose is two logs from the true LD50 (1/100 LD50) while the lower curves show the number of animals used if the true LD50 is used as a starting dose. The percentage of animals saved when the starting dose equals the true LD50 value is about 30% in Figure 2.4a, and independent of the dose mortality slope; whereas in the case of the LR stopping rule (Figure 2.4b), 25 to 40% fewer animals may be used, depending on the slope.

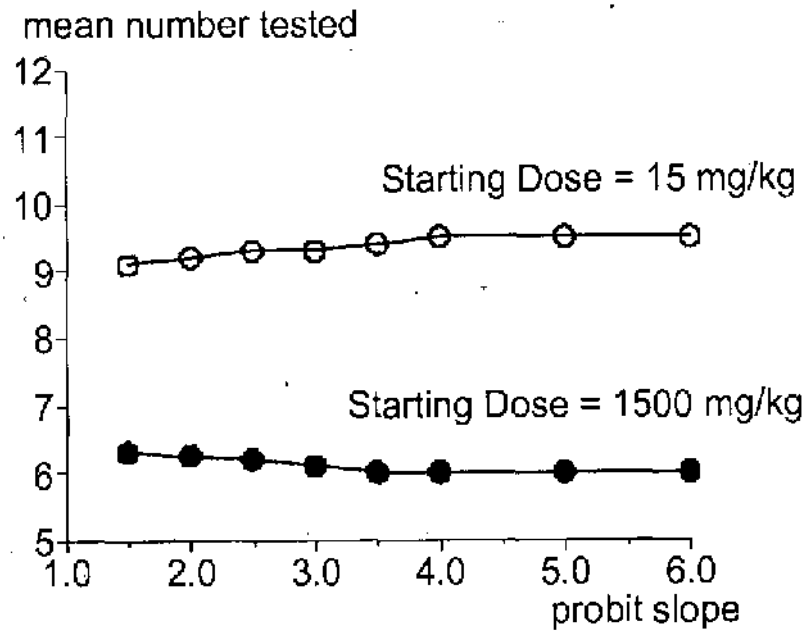

Figure 2.4a.Number of animals needed in relation to the starting dose for UDP adopted TG 425 (OECD 1998b) for LD50 = 1,500 mg/kg b.w.
The figure shows the number of animals needed if the LD50 is used as starting dose (lower curve), or if 1/100 of the LD50 is used as starting dose (upper curve). For details on the stopping rule applied see text.

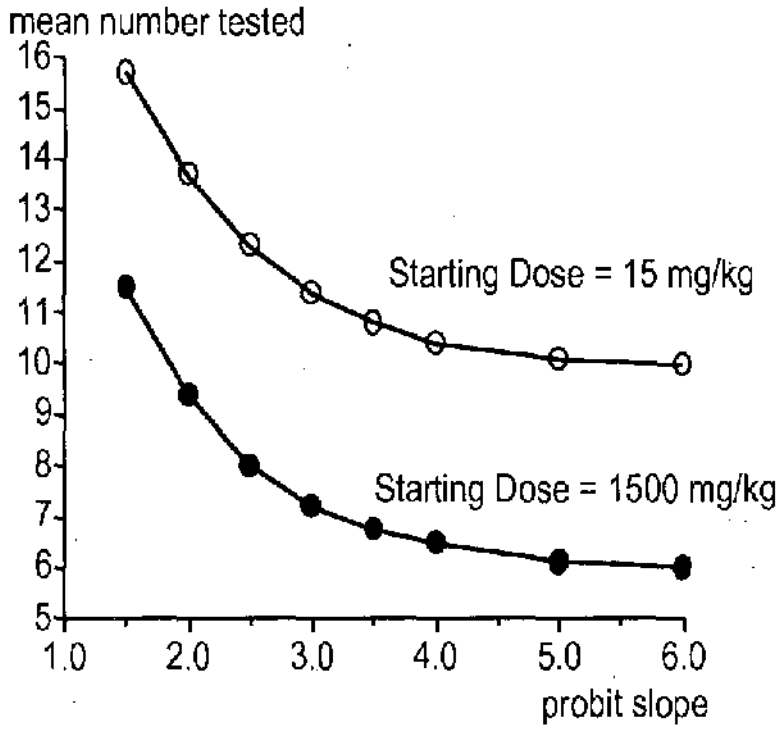

Figure 2.4b. Number of animals needed in relation to the starting dose for UDP draft TG 425 (OECD, 2000) for LD50 = 1,500 mg/kg b.w.
Figure shows the number of animals needed if the LD50 is used as starting dose (lower curve), or, if 1/100 of the LD50 is used as starting dose (upper curve) if the LR stopping rule singularly applies. For details see text.

2.2.5 *Prediction of a Limit Test Value from Basal Cytotoxicity Data*

According to a personal communication (Ingrid Gerner, BgVV) published by Spielmann et al. (1999), the notification process of new chemicals in the EU since 1982 revealed an unbalanced frequency distribution of the toxicity of industrial chemicals. No chemicals were classified "very toxic" (LD50 $\leq$ 25 mg/kg). Only 3% of the chemicals were classified "toxic" (LD50 > 25-200 mg/kg), while 21% were classified "harmful" (LD50 >200-2000 mg/kg), and the vast majority (76%) remained unclassified (LD50 > 2000 mg/kg). In other words, in the world of new industrial chemicals a clear majority are candidates for performing a 'limit test' where only the defined highest dose (2000 mg/kg most often, and occasionally 5000 mg/kg) is applied and no or marginal mortality occurs. Limit tests are defined in all OECD guidelines for acute oral toxicity testing (TG 401, TG 420, TG 423, and TG 425).

It must be emphasized that, if the limit dose defined in these guidelines is applied to all chemicals without knowledge of their toxicity, it would be correct for 76% of the chemicals, while 24% of the chemicals would cause avoidable deaths. It is therefore recommended to perform a limit test only if the prediction from a preceding basal cytotoxicity test suggests an LD50 value larger than the defined limit test dose. Special notice should be given to the fact that the precision of the prediction of low systemic toxicity from cytotoxicity data is much better than the precision of high systemic

toxicity. This is empirically supported by data from the RC (Halle, 1998) shown in Figure 2.1. The main factors affecting a strict log-linear relationship between basal cytotoxicity and systemic toxicity, bioavailability, and in some cases, biotransformation, play a minor role if a chemical is of low basal cytotoxicity.

2.2.6 *Evaluation of a Cytotoxicity Test Intended to be Used for Prediction of a Starting Dose*

This section describes how basal cytotoxicity data can be used to predict a starting dose for an *in vivo* lethality assay. Theoretically, any *in vitro* test that is capable of determining basal cytotoxicity could be used for determining the best estimate of a starting dose for acute testing in the UDP and ATC method. In addition, if the LD50 value predicted from cytotoxicity is high (≥ 2000 mg/kg b.w.), any of the currently used *in vivo* test protocols, including the FDP (OECD, 1992), would allow for performing an *in vivo* limit test without a proceeding sighting study.

In order to apply predictions of LD50 values obtained with experimental cytotoxicity data in the proposed tiered testing strategy as starting doses for the ATC or UDP methods, Spielmann et al. (1999) suggested a procedure shown in Figure 2.5. The authors suggested selecting 10-20 reference chemicals from the RC (Halle, 1998) and testing them in a standardized cytotoxicity test (Figure 2.5, Step 1). A promising candidate would be the BALB/c 3T3 NRU test that has proved robust in several validation studies. To allow comparison of the regression obtained with the in-house test (Figure 2.5, Step 2), reference chemicals should be selected to cover the entire range of cytotoxicity and to be as close as possible to the RC regression line.

Next, the in-house regression equation should be calculated by linear regression (least square method) using the new in-house IC50 values for the reference chemicals and the corresponding LD50 values from the RC. The resulting regression is then compared with the RC regression (Figure 2.5, Step 3). If the regression function obtained with the in-house cytotoxicity test is parallel to the RC regression and within the defined prediction interval, then the test is regarded suitable to be used without modification in applying the RC regression for future predictions of starting doses (Figure 2.5, Step 4). If the in-house regression shows a significantly higher or lower slope, then it may be possible to adjust the in-house test to a higher or lower sensitivity. However, it is likely that a more efficient approach would be to use a cell line and protocol, which have produced results that closely reproduce the RC data (recommended in the Guidance Document, ICCVAM, 2001).

The procedure of evaluating the usability of an in-house cytotoxicity test is explained in full detail in a special Guidance Document from this Workshop (ICCVAM, 2001), in which a set of 11 well-selected reference chemicals from the RC is recommended, and new experimental data obtained by testing the chemicals are presented. The data confirm that an in-house NRU cytotoxicity test, performed either with normal human keratinocytes (NHK) or with BALB/c 3T3 mouse cells, produces a regression line which matched the RC regression line ($R^2 > 0.9$).

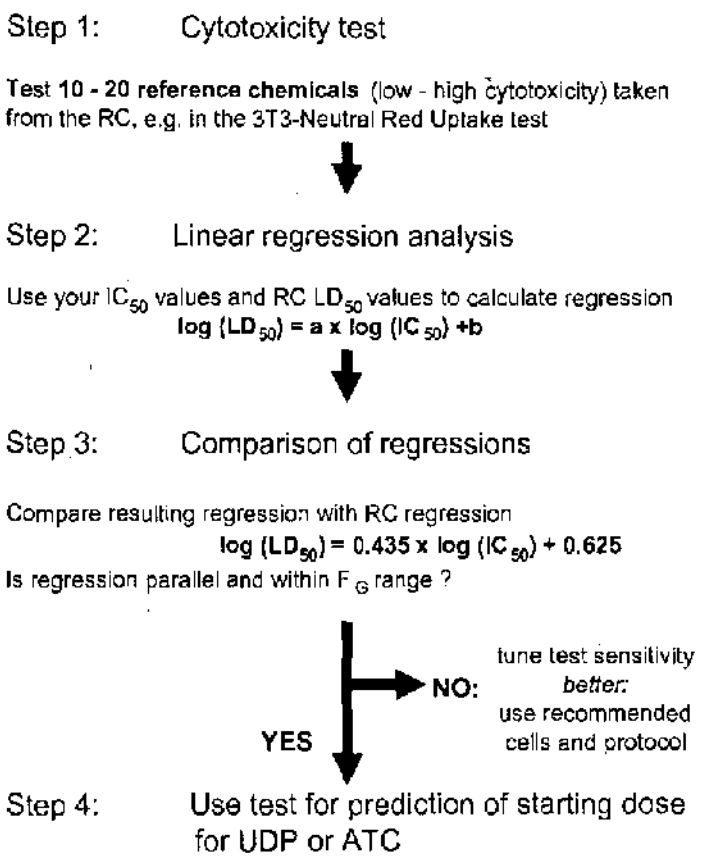

Figure 2.5. Procedure for evaluating a cytotoxicity test for tiered *in vitro*/*in vivo* testing for acute oral toxicity testing (slightly modified version of the scheme presented by Spielmann and colleagues). Note: based on the expectation that many valid cytotoxicity tests would match with the RC regression, Spielmann et al. (1999) defined only the "yes" option between steps 3 and 4. A "no" option has been added here for clarity.

2.2.7 *Multicenter Evaluation of In Vitro Cytotoxicity (MEIC Approach)*

The MEIC program was established by the Scandinavian Society for Cell Toxicology in 1989. The intention of the program was to investigate the relevance of *in vitro* test results for predicting the acute toxic action of chemicals in humans directly rather than in rodents. Batteries of existing *in vitro* tests that have the potential to serve as replacements for acute toxicity tests were identified. The program was designed as an open study with all interested laboratories worldwide invited to participate and test 50 preselected reference chemicals in their particular *in vitro* toxicity assays (Bondesson et al., 1989). Minimal methodological directives were provided in order to maximize protocol diversity among the laboratories. Eventually, some 96 laboratories participated in this voluntary undertaking.

The 50 reference chemicals were selected to represent different classes of chemicals, with the availability of good data on acute toxicity (lethal blood [or serum] concentrations [LC] in humans; oral LD50 values in rats and mice) being a key determinant. Since the LC data available from clinical toxicology handbooks are average values with a wide variation, they were found to be sub-optimal for comparative purposes. Therefore, during 1995-97, the MEIC management team collected case reports from human poisonings with the reference

chemicals to provide LC data with known times between ingestion and sampling/death. The aim was to compile enough case reports to be able to construct time-related LC curves for comparison with the IC50 values for different incubation times *in vitro*. The results were presented and analyzed in a series of 50 MEIC Monographs (referred to as the MEMO subproject by the organizers).

When the MEIC project finished in 1996, all 50 reference chemicals had been tested in 61 different *in vitro* assays. Twenty of these assays used human-derived cells, 18 of which were cell lines and two were primary cell cultures. In 21 of the assays, the cells were of animal origin (12 cell lines and 9 primary cell cultures). Eighteen of the assays were ecotoxicological tests, and two were cell-free test systems. The majority of the assays were based on measurement of effects on cell viability or cell growth (or a combination of the two).

The test results submitted to MEIC were analyzed statistically using analysis of variance (ANOVA), principal component analysis (PCA), and partial least square analysis (PLS) techniques. The analyses conducted were based on *in vitro* cytotoxicity data presented as IC50 values. The predictability of *in vivo* acute toxicity from the *in vitro* IC50 data was assessed against human lethal blood concentrations compiled from three different data sets: clinically measured acute lethal serum concentrations, acute lethal blood concentrations measured post-mortem, and peak lethal concentrations derived from approximate LC50 curves over time after exposure (Ekwall et al., 1998a).

Statistical analysis of results from the 61 assays using the PLS model predicted the three sets of lethal blood concentrations well (R^2 = 0.77, 0.76 and 0.83, Q^2 = 0.74, 0.72, and 0.81, respectively, where R^2 is the determination coefficient and Q^2 is the predicted variance according to cross-validation in the PLS model used) (Ekwall et al., 2000). A two-component PLS model of the prediction of lethal doses in humans from published oral rodent LD50 values for the 50 MEIC compounds was less effective (R^2 = 0.65, Q^2 = 0.64) (Ekwall et al., 1998a; Ekwall et al., 2000).

The analysis showed that *in vitro* assays that were among the most predictive generally used human cell lines (6 of the 18 assays using them gave the highest determination coefficients, vs. 1 of 12 rat cell line assays that performed comparably). Two of 9 non-human primary cell assays analyzed also performed well. Assays that did not perform well were primarily ecotoxicological assays using bacteria or plant cells and, in general, assays with very short exposure times (up to a few hours). Two human primary cell assays, both of which utilized PMN leukocytes and involved 3-hour exposure times, also performed relatively poorly. These results led the authors to note that human-derived cells appeared to be the most predictive for human acute toxicity.

The exposure time for the *in vitro* assays was most often 24 hours, but ranged from 5 minutes to 6 weeks. For 22 of the 50 reference chemicals, the toxicity *in vitro* increased with increasing exposure time. However, high predictivity was generally observed in vertebrate cell assays with 24 to 168 hours exposure. The actual endpoint measurements (cell viability assays) used with the *in vitro* tests were not crucial. Typically, different endpoint measurements gave approximately the same result, suggesting that basal (general) cytotoxicity can be assessed using many mammalian cell lines and almost any growth/viability endpoint.

To select an optimal battery for predicting acute toxicity in humans, the MEIC management team further evaluated various combinations of assays using PLS models and 38 chemicals deemed to have the most reliable and relevant lethal peak concentration data (see Ekwall et al., 2000, for the detailed procedure). From their analysis, the most predictive and cost-effective test battery consisted of four endpoints/two exposure times (protein content/24 hours; ATP content/24 hours; inhibition of elongation of cells/24 hours; pH change/7 days) in three human cell line tests. The test battery (designated 1,5,9/16) was found to be highly predictive of the peak human lethal blood concentrations of all 50 chemicals (R^2 = 0.79, Q^2 = 0.76) when incorporated into an algorithm developed by the team. The R^2 value was further improved to 0.83 when information

on BBB penetration was added to the battery results.

It was noted that passage across the BBB can be predicted from the chemical formula and/or physico-chemical properties, or from *in vitro* tests in appropriate model systems; however those methods were not used in the MEIC analysis. The MEIC team proposed that the cell battery they identified could be used immediately for many non-regulatory purposes in a multistep testing strategy and urged its formal validation (and/or that of other promising cell assays also identified in the MEIC program) as soon as possible (Ekwall et al., 2000). Test protocols for evaluating the proposed assays in a validation exercise remain to be developed and optimized.

In summarizing, the MEIC team concluded that their study yielded a limited battery of *in vitro* assays using human cell lines that showed very good performance and were cost effective for predicting acute lethality in humans (Ekwall et al., 2000). However, to further improve the predictive capability of this proposed battery, and to take into account non-basal cytotoxicity factors as a full replacement for acute animal tests, further, targeted development of *in vitro* methods for other particular endpoints is needed. An evaluation-guided development of new *in vitro* tests (EDIT) has been proposed to address these requirements (Ekwall et al., 1999), which includes, as most urgently needed, *in vitro* assays for:

- Assessing passage through the BBB;
- Predicting gut absorption;
- Distribution volume;
- Biotransformation.

The results of the MEIC program have appeared in a series of publications in the open literature (Clemedson et al., 1996a; Clemedson et al., 1996b; Clemedson et al., 1998a; Clemedson et al., 1998b; Ekwall et al., 1998a; Ekwall et al., 1998b; Ekwall et al., 1999; Clemedson et al., 2000; Ekwall et al., 2000). Additional information about MEIC, MEMO and EDIT, as well as the MEMO database, can be found at the following Internet address:

http://www.ectoxconsulting.a.se/nica.htm

2.3 Identifying Needs

In the area of human health effects, the overall aim is to reliably and accurately predict the potential for human acute toxicity. The Breakout Group noted that there is extensive documentation showing that human outcomes from chemical exposure are not predicted well by studies in rodent species (see, e.g., Ekwall et al. [2000] and the recent survey by Olson et al. [2000] on target organ toxicity). Consequently, it was agreed that the long-term goal (the ideal approach) should be the use a battery of *in vitro* tests employing human (rather than rodent or other animal) cells and tissues to provide data which when combined with information derived from other sources (e.g., on key physico-chemical parameters, kinetics, and dynamics) could more accurately predict human acute toxic effects including lethality. However, in the near term, the Breakout Group considered it appropriate and more pragmatic to concentrate on ways to reduce and replace animal use in acute oral toxicity tests as detailed in OECD TG401, TG420, TG423, and TG425.

The Breakout Group was fully aware that rather more information than just an (approximate) LD50 value can be obtained and used from a properly conducted rodent acute toxicity test (such as clinical signs, dose-response relationships, possible target organs, etc.); however, it received reassurance from the U.S. regulatory agencies represented at the Workshop that if there was a validated *in vitro* cytotoxicity test which could accurately predict the approximate rodent LD50 value *in vivo*, then its implementation would result in a significant reduction in animal use. Thus, the primary focus of Breakout Group 1 was to identify and evaluate candidate *in vitro* cytotoxicity tests that could possibly serve as reduction and replacement alternatives for current rodent acute oral toxicity tests for determining LD50 values.

2.3.1 *Near-term (< 2 years) Goals and Potentially Attainable Objectives*

The Breakout Group participants started from the premise that it is biologically plausible that

cell death (cytotoxicity) *in vitro* could be used to predict acute lethality. The many studies that show relatively good correlations between *in vitro* IC50 values and *in vivo* LD50 data support this view (e.g., Phillips et al., 1990; Garle et al., 1994). Thus, the near-term focus should be on conducting studies aimed at reducing and replacing animal use for determining LD50 values of chemical substances.

The Breakout Group agreed that standardized *in vitro* test protocols were available but probably not optimized, and that prediction models were needed for predicting acute oral LD50 values. Consequently, a prevalidation study, which would include several promising candidate *in vitro* cytotoxicity tests, would have to be undertaken in order to determine which tests should go forward to the validation stage. Partly because of this, the development of a practical replacement test will take time. As a parallel activity, the ZEBET method for generating cytotoxicity data to help establish the starting dose for *in vivo* testing of new chemical substances (Spielmann et al., 1999) should be seriously considered as an interim measure to potentially reduce the numbers of animals used in the *in vivo* tests.

2.3.2 *In Vitro Endpoints for Assessing In Vivo Acute Toxicity*

There is considerable literature covering a large variety of endpoints and endpoint measurements that have been evaluated for *in vitro* cytotoxicity testing (e.g., Phillips et al., 1990; Balls and Fentem, 1992; Garle et al., 1994; Itagaki et al., 1998a; 1998b; Ohno et al., 1998a; 1998b; 1998c; Tanaka et al., 1998; Clemedson and Ekwall, 1999; Ekwall, 1999). Some of these citations were provided to the Breakout Group members for reference, but time did not allow a systematic assessment of the literature on this topic. It was noted nevertheless that, in practice, basal function endpoints (such as NRU or MTT reduction and/or inhibition of cell proliferation), even though they may measure different cellular functions, have been commonly used with a reasonable degree of success; where cell lines are concerned, the endpoints typically assess a combination of both cell death and cell growth/proliferation. Since the events are based on cellular events that have circumstantial if not direct relevance to cellular responses to chemicals *in vivo*, model cell systems incorporating these "nonspecific" endpoints may satisfy requirements for fidelity and discrimination for alternative methods that have been set forth earlier (Blaauboer et al., 1998). The need for cell-specific or functional endpoints in acute toxicity assays was considered to be on a case-by-case basis and more relevant to studying target organ-specific toxicities (Breakout Group 3's charge).

2.3.3 *Other Issues for Selecting Protocols*

The key components of the protocols for *in vitro* cytotoxicity tests were considered to be the appropriate choice of: (a) cell type (human or animal, cell line or primary cultures) and its characteristics (stability, origin, characterization, availability); (b) exposure period(s) – (i.e., duration cells are exposed to the test chemical); and (c) endpoint measurement(s) – (i.e., cell viability assays such as NRU, lactate dehydrogenase [LDH] leakage, ATP content) (Borenfreund and Puerner, 1986; Riddell et al., 1986; Phillips et al., 1990; Balls and Fentem, 1992; Garle et al., 1994; Ekwall, 1999; Ohno et al., 1998a; Ekwall, 1999; Ekwall et al., 2000). In addition, the inclusion of a prediction model, evidence of repeatability, and facility of transfer between laboratories are important considerations (Balls et al., 1995; Bruner et al., 1996; Archer et al., 1997; ICCVAM, 1997). Ease of automation/high throughput where applicable should offer attractive additional cost benefits but is not a requirement for validation purposes.

2.3.4 *QSAR Models for Predicting Acute Toxicity*

The Breakout Group was requested to assess the role of QSAR, or related models such as structure-activity relationships (SAR) in predicting acute toxicity. While SAR methods involve qualitative assessment of chemical features that confer biological properties, QSAR approaches develop a quantitative relationship between physico-chemical or structural properties and biological activity (Albert, 1985; Barratt et al., 1995). QSAR models are usually developed for sets of chemically similar

compounds on the assumption that they will have the same mechanism of action. Any compounds that do not act by the same mechanism are likely to fit the correlation poorly, and thus their effects would not be predicted accurately. Although defining chemical classes or commonality of mechanisms of action are not trivial due to the multidimensional nature of both characteristics, a review of QSAR studies for predicting LD50 values concluded that QSAR methods have shown some success in relating LD50 values to certain physico-chemical properties of a compound, especially lipophilicity (Phillips et al., 1990).

In contrast, QSAR approaches appear to be less successful in correlating electronic properties of molecules (related to reactivity), or structural variables, with LD50 values, and their use with certain important chemical classes, (e.g., pesticides), is problematic. However, the Breakout Group felt that it lacked sufficient expertise in the field to evaluate the potential of QSAR as a replacement test for lethality and suggested that the topic be reviewed more thoroughly by a more appropriate scientific body. The review should include coverage of commercially available models (e.g., TOPKAT, CASE).

The Breakout Group did recognize that these methods might play key roles as adjuncts to improve LD50 predictions and to reduce animal usage. As noted by others (e.g., Barratt et al., 1998; Lipnick et al., 1995b), QSAR can aid in a number of areas, including the selection of test chemicals for validation studies, the interpretation of outliers, and the grouping of chemicals by structure and biological mechanisms. In addition, looking to future requirements to improve the predictive capability of *in vitro* cytotoxicity data for *in vivo* LD50 values, the Breakout Group agrees with Breakout Group 2 in recommending a more thorough evaluation of QSARs for predicting gut absorption and passage across the BBB. These applications were discussed at length by Breakout Group 2.

The Breakout Group noted that, in principle, expert systems, neural networks, and classical structure-activity approaches might be developed and validated for predicting specific systemic effects (Barratt, 2000; Dearden et al., 1997; Phillips et al., 1990). Requirements for the successful development and use of QSAR methods have been identified and include the following:

- A well-defined mechanism of action for the compound(s) used to derive the QSAR model;
- Use of congeneric, pure compounds and not mixtures;
- A common site of action for the biological effect;
- For comparative purposes, expressing concentrations or doses in molar (not weight) units;
- Validation of each model by investigating its predictive capability using a different set of compounds from its learning (i.e., training) set;
- Use of the same ranges of parameter space as the original test chemicals; and
- The QSAR should not be applied outside of its domain of validity (Phillips et al., 1990; Barratt et al., 1995; Worth et al., 1998).

The limitations or general applicability of each model for different chemical classes will need to be established. The application of QSAR procedures for identifying potential systemic effects was considered by Breakout Group 2.

2.4 Current Status

Many investigations of the relationship between *in vitro* cytotoxicity and acute toxicity *in vivo* have been reported. It was not possible to critically review and discuss all of the literature during the course of the Workshop, so the Workshop organizers made a selection of recent key activities and reports for consideration by Breakout Group 1. The Breakout Group made note of the fact that many of these recent initiatives build upon the conclusions of studies conducted, in particular, during the 1980s (e.g., Balls et al., 1992; Balls and Clothier, 1992; Balls and Fentem, 1992; Borenfreund and Puerner, 1986; Clothier et al., 1987; Dierickx, 1989; Ekwall, 1983; Ekwall et al., 2000; Fentem et al., 1993; Fry et al., 1988; Fry et al., 1990; Garle et al., 1987; Garle et al., 1994;

Gülden et al., 1994; Guzzie, 1994; Halle and Spielmann, 1992; Hopkinson et al., 1993; Hulme et al., 1987; Ohno et al., 1998a; Phillips et al., 1990; Riddell et al., 1986; Seibert et al., 1996; Spielmann et al., 1999; Wakuri et al., 1993; Zanetti et al., 1992).

The studies and approaches considered were:

- Studies conducted by FRAME and partners (e.g., Balls et al., 1992; Fry et al., 1990; Hulme et al., 1987; Riddell et al., 1986);
- The MEIC scheme (e.g., Clemedson and Ekwall, 1999; Ekwall et al., 2000);
- Japanese Society of Alternatives to Animal Experiments (JSAAE) activities (e.g., Ohno et al., 1998a);
- The ZEBET approach for predicting *in vivo* starting doses (Halle et al., 2000; Halle and Goeres, 1988; Spielmann et al., 1999);
- Testing strategy outlined in ECVAM Workshop Report 16 (Seibert et al., 1996);
- Testing framework proposed under the auspices of SGOMSEC (Curren et al., 1998);
- TestSmart acute systemic toxicity initiative to determine whether cellular changes can predict acute system failure *in vivo* (A. Goldberg, personal communication).

The MEIC and ZEBET approaches were presented to the Breakout Group as specific proposals for adoption as alternative methodologies by regulatory authorities, and therefore received the most attention.

2.4.1 *In Vitro Methods for Estimating Acute In Vivo Toxicity*

There are more than 80 variations of *in vitro* basal cytotoxicity tests, employing a variety of cell lines (e.g., HeLa, HL-60, BALB/c 3T3, Chang cells) and endpoint measurements (e.g., MTT reduction, NRU, ATP content, LDH leakage). From the results of the MEIC and ZEBET programs it appears that basal cytotoxicity can be determined using almost any cell line and almost any toxicity endpoint measurement that correlates well with cell death and/or growth inhibition. Standard protocols are available for some of these methods (e.g., via the *INVITTOX* database run by ECVAM, from the JSAAE validation study, and by slight modification of test protocols used for other purposes such as phototoxicity or eye irritation testing), but these have not necessarily been optimized for predicting rodent oral LD50 values.

Typically, prediction models have not been explicitly defined, although they are usually based on the IC50 value derived in the *in vitro* cytotoxicity assay. Some of these initiatives made note of that and tried to define useful testing strategies that incorporated *in vitro* assays. An example was the ECVAM Workshop report, which to some extent was based on work from the University of Kiel, recognizing the importance of including biokinetic parameters alongside *in vitro* cytotoxicity data to improve the predictions (Seibert et al., 1996).

2.4.2 *Strengths and Limitations of Available In Vitro Cytotoxicity Assays*

Sufficient information was presented to the Breakout Group for evaluating the merits of the MEIC and ZEBET proposals and the JSAAE study in that the information could be adapted and utilized for evaluating assays designed to predict acute lethality.

The MEIC proposal was that a battery of three human cell-based tests (HepG2, protein content, 24 hr exposure; HL-60, ATP content, 24-hr exposure; Chang liver cell morphology, 24 and 168-hr exposure) could be used to predict human lethal blood concentrations and be a surrogate for the LD50 test (Ekwall et al., 2000). Although the MEIC program was not set up as a validation study and assessing reproducibility was not an objective, the Breakout Group agreed with the following MEIC conclusions:

(1) There is a strong correlation between concentrations of chemicals causing cytotoxicity *in vitro* and human lethal serum concentrations.

(2) Metabolism may not play a role *in vivo* as frequently as thought.

(3) Specificity of action requiring many types of differentiated cells is not as significant a problem as may initially have been envisaged.
(4) Some simple corrections of the data, such as for BBB passage, improve the correlations observed.

The key strengths of the MEIC approach are the comparison of acute cytotoxicity data with human exposure data and the database on human lethal concentrations, kinetic profiles, etc., which has been generated and is available as MEMO monographs for others to evaluate and use. The Breakout Group agreed that attempts be made to extend this human database, and that it should be subjected to independent peer review. The outcome of the MEIC program in general was considered to provide strong support for the concept of basal cytotoxicity first proposed by Ekwall in 1983.

Several issues were raised concerning the MEIC proposal and the use of such an approach as an alternative to animal tests. Various limitations of the approach were cited, including the following:

(1) Because the program was not intended to be a validation study, it was not conducted under controlled conditions.
(2) Replicate assays were generally not performed, hence there is limited information on intra-laboratory assay repeatability and inter-laboratory reproducibility. Nevertheless, there is a large body of evidence from other validation studies that *in vitro* cytotoxicity assays are highly reproducible and relatively easy to transfer between laboratories.
(3) The chemicals tested in the different laboratories were probably from different batches and sources (allowed by MEIC for practical purposes, and because the human case exposures likely involved different materials and sources also).
(4) Statistical analyses were often performed on groups of tests rather than on individual assays.
(5) In many of the assays, not all 50 chemicals were tested. This impacts on the conclusions being made on the basis of correlation coefficients;
(6) There is a tendency for the data to be over-interpreted and some of the conclusions have been over-stated in the publications.
(7) Prediction models were not defined for any of the *in vitro* assays. This would be a pre-requisite for a validation study.

There were also specific confounding factors in relation to the 1, 9, 5/16 battery proposed by Ekwall and colleagues (Ekwall et al., 2000). The assay battery was selected using data from 38 of the 50 MEIC chemicals, and the predictivity for all 50 chemicals reassessed by PLS analysis. The values obtained were: R^2=0.84, 38 chemicals; R^2=0.77, 50 chemicals; R^2=0.88, 38 chemicals + BBB correction; R^2=0.83, 50 chemicals + BBB correction. However, it was noted that: (a) results for test 1 were reported for only 45 chemicals, and 3 of the missing 5 results were for chemicals included in the first set of 38, thus n=35 and n=45; in addition, three other *in vitro* tests employing HepG2 cells and a 24-hr exposure time were evaluated in the MEIC program, and the data vary considerably, particularly for some of the reference chemicals; (b) results for test 9 were reported for only 46 chemicals, and all 4 of the missing results are for chemicals included in the first set of 38, thus n=34 and n=46; and (c) tests 5/16 used Chang liver cells, which are known to possess several HeLa markers. In addition, only single data points for each combination of *in vitro* test and chemical have been reported, meaning that there is no way to evaluate the variability in the assay results which would necessarily impact upon the robustness of the conclusions drawn by the MEIC management team.

A major strength of the ZEBET RC approach is the extensive database underpinning the strategy proposed (Spielmann et al., 1999). The database includes IC50 values derived from numerous *in vitro* cytotoxicity tests on more than 300 chemicals. The actual data are used in a very defined way in trying to predict starting doses for *in vivo* testing, and the simplicity of the concept, flexibility in choice of potentially useful cell systems, and ease of validating and applying the cell systems in practice are attractive features of the approach.

One disadvantage of the ZEBET approach at the present time is the lack of information on the variability in both the *in vitro* and *in vivo* data. In addition, the use of LD50 values from RTECS is perhaps a problem because of this. The Breakout Group suggested that several follow-up actions be undertaken immediately after the Workshop to update and improve the understanding of the applicability of this approach: (a) the examples shown for using *in vitro* cytotoxicity data to identify the starting dose for the ATC or UDP *in vivo* study should be updated to bring them in line with the new draft guidelines, which have now been modified to incorporate the OECD harmonized hazard classification system (OECD, 1998a); and (b) additional simulation modeling should be undertaken to demonstrate the actual reduction in animal use which is expected to be achieved by implementing the approach, and real-life worked examples should be provided to serve as guidance for those adopting and evaluating the approach in the future (See Section 2.6).

2.4.3 *Validation Status of Available In Vitro Screening Methods*

The Breakout Group considered the validation status of the *in vitro* cytotoxicity assays evaluated in the MEIC program, and those used to generate the data included in the RC, relative to the ICCVAM Validation Criteria (ICCVAM, 1997) and the ICCVAM Evaluation Guidelines (ICCVAM, 1999; Section 11, Appendix E). It was concluded that no single *in vitro* cytotoxicity test, or test battery, has yet been formally validated for the specific purpose of replacing the rodent LD50 test. Upon completion of the MEIC study, Ekwall suggested that the battery of three tests proposed should now undergo formal validation (Ekwall et al., 2000). Typically, data on the intra- and inter-laboratory reproducibility of the *in vitro* assays, generated in a structured manner, are lacking, and further work is still needed to fully evaluate the predictive ability of *in vitro* cytotoxicity tests for acute toxicity *in vivo*.

Since several *in vitro* cytotoxicity assays have been included in formal validation studies on eye irritation and phototoxicity (e.g., various test protocols using BALB/c 3T3 mouse fibroblasts or keratinocytes and NRU as the endpoint measurement [Balls et al., 1995b; Brantom et al., 1997; Spielmann et al., 1996; Spielmann et al., 1998]), objective data on the intra-laboratory and inter-laboratory reproducibility of these tests are available for test materials which were coded and tested in at least three laboratories. The Breakout Group proposed that a Working Group be established to evaluate this information and to undertake a paper exercise to determine the capability of these particular *in vitro* cytotoxicity tests for predicting rodent LD50 values rather than Draize rabbit eye irritation scores. It was envisaged that LD50 data would be available for most of the chemicals tested in the EC/HO and BgVV eye irritation validation studies.

A validation study on five *in vitro* cytotoxicity tests (endpoint measurements: colony formation, crystal violet staining, LDH release, MTT, and NRU) has been conducted under the auspices of the JSAAE (Ohno et al., 1998a). Six chemicals (Tween 20, Tween 80, sucrose fatty acid ester, propylene glycol, cetylpyridinium chloride, and sodium lauryl sulfate) were tested. The LDH release endpoint measurement was not reproducible, and the crystal violet staining assay was deemed to be the most reliable of the *in vitro* cytotoxicity tests evaluated (Ohno et al., 1998a). The colony formation assay in HeLa S3 (SC) and BALB/c 3T3 A31-1-1 cell lines was reported to be the most sensitive, but also showed the largest variation (Tanaka et al., 1998).

Disadvantages of the colony formation assay are that it is time-consuming (7 to 13 days culture time, depending on the cell line) and cannot be conducted in 96-well plates and, hence, cannot be readily automated. Although the focus of the study was on comparisons with Draize eye irritation scores and not acute lethality *in vivo*, the study does provide another source of objective information on the general reproducibility and transferability of *in vitro* cytotoxicity tests (Ohno et al., 1998a). In that sense, the Working Group should also examine the data from this study for how well they predict rodent LD50 values for the test chemicals.

Based on consideration of the studies referred to in previous sections, it was concluded that none of the available *in vitro* methods or proposed

testing strategies had been adequately evaluated for implementation to reduce and/or replace animal use for acute systemic toxicity testing. However, it was suggested that the ZEBET approach, using *in vitro* cytotoxicity data to predict *in vivo* starting doses, should be implemented relatively quickly once a guidance document had been prepared (see Section 2.6). The rapid adoption of the ZEBET approach into general practice would enable data to be generated in a relatively short time to fully establish its usefulness and accuracy with a large number of test chemicals.

2.4.4 *Selection of the Most Appropriate Cell Type*

The selection of the most appropriate cell type depends on the objective. Thus, for the prediction of rodent LD50 values in a replacement test, one would conceptually favor a rodent cell line; for the human situation, human cell lines would be more appropriate. Although the MEIC results tend to support this view, the Breakout Group did not feel the data were strong enough (for the reasons given above) to come to a definitive conclusion on this point. Further evidence of this was provided by an analysis of the ZEBET RC data relative to IC50 data generated using a human cell line evaluated in the MEIC program (Clemedson et al., 1998a; Clemedson et al., 1998b). The correlation between the IC50*x* (RC) and IC50*m* (MEIC human cell line) values for the 50 MEIC chemicals was extremely high (R^2=0.90; see Addendum to this report). Consequently, where the objective is to reduce animal numbers required for lethality tests, the apparent difference is too small to rule out the use of a human cell line if that cell line offers other particular advantages or performs acceptably for that purpose.

The current *in vitro* basal cytotoxicity tests do not take into account metabolism-mediated toxicity. It is widely accepted that simple predictive systems (*in vitro* or *in silico*) will need to be developed for early identification of those substances likely to be metabolized to more toxic or less toxic species than the parent chemical (e.g., Fentem et al., 1993; Seibert et al., 1996; Curren et al., 1998; Ekwall et al., 1999). It should be noted that in Ekwall's early studies, approximately 20% of the chemicals assayed in HeLa cell cultures did not fit the basal cytotoxicity concept (Ekwall, 1983). It is expected from the existing literature that "biotransformation screens" will provide valuable data to supplement *in vitro* cytotoxicity results for improving predictions of LD50 values for a significant fraction of those chemicals.

2.5 Future Directions

The Breakout Group concentrated its efforts mainly on short-term approaches to reduce and replace animal use in acute oral toxicity tests, leaving the discussion of longer-term research needs and priorities to Breakout Groups 2 (biokinetics) and 3 (specific organ toxicity and mechanisms). However, it was agreed that the long-term goal (i.e., the ideal approach) should be to develop and use a battery of *in vitro* tests employing human cells and tissues, and integrate this information with that derived from other sources (e.g., on key physico-chemical parameters, kinetics, and dynamics) to predict human acute toxicity, including systemic target organ effects.

2.5.1 *Most Promising In Vitro Methods for Further Evaluation to Reduce and/or Refine Animal Use for Acute Toxicity*

The Breakout Group considered that, in the absence of other information which enables the dose to be set with confidence (e.g., acute toxicity data on structurally related chemicals, physico-chemical or other information), *in vitro* cytotoxicity data generated using the proposed ZEBET approach should be useful for predicting starting doses for *in vivo* studies. The proponents presented supporting data indicating that this approach would result in a further reduction and refinement in animal use for acute toxicity testing. By judicious use of time and resources, initial cytotoxicity assays need not slow the overall developmental or evaluation processes and in fact may actually expedite it where several chemicals can be tested *in vitro* at the same time.

To use the approach, test laboratories should evaluate and compare the performance of several *in vitro* cytotoxicity tests with the

existing RC data (Figure 2.1). For example, a protocol employing the BALB/c 3T3 mouse fibroblast cell line, a 24-hour exposure time, and NRU as the endpoint measurement is appropriate, but other cell lines and cell viability assays could serve the same purpose equally well. The main considerations are:

- The selection of cell type for assessing general cytotoxicity (e.g., rodent fibroblast cell line, human epithelial cell line; monolayer or suspension [e.g., HL60 human acute leukemia cell line] cultures);
- Exposure period (a minimum of 24 hours, but consideration of longer exposures [e.g., 72 hours] as well, if appropriate);
- Endpoint (cell viability/growth);
- Endpoint measurement (e.g., NRU, MTT, ATP, protein).

Since the choice of endpoint measurement does not appear to be critical to the correlative power of the tests (Garle et al., 1994; Ohno et al., 1998a; Spielmann et al., 1999; Ekwall et al., 2000), the simplest, cheapest, most reproducible, with least interference by test chemicals, and, especially where large numbers of chemicals or materials are to be tested, most easily automated endpoint measurements would be the most practical option.

An *in vitro* cytotoxicity test could be implemented in a tiered testing strategy (in the context of predicting starting doses for a subsequent *in vivo* test) in the short-term, without needing to await the outcome of formal validation activities (Section 2.5.2; see below). The main prerequisite would be the production of a guidance document, including details of test protocols considered to be appropriate, and worked examples illustrating the practical application of the strategy.

2.5.2 *Most Promising In Vitro Methods for Further Evaluation to Replace In Vivo Acute Toxicity Test Methods*

The Breakout Group did not evaluate individual test protocols or proposals as candidates for replacement of *in vivo* acute toxicity tests and therefore could not address this question directly. As noted earlier, *in vitro* tests do not currently provide all the information that can be obtained from an *in vivo* study. However, the accumulated results of many cytotoxicity studies and the ZEBET/MEIC initiatives do suggest that, in general, we may be able to obtain reasonable estimates of LD50 values if this parameter is the primary one required for regulatory decisions. Certainly by applying one or more reasonably predictive assays of the LD50 to test the considerable number of chemicals on which such risk assessment data are needed, (e.g., high production volume [HPV] chemicals), it should be possible to make a truly significant reduction in animal usage.

The Breakout Group agreed that a prevalidation study should be initiated at the earliest possible date to identify the most promising *in vitro* cytotoxicity tests for further validation. The study should include a comparison of different cell types (as a minimum, one rodent and one human cell line), exposure periods, and endpoint measurements. Regarding exposure times to evaluate, it was evident from the data available that a minimum exposure of 24 hours should be recommended (Garle et al., 1994; Hopkinson et al., 1993; Riddell et al., 1986), plus an additional "expression" period during which the previously treated cells are cultured in the absence of test material. There may be a need to evaluate several exposure times, as the most appropriate will depend on the cell type chosen, the kinetics of the test chemical, and the sensitivity of the endpoint measured (e.g., Ohno et al., 1998a).

The Breakout Group urged that a Working Group be established to follow up on its conclusions and recommendations at this Workshop (Section 2.6), and specifically, to define the details of the test protocols to be included in any prevalidation study. The selection of basal cytotoxicity tests to be included should be justified with reference to the scientific literature. It was also suggested that the statistical analyses of the MEIC program results be reviewed, so that the basis for the selection of the test battery is fully transparent.

The Breakout Group anticipates that the general performance of the assay or combination/battery of cytotoxicity assays determined from the validation study to be the

best predictor of *in vivo* lethality can be enhanced further by supplementation with other information or data. In this respect, immediate research and development needs of particular importance relate to identifying, standardizing, and validating simple predictive systems for gut absorption, BBB passage, kinetics, and metabolism. These are all important parameters which have been identified as improving the predictive ability of *in vitro* cytotoxicity data for *in vivo* LD50 values (Curren et al., 1998; Seibert et al., 1996; Ekwall et al., 1999). A new initiative on acute systemic toxicity, being undertaken as part of the TestSmart activities, has been established to address the question "can one measure cellular changes that will predict acute system failure?" The successful development of this system would complement basal cytotoxicity assays for predicting acute toxicity *in vivo* (Goldberg, personal communication).

In the longer-term, preferably undertaken as a parallel activity, the focus should be on the development and validation of human test systems for predicting human acute toxicity, integrating the approaches suggested by Breakout Groups 2 and 3. In this respect, there are numerous mechanism-based endpoints that need to be identified and evaluated in future studies.

The Breakout Group recognizes the potential impact genomics and proteomics technologies may have in many areas of toxicology, but feels these technologies could only lead to the identification of new endpoints and screening methods in the long-term, and that acute toxicity testing is not currently an area of high priority for the application of these new technologies. Investigations of changes in gene expression (e.g., using microarrays) are better targeted to more specific toxicological effects rather than general responses such as acute lethality.

2.5.3 *Ways to Evaluate the Usefulness of In Vitro Assays in an Overall Acute Toxicity Testing Strategy*

The evaluation of the usefulness of *in vitro* cytotoxicity assays in the overall testing strategy can be achieved in two ways, as indicated above. Firstly, a prospective evaluation "in practice" (in this case by implementing the use of an *in vitro* cytotoxicity test in the strategy proposed by ZEBET [Spielmann et al., 1999]) can be made once the necessary guidance document, including worked examples, has been produced. Once a sufficient body of data has been collected, the *in vitro* cytotoxicity tests can be evaluated retrospectively to determine the validity and practical usefulness of the strategy and to assess whether the predicted starting dose for an *in vivo* study is accurate for a sufficiently large enough percentage of test chemicals to continue its use.

Secondly, a formal validation activity (of which prevalidation would be an initial step; Curren et al., 1995; ICCVAM, 1997) could be conducted in which the test protocols and prediction models are evaluated independently in a multi-laboratory study involving testing of coded chemicals for the reproducibility of their responses, within and among laboratories, and the ability to predict rodent LD50 values (Balls et al., 1995a; ICCVAM, 1997).

2.6 Summary

2.6.1 *Conclusions*

The Breakout Group agreed that its primary objective was to identify and evaluate candidate *in vitro* cytotoxicity tests that could possibly serve as reduction and replacement alternatives for rodent acute oral toxicity tests for determining LD50 values. Despite the considerable research efforts by a large number of laboratories from different sectors, no standardized *in vitro* cytotoxicity assays, with optimized protocols and prediction models for the determination of LD50 values, have yet been validated. It appears from the number of studies showing positive correlations between cytotoxicity results *in vitro* and acute toxic effects *in vivo* that the application of such *in vitro* methods does have the potential to reduce and refine, and, if properly developed, ultimately replace the use of laboratory animals in acute lethality tests.

A strategy was devised by the Breakout Group that was considered to offer realistic short-term and long-term solutions to address the need for

prevalidation and validation of *in vitro* cytotoxicity tests (Figure 2.6). In the short-term, the Breakout Group concluded that the ZEBET approach (Section 2.2.1) had the potential to produce modest reductions in animal use in the ATC and UDP (OECD TG 423 and TG 425) *in vivo* tests (and in the FDP [OECD TG 420] to obviate the need for any initial sighting study). Thus, it is suggested that an *in vitro* cytotoxicity test be used in a tiered testing scheme as proposed by Spielmann et al, (1999).

The Breakout Group concluded that a guidance document with test protocol details, supporting information, and worked examples should be produced and disseminated as quickly as possible. The testing strategy should be implemented as soon as this guidance was available, without the need for a validation study. This conclusion is based on the Breakout Group's awareness of the large database on *in vitro* cytotoxicity and its demonstrated correlative power with rat acute oral LD50 values, particularly the MEIC and RC' approaches. The validity of the *in vitro* cytotoxicity data in establishing appropriate starting doses for *in vivo* studies (and hence its direct predictive capability for the LD50) should be assessed retrospectively by evaluating the data generated on a sufficiently large number of substances according to pre-defined criteria for judging the acceptability of the approach. The implementation of such a testing strategy was considered to be relatively inexpensive and simple, and would not compromise the actual outcome of the *in vivo* test.

In vitro assays to replace animal tests for acute lethality will require more time to implement. The information and time available to the Breakout Group was inadequate to recommend specific cytotoxicity assays for prevalidation and validation, although the major considerations and suggestions for possible assays (e.g., a BALB/c 3T3 mouse fibroblast NRU assay) have been documented (Section 2.5.1). An additional Working Group will need to be convened for this purpose at the earliest possible date to maintain momentum and to make progress in the near term.

The scheme conceptualizing the Breakout Group's conclusions as to how cytotoxicity tests can reduce/refine and ultimately replace animal use for acute toxicity (LD50) testing (Figure 2.6) indicates what needs to be done and the projected timings for reaching that point. Each pathway involves a stepwise approach to addressing the issue. Step 1 in any testing scheme would be the collection and integration of information on the physical/chemical properties of a compound, including literature reviews and analysis of structure-activity relationships whenever possible. Most companies currently do this as a preliminary step in their evaluation of new candidate compounds for commercial development. In addition, the likelihood that acute toxicity could be metabolism-mediated needs to be considered at this early stage, and here it would be useful to integrate data derived from simple *in vitro* or *in silico* screens for biotransformation (bioactivation or detoxification). Step 2 would involve conducting an *in vitro* basal cytotoxicity test to provide data, either for correct selection of the *in vivo* starting dose (enabling an immediate reduction and refinement of animal use in the interim) or in lieu of animal testing for estimating rodent LD50 values (once the battery of *in vitro* tests required to do this had been validated for this purpose).

In the left-hand pathway in Figure 2.6, *in vivo* studies are still performed and provide supplementary information on dose response, clinical signs, and target organ effects from acute exposure for those agencies or organizations that need this additional information. However, it is anticipated that conducting a preliminary cytotoxicity test for starting dose selection would result in a modest, but cumulatively appreciable, reduction in animal numbers at minimal cost and with negligible impact on chemical or product development time. It is further projected that the ZEBET approach can be proved effective in a straightforward exercise, and Guidance for applying the approach prepared within a short period of time (i.e., 2 to 3 months).

In the right-hand pathway of Figure 2.6, the steps required for validating one or more *in vitro* cytotoxicity assays to replace animal testing for acute lethality are shown (Balls et al., 1995;

ICCVAM, 1997). This goal will take longer to achieve in light of the current state of the art. It will first be necessary to design and conduct a prevalidation study on those *in vitro* assays that are considered promising (Curren et al., 1995). Then the *in vitro* test protocol(s) and prediction models would be subjected to full validation studies to provide the necessary supporting data for assay evaluation, and eventual regulatory acceptance.

It was considered that, if the commitment to conducting a formal validation study was strong enough, the scientific resources could be harnessed for this effort with facility and the *in vitro* tests studied proved good enough, a replacement test battery might be achieved in as short a time as 2-3 years. However, past experience indicates that the formal acceptance of this battery might require substantial additional time. All prevalidation and validation studies should be conducted in compliance with the ICCVAM and ECVAM guidelines (Balls et al., 1995; ICCVAM, 1997), following the designs of similar validation studies conducted on *in vitro* tests for eye irritation (e.g., Brantom et al., 1997), skin corrosion (Fentem et al., 1998) phototoxicity (Spielmann et al., 1998), and a prevalidation study for skin irritation (Fentem et al., 2001).

In summary, it was concluded that initially a prevalidation study should be undertaken for several promising candidate *in vitro* cytotoxicity tests. Meanwhile, as a parallel activity, the generation of *in vitro* cytotoxicity data to help establish the starting dose for *in vivo* testing of new chemical substances (Spielmann et al., 1999) should be strongly encouraged as a means to potentially reduce the numbers of animals used in LD50 tests (Figure 2.6).

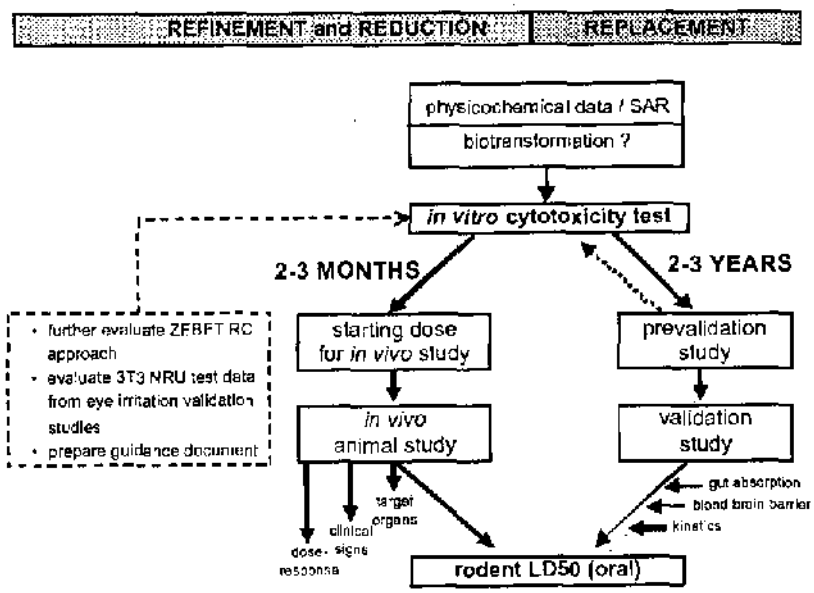

Figure 2.6. Strategy for the reduction, refinement and replacement of animals in acute LD50 testing

2.7 Recommendations

Breakout Group 1 made the following recommendations for the prevalidation, validation, and future development of *in vitro* assays for acute lethal toxicity.

2.7.1 *Short-term Activities*

- A guidance document on the application of *in vitro* cytotoxicity data for predicting *in vivo* starting doses, to include details of current test protocols considered appropriate and their application, and worked examples, should be prepared.
- A Working Group of scientific experts should be established to identify and/or define specific test protocols for inclusion in a prevalidation study. The Working Group should design and plan the study in detail. This Group should take into account the suggestions on cell type, exposure period, and endpoint measurement made by BG1 in this report.

2.7.2 *Intermediate-term Activities*

- It is anticipated that simple systems that predict gut absorption, BBB passage, key kinetic parameters, and metabolism will be needed to improve the capability of *in vitro* cytotoxicity assays to predict rodent LD50 values, or any *in vivo* toxic effects. Continued development and optimization of such systems for this application is encouraged and should receive regulatory support.
- QSAR approaches, including expert systems and neural networks, could be developed and validated as adjunct systems for predicting acute systemic toxicity. The development of commercial QSAR packages should be encouraged. As an initial step in the development of these approaches, an up-to-date review of current QSAR systems for predicting rodent oral LD50 values should be undertaken. In addition, QSARs for predicting gut absorption, metabolism, and BBB passage should be developed and evaluated.

2.7.3 *Longer-term Activities*

- The ultimate objective is the prediction of acute toxicity in humans. For this purpose, the development of simple predictive models for human acute toxicity should be a major focus.
- The evaluation and ultimate acceptance of *in vitro* assays for human acute toxicity will need a larger reference database than is presently available for validation purposes. The MEIC human database should be peer-reviewed, modified if needed, and expanded as soon as possible in order to have the data available for future validation studies.
- Other mechanism-based *in vitro* methods or endpoints, in particular resulting from the application of genomics/proteomics, may provide data that enhances the information that can be derived from cytotoxicity tests. Such research efforts should continue to be encouraged and financially supported.

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ADDENDUM

Combined analyses of the ZEBET Register of Cytotoxicity (RC) and MEIC data

The predictions of acute lethality *in vivo* from the RC and MEIC cytotoxicity data have been analyzed. The correlation for the 50 MEIC chemicals (IC50 *in vitro* vs rodent oral LD50 *in vivo*), including the RC cytotoxicity data for various mammalian cell lines (dark triangles, dark linear regression line) and the MEIC program cytotoxicity data for various human cell lines (circles, gray linear regression line; taken from Clemedson et al., 1998a; Clemedson et al., 1998b), are shown in Figure A.1. Similar standard regression lines, with comparable data fits, were obtained for the RC values (mean IC50*x* data) and the MEIC values (IC50m) for the 50 chemicals (Table A.1).

A similar comparison of the correlations for the 50 MEIC chemicals (RC mammalian *in vitro* values and MEIC human *in vitro* values from Clemedson et al. [1998a; 1998b]) was also undertaken for *in vitro* IC50 vs. human peak lethal blood concentrations *in vivo* (Ekwall et al., 1998a). Again, similar standard regression lines, with comparable fits, were obtained (Table A.1):

RC: log (peak concentration) =
0.822 x log (IC50*x*) - 0.437; r=0.81; R^2=0.66

MEIC: log (peak concentration) =
0.913 x log (IC50*m*) - 0.702; r=0.86; R^2=0.74

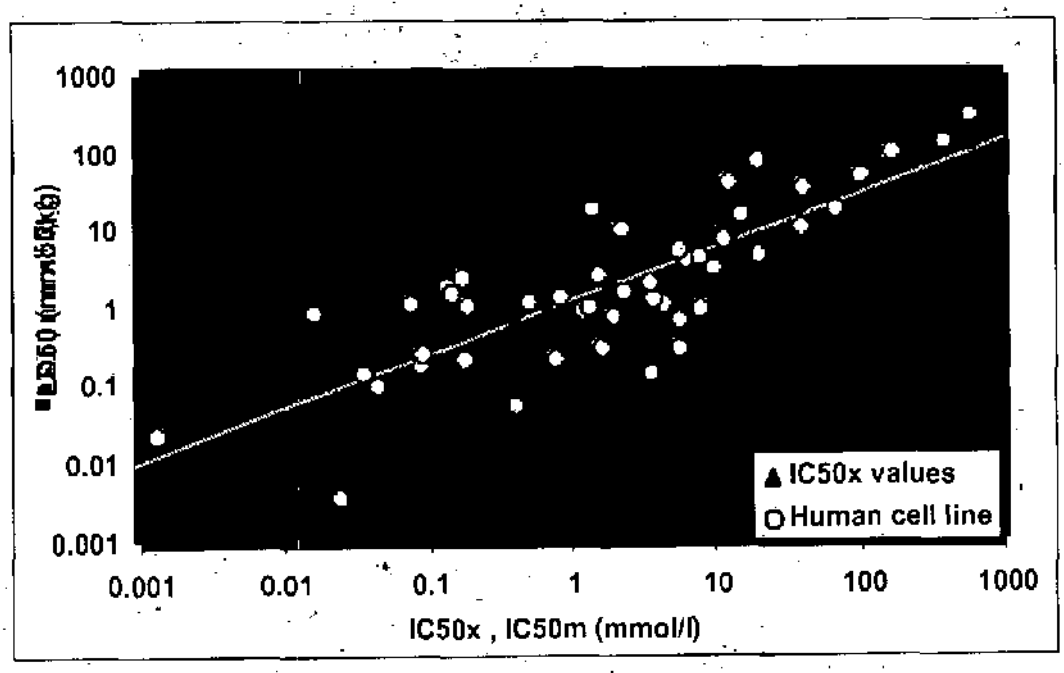

Figure A.1. Regression between Cytotoxicity (IC50) and rodent acute oral LD50 for the 50 MEIC chemicals
RC: log (LD50) = 0.689 x log (IC50*x*) + 0.276; r=0.84; R^2=0.71
MEIC: log (LD50) = 0.690 x log (IC50*m*) + 0.080; r=0.81; R^2=0.66

Table A.1. Summary of linear regression analyses (RC vs MEIC)

Chemicals	x	y	slope	constant	r	R^2
347 non-selected (RC)	IC50*x*	LD50	0.435	0.625	0.67	0.45
50 MEIC (RC)	IC50*x*	LD50	0.689	0.276	0.84	0.71
50 MEIC (human cell lines)	IC50*m*	LD50	0.690	0.080	0.81	0.66
50 MEIC (RC)	IC50*x*	human lethal	0.822	- 0.437	0.81	0.66
50 MEIC (human cell lines)	IC50*m*	human lethal	0.913	- 0.702	0.86	0.74
50 MEIC	LD50	human lethal	0.879	- 0.669	0.71	0.50

To set these results in context, the predictivity of the rat LD50 for human peak lethal concentration was assessed for the MEIC chemicals (Figure A.2; Table A.1). The correlation was not as good as that found with the IC50 values.

The 50 MEIC chemicals are a subset of the RC; the overall predictivity of the entire RC (347 chemicals) for rodent LD50 values is lower than that of the 50 MEIC chemicals (Figure A.3; Table A.1). The relationship between *in vitro* IC50 values and *in vivo* LD50 values should be investigated further by employing multiple regression techniques rather than simple linear regression. In addition, cluster analysis could also be undertaken.

To investigate how basal cytotoxicity data obtained from various human cell lines (IC50m) in the MEIC program (part III and IV) compares with basal cytotoxicity data from various mammalian cell lines (IC50*x*), the correlation between IC50*x* and IC50m is shown in Figure A.4. The correlation is judged very high by R^2 = 0.90, and suggests that basal cytotoxicity data obtained with either human cells or other mammalian cells may be similar and equivalent for the prediction of *in vivo* lethality measures.

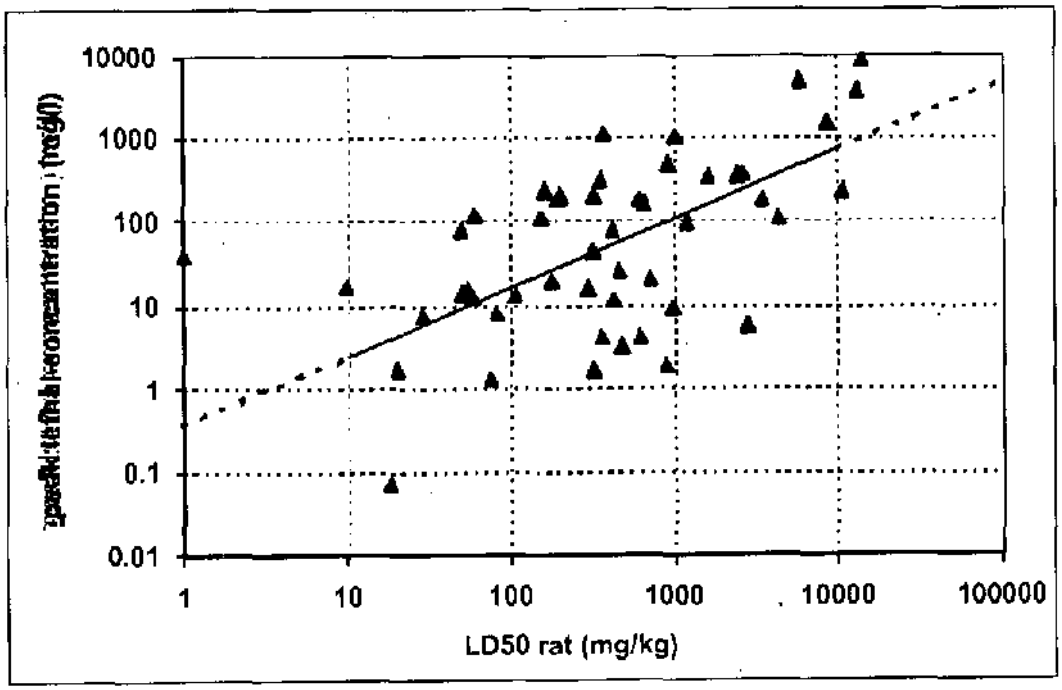

Figure A.2. Regression between rodent acute oral LD50 values and human peak lethal concentrations for the 50 MEIC chemicals.
Regression equation: log (peak conc.) = 0.879 x log (LD50) – 0.669; r=0.71; R^2=0.50.

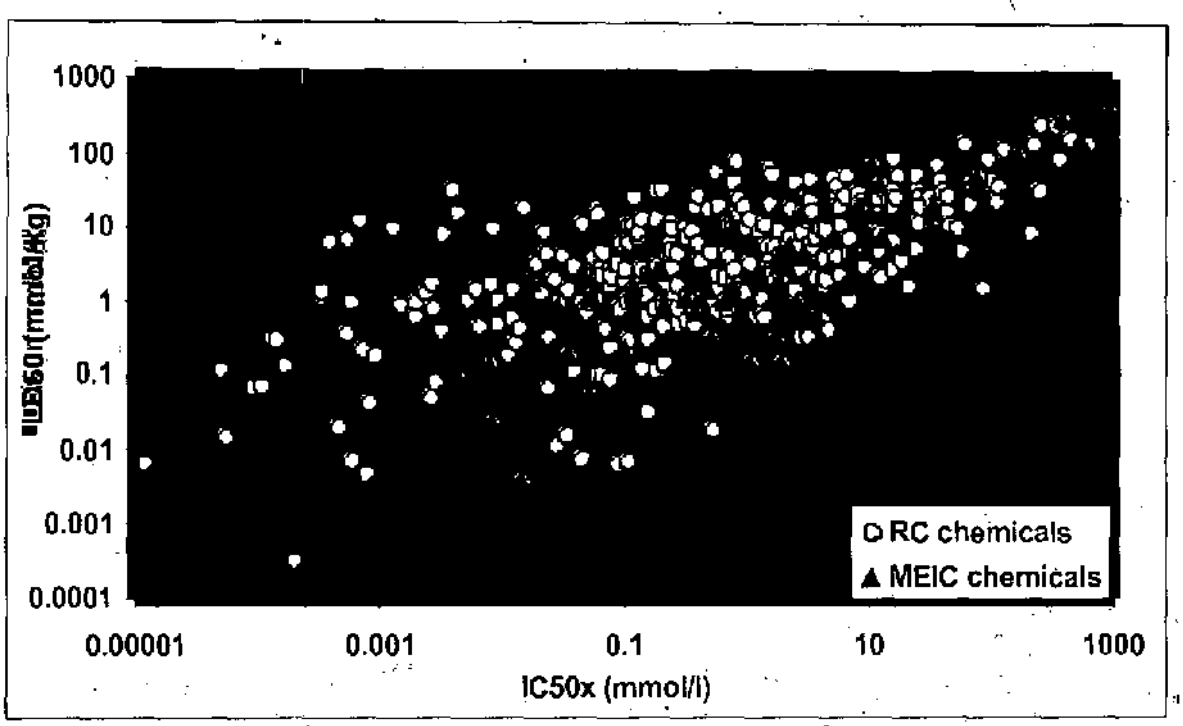

Figure A.3. Regression between Cytotoxicity (IC50) and rodent acute oral LD50 values for the RC database showing the 50 MEIC chemicals as a subset of the 347 chemicals in the RC

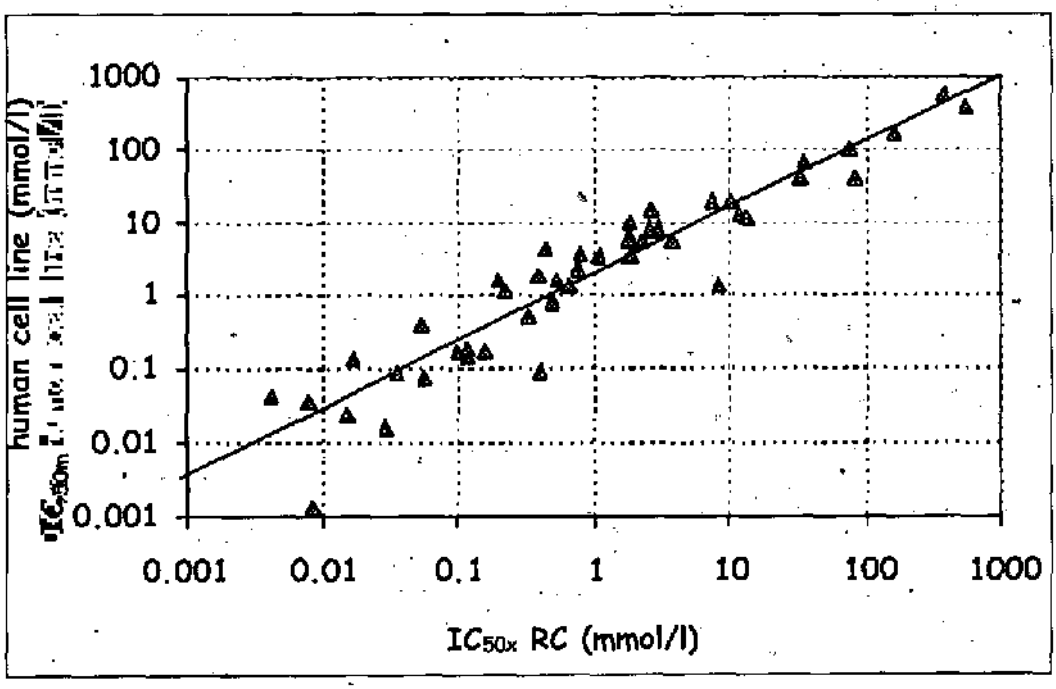

Figure A.4. Correlation between IC50x (averaged from various mammalian cell lines) of the RC and IC50m (from various human cell lines) is shown for the 50 MEIC chemicals
The linear correlation coefficient is high (r = 0.95) and judged by an $R^2 = 0.90$.

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3.0 *IN VITRO* METHODS FOR ASSESSING ACUTE TOXICITY: BIOKINETIC DETERMINATIONS

3.1 Introduction

The biokinetics determinations Breakout Group (Breakout Group 2) was given the task of discussing and evaluating the capabilities of *in vitro* methods for providing biokinetic information (i.e., on absorption, distribution, metabolism, and excretion) that can be used to estimate target-organ dosimetry for acute toxicity testing. The Breakout Group was asked to identify future research needs in the area of biokinetics that will enable *in vitro* methods to more accurately predict acute toxicity *in vivo*. The role of quantitative structure-activity relationships (QSAR) and quantitative structure-property relationships (QSPR) in biokinetic determinations was also to be considered.

The Breakout Group was asked to answer a number of questions in three areas:

(1) The identification of the need for specific knowledge in the field of biokinetics;
(2) The current status of knowledge and technology in the field;
(3) Future directions for research.

The group discussions followed general lectures given in the Workshop's opening plenary session. A presentation to the Breakout Group entitled "An integrated approach for predicting systemic toxicity" was particularly relevant to the Breakout Group's responsibilities, demonstrating the central role of biokinetic modeling in the prediction of systemic toxicity using *in vitro* data (Blaauboer et al., 2000).

3.1.1 *General Discussion*

The goals for the Workshop were presented and the following specific questions were posed:

(1) What *in vitro* systems are available and how can these systems be applied and/or improved?
(2) What research requirements can be formulated?
(3) Which priorities can be set for research?

The discussions of the Breakout Group centered on the role of the kinetics of a chemical *in vivo* in its acute systemic toxicity. The following summary was developed as a point of departure for the Breakout Group's deliberations:

> Results obtained from *in vitro* studies in general are often not directly applicable to the *in vivo* situation. One of the most obvious differences between the situation *in vitro* and *in vivo* is the absence of processes regarding absorption, distribution, metabolism and excretion (i.e., biokinetics) that govern the exposure of the target tissue in the intact organism. The concentrations to which *in vitro* systems are exposed may not correspond to the actual situation at the target tissue after *in vivo* exposure. In addition, the occurrence of metabolic activation and/or saturation of specific metabolic pathways or absorption and elimination mechanisms may also become relevant for the toxicity of a compound *in vivo*. This may lead to misinterpretation of *in vitro* data if such information is not taken into account. Therefore, predictive studies on biological activity of compounds require the integration of data on the mechanisms of action with data on biokinetic behavior. Over the last decade, the feasibility of using mathematical models for interpretation of *in vivo* biokinetics has grown substantially. This development has been facilitated by the increasing availability of computer-based techniques for numerical solution of differential equation sets that characterize biokinetic processes (Blaauboer et al., 2000).

The Breakout Group also reached consensus on some terminology: the word "toxicokinetics" should be replaced by "kinetics" or "biokinetics". Problem areas in predicting kinetics of chemicals were noted in: (a) biotransformation (value of *in vitro* systems for determining biotransformation,

interpretation of *in vitro* data, scaling up to the *in vivo* situation); and (b) the passage across special barrier systems (e.g., in the gastrointestinal [GI] tract, the blood-brain barrier [BBB], and the kidney).

Short presentations on the following were provided as a focal point for Breakout Group discussions:

- Biokinetic modeling of acute exposure;
- QSAR/QSPR;
- BBB;
- Kidney barrier systems;
- Intestinal barrier;
- Metabolic activation, including different systems available for the liver (and extrahepatic tissue);
- Skin as a barrier;
- Microarray alternatives;
- Information from NIEHS Microarray Center;
- Expert systems for making predictions of a compound's partitioning and toxicity.

After the presentation on the use of Physiologically-Based Biokinetic (PBBK) models, the Breakout Group concluded that kinetics play a crucial role in estimating a compound's acute systemic toxicity. The use of these physiologically determined models has proven to be very useful in many aspects. Over the last ten years, the feasibility of this modeling approach has been greatly enhanced due to the availability of computer techniques that allow for the simultaneous numerical solution of differential equations. While species-specific anatomical and physiological data are generally available from the literature (e.g., Arms and Travis, 1988; Brown et al., 1997), compound-specific parameters for PBBK models (e.g., tissue-blood partition coefficients and the Michaelis-Menten constants Vmax and Km) are often still obtained by fitting these parameters to experimental data obtained *in vivo*. Proper use of PBBK models in itself can contribute to reduction and refinement of animal studies by optimization of study design through identification of critical parameters and time frames in kinetic behavior. In addition, incorporation of *in vitro*-derived parameters will lead to a further reduction of large-scale animal studies for quantitative assessment of the biological activity of xenobiotics.

The Breakout Group concluded that a distinction can be made between the goals to be achieved:

- Short-term: improvement of the interpretation of *in vitro* toxicity data for estimating rodent LD50 values;
- Long-term: using *in vitro* data for estimating/predicting sublethal acute toxic effects caused by chemicals in humans (e.g., represented by a TD10 value, i.e., the dose at which mild toxicity could be expected in no more than 10% of the exposed humans).

It will be obvious that the latter goal is of greater interest for the risk evaluation of chemicals, where the protection of humans with regard to toxic effects is the highest priority.

These different goals need different scientific activities; different groups of chemicals will need different approaches for modeling the kinetics. In some cases, a great deal of information is available (e.g., on low molecular weight; volatile lipophilic compounds). For these compounds, reasonable estimates can be obtained for their partitioning in the organism based on their physico-chemical properties. Many kinetic parameters (e.g., Vd and k_e) are also determined by the size of the dose (i.e., the amount of compound available for systemic circulation) because of capacity-limited processes in metabolism and transport.

3.1.2 *Subjects of Discussion*

The intestinal barriers, the role of the gut flora, first pass metabolism, and (counter) transport systems were discussed. A number of cell lines are available to estimate absorption through the gut barrier. BBB and skin absorption models were also addressed. *In vitro* methods for these systems exist, but none reflects the full metabolic and transport capacity seen *in vivo*.

The current status of systems to estimate the kidney epithelia as a barrier was discussed. These

systems include the use of renal cell lines, such as LLC-PK$_1$ cells and MDCK cells. The former cells form low resistance epithelial monolayers when grown on permeable supports; the latter form extremely high resistance. However, these cell lines do not express all the relevant transporters found *in vivo*. The lack of the organic anion transporter is particularly problematic and cell lines transfected with these transporters may be more appropriate. Currently, an ECVAM prevalidation study is under way of trans-epithelial resistance and inulin permeability as endpoints in *in vitro* nephrotoxicity testing.

The ability to estimate biotransformation reactions of chemicals is of particular interest since acute toxicity may be mediated through the bioactivation or deactivation of chemicals. *In vitro* systems designed to address this possibility include:

- Liver homogenates;
- Microsomal preparations;
- Isolated cells;
- Primary monolayer cultures;
- More complicated cell cultures (co-cultures, 3D cultures);
- Transgenic cell lines.

QSAR systems have also been proposed for modeling the metabolic biotransformation of chemicals. The use of QSAR/QSPR and the development of software systems to predict "chemical functionalities" of compounds which may be used to estimate kinetic behavior (including protein binding) and the toxicodynamics were also discussed.

3.2 Identifying Needs

3.2.1 *In Vitro Methods for Evaluating Chemical Kinetics*

As mentioned above, the Breakout Group recognized a short-term and a longer-term goal for using *in vitro* or other non-animal techniques for predicting acute systemic toxicity. First, one focuses on the longer-term goal: how to use these techniques for the evaluation of a chemical's kinetics and the ultimate prediction of sublethal acute toxic effects in humans. Section 3.4.4 concentrates on the short-term (interim) goal: how to improve the prediction of acute lethal effects in rodents. *In vitro* methods, in combination with knowledge of a chemical's structural properties, can be used to predict/determine the chemical's absorption, distribution, metabolism, and elimination in an intact organism. However, it will be a major challenge for the field of *in vitro* toxicology to identify the particular target tissue(s) or cells and the time course of clinical toxicity in the absence of *in vivo* observations.

In the short-term, physico-chemical properties can be used to predict/determine partition. QSAR (or QPPR) can be helpful for this determination (DeJongh et al., 1997). *In vitro* determinations of rates of metabolism and of passage of a chemical across membrane barriers (e.g., GI ⇒ blood; blood ⇒ brain) will improve the kinetic modeling. Taken together, these may be able to be used to calculate an LD50 value (as administered to an intact organism) from the LC50 value in a basal cytotoxicity test. Presentation of any such predicted LD50 value also requires concurrent presentation of the quantitative uncertainties attendant to that value. In the long-term, knowledge of a chemical's kinetics will need to include a comparison of the kinetic and the toxicodynamic time-profiles. Moreover, knowledge of kinetics assists in determining the mode of toxic action and vice versa (Ekwall et al., 2000; Liebsch et al., 2000). [see MEIC evaluation of acute systemic toxicity, Appendix E].

3.2.2 *Biokinetics in the Overall Toxicological Evaluation*

Biokinetics is essential for relating administered dose of toxicant to concentration at the target tissue(s). Tissue-specific concentration of the toxicant is one of the mechanisms that can result in organ-selective toxicity. In addition, biokinetics can establish whether metabolism plays a role in modulating the toxicity. Such modulation can either attenuate or enhance the toxicity.

3.2.3 *Biokinetic Techniques as In Vitro Assays*

The following are techniques that need further development:

(1) *In vitro* determination of partition coefficients, metabolism, protein binding, and stability;
(2) Characterization of biotransformation enzymology;
(3) Structural knowledge and its translation into "chemical functionalities"; estimation of partition coefficients, metabolism, etc. ("*in silico*", including QSAR/QSPR);
(4) Biokinetic modeling, including the integration of toxicodynamic and biokinetic modeling in predicting systemic toxicity.

3.3 Current Status

3.3.1 *Prediction of Biotransformation*

Biotransformation can be carried out using human or animal hepatic subcellular fractions, human or animal primary hepatocytes, or human or animal hepatic precision-cut slices. The use of primary human hepatocytes in suspensions or culture requires specific expertise and may not be appropriate for use in all laboratories. Human or animal hepatic subcellular fractions can be cryopreserved and used at a later time to provide qualitative kinetic data, but these fractions may not reflect the integrated routes (activation and detoxification) of metabolism of a compound.

The selective use of cofactors can aid the determination of routes of metabolism. There is a need for standardization of the conditions for the preparation and incubation of rat hepatocytes. Rat hepatocyte incubations may overestimate the metabolic clearance of a compound. It is essential to quantify the rate of disappearance of the parent compound and desirable to quantify the rate of metabolite formation.

3.3.2 *Systems for Estimating Gastrointestinal Absorption*

Apparent membrane permeability and aqueous solubility are reasonably predictive of the fraction of a dose that will be absorbed through the GI tract. Several *in vitro* systems for measuring intestinal absorption include measuring apparent permeability constants in either intestinal tissue segments or cell monolayers that have been grown on a porous support. Cell lines used for this purpose include the human colon carcinoma cell line Caco-2, the canine kidney cell line MDCK, and the porcine kidney cell line LLC-PK_1. All systems are widely used in the pharmaceutical industry in the oral drug discovery process. Each system has advantages and disadvantages which may or may not be relevant depending on the chemical under study.

Cell lines do not require the use of animals. However, they often lack or have non-physiological levels of uptake and efflux transporters that are present *in vivo*. These transporters can dramatically affect the extent of bioavailability at low doses. The nature and extent of species differences in transporter activity/affinity is presently unknown. The Breakout Group consensus was that in the absence of data to the contrary, it would be appropriate to assume that an administered dose would be completely absorbed. This is a public health conservative approach. For those compounds where such an assumption is not appropriate, the above-mentioned *in vitro* systems can be used to provide experimental data on the fraction absorbed.

3.3.3 *Prediction of Renal Clearance/Accumulation*

Glomerular filtration and reabsorption in the proximal tubule determine the renal excretion of most compounds. These parameters can be predicted from the physico-chemical properties of the compound and its plasma protein (albumin) binding. These parameters are less predictable where active secretion or reabsorption and saturation kinetics are involved. Many of the currently available renal cell lines or renal cell primary cultures lack specific transporters (in particular, the organic anion transporter) which are implicated in the accumulation of several nephrotoxic compounds. The substrate specificity of other proximal tubular transporters is poorly defined.

3.4 Future Directions

3.4.1 *Proposed Approach for Consideration of Kinetics in the Estimation of Acute Oral Toxicity*

The diagram presented in Figure 3.1 illustrates a conceptual structure for the use of kinetic information in the estimation of acute oral toxicity. Under this scheme, available *in vitro* data on the absorption, tissue partitioning, metabolism, and excretion of a test material would be used to parameterize a chemical-specific biokinetic model (Clewell, 1993). In many cases, currently available QSPR/QSAR techniques could be used to estimate chemical properties and kinetics when the specific data for that chemical is lacking. For example, simple empirical correlations have been developed for estimating the tissue partitioning of a chemical from its water solubility, vapor pressure, and octanol/water partitioning (Paterson and Mackay, 1989; DeJongh et al., 1997). Emerging QSAR techniques (e.g., knowledge-based systems) may eventually prove useful in predicting potential target tissues for toxicity so that the appropriate assays of *in vitro* dynamics (response) could be selected. These target tissue assays would, in turn, provide information on the nature and location of the toxicity produced by the chemical (DeJongh et al., 1999).

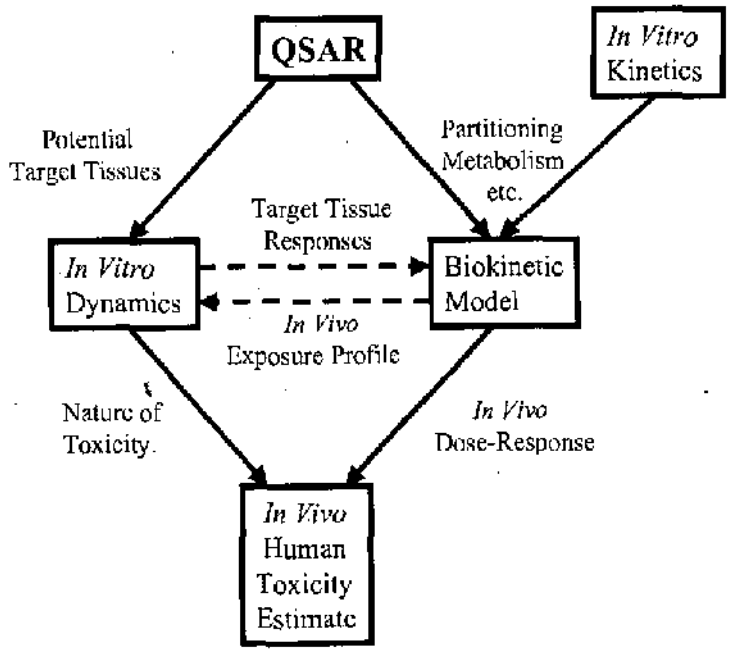

Figure 3.1. A recommended scheme for incorporation of QSAR (QSPR) data, *in vitro* data on kinetics and dynamics, and kinetic modeling in the estimation of human (or animal) toxicity

3.4.2 *Classification of Compounds Based on Their Physico-Chemical Properties*

The complexity of the biokinetic model would depend on the physico-chemical and biochemical characteristics of the chemical. In the specific case of acute toxicity, a simple one-compartment description of the administered chemical may suffice for many chemicals. The volume of distribution for such a model could be estimated

from the volume-weighted average of the estimated partitioning into various tissues, and estimates of fractional absorption and rate of clearance could be based on data for structurally similar compounds.

Each of these assumptions or predictions, however, introduces its own associated uncertainty into the result of the lethality risk estimate. Even with such a simple model, it may be possible to estimate the systemic concentrations that could be expected to result from an *in vivo* exposure to a given dose (DeJongh et al., 1999). Thus, the model could be used to relate the concentrations at which toxicity is observed in an *in vitro* toxicity assay to the equivalent dose that would be expected to be associated with toxicity for *in vivo* exposure. These models can also provide information on the temporal profile for tissue exposure *in vivo*, which can then be used in the design of the most appropriate *in vitro* experimental protocol (Blaauboer et al., 1999).

There are chemical classes for which a one-compartment description would not be expected to be adequate. However, the physiological mammalian structure (tissue volumes, blood flows, ventilation rate, glomerular filtration rate, etc.) is well characterized, and there is no difficulty in describing tissues separately. As mentioned above, techniques exist for estimating tissue-specific partitioning. Other data required would depend on the class of chemical. For volatile chemicals, ventilatory clearance can be estimated from the blood-air partition. For water-soluble chemicals, urinary clearance can be estimated from the glomerular filtration rate or the renal blood flow (for secreted compounds). For some classes of chemicals, it would also be necessary to determine the fractional binding of the chemical to plasma proteins or the partitioning of the chemical into red blood cells.

The greatest challenge in parameterizing the biokinetic model remains the estimation of metabolic clearance. The possibility is increasing to use *in vitro*-determined metabolic parameters (Vmax and Km) in order to accurately predict total body metabolic clearance (Houston and Carlile, 1997). Currently, it would be necessary to perform *in vitro* assays of the dose-response (capacity and affinity) for metabolic clearance (Kedderis, 1997; Kedderis and Held 1996; Kedderis et al., 1993). These assays are generally more expensive than the dynamic (toxicity) assays, since they necessarily involve the development of an analytical method for quantifying the concentration of the parent compound and its metabolite(s) in each tissue of interest over time. Quantification of the concentration of compound in the dynamic assays should also be preferred, but it is not absolutely necessary in that case. Eventually, as data accumulate for a large number of structurally-diverse materials, it might be possible to predict metabolism and disposition using knowledge-based systems.

An important underpinning of this process is that the kind of information necessary for a particular test material depends on its structure and physico-chemical properties. It seems reasonable to expect that chemicals could be categorized into classes based on their properties, and that this categorization would simplify the process of determining the data needed for a particular compound. This concept is illustrated in Figure 3.2. As noted above, the key physico-chemical properties of a test material involves its volatility (reflected in its blood-air partition, Hb/g), its water solubility (Sw), and its lipophilicity (reflected in its octanol-water partition, Ko/w). Compounds with similar properties can be grouped, and data from similar compounds can be used to fill gaps in the knowledge of a particular compound.

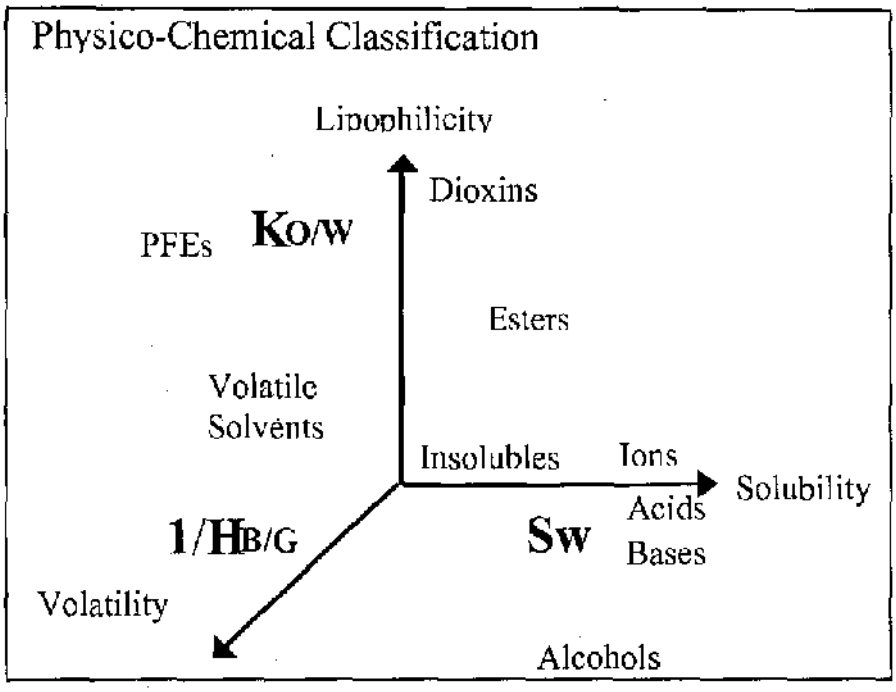

Figure 3.2. Classification of compounds based on their physico-chemical properties

There are two advantages of this *in vitro*/modeling approach over the traditional *in vivo* LD50 test. First, the *in vitro*/modeling approach can provide more extensive information than a traditional oral LD50 value provides. As information accumulates across chemicals, QSAR techniques could play a correspondingly greater role in the prediction of both kinetic and dynamic information. It is likely that QSAR techniques would be more successful for these fundamental processes and simple *in vitro* assays than they have been for the prediction of the *in vivo* assay. Secondly, all of these assays should be performed using human cell systems. The Breakout Group consensus was that *in vitro* testing should, when possible, be performed with human cells rather than rodent cells. This obviates the need, inherent in the rodent LD50 test, to extrapolate from rodents to humans. The uncertainties with the current approach of extrapolating *in vitro* derived data employing human cell cultures to the situation in the intact situation in humans will generally be smaller than those uncertainties for extrapolating data from animal cell experiments to humans.

Classification of chemicals according to their physico-chemical properties has been done extensively in the past. This approach has proven to be useful to predict effects, particularly within closely related classes of chemicals. However, this approach has limitations; it should not be used outside the boundaries of the prediction model used (i.e., the effects that can be predicted should be within the scope of the model assumptions).

If the focus is on the use of *in vitro*-derived data, then the importance of using specific cell systems becomes more important if one is looking at more specific forms of toxicity. Then the biological properties of the cells used become more important. Ultimately, there are two questions that coexist all the time: What does the chemical do to the cell?; and what does the cell do to the chemical? From this conceptual point of departure, the rate-determining step and more

often the rate-limiting steps need to be identified for mathematical modeling.

This problem and part of its solution can be illustrated based on central nervous system (CNS) vs. liver effects of solvents (limit it to small molecular weight chlorinated aliphatics). It is known from the Meyer-Overton rule (Meyer, 1937) that these anesthetic chemicals are very predictive of one another's CNS effects *in vivo*. However, these predictions do not hold for chronic liver effects and vice versa. This is understandable since the two effects have nothing in common, kinetics being the rate-determining step for anesthesia (wake-up driven by elimination of the chemical) vs. dynamics being the rate-determining step for liver cancer (slow reversibility of preneoplastic foci after complete elimination of the solvent). However, an acute endpoint such as reduced flicker fusion reflex is a much more sensitive endpoint of impairment than is chronic liver cancer. Therefore, people will be protected from cancer if regulation is based on the acute effect without the need for elaborate PBBK models based on metabolism in the liver.

The acute toxicity of all these solvents consists of CNS depression leading to respiratory failure without regard to the route of administration. These considerations will become more important when one moves away from the prediction of acute lethal toxicity towards predicting more subtile sublethal (acute) effects. However, these points are essential for modeling (sub)-chronic toxicity.

3.4.3 *Kinetic Support of Interim Rat LD50 Estimate*

In developing the approach just described, the focus of the Breakout Group was on the prediction of human TD10 values (i.e., the dose at which mild toxicity could be expected in no more than 10% of exposed humans). However, the Breakout Group acknowledged that there will be a need in the short-term for the estimation of rodent LD50 values under the HPV chemical program. The following discussion describes the application of the approach described above for this latter need.

3.4.3.1 *Research and Development Needs*

In the first step, estimates of key kinetic parameters can be obtained either from data available on the chemical or from the use of QSPR techniques (which are based on physico-chemical properties of the compound). QSPR techniques can be used as a first approximation of key kinetic parameters such as absorption, partition, etc. If one can use kinetic data that are actually measured, then these data will prevail.

- Octanol/water partition coefficient;
- Water solubility;
- Saturation vapor pressure or blood-air partition;
- PKa;
- Molecular weight/volume (for estimating gastrointestinal absorption);
- Hydrogen bond donors/acceptors (for estimating gastrointestinal absorption).

This prior knowledge on kinetic parameters or the estimation on the basis of QSPR data can then be used to evaluate the *in vitro* LC50 values for a chemical. The assumption is that this LC50 value is equal to the concentration in the intact organism at which cells die *in vivo*. Depending on the chemical's physico-chemical properties, the kinetic model to be used for this estimation may be simple or more complex. For many (e.g., water-soluble compounds) a simple one-compartment model can be used to estimate the oral dose that would result in an average systemic exposure equivalent to the *in vitro* LC50 value over the time period of interest. The key factors needed for the model would be estimates of the oral bioavailability, tissue partitioning (to obtain the volume of distribution), and total clearance. Depending on the properties of the compound, the clearance could be dominated by metabolism, urinary excretion, or pulmonary ventilation. In most cases, metabolic clearance will have to be determined empirically.

A key problem for this near-term application is that many HPV chemicals may not have adequate analytical methods yet developed. Therefore, metabolism assays may be too expensive and time-consuming for high-throughput LD50

estimation. However, a simple, conservative estimate for the oral dose resulting in systemic exposure equivalent to an *in vitro* LC50 value could be obtained by assuming 100% bioavailability, ignoring metabolic clearance, and simply estimating tissue partitioning to obtain the volume of distribution (Vd). For example, a commonly used default for the volume of distribution for water-soluble chemicals as a function of body weight (b.w.) is:

$$Vd = 0.65 * b.w.$$

In this simple approximation, the relationship between the *in vivo* and *in vitro* assays could be described by the formula:

$$LD50 = LC50 * Vd / b.w..$$

Other adjustments could be made to this approach for chemicals where ventilatory or urinary clearance would be important, as described in the previous section. In addition, if data on bioavailability are available, such information could be factored in to obtain a more accurate LD50 estimate. An additional benefit of this approach is that similar calculations could be used to convert the *in vitro* LC50 value to an *in vivo* LC50 value for acute inhalation. These assumptions, however, introduce inherent uncertainties into the resulting calculation of the oral LD50 value and depending upon the material of concern, may result in substantial inaccuracies.

It is not certain that the approach described here is actually viable; in particular, it needs to be determined whether sufficient information is available on the compounds of interest to support the necessary calculations. A first step would be to characterize the HPV chemicals in terms of their physico-chemical properties and determining the range and most frequent combinations of physico-chemical properties. This would provide a basis for the selection of "proof of concept" chemicals (not necessarily HPV chemicals) that could be used to evaluate the kinetic parameter estimation paradigm described here.

Another useful exercise would be to identify the compounds that represent the outliers in the RC correlations of *in vitro* basal cytotoxicity assays with LD50 values. By determining the physico-chemical properties of these compounds, and knowing their target tissues, it might be possible to identify factors that could improve the correlation (e.g., consideration of BBB penetration) between predicted oral LD50 values in rodents and empirical values. In this way it might be possible to define a "predictive range" for various chemical properties over which the *in vitro* assay might be expected to provide reasonable LD50 estimates. Also, exclusion rules for identifying compounds for which the results of the *in vitro* assay should not be relied upon might be defined.

3.4.3.2 *Tiered Approach for Evaluating Acute Toxicity*

A particular problem area in terms of the predictive value of the currently available *in vitro* toxicity assays is where toxicity is secondary to metabolic activation. In particular, it is possible that rapid oxidative or reductive metabolism could result in acute liver toxicity from oral exposure. Examples of such toxicity is the production of phosgene by the oxidative metabolism of chloroform and the acute liver necrosis seen after carbon tetrachloride exposure. Such toxicity would not be observed in *in vitro* assays using basal cells with little or no metabolic competence.

One possible approach for dealing with this problem is illustrated in Figure 3.3. The first step would be to estimate hepatocyte metabolism at a relatively low concentration (e.g., 10 micromolar). If the rate of metabolism (Vmax/Km) observed is low, then the basal cell LC50 value could be relied upon. If, however, the rate is high, then it would be necessary to identify the responsible enzyme system. This identification could be performed, for example, by using a microsomal (S9) fraction with selective addition of cofactors or inhibitors. If these studies indicate that the primary enzyme system is oxidative or reductive, then the possibility of toxicity associated with metabolic activation exists. In this case it would be necessary to perform a hepatocyte cytotoxicity assay. If the LC50 value for the hepatocytes was much lower than for the basal cells, it would be necessary to characterize the concentration-response for metabolism in order to predict the *in vivo* doses that might be associated with toxicity. On the other hand, if the primary metabolism represents detoxication (conjugation, sulfation, etc.), then the (acute) toxicity of the metabolites

will generally be much lower and, therefore, the basal cell assay results for the parent compound could be used with some confidence to calculate the LD50 value.

An alternative approach, suggested by Breakout Group 3, would be to begin with a basal cell cytotoxicity assay (to screen out highly toxic compounds) and then perform a toxicity assay with a hepatocyte primary culture. If similar LC50 values were obtained in both assays, the concern for toxicity secondary to metabolic activation could be effectively ruled out. In such cases, a much less extensive characterization of metabolism would be needed to support an estimate of clearance. On the other hand, if the toxicity in the hepatocyte assay was strikingly greater than that for the basal cells, the more complete characterization of metabolism discussed above would be justified.

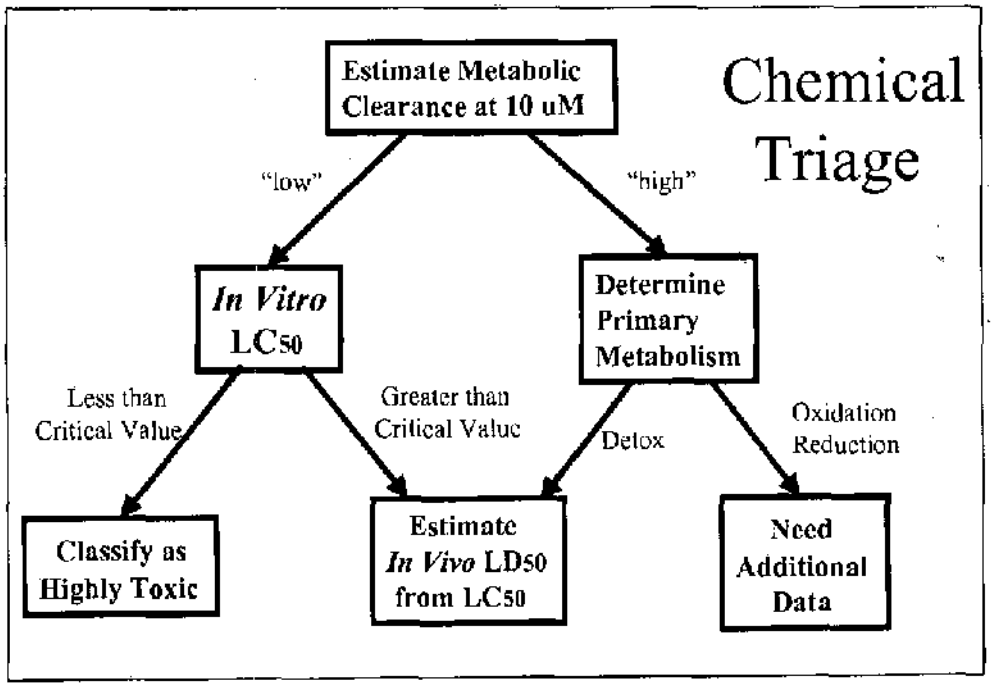

Figure 3.3: Tiered approach for evaluating acute toxicity

3.5 Recommendations

Table 3.1 (Section 3.5.2) lists a number of specific research areas in the area of biokinetics that the Breakout Group felt would improve the ability to use *in vitro* information in the prediction of acute toxicity. The following discussion highlights some of these research areas and illuminates some concerns emphasized by the Breakout Group.

3.5.1 *Long-Term Research Needs*

3.5.1.1 *Metabolites and Acute Toxicity*

In some cases, a circulating metabolite can be responsible for acute toxicity in a tissue remote from its generation. Kidney toxicity from some chlorinated alkenes has been shown to result from the production of a GST conjugate (in the liver) which is converted to the cysteine conjugate in the

kidney, and then activated to a toxic mercaptan by beta-lyase. Another example: the CNS effects of chloral hydrate result from the metabolite trichlorethanol, which is produced in the liver. In cases such as these, metabolite-specific kinetic data are necessary to estimate target tissue exposure, and *in vitro* toxicity assays would have to be conducted with the metabolite(s) responsible for the observed toxicities. The latter, requires structural identification and synthesis of the metabolite(s) of concern in sufficient quantities to conduct these studies.

Other important research areas include the development of validated, stable human hepatocyte systems, as well as *in vitro* systems for key transporters (renal, biliary, etc.). A long-range goal should be the development of template PBBK models for the various classes of chemicals. Target tissues evaluated by *in vitro* assays would be included explicitly in the physiological structure of these models. The models would provide a mechanistic description of barrier functions (gut, bile, kidney, blood-brain barrier, skin), so that the data obtained from transporter assays could be readily incorporated.

3.5.1.2 *QSPR Applications*

At the same time, specific QSPR applications need to be developed to provide the kind of information required by PBBK models (metabolism constants, binding, etc.). Unfortunately, the principal limitation in the development of useful QSPR applications appears to be the dearth of suitable data available for training knowledge-based systems.

3.5.1.3 *Kinetics and Dynamics*

The interaction between kinetics and dynamics needs to be explored. For example, the effect of toxicity on the metabolism and excretion of a chemical or, conversely, the effect of metabolism or reabsorption on the toxicity of a chemical must be taken into account. Rigorous analyses of the time dimension in the conduct of these assays to account for duration and frequency of exposure is also an area that needs to be addressed. Because of cell viability issues, it may not be possible to reproduce the time frame of *in vivo* tissue exposure using *in vitro* systems. Also, the time frame for the appearance of toxicity may be quite different from the time frame for exposure to the chemical (Soni et al., 1999).

It is important to recognize that the proposed schemes (Figures. 3.1 and 3.2), and the discussion above, concern only the approximation and prediction of acute oral toxicity. It was neither the intent nor the purpose of the Breakout Group that these conclusions could be extended in any way to other types of toxicity that are relevant to public health risk assessment (e.g., developmental toxicity, sensitization, carcinogenesis, etc.). In the final analysis, *in vivo* exposure captures the effects of many potentially complex interactions that may be difficult to reproduce with *in vitro* systems.

3.5.2 *Research Needs for the Application of In Vitro Methods to the Prediction of Acute Chemical Toxicity*

Table 3.1 Biokinetic Research Needs

Kinetics	Kinetics-Dynamics Interface (Feedback)	Dynamics	Extrapolation
Understand the relationship between molecular structure, physical-chemical properties, and kinetic behavior of chemicals in biological systems. Develop mathematical modeling techniques to describe complex kinetic systems. Develop mathematical modeling techniques for tissue modeling (anatomically correct models). Develop algorithms to determine the optimum kinetic model for a particular chemical. Conduct research on modeling of fundamental kinetic mechanisms. Develop an optimal battery of *in vitro* assays to evaluate chemical-specific kinetic parameters. Develop QSAR models to predict kinetic parameters. Develop a library of generic models that are acceptable for regulatory risk assessments. Establish a database of chemical-independent parameters (mouse, rat, human).	Understand and model the mechanisms regulating the expression of proteins involved in kinetic processes – (metabolizing enzymes, transport enzymes, metallothionein, membrane channels, etc.). Understand and model effects of changes in physiological processes on kinetics of chemicals.	Develop *in vitro* biological models that are equivalent to *in vivo* tissues (i.e., models that maintain specified differentiated functions that are important for the toxicological phenomena under study). Develop mathematical modeling techniques to describe individual variability (genetic background). Develop mathematical modeling techniques to describe complex dynamic systems and genetic networks at the cellular and at the systemic level. Establish lines of differentiated human cells (e.g., derived from stem cells). Understand and model mechanisms of multi-cellular interactions in development of toxic responses (co-cultures). Understand and model relationships between cellular responses and biomarkers of systemic responses.	Inter- and intra-species extrapolation; comparison of genomic differences, or species-specific expression differences between species and within one species (e.g. polymorphisms in biotransformation enzymes). High dose - low dose extrapolation

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4.0 *IN VITRO* METHODS FOR ORGAN-SPECIFIC TOXICITY

4.1 Introduction

Breakout Group 3 reviewed *in vitro* methods that can be used to predict specific organ toxicity and toxicity associated with alteration of specific cellular or organ functions. The Breakout Group then developed recommendations for priority research efforts necessary to support the development of methods that can accurately assess acute target organ toxicity.

Knowledge of the effects of acute exposure to unknown materials is needed early in the development of new products and chemicals. Researchers who are using new chemicals in the laboratory need to know what types of safety precautions they need to take when handling these materials. Manufacturers must have some idea of the safe levels of exposure before they can develop the processes and build the facilities to safely manufacture the materials. The toxic doses also define precautions that must be taken when shipping materials, and govern the appropriate response of emergency personnel in case of accidental spills. Planned or inadvertent single-dose exposure of specific human or other populations may also occur, such as from accidental ingestion of common household materials, application of single use pesticides, and some pharmaceuticals.

The Breakout Group was asked to review *in vitro* methods for predicting specific target organ toxicity. Specifically the Breakout Group was asked to do the following: (a) identify the most important areas where *in vitro* methods are needed; (b) review and comment on the current status of *in vitro* methods to predict target organ toxicity; and (c) prioritize the need for future research in this area. In addition, the Breakout Group considered where it would be necessary to include prediction of specific target organ toxicity in developing an *in vitro* program to replace the current acute oral toxicity assays used in hazard classification systems.

The scope of the remit was very broad and the Breakout Group proceeded by identifying the organ systems where failure could lead to lethality after acute exposure. The Breakout Group reviewed each system individually, and then proposed a scheme for including the important endpoints identified into a replacement test battery for acute toxicity.

4.1.1 *Regulation of Industrial Chemicals and Pesticides*

A representative (Dr. Karen Hamernik) of the U.S. EPA related the needs of an agency that regulates industrial/commodity chemicals and pesticides. In addition to their use in assigning an international hazard classification, the results of acute toxicity tests are used to set doses for *in vivo* cytogenetics assays, acute neurotoxicity tests, and, occasionally, for other types of rodent tests. Dose setting may utilize LD50 information and dose response data over a range of doses for a given test material. In addition, information on the effect of single exposures is gathered during acute neurotoxicity tests, developmental toxicity tests, and metabolism studies. In these tests, multiple endpoints may be measured and the results can be used for hazard and risk assessments for single-exposure scenarios.

The U.S. EPA is concerned with organ-specific effects -- including their severity, onset, and duration -- that become apparent from various test material exposure scenarios including acute, sub-chronic, or chronic exposure. Some study protocols provide reversibility-of-effects information. Information on organ-specific effects may have an impact, at least in part, on risk assessment methods depending on the effect of concern, whether a mechanism for toxicity can be proposed or identified, and on the available dose-response information. For instance, organ-specific effects may impact decisions on whether to regulate based on cancer or non-cancer endpoints, to use linear or non-linear models, and whether to use dose-response data or benchmark dose approaches.

How organ-specific effects impact risk assessment depends to some extent on where the effects occur on the dose-response curve, what types of effects are seen and their severity, and the nature of the exposure.

Examples include the presence of clear toxic effects such as necrosis and changes in enzyme activities or elevations in hormone levels that may be considered precursors to possible longer-term toxic, or even carcinogenic, effects. The impact of these effects may depend upon whether they are seen only in adult animals, young or adolescent animals, or during *in utero* exposure. Toxicity data are used for human risk assessment and to provide clues for potential concerns for effects in wildlife.

In the United States, organ-specific effects seen in toxicity studies may trigger Food Quality Protection Act-related issues such as the possibility of grouping chemicals with common modes of action or mechanisms for cumulative risk assessment. Certain organ-specific effects may serve as a starting point to look at questions related to human relevance. The presence of such findings may trigger the need for additional studies to support the suspected toxicological mechanism.

4.1.2 *Regulation of Pharmaceuticals*

A representative (Dr. David Lester) of FDA/CEDR related the needs of an agency that regulates pharmaceutical materials. CEDR does not ask for, nor regulate, non-clinical toxicity testing, and does not use estimates of the LD50 value in its assessments. In general, the agency does not find identification of specific organ toxicity after single-dose acute exposure useful since most pharmaceuticals are given as multiple doses.

The results of acute toxicity tests are not useful in establishing dosing regimes because most pharmaceuticals are developed for multiple use. Acute effects are more important for oncologic drugs because the margins of safety may be smaller. Single-dose studies may also be useful for developing imaging agents where it is important to understand tissue distribution after a single exposure.

In vitro studies are often performed in drug development as part of the effort to understand the disease process or to understand the actions of the drugs on specific cells. In drug development, the risk assessments are based on the total dose of the material given and not on the tissue concentration. *In vitro* studies have been used in setting doses for initial human exposure to cancer therapeutics, but otherwise are rarely used for dose setting because current methods cannot extrapolate from the *in vitro* concentration to the dose that must be given to achieve similar effects *in vivo*. Animal studies may be used for initial dose setting for early clinical studies, but these are usually not acute, single-exposure studies.

4.1.3 *U.S. National Toxicology Program (NTP)*

The Breakout Group also heard a presentation (from Dr. Rajendra Chhabra) on the use of acute oral toxicity data by the National Toxicology Program (NTP). The NTP does not find it necessary to use acute studies to set doses for subchronic studies; instead, researchers go directly to 14- or 90-day studies. If there are sufficient data on the chemical of interest, then they are often able to avoid a 14-day study. The results of 90-day studies in rodents are used to set doses for chronic studies and also to determine what specific types of additional studies may be needed (i.e., reproductive, cancer, neurotoxicology, etc.). To facilitate decision making and reduction of animal use, the NTP adds several endpoints to the 90-day study including sperm morphology, immunotoxicology, neurotoxicology, and a micronucleus test.

The NTP is evaluating a battery of *in vitro* tests that might reduce the need for 14-day dermal toxicity studies. The tests include:

- The bovine corneal opacity test;
- The skin permeability assays;
- The EpiDerm™ model for dermal irritation/corrosivity;
- A neutral red uptake (NRU) assay for systemic toxicity;
- A primary rat hepatocyte assay for hepatic toxicity.

Five chemicals have been tested in this battery. The 14-day *in vivo* rodent study costs about $150,000, uses 120 animals, and takes about six months to perform. An accurate battery of *in vitro* tests would be less expensive in both time and cost.

4.1.4 *Initial Considerations*

The Breakout Group agreed for the purposes of this exercise to define acute toxicity as *"toxicity occurring within 14 days of a single exposure or multiple exposures within 24 hours"*. For evaluating chemicals for acute toxicity, the Breakout Group identified the following major organ systems as the ones that need to be considered:

- Liver;
- Central nervous system;
- Kidney;
- Heart;
- Hematopoietic system;
- Lung.

Damage significant enough to cause death can occur to these systems after a single acute exposure. The Breakout Group recognized that local effects of xenobiotics on the skin, gastrointestinal tract, and eye may also be important, but agreed to focus on systemic effects rather than local effects. The Breakout Group also recognized that the developing embryo may suffer serious, even lethal, consequences after a single acute exposure to a xenobiotic. However, the Breakout Group felt these effects are adequately evaluated by the standard battery of tests for reproductive and developmental effects and do not need to be included as part of an *in vitro* battery to replace the acute toxicity tests.

The Breakout Group discussed the use of rodent cell cultures as the basis of *in vitro* tests to predict acute toxicity. The work of Ekwall (Ekwall et al., 2000) indicates that for general cytotoxicity cells of human origin correlate best with human acute lethal blood concentrations. There are well recognized species differences in response to many classes of xenobiotics that must be taken into account as systems are developed to predict effects specific to individual organ systems. Considering the species differences currently recognized and other differences that might not yet be identified, the Breakout Group recommends that every effort should be made to use human-derived cells and tissues, preferably normal, as the basis for *in vitro* assays since data from the *in vitro* studies will ultimately be used to predict toxicity in humans.

4.2 Review of a Proposed Screen to Elucidate Mechanism of Injury

The Breakout Group examined specific endpoints or organ systems. Both *in vivo* and *in vitro* systems are used extensively in industry and academia to aid in the understanding and prediction of mechanisms of toxicity. The review attempted to highlight situations where *in vitro* studies provide information at least as useful and often more useful than *in vivo* studies and to identify areas where further research is needed before *in vitro* techniques will be able to replace whole animal studies.

The Breakout Group first reviewed a program using eight different normal, human epithelial cell lines or primary cells for initial toxicity screening to elucidate mechanisms of injury by measuring comparative tissue-specific cytotoxicity of cancer preventive agents (Elmore, 2000; Elmore, in press). Tissue-specific cytotoxicity was assessed using cell proliferation at three days and five days, mitochondrial function, and PCNA or albumin synthesis (hepatocytes only) as endpoints. The cells used were early passage cell lines following cryopreservation or were primary cultures (hepatocytes) and included liver, skin, prostate, renal, bronchial, oral mucosa, cervix, and mammary tissues.

The results suggest that different chemicals induced unique tissue-specific patterns of toxicity. Changes in toxicity following three and five day exposures provide additional information on both delayed toxicity and the potential for recovery. Confirmation of the predictive trends was confirmed with several agents in keratinocytes using 14-day cultures with multiple exposures. Ongoing studies will compare the *in vitro* data with blood levels from preclinical animal studies, and plasma levels and observed side effects from clinical trials.

4.3 *In Vitro* Methods for Determination of Acute Liver Toxicity

Adequate liver function is critical to the survival of an organism. The liver is at high risk for injury because it is actively involved in

metabolizing xenobiotics, and because the liver is exposed first to materials absorbed from the gastrointestinal tract. The liver also excretes many materials via the bile and this puts the biliary system at risk for toxicity as well. For these reasons, one of the highest priority needs is for a test system that can accurately evaluate the effects of xenobiotics on the liver. Test systems need to be able to assess both the potential for hepatic toxicity and whether the liver will be able to metabolize the test chemical either to a more or less toxic moiety. Xenobiotics may also affect the biliary tract, and an *in vitro* system to investigate these effects will also be needed.

4.3.1 *Available Non-Animal Models*

Available non-animal models include metabolically competent animal or human liver cells. Such cells have been cryopreserved and cryopreserved human cells are available commercially. The cells of human origin have a short life span, but they can be obtained with certain well-characterized metabolic profiles including specific active P450 systems. Immortalized human cell lines, some of which have been transfected to express specific recombinant phase I or II enzymes are also available, but most cell lines are limited to expressing only one enzyme.

Assessment of the potential for hepatic metabolism is possible using isolated hepatocytes (Cross and Bayliss, 2000; Guillouzo, 1997) and cell lines. Liver microsomes are used in high throughput screening assay systems to determine the extent of metabolism of a parent compound. Whole liver homogenates, subcellular fractions, and liver slices are also commonly used in basic research on hepatic function and toxicology (Guillouzo, 1998; Parrish, et al., 1995; Ulrich et al., 1995; Waring and Ulrich, 2000). A report on the ECVAM Workshop on the Use of Tissue Slices for Pharmacotoxicology Studies includes a comprehensive review of the use of liver slices in toxicology (Bach et al., 1996). These systems can be robust, but the supply of human liver tissue is limited and is decreasing as more donor liver is being used for transplantation

Recently, more complex systems have been developed in an attempt to better mimic hepatic function. Cell culture techniques that involve sandwiching liver cells between layers of collagen can be used to study induction of metabolic function, but it is difficult to examine the hepatocytes after treatment because of the collagen in the system. Liver cells can also be cultured as small compact spheres of cells. As these spheroids grow, they tend to become necrotic in the center so their usefulness in toxicology needs to be established.

There have been some attempts to develop *in vitro* systems to study effects on biliary function. A couplet system made up of two hepatocytes with bile canaliculi attached has been described. This system is very labor intensive and currently would not be viable as a routine test system but is useful as a way to study mechanisms of cholestasis. In addition, liver fibroblasts can be cultured for the study of mechanism of hepatic cirrhosis.

4.3.2 *Specific Endpoint Measurements*

As *in vitro* systems for hepatic function are developed to replace animals in acute toxicity studies, the specific endpoints which should be considered are changes in enzyme systems, membrane damage, changes in mitochondrial function, changes in albumin synthesis, and possibly cell detachment. It will be important to identify systems that express the most important metabolic systems present in normal human liver. The Breakout Group discussed the need for multiple cell lines to represent the known diversity of enzyme systems expressed by the human population. While such systems are very useful in drug development, the Breakout Group recognized that this degree of sophistication is not available with the current *in vivo* systems and should not be required for a replacement system for acute toxicity.

4.3.3 *Future Needs*

Future work in the area of hepatic toxicology will depend upon the development of more robust models that are as metabolically competent as mature human hepatocytes *in vivo*. Pharmaceutical companies are currently using *in vitro* assays of hepatic function for screening new drugs and as their methods become more readily available, they may be useful in acute toxicity testing. An ILSI HESI

Genomics Subcommittee is assessing changes in gene expression that occur in response to several prototypic chemicals, including hepatotoxicants, and will be attempting to correlate the gene expression changes with changes in various biological and toxicological parameters.

Two methodological issues need to be addressed as *in vitro* methods are developed and evaluated. First, when culturing liver cells, it is vital that the cells are constantly monitored to ensure they are still expressing the desired characteristics and this monitoring must be built into protocols. Second, there is considerable variability in enzyme function between cells from different individual donors, and for toxicity testing it will be necessary to agree upon the cell characteristics needed for an appropriate test system that will best represent the overall human population.

There is a high-priority need to develop a system for regulatory use that will be able to recognize which compounds the liver will metabolize to another compound or compounds. To replace whole animal, systems must be devised that can also determine the effect of the product or products of hepatic metabolism on other organ systems in a dose responsive manner.

There is a need for a worldwide database comparing human *in vitro* and *in vivo* data for hepatic toxicity. Scientists attempting to develop hepatic systems for toxicity testing are encouraged to share methodology and cell lines. Collaboration among laboratories would increase the pace of research and avoid development of multiple and competing test methods.

4.4 *In Vitro* Methods for the Determination of Acute Central Nervous System (CNS) Toxicity

Neurotoxic effects after a single dose are often expressed as either overall CNS depression resulting in sedation, or excitation, generating seizures or convulsions. The molecular mechanisms for these states may be related to very specific toxicant-target interaction, or the targets may be general for all cell types but are involved in critical functions in neurons. Because CNS effects can lead to acute lethality, a neurotoxicological screen should be performed when certain criteria in the tiered test battery, as described in Section 4.10.1, have been fulfilled. Briefly, the steps are physico-chemical or other information indicating that the toxicant can pass the BBB, low basal cytotoxicity (high EC20 or EC50 values) in non-neuronal cells, low hepatotoxicity, and no evidence of impaired energy metabolism at non-cytotoxic conditions. If these initial criteria are fulfilled, investigations of the neurotoxic potential of the test material must be carried out. The cellular targets can be either general or very specific functions.

4.4.1 *Important General Cellular Functions for CNS Toxicity*

Examples of important general cellular functions that upon impairment may cause severe brain damage after acute exposure are decreases in resting cell membrane potential, increases in intracellular free calcium concentration ($[Ca^{2+}]i$), and formation of free radicals and reactive oxygen species (ROS). Cytotoxicity may, eventually, occur as a result of severe insult to these cellular functions. In some cases, astrocytes are the immediate target and the toxic reaction may appear as astrocyte activation and formation of neurotoxic cytokines. An early marker for acute astrocyte activation is increased glial fibrillary acidic protein (GFAP) expression.

4.4.1.1 *General Endpoints*

Endpoints that can be assessed include cell membrane potential, increased $[Ca^{2+}]i$, and free radical formation that can easily be measured by fluorescent probes or by simple spectrophotometry. Cytokines and GFAP levels can be determined by immunochemical techniques, such as ELISA, or by mRNA quantification (e.g., *in situ* hybridization, RT-PCR, or gene array analysis). Most assays can be performed on adherent cells in microtitre plates, which make them useful for high throughput screening.

4.4.1.2 *Cell Models for General Functions*

Several cell models are available. General cell functions can be studied in cell types that

possess a near normal cell membrane potential and aerobic energy metabolism. Certain differentiated human neuroblastoma cell lines, such as SH-SY5Y, fulfill these criteria and are easy to obtain, culture, and differentiate. Human brain neural progenitor cell lines (e.g., NHNP and NT2) are now widely available. The NHNP cell line has the advantage that in culture it differentiates into a mixture of neurons and glia. It can be passed through numerous passages and forms spheroids in suspension (Svendsen et al., 1997). Glial cell lines are generally poorly differentiated even though there are reports of some GFAP-expressing human cell lines (Izumi et al., 1994; Matsumura and Kawamoto, 1994). Rat glioma 9L cells have been reported to manifest astrogliosis upon chemical exposure (Malhotra et al., 1997). Nevertheless, primary rat astrocyte cultures are used in most studies on astrocyte activation.

4.4.2 *Important Specific Functions for CNS Toxicity*

Specific functions can be measured by assessing neuronal targets that will cause acute CNS depression or excitation if their functions are impaired. These functions are voltage operated Na^+, K^+, and Ca^{2+} channels and the ionotropic glutamate NMDA, $GABA_A$, and nicotinergic acetylcholine (nACh) receptors. Furthermore, severe intoxication may occur after acute exposure to cholinesterase inhibitors. Besides the acute effect on cholinesterase function, delayed neuropathy may also be evident after a single dose.

4.4.2.1 *Specific Endpoints*

Ion fluxes over the cellular membrane can be estimated by using various ion-selective fluorescent probes. However, upon stimulation, effects on ion channels or receptors change the net membrane potential. Eventually, this will result in altered Ca^{2+} fluxes and $[Ca^{2+}]i$, which in turn will affect transmitter release. Therefore, effects of toxicants on receptor and ion channel functions may be detected as increased or decreased $[Ca^{2+}]i$ (Forsby et al., 1995) or neurotransmitter release (Andres et al., 1997; Nakamura et al., 2000; Smith and Hainsworth, 1998; Wade et al., 1998). The effects may be evident directly by the toxicant itself, but also after applied stimuli such as potassium-evoked cell membrane depolarization, possibly in the presence of receptor agonists. Acetylcholine esterase (AChE) activity in neuronal cells can be measured in differentiated cells such as SH-SY5Y cells. Evaluating changes in the ratio between AChE and neuropathy target esterase (NTE) has been proposed as a method for estimating the risk for delayed neuropathy (Ehrich et al., 1997).

4.4.2.2 *Cell Models for Specific CNS Functions*

Cell models for studies on specific CNS functions should be of human origin, mainly because certain enzyme structures and receptor sub-unit expressions differ among different species. Furthermore, the level of cellular differentiation is crucial. The cell lines must, in most cases, be treated with differentiating agents such as retinoic acid to express features of normal, adult neurons. Cells that are transfected with genes expressing specific receptor and ion channel proteins can also be useful for studies on specific functions.

One example of non-primary neuronal cells is the human neuronal progenitor NT2 cells derived from a teratocarcinoma. The NT2 cells can be terminally differentiated to NT2-N cells after treatment with retinoic acid and mitosis-arresting agents after months in culture. NT2-N cells express functional NMDA and $GABA_A$ receptors (Younkin et al., 1993; Munir et al., 1996; Neelands et al., 1998). The previously cited NHNP neural human brain progenitor cell line could also serve as an important model system for neurotoxicity screening (Svendsen et al., 1997). It is not as well characterized as the NT2 line but deserves investigation. Alternatives to NT2-N may be native or differentiated human neuroblastoma cell lines (e.g., SH-SY5Y, IMR32 and CPH100). However, their receptor sub-unit expression and receptor function may vary from normal receptors present in adult brain tissue.

Co-cultures of neuronal and glial cells may be used for studies on interactions between neurons and glia cells. For instance, NT2 cells differentiate and establish functional synapses

when they are cultured on astrocytes (Hartely et al., 1999). Upon differentiation, the NHNP cell line cultures contain a mixture of astrocytes and neurons varying in ratio from 1:9 to 2:3. In suspension, the NHNP cells form spheroids (see Clonetics web site). Reaggregated embryonic brain cultures have been recommended for screening of neurotoxic compounds (Atterwill, 1994) but significant further work on this promising model is needed before it can be used as a standard test method.

4.4.3 *Future Needs*

Some endpoints, assays, and cell models for the more general endpoints have been studied and used extensively, which make them ready for formal validation. However, most assays and cell models determining effects on special functions still need significant basic research before they will be useful in screening systems.

4.5 *In Vitro* Methods to Assess Blood-Brain Barrier (BBB) Function

The CNS is dependent on a very stable internal environment. The BBB helps maintain this stable environment by regulating all uptake into and release from the brain of substances involved in CNS metabolism. The barrier acts as a functional interface between the blood and the brain, rather than as a true barrier, and this function is localized to the brain capillary endothelial cells. These cells differ from endothelial cells in other organs in that they form tight junctions. They have a higher turnover of energy and thus contain numerous mitochondria; they have a low endocytotic activity. Furthermore, they express specific transport proteins and enzymes. Water, gases, and lipid-soluble substances may pass the BBB by simple diffusion whereas glucose, monocarboxylic acids, neutral and basic amino acids, and choline are taken up from the blood by active processes. Ions pass the BBB very slowly and proteins generally not at all. Weak organic acids, halides, and potassium ions are actively transported out of the CNS.

From a toxicological viewpoint, three aspects of the BBB are of interest: (a) the BBB regulates uptake and release of endogenous substances and also xenobiotics, (b) toxic substances may interfere with the structural and functional properties of the BBB, and (c) certain parts of the CNS (e.g., areas in the hypothalamus and the choroid plexa), have poorly developed BBB functions. The latter is also true for all parts of the embryonic and juvenile brains.

Several authors and working parties have identified the need for a reliable *in vitro* model of BBB functions as being essential for the development of alternative methods for use in tests of acute systemic toxicity, neurotoxicity, and in drug development (Balls and Walum, 1999; Ekwall et al., 1999; Janigro et al., 1999; the ECVAM workshop on *In Vitro* Neurotoxicity [Atterwill et al., 1994], the ECVAM Neurotoxicity Task Force, [1996, unpublished], and the BTS Working Party Report on *In Vitro* Toxicology, [Combes and Earl, 1999]). ECVAM is currently supporting a prevalidation study of *in vitro* models for the BBB. The study largely follows the recommendations published by Garberg (1998).

4.5.1 *Endpoints for Acute Toxic Effects*

For acute toxic effects, there are two endpoints for toxic insult to the blood brain barrier: (a) partial or complete breakdown of the barrier function (i.e., effects on the ability of the BBB to exclude endogenous and exogenous substances) and (b) changes in the specific transport capacity of the BBB. There is a need to measure the ability of the normal BBB to transport toxicants into or out of the brain.

4.5.2 *Models*

Models currently being assessed in the ECVAM-sponsored prevalidation study include:

- Immortalized endothelial cell lines of both human and animal origin;
- Primary bovine endothelial cells co-cultured with glial cells;
- Barrier-forming continuous cell lines of non-endothelial origin.

Preliminary results from the ECVAM prevalidation study, as well as previously published results, show that the rate of penetration of compounds that pass the BBB by simple diffusion can be estimated by the

determination of log P, or by the use of any cell system that forms a barrier (e.g., MDCK or CaCo2 cells). This means that the distribution of lipophilic compounds over the BBB can be determined simply, and that the first aspect of acute toxic effects (i.e., impairment of the barrier function [see above]) can be studied in continuous cell lines, provided they are able to form tight junctions.

With respect to the second endpoint, impairment of the transporter functions and the transport-mediated brain uptake, the situation is different. The modeling of these features of the BBB ideally requires an *in vitro* system with a high degree of differentiation, including the significant expression of all transporter proteins representing species-specific properties. At present, this can only be achieved in primary cultures of brain endothelial cells co-cultured with brain glial cells.

A model presented by Stanness et al. (1997) shows development of a dynamic, tri-dimensional *in vitro* culture system (DIV-BBB) that mimics the *in vivo* BBB phenotype more closely than other models in use. In this system, cerebral endothelial cells are cultured in the presence of astrocytes using a hollow fiber technique. The fiber cartridge, representing artificial capillaries, is exposed to a luminal pulsatile flow of medium. Although a very good model for the *in vivo* situation, the DIV-BBB model may be too resource intensive to be of practical use in a screening situation.

4.6 *In Vitro* Systems to Study Kidney Toxicity

The major effect seen in the kidney after acute exposure to a nephrotoxin is acute tubular necrosis. In approximately 90% of the cases, the changes are seen in the proximal tubular cells (proximal to the convoluted tubules). These cells have high metabolic activity and a significant concentrating function, both of which put them at increased risk for damage. There are a much smaller number of substances that are toxic to the distal tubular cells. While acute toxicity in tubular cells is highly significant and can be fatal, it is important to recognize that these cells have great regenerative capacity and with adequate treatment and time will repopulate and replace the destroyed cells.

There are a few substances that cause direct glomerular damage which is more serious because glomerular damage is permanent resulting in the loss of the affected nephron. Although the kidney has a considerable reserve capacity of nephrons, it is important to understand the effects of a reduction of this reserve capacity particularly in individuals, such as the elderly, who may already have a reduced number of nephrons.

A comprehensive review of the use of *in vitro* systems to assess nephrotoxicity has been completed by ECVAM and was used as the basis for the discussion (Hawksworth et al., 1995). *In vitro* systems will need to utilize metabolically competent kidney tubular cells. This should not be as difficult as liver systems since much is known about the metabolic function of renal tubular cells, and there does not appear to be significant variability between individuals. In addition to direct cytotoxicity, *in vitro* systems must be able to evaluate the barrier function of the kidney. A system to assess this parameter is currently being studied in Europe, with support from ECVAM. In addition, *in vitro* systems may need to assess transport functions. At this time it is not clear how important these functions are in acute toxicity. It is also not known how much variability exists in these functions from one individual to another. The specific transport functions are not completely characterized and more basic research is needed before test systems can be developed.

It is possible to measure kidney function in a non-invasive fashion in humans who are exposed to low levels of xenobiotics, for instance, in occupational exposures. It would be valuable to evaluate the correlation of the results from *in vitro* toxicity tests with information from humans.

4.7 *In Vitro* Methods to Assess Cardiotoxicity

Cardiovascular toxicity can result from excessive accumulation of toxic chemicals within the tissue, cardiovascular-specific bioactivation of protoxicants, and/or chemical

interference with specialized cellular functions. Because a cardiotoxic insult interferes with the ability of the heart to pump blood through the vasculature, blood flow to major organs is often compromised. Vascular toxicities are often characterized by slow onsets and long latency periods and are not usually important in acute toxicity; however, changes in arterial pressure and blood flow control may be significant in acute effects.

The pathogenesis of cardiovascular injury often involves the elucidation of oxidative mechanisms and many cardiovascular disorders are characterized by loss of redox homeostasis. The central role for oxidant mechanisms is consistent with studies which show evidence of beneficial effects of antioxidants provided to patients with coronary heart disease (Napoli, 1997). The vascular production of reactive oxygen metabolites increases substantially in disease states (Harrison, 1997). Links between cardiovascular and cerebro-vascular disorders have also been established. During periods of emotional stress, adrenaline toxicity to vascular endothelial cells may involve its deamination by monoamine oxidase A to form methylamine, a product further deaminated by semicarbazide-sensitive amine oxidase to formaldehyde, hydrogen peroxide, and ammonia (Yu et al., 1997).

4.7.1 *Perfused Organ Preparations*

Perfused organ preparations are currently the most representative of the *in vivo* situation. Aortic preparations are most preferred; they can be readily excised, perfused, and super-perfused with appropriate buffers, (Crass et al., 1988). Perfused preparations are advantageous because they retain the level of structural organization found *in vivo*. Toxin-induced changes in physiologic/pharmacologic sensitivity and changes in excitability and/or contractility can be readily evaluated. The biological actions of nitric oxide, a soluble gas synthesized by the endothelium, was first discovered using perfused preparations. Because perfused organ preparations require harvesting fresh tissue, better methods are still needed. In addition, significant limitations of perfused preparations in toxicity testing include the small number of replicates that can be processed, the time required for isolation, and the provision that the system can only be used for short periods of time because of rapid loss of viability. Parameters measured include: (a) time to peak tension, (b) maximal rate of tension development, and (c) tension development. Oxygen concentration of the perfusate provides an index of myocardial oxygen consumption. Pin electrodes can be used to obtain electrocardiographic readings. Measurements of contractility and stress development can be used to evaluate effects of drugs and chemicals.

4.7.2 *Isolated Muscle Preparations*

Isolated muscle preparations consisting of strips of atrial, ventricular or papillary muscles (Foex, 1988), or segments from vascular beds (Hester and Ramos, 1991) can be super-perfused with oxygenated physiologic solutions for measurements of tension development. The pre-load and after-load placed on the tissue can be controlled accurately to evaluate isometric force development, isotonic force development, and quick-release contractions. Oxygenation of the tissue is a function of diffusion, and the thickness of the strips and oxygen concentration in the solution bath must be carefully monitored. The stability of these muscle strips is limited to short time periods. Because many preparations can be made from each animal, these systems use less numbers of animals than perfused organ preparations.

Isolated preparations have been used to examine the angiotoxic effects of ethanol (Rhee et al., 1995), acetaldehyde (Brown and Savage, 1996), palytoxin (Taylor et al., 1995), and cadmium (Ozdem and Ogutman, 1997). Regional differences in physiologic and pharmacologic responsiveness must be considered in developing strategies that examine vasculotoxic responses. Aortic rings exhibit higher sensitivity to norepinephrine than mesenteric artery rings, while the reverse effects are found with serotonin. However, no differences in sensitivity to KCl and $CaCl_2$ were observed (Adegunolye and Sofola, 1997). Differences between the two vessels appear dependent on agonist ability to mobilize calcium from intracellular stores.

4.7.3 *Organ Culture Preparations*

Organ culture preparations offer long-term stability as compared to other *in vitro* preparations. Whole fetal hearts from mice and chicks have allowed the study of processes associated with myocardial cell injury (Ingwall et al., 1975; Speralakis and Shigenoubu, 1974). Organ-cultured blood vessels have led to elucidation of structural/functional relationships of the vessel wall matrix (Koo and Gottlieb, 1992). However, organ culture of rat aortic rings results in significant loss of contractile responsiveness to different agonists within 24 hour (Wang et al., 1997).

4.7.4 *Tissue Slice Preparations*

Tissue slice preparations of cardiac tissue have been characterized as models to evaluate toxicity of xenobiotics (Gandolfi et al., 1995) and could be useful in toxicity testing applications (Parrish et al., 1995).

4.7.5 *Single-Cell Suspensions*

Single-cell suspensions of embryonic or neonatal cells that are derived from ventricular, atrial, or whole heart tissue can be easily prepared by enzymatic and/or mechanical dissociation of the tissues. Adult hearts can also be dissociated by a modified recirculating Langerdorff perfusion that yields a large proportion of cells which remain rod shaped and are quiescent in medium containing physiologic calcium levels (Piper et al., 1982). The anatomic distribution of cells within the walls of large and medium-sized mammalian vessels facilitates the isolation of relatively pure suspensions of fibroblastic, endothelial, or smooth muscle cells. In contrast to cardiac preparations, vascular cells from embryonic, neonatal, and adult vessels can be efficiently isolated in calcium- and magnesium-containing solutions.

Myocardial cell suspensions represent a heterogeneous population of muscle and non-muscle cells. Neonatal myocytes are remarkably resistant to injury and exhibit variable degrees of beating shortly after isolation. In contrast, spontaneous beating of adult cardiac myocytes is thought to be due to uncontrolled leakage of calcium through a permeable plasma membrane. Adult cardiac myocytes are mechanically at rest when properly isolated suggesting that functional differences in regulation exist between adult and neonatal cells. Isolated cells can be microinjected with fluorescent dyes for the assessment of multiple cellular functions following exposure to toxic chemicals. The viability of cells in suspension decreases rapidly as a function of time. Investigators rarely use these cell suspensions for more than four hours.

Changes in cell function or contractility can be assessed using these models. Because heart failure, in some instances, is characterized by contractile dysfunction of the myocardium and elevated sympathetic activity, cell function or contractility is of concern (Satoh et al., 2000). It has been demonstrated that adult rat ventricular myocytes in culture show signs of decreased contractility when exposed to adrenergic stimulation by norepinephrine + propanolol for 48 hours. This result seemed to be due to decreased Ca (2+)-ATPase. Consequently, sympathomimetic agents or other chemicals that decrease Ca (2+)-ATPase would have similar activity.

A number of anthracycline antineoplastic agents are known to cause cardiac cytotoxicity that can be severe and often irreversible. Doxorubicin and 4'-epirubicin significantly depress myocyte contractility in isolated neonatal and adult rat ventricular myocytes (Chan et al., 1996) but the etiology of the toxicity has not been determined definitively (Sawyer et al., 1999). The effect can be assessed by visualizing the beating of the myocytes (Jahangiri et al., 2000) or by measuring calcium flux using fluorescent dyes (Trollinger et al., 2000). Cultured fetal chick cardiac myocytes have also been used to study the toxicity of hydrogen peroxide and certain agents which can protect against such toxicity (Horwitz et al., 1996).

4.7.6 *Models Using Cell Lines*

Cardiac cell lines are generally preferred for the evaluation of chemical toxicity following prolonged exposures or following multiple challenges *in vitro*. Primary cultures can be established with relative ease from cell

suspensions of cardiac and vascular tissue. However, they must be characterized at the morphologic, ultrastructural, biochemical, and functional levels before being used in cytotoxicity testing applications because they undergo variable degrees of dedifferentiation, including loss of defined features and cell-specific functions. Vascular endothelial and smooth muscle cultures can also be established using explant methods, but the explant method selects cells with a growth advantage. Neonatal and embryonic cells of cardiac origin proliferate readily under appropriate *in vitro* conditions. Although adult cardiac myocytes do not divide in culture, the ability of cardiac myocytes to divide is only repressed and not completely lost (Barnes, 1988). A human fetal cardiac myocyte cell line was developed by transfection with the SV40 large T antigen to stimulate myocardial cell division, and many of the morphologic and functional features of human fetal cardiac myocytes were preserved (Wang et al., 1991).

4.7.7 *Endpoints That Can Be Assessed In Vitro*

Flow cytometry and computerized evaluation of cell images have added to toxicity evaluations of cardiac myocytes. Toxicity can also be evaluated based on the arrhythmogenic potential of chemicals (Aszalos et al., 1984). Ionic homeostasis can be used as an index of disturbances in the structural and functional integrity of the plasma membrane. Use of co-cultures of myocytes and endothelial cells or smooth muscle cells in the progression of the toxic response emphasizes the importance of cell-cell interactions (Saunders and D'Amore, 1992).

4.7.8 *Future Research Needs*

Vasculitis may need to be assessed by *in vitro* methods. It can be present in numerous forms such as lymphocytic vasculitis and leukocytoclastic vasculitis, the latter usually affecting the skin (Gupta et al., 2000). The most common type of vasculitis is Giant cell arteritis (Gonzalez-Gay et al, 2000), which generally involves large and medium-sized blood vessels. Further work will be needed to identify *in vitro* systems to assess this endpoint.

Certain drugs have the potential to alter the QT interval in the heart, producing ventricular arrhythmias and it will be necessary to develop systems to detect this effect. Halofantrine, an antimalarial drug, has been reported to produce such effects, and some drugs have been implicated in the sudden death of patients from ventricular arrhythmias (Champeroux et al., 2000). In a review by Champeroux (2000), different methodologies have been investigated as possible ways of examining this potential -- *in vitro* as well as *in vivo*. These include isolated cardiac tissues, Purkinje fibers, or papillary muscles. Wesche (2000) also used an isolated perfused heart model and isolated ventricular myocytes to determine potential cardiotoxicity associated with antimalarial drugs (Wesche et al., 2000).

A final important effect of acute exposure to xenobiotics is aseptic shock, which is associated with a fall in blood pressure. This is a systemic effect and no method of measuring or modeling this effect *in vitro* could be identified at this time. Further work to elucidate the exact causes of this effect may allow modeling of the change *in vitro*.

To the Breakout Group's knowledge, none of the cardiovascular toxicity models have been validated. After reviewing the literature, the likely candidate *in vitro* systems for an acute cardiotoxicity-testing scheme after chemical exposure could include the following:

- Short-term single-cell suspensions of adult rat myocytes to measure products of oxidation;
- Primary cultures of neonatal myocytes to measure changes in beating rates and plasma membrane potentials;
- Co-culture of smooth muscle cells or endothelial cells with macrophages, for example, to examine rate of wound healing (DNA synthesis);
- An immortalized cell line (e.g., the human fetal cardiac myocyte line) to measure classical cytotoxic endpoints.

It also may be important to include the perfused heart preparation, in spite of its limitations, for a comparison with the other *in vitro* models,

because this system is the most representative of the *in vivo* situation.

4.8 *In Vitro* Methods to Study Hematopoietic Toxicity

Hematopoietic toxicity issues were recently reviewed by Gribaldo. [Progress in the Reduction, Refinement and Replacement of Animal Experiments, ed. M. Balls, A-M. van Zeller & M.E. Halder, pp. 671-677. Elsevier, Amsterdam, The Netherlands, 2000.] Xenobiotics can affect both the production and function of the various circulating cell populations, as well as the circulatory system that supports and helps maintain these cells. Acute effects on blood itself can also include the binding of materials to hemoglobin resulting in a loss of oxygen carrying capacity and cell lysis. Both of these latter endpoints should be easily modeled by *in vitro* systems if exposure conditions can be modeled.

During preclinical drug development it is often important to determine the following:

- Whether a new agent will be clinically toxic to the bone marrow cells;
- Whether the toxicity will be specific to one cell lineage (lymphocytes, neutrophils, megakaryocytes or erythrocytes);
- At what dose or plasma level the drug will be toxic;
- Which model best predicts the clinical situation, and
- When the onset and nadir of cytopenia and recovery will be likely to occur.

Validated *in vitro* tests using human cell systems are particularly important in this area as the prediction of human effects from animal systems are unreliable and necessitate the use of larger safety factors in human studies. *In vitro* colony-forming assays to study the growth and differentiation of various hematopoietic cell populations have been developed and perfected over the last twenty years, but none have yet been validated for use in regulatory toxicology testing. A validation study of the use of colony-forming assays to test for the possible development of neutropenia is being supported by ECVAM. Methods to assess effects on thrombocytopoiesis and erythropoiesis are also available and can be considered for validation.

Associated projects have been also been carried out, such as the optimization of a protocol for detecting apoptosis using FACS analysis with fluorescent antibodies against Annexin V (Vermes et al., 1995). Using this assay, the induction of apoptosis in established stromal cells (SR-4897) (Pessina et al., 1997) and in murine and human leukemia cells (WEHI-3B; HL-60), following exposure to anti-neoplastic agents, has been investigated in relation to the cell cycle. The relationship between these observations and chromosome damage during mitosis is under evaluation. The drug sensitivities of myeloid progenitors from fresh murine bone marrow and from long-term cultures have been investigated by many authors including (Gribaldo et al., 1998a) as well as the role of the microenvironment in the modulation of anti-cancer drug activity (Pessina et al., 1999; Gribaldo et al., 1999).

In the session on hematotoxicity at the *3rd World Congress on Alternatives and Animal Use in the Life Sciences*, results were described for possible new endpoints (Balls et al., 2000). For example, the toxic effects of drugs on the proliferation of erythroblastic progenitors were evaluated using human and murine progenitors from long-term bone marrow cultures. Two kinds of tests were employed: (a) continuous exposure of human cord blood cells (CBC) and murine bone marrow cells (BMC) during the assay, and (b) pretreatment of long-term murine bone marrow cultures (for 24 hours and 96 hours), with subsequent testing of the clonogenic capacity of progenitor cells collected in the absence of the drug. The classes of drugs of interest in the study were: antivirals (3'-azido-3'-deoxythymidine), antidiabetics (chlorpropamide), and heme-analogous compounds (protophorphirin IX/zinc [II]). The results indicate that all these drugs interfere with the normal hematopoietic process, causing a selective toxicity to the erythroid progenitors via different mechanisms, and that human and murine progenitors have similar drug sensitivities. Moreover, the drugs exerted different toxicities based on the time of exposure.

Another aspect of hematotoxicology is in relation to the use of *in vitro* colony assays to support the risk assessment of industrial and food chemicals and pesticides. Some of these chemicals and formulations may interfere with the proliferative activity of the hematopoietic tissue and cause myelosuppression (Gribaldo et al., 1998b). One of the major difficulties in food toxicology is to establish the relationship between the consumption of a food contaminated by a toxin and the occurrence of a particular pathology. Clonogenic assays are a useful tool for establishing this relationship and for elucidating the mechanisms involved.

Three different clonogenic assays, with BFU-E (Burst-forming unit – erythrocytes), CFU-GM (Colony-forming unit granulocyte/macrophage), and CFU-MK (Colony-forming unit – megakaryocytes) cultures, have been used in toxicological investigations to detect or to confirm food-related hematotoxicity (Parent-Massin, 2000). By using these clonogenic assays, it has been possible to determine:

- The origin of neutropenia and hemorrhage induced by the consumption of trichothecene mycotoxin;
- The safety of a new process for manufacturing food additives;
- The mechanism of lead-induced hematotoxicity;
- The myelotoxicity of phycotoxins present in shellfish; and
- The risk to consumers and agricultural workers of hematological problems caused by pesticides (Parent-Massin and Thouvenot, 1995, 1993).

ECVAM is providing financial and organizational support to a new project on the development and prevalidation of *in vitro* assays for the prediction of thrombocytopenia. The continuous maintenance of an adequate supply of circulating platelets is essential for sustaining life. Since neither platelets nor megakaryocytes are capable of regeneration, their production is dependent on a continuous generative process from self-replicating precursors. The CFU-MK is the progenitor cell thought to be immediately responsible for the production of megakaryocytes and is therefore being evaluated for its ability to predict thrombocytopenia.

Drug effects are by far the most common cause of platelet suppression in the bone marrow (Miescher, 1980). In many instances, thrombocytopenia is the first evidence of drug-induced toxicity, and continued administration of the drug produces total aplasia. Cytotoxic agents, such as 5-fluorouracil, vincristine, and cytosine arabinoside, cause perturbation of the bone marrow, with changes within the proliferating compartments, as well as effects on the maturing cell pool. In contrast, the thiazide diuretics, estrogens, and alcohol appear to have specific effects on platelet production. In addition, solvents, including benzene, insecticides (DDT, chlordane, lindane), spot removers, and model airplane glue, have all been associated with marrow-related thrombocytopenia (Amess, 1993).

Following bone marrow transplantation, the restoration of a normal platelet count occurs as a result of a compensatory adjustment in megakaryocytopoiesis (Vainchenker, 1995). For these reasons, appropriate *in vitro* endpoints for megakaryocytopoiesis that correlate well with platelet levels *in vivo* should be identified. A preliminary study carried out in ECVAM's laboratories to optimize an *in vitro* CFU-MK permitted a comparison of the suitability and drug-sensitivities of human BMC and CBC. The percentage of enrichment in $CD34^+/CD38^-$ cells from both populations was measured by using a negative selection system, and their clonogenicity was evaluated. Furthermore, the effects on megakaryocyte colony formation of busulphan, a cytotoxic drug, and the non-cytotoxic drugs, quinidine-sulphate, D-penicillamine, sodium valproate, and indomethacin were investigated by using both the whole cell populations and selected cells from the two sources. The data analyses confirmed the usefulness of the *in vitro* test as a potential tool for screening drug toxicity to megakaryocyte progenitors. The *in vitro* test showed that human CBC can be used as a human target source, was more suitable for this purpose, and provided a means of avoiding ethical problems that exist in some countries connected with the collection of human BMC.

Up until now, primary cells have been more-reliable and more-relevant targets for clonogenic assays than the immortalized cell

lines, but in the future, attempts should be made to establish standardized cell populations for *in vitro* tests, and in particular, for screening purposes. This may help to avoid the technical problems related to the absence of primary cell repositories, and to avoid the problem of inter-individual variability of the donors, in terms of drug sensitivity. A future topic will be the automated scoring of colonies in the clonogenic assays, which will provide the opportunity to refine the performance of the assays in terms of accuracy and repeatability, and to reduce personnel costs.

4.9 *In Vitro* Methods to Study Respiratory System Toxicity

The lungs fulfill the vital function of exchanging oxygen and carbon dioxide and a secondary function of protecting the organism from noxious or irritating inhaled stimuli. As such, the nasal and pulmonary airways represent a crucial organ-system that is likely to debilitate the organism if injured or irritated. The airways are particularly difficult to evaluate in *in vitro* because of their complexity. The following is a discussion of relevant airway cells and target-specific endpoints that should be considered in an *in vitro* battery for target-specific acute toxicity.

4.9.1 *Cell Types*

The tracheal-bronchial epithelial lining consists of stratified epithelium and diverse populations of other cell types including ciliated, secretory (mucous, Clara, serous), and non-secretory cells. The cells lining the airways may be represented by various human cell lines such as CCL-30 (nasal septum) (Poliquin et al., 1985) and BEAS-2B (bronchial-tracheal epithelia/transformed) (Noah et al., 1991; Reddel et al., 1988). More distally, alveolar Type II epithelia (A549) function in conjunction with capillary endothelial cells for O_2:CO_2 exchange in the lower alveolar regions. This cell line can be used to show induction of P450 enzymes such as 1A1, 1B1, and 3A5 (Hukkanen et al., 2000), and to assess mucin production (Rose et al., 2000). The H441 cell line has been used in studies to evaluate toxicant effects on surfactant production *in vitro*. Various scavenger cells (alveolar macrophages) are present to engulf microbiological or foreign debris and destroy it. Several human alveolar macrophage cell lines exist which display the oxidative burst in response to irritants and biological debris (Marom et al., 1984). Neutrophils and eosinophils function as cellular sentinels of inflammation.

4.9.2 *Endpoint Markers*

A variety of endpoint markers valid for pulmonary cytotoxicity and irritation are available. ELISA-based assays can be used to quantitate many of these markers (e.g., cytokine, LDH), thus reducing the technical investment. The most useful markers will relate to the basic mechanisms by which airway epithelia respond to toxic exposure. LDH, a cytoplasmic enzyme released from damaged or lysed cells, is useful as a general marker of cytotoxicity. Mucous glycoprotein stain is a marker for alteration of mucous cells. Other possible endpoints include:

- Ciliary beat frequency (epithelial viability and function);
- Attachment (viability);
- Electrical resistance (to measure the integrity of the epithelial layer);
- Evans blue (to measure endothelial leakage);
- IL-8, IL-6, and TNFa (cytokine endpoints of inflammation).

As *in vitro* systems are developed and evaluated, biochemical markers of damage can be assayed in the lavaged fluid and directly compared to changes in similar markers in *in vitro* systems. Like the kidney, utilization of these comparisons will facilitate the development of predictive *in vitro* systems.

In vitro systems are available that can be used to indicate chemical-induced cell damage/death. The cells of the airways from animals or humans are relatively accessible to brushing, biopsy, and lavage, and therefore lend themselves for harvesting and use as primary cells (Larivee et al., 1990; Werle et al., 1994). Lung slices have been investigated for use in toxicology (Parrish, et al., 1995). The most useful markers are those that relate to the basic mechanisms by which airway epithelia respond to toxic exposure. However, most assays and

cell models determining effects on special functions still need significant basic research before they will be useful as screening systems. The use of *in vitro* systems in respiratory toxicology was a subject of an ECVAM Workshop 18 (Lambre, et al. 1996).

4.10 Conclusions on the Use of *In Vitro* Systems for Assessing Organ-Specific Effects of Acute Exposure

There are significant ongoing advances in both technology and our understanding of biology that will have major effects on our ability to predict whole-animal (or human) toxic effects from non-whole animal model systems. For instance, toxicogenomics and proteonomics provide rapid identification of early changes in cells *in vitro* or from individual animals and humans. However, these systems are very early in development and significant work will be needed to understand how the changes seen relate to whole animal toxicity, and particularly which changes are the direct result of exposure and which are due to secondary effects as the cells and tissues react to the primary injury. Because these systems appear to be very sensitive, it will also be important to determine how the assays can be used in the prediction of dose-response information for toxicology.

In recognition of the possible importance of advances in toxicogenomics to toxicology, the Breakout Group recommends that some effort be put toward preserving samples from animal studies for future evaluation so as to avoid having to repeat these studies at a future time.

It is very important that the proper quality control procedures be built into any *in vitro* test system developed for use in screening such as:

- Stability of the test material;
- Reactions of the test material with plastic in culture dishes and laboratory ware;
- Measurement(s) of test material concentration in the test vehicle;
- Non-specific binding to proteins in the culture medium;
- Reactive compounds;
- Ensuring that the cells reliably express the necessary metabolic systems.

Each individual test system will need to have a complete, standardized protocol developed, evaluated, and validated. All test schemes that are developed will then build on these validated tests. The prediction model for the entire scheme may also need to be evaluated and validated.

4.10.1 *Proposed Scheme for Assessing Acute Toxicity Using Non-Whole Animal Methods*

For the assessment of acute systemic toxicity for the purposes of setting hazard and risk levels for chemicals and products, data on specific organ toxicity are usually not needed. The need is for a system to appropriately classify the hazard of materials that may cause death after acute exposure irrespective of the specific organ damage. For such a system, the routine use of *in vitro* models to evaluate all possible organ effects would be impractical from both a time and money standpoint and evaluation of the effects of xenobiotics on specific organ function is not included in the current assays for acute toxicity. Current acute toxicity assay systems utilize young adult animals, often of only one sex, and only recognize observable effects within 14 days. Currently standard assays do not evaluate effects in different sub-populations or the long-term effects of single acute exposures.

Acute toxicity assays are primarily used to predict the toxicity of materials to humans. For this reason, where species differences are known, the Breakout Group recommends that screening systems be developed that will predict effects in humans.

Breakout Group 3 discussed what additional assay systems would be required, in addition to the basic cytotoxicity assay discussed by Breakout Group 1, in order to replace the current acute oral toxicity assays for regulatory purposes. Breakout Group 3 developed a stepwise approach to address those effects identified in the discussions of the specific organ systems that were highly relevant to the prediction of acute toxicity and would not be elucidated by a simple basal cytotoxicity test. This scheme is shown in Figure 4.1. The

scheme includes a process for determining when additional specific effects need be evaluated, and gives some guidance on how to do so. The scheme includes steps proposed earlier by a expert workshop hosted by ECVAM and by Bjorn Ekwall in his series of papers.

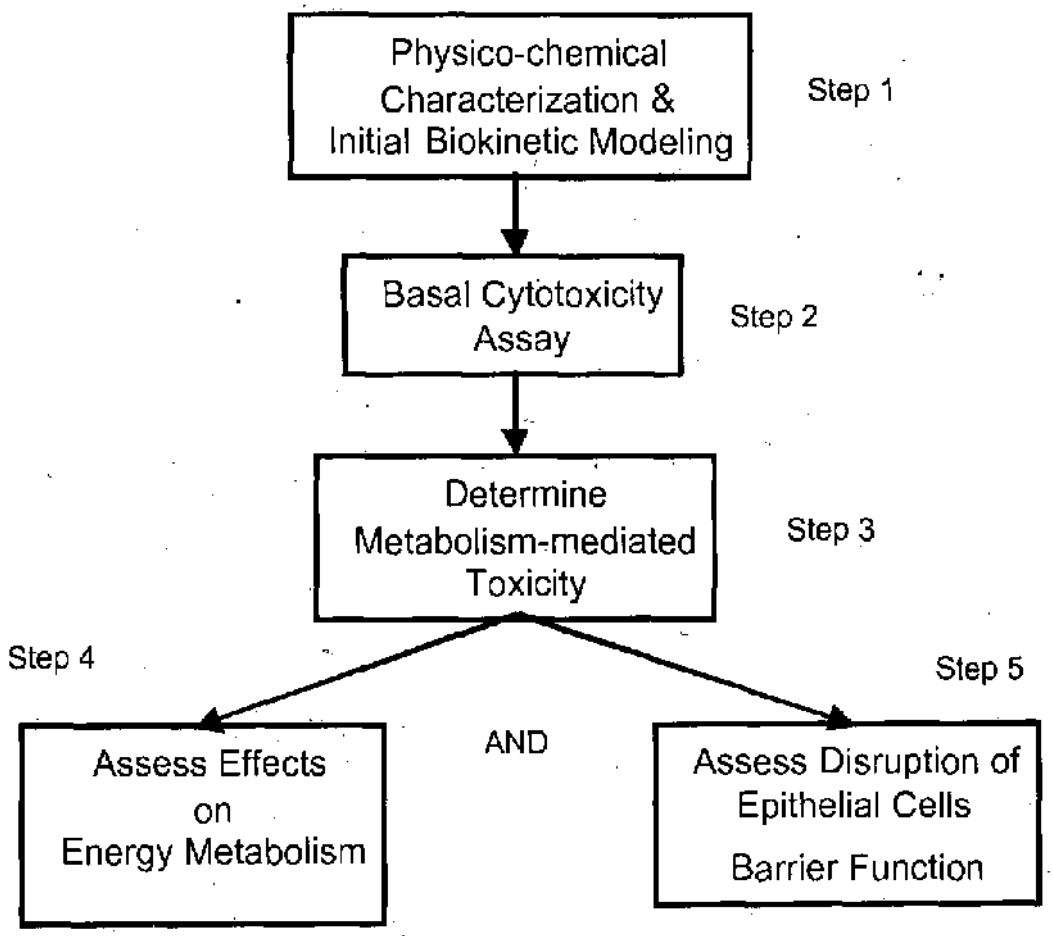

Figure 4.1 Proposed scheme for assessing acute toxicity using non-animal methods

(1) Step 1

- Perform physico-chemical characterization and initial biokinetic modeling (BG2 output). This information will be used for comparison with chemicals with similar structures or properties that have existing toxicity data. The information may also be useful in predicting organ distribution. It may be possible to accurately predict the toxicity effects of some chemicals from this step alone.

(2) Step 2

- Conduct a basal cytotoxicity assay (BG1 output).

(3) Step 3

Determine the potential that metabolism will mediate the effect seen in Step 2:

- Use HEPG2 cells transfected with major metabolizing enzymes – at this time at least four different cell lines, each containing one of the four major metabolic enzymes will be needed. A secondary, and perhaps more relevant, possibility would be to use metabolically competent, primary human hepatocytes, but cell lines would allow a more standardized approach for regulatory purposes.

- Both cytotoxicity and, ideally, some measure of metabolism of the test substance, must be determined, either by detecting a decrease in the parent compound or by some method that directly detects metabolites.

  A. If the material is more cytotoxic in the hepatocyte test system compared to that measured in Step 2, then assume the compound is metabolized to a toxic substance. In this case, the measure of cytotoxicity would use the value obtained from the metabolically active system instead of the value obtained in Step 2.

  B. If the material is less cytotoxic than seen in Step 2, then it is assumed there is detoxification, and in those exposure scenarios where it can be shown the materials will pass through the liver before the rest of the body is exposed (first pass effect) it may be possible to reduce the prediction of toxicity accordingly.

  C. If the cytotoxicity is similar to the basic cytotoxicity measured in Step 2, then the possibility of metabolite formation still must be assessed to assure the metabolite will not have an effect on some other cells that do not have the metabolic capabilities of hepatocytes.

     1. If there is no evidence of metabolism then the value used in Step 2 can be used.

     2. If there is evidence of metabolism, Step 2 must be repeated after exposure to the metabolite(s) either by directly identifying the metabolites and using them in the system, or by some other undetermined systems such as co-cultures or conditioned media; exact protocols will need to be determined. The system that is developed must be able quantitatively asses the effects of the initial toxicant. For instance, according to Breakout Group 2, co-cultures will not enable the biokinetic modelers to predict systemic toxicity in a quantitative manner.

(4) Step 4 (note: Steps 4 and 5 can be done in either order)

- Assess the test substance effect on energy metabolism by using a neuronal cell line that expresses good aerobic energy metabolism function. This system will help determine if the nervous or cardiovascular systems, both of which require high-energy metabolism, are likely target organs.

- The endpoints would be measurement of energy metabolism using a variety of specific probes of energy change, or oxygen consumption, or possibly mitochondrial function. The exact endpoint needs to be determined.

- If there is evidence of metabolism in Step 3, these tests must be done with both the parent compound and the metabolite(s).

(5) Step 5 (note: Steps 4 and 5 can be done in either order)

- Assess the ability of the compound to disrupt epithelial cell barrier function using a transepithelial resistance assay across a membrane, such as MDCK cells. The endpoint used could be dye leakage. This system will help in determining if organs dependent on epithelial barrier function for defense against toxic insult (e.g., brain, kidney) are likely target organs.

- If the compound causes disruption of barrier function at a value lower than the basal cytotoxicity, the endpoint used in determining the effect on the organism might need to be lowered to take this into consideration. [Note: Barrier disruption values will likely be lower than those that cause basal cytotoxicity.]

- If there is evidence of metabolism in Step 3, this test must be done with both the parent compound and the metabolite(s).

Next Steps

Before this system can be evaluated for implementation there is a need to:

- Identify the best cell culture systems to use based on accuracy, reproducibility, cost, and availability;

- Develop complete protocols for all the five steps and validate each assay;

- Develop prediction models for the prediction of relevant human toxic levels as required by regulatory agencies. Prediction of No Observed Adverse Effect Levels (NOAELs) would be addressed at this step;

- Evaluate the scheme with a number of test compounds covering all endpoints and then with enough compounds to develop a prediction model;

- Validate the entire scheme and prediction model.

The Breakout Group recommends that this work be done with the input and cooperation of the regulatory agencies and industries who have a need to use acute toxicity data in order to ensure the final result will meet everyone's needs.

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5.0 CHEMICAL DATA SETS FOR VALIDATION OF *IN VITRO* TOXICITY TESTS

5.1 Introduction

Breakout Group 4 discussed the selection of chemical data sets for validation of *in vitro* toxicity tests. The Breakout Group agreed that it would not develop specific lists of chemicals but would concentrate upon principles for the development of a database of chemicals that could be used in validation of individual tests or prediction models, and strategies for selection of the chemicals to be included in the database. Primary database development will most likely come from existing databases such as those available at the U.S. EPA, FDA, NCI, NTP, DOT, Galileo, Euclid, and others that are to be identified.

In addition to establishing criteria for primary database development, a set of criteria was developed for selecting chemicals for subset development. The chemicals in the subsets will be chosen from the primary database and will be used to validate individual tests or prediction models. The primary assumption in establishing criteria for subset development is that the purpose and proposed use of the test, the endpoint measured, the range of testable chemicals, and the prediction model must be clearly defined before chemical selection begins. Criteria that were considered important in selecting a set of reference chemicals were developed, as well as a set of fields considered relevant for the chemical reference database.

Lastly, the Breakout Group assembled a list of recommended actions that was divided into two parts: one that was database specific and one that was human toxicity specific.

5.2 Objectives

Before beginning a discussion of the primary database development, the Breakout Group defined some common points of reference and some points of agreement that would serve as the basis for discussions during the meeting. These are presented in the next sections.

5.2.1 *Points of Reference*

(1) The main function of the Breakout Group was to develop a set of general principles that would be useful for choosing test chemicals for validation.

(2) The Breakout Group would attempt to identify databases, and other sources that contain the information necessary to choose the test chemicals, and define their uses and limitations.

(3) The Breakout Group agreed that it would not identify specific chemicals or develop lists of chemicals at this time.

5.2.2 *Points of Agreement*

In addition to the three reference points, several items were set out by the Breakout Group to ensure that all members understood the exact aim of the discussion and their charge to the Breakout Group.

(1) It was agreed that the aim of the Breakout Group was to identify chemicals and supporting chemical information that can be used to validate replacement test(s) for acute toxicity tests.

(2) The chemicals used to validate a replacement test should cover the entire range of responses of the LD50 values. They should not be chosen to bracket just the range of classification used in the internationally agreed upon classification scheme(s).

(3) In addition to covering the entire range of responses, the chemicals chosen for use in a validation study should be uniformly distributed across that range, (i.e., there should not be a preponderance of either very toxic or non-toxic chemicals among those used).

(4) Identification of "chemical classes" is problematic. The basis for classification is the most significant issue. There was an unresolved discussion within the Breakout Group as to whether

classification should be done on the basis of chemical structure or mechanism of biological action. There was some discussion also about classifying according to use, such as "pesticide" or "food additive".

(5) The Breakout Group agreed that it is not necessary to be restricted to only one classification scheme. Chemicals could be classified by structure and by biological activity and/or use class. The classification approach would, by necessity, vary according to the type of test and its proposed uses.

(6) There are many public databases from which to draw information. These databases contain chemicals of concern to society. Investigators may not need, therefore, to use the proprietary databases such as the U.S. EPA OPP pesticides database or the FDA drug database to get the information and identify chemicals for use in tests for validation, but it would be helpful if information from those databases could be made available.

(7) There is a need for training sets of chemicals that can be used for method development, and validation sets of chemicals that can be used for confirming the predictive capacity of the tests.

(8) In selecting chemicals for use in validation studies, investigators need to consider the user community(ies) and assure that chemicals are chosen that meet their needs.

(9) The performance parameters of the *in vivo* tests must be clearly defined prior to chemical selection if the results of these tests are to serve as a baseline for judging success.

5.2.3 *Definition of Responsibility*

Breakout Group 4 defined its responsibility as follows:

- To define what chemical data sets are required for validation studies;
- To define the information to be included as part of the data set;
- To identify existing resources;
- To recommend approaches for using existing data sets;
- To recommend approaches for developing new data sets.

The Breakout Group explored the possible use of such databases as the HPV database, the U.S. EPA pesticides database, the NTP chemical database, the FDA database of drugs and food additive chemicals, and the use of QSAR to predict toxicity of chemicals.

5.3 Current Status: Discussions Regarding the Use of the NTP and HPV Databases, and the Use of QSAR

5.3.1 *The NTP Database*

The NTP chemicals were not tested for acute toxicity and therefore no LD50 data were developed. However, many were tested in 90-day studies, and some in 14-day studies, and these have associated target-organ toxicity data, as do the 2-year carcinogenicity studies. This information would be useful in validating *in vitro* tests for target-organ toxicity. The NTP database would be a useful component of any primary database of chemicals for validation.

Both the U.S. EPA pesticides database and the FDA drugs and food additive databases have associated LD50 data of good quality. However, there was some question about the ultimate accessibility of these data because of claims of confidentiality by the sponsors. Ease of access was a concern even where the data are not claimed to be confidential. Access through the Freedom of Information Act (FOIA) was discussed as a possibility, but this is a slow process and members of the Breakout Group expressed the desire that sources of unencumbered data should be used if they were available. Also, this approach may not provide the supporting information deemed necessary by the Breakout Group.

5.3.2 *The HPV Database*

There was a short presentation of the classification of the chemicals that are part of the HPV Program of the U.S. EPA OPPT. Using only

696 pure chemicals on the list and classifying them according to chemical structure, a list of 45 chemical classes with from 4 to 72 chemicals per class was developed. This classification is based solely upon chemical structure and each chemical is assigned to one class only. There is no indication of how many of these chemicals fall into more than one class. There is also no indication of which of these chemicals have LD50 data, the quality of these data where they exist, or the range of responses that is covered. Without this information, it is impossible to tell which of the HPV chemicals would be useful as validation chemicals. In addition, the chemicals on the HPV list are primarily industrial chemicals and their use as validation chemicals might not meet the needs of all user communities.

5.3.3 *QSAR Methods and Structure-Activity Methods for Toxicity*

QSAR methods can be applied to the problem of developing models to predict toxicity endpoints or toxic classes given sufficient quantity and quality of data.

The basis for the prediction of toxicity from chemical structure is that the properties of a chemical are implicit in its molecular structure. Biological activity can be expressed as a function of partition and reactivity. For a chemical to be able to express its toxicity, it must be transported from its site of administration to its site of action and then it must bind to or react with its receptor or target. This process may also involve metabolic transformation(s) of the chemical and its metabolites.

The application of QSAR principles to the prediction of the toxicity of new or untested chemicals has been achieved in a number of different ways and covers a wide range of complexity. The common feature of these approaches is that their starting point is a mechanistic hypothesis linking chemical structure and/or functionality with the toxicological endpoint of interest. A number of such "*in silico*" methodologies have also been applied with varying degrees of success to the evaluation of LD50 values and MTDs, and some are available commercially (e.g., DEREK, MCASE, and TOPKAT).

The prediction of toxicity from chemical structure and physical properties can make a valuable contribution to the reduction of animal usage in the screening out of potentially toxic chemicals at an early stage and in providing data for making positive classifications of toxicity. However, such methods should also be validated, using protocols similar to those described in these pages, so as to assess their potential effectiveness in assessing acute toxicity.

5.4 Identification of Needs

5.4.1 *Selection of Test Chemicals for Validation of In Vitro Tests*

In the context of using *in vitro* tests to replace or reduce animal usage, the performance of an *in vitro* test or an *in silico* test is assessed by its capability of correctly predicting the *in vivo* response. However, it is unreasonable to expect that the *in vitro* test will be able to predict the result of an *in vivo* test with any more accuracy than would a repeat *in vivo* test.

The assessment of any new test would be best accomplished by selecting a series of reference chemicals that cover the full range of responses, from negative, to weak, to intermediate, to strong. Selection of only strongly active chemicals will not provide information on the discriminating ability of a test, or its ability to detect the weakly active chemicals. The absence of chemicals known to be inactive will not allow a determination of the ability of the test to identify chemicals without activity, or of the false positive rate of the test.

5.4.2 *Evaluating the Quality of Data Used to Develop the Chemical Data Set*

A major challenge facing researchers developing either *in vitro* or *in silico* models is the sparse availability of high quality data derived from experiments with animals, or from human monitoring studies and clinical reports. Biological data which do not meet today's stringent requirements of acceptability,

particularly historical data generated prior to the advent of standardized test guidelines, but which are nevertheless of acceptable quality, can be used to validate newly developed test methods.

The Breakout Group discussed the establishment of a primary database from which sets of chemicals could be drawn for use as validation chemicals for specific tests or prediction models. In addition to the need to establish criteria for primary database development, a set of criteria for selecting chemicals for subset development should be developed.

5.5 Conclusions

5.5.1 *Primary Assumption for Data Set Development*

The primary assumption in establishing criteria for data set development is:

- The purpose and proposed use of the test, the endpoint measured, the range of testable chemicals, and the prediction model must be clearly defined before chemical selection begins.

Such information is used as the guide for choosing the most appropriate materials for evaluating whether or not the test method would satisfy its proposed uses.

5.5.2 *Criteria for Data Set Development*

The following criteria were established for data set development.

(1) The chemicals selected must be consistent with the test protocol and its prediction model.
- The chemicals selected must be physically and chemically compatible with the test system.
- The relevant chemical classes must be included.
  - — The definition of chemical class is context-specific.
  - — The developers of the test must specify the parameters that define the class.
  - — The chemicals must be independently chosen.

(2) The toxicity must cover the range of response with uniform distribution.

(3) The number of chemicals used in the subset will depend on the nature of the test and the questions being asked, and should be determined with statistical advice.

5.5.3 *Primary Data Base Development*

Primary database development will most likely come from existing databases such as those available at the EPA, FDA, NCI, NTP, DOT, Galileo, Euclid, and others that are to be identified. As noted above, the more publicly available the database, the easier it will be to access the data. The problem, of course, is quality control of the data that goes into the database. The two most important considerations in assembling the primary set of reference chemicals are: (a) *in vivo* data must be of high quality, cover the range of response, and be uniformly distributed over that range and (b) the chemicals selected must be commercially available and their specifications (including purity) must be available.

The Breakout Group noted that there were some unresolved questions surrounding the issue of quality control. The first concerned protocol and, specifically, route of administration. There was some discussion about whether to accept tests done by all routes of administration or to limit the database to the oral route. It was decided that oral and inhalation routes were acceptable and that the dermal route while important for some purposes, was not of primary concern for most acute toxicity studies. However, the Breakout Group agreed, that if data were available from all routes, such data should be included in the database.

The Breakout Group agreed that, where possible, the data used should be derived from generally recognized test guidelines, such as those from the U.S. EPA, OECD, ICH, etc., because data from these guidelines carry a higher degree of assurance than data from an undefined or novel protocol. An issue that was not resolved was whether or not to require that the data used in the

database be from a study done according to Good Laboratory Practices (GLPs).

5.5.4 *Criteria for Choosing Reference Chemicals: Reference Test Data*

The following criteria were considered of prime importance in selecting a set of reference chemicals.

(1) The reference data for the endpoint predicted are available.

(2) The performance characteristics of the reference test must be defined.
- Variation will be introduced by protocol (including animal strain) differences.
- Different agencies use different protocols.
- The between-laboratory reproducibility of the test must be determined.
- The limitations of the reference test must be known.

(3) The reference test data must be of high quality.

(4) The protocol used must be available for review.

(5) Generally accepted methods (e.g., OECD, EPA, FDA, ICH guidelines) should have been used to generate the data.

(6) Details of the study should be available and ideally should satisfy ICCVAM and ECVAM Submission Guidelines.

(7) Study has sufficient supporting information. Ideally, GLPs should have been followed in study development.

(8) Other important considerations:
- The chemicals should be drawn from a wide range of structural and use classes.
- They should not be highly reactive, corrosive, or controlled substances.

5.5.5 *Database Fields*

The Breakout Group defined some of the information fields it considered relevant for the chemical reference database. These fields should include information about the identity, purities, and properties of the chemicals, and detailed reference test data.

(1) Chemical Information
- Name and Chemical Abstract Service (CAS) Number;
- Structure (coded, e.g., using Simplified Molecular Input Line Entry Specification [SMILES] nomenclature);
- Physical chemical characteristics (e.g., K_{ow}, pKa, water solubility, molecular weight., physical state);
- Purity;
- Chemical class (e.g., The International Union of Pure and Applied Chemistry [IUPAC] and use).

(2) Reference Test Data
- Specifications of chemical used in reference test;
- Information concerning the protocol used to generate the data;
- Endpoint value (e.g., LD50) and variance term (e.g., confidence interval), if available;
- Species, strain, sex;
- Route of exposure; duration of exposure;
- Information needed by Breakout Groups 2 and 3 should also be included.

5.6 Recommended Actions

5.6.1 *Rodent Toxicity Database*

(1) A study should be undertaken of existing databases to determine:
- The variation in the rodent LD50 introduced by differences in protocols;
- The within- and between-laboratory reproducibility of the rodent LD50 test and other acute toxicity tests that will be used as reference tests.

(2) An expert committee should be convened that will assemble a reference set of test chemicals from existing databases according to the criteria specified.

5.6.2 *Human Toxicity Database*

(1) There is a need to build upon the foundations of the MEIC and MEMO exercises.

(2) An expert panel should review the MEIC/MEMO approach for measuring acute toxicity parameters in humans.

(3) A consensus standard approach for measuring acute toxicity parameters is necessary.

(4) Existing sources of information need to be carefully searched in order to assure all relevant human data are obtained.

(5) A mechanism prospectively should be established to: (a) gather human toxicity data from hospital/Poison Control Center (PCC) sources; (b) retrieve existing human toxicity data; (c) collect and organize human toxicity data as accidents occur. Biomonitoring data should also be collected. Such information could define sub- or non-toxic levels, and be used to see if they overlap with the range of reported toxic levels.

6.0 GLOSSARY

Note: These definitions are based on (1) definitions used by one or more Breakout Groups at the *In vitro* Workshop or (2) a commonly used interpretation or definition.

Acute Toxic Class Method (ATC): An *in vivo* approach to assessing acute toxicity that tests animals in a step-wise fashion. Based on mortality and/or morbidity (or absence thereof), testing continues at the next highest (or lowest) fixed dose until an adequate assessment can be made. The method usually entails testing at two to four step-wise doses.

Acute Toxicity: The adverse effects occurring within a relatively short time after administration of a single dose of a substance or multiple doses within a 24-hour period. BG3 added: *"toxicity occurring within 14 days of a single exposure or multiple exposures within 24 hours".*

Acute Systemic Toxicity: Acute effects that require absorption and distribution of the toxic agent from its entry point to a distant site at which adverse effects are produced vs. acute local toxicity.

ADAPT: (Automated Data Analysis by Pattern recognition Techniques); commercially available QSAR system for the evaluation of LD50s and MTDs; available from the laboratory of Peter Jurs, Penn State University.

ADME: biokinetic information on Absorption, Distribution, Metabolism, and Excretion.

Biotransformation: the series of chemical reactions of a compound in a biological system occurring within the body usually due to enzymatic metabolic reactions.

CASE: (Computer Automated Structure Evaluation); commercially available QSAR software

Cytotoxicity: The adverse effects of interference with structures and/or processes essential for cell survival, proliferation, and/or function. These effects may involve the integrity of membranes and the cytoskeleton, metabolism, the synthesis and degradation or release of cellular constituents or products, ion regulation, and cell division.

Basal cytotoxicity: Involves one or more of the above mentioned structures or processes that would be expected to be intrinsic to all cell types. Sometimes called general cytotoxicity.

Selective cytotoxicity: Occurs when some types of differentiated cells are more sensitive to the effects of a particular toxicant than others, potentially as a result of, for example, biotransformation, binding to specific receptors, or uptake by a cell type specific mechanism.

Cell specific function cytotoxicity: Occurs when the toxicant affects structures or processes that may not be critical for the affected cells themselves, but which are critical for the organism as a whole. For example, such toxicity can involve effects on cell to cell communication, via the synthesis, release, binding and degradation of cytokines, hormones and transmitters

DEREK: (Deduction of Risk from Existing Knowledge); commercially available knowledge-based QSAR expert system.

EUCLID: (Electronically Useful Chemistry Laboratory Instructional Database); database of industrial chemicals tested in Europe maintained by the European Union.

Fixed Dose Procedure (FDP): An *in vivo* approach to assessing acute toxicity that avoids using death of animals as an endpoint, but instead uses the observation of clear signs of toxicity at one of a series of fixed dose levels. Instead of providing an LD50 value, this method estimates a range in which the LD50 of the test substance is estimated to occur.

Galileo: A publicly available database of chemicals that have been tested for toxicity (from alternative studies, mostly related to cosmetics testing).

Globally Harmonized System (GHS): Co-ordinating Group for the Harmonization of Chemical Classification Systems (CG/HCCS) was

established to promote and oversee the work to develop a GHS. The group would integrate the harmonized classification scheme with a harmonized hazard communication system to give an overall Globally Harmonized Classification and labeling System (GHS): OECD-sponsored.

IC50: (Inhibitory Concentration 50); the concentration of a material estimated to inhibit the biological endpoint of interest (e.g., cell growth, ATP levels) by 50%.

LD50: (Median Lethal Dose); a statistically derived single dose of a substance that can be expected to cause death in 50% of animals. This value is expressed in terms of the weight of the test substance per unit weight of the test animal.

LD50 Test, Conventional: An *in vivo* approach to assessing acute toxicity that tests several dose levels using groups of animals. Doses selected are often determined from a range-finding study. Observations of mortality and morbidity, as well as effects, are made for each dose group, and the LD50 is derived based on those observations.

MCASE: (Model-based Computer Automated Structure Evaluation); commercially available QSAR system for the evaluation of LD50s and MTDs available from Multicase, Inc.

Moribund: A clinical condition of a test animal that is indicative of impending death. Animals in the moribund state are humanely killed and are considered for acute toxicity testing purposes in the same way as animals that died.

MEIC: Multicenter Evaluation of In Vitro Cytotoxicity. Established by the Scandinavian Society for Cell Toxicology in 1989 to investigate the relevance of *in vitro* test results for predicting the acute toxic action of chemicals in humans directly rather than in rodents.

MEIC approach: The MEIC team collected case reports from human poisonings with the 50 reference chemicals to provide LC data with known times between ingestion and sampling/death. Constructed time-related LC curves for comparison with the IC50 values for different incubation times *in vitro* (*see*, 50 MEIC Monographs [MEMO]). Analyses of test results were based on *in vitro* cytotoxicity data presented as IC50 values. The predictability of *in vivo* acute toxicity from the *in vitro* IC50 data was assessed against human lethal blood concentrations compiled from three different data sets: clinically measured acute lethal serum concentrations, acute lethal blood concentrations measured post-mortem, and peak lethal concentrations derived from approximate LC50 curves over time. The analysis showed that *in vitro* assays that were among the most predictive generally used human cell lines. Human-derived cells appeared to be the most predictive for human acute toxicity. The most predictive and cost-effective test battery consisted of four endpoints/two exposure times (protein content/24 hours; ATP content/24 hours; inhibition of elongation of cells/24 hours; pH change/7 days) in three human cell line tests. The test battery was found to be highly predictive of the peak human lethal blood concentrations of all 50 chemicals when incorporated into an algorithm developed by the team.

Mortality: Death of the test animals presumably due to the toxicity of the test material.

Predictive range: Range for various chemical properties over which the *in vitro* assay might be expected to provide reasonable LD50 estimates.

Quantitative Structure Activity Relationships (QSAR): The measurable biological activity of a series of similar compounds based on one or more physicochemical or structural properties of the compounds.

Registry of Cytotoxicity (RC): ZEBET database of acute oral LD50 data from rats and mice (taken from the NIOSH Registry of Toxic Effects of Chemical Substances [RTECS]) and IC50*x* values of chemicals and drugs from *in vitro* cytotoxicity assays. Currently contains data on 347 chemicals.

TOPKAT: (The Open Practical Knowledge Acquisition Toolkit); commercially available QSAR software.

Toxicokinetics: kinetics or biokinetics (BG2 definition).

Up-and-Down Procedure (UDP): An *in vivo* approach to assessing acute toxicity. Animals are dosed, one at a time, at 48-hour intervals. The first animal receives a dose at the investigator's best estimate of the LD50, and subsequent animals are given a higher or lower dose depending on the survival of the previous animal. After reaching the point where an increasing (or decreasing) dose pattern is reversed by giving a small (or higher dose), four additional animals are dosed following the same method, and the LD_{50} is calculated using the method of maximum likelihood.

ZEBET approach: Strategy to reduce the number of animals required for acute oral toxicity testing; Strategy involves using *in vitro* cytotoxicity data to determine the starting dose for *in vivo* testing. Researchers report the findings of an initial study conducted to assess the feasibility of applying the standard regression between mean IC50 values (i.e., IC50x, the mean concentration estimated to affect the endpoint in question by 50%) and acute oral LD50 data included in the Register of Cytotoxicity (RC) to estimate the LD50 value which can then be used to determine the *in vivo* starting dose.

ZEBET: Zentralstelle zur Erfassung und Bewertungvon Ersatz- und Ergänzungsmethoden zum Tierversuch (Centre for Documentation and Evaluation of Alternative Methods to Animal Experiments)

7.0 REGISTRY OF CYTOTOXICITY (RC) DATA (ZEBET)

7.1 The ZEBET Database

ZEBET was established in Germany in 1989 at the Federal Institute for Consumer Health Protection and Veterinary Medicine (BgVV; http://www.bgvv.de). The ZEBET database contains evaluated information from the field of biomedicine and related fields on alternative methods that address the 3Rs concept of research that involves animals: refinement of animal use in experimentation, reduction of animal use, and replacement of animals. The database information was obtained from approximately 800 different documents (e.g., books, journals, monographs, etc.). The RC is part of the database and provides *in vitro* IC50 values as well as acute oral toxicity data (LD50) for rats and mice for 347 chemicals. The LD50 values come from the RTECS database at NIOSH. The ZEBET database also includes data for the 50 chemicals from the MEIC database. The German Institute for Medical Documentation and Information (DIMDI) provides access to the ZEBET database (http://www.dimdi.de).

7.1.1 *Tables*

Table 7.1: IC50 values in ascending order (all RC chemicals)
Table 7.2: Rat LD50 oral values in descending order (all RC chemicals)
Table 7.3: Alphabetical order (all RC chemicals)
Table 7.4: Rat LD50 oral values in descending order (MEIC chemicals)

The acute oral toxicity values are provided in mg/kg and mmol/l for rats and mice. Regression calculation values are in the last column of the data sheets. Rat LD50 values were used for the calculations if they were available; if not, then mouse LD50 values were used.

7.1.2 *Figures*

Regression calculations between cytotoxicity and acute oral toxicity are illustrated in the figures following the data.

Figure 7.1: Regression between RC values (IC50x) and acute oral LD50 values (MEIC chemicals)
Figure 7.2: Regression between human cell lines (IC50m) and acute oral LD50 values (MEIC chemicals)

7.1.3 *German Organizational Names*

ZEBET: Zentralstelle zur Erfassung und Bewertungvon Ersatz- und Ergänzungsmethoden zum Tierversuch
(German Centre for the Documentation and Validation of Alternative Methods [at BgVV])

DIMDI: Deutsches Institut für Medizinische Dokumentation und Information
(The German Institute for Medical Documentation and Information)

BgVV: Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin
(Federal Institute for Health Protection of Consumers and Veterinary Medicine)

Section 7.2
Table 7.1
Chemical Data from the Registry of Cytotoxicity Database (Sorted by IC50x mmol/l)

RC #	MEIC #	Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE		MW	Rodent LD50 (mmol/kg)
				mg/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg		for Regression
1		Trenimon	68-76-8	0.00	0.0000033	NA	NA	NA	NA	231.28	
2		Actinomycin D	50-76-0	0.01	0.0000081	7.2	0.0057	12.6	0.01	1255.6	0.0057
3		Aminopterin	54-62-6	0.01	0.000012	NA	NA	3.0	0.0068	440.47	0.0068
4		Vincristine sulfate	2068-78-2	0.01	0.000015	NA	NA	NA	NA	923.14	
5	K-	Strophantin		0.03	0.000044	NA	NA	NA	NA	710.9	
132		Triphenyltin hydroxide	76-87-9	0.02	0.000049	44.0	0.12	245.9	0.67	367.03	0.12
6		Colchicine	64-86-8	0.02	0.000054	NA	NA	6.0	0.015	399.48	0.015
7		Ouabain	630-60-4	0.04	0.000072	NA	NA	NA	NA	584.73	
133		Cytochalasin D	22144-77-0	0.05	0.000092	NA	NA	36.0	0.071	507.68	0.071
8		Digitoxin	71-63-6	0.08	0.00011	55.8	0.073	NA	NA	765.05	0.073
134		Rotenone	83-79-4	0.05	0.00013	130.2	0.33	351.1	0.89	394.45	0.33
9		Amethopterin	59-05-2	0.06	0.00014	136.4	0.3	145.4	0.32	454.5	0.3
10		Emetine	483-18-1	0.08	0.00016	67.3	0.14	NA	NA	480.71	0.14
135	2,3,7,8-	Tetrachloro-dibenzo-p-dioxin	1746-01-6	0.06	0.0002	NA	NA	0.1	0.00035	321.96	0.00035
11		Doxorubicin * HCl	25316-40-9	0.19	0.00033	NA	NA	696.0	1.2	580.03	1.2
12		Puromycin	53-79-2	0.16	0.00033	NA	NA	674.4	1.43	471.58	1.43
136		Diethyldithiocarbamate sodium* 3H20	20624-25-3	0.09	0.00039	1500.7	6.66	1500.7	6.66	225.33	6.66
137		Triethyltin chloride	994-31-0	0.11	0.00046	5.1	0.021	NA	NA	241.35	0.021
138		Tributyltin chloride	1461-22-9	0.18	0.00054	120.4	0.37	NA	NA	325.53	0.37
139		Retinol	68-26-8	0.15	0.00054	1999.8	6.98	4011.0	14	286.5	6.98
140	6-	Thioguanine	154-42-7	0.10	0.00057	NA	NA	160.5	0.96	167.21	0.96
13		Cycloheximide	66-81-9	0.17	0.00059	2.0	0.0071	132.3	0.47	281.39	0.0071
141		Cytosine arabinoside	147-94-4	0.17	0.00068	NA	NA	3137.9	12.9	243.25	12.9
142		Methylmercury chloride	115-09-3	0.18	0.00071	NA	NA	57.7	0.23	251.08	0.23
143		Triethylene melamine	51-18-3	0.16	0.00078	1.0	0.005	14.9	0.073	204.27	0.005
14		Mitomycin C	50-07-7	0.28	0.00084	14.0	0.042	17.1	0.051	334.37	0.042
144		Sodium bichromate VI	10588-01 9	0.24	0.00093	49.8	0.19	NA	NA	261.98	0.19
15	8-	Azaguanine	134-58-7	0.20	0.0013	NA	NA	1500.1	9.86	152.14	9.86
145		Potassium chromate VI	7789-00-6	0.29	0.0015	NA	NA	180.6	0.93	194.2	0.93
16		Azaserine	115-02-6	0.35	0.002	169.7	0.98	150.6	0.87	173.15	0.98
146		Potassium bichromate VI		0.59	0.002	NA	NA	191.2	0.65	294.2	0.65
147		Mitoxantrone	65271-80-9	1.07	0.0024	586.8	1.32	NA	NA	444.54	1.32
148		Nitrogen mustard * HCl	55-86-7	0.50	0.0026	10.0	0.052	19.3	0.1	192.53	0.052
17	5-	Fluorouracil	51-21-8	0.34	0.0026	230.3	1.77	114.5	0.88	130.09	1.77
149		Chromium VI trioxide	1333-82-0	0.27	0.0027	80.0	0.8	127.0	1.27	100	0.8
150		Cis-platinum	15663-27-1	0.84	0.0028	25.8	0.086	33.0	0.11	300.07	0.086
151		Hexachlorocyclopentadiene	77-47-4	0.85	0.0031	111.8	0.41	NA	NA	272.75	0.41
152	8-	Hydroxyquinoline	148-24-3	0.48	0.0033	1200.6	8.27	NA	NA	145.17	8.27
18		Captan	133-06-2	1.17	0.0039	10009.6	33.3	7003.7	23.3	300.59	33.3

Section 7.2
Table 7.1
Chemical Data from the Registry of Cytotoxicity Database (Sorted by IC50x mmol/l)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
153	26		Arsenic III trioxide	1327-53-3	0.83	0.0042	19.8	0.1	45.5	0.23	197.84	0.1
154			Maneb	12427-38-2	1.12	0.0042	4500.6	16.9	3994.7	15	266.31	16.9
19			Cytochalasin B	14930-96-2	2.40	0.005	NA	NA	NA	NA	479.67	
155			Benzalkonium chloride	8001-54-5	1.90	0.0052	401.5	1.1	339.5	0.93	365	1.1
156			Stearyltrimethylammoniumchloride	112-03-8	2.09	0.006	NA	NA	536.1	1.54	348.13	1.54
20			Cadmium II chloride	10108-64-2	1.17	0.0064	88.0	0.48	174.1	0.95	183.3	0.48
157	38		Hexachlorophene	70-30-4	3.21	0.0079	61.0	0.15	65.1	0.16	406.89	0.15
21		6-	Mercaptopurine	50-44-2	1.22	0.008	NA	NA	280.0	1.84	152.19	1.84
158			Dichlorophene	97-23-4	2.23	0.0083	2691.3	10	1001.2	3.72	269.13	10
22	6		Digoxin	20830-75-5	6.64	0.0085	NA	NA	18.0	0.023	781.05	0.023
159			Hexadecyltrimethylammoniumbromide	57-09-0	3.24	0.0089	408.3	1.12	NA	NA	364.53	1.12
23			Daraprim	58-14-0	2.21	0.0089	NA	NA	126.9	0.51	248.74	0.51
24			Ethylenediamine-tetraacetic acid	60-00-4	2.92	0.01	NA	NA	NA	NA	292.28	
25			Thio-TEPA	52-24-4	2.08	0.011	NA	NA	37.8	0.2	189.24	0.2
160		N-	Methyl-N'-nitro-N-nitroso- guanidine	70-25-7	1.77	0.012	89.7	0.61	NA	NA	147.12	0.61
26			Kelthane	115-32-2	4.45	0.012	574.2	1.55	418.6	1.13	370.48	1.55
161			Silver I nitrate	7761-88-8	2.21	0.013	NA	NA	49.3	0.29	169.88	0.29
27			Chlorpromazine	50-53-3	4.46	0.014	140.3	0.44	261.5	0.82	318.89	0.44
28			Aldosterone	52-39-1	5.05	0.014	NA	NA	NA	NA	360.44	
29	28		Mercury II chloride	7487-94-7	4.07	0.015	1.0	0.0037	10.0	0.037	271.49	0.0037
162			Chlorhexidine	55-56-1	7.58	0.015	9200.5	18.2	9857.6	19.5	505.52	18.2
30			Sodium arsenate, dibasic	7778-43-0	2.79	0.015	NA	NA	NA	NA	185.91	
31	41		Chloroquine diphosphate	50-63-5	8.77	0.017	969.9	1.88	500.4	0.97	515.92	1.88
164			Oxatomide	60607-34-3	8.11	0.019	1412.1	3.31	9598.7	22.5	426.61	3.31
163			Cetyltrimethylammonium chloride	112-02-7	7.61	0.021	474.4	1.31	NA	NA	362.16	1.31
165			Isoproterenol * HCl	51-30-9	5.45	0.022	2219.8	8.96	NA	NA	247.75	8.96
32			Hydrocortisone	50-23-7	7.98	0.022	NA	NA	NA	NA	362.51	
166			Triisooctylamine	2757-28-0	8.14	0.023	1620.2	4.58	NA	NA	353.76	4.58
167		p,p'	DDD	72-54-8	7.68	0.024	112.0	0.35	NA	NA	320.04	0.35
33		p-	Chloromercuribenzoic acid	59-85-8	8.57	0.024	NA	NA	25.0	0.07	357.16	0.07
34			Diethylstilbestrol	56-53-1	6.71	0.025	NA	NA	NA	NA	268.38	
168			Dicoumarol	66-76-2	9.08	0.027	709.6	2.11	232.1	0.69	336.31	2.11
169			Epinephrine bitartrate	51-42-3	9.33	0.028	NA	NA	4.0	0.012	333.33	0.012
35			Flufenamic acid	530-78-9	8.16	0.029	272.8	0.97	714.4	2.54	281.25	0.97
170	29		Thioridazine * HCl	130-61-0	11.81	0.029	NA	NA	358.2	0.88	407.07	0.88
36			Progesterone	57-83-0	9.44	0.03	NA	NA	NA	NA	314.51	
171			Fumagillin	297-95-0	14.22	0.031	NA	NA	1999.5	4.36	458.6	4.36
37			Aflatoxin B1	1162-65-8	10.62	0.034	5.0	0.016	9.1	0.029	312.29	0.016
172			Nabam	142-59-6	8.97	0.035	394.8	1.54	579.3	2.26	256.34	1.54

Section 7.2
Table 7.1
Chemical Data from the Registry of Cytotoxicity Database (Sorted by IC50x mmol/l)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
173	39		Pentachlorophenol	87-86-5	9.59	0.036	50.6	0.19	NA	NA	266.32	0.19
174			Ambazone	539-21-9	9.02	0.038	749.9	3.16	999.1	4.21	237.32	3.16
175			Norepinephrine	51-41-2	6.60	0.039	NA	NA	20.3	0.12	169.2	0.12
46			Lead II chloride	7758-95-4	11.96	0.043	NA	NA	NA	NA	278.09	
176			Papaverine	58-74-2	15.27	0.045	325.8	0.96	230.8	0.68	339.42	0.96
177			Busulphan	55-98-1	11.33	0.046	1.9	0.0076	199.5	0.81	246.32	0.0076
178			Salicylanilide	87-17-2	9.81	0.046	NA	NA	2409.7	11.3	213.25	11.3
179			Acrolein	107-02-8	2.64	0.047	46.0	0.82	39.8	0.71	56.07	0.82
180		p-	Phenylenediamine	106-50-3	5.41	0.05	80.0	0.74	NA	NA	108.16	0.74
38			Imipramine * HCl	113-52-0	17.11	0.054	304.2	0.96	374.0	1.18	316.91	0.96
181	30		Thallium I sulfate	7446-18-6	27.26	0.054	NA	NA	28.8	0.057	504.8	0.057
39		2,4-	Dichlorophenol	120-83-2	8.97	0.055	580.3	3.56	1600.7	9.82	163	3.56
182			Triton X-100	9002-93-1	35.59	0.055	1798.7	2.78	NA	NA	647	2.78
183	5		Amitriptyline	50-48-6	15.54	0.056	319.1	1.15	147.0	0.53	277.44	1.15
184			Butylated hydroxytoluene	128-37-0	12.34	0.056	890.4	4.04	1040.2	4.72	220.39	4.04
185			Heptachlor	76-44-8	22.02	0.059	41.1	0.11	67.2	0.18	373.3	0.11
186			Zineb	12122-67-7	16.27	0.059	5211.3	18.9	7610.1	27.6	275.73	18.9
40			Chlordan	57-74-9	24.59	0.06	458.9	1.12	NA	NA	409.76	1.12
41			Chloroquine sulfate	132-73-0	25.08	0.06	1086.8	2.6	NA	NA	418	2.6
42		p-	Aminophenol	23-30-8	6.77	0.062	1658.9	15.2	NA	NA	109.14	15.2
187		4-	Hexylresorcinol	136-77-6	12.44	0.064	549.9	2.83	NA	NA	194.3	2.83
43			Aldrin	309-00-2	24.45	0.067	40.1	0.11	43.8	0.12	364.9	0.11
44			Hydroxyzine * HCl	1244-76-4	27.56	0.067	950.4	2.31	NA	NA	411.41	2.31
188		t-	Butyl hydroquinone	1948-33-0	11.47	0.069	799.6	4.81	1000.8	6.02	166.24	4.81
189			Antimycin	11118-72-2	17.52	0.07	NA	NA	112.6	0.45	250.27	0.45
45			Quinine * HCl	130-89-2	27.07	0.075	620.8	1.72	1158.6	3.21	360.92	1.72
190			Chlorambucil	305-03-3	23.12	0.076	76.1	0.25	100.4	0.33	304.24	0.25
191			Dimenhydrinate	523-87-5	35.72	0.076	1320.8	2.81	202.1	0.43	470.02	2.81
192		1,3-	Bis(2-chloroethyl)- 1-nitrosourea	154-93-8	16.70	0.078	19.9	0.093	19.1	0.089	214.07	0.093
193		5-	Azacytidine	320-67-2	19.29	0.079	NA	NA	571.5	2.34	244.24	2.34
47			Naftipramide	1505-95-9	25.07	0.084	1029.7	3.45	1086.4	3.64	298.47	3.45
48			Mefenamic acid	61-68-7	20.99	0.087	789.1	3.27	629.8	2.61	241.31	3.27
49			Parathion	56-38-2	27.09	0.093	2.0	0.0069	6.1	0.021	291.28	0.0069
194		p-	Toluylendiamine	95-70-5	11.49	0.094	101.4	0.83	NA	NA	122.19	0.83
50			Trypan blue	72-57-1	91.66	0.095	6204.2	6.43	NA	NA	964.88	6.43
195		p,p'	DDA	83-05-6	27.83	0.099	NA	NA	590.4	2.1	281.14	2.1
196	40		Verapamil HCl	152-11-4	49.11	0.1	108.0	0.22	162.1	0.33	491.13	0.22
197		p,p'	DDE	72-55-9	31.80	0.1	880.9	2.77	NA	NA	318.02	2.77
51			Disulfoton	298-04-4	30.19	0.11	2.0	0.0073	5.5	0.02	274.42	0.0073

Section 7.2
Table 7.1
Chemical Data from the Registry of Cytotoxicity Database (Sorted by IC50x mmol/l)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
198			Ioxynil	1689-83-4	40.80	0.11	111.3	0.3	NA	NA	370.91	0.3
199			Cupric chloride	7447-39-4	14.79	0.11	139.8	1.04	189.6	1.41	134.44	1.04
200			Dimethylaminoethyl methacrylate (polymer)	2867-47-2	17.30	0.11	1745.4	11.1	NA	NA	157.24	11.1
52			all-trans-Retinoic acid	302-79-4	33.05	0.11	2001.2	6.66	NA	NA	300.48	6.66
53	43		Quinidine sulfate	50-54-4	50.70	0.12	456.3	1.08	595.8	1.41	422.54	1.08
202			Formaldehyde	50-00-0	3.60	0.12	798.8	26.6	NA	NA	30.03	26.6
54	23		Propranolol * HCl	318-98-9	35.50	0.12	NA	NA	470.4	1.59	295.84	1.59
201		13-cis-	Retinoic acid	4759-48-2	36.06	0.12	NA	NA	3395.4	11.3	300.48	11.3
55			Zinc II chloride	7646-85-7	17.72	0.13	350.2	2.57	350.2	2.57	136.27	2.57
56			Manganese IIchloride *4 H2O	13446-34-9	25.73	0.13	1484.4	7.5	NA	NA	197.92	7.5
57		L-	Dopa	59-92-7	25.64	0.13	1780.8	9.03	2366.5	12	197.21	9.03
204			Azathioprine	446-86-6	38.82	0.14	535.2	1.93	1389.2	5.01	277.29	1.93
58			Dihydralazine sulfate	7327-87-9	40.36	0.14	818.8	2.84	400.8	1.39	288.32	2.84
59			Tetracycline * HCl	64-75-5	67.33	0.14	6444.6	13.4	NA	NA	480.94	13.4
203			Thallium I acetate	563-68-8	36.88	0.14	NA	NA	34.2	0.13	263.42	0.13
205			Versalide	88-29-9	38.77	0.15	315.3	1.22	NA	NA	258.44	1.22
60			Indomethacin	53-86-1	57.25	0.16	12.2	0.034	19.0	0.053	357.81	0.034
62			Cobalt II chloride	7646-79-9	20.77	0.16	80.5	0.62	80.5	0.62	129.83	0.62
61		p,p'	DDT	50-29-3	56.72	0.16	113.4	0.32	134.7	0.38	354.48	0.32
206			Diquat dibromide	85-00-7	55.05	0.16	230.5	0.67	234.0	0.68	344.08	0.67
63	4		Diazepam	439-14-5	45.56	0.16	709.1	2.49	535.3	1.88	284.76	2.49
207			Dieldrin	60-57-1	68.56	0.18	45.7	0.12	38.1	0.1	380.9	0.12
64			Bendiocarb	22781-23-3	40.19	0.18	178.6	0.8	NA	NA	223.25	0.8
208			Undecylenic acid	112-38-9	33.18	0.18	2506.6	13.6	8496.7	46.1	184.31	13.6
209			Propylparaben	94-13-3	32.44	0.18	NA	NA	6325.7	35.1	180.22	35.1
65			Oxyphenbutazone	129-20-4	61.64	0.19	999.2	3.08	480.1	1.48	324.41	3.08
66			Cortisone	53-06-5	68.49	0.19	NA	NA	NA	NA	360.49	
210		p-	Nitrophenol	100-02-7	27.82	0.2	350.6	2.52	467.4	3.36	139.12	2.52
67	15		Malathion	121-75-5	66.08	0.2	885.4	2.68	776.4	2.35	330.38	2.68
211			Catechol	120-80-9	22.02	0.2	3887.2	35.3	259.9	2.36	110.12	35.3
68		2,4-	Dinitrophenol	51-28-5	38.67	0.21	29.5	0.16	44.2	0.24	184.12	0.16
69			Secobarbital sodium	309-43-3	54.66	0.21	124.9	0.48	NA	NA	260.3	0.48
212		p-	Cresol	106-44-5	23.79	0.22	206.6	1.91	343.9	3.18	108.15	1.91
70	49		Atropine sulfate	55-48-1	148.92	0.22	622.7	0.92	764.9	1.13	676.9	0.92
213			Ammonium persulfate	7727-54-0	52.49	0.23	819.3	3.59	NA	NA	228.22	3.59
214			Thymol	89-83-8	34.56	0.23	979.6	6.52	1802.9	12	150.24	6.52
71			Diphenhydramine * HCl	147-24-0	70.04	0.24	855.1	2.93	113.8	0.39	291.85	2.93
72			Butylated hydoxyanisole	8003-24-5	43.26	0.24	2199.3	12.2	2001.0	11.1	180.27	12.2
215			Chlorotetracycline	57-62-5	114.94	0.24	NA	NA	2500.0	5.22	478.92	5.22

Section 7.2
Table 7.1
Chemical Data from the Registry of Cytotoxicity Database (Sorted by IC50x mmol/l)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE		Rodent LD50 (mmol/kg)	
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
216			Refortan		78.28	0.25	3162.3	10.1	NA	NA	313.1	10.1
73			Carbaryl	63-25-2	52.32	0.26	249.5	1.24	438.7	2.18	201.24	1.24
74			Nickel II chloride	7718-54-9	34.99	0.27	105.0	0.81	NA	NA	129.61	0.81
75			Trichlorfon	52-68-6	69.51	0.27	450.5	1.75	298.6	1.16	257.44	1.75
76			Sodium dodecyl sulfate	151-21-3	78.15	0.27	1288.0	4.45	NA	NA	289.43	4.45
77			Cinchophen	132-60-5	67.31	0.27	NA	NA	NA	NA	249.28	
217			Amrinone	60719-84-8	52.42	0.28	101.1	0.54	288.3	1.54	187.22	0.54
218		o-	Phenylenediamine	95-54-5	33.53	0.31	1069.7	9.89	NA	NA	108.16	9.89
78		6-	Methylcoumarin	92-48-8	49.66	0.31	1681.9	10.5	NA	NA	160.18	10.5
79			Phenylbutazone	50-33-9	98.69	0.32	376.3	1.22	441.0	1.43	308.41	1.22
80		2-	Thiouracil	141-90-2	41.01	0.32	999.6	7.8	NA	NA	128.16	7.8
219			Hydralazine	86-54-4	52.87	0.33	89.7	0.56	121.8	0.76	160.2	0.56
81	27		Cupric sulfate * 5 H2O	7758-99-8	82.40	0.33	299.6	1.2	NA	NA	249.7	1.2
238			Imidazolidinyl urea	39236-46-9	100.17	0.36	2598.9	9.34	3700.9	13.3	278.26	9.34
220		m-	Dinitrobenzene	99-65-0	65.57	0.39	82.4	0.49	NA	NA	168.12	0.49
221		2-	Nitro-p-phenylene-diamine	5307-14-2	59.73	0.39	3078.5	20.1	NA	NA	153.16	20.1
82	44		Diphenylhydantoin	57-41-0	98.39	0.39	NA	NA	199.3	0.79	252.29	0.79
222			Glibenclamide	10238-21-8	197.62	0.4	NA	NA	3250.8	6.58	494.05	6.58
223	32		Lindane	58-89-9	119.24	0.41	75.6	0.26	87.2	0.3	290.82	0.26
224		n-	Butyl benzoate	136-60-7	73.08	0.41	5133.6	28.8	NA	NA	178.25	28.8
225			Ammonium sulfide	12135-76-1	21.47	0.42	168.2	3.29	NA	NA	51.12	3.29
226			Dodecylbenzene sodiumsulfonate	25155-30-0	146.38	0.42	1261.6	3.62	2000.5	5.74	348.52	3.62
227	46		Sodium oxalate	62-76-0	58.96	0.44	155.4	1.16	NA	NA	134	1.16
228		2,4,5-	Trichlorophen- oxyacetic acid	93-76-5	112.41	0.44	298.9	1.17	388.3	1.52	255.48	1.17
229	22		Dextropropoxyphene * HCl	1639-60-7	184.23	0.49	82.7	0.22	82.7	0.22	375.98	0.22
230	42		Orphenadrine * HCl	341-69-5	149.88	0.49	425.2	1.39	125.4	0.41	305.88	1.39
231			Tween 80	9005-65-6	641.90	0.49	NA	NA	25021.0	19.1	1310	19.1
232		o-	Cresol	95-48-7	56.24	0.52	121.1	1.12	343.9	3.18	108.15	1.12
233			Ibuprofen	15687-27-1	107.28	0.52	1008.9	4.89	980.0	4.75	206.31	4.89
234			Phenylthiourea	103-85-5	82.20	0.54	3.0	0.02	10.0	0.066	152.23	0.02
235	25		Paraquat	4685-14-7	100.58	0.54	57.7	0.31	195.6	1.05	186.25	0.31
83			Thiopental	76-75-5	133.30	0.55	NA	NA	601.1	2.48	242.37	2.48
84			Amobarbital	57-43-2	126.73	0.56	NA	NA	344.0	1.52	226.31	1.52
236			Hydrogen peroxide 90%	7722-84-1	19.05	0.56	NA	NA	2000.4	58.8	34.02	58.8
85			Metamizol	68-89-3	193.94	0.58	7189.2	21.5	NA	NA	334.38	21.5
237			Beryllium II sulfate	13510-49-1	64.09	0.61	82.0	0.78	79.9	0.76	105.07	0.78
239		m-	Cresol	108-39-4	71.38	0.66	242.3	2.24	828.4	7.66	108.15	2.24
240			Pentoxifylline	6493-05-6	183.71	0.66	NA	NA	1386.2	4.98	278.35	4.98
86	31		Warfarin	81-81-2	206.59	0.67	323.8	1.05	373.1	1.21	308.35	1.05

Section 7.2
Table 7.1
Chemical Data from the Registry of Cytotoxicity Database (Sorted by IC50x mmol/l)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE		Rodent LD50 (mmol/kg)	
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
241			Sodium azide	26628-22-8	46.16	0.71	44.9	0.69	27.3	0.42	65.02	0.69
87			Pentobarbital sodium	57-33-0	176.29	0.71	201.1	0.81	280.6	1.13	248.29	0.81
242		1,2,4-	Trichlorobenzene	120-82-1	128.82	0.71	756.6	4.17	765.7	4.22	181.44	4.17
243		p-	Anisidine	104-94-9	89.91	0.73	1404.1	11.4	NA	NA	123.17	11.4
244			Doxylamine succinate	562-10-7	291.38	0.75	NA	NA	470.1	1.21	388.51	1.21
88			Dibutyl phthalate	84-74-2	211.57	0.76	11998.2	43.1	NA	NA	278.38	43.1
89	16	2,4-	Dichlorophenoxy- acetic acid	94-75-7	170.20	0.77	369.1	1.67	366.9	1.66	221.04	1.67
90			Iproniazid	54-92-2	141.61	0.79	365.7	2.04	681.2	3.8	179.25	2.04
91	45		Chloramphenicol	56-75-7	255.29	0.79	3393.1	10.5	2640.1	8.17	323.15	10.5
245			Resorcinol	108-46-3	88.10	0.8	300.6	2.73	NA	NA	110.12	2.73
246	37		Barium II nitrate	10022-31-8	211.70	0.81	355.4	1.36	NA	NA	261.36	1.36
247		(+)-	Thalidomide	731-40-8	209.18	0.81	NA	NA	400.3	1.55	258.25	1.55
92			Di(2-ethylhexyl)phthalate	117-81-7	328.12	0.84	31015.2	79.4	29999.6	76.8	390.62	79.4
93			Sulfisoxazole	127-69-5	227.23	0.85	NA	NA	6790.2	25.4	267.33	25.4
248		m-	Aminophenol	591-27-5	93.86	0.86	1658.9	15.2	NA	NA	109.14	15.2
94			Menthol	89-78-1	148.49	0.95	3172.9	20.3	NA	NA	156.3	20.3
249		3-	Cyano-2-morpholino-5-(pyrid-4-yl)-pyridine (Chemical 122)		255.66	0.96	346.2	1.3	NA	NA	266.31	1.3
250			Valproate sodium	1069-66-5	166.22	1	NA	NA	1695.4	10.2	166.22	10.2
251			Scopolamine * HBr	6533-68-2	415.05	1.08	1268.2	3.3	1879.3	4.89	384.31	3.3
95			Salicylamide	65-45-2	148.12	1.08	1892.7	13.8	1398.9	10.2	137.15	13.8
252	19		Potassium cyanide	151-50-8	72.93	1.12	9.8	0.15	8.5	0.13	65.12	0.15
96			Cygon	60-51-5	284.29	1.24	151.3	0.66	59.6	0.26	229.27	0.66
97			Phenacetin	62-44-2	227.63	1.27	1650.8	9.21	1220.6	6.81	179.24	9.21
253			Isoxepac	55453-87-7	356.81	1.33	198.5	0.74	NA	NA	268.28	0.74
254			Buflomedil	55837-25-7	415.03	1.35	365.8	1.19	NA	NA	307.43	1.19
98			Methylparaben	99-76-3	216.07	1.42	NA	NA	1749.8	11.5	152.16	11.5
255			Sodium monochloroacetate	3926-62-3	168.90	1.45	75.7	0.65	NA	NA	116.48	0.65
99			Nalidixic acid	389-08-2	348.39	1.5	1349.4	5.81	571.4	2.46	232.26	5.81
256			Tin II chloride	7772-99-8	286.28	1.51	699.6	3.69	1200.1	6.33	189.59	3.69
257			Isononylaldehyde	5435-64-3	216.25	1.52	3243.8	22.8	NA	NA	142.27	22.8
100		L-	Ascorbic acid	50-81-7	267.73	1.52	11907.1	67.6	3364.3	19.1	176.14	67.6
101			Glutethimide	77-21-4	338.97	1.56	599.7	2.76	360.7	1.66	217.29	2.76
102			Acrylamide	79-06-1	114.45	1.61	169.9	2.39	169.9	2.39	71.09	2.39
258			Diethyl sebacate	110-40-7	421.19	1.63	14470.4	56	NA	NA	258.4	56
259			Methyl salicylate	119-36-8	258.67	1.7	887.1	5.83	NA	NA	152.16	5.83
260			Coumarin	91-64-5	249.92	1.71	292.3	2	195.8	1.34	146.15	2
103	18		Nicotine	54-11-5	290.45	1.79	50.3	0.31	24.3	0.15	162.26	0.31
104			Tolbutamide	64-77-7	489.39	1.81	NA	NA	2601.1	9.62	270.38	9.62
105	21		Theophylline	58-55-9	329.75	1.83	NA	NA	600.0	3.33	180.19	3.33

Section 7.2
Table 7.1
Chemical Data from the Registry of Cytotoxicity Database (Sorted by IC50x mmol/l)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
106	14		Sodium I fluoride	7681-49-4	77.68	1.85	180.1	4.29	NA	NA	41.99	4.29
261	3		Ferrous sulfate	7720-78-7	281.03	1.85	319.0	2.1	978.3	6.44	151.91	2.1
262	47		Amphetamine sulfate	60-13-9	726.02	1.97	55.3	0.15	24.0	0.065	368.54	0.15
107	2		Acetylsalicylic acid	50-78-2	408.99	2.27	999.9	5.55	814.4	4.52	180.17	5.55
108			Gibberellic acid	77-06-5	796.74	2.3	6304.7	18.2	NA	NA	346.41	18.2
109			Frusemide	54-31-9	770.67	2.33	2599.8	7.86	4597.6	13.9	330.76	7.86
110			Acrylonitrile	107-13-1	128.43	2.42	81.7	1.54	27.1	0.51	53.07	1.54
263			Acetaldehyde	75-07-0	107.95	2.45	1929.8	43.8	NA	NA	44.06	43.8
111			Clofibric acid	882-09-7	560.26	2.61	1249.3	5.82	1169.9	5.45	214.66	5.82
112	48		Caffeine	58-08-2	512.74	2.64	192.3	0.99	619.6	3.19	194.22	0.99
264			Chloral hydrate	302-17-0	438.31	2.65	479.7	2.9	1101.6	6.66	165.4	2.9
113	1		Acetaminophen	103-90-2	409.70	2.71	2403.8	15.9	338.6	2.24	151.18	15.9
265			Streptomycin sulfate	298-39-5	3979.25	2.73	NA	NA	495.6	0.34	1457.6	0.34
114			Natulan * HCl	366-70-1	706.37	2.74	783.7	3.04	NA	NA	257.8	3.04
266			Potassium hexacyanoferrate III	13746-66-2	928.54	2.82	NA	NA	2970.0	9.02	329.27	9.02
267		p-	Hydroxybenzoic acid	99-96-7	403.34	2.92	NA	NA	2196.3	15.9	138.13	15.9
115	12		Phenol	108-95-2	283.30	3.01	414.1	4.4	300.2	3.19	94.12	4.4
268		1-	Octanol	111-87-5	398.60	3.06	NA	NA	1784.6	13.7	130.26	13.7
116			Cyclophosphamide * H2O	6055-19-2	870.89	3.12	94.9	0.34	136.8	0.49	279.13	0.34
269			Potassium I fluoride	7789-23-3	181.85	3.13	245.2	4.22	NA	NA	58.1	4.22
117			Di(2-ethylhexyl)adipate	103-23-1	1167.52	3.15	9117.7	24.6	NA	NA	370.64	24.6
270			Propionaldehyde	123-38-6	188.79	3.25	1411.6	24.3	NA	NA	58.09	24.3
271			Styrene	100-42-5	343.73	3.3	4999.7	48	315.6	3.03	104.16	48
272			Salicylic acid	69-72-7	466.88	3.38	890.9	6.45	479.3	3.47	138.13	6.45
273			Bromobenzene	108-86-1	543.29	3.46	2700.7	17.2	NA	NA	157.02	17.2
274		L-	Cysteine	52-90-4	431.37	3.56	NA	NA	660.4	5.45	121.17	5.45
275			Nitrilotriacetic acid	139-13-9	690.09	3.61	1470.0	7.69	3154.1	16.5	191.16	7.69
276			Ambuphylline	5634-34-4	988.51	3.67	NA	NA	600.7	2.23	269.35	2.23
118	24		Phenobarbital	50-06-6	884.91	3.81	162.6	0.7	167.2	0.72	232.26	0.7
277			Potassium cyanate	590-28-3	335.84	4.14	NA	NA	843.6	10.4	81.12	10.4
278			Phenylephrine * HCl	939-38-8	847.35	4.16	350.3	1.72	120.2	0.59	203.69	1.72
279			Thioacetamide	62-55-5	313.33	4.17	301.3	4.01	NA	NA	75.14	4.01
280			Theophylline sodium acetate	8002-89-9	1098.74	4.19	582.2	2.22	NA	NA	262.23	2.22
281		1,2-	Dibromomethane	106-93-4	730.17	4.2	107.8	0.62	NA	NA	173.85	0.62
119			Sodium salicylate	54-21-7	693.28	4.33	1599.5	9.99	899.8	5.62	160.11	9.99
282		(-)-	Phenylephrine	59-42-7	744.17	4.45	349.5	2.09	NA	NA	167.23	2.09
283			Milrinone	78415-72-2	1007.61	4.77	90.8	0.43	137.3	0.65	211.24	0.43
120		5-	Aminosalicylic acid	89-57-6	776.47	5.07	NA	NA	7749.4	50.6	153.15	50.6
121			Aminophenazone	58-15-1	1246.87	5.39	999.3	4.32	358.6	1.55	231.33	4.32

Section 7.2
Table 7.1
Chemical Data from the Registry of Cytotoxicity Database (Sorted by IC50x (mmol/l)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
284			Ammonium chloride	12125-02-9	295.32	5.52	1647.8	30.8	NA	NA	53.5	30.8
122			Diethyl phthalate	84-66-2	1226.88	5.52	8601.5	38.7	6178.8	27.8	222.26	38.7
285			Caffeine sodium benzoate	8000-95-1	1918.33	5.67	859.4	2.54	798.5	2.36	338.33	2.54
286			Benzylpenicillin sodium	69-57-8	2042.17	5.73	6914.2	19.4	NA	NA	356.4	19.4
287			Benzylalcohol	100-51-6	628.35	5.81	1232.9	11.4	1579.0	14.6	108.15	11.4
288		1-	Heptanol	111-70-6	726.44	6.25	3254.4	28	1499.4	12.9	116.23	28
289			Tetrachloroethene	127-18-4	1084.46	6.54	8854.8	53.4	8092.0	48.8	165.82	53.4
290			Sodium sulfite	7757-83-7	854.55	6.78	NA	NA	820.5	6.51	126.04	6.51
291			Aniline	62-53-3	642.67	6.9	439.6	4.72	439.6	4.72	93.14	4.72
292			Allylalcohol	107-18-6	403.14	6.94	63.9	1.1	95.8	1.65	58.09	1.1
293			Diisopropylamine dichloroacetate	660-27-5	1611.12	7	NA	NA	1700.9	7.39	230.16	7.39
123	35		Isoniazid	54-85-3	1027.33	7.49	650.1	4.74	NA	NA	137.16	4.74
294			Trichloroacetic acid	76-03-9	1338.08	8.19	4999.4	30.6	5636.6	34.5	163.38	30.6
295		2,5-	Hexanedione	110-13-4	964.65	8.45	2705.6	23.7	NA	NA	114.16	23.7
124			Acetazolamide	59-66-5	1886.99	8.49	NA	NA	4289.6	19.3	222.26	19.3
125	34		Carbon tetrachloride	56-23-5	1308.92	8.51	2799.3	18.2	12797.0	83.2	153.81	18.2
296			Homatropine methylbromide	80-49-9	3332.97	9	1199.9	3.24	1399.8	3.78	370.33	3.24
297	11	1,1,1-	Trichloroethane	71-55-6	1374.02	10.3	10298.5	77.2	11245.6	84.3	133.4	77.2
298			Dichloroacetic acid	79-43-6	1482.81	11.5	2823.8	21.9	5518.6	42.8	128.94	21.9
299			Imidazole	288-32-4	783.04	11.5	NA	NA	1879.3	27.6	68.09	27.6
300			Antipyrine	60-80-0	2183.70	11.6	1799.7	9.56	1699.9	9.03	188.25	9.56
301	17		Xylene	1330-20-7	1274.16	12	4300.3	40.5	NA	NA	106.18	40.5
302			Nitrobenzene	98-95-3	1502.06	12.2	640.2	5.2	NA	NA	123.12	5.2
304			Calcium II chloride	10043-52-4	1376.15	12.4	999.9	9.01	NA	NA	110.98	9.01
303			Theophylline sodium	3485-82-3	2519.43	12.4	NA	NA	445.0	2.19	203.18	2.19
305		n-	Butanal	123-72-8	923.14	12.8	2488.1	34.5	NA	NA	72.12	34.5
306			Anisole	100-66-3	1427.58	13.2	3698.7	34.2	NA	NA	108.15	34.2
307		2-	Ethylbutanal	97-96-1	1322.38	13.2	3977.1	39.7	NA	NA	100.18	39.7
308	33		Chloroform	67-66-3	1599.56	13.4	908.4	7.61	25.8	0.3	119.37	7.61
309			Isobutanal	78-84-2	973.62	13.5	2812.7	39	NA	NA	72.12	39
126			Triethyl citrate	77-93-0	4061.90	14.7	6990.9	25.3	NA	NA	276.32	25.3
310			Tributylamine	102-82-9	2855.16	15.4	539.5	2.91	NA	NA	185.4	2.91
311		1-	Hexanol	111-27-3	1573.88	15.4	719.5	7.04	1952.0	19.1	102.2	7.04
312			Benzoic acid	65-85-0	1917.44	15.7	2528.1	20.7	2369.3	19.4	122.13	20.7
313			Xanthinol nicotinate	437-74-1	6865.26	15.8	14121.6	32.5	17336.9	39.9	434.51	32.5
314			Saccharin	81-07-2	3004.32	16.4	NA	NA	17000.0	92.8	183.19	92.8
315			Isobenzoic furano dione		2518.04	17	4014.1	27.1	1999.6	13.5	148.12	27.1
316			Toluene	108-88-3	1575.77	17.1	5003.7	54.3	NA	NA	92.15	54.3
317			Barbital sodium	144-02-5	3835.32	18.6	NA	NA	800.1	3.88	206.2	3.88

Section 7.2
Table 7.1
Chemical Data from the Registry of Cytotoxicity Database (Sorted by IC50x mmol/l)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
318			Trifluoroacetic acid	76-05-1	2337.62	20.5	199.6	1.75	NA	NA	114.03	1.75
127			Dimethyl phthalate	131-11-3	4544.28	23.4	6894.1	35.5	7204.8	37.1	194.2	35.5
319			Methylpentinol	77-75-8	2336.21	23.8	NA	NA	525.2	5.35	98.16	5.35
320		N,N-	Dimethylacetamide	127-19-5	2108.79	24.2	5089.0	58.4	4618.4	53	87.14	58.4
321			Acetic acid	64-19-7	1459.46	24.3	3309.3	55.1	4961.0	82.6	60.06	55.1
322		1-	Pentanol	71-41-0	2195.43	24.9	3033.0	34.4	200.1	2.27	88.17	34.4
323			Urethan	51-79-6	2307.95	25.9	NA	NA	2504.0	28.1	89.11	28.1
324		2-	Butoxyethanol	111-76-2	3073.20	.26	1477.5	12.5	1229.3	10.4	118.2	12.5
325			Cyclohexanol	108-93-0	2634.73	26.3	2063.7	20.6	NA	NA	100.18	20.6
326			Halothane	151-67-7	6138.83	31.1	5684.8	28.8	NA	NA	197.39	28.8
327	20		Lithium I sulfate	10377-48-7	3704.98	33.7	NA	NA	1187.4	10.8	109.94	10.8
328	36		Dichloromethane	75-09-2	2964.06	34.9	1596.7	18.8	NA	NA	84.93	18.8
329			Sodium cyclamate	139-05-9	7123.90	35.4	15254.0	75.8	17004.8	84.5	201.24	75.8
330			Sulfuric acid	7664-93-9	3530.88	36	2138.1	21.8	NA	NA	98.08	21.8
331			Strontium II chloride	10476-85-4	5770.13	36.4	2251.0	14.2	3107.0	19.6	158.52	14.2
332		1,4-	Dioxane	123-91-1	3357.37	38.1	4203.3	47.7	5701.4	64.7	88.12	47.7
333			Lithium I chloride	7447-41-8	1636.25	38.6	758.8	17.9	1165.7	27.5	42.39	17.9
334			Isobutanol	78-83-1	2973.01	40.1	2461.4	33.2	NA	NA	74.14	33.2
335			Potassium hexacyano- ferrate II	13943-58-3	15582.05	42.3	6409.6	17.4	5009.8	13.6	368.37	17.4
336			Nicotinamide	98-92-0	5423.02	44.4	3505.4	28.7	NA	NA	122.14	28.7
337			Pyridine	110-86-1	3710.26	46.9	893.9	11.3	NA	NA	79.11	11.3
338		1-	Butanol	71-36-3	3892.35	52.5	793.3	10.7	NA	NA	74.14	10.7
339		1-	Nitropropane	79-46-9	5159.47	57.9	455.4	5.11	NA	NA	89.11	5.11
340			Diethylene glycol	111-46-6	6591.29	62.1	14753.5	139	23669.2	223	106.14	139
341			Lactic acid	598-82-3	5945.94	66	3729.7	41.4	4873.9	54.1	90.09	41.4
342			Piperazine	110-85-0	5789.95	67.2	1904.1	22.1	1438.9	16.7	86.16	22.1
343			Magnesium II chloride * 6 H2O	7791-18-6	14314.43	70.4	8092.5	39.8	NA	NA	203.33	39.8
344	13		Sodium chloride	7647-14-5	4435.60	75.9	2998.0	51.3	3997.3	68.4	58.44	51.3
345			Sodium I bromide	7647-15-6	8120.81	77.4	3504.3	33.4	6998.2	66.7	104.92	33.4
346	50		Potassium I chloride	7447-40-7	6113.10	82	2601.8	34.9	1498.5	20.1	74.55	34.9
347			Thiourea	62-56-6	6547.18	86	124.9	1.64	8526.6	112	76.13	1.64
348		1-	Propanol	71-23-8	5800.62	96.5	5397.9	89.8	NA	NA	60.11	89.8
349			Ethyl methyl ketone	78-93-3	7500.48	104	3396.9	47.1	NA	NA	72.12	47.1
350			Tetrahydrofurfuryl alcohol	97-99-4	11338.65	111	2502.7	24.5	2298.4	22.5	102.15	24.5
351			Dimethylformamide	68-12-2	8334.54	114	2800.1	38.3	3750.5	51.3	73.11	38.3
352		1,2,6-	Hexanetriol	106-69-4	16506.60	123	15969.8	119	NA	NA	134.2	119
353			Ethyl acetate	141-78-6	11279.36	128	11015.0	125	NA	NA	88.12	125
128	10	2-	Propanol	67-63-0	10038.37	167	5842.7	97.2	NA	NA	60.11	97.2
354		1,3,5-	Trioxane	110-88-3	19189.17	213	800.0	8.88	NA	NA	90.09	8.88

Section 7.2
Table 7.1
Chemical Data from the Registry of Cytotoxicity Database (Sorted by IC50x mmol/l)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
355			D-Glucose	50-99-7	40720.68	226	25765.7	143	NA	NA	180.18	143
356			2-Methoxyethanol	109-86-4	19103.61	251	2458.4	32.3	NA	NA	76.11	32.3
129			Dimethyl sulfoxide	75-18-3	19691.28	252	19691.3	252	16487.5	211	78.14	252
357			Propylene glycol	57-55-6	26029.62	342	20016.9	263	23974.7	315	76.11	263
358			Acetonitrile	75-05-8	15110.08	368	3798.1	92.5	NA	NA	41.06	92.5
130	9		Ethanol	64-17-5	17464.32	379	14008.3	304	7787.5	169	46.08	304
359			Acetone	67-64-1	25791.96	444	9759.1	168	NA	NA	58.09	168
360	7		Ethylene glycol	107-21-1	34454.40	555	8567.0	138	7511.7	121	62.08	138
131			Glycerol	56-81-5	57476.64	624	12619.1	137	25975.0	282	92.11	137
361	8		Methanol	67-56-1	29806.50	930	13012.3	406	NA	NA	32.05	406

Section 7.2
Table 7.2
Chemical Data from the Registry of Cytotoxicity Database (Sorted by Rat LD50 Oral mg/kg)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
29	28		Mercury II chloride	7487-94-7	4.07	0.015	1.0	0.0037	10.0	0.037	271.49	0.0037
143			Triethylene melamine	51-18-3	0.16	0.00078	1.0	0.005	14.9	0.073	204.27	0.005
177			Busulphan	55-98-1	11.33	0.046	1.9	0.0076	199.5	0.81	246.32	0.0076
13			Cycloheximide	66-81-9	0.17	0.00059	2.0	0.0071	132.3	0.47	281.39	0.0071
51			Disulfoton	298-04-4	30.19	0.11	2.0	0.0073	5.5	0.02	274.42	0.0073
49			Parathion	56-38-2	27.09	0.093	2.0	0.0069	6.1	0.021	291.28	0.0069
234			Phenylthiourea	103-85-5	82.20	0.54	3.0	0.02	10.0	0.066	152.23	0.02
37			Aflatoxin B1	1162-65-8	10.62	0.034	5.0	0.016	9.1	0.029	312.29	0.016
137			Triethyltin chloride	994-31-0	0.11	0.00046	5.1	0.021	NA	NA	241.35	0.021
2			Actinomycin D	50-76-0	0.01	0.0000081	7.2	0.0057	12.6	0.01	1255.6	0.0057
252	19		Potassium cyanide	151-50-8	72.93	1.12	9.8	0.15	8.5	0.13	65.12	0.15
148			Nitrogen mustard * HCl	55-86-7	0.50	0.0026	10.0	0.052	19.3	0.1	192.53	0.052
60			Indomethacin	53-86-1	57.25	0.16	12.2	0.034	19.0	0.053	357.81	0.034
14			Mitomycin C	50-07-7	0.28	0.00084	14.0	0.042	17.1	0.051	334.37	0.042
153	26		Arsenic III trioxide	1327-53-3	0.83	0.0042	19.8	0.1	45.5	0.23	197.84	0.1
192		1,3-	Bis(2-chloroethyl)- 1-nitrosourea	154-93-8	16.70	0.078	19.9	0.093	19.1	0.089	214.07	0.093
150			Cis-platinum	15663-27-1	0.84	0.0028	25.8	0.086	33.0	0.11	300.07	0.086
68		2,4-	Dinitrophenol	51-28-5	38.67	0.21	29.5	0.16	44.2	0.24	184.12	0.16
43			Aldrin	309-00-2	24.45	0.067	40.1	0.11	43.8	0.12	364.9	0.11
185			Heptachlor	76-44-8	22.02	0.059	41.1	0.11	67.2	0.18	373.3	0.11
132			Triphenyltin hydroxide	76-87-9	0.02	0.000049	44.0	0.12	245.9	0.67	367.03	0.12
241			Sodium azide	26628-22-8	46.16	0.71	44.9	0.69	27.3	0.42	65.02	0.69
207			Dieldrin	60-57-1	68.56	0.18	45.7	0.12	38.1	0.1	380.9	0.12
179			Acrolein	107-02-8	2.64	0.047	46.0	0.82	39.8	0.71	56.07	0.82
144			Sodium bichromate VI	10588-01-9	0.24	0.00093	49.8	0.19	NA	NA	261.98	0.19
103	18		Nicotine	54-11-5	290.45	1.79	50.3	0.31	24.3	0.15	162.26	0.31
173	39		Pentachlorophenol	87-86-5	9.59	0.036	50.6	0.19	NA	NA	266.32	0.19
262	47		Amphetamine sulfate	60-13-9	726.02	1.97	55.3	0.15	24.0	0.065	368.54	0.15
8			Digitoxin	71-63-6	0.08	0.00011	55.8	0.073	NA	NA	765.05	0.073
235	25		Paraquat	4685-14-7	100.58	0.54	57.7	0.31	195.6	1.05	186.25	0.31
157	38		Hexachlorophene	70-30-4	3.21	0.0079	61.0	0.15	65.1	0.16	406.89	0.15
292			Allylalcohol	107-18-6	403.14	6.94	63.9	1.1	95.8	1.65	58.09	1.1
10			Emetine	483-18-1	0.08	0.00016	67.3	0.14	NA	NA	480.71	0.14
223	32		Lindane	58-89-9	119.24	0.41	75.6	0.26	87.2	0.3	290.82	0.26
255			Sodium monochloroacetate	3926-62-3	168.90	1.45	75.7	0.65	NA	NA	116.48	0.65
190			Chlorambucil	305-03-3	23.12	0.076	76.1	0.25	100.4	0.33	304.24	0.25
149			Chromium VI trioxide	1333-82-0	0.27	0.0027	80.0	0.8	127.0	1.27	100	0.8
180		p-	Phenylenediamine	106-50-3	5.41	0.05	80.0	0.74	NA	NA	108.16	0.74
62			Cobalt II chloride	7646-79-9	20.77	0.16	80.5	0.62	80.5	0.62	129.83	0.62

Section 7.2
Table 7.2
Chemical Data from the Registry of Cytotoxicity Database (Sorted by Rat LD50 Oral mg/kg)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
110			Acrylonitrile	107-13-1	128.43	2.42	81.7	1.54	27.1	0.51	53.07	1.54
237			Beryllium II sulfate	13510-49-1	64.09	0.61	82.0	0.78	79.9	0.76	105.07	0.78
220		m-	Dinitrobenzene	99-65-0	65.57	0.39	82.4	0.49	NA	NA	168.12	0.49
229	22		Dextropropoxyphene * HCl	1639-60-7	184.23	0.49	82.7	0.22	82.7	0.22	375.98	0.22
20			Cadmium II chloride	10108-64-2	1.17	0.0064	88.0	0.48	174.1	0.95	183.3	0.48
219			Hydralazine	86-54-4	52.87	0.33	89.7	0.56	121.8	0.76	160.2	0.56
160		N-	Methyl-N'-nitro-N-nitroso- guanidine	70-25-7	1.77	0.012	89.7	0.61	NA	NA	147.12	0.61
283			Milrinone	78415-72-2	1007.61	4.77	90.8	0.43	137.3	0.65	211.24	0.43
116			Cyclophosphamide * H2O	6055-19-2	870.89	3.12	94.9	0.34	136.8	0.49	279.13	0.34
217			Amrinone	60719-84-8	52.42	0.28	101.1	0.54	288.3	1.54	187.22	0.54
194		p-	Toluylendiamine	95-70-5	11.49	0.094	101.4	0.83	NA	NA	122.19	0.83
74			Nickel II chloride	7718-54-9	34.99	0.27	105.0	0.81	NA	NA	129.61	0.81
281		1,2-	Dibromomethane	106-93-4	730.17	4.2	107.8	0.62	NA	NA	173.85	0.62
196	40		VerapamilHCl	152-11-4	49.11	0.1	108.0	0.22	162.1	0.33	491.13	0.22
198			Ioxynil	1689-83-4	40.80	0.11	111.3	0.3	NA	NA	370.91	0.3
151			Hexachlorocyclopentadiene	77-47-4	0.85	0.0031	111.8	0.41	NA	NA	272.75	0.41
167		p,p'	DDD	72-54-8	7.68	0.024	112.0	0.35	NA	NA	320.04	0.35
61		p,p'	DDT	50-29-3	56.72	0.16	113.4	0.32	134.7	0.38	354.48	0.32
138			Tributyltin chloride	1461-22-9	0.18	0.00054	120.4	0.37	NA	NA	325.53	0.37
232		o-	Cresol	95-48-7	56.24	0.52	121.1	1.12	343.9	3.18	108.15	1.12
347			Thiourea	62-56-6	6547.18	86	124.9	1.64	8526.6	112	76.13	1.64
69			Secobarbital sodium	309-43-3	54.66	0.21	124.9	0.48	NA	NA	260.3	0.48
134			Rotenone	83-79-4	0.05	0.00013	130.2	0.33	351.1	0.89	394.45	0.33
9			Amethopterin	59-05-2	0.06	0.00014	136.4	0.3	145.4	0.32	454.5	0.3
199			Cupric chloride	7447-39-4	14.79	0.11	139.8	1.04	189.6	1.41	134.44	1.04
27			Chlorpromazine	50-53-3	4.46	0.014	140.3	0.44	261.5	0.82	318.89	0.44
96			Cygon	60-51-5	284.29	1.24	151.3	0.66	59.6	0.26	229.27	0.66
227	46		Sodium oxalate	62-76-0	58.96	0.44	155.4	1.16	NA	NA	134	1.16
118	24		Phenobarbital	50-06-6	884.91	3.81	162.6	0.7	167.2	0.72	232.26	0.7
225			Ammonium sulfide	12135-76-1	21.47	0.42	168.2	3.29	NA	NA	51.12	3.29
16			Azaserine	115-02-6	0.35	0.002	169.7	0.98	150.6	0.87	173.15	0.98
102			Acrylamide	79-06-1	114.45	1.61	169.9	2.39	169.9	2.39	71.09	2.39
64			Bendiocarb	22781-23-3	40.19	0.18	178.6	0.8	NA	NA	223.25	0.8
106	14		Sodium I fluoride	7681-49-4	77.68	1.85	180.1	4.29	NA	NA	41.99	4.29
112	48		Caffeine	58-08-2	512.74	2.64	192.3	0.99	619.6	3.19	194.22	0.99
253			Isoxepac	55453-87-7	356.81	1.33	198.5	0.74	NA	NA	268.28	0.74
318			Trifluoroacetic acid	76-05-1	2337.62	20.5	199.6	1.75	NA	NA	114.03	1.75
87			Pentobarbital sodium	57-33-0	176.29	0.71	201.1	0.81	280.6	1.13	248.29	0.81
212		p-	Cresol	106-44-5	23.79	0.22	206.6	1.91	343.9	3.18	108.15	1.91

Section 7.2
Table 7.2
Chemical Data from the Registry of Cytotoxicity Database (Sorted by Rat LD50 Oral mg/kg)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE		MW	Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg		for Regression
17		5-	Fluorouracil	51-21-8	0.34	0.0026	230.3	1.77	114.5	0.88	130.09	1.77
206			Diquat dibromide	85-00-7	55.05	0.16	230.5	0.67	234.0	0.68	344.08	0.67
239		m-	Cresol	108-39-4	71.38	0.66	242.3	2.24	828.4	7.66	108.15	2.24
269			Potassium I fluoride	7789-23-3	181.85	3.13	245.2	4.22	NA	NA	58.1	4.22
73			Carbaryl	63-25-2	52.32	0.26	249.5	1.24	438.7	2.18	201.24	1.24
35			Flufenamic acid	530-78-9	8.16	0.029	272.8	0.97	714.4	2.54	281.25	0.97
260			Coumarin	91-64-5	249.92	1.71	292.3	2	195.8	1.34	146.15	2
228		2,4,5-	Trichlorophen- oxyacetic acid	93-76-5	112.41	0.44	298.9	1.17	388.3	1.52	255.48	1.17
81	27		Cupric sulfate * 5 H2O	7758-99-8	82.40	0.33	299.6	1.2	NA	NA	249.7	1.2
245			Resorcinol	108-46-3	88.10	0.8	300.6	2.73	NA	NA	110.12	2.73
279			Thioacetamide	62-55-5	313.33	4.17	301.3	4.01	NA	NA	75.14	4.01
38			Imipramine * HCl	113-52-0	17.11	0.054	304.2	0.96	374.0	1.18	316.91	0.96
205			Versalide	88-29-9	38.77	0.15	315.3	1.22	NA	NA	258.44	1.22
261	3		Ferrous sulfate	7720-78-7	281.03	1.85	319.0	2.1	978.3	6.44	151.91	2.1
183	5		Amitriptyline	50-48-6	15.54	0.056	319.1	1.15	147.0	0.53	277.44	1.15
86	31		Warfarin	81-81-2	206.59	0.67	323.8	1.05	373.1	1.21	308.35	1.05
176			Papaverine	58-74-2	15.27	0.045	325.8	0.96	230.8	0.68	339.42	0.96
249		3-	Cyano-2-morpholino-5-(pyrid-4-yl)-pyridine (Chemical 122)		255.66	0.96	346.2	1.3	NA	NA	266.31	1.3
282		(-)-	Phenylephrine	59-42-7	744.17	4.45	349.5	2.09	NA	NA	167.23	2.09
55			Zinc II chloride	7646-85-7	17.72	0.13	350.2	2.57	350.2	2.57	136.27	2.57
278			Phenylephrine * HCl	939-38-8	847.35	4.16	350.3	1.72	120.2	0.59	203.69	1.72
210		p-	Nitrophenol	100-02-7	27.82	0.2	350.6	2.52	467.4	3.36	139.12	2.52
246	37		Barium II nitrate	10022-31-8	211.70	0.81	355.4	1.36	NA	NA	261.36	1.36
90			Iproniazid	54-92-2	141.61	0.79	365.7	2.04	681.2	3.8	179.25	2.04
254			Buflomedil	55837-25-7	415.03	1.35	365.8	1.19	NA	NA	307.43	1.19
89	16	2,4-	Dichlorophenoxy- acetic acid	94-75-7	170.20	0.77	369.1	1.67	366.9	1.66	221.04	1.67
79			Phenylbutazone	50-33-9	98.69	0.32	376.3	1.22	441.0	1.43	308.41	1.22
172			Nabam	142-59-6	8.97	0.035	394.8	1.54	579.3	2.26	256.34	1.54
155			Benzalkonium chloride	8001-54-5	1.90	0.0052	401.5	1.1	339.5	0.93	365	1.1
159			Hexadecyltrimethylammoniumbromide	57-09-0	3.24	0.0089	408.3	1.12	NA	NA	364.53	1.12
115	12		Phenol	108-95-2	283.30	3.01	414.1	4.4	300.2	3.19	94.12	4.4
230	42		Orphenadrine * HCl	341-69-5	149.88	0.49	425.2	1.39	125.4	0.41	305.88	1.39
291			Aniline	62-53-3	642.67	6.9	439.6	4.72	439.6	4.72	93.14	4.72
75			Trichlorfon	52-68-6	69.51	0.27	450.5	1.75	298.6	1.16	257.44	1.75
339		1-	Nitropropane	79-46-9	5159.47	57.9	455.4	5.11	NA	NA	89.11	5.11
53	43		Quinidine sulfate	50-54-4	50.70	0.12	456.3	1.08	595.8	1.41	422.54	1.08
40			Chlordan	57-74-9	24.59	0.06	458.9	1.12	NA	NA	409.76	1.12
163			Cetyltrimethylammonium chloride	112-02-7	7.61	0.021	474.4	1.31	NA	NA	362.16	1.31
264			Chloral hydrate	302-17-0	438.31	2.65	479.7	2.9	1101.6	6.66	165.4	2.9

Section 7.2
Table 7.2
Chemical Data from the Registry of Cytotoxicity Database (Sorted by Rat LD50 Oral mg/kg)

RC #	MEIC #	Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE		MW	Rodent LD50 (mmol/kg)
				ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg		for Regression
204		Azathioprine	446-86-6	38.82	0.14	535.2	1.93	1389.2	5.01	277.29	1.93
310		Tributylamine	102-82-9	2855.16	15.4	539.5	2.91	NA	NA	185.4	2.91
187		4-Hexylresorcinol	136-77-6	12.44	0.064	549.9	2.83	NA	NA	194.3	2.83
26		Kelthane	115-32-2	4.45	0.012	574.2	1.55	418.6	1.13	370.48	1.55
39		2,4 Dichlorophenol	120-83-2	8.97	0.055	580.3	3.56	1600.7	9.82	163	3.56
280		Theophylline sodium acetate	8002-89-9	1098.74	4.19	582.2	2.22	NA	NA	262.23	2.22
147		Mitoxantrone	65271-80-9	1.07	0.0024	586.8	1.32	NA	NA	444.54	1.32
101		Glutethimide	77-21-4	338.97	1.56	599.7	2.76	360.7	1.66	217.29	2.76
45		Quinine * HCl	130-89-2	27.07	0.075	620.8	1.72	1158.6	3.21	360.92	1.72
70	49	Atropine sulfate	55-48-1	148.92	0.22	622.7	0.92	764.9	1.13	676.9	0.92
302		Nitrobenzene	98-95-3	1502.06	12.2	640.2	5.2	NA	NA	123.12	5.2
123	35	Isoniazid	54-85-3	1027.33	7.49	650.1	4.74	NA	NA	137.16	4.74
256		Tin II chloride	7772-99-8	286.28	1.51	699.6	3.69	1200.1	6.33	189.59	3.69
63	4	Diazepam	439-14-5	45.56	0.16	709.1	2.49	535.3	1.88	284.76	2.49
168		Dicoumarol	66-76-2	9.08	0.027	709.6	2.11	232.1	0.69	336.31	2.11
311		1-Hexanol	111-27-3	1573.88	15.4	719.5	7.04	1952.0	19.1	102.2	7.04
174		Ambazone	539-21-9	9.02	0.038	749.9	3.16	999.1	4.21	237.32	3.16
242		1,2,4-Trichlorobenzene	120-82-1	128.82	0.71	756.6	4.17	765.7	4.22	181.44	4.17
333		Lithium I chloride	7447-41-8	1636.25	38.6	758.8	17.9	1165.7	27.5	42.39	17.9
114		Natulan * HCl	366-70-1	706.37	2.74	783.7	3.04	NA	NA	257.8	3.04
48		Mefenamic acid	61-68-7	20.99	0.087	789.1	3.27	629.8	2.61	241.31	3.27
338		1-Butanol	71-36-3	3892.35	52.5	793.3	10.7	NA	NA	74.14	10.7
202		Formaldehyde	50-00-0	3.60	0.12	798.8	26.6	NA	NA	30.03	26.6
188		t-Butyl hydroquinone	1948-33-0	11.47	0.069	799.6	4.81	1000.8	6.02	166.24	4.81
354		1,3,5-Trioxane	110-88-3	19189.17	213	800.0	8.88	NA	NA	90.09	8.88
58		Dihydralazine sulfate	7327-87-9	40.36	0.14	818.8	2.84	400.8	1.39	288.32	2.84
213		Ammonium persulfate	7727-54-0	52.49	0.23	819.3	3.59	NA	NA	228.22	3.59
71		Diphenhydramine * HCl	147-24-0	70.04	0.24	855.1	2.93	113.8	0.39	291.85	2.93
285		Caffeine sodium benzoate	8000-95-1	1918.33	5.67	859.4	2.54	798.5	2.36	338.33	2.54
197		p,p' DDE	72-55-9	31.80	0.1	880.9	2.77	NA	NA	318.02	2.77
67	15	Malathion	121-75-5	66.08	0.2	885.4	2.68	776.4	2.35	330.38	2.68
259		Methyl salicylate	119-36-8	258.67	1.7	887.1	5.83	NA	NA	152.16	5.83
184		Butylated hydroxytoluene	128-37-0	12.34	0.056	890.4	4.04	1040.2	4.72	220.39	4.04
272		Salicylic acid	69-72-7	466.88	3.38	890.9	6.45	479.3	3.47	138.13	6.45
337		Pyridine	110-86-1	3710.26	46.9	893.9	11.3	NA	NA	79.11	11.3
308	33	Chloroform	67-66-3	1599.56	13.4	908.4	7.61	35.8	0.3	119.37	7.61
44		Hydroxyzine * HCl	1244-76-4	27.56	0.067	950.4	2.31	NA	NA	411.41	2.31
31	41	Chloroquine diphosphate	50-63-5	8.77	0.017	969.9	1.88	500.4	0.97	515.92	1.88
214		Thymol	89-83-8	34.56	0.23	979.6	6.52	1802.9	12	150.24	6.52

Section 7.2
Table 7.2
Chemical Data from the Registry of Cytotoxicity Database (Sorted by Rat LD50 Oral mg/kg)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
65			Oxyphenbutazone	129-20-4	61.64	0.19	999.2	3.08	480.1	1.48	324.41	3.08
121			Aminophenazone	58-15-1	1246.87	5.39	999.3	4.32	358.6	1.55	231.33	4.32
80		2-	Thiouracil	141-90-2	41.01	0.32	999.6	7.8	NA	NA	128.16	7.8
304			Calcium II chloride	10043-52-4	1376.15	12.4	999.9	9.01	NA	NA	110.98	9.01
107	2		Acetylsalicylic acid	50-78-2	408.99	2.27	999.9	5.55	814.4	4.52	180.17	5.55
233			Ibuprofen	15687-27-1	107.28	0.52	1008.9	4.89	980.0	4.75	206.31	4.89
47			Naftipramide	1505-95-9	25.07	0.084	1029.7	3.45	1086.4	3.64	298.47	3.45
218		o-	Phenylenediamine	95-54-5	33.53	0.31	1069.7	9.89	NA	NA	108.16	9.89
41			Chloroquine sulfate	132-73-0	25.08	0.06	1086.8	2.6	NA	NA	418	2.6
296			Homatropine methylbromide	80-49-9	3332.97	9	1199.9	3.24	1399.8	3.78	370.33	3.24
152		8-	Hydroxyquinoline	148-24-3	0.48	0.0033	1200.6	8.27	NA	NA	145.17	8.27
287			Benzylalcohol	100-51-6	628.35	5.81	1232.9	11.4	1579.0	14.6	108.15	11.4
111			Clofibric acid	882-09-7	560.26	2.61	1249.3	5.82	1169.9	5.45	214.66	5.82
226			Dodecylbenzene sodiumsulfonate	25155-30-0	146.38	0.42	1261.6	3.62	2000.5	5.74	348.52	3.62
251			Scopolamine * HBr	6533-68-2	415.05	1.08	1268.2	3.3	1879.3	4.89	384.31	3.3
76			Sodium dodecyl sulfate	151-21-3	78.15	0.27	1288.0	4.45	NA	NA	289.43	4.45
191			Dimenhydrinate	523-87-5	35.72	0.076	1320.8	2.81	202.1	0.43	470.02	2.81
99			Nalidixic acid	389-08-2	348.39	1.5	1349.4	5.81	571.4	2.46	232.26	5.81
243		p-	Anisidine	104-94-9	89.91	0.73	1404.1	11.4	NA	NA	123.17	11.4
270			Propionaldehyde	123-38-6	188.79	3.25	1411.6	24.3	NA	NA	58.09	24.3
164			Oxatomide	60607-34-3	8.11	0.019	1412.1	3.31	9598.7	22.5	426.61	3.31
275			Nitrilotriacetic acid	139-13-9	690.09	3.61	1470.0	7.69	3154.1	16.5	191.16	7.69
324		2-	Butoxyethanol	111-76-2	3073.20	26	1477.5	12.5	1229.3	10.4	118.2	12.5
56			Manganese IIchloride *4 H2O	13446-34-9	25.73	0.13	1484.4	7.5	NA	NA	197.92	7.5
136			Diethyldithiocarbamate sodium* 3H20	20624-25-3	0.09	0.00039	1500.7	6.66	1500.7	6.66	225.33	6.66
328	36		Dichloromethane	75-09-2	2964.06	34.9	1596.7	18.8	NA	NA	84.93	18.8
119			Sodium salicylate	54-21-7	693.28	4.33	1599.5	9.99	899.8	5.62	160.11	9.99
166			Triisooctylamine	2757-28-0	8.14	0.023	1620.2	4.58	NA	NA	353.76	4.58
284			Ammonium chloride	12125-02-9	295.32	5.52	1647.8	30.8	NA	NA	53.5	30.8
97			Phenacetin	62-44-2	227.63	1.27	1650.8	9.21	1220.6	6.81	179.24	9.21
248		m-	Aminophenol	591-27-5	93.86	0.86	1658.9	15.2	NA	NA	109.14	15.2
42		p-	Aminophenol	23-30-8	6.77	0.062	1658.9	15.2	NA	NA	109.14	15.2
78		6-	Methylcoumarin	92-48-8	49.66	0.31	1681.9	10.5	NA	NA	160.18	10.5
200			Dimethylaminoethyl methacrylate (polymer)	2867-47-2	17.30	0.11	1745.4	11.1	NA	NA	157.24	11.1
57		L-	Dopa	59-92-7	25.64	0.13	1780.8	9.03	2366.5	12	197.21	9.03
182			Triton X-100	9002-93-1	35.59	0.055	1798.7	2.78	NA	NA	647	2.78
300			Antipyrine	60-80-0	2183.70	11.6	1799.7	9.56	1699.9	9.03	188.25	9.56
95			Salicylamide	65-45-2	148.12	1.08	1892.7	13.8	1398.9	10.2	137.15	13.8
342			Piperazine	110-85-0	5789.95	67.2	1904.1	22.1	1438.9	16.7	86.16	22.1

Section 7.2
Table 7.2
Chemical Data from the Registry of Cytotoxicity Database (Sorted by Rat LD50 Oral mg/kg)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
263			Acetaldehyde	75-07-0	107.95	2.45	1929.8	43.8	NA	NA	44.06	43.8
139			Retinol	68-26-8	0.15	0.00054	1999.8	6.98	4011.0	14	286.5	6.98
52			all-trans-Retinoic acid	302-79-4	33.05	0.11	2001.2	6.66	NA	NA	300.48	6.66
325			Cyclohexanol	108-93-0	2634.73	26.3	2063.7	20.6	NA	NA	100.18	20.6
330			Sulfuric acid	7664-93-9	3530.88	36	2138.1	21.8	NA	NA	98.08	21.8
72			Butylated hydoxyanisole	8003-24-5	43.26	0.24	2199.3	12.2	2001.0	11.1	180.27	12.2
165			Isoproterenol * HCl	51-30-9	5.45	0.022	2219.8	8.96	NA	NA	247.75	8.96
331			Strontium II chloride	10476-85-4	5770.13	36.4	2251.0	14.2	3107.0	19.6	158.52	14.2
113	1		Acetaminophen	103-90-2	409.70	2.71	2403.8	15.9	338.6	2.24	151.18	15.9
356		2-	Methoxyethanol	109-86-4	19103.61	251	2458.4	32.3	NA	NA	76.11	32.3
334			Isobutanol	78-83-1	2973.01	40.1	2461.4	33.2	NA	NA	74.14	33.2
305		n-	Butanal	123-72-8	923.14	12.8	2488.1	34.5	NA	NA	72.12	34.5
350			Tetrahydrofurfuryl alcohol	97-99-4	11338.65	111	2502.7	24.5	2298.4	22.5	102.15	24.5
208			Undecylenic acid	112-38-9	33.18	0.18	2506.6	13.6	8496.7	46.1	184.31	13.6
312			Benzoic acid	65-85-0	1917.44	15.7	2528.1	20.7	2369.3	19.4	122.13	20.7
238			Imidazolidinyl urea	39236-46-9	100.17	0.36	2598.9	9.34	3700.9	13.3	278.26	9.34
109			Frusemide	54-31-9	770.67	2.33	2599.8	7.86	4597.6	13.9	330.76	7.86
346	50		Potassium Chloride	7447-40-7	6113.10	82	2601.8	34.9	1498.5	20.1	74.55	34.9
158			Dichlorophene	97-23-4	2.23	0.0083	2691.3	10	1001.2	3.72	269.13	10
273			Bromobenzene	108-86-1	543.29	3.46	2700.7	17.2	NA	NA	157.02	17.2
295		2,5-	Hexanedione	110-13-4	964.65	8.45	2705.6	23.7	NA	NA	114.16	23.7
125	34		Carbon tetrachloride	56-23-5	1308.92	8.51	2799.3	18.2	12797.0	83.2	153.81	18.2
351			Dimethylformamide	68-12-2	8334.54	114	2800.1	38.3	3750.5	51.3	73.11	38.3
309			Isobutanal	78-84-2	973.62	13.5	2812.7	39	NA	NA	72.12	39
298			Dichloroacetic acid	79-43-6	1482.81	11.5	2823.8	21.9	5518.6	42.8	128.94	21.9
344	13		Sodium chloride	7647-14-5	4435.60	75.9	2998.0	51.3	3997.3	68.4	58.44	51.3
322		1-	Pentanol	71-41-0	2195.43	24.9	3033.0	34.4	200.1	2.27	88.17	34.4
221		2-	Nitro-p-phenylene-diamine	5307-14-2	59.73	0.39	3078.5	20.1	NA	NA	153.16	20.1
216			Refortan		78.28	0.25	3162.3	10.1	NA	NA	313.1	10.1
94			Menthol	89-78-1	148.49	0.95	3172.9	20.3	NA	NA	156.3	20.3
257			Isononylaldehyde	5435-64-3	216.25	1.52	3243.8	22.8	NA	NA	142.27	22.8
288		1-	Heptanol	111-70-6	726.44	6.25	3254.4	28	1499.4	12.9	116.23	28
321			Acetic acid	64-19-7	1459.46	24.3	3309.3	55.1	4961.0	82.6	60.06	55.1
91	45		Chloramphenicol	56-75-7	255.29	0.79	3393.1	10.5	2640.1	8.17	323.15	10.5
349			Ethyl methyl ketone	78-93-3	7500.48	104	3396.9	47.1	NA	NA	72.12	47.1
345			Sodium Bromide	7647-15-6	8120.81	77.4	3504.3	33.4	6998.2	66.7	104.92	33.4
336			Nicotinamide	98-92-0	5423.02	44.4	3505.4	28.7	NA	NA	122.14	28.7
306*			Anisole	100-66-3	1427.58	13.2	3698.7	34.2	NA	NA	108.15	34.2
341			Lactic acid	598-82-3	5945.94	66	3729.7	41.4	4873.9	54.1	90.09	41.4

Section 7.2
Table 7.2
Chemical Data from the Registry of Cytotoxicity Database (Sorted by Rat LD50 Oral mg/kg)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
358			Acetonitrile	75-05-8	15110.08	368	3798.1	92.5	NA	NA	41.06	92.5
211			Catechol	120-80-9	22.02	0.2	3887.2	35.3	259.9	2.36	110.12	35.3
307		2-	Ethylbutanol	97-96-1	1322.38	13.2	3977.1	39.7	NA	NA	100.18	39.7
315			Isobenzoic furano dione		2518.04	17	4014.1	27.1	1999.6	13.5	148.12	27.1
332		1,4-	Dioxane	123-91-1	3357.37	38.1	4203.3	47.7	5701.4	64.7	88.12	47.7
301	17		Xylene	1330-20-7	1274.16	12	4300.3	40.5	NA	NA	106.18	40.5
154			Maneb	12427-38-2	1.12	0.0042	4500.6	16.9	3994.7	15	266.31	16.9
294			Trichloroacetic acid	76-03-9	1338.08	8.19	4999.4	30.6	5636.6	34.5	163.38	30.6
271			Styrene	100-42-5	343.73	3.3	4999.7	48	315.6	3.03	104.16	48
316			Toluene	108-88-3	1575.77	17.1	5003.7	54.3	NA	NA	92.15	54.3
320		N,N-	Dimethylacetamide	127-19-5	2108.79	24.2	5089.0	58.4	4618.4	53	87.14	58.4
224		n-	Butyl benzoate	136-60-7	73.08	0.41	5133.6	28.8	NA	NA	178.25	28.8
186			Zineb	12122-67-7	16.27	0.059	5211.3	18.9	7610.1	27.6	275.73	18.9
348		1-	Propanol	71-23-8	5800.62	96.5	5397.9	89.8	NA	NA	60.11	89.8
326			Halothane	151-67-7	6138.83	31.1	5684.8	28.8	NA	NA	197.39	28.8
128	10	2-	Propanol	67-63-0	10038.37	167	5842.7	97.2	NA	NA	60.11	97.2
50			Trypan blue	72-57-1	91.66	0.095	6204.2	6.43	NA	NA	964.88	6.43
108			Gibberellic acid	77-06-5	796.74	2.3	6304.7	18.2	NA	NA	346.41	18.2
335			Potassium hexacyano- ferrate II	13943-58-3	15582.05	42.3	6409.6	17.4	5009.8	13.6	368.37	17.4
59			Tetracycline * HCl	64-75-5	67.33	0.14	6444.6	13.4	NA	NA	480.94	13.4
127			Dimethyl phthalate	131-11-3	4544.28	23.4	6894.1	35.5	7204.8	37.1	194.2	35.5
286			Benzylpenicillin sodium	69-57-8	2042.17	5.73	6914.2	19.4	NA	NA	356.4	19.4
126			Triethyl citrate	77-93-0	4061.90	14.7	6990.9	25.3	NA	NA	276.32	25.3
85			Metamizol	68-89-3	193.94	0.58	7189.2	21.5	NA	NA	334.38	21.5
343			Magnesium II chloride * 6 H2O	7791-18-6	14314.43	70.4	8092.5	39.8	NA	NA	203.33	39.8
360	7		Ethylene glycol	107-21-1	34454.40	555	8567.0	138	7511.7	121	62.08	138
122			Diethyl phthalate	84-66-2	1226.88	5.52	8601.5	38.7	6178.8	27.8	222.26	38.7
289			Tetrachloroethene	127-18-4	1084.46	6.54	8854.8	53.4	8092.0	48.8	165.82	53.4
117			Di(2-ethylhexyl)adipate	103-23-1	1167.52	3.15	9117.7	24.6	NA	NA	370.64	24.6
162			Chlorhexidine	55-56-1	7.58	0.015	9200.5	18.2	9857.6	19.5	505.52	18.2
359			Acetone	67-64-1	25791.96	444	9759.1	168	NA	NA	58.09	168
18			Captan	133-06-2	1.17	0.0039	10009.6	33.3	7003.7	23.3	300.59	33.3
297	11	1,1,1	Trichloroethane	71-55-6	1374.02	10.3	10298.5	77.2	11245.6	84.3	133.4	77.2
353			Ethyl acetate	141-78-6	11279.36	128	11015.0	125	NA	NA	88.12	125
100		L-	Ascorbic acid	50-81-7	267.73	1.52	11907.1	67.6	3364.3	19.1	176.14	67.6
88			Dibutyl phthalate	84-74-2	211.57	0.76	11998.2	43.1	NA	NA	278.38	43.1
131			Glycerol	56-81-5	57476.64	624	12619.1	137	25975.0	282	92.11	137
361	8		Methanol	67-56-1	29806.50	930	13012.3	406	NA	NA	32.05	406
130	9		Ethanol	64-17-5	17464.32	379	14008.3	304	7787.5	169	46.08	304

Section 7.2
Table 7.2
Chemical Data from the Registry of Cytotoxicity Database (Sorted by Rat LD50 Oral mg/kg)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE		MW	Rodent LD50 (mmol/kg) for Regression
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg		
313			Xanthinol nicotinate	437-74-1	6865.26	15.8	14121.6	32.5	17336.9	39.9	434.51	32.5
258			Diethyl sebacate	110-40-7	421.19	1.63	14470.4	56	NA	NA	258.4	56
340			Diethylene glycol	111-46-6	6591.29	62.1	14753.5	139	23669.2	223	106.14	139
329			Sodium cyclamate	139-05-9	7123.90	35.4	15254.0	75.8	17004.8	84.5	201.24	75.8
352		1,2,6-	Hexanetriol	106-69-4	16506.60	123	15969.8	119	NA	NA	134.2	119
129			Dimethyl sulfoxide	75-18-3	19691.28	252	19691.3	252	16487.5	211	78.14	252
357			Propylene glycol	57-55-6	26029.62	342	20016.9	263	23974.7	315	76.11	263
355			D-Glucose	50-99-7	40720.68	226	25765.7	143	NA	NA	180.18	143
92			Di(2-ethylhexyl)phthalate	117 81-7	328.12	0.84	31015.2	79.4	29999.6	76.8	390.62	79.4
124			Acetazolamide	59 66-5	1886.99	8.49	NA	NA	4289.6	19.3	222.26	19.3
28			Aldosterone	52-39-1	5.05	0.014	NA	NA	NA	NA	360.44	
276			Ambuphylline	5634-34-4	988.51	3.67	NA	NA	600.7	2.23	269.35	2.23
3			Aminopterin	54-62-6	0.01	0.000012	NA	NA	3.0	0.0068	440.47	0.0068
120		5-	Aminosalicylic acid	89-57-6	776.47	5.07	NA	NA	7749.4	50.6	153.15	50.6
84			Amobarbital	57-43-2	126.73	0.56	NA	NA	344.0	1.52	226.31	1.52
189			Antimycin	11118-72-2	17.52	0.07	NA	NA	112.6	0.45	250.27	0.45
193		5-	Azacytidine	320-67-2	19.29	0.079	NA	NA	571.5	2.34	244.24	2.34
15		8-	Azaguanine	134-58-7	0.20	0.0013	NA	NA	1500.1	9.86	152.14	9.86
317			Barbital sodium	144 02-5	3835.32	18.6	NA	NA	800.1	3.88	206.2	3.88
33		p-	Chloromercuribenzoic acid	59-85-8	8.57	0.024	NA	NA	25.0	0.07	357.16	0.07
215			Chlorotetracycline	57-62-5	114.94	0.24	NA	NA	2500.0	5.22	478.92	5.22
77			Cinchophen	132-60-5	67.31	0.27	NA	NA	NA	NA	249.28	
6			Colchicine	64-86-8	0.02	0.000054	NA	NA	6.0	0.015	399.48	0.015
66			Cortisone	53-06-5	68.49	0.19	NA	NA	NA	NA	360.49	
274		L-	Cysteine	52-90-4	431.37	3.56	NA	NA	660.4	5.45	121.17	5.45
19			Cytochalasin B	14930 96-2	2.40	0.005	NA	NA	NA	NA	479.67	
133			Cytochalasin D	22144-77-0	0.05	0.000092	NA	NA	36.0	0.071	507.68	0.071
141			Cytosine arabinoside	147-94-4	0.17	0.00068	NA	NA	3137.9	12.9	243.25	12.9
23			Daraprim	58-14 0	2.21	0.0089	NA	NA	126.9	0.51	248.74	0.51
195		p,p'	DDA	83-05-6	27.83	0.099	NA	NA	590.4	2.1	281.14	2.1
34			Diethylstilbestrol	56-53-1	6.71	0.025	NA	NA	NA	NA	268.38	
22	6		Digoxin	20830-75-5	6.64	0.0085	NA	NA	18.0	0.023	781.05	0.023
293			Diisopropylamine dichloroacetate	660-27-5	1611.12	7	NA	NA	1700.9	7.39	230.16	7.39
82	44		Diphenylhydantoin	57-41-0	98.39	0.39	NA	NA	199.3	0.79	252.29	0.79
11			Doxorubicin * HCl	25316-40-9	0.19	0.00033	NA	NA	696.0	1.2	580.03	1.2
244			Doxylamine succinate	562-10-7	291.38	0.75	NA	NA	470.1	1.21	388.51	1.21
169			Epinephrine bitartrate	51-42-3	9.33	0.028	NA	NA	4.0	0.012	333.33	0.012
24			Ethylenediamine-tetraacetic acid	60-00-4	2.92	0.01	NA	NA	NA	NA	292.28	
171			Fumagillin	297-95-0	14.22	0.031	NA	NA	1999.5	4.36	458.6	4.36

Section 7.2
Table 7.2
Chemical Data from the Registry of Cytotoxicity Database (Sorted by Rat LD50 Oral mg/kg)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
222			Glibenclamide	10238-21-8	197.62	0.4	NA	NA	3250.8	6.58	494.05	6.58
32			Hydrocortisone	50-23-7	7.98	0.022	NA	NA	NA	NA	362.51	
236			Hydrogen peroxide 90%	7722-84-1	19.05	0.56	NA	NA	2000.4	58.8	34.02	58.8
267		p-	Hydroxybenzoic acid	99-96-7	403.34	2.92	NA	NA	2156.3	15.9	138.13	15.9
299			Imidazole	288-32-4	783.04	11.5	NA	NA	1879.3	27.6	68.09	27.6
46			Lead II chloride	7758-95-4	11.96	0.043	NA	NA	NA	NA	278.09	
327	20		Lithium I sulfate	10377-48-7	3704.98	33.7	NA	NA	1187.4	10.8	109.94	10.8
21		6-	Mercaptopurine	50-44-2	1.22	0.008	NA	NA	280.0	1.84	152.19	1.84
142			Methylmercury chloride	115-09-3	0.18	0.00071	NA	NA	57.7	0.23	251.08	0.23
98			Methylparaben	99-76-3	216.07	1.42	NA	NA	1749.8	11.5	152.16	11.5
319			Methylpentinol	77-75-8	2336.21	23.8	NA	NA	525.2	5.35	98.16	5.35
175			Norepinephrine	51-41-2	6.60	0.039	NA	NA	20.3	0.12	169.2	0.12
268		1-	Octanol	111-87-5	398.60	3.06	NA	NA	1784.6	13.7	130.26	13.7
7			Ouabain	630-60-4	0.04	0.000072	NA	NA	NA	NA	584.73	
240			Pentoxifylline	6493-05-6	183.71	0.66	NA	NA	1386.2	4.98	278.35	4.98
146			Potassium bichromate VI	7778-50-9	0.59	0.002	NA	NA	191.2	0.65	294.2	0.65
145			Potassium chromate VI	7789-00-6	0.29	0.0015	NA	NA	180.6	0.93	194.2	0.93
277			Potassium cyanate	590-28-3	335.84	4.14	NA	NA	843.6	10.4	81.12	10.4
266			Potassium hexacyanoferrate III	13746-66-2	928.54	2.82	NA	NA	2970.0	9.02	329.27	9.02
36			Progesterone	57-83-0	9.44	0.03	NA	NA	NA	NA	314.51	
54	23		Propranolol * HCl	318-98-9	35.50	0.12	NA	NA	470.4	1.59	295.84	1.59
209			Propylparaben	94-13-3	32.44	0.18	NA	NA	6325.7	35.1	180.22	35.1
12			Puromycin	53-79-2	0.16	0.00033	NA	NA	674.4	1.43	471.58	1.43
201		13-cis-	Retinoic acid	4759-48-2	36.06	0.12	NA	NA	3395.4	11.3	300.48	11.3
314			Saccharin	81-07-2	3004.22	16.4	NA	NA	17000.0	92.8	183.19	92.8
178			Salicylanilide	87-17-2	9.81	0.046	NA	NA	2409.7	11.3	213.25	11.3
161			Silver I nitrate	7761-88-8	2.21	0.013	NA	NA	49.3	0.29	169.88	0.29
30			Sodium arsenate, dibasic	7778-43-0	2.79	0.015	NA	NA	NA	NA	185.91	
290			Sodium sulfite	7757-83-7	854.55	6.78	NA	NA	820.5	6.51	126.04	6.51
156			Stearyltrimethylammoniumchloride	112-03-8	2.09	0.006	NA	NA	536.1	1.54	348.13	1.54
265			Streptomycin sulfate	298-39-5	3979.25	2.73	NA	NA	495.6	0.34	1457.6	0.34
5		K-	Strophantin		0.03	0.000044	NA	NA	NA	NA	710.9	
93			Sulfisoxazole	127-69-5	227.23	0.85	NA	NA	6790.2	25.4	267.33	25.4
135		2,3,7,8-	Tetrachloro-dibenzo-p-dioxin	1746-01-6	0.06	0.0002	NA	NA	0.1	0.00035	321.96	0.00035
247		(+)-	Thalidomide	731-40-8	209.18	0.81	NA	NA	400.3	1.55	258.25	1.55
203			Thallium I acetate	563-68-8	36.88	0.14	NA	NA	34.2	0.13	263.42	0.13
181	30		Thallium I sulfate	7446-18-6	27.26	0.054	NA	NA	28.8	0.057	504.8	0.057
105	21		Theophylline	58-55-9	329.75	1.83	NA	NA	600.0	3.33	180.19	3.33
303			Theophylline sodium	3485-82-3	2519.43	12.4	NA	NA	445.0	2.19	203.18	2.19

Section 7.2
Table 7.2
Chemical Data from the Registry of Cytotoxicity Database (Sorted by Rat LD50 Oral mg/kg)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
25			Thio-TEPA	52-24-4	2.08	0.011	NA	NA	37.8	0.2	189.24	0.2
140		6	Thioguanine	154-42-7	0.10	0.00057	NA	NA	160.5	0.96	167.21	0.96
83			Thiopental	76-75-5	133.30	0.55	NA	NA	601.1	2.48	242.37	2.48
170	29		Thioridazine * HCl	130-61-0	11.81	0.029	NA	NA	358.2	0.88	407.07	0.88
104			Tolbutamide	64-77-7	489.39	1.81	NA	NA	2601.1	9.62	270.38	9.62
1			Trenimon	68-76-8	0.00	0.0000033	NA	NA	NA	NA	231.28	
231			Tween 80	9005-65-6	641.90	0.49	NA	NA	25021.0	19.1	1310	19.1
323			Urethan	51-79-6	2307.95	25.9	NA	NA	2504.0	28.1	89.11	28.1
250			Valproate sodium	1069-66-5	166.22	1	NA	NA	1695.4	10.2	166.22	10.2
4			Vincristine sulfate	2068-78-2	0.01	0.000015	NA	NA	NA	NA	923.14	

Section 7.2
Table 7.3
Chemical Data from the Registry of Cytotoxicity Data Bank (Alphabetical))

RC #	MEIC #		Chemical	CAS #	IC50s		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
263			Acetaldehyde	75-07-0	107.95	2.45	1929.8	43.8	NA	NA	44.06	43.8
113	1		Acetaminophen	103-90-2	409.70	2.71	2403.8	15.9	338.6	2.24	151.18	15.9
124			Acetazolamide	59-66-5	1886.99	8.49	NA	NA	4289.6	19.3	222.26	19.3
321			Acetic acid	64-19-7	1459.46	24.3	3309.3	55.1	4961.0	82.6	60.06	55.1
359			Acetone	67-64-1	25791.96	444	9759.1	168	NA	NA	58.09	168
358			Acetonitrile	75-05-8	15110.08	368	3798.1	92.5	NA	NA	41.06	92.5
107	2		Acetylsalicylic acid	50-78-2	408.99	2.27	999.9	5.55	814.4	4.52	180.17	5.55
179			Acrolein	107-02-8	2.64	0.047	46.0	0.82	39.8	0.71	56.07	0.82
102			Acrylamide	79-06-1	114.45	1.61	169.9	2.39	169.9	2.39	71.09	2.39
110			Acrylonitrile	107-13-1	128.43	2.42	81.7	1.54	27.1	0.51	53.07	1.54
2			Actinomycin D	50-76-0	0.01	0.0000081	7.2	0.0057	12.6	0.01	1255.6	0.0057
37			Aflatoxin B1	1162 65-8	10.62	0.034	5.0	0.016	9.1	0.029	312.29	0.016
28			Aldosterone	52-39-1	5.05	0.014	NA	NA	NA	NA	360.44	
43			Aldrin	309-00-2	24.45	0.067	40.1	0.11	43.8	0.12	364.9	0.11
52			all-trans-Retinoic acid	302-79-4	33.05	0.11	2001.2	6.66	NA	NA	300.48	6.66
292			Allylalcohol	107-18-6	403.14	6.94	63.9	1.1	95.8	1.65	58.09	1.1
174			Ambazone	539-21-9	9.02	0.038	749.9	3.16	999.1	4.21	237.32	3.16
276			Ambuphylline	5634-34-4	988.51	3.67	NA	NA	600.7	2.23	269.35	2.23
9			Amethopterin	59-05 2	0.06	0.00014	136.4	0.3	145.4	0.32	454.5	0.3
121			Aminophenazone	58-15-1	1246.87	5.39	999.3	4.32	358.6	1.55	231.33	4.32
248		m-	Aminophenol	591-27-5	93.86	0.86	1658.9	15.2	NA	NA	109.14	15.2
42		p-	Aminophenol	23-30-8	6.77	0.062	1658.9	15.2	NA	NA	109.14	15.2
3			Aminopterin	54-62-6	0.01	0.000012	NA	NA	> 3.0	0.0068	440.47	0.0068
120		5-	Aminosalicylic acid	89-57-6	776.47	5.07	NA	NA	7749.4	50.6	153.15	50.6
183	5		Amitriptyline	50-48-6	15.54	0.056	319.1	1.15	147.0	0.53	277.44	1.15
284			Ammonium chloride	12125-02 9	295.32	5.52	1647.8	30.8	NA	NA	53.5	30.8
213			Ammonium persulfate	7727-54-0	52.49	0.23	819.3	3.59	NA	NA	228.22	3.59
225			Ammonium sulfide	12135-76-1	21.47	0.42	168.2	3.29	NA	NA	51.12	3.29
84			Amobarbital	57-43-2	126.73	0.56	NA	NA	344.0	1.52	226.31	1.52
262	47		Amphetamine sulfate	60-13-9	726.02	1.97	55.3	0.15	24.0	0.065	368.54	0.15
217			Amrinone	60719-84-8	52.42	0.28	101.1	0.54	288.3	1.54	187.22	0.54
291			Aniline	62-53-3	642.67	6.9	439.6	4.72	439.6	4.72	93.14	4.72
243		p-	Anisidine	104-94-9	89.91	0.73	1404.1	11.4	NA	NA	123.17	11.4
306			Anisole	100-66-3	1427.58	13.2	3698.7	34.2	NA	NA	108.15	34.2
189			Antimycin	11118-72-2	17.52	0.07	NA	NA	112.6	0.45	250.27	0.45
300			Antipyrine	60-80-0	2183.70	11.6	1799.7	9.56	1699.9	9.03	188.25	9.56
153	26		Arsenic III trioxide	1327-53-3	0.83	0.0042	19.8	0.1	45.5	0.23	197.84	0.1
100		L-	Ascorbic acid	50-81-7	267.73	1.52	11907.1	67.6	3364.3	19.1	176.14	67.6
70	49		Atropine sulfate	55-48-1	148.92	0.22	622.7	0.92	764.9	1.13	676.9	0.92

Section 7.2
Table 7.3
Chemical Data from the Registry of Cytotoxicity Data Bank (Alphabetical))

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
193		5-	Azacytidine	320-67-2	19.29	0.079	NA	NA	571.5	2.34	244.24	2.34
15		8-	Azaguanine	134-58-7	0.20	0.0013	NA	NA	1500.1	9.86	152.14	9.86
16			Azaserine	115-02-6	0.35	0.002	169.7	0.98	150.6	0.87	173.15	0.98
204			Azathioprine	446-86-6	38.82	0.14	535.2	1.93	1389.2	5.01	277.29	1.93
317			Barbital sodium	144-02-5	3835.32	18.6	NA	NA	800.1	3.88	206.2	3.88
246	37		Barium II nitrate	10022-31-8	211.70	0.81	355.4	1.36	NA	NA	261.36	1.36
64			Bendiocarb	22781-23-3	40.19	0.18	178.6	0.8	NA	NA	223.25	0.8
155			Benzalkonium chloride	8001-54-5	1.90	0.0052	401.5	1.1	339.5	0.93	365	1.1
312			Benzoic acid	65-85-0	1917.44	15.7	2528.1	20.7	2369.3	19.4	122.13	20.7
287			Benzylalcohol	100-51-6	628.35	5.81	1232.9	11.4	1579.0	14.6	108.15	11.4
286			Benzylpenicillin sodium	69-57-8	2042.17	5.73	6914.2	19.4	NA	NA	356.4	19.4
237			Beryllium II sulfate	13510-49-1	64.09	0.61	82.0	0.78	79.9	0.76	105.07	0.78
192		1,3-	Bis(2-chloroethyl)- 1-nitrosourea	154-93-8	16.70	0.078	19.9	0.093	19.1	0.089	214.07	0.093
273			Bromobenzene	108-86-1	543.29	3.46	2700.7	17.2	NA	NA	157.02	17.2
254			Busulfan	55837-25-7	415.03	1.35	365.8	1.19	NA	NA	307.43	1.19
177			Busulphan	55-98-1	11.33	0.046	1.9	0.0076	199.5	0.81	246.32	0.0076
305		n-	Butanal	123-72-8	923.14	12.8	2488.1	34.5	NA	NA	72.12	34.5
338		1-	Butanol	71-36-3	3892.35	52.5	793.3	10.7	NA	NA	74.14	10.7
324		2-	Butoxyethanol	111-76-2	3073.20	26	1477.5	12.5	1229.3	10.4	118.2	12.5
224		n-	Butyl benzoate	136-60-7	73.08	0.41	5133.6	28.8	NA	NA	178.25	28.8
188		t-	Butyl hydroquinone	1948-33-0	11.47	0.069	799.6	4.81	1000.8	6.02	166.24	4.81
72			Butylated hydoxyanisole	8003-24-5	43.26	0.24	2199.3	12.2	2001.0	11.1	180.27	12.2
184			Butylated hydroxytoluene	128-37-0	12.34	0.056	890.4	4.04	1040.2	4.72	220.39	4.04
20			Cadmium II chloride	10108-64-2	1.17	0.0064	88.0	0.48	174.1	0.95	183.3	0.48
112	48		Caffeine	58-08-2	512.74	2.64	192.3	0.99	619.6	3.19	194.22	0.99
285			Caffeine sodium benzoate	8000 95-1	1918.33	5.67	859.4	2.54	798.5	2.36	338.33	2.54
304			Calcium II chloride	10043-52-4	1376.15	12.4	999.9	9.01	NA	NA	110.98	9.01
18			Captan	133-06-2	1.17	0.0039	10009.6	33.3	7003.7	23.3	300.59	33.3
73			Carbaryl	63-25-2	52.32	0.26	249.5	1.24	438.7	2.18	201.24	1.24
125	34		Carbon tetrachloride	56-23-5	1308.92	8.51	2799.3	18.2	12797.0	83.2	153.81	18.2
211			Catechol	120-80-9	22.02	0.2	3887.2	35.3	259.9	2.36	110.12	35.3
163			Cetyltrimethylammonium chloride	112-02-7	7.61	0.021	474.4	1.31	NA	NA	362.16	1.31
264			Chloral hydrate	302-17-0	438.31	2.65	479.7	2.9	1101.6	6.66	165.4	2.9
190			Chlorambucil	305-03-3	23.12	0.076	76.1	0.25	100.4	0.33	304.24	0.25
91	45		Chloramphenicol	56-75-7	255.29	0.79	3393.1	10.5	2640.1	8.17	323.15	10.5
40			Chlordan	57-74-9	24.59	0.06	458.9	1.12	NA	NA	409.76	1.12
162			Chlorhexidine	55-56-1	7.58	0.015	9200.5	18.2	9857.6	19.5	505.52	18.2
308	33		Chloroform	67-66-3	1599.56	13.4	908.4	7.61	35.8	0.3	119.37	7.61
33		p-	Chloromercuribenzoic acid	59-85-8	8.57	0.024	NA	NA	25.0	0.07	357.16	0.07

Section 7.2
Table 7.3
Chemical Data from the Registry of Cytotoxicity Data Bank (Alphabetical))

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
31	41		Chloroquine diphosphate	50-63-5	8.77	0.017	969.9	1.88	500.4	0.97	515.92	1.88
41			Chloroquine sulfate	132-73-0	25.08	0.06	1086.8	2.6	NA	NA	418	2.6
215			Chlorotetracycline	57-62-5	114.94	0.24	NA	NA	2500.0	5.22	478.92	5.22
27			Chlorpromazine	50-53-3	4.46	0.014	140.3	0.44	261.5	0.82	318.89	0.44
149			Chromium VI trioxide	1333-82-0	0.27	0.0027	80.0	0.8	127.0	1.27	100	0.8
77			Cinchophen	132-60-5	67.31	0.27	NA	NA	NA	NA	249.28	
150			Cis-platinum	15663-27-1	0.84	0.0028	25.8	0.086	33.0	0.11	300.07	0.086
111			Clofibric acid	882-09-7	560.26	2.61	1249.3	5.82	1169.9	5.45	214.66	5.82
62			Cobalt II chloride	7646-79-9	20.77	0.16	80.5	0.62	80.5	0.62	129.83	0.62
6			Colchicine	64-86-8	0.02	0.000054	NA	NA	6.0	0.015	399.48	0.015
66			Cortisone	53-06-5	68.49	0.19	NA	NA	NA	NA	360.49	
260			Coumarin	91-64-5	249.92	1.71	292.3	2	195.8	1.34	146.15	2
239		m-	Cresol	108-39-4	71.38	0.66	242.3	2.24	828.4	7.66	108.15	2.24
232		o-	Cresol	95-48-7	56.24	0.52	121.1	1.12	343.9	3.18	108.15	1.12
212		p-	Cresol	106-44-5	23.79	0.22	206.6	1.91	343.9	3.18	108.15	1.91
199			Cupric chloride	7447-39-4	14.79	0.11	139.8	1.04	189.6	1.41	134.44	1.04
81	27		Cupric sulfate * 5 H2O	7758-99-8	82.40	0.33	299.6	1.2	NA	NA	249.7	1.2
249		3-	Cyano-2-morpholino-5-(pyrid-4-yl)-pyridine (Chemical 122)		255.66	0.96	346.2	1.3	NA	NA	266.31	1.3
325			Cyclohexanol	108-93-0	2634.73	26.3	2063.7	20.6	NA	NA	100.18	20.6
13			Cycloheximide	66-81-9	0.17	0.00059	2.0	0.0071	132.3	0.47	281.39	0.0071
116			Cyclophosphamide * H2O	6055-19-2	870.89	3.12	94.9	0.34	136.8	0.49	279.13	0.34
96			Cygon	60-51-5	284.29	1.24	151.3	0.66	59.6	0.26	229.27	0.66
274		L-	Cysteine	52-90-4	431.37	3.56	NA	NA	660.4	5.45	121.17	5.45
19			Cytochalasin B	14930-96-2	2.40	0.005	NA	NA	NA	NA	479.67	
133			Cytochalasin D	22144-77-0	0.05	0.000092	NA	NA	36.0	0.071	507.68	0.071
141			Cytosine arabinoside	147-94-4	0.17	0.00068	NA	NA	3137.9	12.9	243.25	12.9
355			D-Glucose	50-99-7	40720.68	226	25765.7	143	NA	NA	180.18	143
23			Daraprim	58-14-0	2.21	0.0089	NA	NA	126.9	0.51	248.74	0.51
195		p,p'	DDA	83-05-6	27.83	0.099	NA	NA	590.4	2.1	281.14	2.1
167		p,p'	DDD	72-54-8	7.68	0.024	112.0	0.35	NA	NA	320.04	0.35
197		p,p'	DDE	72-55-9	31.80	0.1	880.9	2.77	NA	NA	318.02	2.77
61		p,p'	DDT	50-29-3	56.72	0.16	113.4	0.32	134.7	0.38	354.48	0.32
229	22		Dextropropoxyphene * HCl	1639-60-7	184.23	0.49	82.7	0.22	82.7	0.22	375.98	0.22
117			Di(2-ethylhexyl)adipate	103-23-1	1167.52	3.15	9117.7	24.6	NA	NA	370.64	24.6
92			Di(2-ethylhexyl)phthalate	117-81-7	328.12	0.84	31015.2	79.4	29999.6	76.8	390.62	79.4
63	4		Diazepam	439-14-5	45.56	0.16	709.1	2.49	535.3	1.88	284.76	2.49
281		1,2-	Dibromomethane	106-93-4	730.17	4.2	107.8	0.62	NA	NA	173.85	0.62
88			Dibutyl phthalate	84-74-2	211.57	0.76	11998.2	43.1	NA	NA	278.38	43.1
298			Dichloroacetic acid	79-43-6	1482.81	11.5	2823.8	21.9	5518.6	42.8	128.94	21.9

Section 7.2
Table 7.3
Chemical Data from the Registry of Cytotoxicity Data Bank (Alphabetical))

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
328	36		Dichloromethane	75-09-2	2964.06	34.9	1596.7	18.8	NA	NA	84.93	18.8
158			Dichlorophene	97-23-4	2.23	0.0083	2691.3	10	1001.2	3.72	269.13	10
39		2,4-	Dichlorophenol	120-83-2	8.97	0.055	580.3	3.56	1600.7	9.82	163	3.56
89	16	2,4-	Dichlorophenoxy- acetic acid	94-75-7	170.20	0.77	369.1	1.67	366.9	1.66	221.04	1.67
168			Dicoumarol	66-76-2	9.08	0.027	709.6	2.11	232.1	0.69	336.31	2.11
207			Dieldrin	60-57-1	68.56	0.18	45.7	0.12	38.1	0.1	380.9	0.12
122			Diethyl phthalate	84-66-2	1226.88	5.52	8601.5	38.7	6178.8	27.8	222.26	38.7
258			Diethyl sebacate	110-40-7	421.19	1.63	14470.4	56	NA	NA	258.4	56
136			Diethyldithiocarbamate sodium* 3H20	20624-25-3	0.09	0.00039	1500.7	6.66	1500.7	6.66	225.33	6.66
340			Diethylene glycol	111-46-6	6591.29	62.1	14753.5	139	23669.2	223	106.14	139
34			Diethylstilbestrol	56-53-1	6.71	0.025	NA	NA	NA	NA	268.38	
8			Digitoxin	71-63-6	0.08	0.00011	55.8	0.073	NA	NA	765.05	0.073
22	6		Digoxin	20830-75-5	6.64	0.0085	NA	NA	18.0	0.023	781.05	0.023
58			Dihydralazine sulfate	7327-87-9	40.36	0.14	818.8	2.84	400.8	1.39	288.32	2.84
293			Diisopropylamine dichloroacetate	660-27-5	1613.12	7	NA	NA	1700.9	7.39	230.16	7.39
191			Dimenhydrinate	523-87-5	35.72	0.076	1320.8	2.81	202.1	0.43	470.02	2.81
127			Dimethyl phthalate	131-11-3	4544.28	23.4	6894.1	35.5	7204.8	37.1	194.2	35.5
129			Dimethyl sulfoxide	75-18-3	19691.28	252	19691.3	252	16487.5	211	78.14	252
320		N,N-	Dimethylacetamide	127-19-5	2108.79	24.2	5089.0	58.4	4618.4	53	87.14	58.4
200			Dimethylaminoethyl methacrylate (polymer)	2867-47-2	17.30	0.11	1745.4	11.1	NA	NA	157.24	11.1
351			Dimethylformamide	68-12-2	8334.54	114	2800.1	38.3	3750.5	51.3	73.11	38.3
220		m-	Dinitrobenzene	99-65-0	65.57	0.39	82.4	0.49	NA	NA	168.12	0.49
68		2,4-	Dinitrophenol	51-28-5	38.67	0.21	29.5	0.16	44.2	0.24	184.12	0.16
332		1,4-	Dioxane	123-91-1	3357.37	38.1	4203.3	47.7	5701.4	64.7	88.12	47.7
71			Diphenhydramine * HCl	147-24-0	70.04	0.24	855.1	2.93	113.8	0.39	291.85	2.93
82	44		Diphenylhydantoin	57-41-0	98.39	0.39	NA	NA	199.3	0.79	252.29	0.79
206			Diquat dibromide	85-00-7	55.05	0.16	230.5	0.67	234.0	0.68	344.08	0.67
51			Disulfoton	298-04-4	30.19	0.11	2.0	0.0073	5.5	0.02	274.42	0.0073
226			Dodecylbenzene sodiumsulfonate	25155-30-0	146.38	0.42	1261.6	3.62	2000.5	5.74	348.52	3.62
57		L-	Dopa	59-92-7	25.64	0.13	1780.8	9.03	2366.5	12	197.21	9.03
11			Doxorubicin * HCl	25316-40-9	0.19	0.00033	NA	NA	696.0	1.2	580.03	1.2
244			Doxylamine succinate	562-10-7	291.38	0.75	NA	NA	470.1	1.21	388.51	1.21
10			Emetine	483-18-1	0.08	0.00016	67.3	0.14	NA	NA	480.71	0.14
169			Epinephrine bitartrate	51-42-3	9.33	0.028	NA	NA	4.0	0.012	333.33	0.012
130	9		Ethanol	64-17-5	17464.32	379	14008.3	304	7787.5	169	46.08	304
353			Ethyl acetate	141-78-6	11279.36	128	11015.0	125	NA	NA	88.12	125
349			Ethyl methyl ketone	78-93-3	7500.48	104	3396.9	47.1	NA	NA	72.12	47.1
307		2-	Ethylbutanal	97-96-1	1322.38	13.2	3977.1	39.7	NA	NA	100.18	39.7
360	7		Ethylene glycol	107-21-1	34454.40	555	8567.0	138	7511.7	121	62.08	138

Section 7.2
Table 7.3
Chemical Data from the Registry of Cytotoxicity Data Bank (Alphabetical))

RC #	MEIC #	Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
				ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
24		Ethylenediamine-tetraacetic acid	60-00-4	2.92	0.01	NA	NA	NA	NA	292.28	
261	3	Ferrous sulfate	7720-78-7	281.03	1.85	319.0	2.1	978.3	6.44	151.91	2.1
35		Flufenamic acid	530-78-9	8.16	0.029	272.8	0.97	714.4	2.54	281.25	0.97
17		5-Fluorouracil	51-21-8	0.34	0.0026	230.3	1.77	114.5	0.88	130.09	1.77
202		Formaldehyde	50-00-0	3.60	0.12	798.8	26.6	NA	NA	30.03	26.6
109		Frusemide	54-31-9	770.67	2.33	2599.8	7.86	4597.6	13.9	330.76	7.86
171		Fumagillin	297-95-0	14.22	0.031	NA	NA	1999.5	4.36	458.6	4.36
108		Gibberellic acid	77-06-5	796.74	2.3	6304.7	18.2	NA	NA	346.41	18.2
222		Glibenclamide	10238-21-8	197.62	0.4	NA	NA	3250.8	6.58	494.05	6.58
101		Glutethimide	77-21-4	338.97	1.56	599.7	2.76	360.7	1.66	217.29	2.76
131		Glycerol	56-81-5	57476.64	624	12619.1	137	25975.0	282	92.11	137
326		Halothane	151-67-7	6138.83	31.1	5684.8	28.8	NA	NA	197.39	28.8
185		Heptachlor	76-44-8	22.02	0.059	41.1	0.11	67.2	0.18	373.3	0.11
288		1-Heptanol	111-70-6	726.44	6.25	3254.4	28	1499.4	12.9	116.23	28
151		Hexachlorocyclopentadiene	77-47-4	0.85	0.0031	111.8	0.41	NA	NA	272.75	0.41
157	38	Hexachlorophene	70-30-4	3.21	0.0079	61.0	0.15	65.1	0.16	406.89	0.15
159		Hexadecyltrimethylammoniumbromide	57-09-0	3.24	0.0089	408.3	1.12	NA	NA	364.53	1.12
295		2,5-Hexanedione	110-13-4	964.65	8.45	2705.6	23.7	NA	NA	114.16	23.7
352		1,2,6-Hexanetriol	106-69-4	16506.60	123	15969.8	119	NA	NA	134.2	119
311		1-Hexanol	111-27-3	1573.88	15.4	719.5	7.04	1952.0	19.1	102.2	7.04
187		4-Hexylresorcinol	136-77-6	12.44	0.064	549.9	2.83	NA	NA	194.3	2.83
296		Homatropine methylbromide	80-49-9	3332.97	9	1199.9	3.24	1399.8	3.78	370.33	3.24
219		Hydralazine	86-54-4	52.87	0.33	89.7	0.56	121.8	0.76	160.2	0.56
32		Hydrocortisone	50-23-7	7.98	0.022	NA	NA	NA	NA	362.51	
236		Hydrogen peroxide 90%	7722-84-1	19.05	0.56	NA	NA	2000.4	58.8	34.02	58.8
267		p-Hydroxybenzoic acid	99-96-7	403.34	2.92	NA	NA	2196.3	15.9	138.13	15.9
152		8-Hydroxyquinoline	148-24-3	0.48	0.0033	1200.6	8.27	NA	NA	145.17	8.27
44		Hydroxyzine * HCl	1244-76-4	27.56	0.067	950.4	2.31	NA	NA	411.41	2.31
233		Ibuprofen	15687-27-1	107.28	0.52	1008.9	4.89	980.0	4.75	206.31	4.89
299		Imidazole	288-32-4	783.04	11.5	NA	NA	1879.3	27.6	68.09	27.6
238		Imidazolidinyl urea	39236-46-9	100.17	0.36	2598.9	9.34	3700.9	13.3	278.26	9.34
38		Imipramine * HCl	113-52-0	17.11	0.054	304.2	0.96	374.0	1.18	316.91	0.96
60		Indomethacin	53-86-1	57.25	0.16	12.2	0.034	19.0	0.053	357.81	0.034
198		Ioxynil	1689-83-4	40.80	0.11	111.3	0.3	NA	NA	370.91	0.3
90		Iproniazid	54-92-2	141.61	0.79	365.7	2.04	681.2	3.8	179.25	2.04
315		Isobenzoic furano dione		2518.04	17	4014.1	27.1	1999.6	13.5	148.12	27.1
309		Isobutanal	78-84-2	973.62	13.5	2812.7	39	NA	NA	72.12	39
334		Isobutanol	78-83-1	2973.01	40.1	2461.4	33.2	NA	NA	74.14	33.2
123	35	Isoniazid	54-85-3	1027.33	7.49	650.1	4.74	NA	NA	137.16	4.74

Section 7.2
Table 7.3
Chemical Data from the Registry of Cytotoxicity Data Bank (Alphabetical))

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
257			Isononylaldehyde	5435-64-3	216.25	1.52	3243.8	22.8	NA	NA	142.27	22.8
165			Isoproterenol * HCl	51-30-9	5.45	0.022	2219.8	8.96	NA	NA	247.75	8.96
253			Isoxepac	55453-87-7	356.81	1.33	198.5	0.74	NA	NA	268.28	0.74
26			Kelthane	115-32-2	4.45	0.012	574.2	1.55	418.6	1.13	370.48	1.55
341			Lactic acid	598-82-3	5945.94	66	3729.7	41.4	4873.9	54.1	90.09	41.4
46			Lead II chloride	7758-95-4	11.96	0.043	NA	NA	NA	NA	278.09	
223	32		Lindane	58-89-9	119.24	0.41	75.6	0.26	87.2	0.3	290.82	0.26
333			Lithium I chloride	7447-41-8	1636.25	38.6	758.8	17.9	1165.7	27.5	42.39	17.9
327	20		Lithium I sulfate	10377-48-7	3704.98	33.7	NA	NA	1187.4	10.8	109.94	10.8
343			Magnesium II chloride * 6 H2O	7791-18-6	14314.43	70.4	8092.5	39.8	NA	NA	203.33	39.8
67	15		Malathion	121-75-5	66.08	0.2	885.4	2.68	776.4	2.35	330.38	2.68
154			Maneb	12427-38-2	1.12	0.0042	4500.6	16.9	3994.7	15	266.31	16.9
56			Manganese IIchloride *4 H2O	13446-34-9	25.73	0.13	1484.4	7.5	NA	NA	197.92	7.5
48			Mefenamic acid	61-68-7	20.99	0.087	789.1	3.27	629.8	2.61	241.31	3.27
94			Menthol	89-78-1	148.49	0.95	3172.9	20.3	NA	NA	156.3	20.3
21		6-	Mercaptopurine	50-44-2	1.22	0.008	NA	NA	280.0	1.84	152.19	1.84
29	28		Mercury II chloride	7487-94-7	4.07	0.015	1.0	0.0037	10.0	0.037	271.49	0.0037
85			Metamizol	68-89-3	193.94	0.58	7189.2	21.5	NA	NA	334.38	21.5
361	8		Methanol	67-56-1	29806.50	930	13012.3	406	NA	NA	32.05	406
356		2-	Methoxyethanol	109-86-4	19103.61	251	2458.4	32.3	NA	NA	76.11	32.3
259			Methyl salicylate	119-36-8	258.67	1.7	887.1	5.83	NA	NA	152.16	5.83
160		N-	Methyl-N'-nitro-N-nitroso- guanidine	70-25-7	1.77	0.012	89.7	0.61	NA	NA	147.12	0.61
78		6-	Methylcoumarin	92-48-8	49.66	0.31	1681.9	10.5	NA	NA	160.18	10.5
142			Methylmercury chloride	115-09-3	0.18	0.00071	NA	NA	57.7	0.23	251.08	0.23
98			Methylparaben	99-76-3	216.07	1.42	NA	NA	1749.8	11.5	152.16	11.5
319			Methylpentinol	77-75-8	2336.21	23.8	NA	NA	525.2	5.35	98.16	5.35
283			Milrinone	78415-72-2	1007.61	4.77	90.8	0.43	137.3	0.65	211.24	0.43
14			Mitomycin C	50-07-7	0.28	0.00084	14.0	0.042	17.1	0.051	334.37	0.042
147			Mitoxantrone	65271-80-9	1.07	0.0024	586.8	1.32	NA	NA	444.54	1.32
172			Nabam	142-59-6	8.97	0.035	394.8	1.54	579.3	2.26	256.34	1.54
47			Naftipramide	1505-95-9	25.07	0.084	1029.7	3.45	1086.4	3.64	298.47	3.45
99			Nalidixic acid	389-08-2	348.39	1.5	1349.4	5.81	571.4	2.46	232.26	5.81
114			Narulan * HCl	366-70-1	706.37	2.74	783.7	3.04	NA	NA	257.8	3.04
74			Nickel II chloride	7718-54-9	34.99	0.27	105.0	0.81	NA	NA	129.61	0.81
336			Nicotinamide	98-92-0	5423.02	44.4	3505.4	28.7	NA	NA	122.14	28.7
103	18		Nicotine	54-11-5	290.45	1.79	50.3	0.31	24.3	0.15	162.26	0.31
275			Nitrilotriacetic acid	139-13-9	690.09	3.61	1470.0	7.69	3154.1	16.5	191.16	7.69
221		2-	Nitro-p-phenylene-diamine	5307-14-2	59.73	0.39	3078.5	20.1	NA	NA	153.16	20.1
302			Nitrobenzene	98-95-3	1502.06	12.2	640.2	5.2	NA	NA	123.12	5.2

Section 7.2
Table 7.3
Chemical Data from the Registry of Cytotoxicity Data Bank (Alphabetical))

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE		Rodent LD50 (mmol/kg)	
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
148			Nitrogen mustard * HCl	55-86-7	0.50	0.0026	10.0	0.052	19.3	0.1	192.53	0.052
210		p-	Nitrophenol	100-02-7	27.82	0.2	350.6	2.52	467.4	3.36	139.12	2.52
339		1-	Nitropropane	79-46-9	5159.47	57.9	455.4	5.11	NA	NA	89.11	5.11
175			Norepinephrine	51-41-2	6.60	0.039	NA	NA	20.3	0.12	169.2	0.12
268		1-	Octanol	111-87-5	398.60	3.06	NA	NA	1784.6	13.7	130.26	13.7
230	42		Orphenadrine * HCl	341-69-5	149.88	0.49	425.2	1.39	125.4	0.41	305.88	1.39
7			Ouabain	630-60-4	0.04	0.000072	NA	NA	NA	NA	584.73	
164			Oxatomide	60607-34-3	8.11	0.019	1412.1	3.31	9598.7	22.5	426.61	3.31
65			Oxyphenbutazone	129-20-4	61.64	0.19	999.2	3.08	480.1	1.48	324.41	3.08
176			Papaverine	58-74-2	15.27	0.045	325.8	0.96	230.8	0.68	339.42	0.96
235	25		Paraquat	4685-14-7	100.58	0.54	57.7	0.31	195.6	1.05	186.25	0.31
49			Parathion	56-38-2	27.09	0.093	2.0	0.0069	6.1	0.021	291.28	0.0069
173	39		Pentachlorophenol	87-86-5	9.59	0.036	50.6	0.19	NA	NA	266.32	0.19
322		1-	Pentanol	71-41-0	2195.43	24.9	3023.0	34.4	200.1	2.27	88.17	34.4
87			Pentobarbital sodium	57-33-0	176.29	0.71	201.1	0.81	280.6	1.13	248.29	0.81
240			Pentoxifylline	6493-05-6	183.71	0.66	NA	NA	1386.2	4.98	278.35	4.98
97			Phenacetin	62-44-2	227.63	1.27	1650.8	9.21	1220.6	6.81	179.24	9.21
118	24		Phenobarbital	50-06-6	884.91	3.81	162.6	0.7	167.2	0.72	232.26	0.7
115	12		Phenol	108-95-2	283.30	3.01	414.1	4.4	300.2	3.19	94.12	4.4
79			Phenylbutazone	50-33-9	98.69	0.32	376.3	1.22	441.0	1.43	308.41	1.22
218		o-	Phenylenediamine	95-54-5	33.53	0.31	1069.7	9.89	NA	NA	108.16	9.89
180		p-	Phenylenediamine	106-50-3	5.41	0.05	80.0	0.74	NA	NA	108.16	0.74
282		(-)-	Phenylephrine	59-42-7	744.17	4.45	349.5	2.09	NA	NA	167.23	2.09
278			Phenylephrine * HCl	939-38-8	847.35	4.16	350.3	1.72	120.2	0.59	203.69	1.72
234			Phenylthiourea	103-85-5	82.20	0.54	3.0	0.02	10.0	0.066	152.23	0.02
342			Piperazine	110-85-0	5789.95	67.2	1904.1	22.1	1438.9	16.7	86.16	22.1
146			Potassium bichromate VI	7778-50-9	0.59	0.002	NA	NA	191.2	0.65	294.2	0.65
145			Potassium chromate VI	7789-00-6	0.29	0.0015	NA	NA	180.6	0.93	194.2	0.93
277			Potassium cyanate	590-28-3	335.84	4.14	NA	NA	843.6	10.4	81.12	10.4
252	19		Potassium cyanide	151-50-8	72.93	1.12	9.8	0.15	8.5	0.13	65.12	0.15
335			Potassium hexacyno- ferrate II	13943-58-3	15582.05	42.3	6409.6	17.4	5009.8	13.6	368.37	17.4
266			Potassium hexacyanoferrate III	13746-66-2	928.54	2.82	NA	NA	2970.0	9.02	329.27	9.02
346	50		Potassium I chloride	7447-40-7	6113.10	82	2601.8	34.9	1498.5	20.1	74.55	34.9
269			Potassium I fluoride	7789-23-3	181.85	3.13	245.2	4.22	NA	NA	58.1	4.22
36			Progesterone	57-83-0	9.44	0.03	NA	NA	NA	NA	314.51	
348		1-	Propanol	71-23-8	5800.62	96.5	5397.9	89.8	NA	NA	60.11	89.8
128	10	2-	Propanol	67-63-0	10038.37	167	5842.7	97.2	NA	NA	60.11	97.2
270			Propionaldehyde	123-38-6	188.79	3.25	1411.6	24.3	NA	NA	58.09	24.3
54	23		Propranolol * HCl	318-98-9	35.50	0.12	NA	NA	470.4	1.59	295.84	1.59

Section 7.2
Table 7.3
Chemical Data from the Registry of Cytotoxicity Data Bank (Alphabetical))

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
357			Propylene glycol	57-55-6	26029.62	342	20016.9	263	23974.7	315	76.11	263
209			Propylparaben	94-13-3	32.44	0.18	NA	NA	6325.7	35.1	180.22	35.1
12			Puromycin	53-79-2	0.16	0.00033	NA	NA	674.4	1.43	471.58	1.43
337			Pyridine	110-86-1	3710.26	46.9	893.9	11.3	NA	NA	79.11	11.3
53	43		Quinidine sulfate	50-54-4	50.70	0.12	456.3	1.08	595.8	1.41	422.54	1.08
45			Quinine * HCl	130-89-2	27.07	0.075	620.8	1.72	1158.6	3.21	360.92	1.72
216			Refortan		78.28	0.25	3162.3	10.1	NA	NA	313.1	10.1
245			Resorcinol	108-46-3	88.10	0.8	300.6	2.73	NA	NA	110.12	2.73
201		13-cis-	Retinoic acid	4759-48-2	36.06	0.12	NA	NA	3395.4	11.3	300.48	11.3
139			Retinol	68-26-8	0.15	0.00054	1990.8	6.98	4011.0	14	286.5	6.98
134			Rotenone	83-79-4	0.05	0.00013	130.2	0.33	351.1	0.89	394.45	0.33
314			Saccharin	81-07-2	3004.32	16.4	NA	NA	17000.0	92.8	183.19	92.8
95			Salicylamide	65-45-2	148.12	1.08	1892.7	13.8	1398.9	10.2	137.15	13.8
178			Salicylanilide	87-17-2	9.81	0.046	NA	NA	2409.7	11.3	213.25	11.3
272			Salicylic acid	69-72-7	466.88	3.38	890.9	6.45	479.3	3.47	138.13	6.45
251			Scopolamine * HBr	6533-68-2	415.05	1.08	1268.2	3.3	1879.3	4.89	384.31	3.3
69			Secobarbital sodium	309-43-3	54.66	0.21	124.9	0.48	NA	NA	260.3	0.48
161			Silver I nitrate	7761-88-8	2.21	0.013	NA	NA	49.3	0.29	169.88	0.29
30			Sodium arsenate, dibasic	7778-43-0	2.79	0.015	NA	NA	NA	NA	185.91	
241			Sodium azide	26628-22-8	46.16	0.71	44.9	0.69	27.3	0.42	65.02	0.69
144			Sodium bichromate VI	10588-01-9	0.24	0.00093	49.8	0.19	NA	NA	261.98	0.19
344	13		Sodium chloride	7647-14-5	4435.60	75.9	2998.0	51.3	3997.3	68.4	58.44	51.3
329			Sodium cyclamate	139-05-9	7123.90	35.4	15254.0	75.8	17004.8	84.5	201.24	75.8
76			Sodium dodecyl sulfate	151-21-3	78.15	0.27	1288.0	4.45	NA	NA	289.43	4.45
345			Sodium I bromide	7647-15-6	8120.81	77.4	3504.3	33.4	6998.2	66.7	104.92	33.4
106	14		Sodium I fluoride	7681-49-4	77.68	1.85	180.1	4.29	NA	NA	41.99	4.29
255			Sodium monochloroacetate	3926-62-3	168.90	1.45	75.7	0.65	NA	NA	116.48	0.65
227	46		Sodium oxalate	62-76-0	58.96	0.44	155.4	1.16	NA	NA	134	1.16
119			Sodium salicylate	54-21-7	693.28	4.33	1599.5	9.99	899.8	5.62	160.11	9.99
290			Sodium sulfite	7757-83-7	854.55	6.78	NA	NA	820.5	6.51	126.04	6.51
156			Stearyltrimethylammoniumchloride	112-03-8	2.09	0.006	NA	NA	536.1	1.54	348.13	1.54
265			Streptomycin sulfate	298-39-5	3979.25	2.73	NA	NA	495.6	0.34	1457.6	0.34
331			Strontium II chloride	10476-85-4	5770.13	36.4	2251.0	14.2	3107.0	19.6	158.52	14.2
5		K-	Strophantin		0.03	0.000044	NA	NA	NA	NA	710.9	
271			Styrene	100-42-5	343.73	3.3	4999.7	48	315.6	3.03	104.16	48
93			Sulfisoxazole	127-69-5	227.23	0.85	NA	NA	6790.2	25.4	267.33	25.4
330			Sulfuric acid	7664-93-9	3530.88	36	2138.1	21.8	NA	NA	98.08	21.8
135		2,3,7,8-	Tetrachloro-dibenzo-p-dioxin	1746-01-6	0.06	0.0002	NA	NA	0.1	0.00035	321.96	0.00035
289			Tetrachloroethene	127-18-4	1084.46	6.54	8854.8	53.4	8092.0	48.8	165.82	53.4

Section 7.2
Table 7.3
Chemical Data from the Registry of Cytotoxicity Data Bank (Alphabetical))

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
59			Tetracycline * HCl	64-75-5	67.33	0.14	6444.6	13.4	NA	NA	480.94	13.4
350			Tetrahydrofurfuryl alcohol	97-99-4	11338.65	111	2502.7	24.5	2298.4	22.5	102.15	24.5
247		(+)-	Thalidomide	731-40-8	209.18	0.81	NA	NA	400.3	1.55	258.25	1.55
203			Thallium I acetate	563-68-8	36.88	0.14	NA	NA	34.2	0.13	263.42	0.13
181	30		Thallium I sulfate	7446-18-6	27.26	0.054	NA	NA	28.8	0.057	504.8	0.057
105	21		Theophylline	58-55-9	329.75	1.83	NA	NA	600.0	3.33	180.19	3.33
303			Theophylline sodium	3485-82-3	2519.43	12.4	NA	NA	445.0	2.19	203.18	2.19
280			Theophylline sodium acetate	8002-89 9	1098.74	4.19	582.2	2.22	NA	NA	262.23	2.22
25			Thio-TEPA	52-24-4	2.08	0.011	NA	NA	37.8	0.2	189.24	0.2
279			Thioacetamide	62-55-5	313.33	4.17	301.3	4.01	NA	NA	75.14	4.01
140		6-	Thioguanine	154-42-7	0.10	0.00057	NA	NA	160.5	0.96	167.21	0.96
83			Thiopental	76-75-5	133.30	0.55	NA	NA	601.1	2.48	242.37	2.48
170	29		Thioridazine * HCl	130-61-0	11.81	0.029	NA	NA	358.2	0.88	407.07	0.88
80		2-	Thiouracil	141-90-2	41.01	0.32	999.6	7.8	NA	NA	128.16	7.8
347			Thiourea	62-56-6	6547.18	86	124.9	1.64	8526.6	112	76.13	1.64
214			Thymol	89-83-8	34.56	0.23	979.6	6.52	1802.9	12	150.24	6.52
256			Tin II chloride	7772-99-8	286.28	1.51	699.6	3.69	1200.1	6.33	189.59	3.69
104			Tolbutamide	64-77-7	489.39	1.81	NA	NA	2601.1	9.62	270.38	9.62
316			Toluene	108-88 3	1575.77	17.1	5003.7	54.3	NA	NA	92.15	54.3
194		p-	Toluylendiamine	95-70-5	11.49	0.094	101.4	0.83	NA	NA	122.19	0.83
1			Trenimon	68-76-8	0.00	0.0000033	NA	NA	NA	NA	231.28	
310			Tributylamine	102-82-9	2855.16	15.4	539.5	2.91	NA	NA	185.4	2.91
138			Tributyltin chloride	1461-22-9	0.18	0.00054	120.4	0.37	NA	NA	325.53	0.37
75			Trichlorfon	52-68-6	69.51	0.27	450.5	1.75	298.6	1.16	257.44	1.75
294			Trichloroacetic acid	76-03-9	1338.08	8.19	4999.4	30.6	5636.6	34.5	163.38	30.6
242		1,2,4-	Trichlorobenzene	120-82-1	128.82	0.71	756.6	4.17	765.7	4.22	181.44	4.17
297	11	1,1,1-	Trichloroethane	71-55-6	1374.02	10.3	10298.5	77.2	11245.6	84.3	133.4	77.2
228		2,4,5-	Trichlorophen- oxyacetic acid	93-76-5	112.41	0.44	298.9	1.17	388.3	1.52	255.48	1.17
126			Triethyl citrate	77-93-0	4061.90	14.7	6990.9	25.3	NA	NA	276.32	25.3
143			Triethylene melamine	51-18-3	0.16	0.00078	1.0	0.005	14.9	0.073	204.27	0.005
137			Triethyltin chloride	994-31-0	0.11	0.00046	5.1	0.021	NA	NA	241.35	0.021
318			Trifluoroacetic acid	76-05-1	2337.62	20.5	199.6	1.75	NA	NA	114.03	1.75
166			Triisooctylamine	2757-28 0	8.14	0.023	1620.2	4.58	NA	NA	353.76	4.58
354		1,3,5-	Trioxane	110-88-3	19189.17	213	800.0	8.88	NA	NA	90.09	8.88
132			Triphenyltin hydroxide	76-87-9	0.02	0.000049	44.0	0.12	245.9	0.67	367.03	0.12
182			Triton X-100	9002-93-1	35.59	0.055	1798.7	2.78	NA	NA	647	2.78
50			Trypan blue	72-57-1	91.66	0.095	6204.2	6.43	NA	NA	964.88	6.43
231			Tween 80	9005-65-6	641.90	0.49	NA	NA	25021.0	19.1	1310	19.1
208			Undecylenic acid	112-38-9	33.18	0.18	2506.6	13.6	8496.7	46.1	184.31	13.6

Section 7.2
Table 7.3
Chemical Data from the Registry of Cytotoxicity Data Bank (Alphabetical))

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
323			Urethan	51-79-6	2307.95	25.9	NA	NA	2504.0	28.1	89.11	28.1
250			Valproate sodium	1069-66-5	166.22	1	NA	NA	1695.4	10.2	166.22	10.2
196	40		VerapamilHCl	152-11-4	49.11	0.1	108.0	0.22	162.1	0.33	491.13	0.22
205			Versalide	88-29-9	38.77	0.15	315.3	1.22	NA	NA	258.44	1.22
4			Vincristine sulfate	2068-78-2	0.01	0.000015	NA	NA	NA	NA	923.14	
86	31		Warfarin	81-81-2	206.59	0.67	323.8	1.05	373.1	1.21	308.35	1.05
313			Xanthinol nicotinate	437-74-1	6865.26	15.8	14121.6	32.5	17336.9	39.9	434.51	32.5
301	17		Xylene	1330-20-7	1274.16	12	4300.3	40.5	NA	NA	106.18	40.5
55			Zinc II chloride	7646 85-7	17.72	0.13	350.2	2.57	350.2	2.57	136.27	2.57
186			Zineb	12122-67-7	16.27	0.059	5211.3	18.9	7610.1	27.6	275.73	18.9

Section 7.2
Table 7.4
Registry of Cytotoxicity Data - MEIC Chemicals (Sorted by Rat LD50 Oral mg/kg)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
29	28		Mercury II chloride	7487-94-7	4.07	0.015	1.0	0.0037	10.0	0.037	271.49	0.0037
252	19		Potassium cyanide	151-50-8	72.93	1.12	9.8	0.15	8.5	0.13	65.12	0.15
153	26		Arsenic III trioxide	1327-53-3	0.83	0.0042	19.8	0.1	45.5	0.23	197.84	0.1
103	18		Nicotine	54-11-5	290.45	1.79	50.3	0.31	24.3	0.15	162.26	0.31
173	39		Pentachlorophenol	87-86-5	9.59	0.036	50.6	0.19	NA	NA	266.32	0.19
262	47		Amphetamine sulfate	60-13-9	726.02	1.97	55.3	0.15	24.0	0.065	368.54	0.15
235	25		Paraquat	4685-14-7	100.58	0.54	57.7	0.31	195.6	1.05	186.25	0.31
157	38		Hexachlorophene	70-30-4	3.21	0.0079	61.0	0.15	65.1	0.16	406.89	0.15
223	32		Lindane	58-89-9	119.24	0.41	75.6	0.26	87.2	0.3	290.82	0.26
229	22		Dextropropoxyphene * HCl	1639-60-7	184.23	0.49	82.7	0.22	82.7	0.22	375.98	0.22
196	40		VerapamilHCl	152-11-4	49.11	0.1	108.0	0.22	162.1	0.33	491.13	0.22
227	46		Sodium oxalate	62-76-0	58.96	0.44	155.4	1.16	NA	NA	134	1.16
118	24		Phenobarbital	50-06-6	884.91	3.81	162.6	0.7	167.2	0.72	232.26	0.7
106	14		Sodium f fluoride	7681-49-4	77.68	1.85	180.1	4.29	NA	NA	41.99	4.29
112	48		Caffeine	58-08-2	512.74	2.64	192.3	0.99	619.6	3.19	194.22	0.99
81	27		Cupric sulfate * 5 H2O	7758-99-8	82.40	0.33	299.6	1.2	NA	NA	249.7	1.2
261	3		Ferrous sulfate	7720-78-7	281.03	1.85	319.0	2.1	978.3	6.44	151.91	2.1
183	5		Amitriptyline	50-48-6	15.54	0.056	319.1	1.15	147.0	0.53	277.44	1.15
86	31		Warfarin	81-81-2	206.59	0.67	323.8	1.05	373.1	1.21	308.35	1.05
246	37		Barium II nitrate	10022-31-8	211.70	0.81	355.4	1.36	NA	NA	261.36	1.36
89	16	2,4-	Dichlorophenoxy- acetic acid	94-75-7	170.20	0.77	369.1	1.67	366.9	1.66	221.04	1.67
115	12		Phenol	108-95-2	283.30	3.01	414.1	4.4	300.2	3.19	94.12	4.4
230	42		Orphenadrine * HCl	341-69-5	149.88	0.49	425.2	1.39	125.4	0.41	305.88	1.39
53	43		Quinidine sulfate	50-54-4	50.70	0.12	456.3	1.08	595.8	1.41	422.54	1.08
70	49		Atropine sulfate	55-48-1	148.92	0.22	622.7	0.92	764.9	1.13	676.9	0.92
123	35		Isoniazid	54-85-3	1027.33	7.49	650.1	4.74	NA	NA	137.16	4.74
63	4		Diazepam	439-14-5	45.56	0.16	709.1	2.49	535.3	1.88	284.76	2.49
67	15		Malathion	121-75-5	66.08	0.2	885.4	2.68	776.4	2.35	330.38	2.68
308	33		Chloroform	67-66-3	1599.56	13.4	908.4	7.61	35.8	0.3	119.37	7.61
31	41		Chloroquine diphosphate	50-63-5	8.77	0.017	969.9	1.88	500.4	0.97	515.92	1.88
107	2		Acetylsalicylic acid	50-78-2	408.99	2.27	999.9	5.55	814.4	4.52	180.17	5.55
328	36		Dichloromethane	75-09-2	2964.06	34.9	1596.7	18.8	NA	NA	84.93	18.8
113	1		Acetaminophen	103-90-2	409.70	2.71	2403.8	15.9	338.6	2.24	151.18	15.9
346	50		Potassium f chloride	7447-40-7	6113.10	82	2601.8	34.9	1498.5	20.1	74.55	34.9
125	34		Carbon tetrachloride	56-23-5	1308.92	8.51	2799.3	18.2	12797.0	83.2	153.81	18.2
344	13		Sodium chloride	7647-14-5	4435.60	75.9	2998.0	51.3	3997.3	68.4	58.44	51.3
91	45		Chloramphenicol	56-75-7	255.29	0.79	3393.1	10.5	2640.1	8.17	323.15	10.5

Section 7.2
Table 7.4
Registry of Cytotoxicity Data - MEIC Chemicals (Sorted by Rat LD50 Oral mg/kg)

RC #	MEIC #		Chemical	CAS #	IC50x		LD50 RAT		LD50 MOUSE			Rodent LD50 (mmol/kg)
					ug/ml	mmol/l	mg/kg	mmol/kg	mg/kg	mmol/kg	MW	for Regression
301	17		Xylene	1330-20-7	1274.16	12	4300.3	40.5	NA	NA	106.18	40.5
128	10	2-	Propanol	67-63-0	10038.37	167	5842.7	97.2	NA	NA	60.11	97.2
360	7		Ethylene glycol	107-21-1	34454.40	555	8567.0	138	7511.7	121	62.08	138
297	11	1,1,1-	Trichloroethane	71-55-6	1374.02	10.3	10298.5	77.2	11245.6	84.3	133.4	77.2
361	8		Methanol	67-56-1	29806.50	930	13012.3	406	NA	NA	32.05	406
130	9		Ethanol	64-17-5	17464.32	379	14008.3	304	7787.5	169	46.08	304
22	6		Digoxin	20830-75-5	6.64	0.0085	NA	NA	18.0	0.023	781.05	0.023
327	20		Lithium Sulfate	10377-48-7	3704.98	33.7	NA	NA	1187.4	10.8	109.94	10.8
105	21		Theophylline	58-55-9	329.75	1.83	NA	NA	600.0	3.33	180.19	3.33
54	23		Propranolol * HCl	318-98-9	35.50	0.12	NA	NA	470.4	1.59	295.84	1.59
170	29		Thioridazine * HCl	130-61-0	11.81	0.029	NA	NA	358.2	0.88	407.07	0.88
181	30		Thallium Sulfate	7446-18-6	27.26	0.054	NA	NA	28.8	0.057	504.8	0.057
82	44		Diphenylhydantoin	57-41-0	98.39	0.39	NA	NA	199.3	0.79	252.29	0.79

Calculation of the Regression Between Cytotoxicity and Acute Oral Toxicity

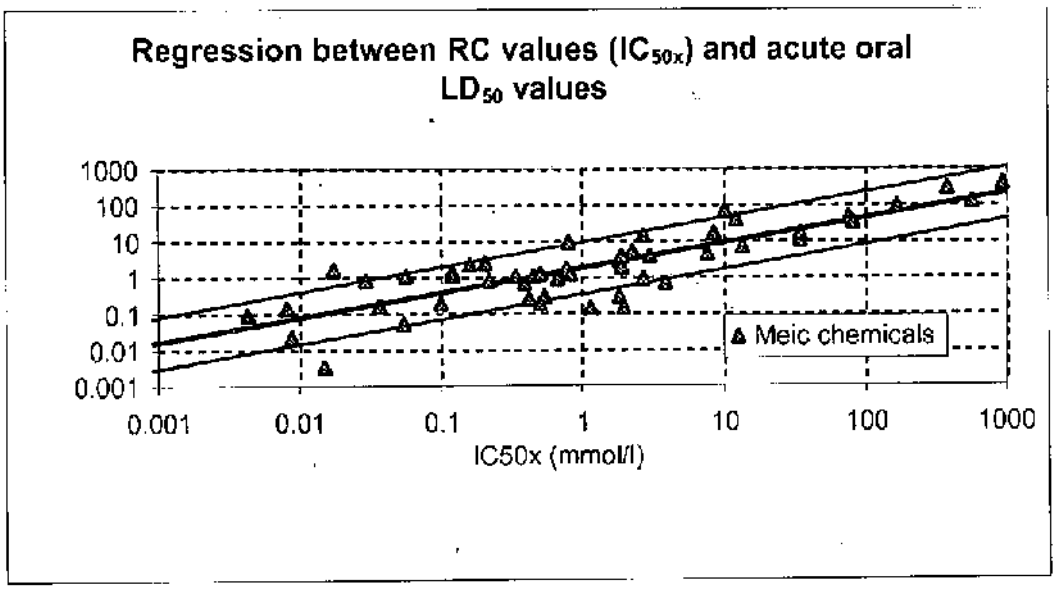

Figure 7.1 Regression between RC values (IC50x) and acute oral LD50 values (MEIC chemicals)

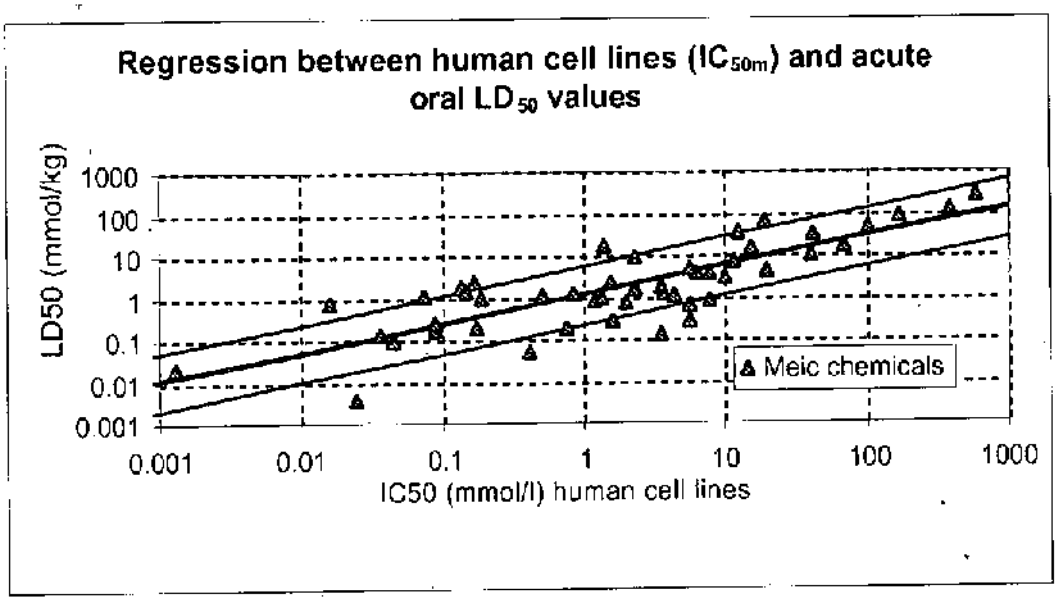

Figure 7.2 Regression between human cell lines (IC50m) and acute oral LD50 values (MEIC chemicals)

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APPENDIX A

Detailed Workshop Agenda

ICCVAM International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity

Hyatt Regency Crystal City Hotel, Arlington, VA

Detailed Workshop Agenda

Tuesday, October 17, 2000

7:30 a.m.	**Registration**
8:30 a.m.	**Opening Plenary Session – Regency Ballroom F**
8:30 a.m.	Welcome from the National Toxicology Program (NTP) Dr. John Bucher, Deputy Director, ETP, NIEHS
8:35 a.m.	Workshop Introduction Dr. Philip Sayre, U.S. EPA, OPPTS, Co-Chair Workshop Organizing Committee
8:45 a.m.	Special Presentation on Dr. Bjorn Ekwall: Contributions to *In Vitro* Toxicology Dr. Erik Walum, Pharmacia & Upjohn AB, Stockholm, Sweden
8:55 a.m.	Role of ICCVAM and the NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) in the Validation and Acceptance of New Methods Dr. William Stokes, NIEHS, Co-Chair ICCVAM
9:10 a.m.	Acute Toxicity: Historical and Current Regulatory Perspectives Dr. Steve Galson, Director, Office of Science Policy and Coordination, U.S. EPA
9:40 a.m.	Acute Toxicity Data: A Clinical Perspective Dr. Jim Cone, Chief, Occupational Health Branch, California Dept. of Health Services
10:10 a.m.	**Coffee Break**
10:30 a.m.	***In Vitro* Approaches to Estimate the Acute Toxicity Potential of Chemicals** Estimating Starting Doses for *In Vivo* Studies using *In Vitro* Data Dr. Manfried Liebsch, ZEBET – Center for Documentation and Evaluation of Alternative Methods to Animal Experiments
11:00 a.m.	An Integrated Approach for Predicting Acute Systemic Toxicity Dr. Bas Blaauboer, Research Institute of Toxicology (RITOX), Utrecht University
11:30 a.m.	Opportunities for Future Progress Dr. Oliver Flint, Bristol-Meyers Squibb
12:00 p.m.	**Public Comment**
12:15 p.m.	**Breakout Group Charges** Dr. John Frazier, DOD Tri-Service Toxicology Lab, USAF, Co-Chair Workshop Organizing Committee
12:30 p.m.	**Lunch Break**
1:45 p.m.	**Breakout Groups: Identify Needs** 1. Screening Methods (Regency Ballroom F) 2. Toxicokinetic Determinations (Arlington Room) 3. Predicting Organ Specific Toxicity and Mechanisms (Fairfax Room) 4. Chemical Data Sets for Validation (Prince William Room)
3:30 p.m.	**Coffee Break**
4:00 p.m.	**Breakout Groups (Cont'd)**
5:30 p.m.	**Adjourn for Day**
6:00 p.m.	**Shuttle Begins between Hyatt Regency and Potowmack Landing Restaurant**
7:00 p.m.	**Dinner (Chart Room) – Pre-registration was required by October 9th.**
8:00 p.m.	**Dinner Speaker – Professor Michael Balls, ECVAM "*In Vitro Toxicology: Perspectives on Past and Future Progress*"**
8:45 p.m.	**Shuttle Begins between Potowmack Landing Restaurant and Hyatt Regency**

Wednesday, October 18, 2000

8:00 a.m.	**Plenary Session – Status Reports by Breakout Group Co-Chairs – Regency Ballroom F** (Moderator: Dr. Philip Sayre, U.S. EPA, OPPTS, Co-Chair Workshop Organizing Committee)
8:40 a.m.	**General Discussion**
9:00 a.m.	**Breakout Group: Current Status** 1. Screening Methods (Regency Ballroom F) 2. Toxicokinetic Determinations (Arlington Room) 3. Predicting Organ Specific Toxicity and Mechanisms (Fairfax Room) 4. Chemical Data Sets for Validation (Prince William Room)
10:30 a.m.	**Coffee Break**
10:45 a.m.	**Breakout Groups (Cont'd)**
12:00 p.m.	**Lunch Break**
1:30 p.m.	**Breakout Groups (Cont'd)**
3:30 p.m.	**Coffee Break**
4:00 p.m.	**Breakout Groups (Cont'd)**
5:30 p.m.	**Adjourn for the Day**

Thursday, October 19, 2000

8:00 a.m.	**Current Status Plenary Session – Status Reports by Breakout Group Co-Chairs – Regency Ballroom F** (Moderator: Dr. John Frazier, DOD Tri-Service Toxicology Lab, USAF, Co-Chair Workshop Organizing Committee)
8:40 a.m.	**General Discussion**
9:00 a.m.	**Breakout Groups: Future Directions** 1. Screening Methods (Regency Ballroom F) 2. Toxicokinetic Determinations (Arlington Room) 3. Predicting Organ Specific Toxicity and Mechanisms (Fairfax Room) 4. Chemical Data Sets for Validation (Prince William Room)
10:30 am	**Coffee Break**
10:45 a.m.	**Breakout Groups (Cont'd)**
12:00 p.m.	**Lunch Break**
1:30 p.m.	**Breakout Groups (Cont'd)**
3:30 p.m.	**Coffee Break**
4:00 p.m.	**Breakout Groups (Cont'd)**
5:30 p.m.	**Adjourn for the Day**

Friday, October 20, 2000

8:00 a.m.	**Closing Plenary Session – Reports by Breakout Group Co-Chairs – Regency Ballroom A/B** (Moderator: Dr. William Stokes, NIEHS, Co-Chair ICCVAM)
8:00 a.m.	Screening Methods *(30 min/15 min discussion)*
8:45 a.m.	Toxicokinetic Determinations *(30 min/15 min discussion)*
9:30 a.m.	Predicting Organ Specific Toxicity and Mechanisms *(30 min/15 min discussion)*
10:15 a.m.	**Coffee Break**
10:45 a.m.	**Closing Plenary Session – Reports by Breakout Group Co-Chairs (Cont'd)**
10:45 a.m.	Chemical Data Sets for Validation of *In Vitro* Testing Methods for Assessing Acute Toxicity *(30 min/15 min discussion)*
11:30 a.m.	**Public Comment**
12:00 p.m.	**Closing Comments**
12:15 p.m.	**Adjourn**

APPENDIX B

Summary of Opening Plenary Session and Public Comments

Summary of Opening Plenary Session and Public Comments

The International Workshop on *In Vitro* Methods for Assessing Acute Toxicity

October 17-20, 2000

Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)

The National Toxicology Program (NTP)
Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

National Institute of Environmental Health Sciences (NIEHS)

Opening Plenary Session

Speakers:

- Dr. John Frazier, USAF/ICCVAM, Workshop Co-Chair
- Dr. Philip Sayre, EPA/OPPT/ICCVAM, Workshop Co-Chair
- Dr. William Stokes, NIEHS/ICCVAM/NICEATM
- Dr. John Bucher, NIEHS
- Dr. Steve Galson, EPA/OPPT
- Dr. James Cone, California Department of Health Services
- Dr. Manfred Liebsch, ZEBET
- Dr. Bas Blaauboer, Research Institute of Toxicology, Utrecht University
- Dr. Oliver Flint, Bristol-Meyers Squibb

Call to Order and Introductions

Dr. William Stokes called the workshop to order at 8:38 a.m. Dr. Stokes explained that the Workshop was organized by ICCVAM and NICEATM and was co-sponsored by the U.S. Environmental Protection Agency (EPA), the National Institute of Environmental Health Sciences (NIEHS) and the National Toxicology Program (NTP). He thanked everyone for their participation and attendance. He discussed the goals of ICCVAM and NICEATM stating that the overall goal is to validate and achieve regulatory acceptance of test methods that will provide improved protection of human health and the environment, while incorporating the three Rs for the use of animals (refinement, reduction and replacement) whenever scientifically feasible. He stated that the purpose of the workshop was to evaluate the validation status of *in vitro* test methods for assessing acute systemic toxicity. He reviewed the functions of ICCVAM, which include the technical evaluation of new methods including independent scientific peer reviews, and organizing expert panel meetings to review test methods at various stages of development and validation. Dr. Stokes concluded by stating that ICCVAM also organizes workshops to identify additional research and validation efforts necessary to develop and further enhance the usefulness of new methods.

Welcome from the National Toxicology Program (NTP)

Dr. Stokes introduced Dr. John Bucher of NIEHS as the next speaker. Dr. Bucher thanked Dr. Stokes and welcomed the participants of the workshop. He conveyed the regrets of Dr. Christopher Portier of NIEHS/NTP who was unable to attend the workshop and then thanked the ICCVAM agencies and the

U.S. EPA for the effort provided for the workshop. Dr. Bucher remarked that the purpose of the workshop was to seek scientific advice and opinion concerning alternative test methods. He expressed hope that the scientists would work to advance alternatives for acute toxicity testing and provide information to move *in vitro* alternative tests forward. He concluded by thanking the workshop participants for their knowledge, experience and time.

Workshop Objectives

Dr. Sayer reintroduced the objectives of the workshop, provided background remarks and listed points for the participants to consider: 1) determine the hazards of chemicals by alternative methods; 2) find non-lethal acute toxicity testing endpoints; and 3) ascertain which *in vitro* methods might be helpful and could be validated. He challenged the scientists to review *in vitro* screening methods for toxicokinetics and specific organ toxicity and to recommend applicable methods for pre-validation and validation studies. Dr. Sayre asked the scientists to recommend validation study designs, to determine lists of reference chemicals and to prioritize *in vitro* methods.

Dr. Sayre discussed the general structure of the workshop. Four breakout groups would investigate their respective topics and the invited expert scientists would lead the discussions. Time would be made available for public comment at the meetings. The workshop would begin each morning with a short plenary session to discuss the previous day's activities and would end each evening with a meeting of the co-chairs and rapporteurs. A final report from each breakout group would be compiled as a workshop report ready for publishing by January 2001. He also said that a workshop monograph could be published by NIEHS' Environmental Health Perspectives Supplements in April 2001. Dr. Sayre concluded his remarks by naming the organizing committee for the workshop and then thanked everyone for their work.

Memoriam for Björn Ekwall

Dr. Stokes thanked Dr. Sayre and continued the session by mentioning the recent untimely death of Dr. Björn Ekwall. He spoke of Dr. Ekwall's extensive contributions and dedication to alternative test method development. Dr. Stokes then introduced Dr. Erik Walum, a close friend and colleague of Dr. Ekwall.

Dr. Walum described Dr. Ekwall as a medical doctor and toxicologist who pushed seriously for implementation of *in vitro* test methods. He discussed Dr. Ekwall's life and work in Uppsala, Sweden and related Dr. Ekwall's belief that the United States must accept *in vitro* alternative testing methods in order for the world to embrace the methodology. Dr. Ekwall established the Scandinavian Cell Toxicology Society whose mission is to gather scientists for meetings and show that chemical effects on cells should translate to *in vivo* effects. He initiated the Multicenter Evaluation of *In Vitro* Cytotoxicity (MEIC) to test 50 chemicals and collect the results. Sixty-five different test methods were employed for testing the chemicals. He introduced the concept to test compounds in simple systems such as cell cultures and to extrapolate the results to human toxicity. He felt that one could break down systems to elementary parts then analyze them by *in vitro* methods. Dr. Walum concluded his remarks by relating that Dr. Ekwall knew that if he were not able to continue his work, then someone else would take over. Dr. Stokes thanked Dr. Walum for his remarks.

The Role of ICCVAM

Dr. Stokes described the evolution, structure, and function of ICCVAM, and its role in facilitating the development and validation of alternative test methods. The driving forces for the establishment and need for ICCVAM were listed: 1) the opportunity to incorporate new science and technologies into toxicological testing practices; 2) the potential benefits of improved prediction of toxicity, improved efficiency and improved animal welfare; 3) legislation including the NIH Revitalization Act of 1993

(Public Law 103-43); and 4) the need for development and validation of test methods for new endpoints of concern, such as the Endocrine Disruptor Screening and Testing Program at EPA. ICCVAM also fulfills other mandates provided to NIEHS by Public Law 103-43, such as alternative test method development and validation.

Dr. Stokes related that ICCVAM began as an ad hoc committee comprised of representatives from 15 Federal regulatory and research agencies in September 1994. The committee developed a report on criteria and processes for the validation and regulatory acceptance of toxicological test methods that was published in 1997. A standing ICCVAM committee was established in May 1997 to implement the Public Law 103-43 mandate that NIEHS establish a process to achieve the regulatory acceptance of scientifically valid alternative methods. The committee evaluates proposed test methods and provides recommendations to Federal agencies, which in turn decide the regulatory acceptability of the methods. He explained that NICEATM is located at NIEHS and provides operational and technical support for ICCVAM by co-organizing workshops and peer reviews of test methods, disseminating information, and developing partnerships with stakeholders.

Dr. Stokes reviewed the prerequisites for using new methods which include: 1) adequate validation, which involves determining the reliability and relevance of test methods for specific purposes, and 2) acceptance, which involves determination of the acceptability for regulatory risk assessment purposes. The evolution process for new testing includes: the review of existing risk assessment methods, research, development, pre-validation, validation, peer review, regulatory acceptance, and implementation. The current ICCVAM/NICEATM role in test method development and validation is to provide information, to evaluate test methods, and to provide recommendations to agencies. The objectives of ICCVAM Workshops include: to evaluate the adequacy of current test methods; to identify toxicological endpoints; to identify promising methods which need further development and validation; to recommend appropriate validation studies; and to recommend research and model development efforts needed to support improved test methods for specific toxicity endpoints. ICCVAM/NICEATM has completed independent peer review evaluations for the following tests: 1) the murine local lymph node assay (LLNA); 2) Corrositex®; 3) FETAX; and 4) the revised UDP. Dr. Stokes concluded his presentation by acknowledging the contributions of the ICCVAM Agency Representatives, the ICCVAM Workshop Organizing Committee, and the NICEATM staff.

Acute Toxicity Testing: Historical and Current Regulatory Perspectives

Dr. Galson began by saying that the workshop represents the working relationship of EPA and NIEHS. He thanked Dr. Richard Hill of the EPA and Dr. Stokes for their work and participation in the workshop. He acknowledged the animal welfare groups for their role in pushing forward the objectives of alternative testing. He also thanked Dr. Amy Rispin of the EPA for her contributions to forwarding alternative testing. Dr. Galson said the EPA committee assures that the 3Rs will be the primary objective of the workshop and the committee will work toward regulatory acceptance with the protection of public health foremost in mind.

Dr. Galson spoke of alternative methods for determining acute toxicity being used by the regulatory agencies to revise acute toxicity studies. The long-term goal is to develop *in vitro* methods to replace animals and recommendations from the workshop participants will move *in vitro* methods forward. He outlined the current methods used for determining acute toxicity as the "classical" LD50 test and OECD Acute Oral Toxicity Tests 401, 420, 423 and 425. He related that OECD 401 test was to be dropped and that U.S. agencies will accept this decision.

Regulatory uses of acute toxicity data include hazard labeling (only EPA requires), hazard classification (LD50 dose points – required by some EPA offices, e.g., Office of Pesticide Programs), and risk

assessment. Dr. Galson listed the regulatory agencies and illustrated how they use hazard labels, and how they receive data and perform risk assessment. It is important to harmonize test methods between the various federal agencies (CPSC, DOT, OSHA, EPA, FDA, NIOSH, and ATSDR). Dr. Galson concluded by urging the workshop participants to revise methods for determining acute toxicity and to meet the scientific challenges. Recommendations of the workshop would be relevant to the federal regulatory agencies, in particular, the EPA for the HPV chemical program. Dr. Stokes thanked Dr. Galson and then introduced Dr. James Cone who would speak about clinical perspectives in occupational health.

Acute Toxicity Data -- A Clinical Perspective

Dr. Cone defined acute toxicity as health effects resulting from exposure over a short period of time. Though no single definition for acute exposure had been agreed upon, he felt that unintended releases of chemicals into the environment and poisonings would constitute a working definition. Many chemicals have acute toxicity human data and he related the clinician's experience with acute toxicity data by listing the available tools: Physicians Desk Reference (PDR), Material Safety Data Sheets (MSDS), poison control centers (PCC), Medline searches, the internet and the telephone. Knowledge is often based on human exposure. The clinician views acute toxicity as an immediate exposure to a substance while chronic toxicity occurs from exposure over a long period of time.

Dr. Cone discussed two incidents of toxic exposure that occurred in California. One incident involved a four-hour release/spill of oleum into the environment and required the evaluation of 20,000 residents at local emergency facilities. A second case study resulted from the release of 19,000 gallons of metam sodium into a river. Problems faced by agencies responding to these incidents included determining: the toxic agent, the acute health effects of the release, medical treatment and whether evacuation of the area was necessary. Exposure assessment was difficult in these cases because of differences in the odor threshold and the irritant threshold. It was important to know whether the substance traveled as a plume or flowed in the waterways. Dr. Cone discussed the examination of personnel close to the spills and the difficulty in detecting acute exposure in the individuals.

Dr. Cone suggested that the clinician's tools for measuring acute toxicity are mostly crude. Data from HSDB may be too old, as are data for threshold limit values (TLV) and legal permissible exposure limits (PELs). The limitations of the existing toxicity data include the lack of acute toxicity data for some chemicals and the lack of toxicity information for exposure to multiple chemicals, which is a common exposure scenario for humans. Dr. Cone also provided sources/websites of acute toxicity data. Dr. Cone stated that the clinician is challenged on how to interpret acute toxicity data on chemicals and on how to keep updated on human data. Dr. Cone ended his presentation by reminding the participants of the Nuremberg Code for Medical Experimentation on Humans. Dr. Stokes thanked Dr. Cone and dismissed the participants for a break.

In Vitro Approaches to Estimate the Acute Toxicity Potential of Chemicals

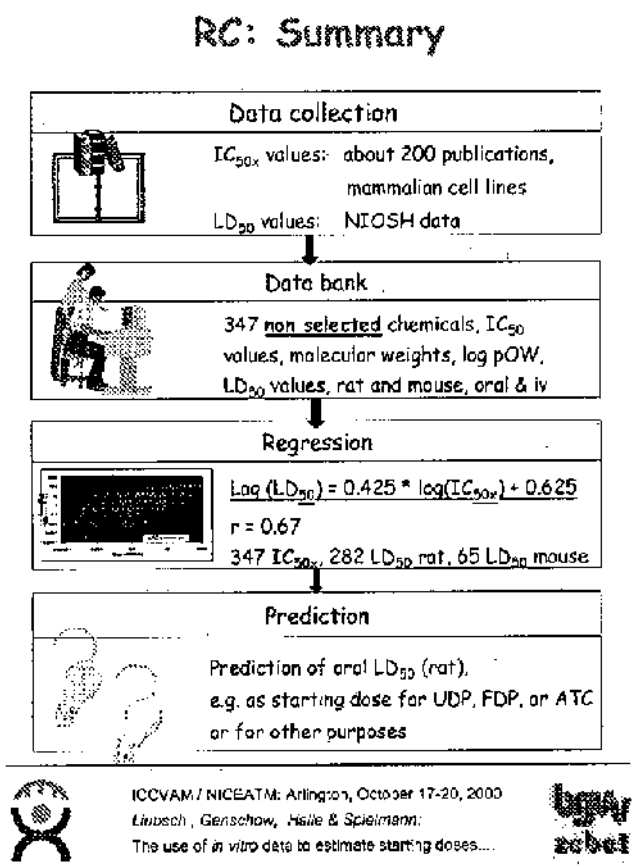
Dr. John Frazier opened the second phase of the plenary session by introducing Dr. Manfred Liebsch from the Center for Documentation and Evaluation of Alternative Methods to Animal Experiments (ZEBET).

Estimating Starting Doses for *In Vivo* Studies using *In Vitro* Data

Dr. Liebsch began his presentation with an overview of ZEBET, which is part of the Federal Institute for Health Protection of Consumers and Veterinary Medicine of Germany. The three divisions of ZEBET are for documentation, evaluation and research. ZEBET uses *in vitro* data for prediction of *in vivo* toxicity. One hundred ten chemicals were evaluated in 1954 and another 15 chemicals were evaluated in 1956

using data from Dr. Willi Halle (Registry of Cytotoxicity) and Dr. Björn Ekwall (MEIC). Dr. Halle produced a monograph, which include a registry of 347 chemicals, in 1998. Dr. Liebsch provided the scheme used for predicting starting doses for acute toxicity tests for these chemicals: NIOSH data → concentration response curve → databank → regression → prediction of starting dose.

The Registry of Cytotoxicity (RC) acceptance criteria includes: 1) *in vitro* IC50 data gathered from the literature; 2) data from mammalian primary cells or cell lines (no hepatocytes); 3) chemical incubation time ≥ 16 hours; and 4) data from two different laboratories or two different cell types or two cytotoxicity endpoints. *In vitro* cytotoxicity endpoints include cell profiles, viability (MTT, Neutral Red, Trypan Blue data) and markers for differentiation. *In vivo* LD50 data includes only values found in NIOSH databases. If more than one LD50 value is available, then the largest value is used. LD50 data from rats and mice (oral and iv route) were collected; rat data are preferred. The ZEBET chemical list was shown and IC50x (i.e., geometric mean of IC50s for each chemical) values were discussed.

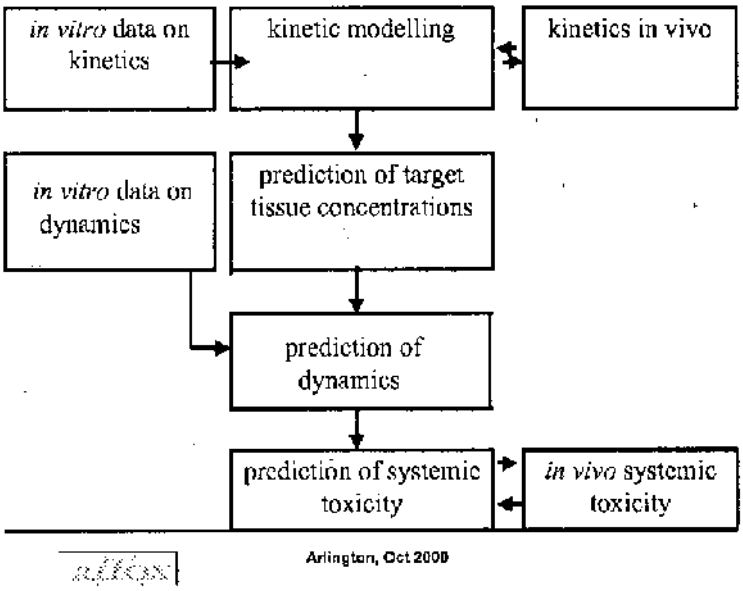


Dr. Liebsch presented the RC method of validation: LD50 = a + b x log IC50x (a = intercept, b = regression coefficient, r = correlation coefficient). Changes in the estimates of a, b, and r were small for the four regression analyses of the RC using 102, 117, 230, and 347 chemicals. The regression analysis provides a better prediction of LD50 for less toxic chemicals. Dr. Liebsch continued by discussing ECVAM Workshop 16 (1994) that produced 10 recommendations for determining starting doses. He discussed the UDP test, which uses sequential dosing starting close to the LD50 value, and said that the RC data could predict acute oral LD50s. One would determine the IC50 in a cytotoxicity test, predict the LD50 using the RC, and then determine the LD50 in the animal. A tiered approach to the LD50, as shown in Dr. Liebsch's slide on the left, would use a cytotoxicity test to determine the starting dose for non-toxic chemicals where only the highest dose is applied (Limit Test). In a classification of 1115 industrial chemicals for acute toxicity in Europe, the majority were found to be non-toxic. Dr. Liebsch concluded his presentation with the following points: 1) the use of basal cytotoxicity to predict the oral LD50 for use as a starting dose will save 30-40% of animals used; 2) basal cytoxicity tests can be used to determine whether a Limit Test should be performed; 3) the increased number of toxicity classes in OECD-HCL guidelines will increase the animal saving effect of the tiered in vitro/in vivo approach; and 4) lower animal use is predicted and validation of animal reduction is needed. His final point was that all of the effort is worth it to reduce animal testing. Dr. Frazier thanked Dr. Liebsch and then introduced Dr. Bas Blaauboer as the next speaker.

An Integrated Approach for Predicting Systemic Toxicity

Dr. Blaauboer introduced his presentation on how to integrate *in vitro* data in predictive toxicology. He challenged the workshop participants to eliminate animal use and discussed the Institute of Risk Assessment Sciences, the development of computer based biokinetic models, and *in vitro* tests. He provided a brief discussion of the ECITTS (ERGATT/CFN Integrated Toxicity Testing Scheme) project.

Dr. Blaauboer explained that the aim of "classical" toxicological risk assessment is to establish safety factors for human exposure. Classical *in vitro* toxicology methods are limited because they find concentration for effect instead of determining dose and it is difficult to extrapolate the data to an intact organism. There is also a lack of biotransformation/kinetics data and the tests concentrate on cytotoxicity rather than on mechanisms of importance *in vivo*. He presented the necessary building blocks to produce integrated models: 1) biokinetic modeling; 2) prediction of tissue concentration; 3) knowledge of effective concentration for relative targets; 4) prediction of these effective concentrations; and 5) calculation of doses relevant for risk assessment. He briefly discussed the European Research Group for Alternatives in Toxicity Testing (ERGATT) and the Swedish National Board for Laboratory Animals (CFN).



The ECITTS project building blocks are: 1) experimental – QSAR and *in vitro* data for biokinetics model; 2) modeling – *in vitro* data for PBBK models, determination of target tissue concentration; and 3) validation – validate against *in vivo* kinetics. The stepwise approach is: 1) determine the relevant parameters for biokinetic model, building model using non-animal data – physiochemical properties (e.g. tissue partition, air/blood partition) and data from cell culture systems (e.g., biotransformation, passage of cellular layers with barrier functions); 2) validate with *in vitro/in vivo* comparisons; 3) use *in vivo* data to construct or improve biokinetic model; extrapolate data from non-toxic doses; 4) estimate tissue concentration especially in target tissues; 5) use *in vitro* assays to get response surrogates; 6) integrate kinetic and dynamic data, as shown in Dr. Blaauboer's slide above; and 7) predict surrogate dose.

Dr. Blaauboer produced a list of compounds tested with a neural aspect (e.g., pesticides) and explained that the test strategy included: determination of basal cytotoxicity and morphological changes; determination of changes in cell physiology and neurochemistry; and determination of neurotoxic concentration (EC20). He illustrated this strategy using acrylamide as an example.

The following schematic would be used for the integrated use of alternative methods in toxicological risk assessment: structure of compound → chemical functionalities → QSAR → *in vitro* testing → classification of compound. This approach would lead to an *in vitro* test battery that could produce EC50 ratios, ultimately leading to limited *in vivo* testing. Dr. Blaauboer concluded that integrating *in vitro* data

in risk evaluation is valid provided biokinetics are taken into account and that the integration of all available data in a stepwise manner will improve risk assessment. Dr. Frazier thanked Dr. Blaauboer and introduced Dr. Oliver Flint.

Opportunities for Future Progress - *In Vitro* Approaches to Predicting Acute Toxicity

Dr. Flint opened his presentation by stating that *in vitro* tests used in a focused way could predict acute toxicity. He provided a test example: Taxol® Neuropathy – Successful *In Vitro* Prediction of Acute Toxicity. The objective was to characterize the neurotoxic effect of Taxol®. The *in vitro* model uses dorsal root ganglia cells and examines cytotoxicity, mitochondrial transport, morphology, and LDH leakage as endpoints. Dr. Flint discussed prediction of lethality as described by the MEIC project. He listed MEIC websites and suggested that mirror sites for the data be established. The basal cytotoxicity hypothesis for lethality using the 50 MEIC compounds correlates with human lethal plasma concentration. Problems with the basal cytotoxicity hypothesis are confounding factors such as interspecies differences in liver toxicity and specific toxicity for cell types; not all cell lines are alike.

He presented lessons in lethality predictions: 1) *in vitro* systems can make general predictions of *in vivo* toxicity; 2) human toxicity is best predicted by human cells; 3) variability is an unavoidable confounding factor; and 4) choosing the right cell is of critical importance. Future directions for predicting acute and other toxicities include computational predictions, molecular biology and *in vitro* systems targeting specific toxicological areas. *In silico* predictive toxicity is good for mutagenicity and carcinogenicity predictions, but weak for acute and reproductive toxicology. Dr. Flint presented the table, on the left, for the changing paradigm illustrating the great reduction of testing time using *in silico* predictions. He also discussed emerging technologies such as transcriptome, proteome, and metabonome and stated the usefulness and limitations of the techniques. Dr. Flint concluded by stating the need to develop new technologies to characterize predictive biomarkers and to investigate transcriptome and proteome for *in vitro* and metabonomics for *in vivo*.

The Changing Paradigm

	MUTA-GENICITY	CARCINO-GENICITY	TERATO-GENICITY
TRADITIONAL	1-Month Ames	2-Year Rodent Bioassay	4-Month Segment II Rodent Assay
PARADIGM SHIFT - *In silico* followed by:	1-Day DNA Damage Assay	2-6 Week Cell Transformation Assay	5-Day Cell Differentiation Assay

Public Comments:

Ms. Mary Beth Sweetland (PETA)

Ms. Sweetland spoke of the January 1997 Scientific Group on Methodologies for the Safety Evaluation of Chemicals (SGOMSEC) conference on alternatives and the focus on the need to increase the rate of development of alternatives for toxicology. She expressed concern for the EPA

endocrine disruptor screening program's use of numerous animals. She appreciated Dr. Galson's assurance that the EPA supported dropping OECD's TG 401 but feels that the ICCVAM validation principles are being applied arbitrarily resulting in a double standard. Ms. Sweetland stated that the non-standardized developmental neurotoxicity test uses up to two-thousand animals and is required by the EPA in the pesticide testing program even though testers can't agree on many points of the test. She believes that the EPA should support and practice full validation of all tests, animal and non-animal. Additionally, she feels that transgenics are not a true reduction method. She expressed frustration at the EPA, FDA and DOT for the agencies' continued use of animals in testing and dismay that *in vitro* cytotoxicity testing was being viewed as a novel concept instead of a time tested one. She again expressed appreciation for Dr. Galson's recommendation that *in vitro* cytotoxicity be used for dose setting as an interim step to total replacement. She urged regulatory agencies and companies to not wait for others to solve the problem and move forward on enhancing the cell tests.

Dr. Andrew Rowan (U.S. Humane Society)
Dr. Rowan explained that the Helsinki Declaration has been significantly revised in terms of animal welfare and appropriate animal testing and thus has been significantly modified from the old Nuremburg Code.

Dr. Giles Klopman (Case Western Reserve University; Multicase, Inc.)
Dr. Klopman stated that computer models wouldn't come into play if the validation is as lax as validation of short-term assays. He predicted that computer models will replace short-term assays and said that the FDA has a database for short-term assays. He was confident that the scientific community would solve the testing problems in the long run.

Adjournment

Dr. Frazier concluded the morning plenary session by restating the charge for the breakout groups and workshop participants. He stated the workshop objectives and described the nature of the four breakout groups. He explained that the workshop was to have the breakout groups answer the prepared questions provided by the Organizing Committee and to produce reports that will eventually be published. The morning session ended at 12:18 p.m.

Closing Plenary Session

Dr. Stokes opened the closing plenary session at 8:04 a.m. and introduced the Co-Chairs of the breakout groups. Co-Chairs presented their workshop reports (See **Sections 2-5**) and an opportunity for public comments was permitted.

Public Comments:

Ms. Jessica Sandler (PETA)
Ms. Sandler spoke of money available for development of non-animal tests: NIEHS committed $1.5 million for fiscal year 2000 and $3.0 million for fiscal year 2001; the EPA committed $0.5 million over two years, and stated that the MEIC study would receive high priority. She expressed concern that the EPA had no single project in development for developing non-animal tests, yet continued requiring massive animal testing programs, in particular the HPV program. Ms. Sandler urged the ICCVAM to take a more aggressive role in developing alternative testing methods. She praised the

workshop for bringing together international and American scientists to persuade government regulators to seriously consider alternative testing methods.

Dr. Martin Stevens (Humane Society of the U.S.)
Dr. Stevens complimented ICCVAM for its role in organizing the workshop and hoped to be involved with ICCVAM in moving forward with the recommendations put forth by the workshop. He spoke of three hurdles in the evolution of replacing the LD50 test: 1) use of cytotoxicity data to accurately predict starting doses to reduce animal use; 2) use of limit tests to confirm non-toxicity; and 3) total replacement of the LD50 test.

Ms. Mary Beth Sweetland (PETA)
Ms. Sweetland made comments directly to ICCVAM concerning European Union acceptance of four validated test methods (three for corrosion and one for phototoxicity): Episkin™, EpiDerm™, rat skin TER, and 3T3 Neutral Red Uptake. She stated that the United States should accept the ECVAM validations and present these methods to the OECD as accepted methods. She concluded by thanking those who put the effort forth for the workshop.

In response to Ms. Sweetland's comments, Dr. Stokes stated that ICCVAM has an interagency Corrosivity Working Group that has provided extensive comments on the OECD proposals for the corrosivity methods mentioned, and U.S. government scientists also provided comments on the phototoxicity method. ICCVAM is currently developing an expedited process by which methods reviewed, validated, and accepted in Europe could be reviewed and considered by U.S. agencies.

Conclusion and Adjournment

Dr. Stokes presented the closing comments for the workshop, stating that the Breakout Groups had made remarkable progress. He thanked the co-chairs of the breakout groups, the agency representatives and the scientists attending the workshop. He stated that the objectives of the workshop had been met or exceeded in all areas, and that the Workshop's advice will lead to refinement in the near term and contribute to progress toward replacement. He stated that a report of the workshop would be published in 2001 and made available to the public. Dr. Stokes also recognized and thanked the ICCVAM Organizing Committee, Dr. Philip Sayre, Dr. John Frazier, and the NICEATM staff. The meeting was adjourned at 12:00 noon.

APPENDIX C

Guidance for Breakout Groups

International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity
October 17-20, 2000
Arlington, VA. U.S.A.

Guidance for Breakout Groups

Breakout Groups will address the applicable Workshop objectives and develop responses to the questions provided for each Breakout Group.

A. Workshop Objectives:

1. Review the status of *in vitro* methods for assessing acute systemic toxicity:
   a. Review the validation status of available *in vitro* screening methods for their usefulness in estimating *in vivo* acute toxicity;
   b. Review in vitro methods for predicting toxicokinetic parameters important to acute toxicity (i.e., absorption, distribution, metabolism, elimination);
   c. Review *in vitro* methods for predicting specific target organ toxicity;
2. Recommend candidate methods for future evaluation in prevalidation and validation studies;
3. Recommend validation study designs that can be used to adequately characterize the usefulness and limitations of proposed *in vitro* methods;
4. Identify reference chemicals that can be used for development and validation of *in vitro* methods for assessing *in vivo* acute toxicity;
5. Identify priority research efforts necessary to support the development of mechanism-based *in vitro* methods to assess acute systemic toxicity. Such efforts might include incorporation and evaluation of new technologies, such as gene microarrays, and development of methods necessary to generate dose response information.

B. Breakout Group Questions

Breakout Group 1: *In Vitro* Screening Methods for Assessing Acute Toxicity

This Breakout Group is asked to evaluate the validation status of available in vitro methods for estimating in vivo acute toxicity. The Group will identify methods and appropriate validation studies that might be completed within the next 1-2 years. The potential uses of QSAR as part of an in vitro strategy will also be evaluated.

Session 1-1: Identifying Needs

1. What are the near-term (< 2 years) goals and potentially attainable objectives for validation and use of *in vitro* methods that might reduce animal use for assessing acute toxicity?
2. What types of in vitro endpoints would be most effective for assessing in vivo acute toxicity; those that relate to general toxicity (e.g., cell death, growth inhibition) or those that are more cell or function specific (e.g., DNA damage/repair/synthesis; mitochondrial functionality; inhibition of other metabolic pathways)?
3. What other issues need to be considered for selecting protocols, e.g., robustness of protocol, reproducibility, stability of cell line?
4. What is the role of QSAR (and other prediction models) in predicting acute toxicity?

Session 1-2: Current Status

1. What are the available in vitro methods that might be useful in estimating acute in vivo toxicity.? Are standardized and/or optimized protocols available?
2. What are the strengths and limitations of available in vitro cytotoxicity assays (e.g., MEIC; ZEBET's validation efforts to extend cytotoxicity data to obtain better starting dose estimations; other mechanism-based cytotoxicity assays)?
3. What is the validation status of available in vitro screening methods (see Validation Criteria)?
4. Have any of these available in vitro methods been adequately evaluated for their usefulness for a specific purpose? If so, is their performance sufficient to recommend their use at this time?
5. What are the relative advantages and disadvantages for the use of human cells/tissues versus human cell lines versus animal cells/tissues versus animal cell lines?

6. To what extent do available methods take into consideration metabolic activation/inactivation of chemicals?
7. How have QSAR and other prediction models been used to estimate acute toxicity? What commercially available software exists? What are their advantages and disadvantages?
8. Are the available toxicity databases adequate to develop useful QSARs for industrial chemicals, consumer products, drugs? If not, what are the data needs?

Session 1-3: Future Directions

1. What are the most promising *in vitro* methods that should be further evaluated for their usefulness in reducing and/or refining animal use for acute toxicity?
   a.. What validation studies would be necessary to adequately evaluate the usefulness and limitations of these proposed methods for their proposed use?
   b. What research and/or developmental needs are required for candidate *in vitro* tests?
   c. What other mechanism-based *in vitro* methods or endpoints should be evaluated in future validation studies (e.g., microarray evaluation of altered gene expression patterns)? If so, which *in vitro* methods or endpoints should be given priority?
2. Which are the most promising *in vitro* methods for further evaluation or validation as replacements for *in vivo* acute toxicity test methods?
   a. What additional validation studies would be necessary to adequately evaluate the usefulness and limitations of these methods as replacements?
   b. What research and/or developmental needs are required for candidate *in vitro* tests?
   c. What other mechanism-based *in vitro* methods or endpoints should be evaluated in future validation studies (e.g., microarray evaluation of altered gene expression patterns)? If so, which *in vitro* methods or endpoints should be given priority?
3. How should individual tests be evaluated to determine their usefulness for integration into an overall acute toxicity testing strategy?
4. What criteria should be used to evaluate QSAR methods? To what extent could QSAR's be improved by an improved understanding of the molecular and cellular mechanisms of action of toxicity? What knowledge gaps exist that should be addressed by future research?

Breakout Group 2: *In Vitro* Methods for Assessing Acute Toxicity –Toxicokinetic Determinations

This Breakout Group will evaluate the capabilities of *in vitro* methods for providing toxicokinetic information (absorption, distribution, metabolism, and elimination) that can be used to estimate target organ dosimetry for acute toxicity testing and to provide recommendations for future research needs to accomplish this goal. The role of QSAR in toxicokinetic determinations will also be explored.

Session 2-1: Identify Needs

1. How can *in vitro* methods for evaluating chemical kinetics in biological systems contribute to the hazard and risk assessment process?
2. What is the role of toxicokinetics in the overall mechanisms by which chemicals illicit acute toxicity?
3. What toxicokinetic techniques should be considered as *in vitro* assays to improve predictivity and increase understanding of toxicity mechanisms? What is the role of QSAR in predicting chemical kinetics?

Session 2-2: Current Status

1. What *in vitro* methods are available for *in vitro* estimations of chemical-specific toxicokinetic parameters in animals and humans?
2. What are the strengths, limitations, and validation status of these available methods?
3. What mathematical approaches are available to predict or model toxicokinetics of

chemicals in mammalian systems based on data from *in vitro* systems?
4. What are the potential strengths and limitations of these approaches?
5. How would the approaches have to be modified/improved to meet acute toxicity testing needs?
6. How effective are the available QSAR systems for predicting *in vivo* toxicokinetic parameters?

Session 2-3: Future Directions

1. Which *in vitro*, QSAR or PBBK methods are the most promising for future use or development?
2. How should candidate methods be further developed/validated?
3. What are the more important issues to focus on in the long run (e.g., GI absorption, blood-brain barrier penetration)?
4. What research and development efforts are needed to achieve the ability to predict chemical kinetics in animals and humans?

Breakout Group 3: *In Vitro* Methods for Assessing Acute Toxicity - Specific Organ Toxicity and Mechanisms

This Breakout Group will review *in vitro* methods that can be used to predict specific organ toxicity or toxicity associated with alteration of specific cellular or organ functions, and develop recommendations for priority research efforts necessary to support the development of methods that can accurately assess target organ toxicity.

Session 3-1: Identify Needs

1. How can *in vitro* methods for assessing target organ toxicity contribute to hazard identification and dose-response assessment processes?
2. What is the relationship between *in vitro* mechanisms of toxicity and mechanisms by which chemicals are acutely toxic to animals and humans?
3. How can *in vitro* toxicity assays be used to predict acute organ-specific toxicity?
4. Can mechanism-based *in vitro* methods be developed to evaluate the range of *in vivo* toxicity processes and estimate those which may lead to injury or lethality?
5. What *in vitro* procedures and endpoints should be considered to improve predictability of *in vivo* effects and increase understanding of toxicity mechanisms?

Session 3-2: Current Status

1. What *in vitro* methods are available for target tissue-based estimations of animal and human responses to chemicals?
2. What is the validation status of these available methods?
3. What are their potential strengths and limitations?
4. How would they have to be modified/improved to enhance their usefulness?
5. Are techniques available to extrapolate *in vitro* cell toxicity data to predict acute systemic responses and ultimately system failure?

Session 3-3: Future Directions

1. Which are the most promising assays or methodologies to evaluate further?
2. How should each one be further developed/validated?
3. What are the research needs to attain the ability to predict acute toxicity in animals and humans?
4. What new methods or approaches are available that might improve mechanism-based in vitro estimations of animal and human responses to chemicals? How should they be developed for acute toxicity testing purposes?
5. How might the potential usefulness of microarray technology/differential gene expression for predicting systemic toxicity be further evaluated?
6. What research needs must be supported to improve QSAR methods for predicting target organ toxicity?

Breakout Group 4: Chemical Data Sets for Validation of *In Vitro* Toxicity Tests

This Breakout Group will have the responsibility of defining what chemical data sets are required for validation studies, identifying existing resources, and recommending approaches for using existing data sets and/or compiling or developing new data sets.

Session 4-1: Identify Needs

1. What are the characteristics of chemical [sets] that should be used in the validation of *in vitro* test methods for acute toxicity? For predicting organ-specific toxicity or toxicity based on specific mechanisms?
2. What criteria should be used for selecting chemical classes and chemicals to validate *in vitro* methods for assessing acute toxicity? Considering the different purposes of various *in vitro* methods, which sets of chemicals should be used to evaluate these different purposes?
3. To what extent and how should product classes/chemical classes (as used by regulatory agencies) be used to guide chemical selection?
4. To what extent and how should mode of action and biological target data be used to identify chemicals for use in validation studies?
5. How can QSAR methods help in the selection of validation chemicals?

Session 4-2: Current Status

1. What chemical data sets are available (e.g., EPA-HPV industrial chemicals, pesticides, drugs, food additives, NTP chemicals) that could be used for the validation of acute toxicity testing methods?
2. Are sufficient toxicity data available on existing chemicals or will additional data need to be obtained.
3. Do the available chemical data sets adequately represent the range of regulatory classifications for toxicity?
4. What QSAR models are currently available for such an effort?

Session 4-3: Future Directions

1. What are the characteristics of chemical data sets that could be used for validation of in vitro tests for in vivo toxicity (e.g., estimation of acute toxicity; identification of organ-specific toxic effects; determination of ADME parameters)?
2. To the extent possible, identify reference chemicals for which sufficient information is available that they should be considered for validation of assays/methodologies for predicting starting doses for in vivo studies, assays, or other assays that can be implemented in the near term? Are existing chemical sets adequate? Are additional chemicals needed, and if yes, are additional in vivo acute toxicity data needed?
3. To the extent possible, which reference chemicals should be used in the development/validation of assays/methods developed to predict in vivo acute toxicity in the longer term? Are different sets of chemicals needed to evaluate methods to predict target organ toxicity?
4. Should there be established chemical data sets for use in validation studies, or should they be selected or developed according to the specific test to be evaluated?
5. What additional chemical data sets need to be compiled or developed?
6. How should these chemical data sets be developed, and by whom?

APPENDIX D

Background Document for Workshop Participants

APPENDIX D

Background Document for Workshop Participants

This document was provided in the Background Materials and Supplemental Information Notebook for the International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity [Section I].

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D.2 Introduction
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D.4 *In Vitro* Screening Methods for Assessing Acute Toxicity (Breakout Group 1)
D.5 *In Vitro* Methods for Assessing Acute Toxicity – Toxicokinetic Determinations (Breakout Group 2)
D.6 *In Vitro* Methods for Assessing Acute Toxicity – Specific Organ Toxicity and Mechanisms (Breakout Group 3)
D.7 Chemical Data Sets for Validation of *In Vitro* Toxicity Tests (Breakout Group 4)
D.8 Relevant General Databases
D.9 References
D.10 Glossary

International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity

October 17-20, 2000
Hyatt Regency Crystal City
Arlington, VA, U.S.A.

Background Document

National Toxicology Program (NTP)
Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)
National Institute of Environmental Health Sciences
Research Triangle Park, NC 27709

September 2000

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LIST OF ABBREVIATIONS AND ACRONYMS

ATC	Acute Toxic Class Method
ATP	Adenosine triphosphate
CFN	Swedish National Board for Laboratory Animals
CPSC	Consumer Product Safety Commission
CTLU	Cytotoxicology Laboratory, Uppsala
DOT	Department of Transportation
ECVAM	European Center for the Validation of Alternative Methods
ECITTS	ERGATT/CFN Integrated Toxicity Testing Scheme
EDIT	Evaluation-Guided Development of *In Vitro* Tests
EPA	Environmental Protection Agency
ERGATT	European Research Group for Alternatives in Toxicity Testing
FDP	Fixed Dose Procedure
IC_{50}	Inhibitory Concentration - the concentration of a material estimated to reduce the biological endpoint (e.g., cell growth, ATP levels) being evaluated as a measure of toxicity by 50%.
$IC_{50}\bar{x}$	Mean of two or more IC_{50} values
ID_{50}	Model body doses that affect the endpoint in question by 50%.
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
i.p.	Intraperitoneal
LD_{50}	Dose producing lethality in 50% of the animals
LDH	Lactate dehydrogenase
MEIC	Multicenter Evaluation of *In Vitro* Cytotoxicity
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H* tetrazolium bromide
NICEATM	NTP Interagency Center for the Evaluation of Alternative Toxicological Methods
NLM	National Library of Medicine
NRU	Neutral Red Uptake
NTP	National Toxicology Program
OECD	Organization for Economic Cooperation and Development
OSHA	Occupational Safety and Health Administration
PBBK	Physiologically Based Biokinetic
PBPK	Physiologically Based Pharmacokinetic
QSAR	Quantitative Structure Activity Relationship
TG	Test Guideline
UDP	Up-and-Down Procedure
U.S.	United States
ZEBET	The German Center for Documentation and Evaluation of Alternative Methods to Animal Experiments

1.0 Preface

[Note: This document has been modified for inclusion in this *In Vitro* Workshop Report.]

This document provides background information to facilitate discussion at the International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity, to be held on October 17-20, 2000, at the Hyatt Regency Crystal City in Arlington, VA, U.S. Undoubtedly, other information on this topic exists. Participants are encouraged to bring relevant information to the attention of NICEATM for consideration at the workshop. The Introduction (**Section 2**) provides information on acute toxicity, the uses of acute toxicity testing data by regulatory authorities and clinicians, and the U.S. and OECD *in vivo* test methods currently used for assessing acute toxicity. **Section 3** discusses general strategies for using *in vitro* test methods to assess *in vivo* toxicity, including the use of quantitative structure activity relationships (QSAR). Sections 4 - 7 provide information relevant to each of the four Workshop Breakout Groups: Breakout Group 1: *In Vitro* Screening Methods for Assessing Acute Toxicity; Breakout Group 2: *In Vitro* Methods for Assessing Acute Toxicity –Toxicokinetic Determinations; Breakout Group 3: *In Vitro* Methods for Assessing Acute Toxicity - Specific Organ Toxicity and Mechanisms; and Breakout Group 4: Chemical Data Sets for Validation of *In Vitro* Toxicity Tests, including lists of relevant publications. Information on potentially useful general databases is provided in **Section 8**, a complete list of references cited is provided in **Section 9**, and a Glossary in **Section 10.**

2.0 Introduction

Acute toxicity testing in animals is typically the initial step in the assessment and evaluation of the health effects characteristics of a test substance, and its primary purpose is to provide information on potential health hazards that may result from a short-term exposure (OECD, 1987). This information is used to properly classify and label materials as to their toxicity in accordance with national and international regulations and guidelines. An internationally harmonized system has also been proposed (OECD, 1998a). Another purpose of such studies is to help guide the design of longer-term health effects studies. Acute oral toxicity is defined as the adverse effects occurring within a short time (i.e., up to a few weeks) of oral administration of a single dose of a substance or multiple doses given within 24 hours (OECD, 1987). It is typically presented as an LD_{50} value, which is a statistically derived estimate of the single dose of a substance that can be expected to cause death in 50 percent of the treated animals. LD_{50} data are expressed in terms of amount of the test substance per unit body weight of the animal (e.g., g or mg/kg). Potential target organ toxicity, toxicokinetic parameters, and dose-response relationships may also be evaluated in acute toxicity studies. While animals are currently used to evaluate acute toxicity, recent studies suggest that *in vitro* methods might be helpful in predicting acute toxicity and in estimating *in vivo* toxic chemical concentrations.

Studies by Spielmann et al. (1999) suggest that *in vitro* cytotoxicity data may be useful in identifying an appropriate starting dose for *in vivo* studies, and thus may potentially reduce the number of animals necessary for such determinations. Other studies (e.g., Ekwall et al., 2000) have indicated an association between chemical concentrations leading to *in vitro* cytotoxicity and human lethal blood concentrations. A program to estimate toxicokinetic parameters and target organ toxicity utilizing *in vitro* methods has been proposed that may provide enhanced predictions of toxicity, and potentially reduce or replace animal use for some tests (Ekwall et. al., 1999). However, many of the necessary *in vitro* methods for this program have not yet been developed. Other methods have not been evaluated for reliability and relevance, and their usefulness and limitations for generating information to meet regulatory requirements for acute toxicity testing have not been assessed.

The International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity will examine the status of available *in vitro* methods for assessing acute toxicity. The methods to be addressed will include screening methods for acute toxicity, such as methods that might be used

to predict the starting dose for *in vivo* animal studies, and methods for generating information on toxicokinetics, target organ toxicity, and mechanisms of toxicity. The Workshop will develop recommendations for validation efforts necessary to characterize the usefulness and limitations of these methods. Recommendations will also be developed for future mechanism-based research and development efforts that might further improve *in vitro* assessments of acute systemic lethal and non-lethal toxicity.

The objectives of the Workshop are to:

- Review the status of *in vitro* methods for assessing acute systemic toxicity:
  a. Review the validation status of available *in vitro* screening methods for their usefulness in estimating *in vivo* acute toxicity;
  b. Review *in vitro* methods for predicting toxicokinetic parameters important to acute toxicity (i.e., absorption, distribution, metabolism, elimination);
  c. Review *in vitro* methods for predicting specific target organ toxicity;
- Recommend candidate methods for further evaluation in prevalidation and validation studies;
- Recommend validation study designs that can be used to adequately characterize the usefulness and limitations of proposed *in vitro* methods;
- Identify reference chemicals that can be used for development and validation of *in vitro* methods for assessing *in vivo* acute toxicity;
- Identify priority research efforts necessary to support the development of mechanism-based *in vitro* methods to assess acute systemic toxicity. Such efforts might include incorporation and evaluation of new technologies, such as gene microarrays, and development of methods necessary to generate dose response information.
-

2.1 Uses of Acute Toxicity Testing Data by Regulatory Authorities

Internationally, the most common use of acute systemic toxicity data is to provide a basis for hazard classification and the labeling of chemicals for their manufacture, transport, and use (**Table 1, OECD, 1998a**). Other, potential uses for acute toxicity testing data include:

- Establish dosing levels for repeated-dose toxicity studies;
- Generate information on the specific organs affected;
- Provide information related to the mode of toxic action;
- Aid in the diagnosis and treatment of toxic reactions;
- Provide information for comparison of toxicity and dose response among substances in a specific chemical or product class;
- Aid in the standardization of biological products;
- Aid in judging the consequences of exposures in the workplace, home, or from accidental release, and
- Serve as a standard for evaluating alternatives to animal tests.

Table 1. OECD Harmonized Integrated Hazard Classification System for Human Health and Environmental Effects of Chemical Substances—Oral Toxicity (OECD, 1998a)

	Class 1	Class 2	Class 3	Class 4	Class 5
Oral (mg/kg)	5	50	300	2000	5000

2.2 Uses of Acute Toxicity Testing Data by Clinicians

In an effort to obtain information on the uses of acute toxicity data by clinicians, NICEATM contacted Ms. Kathy Kirkland, the Director of the Association of Occupational and Environmental Clinics. Ms. Kirkland queried the clinicians within the Association for such information. The following outlines the responses from two physicians.

In a clinic that deals primarily with cases of heavy metal and pesticides exposures, LD_{50} values are used to assess the dose and likelihood of toxic effects in a patient. However, many of the cases deal with mixed or unknown exposures, and LD_{50} values are not available for these materials. *In vitro* cytotoxicity data is utilized in a body of evidence approach to the extent that it is available.

In another clinical practice that treats mainly chronic toxicity cases (e.g., pneumonoconiosis, malignancy, solvent neurotoxicity), the clinicians tend to rely on historical human toxicity data, such as published reports of previous industrial toxicity, for which there is much literature. It was felt that animal toxicity data alone is not very useful in the absence of a clinical database, but that animal studies are helpful in supporting human epidemiological literature for occupational cancer. No specific response was provided on the use of *in vitro* cytotoxicity test data.

2.3 Current *In Vivo* Methods for Assessing Acute Toxicity

The first of the methods described in this section (the conventional LD_{50} test) is the approach used historically to provide acute toxicity data (LD_{50} value, slope of the dose-response curve, confidence interval), and information regarding toxic signs. Compared to other, more recently developed alternative *in vivo* methods for evaluating acute toxicity, the conventional LD_{50} test requires the use of more animals. For this reason, there are considerable international efforts through the OECD to delete the test guideline for this method (Test Guideline [TG] 401). These efforts have prompted a re-assessment of all of the OECD *in vivo* test guidelines for acute toxicity to ensure that regulatory needs are met while minimizing animal usage and maximizing data quality. Each of the OECD *in vivo* test methods is described in this section.

In these *in vivo* test methods, rats are the preferred species, although other rodent species may be used. Oral gavage is the primary route for administration of solid and liquid test substance. Doses that are known to cause marked pain and distress due to corrosive or severely irritant actions are not used. In the draft alternative *in vivo* test method guidelines, animals of a single sex are considered sufficient. Females are given preference because literature surveys of test results using the OECD TG 401 method have shown that although there is little difference in sensitivity between the sexes, in those cases where significant differences were observed, females were more frequently the more sensitive sex.

2.3.1 *The Conventional LD_{50} Test (OECD TG 401)*

OECD TG 401 (OECD, 1987) outlines the conventional LD_{50} test to assess acute oral toxicity. The use of five animals (of the same sex) using at least three dose levels in the toxic/lethal range is recommended. The test often

uses five or more dose levels. When testing is completed in one sex, at least one group of five animals of the other sex is dosed to establish that animals of this sex do not have markedly different sensitivity to the test substance. When testing substances for which no relevant toxicity information is available, a range-finding or sighting study that uses up to five animals must be conducted. Thus, a minimum of 20 to 25 animals would be used in each study. Generally, the test substance is administered to all animals within a study on the same day to eliminate potential differences in preparing the test substance solutions on different days. The goal of the test is to produce at least two dose groups in which at least one, but not all, of the animals is killed by the test substance with 14 days. If this occurs, the LD_{50}, its confidence interval, and the slope of the dose-response curve can be calculated using probit analysis, and a hazard classification determined.

When it is suspected that the test substance may have little or no toxicity, a limit test may be conducted. TG 401 specifies testing five animals of each sex at 2000 mg/kg. If test substance-related mortality is produced, a full study may need to be conducted. If no mortality occurs, the substance is classified as having an LD50 of >2000 mg/kg

2.3.2 *Fixed Dose Procedure (FDP) (Draft OECD TG 420)*

The draft OECD TG 420 (OECD, 1999a) describes the FDP for acute toxicity testing. The method is designed so that only moderately toxic doses are administered (i.e., doses that are expected to be lethal are avoided). The method allows test substances to be ranked and classified according to a globally harmonized system for the classification of chemicals that cause acute toxicity (**Table 1**) (OECD, 1998a).

Specifically, groups of animals of a single sex are dosed in a step-wise procedure using fixed doses of 5, 50, 300, and 2000 mg/kg (exceptionally, an additional fixed dose of 5000 mg/kg may be considered, if required for a specific regulatory purpose). The initial dose for the main study is selected on the basis of a sighting study as the dose expected to produce some signs of toxicity without causing severe toxic effects or mortality. The initial fixed dose selected for the sighting study is one expected to produce evident toxicity based, when possible on evidence from structurally related chemicals. In the absence of such information, the sighting fixed dose is 300 mg/kg; the test substance is administered to a single animal per dose group in a sequential manner, with at least 24 hours allowed between the dosing of each animal. Subsequent animals are dosed at higher or lower fixed doses depending on the absence or presence of toxic signs or mortality, respectively. The procedure continues until the dose causing evident toxicity, or not more than one death, is identified, or when no effects are observed at the limit dose, or when deaths occur at the lowest dose.

In the main test, five animals per dose level are usually used. The animals tested during the sighting study are included in that total. Thus, if an animal had been tested at a specific dose level in the sighting study, only four more animals would be tested at that same dose level, if it were selected as an appropriate dose to test further.

In vivo and modeling studies have shown the FDP to be reproducible (OECD, 1999a). The method is considered advantageous because it:

- Uses fewer animals than OECD TG 401,
- Causes less suffering than tests that primarily use lethality and morbidity as the endpoint, and
- Is able to rank test substances in a similar manner to other *in vivo* alternative acute toxicity test methods (e.g., the Acute Toxic Class Method [ATC]).

The FDP is not intended to allow for the calculation of the LD_{50} value or of a dose-response slope.

2.3.3 *Acute Toxic Class Method (ATC) (Draft OECD TG 423)*

The ATC is a step-wise procedure that uses three animals of a single sex per step (OECD, 1999b). Testing is conducted at defined doses of 5, 50, 300, and 2000 mg/kg (exceptionally, an additional

fixed dose of 5000 mg/kg may be considered, if required for a specific regulatory purpose) that allow a test substance to be ranked and classified according to a globally harmonized system for the classification of chemicals that cause acute toxicity (**Table 1**) (OECD, 1998a). The dose level to be used as the starting dose is selected from one of the four fixed dose levels based on an expectation that mortality would be induced in at least some of the dosed animals. When available information suggests that mortality is unlikely at the limit dose, then a limit test should be conducted. A limit test involves testing three animals of the same sex at the limit dose. When there is no information on a substance to be tested, it is recommended for animal welfare concerns that the starting dose be 300 mg/kg. Depending on the mortality and/or moribund status of the animals, an average of two to four steps may be necessary to allow judgement of the acute toxicity potential of the test substance. The time interval between treatment groups is determined by the onset, duration, and severity of toxic signs. Treatment of animals at the next higher dose should be delayed until one is confident of survival of the previously dosed animals. The number of animals used per test is generally in the range of six to 12. The method is based on biometric evaluations, and has been validated internationally (OECD, 1999b).

The ATC is not intended to allow for the calculation of the LD_{50}, but does allow for the determination of defined exposure ranges where lethality is expected, since death of a proportion of animals is a major endpoint of the test. An LD_{50} can be calculated only when at least two doses result in mortality in some, but not all, animals. The main advantage of this method is that it requires fewer animals than OECD TG 401. In theory, the method also should increase laboratory-to-laboratory reproducibility because the provisions for dose selection and interpretation are specifically set.

2.3.4 *Up-and-Down Procedure (UDP) (U.S. EPA Draft OECD TG 425)*

The U.S. EPA draft of OECD TG 425 (OECD, 1998b) specifies the approach for conducting the UDP. In this procedure, animals are dosed sequentially at 48-hour intervals. The first animal receives a dose at the best estimate of the LD_{50}; when no information is available, an initial dose of 175 mg/kg is recommended. Depending on the outcome for the previous animal, the dose for the next animal is adjusted upwards or downwards by a dose-spacing factor of 3.2 (half-log). If an animal survives, the dose for the next animal is higher; if the animal dies or is moribund, the dose for the next animal is lowered. Dosing continues depending on the fixed-time interval outcomes of all the animals up to that time. The testing stops when (1) three consecutive animals survive at the limit dose (or three consecutive animals die at a predetermined lower limit dose, or (2) five reversals occur in 6 animals started, or (3) at least 4 animals have followed the first reversal and the criteria of the stopping rules based on likelihood-ratios are met (OECD, 1998b). A reversal is a situation where nonresponse is observed at some dose, and a response is observed at the next dose tested. Calculations are made with each dose, following the fourth animal after the first reversal. For a wide variety of combinations of LD50 and slopes as low as 2.5, the stopping rule (i.e., the criteria for terminating the study) will be satisfied with four to six animals after the first reversal. However, for chemicals with a shallow dose-response slope, more animals (but not more than 15) may be needed. When the stopping criteria have been attained after the initial reversal, the estimated LD50 should be calculated from the animal outcomes at test termination using the statistical method described in the Guideline (OECD, 1998b). The LD_{50} is calculated using the method of maximum likelihood.

When weak toxicity is suspected, a limit test may be used. A single animal is tested at the limit dose of 2000 or 5000 mg/kg. Which limit dose is used depends on the regulatory requirement being fulfilled. If the animal survives, then two additional animals receive the same dose. If one or more of these two animals die, a fourth and perhaps a fifth animal is placed on test at the same dose. At 5000 mg/kg, the test is terminated whenever a total of three animals have survived or have died. At 200 mg/kg, all 5 animals must be tested. If three animals survive, the LD50 is above the limit dose; if three animals die, the LD50 is below the limit dose. In situations where

the first animal dies, the UDP main test is conducted. Also, if three animals have died and an LD50 value is required, the UDP main test is conducted.

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the National Toxicology Program (NTP) Interagency Center on the Validation of Alternative Toxicological Methods (NICEATM) recently coordinated a peer review of U.S. EPA draft TG 425; the peer review report for that meeting will be available soon.

3.0 *In Vitro* Test Methods for Predicting *In Vivo* Toxicity—General Strategies

Cytotoxicity is defined as the adverse effects resulting from interference with structures and/or processes essential for cell survival, proliferation, and/or function. These effects may involve the integrity of membranes and the cytoskeleton, cellular metabolism, the synthesis and degradation or release of cellular constituents or products, ion regulation, and cell division. Generally, three principal mechanisms for toxicity have been identified. These include general (also known as basal) toxicity, selective toxicity, and cell-specific function toxicity. General cytotoxicity involves one or more structures or processes that would be expected to be intrinsic to all cell types (e.g., mitochondrial function, membrane integrity). Selective cytotoxicity occurs when some types of differentiated cells are more sensitive to the effects of a particular toxicant than others, potentially as a result of, for example, binding to specific receptors, or uptake by a cell-type specific mechanism. Cell-specific function cytotoxicity occurs when the toxicant affects structures or processes that may not be critical for the affected cells themselves, but which are critical for the organism as a whole. For example, such toxicity can involve effects on cell-to-cell communication, via the synthesis, release, binding and degradation of cytokines, hormones and transmitters.

Numerous assays have been developed for assessing cytotoxicity *in vitro* (see **Table 2**). However, until recently, there has been little emphasis on to how to apply the resulting data to predicting *in vivo* toxicity and to the regulatory decision-making process. Several large scale, international multi-laboratory studies have attempted to address the issue of using *in vitro* toxicity information to predict *in vivo* test substance-induced toxic effects (Fentem et al., 1993; Garle et al., 1994); some of these studies will be discussed in subsequent sections. The goals of these studies have ranged from a complete replacement of *in vivo* acute toxicity tests by *in vitro* tests (e.g., see **Section 4.1**) to reducing animal usage by using *in vitro* cytotoxicity data to identify the optimal starting dose for an *in vivo* acute toxicity test (e.g., see **Section 4.3**), or to determine whether a limit test should be conducted first.

Several work groups have proposed the potential use of *in vitro* cytotoxicity test methods in a tiered testing scheme. For the sake of brevity, only two examples are provided here although other, generally similar approaches have been presented in different forums (e.g., see **Section 6.1**).

In 1996, Seibert et al. reported on an international evaluation of selected *in vitro* toxicity test systems for predicting acute systemic toxicity (see also Fentem et al., 1993). The goal of the evaluation was to identify strategies for using data obtained from *in vitro* tests as a basis for classifying and labelling new chemicals, thereby reducing (and possibly replacing) the need for acute toxicity tests in animals. A diverse group of 42 chemicals were evaluated; the chemicals had been tested in a range of *in vitro* systems (bovine spermatozoa, BALB/c 3T3 cells, rat hepatocytes, rat skeletal muscle cells, hepatocyte/3T3 co-cultures, V79 cells, 3T3-L1 cells, and V79/hepatocyte co-cultures), employing various exposure periods and endpoint measurements. *In vitro* effective concentration values were compared with *in vivo* rodent LD50 values. Based on the recommendations of the participants, the following tiered testing scheme for assessing acute toxicity was proposed.

In Stage 1, basal cytotoxicity is determined using cell proliferation inhibition as the endpoint. In Stage 2, a test is conducted to determine hepatocyte-specific cytotoxicity and to define the role of metabolism in the cytotoxic effects of the

test chemical. Finally, in Stage 3, additional testing is conducted that would provide information on selective cytotoxicity (other than hepatocyte-specific cytotoxicity) as well as an indication of any interference with important specific, but non-vital, cell functions. Many test systems may be appropriate for this level of testing, including the use of cells from the nervous system, heart, or kidney.

More recently, and based also on discussion at a meeting that focused on validation and acute toxicity testing, Curren et al. (1998) also suggested the use of *in vitro* cytotoxicity and other information tests in a tiered testing approach. Step one would be the collection and integration of information on the physical/chemical properties of a compound, including literature reviews and analysis of the structure-activity relationships (when possible). Step two would be the determination of general cytotoxicity using an *in vitro* model system. This Step would include gathering information (via *in vitro* models) on gastrointestinal uptake, the penetration of the blood-brain barrier, and biotransformation. In Step three, general cytotoxicity information could be reinforced and supplemented with computer-based modeling of biokinetic data.

Curren et al. (1998) concluded that these steps might provide sufficient information to estimate the hazard classification for some compounds. In cases where additional information is needed, tests using a limited number of animals might be conducted to supplement the data obtained from literature review, *in vitro* testing, and computer modeling. Curren et al. (1998) recognized also that the use of this tiered testing strategy is currently limited because there is insufficient information on structure-activity relationships with respect to acute systemic toxicity, most likely because of the large number of mechanisms involved in the expression of this type of toxicity. Thus, substantial additional investigation into the cause of chemically induced lethality is needed. Curren concluded that the *in vitro* models used to determine gastrointestinal uptake, blood-brain barrier passage, and biotransformation have not been formally validated.

A variety of *in vitro* tests have been developed to evaluate the types of cytotoxicity (general or basal, selective, cell-specific function) that have the potential to result in acute systemic toxicity, with the greater effort focused on general toxicity. Any strategy used to extrapolate *in vitro* toxicity test results to an *in vivo* toxicity response must consider all of these possibilities, as well as toxicokinetics. To provide some indication of the range of biological endpoints used to assess cytotoxicity *in vitro*, **Table 2** summarizes the *in vitro* toxicity endpoints/test systems used in three large studies. Information on the reliability (intra-laboratory repeatability and inter-laboratory reproducibility) of any *in vitro* toxicity test method was not located. The studies considered for this document evaluated the correlation between *in vitro* test method results and animal LD50 or human lethal blood concentrations; test method reliability was not addressed.

3.1 Quantitative Structure Activity Relationship (QSAR) Methods

The potential uses of QSAR as part of an *in vitro* strategy will need to be evaluated during the Workshop. QSAR methods are models that relate the biological activities of a series of similar compounds to one or more physicochemical or structural properties of the compounds (Barratt et al., 1995). 'Similar' includes compounds that exhibit the same mechanism of action in addition to those that have related chemical structures. However, it is often difficult to determine mechanism of action, whereas it is less difficult to establish chemical similarity. Therefore, QSAR models are usually developed for sets of chemically similar compounds on the assumption that they will have the same mechanism of action. Any compounds that do not act by the same mechanism are likely to poorly fit the correlation, and would thus not be accurately modeled or predicted.

Table 2. Various *In Vitro* Cytotoxicity Endpoints Evaluated in MEIC and Spielmann et al. (1999)

Endpoint	Measured as	Cell Line(s)	Study
Cell viability	ATP content or leakage	ELD cells (mouse); erythrocytes (mouse); LS-L929 cells (mouse); hepatocytes (rat); spermatozoa (bovine); HL-60 cells (human)	MEIC
	Cell morphology	C9 cells (rat); hepatocytes (rat); L2 cells (rat); MDBK cells (bovine); Chang liver cells (human); HeLa cells (human); McCoy cells (human); WI-1003/Hep-G2 cells (human)	MEIC
	Chromium release	LS-L929 cells (mouse)	MEIC
	Creatine kinase activity	Muscle cells (rat)	MEIC
	Hemolysis	Erythrocytes (human)	MEIC
	Killing index (*sic*)	SQ-5 cells (human)	MEIC
	LDH release	3T3 Cells (mouse); hepatocytes (rat, human); Hep-2 cells (human); Hep-G2 cells (human); lymphocytes (human); SQ-5 cells (human)	MEIC
	Neutral Red Uptake	3T3 cells (mouse); L929 cells (mouse); NB41-A3 cells (mouse); BHK cells (hamster); hepatocytes (rat, human); HeLa cells (human); Hep-2 cells (human); keratinocytes (human)	MEIC; Spielmann et al. (1999)
	Plating efficiency	HeLa cells (human)	MEIC
	^{86}Rb leakage	Not designated	MEIC
	Viable cell count	LS-L929 cells (mouse); polymorphonuclear leukocytes (human)	MEIC
Cell growth	Cell cycle distribution	Daudi cells (human), RERF-LC-AI cells (human)	MEIC
	Glucose consumption	Muscle cells (rat)	MEIC
	Macromolecule content	HTC cells (rat); Hep-G2 cells (human)	MEIC
	MTT metabolism	3T3 cells (mouse); L929 cells (mouse); NG108-15 cells (mouse, rat); V79 cells (hamster); hepatocytes (rat, human); Detroit 155, DET dermal fibroblasts (human); FaO cells (human); Hep-G2 cells (human); HFL1 cells (human); 3D Skin^2, Dermal Model ZK1100 keratinocytes (human); lymphocytes (human); RERF-LC-AI cells (human); WS1 cells (human)	MEIC
	pH change	L2 cells (rat); Chang liver cells (human); HeLa cells (human); WI-1003/Hep-G2 cells (human)	MEIC

Endpoint	Measured as	Cell Line(s)	Study
	Protein content	3T3 or 3T3-L1 cells (mouse); Hepa-1c1c7 (mouse); L929 cells (mouse); V79 cells (hamster); hepatocytes (rat); PC12h cells (rat); LLC-PK1 cells (pig); HeLa cells (human); Hep-2 cells (human); Hep-G2 cells (human); MRC-5 cells (human); NB-1 cells (human); Chinese hamster V79 cells	MEIC; Spielmann et al. (1999); Fry et al., 1990
	Tritiated-proline uptake	L2 cells (rat)	MEIC
	Tritiated-thymidine incorporation	Peripheral lymphocytes (human)	MEIC, Spielmann et al. (1999)
	Cell resting membrane potential	NG108-15 (mouse, rat)	MEIC
	Chemotaxis/locomotion stimulated by chemotactic peptide	Polymorphonuclear leukocytes (human)	MEIC
	EOD activity	Hepatocytes (rat)	MEIC
Specialized function effects	Inhibition of NK cell-mediated cytotoxicity activity	Natural killer cells, including over 90% CD16+ or CD56+ cells (human)	MEIC
	Intracellular glycogen content	Hepatocytes (rat)	MEIC
	Motility or velocity	Spermatozoa (bovine)	MEIC
	Spontaneous contractility	Muscle cells (rat)	MEIC

Abbreviations: ATP = Adenosine triphosphate; CR = calorimetric respirometric ratio; EOD = 7-ethoxycoumarin *O*-deethylase; LDH = Lactate dehydrogenase; MTT = 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide; MEIC = Multicenter Evaluation of *In Vitro* Cytotoxicity (see summary in **Appendix 6 [Appendix E *of the In Vitro Workshop Report*]**).

In a review of QSAR studies, Phillips et al. (1990) concluded that QSAR methods have shown some success in relating LD_{50} values to certain physicochemical properties of a compound (especially lipophilicity). However, QSAR appears to be less successful in correlating electronic properties of molecules (related to reactivity) or structural variables with LD_{50} values.

Of the numerous QSAR studies intended to rationalize and predict the *in vivo* mammalian toxicity of chemicals based on properties related to structure, one popular approach is the linear free-energy, extra-thermodynamic method developed by Hansch and colleagues (Phillips et al., 1990). The basic assumption of this approach is that the effect of the substituents on the magnitude of a compound's interaction with biological receptors or other molecules is an additive combination of the substituents' interactions in simpler systems.

A second common approach was developed by Free and Wilson in 1964 (Phillips et al., 1990). It is based on the assumption that, for congeneric

series of compounds with multiple sites of substitutions, the observed activity can be expressed in terms of the mutually independent contributions from the various substituents of the molecule.

Requirements/caveats for the successful development and use of QSAR methods include the following:

- There should be a well-defined mechanism of action for the compound(s) used to derive the QSAR model (Phillips et al., 1990; Barratt et al., 1995);
- The compounds should form part of a congeneric group (Phillips et al., 1990) and should be pure (i.e., not mixtures) (Barratt et al., 1995);
- There should be a common site of action for the biological effect (Phillips et al., 1990);
- As for any comparative purpose, concentrations or doses should be presented in molar (not weight) units (Barratt et al., 1995);
- Each QSAR model should be validated by investigating its predictive ability using a different set of compounds from its learning set, which should cover the same ranges of parameter space as the original test chemicals (Barratt et al., 1995); and
- The QSAR should not be applied outside of its domain of validity (i.e., outside the parameter space covered by the training set) (Barratt et al., 1995).

3.1.1 *Publications Containing Further Information*

Free, S.M., And J.W. Wilson. 1964. A Mathematical Contribution To Structure-Activity Studies. J. Med. Chem. 7: 395-399.

Hansch, C., and T. Fujita. 1964. ρ, σ, π Analysis. A method for the correlation of biological activity and chemical structure. J. Am. Chem. Soc. 86: 1616-1626.

4.0 *In Vitro* Screening Methods for Assessing Acute Toxicity (Breakout Group 1)

This Breakout Group will evaluate the validation status of available *in vitro* methods for estimating *in vivo* acute toxicity. The Group will identify the most promising methods and recommend appropriate validation studies that might be completed within the next one to two years. The potential uses of QSAR as part of an *in vitro* strategy will also be evaluated (see **Section 3.1**). Most of the *in vitro* test method development for assessing cytotoxicity has focused on general (or basal) cytotoxicity. General cytotoxicity is independent of cell type and involves one or more adverse effects that interfere with structures and/or processes essential for cell survival, proliferation, and/or function. These effects may include adverse effects on the integrity of membranes (including the cytoskeleton), general metabolism, ion regulation, and cell division. Studies conducted to evaluate the suitability of *in vitro* general cytotoxicity methods for predicting *in vivo* toxicity are described briefly; more detailed information can be obtained as indicated.

4.1 The Multicenter Evaluation of *In Vitro* Cytotoxicity (MEIC)

Additional details of the MEIC study are reported in the MEIC Summary prepared by NICEATM (**Appendix A [Appendix E of the *In Vitro* Workshop Report]**) and in the list of MEIC-related publications provided in **Section 4.1.4**.

4.1.1 *General Study Description*

The MEIC program was organized by the Scandinavian Society for Cell Toxicology in 1989. The intent of the program was to investigate the relevance of *in vitro* test results for predicting the acute toxic action of chemicals in humans. Given that such relevance was identified, the next goal was to establish batteries of existing *in vitro* toxicity tests that have the potential to serve as replacements for acute toxicity tests using laboratory mammals.

MEIC was a voluntary effort involving 96 international laboratories that evaluated the

effectiveness of *in vitro* cytotoxicity tests originally developed as alternatives to (or supplements for) laboratory mammal tests for acute and/or chronic systemic toxicity, organ toxicity, skin irritancy, or other forms of general toxicity. Minimal methodological directives were provided in order to maximize protocol diversity among the laboratories. The collection of test method data was completed in 1996; to date, 24 publications originating from these studies have been published.

By the end of the project, 39 laboratories had tested the first 30 reference chemicals in 82 *in vitro* assays, while the last 20 chemicals were tested in 67 *in vitro* assays. The primary 82 assays included 20 human cell line assays; seven human primary culture assays utilizing hepatocytes, keratinocytes, and polymorphonuclear leukocytes; 19 animal cell line assays, 18 animal primary culture assays, and 18 ecotoxicological tests utilizing bacteria, rotifer, crustacea, plant, and fish cells. Thirty-eight of these assays were based on viability, 29 on growth, and the remaining assays involved more specific endpoints, such as locomotion, contractility, motility, velocity, bioluminescence, and immobilization. The endpoints assessed were based on exposure durations ranging from five minutes to six weeks. The analyses conducted by the MEIC management team were based on *in vitro* toxicity data presented as IC50 values (i.e., the dose estimated to affect the endpoint in question by 50%). The types of comparative data used to evaluate the predictive accuracy of the *in vitro* IC50 toxicity data for *in vivo* acute toxicity included oral rat and mouse LD50 values, acute oral lethal doses in humans, clinically measured acute lethal serum concentrations in humans, acute lethal blood concentrations in humans measured post-mortem, human pharmacokinetics following single doses, peaks from curves of an ~50% lethal blood/serum concentration over time after ingestion.

4.1.2 *List of Chemicals Tested and Selection Rationale*

The chemical set (50 chemicals) used in the MEIC studies is provided in the MEIC Summary (**Appendix A [Appendix E of the *In Vitro* Workshop Report]**). These chemicals were selected because of the availability of human data on acute toxicity (e.g., lethal blood concentrations).

4.1.3 *Summary Conclusions*

Based on the results obtained, a battery of four endpoints/two exposure times (protein content/24 hours; ATP content/24 hours; inhibition of elongation of cells/24 hours; pH change/7 days) in three human cell line tests was found to be highly predictive of the peak human lethal blood concentrations (LC50) of chemicals when incorporated into an algorithm developed by the MEIC management team. The MEIC management team concluded that the battery could be used directly as a surrogate for a LD50 test. However, since the battery predicts lethal blood concentrations, not lethal oral dosages, it is not a direct counterpart of the animal LD50 test. Thus, the battery must be supplemented with data on gut absorption as well as the distribution volumes of chemicals. Furthermore, in this study, there was no assessment of test method reliability, either within or between laboratories.

4.1.4 *Publications Containing Additional Study Information*

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Hellberg, S., L. Eriksson, J. Jonsson, F. Lindgren, M. Sjöström, S. Wold, B. Ekwall, M.J. Gómez-Lechón, R. Clothier, N.J. Accomando, G. Gimes, F.A. Barile, M. Nordin, C.A. Tyson, P. Dierickx, R.S. Shrivastava, M., Tingsleff-Skaanild, L. Garza-Ocanas, and G. Fiskesjö. 1990. Analogy Models for Prediction of Human Toxicity. ATLA 18: 103-116.

Shrivastava, R., C. Delomenie, A. Chevalier, G. John, B. Ekwall, E. Walum, and R. Massingham. 1992. Comparison of *In Vivo* Acute Lethal

Potency and *In Vitro* Cytotoxicity of 48 Chemicals. Cell Biol. Toxicol. 8(2): 157-170.

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Walum, E., M. Nilsson, C. Clemedson, and B. Ekwall. 1995. The MEIC Program and its Implications for the Prediction of Acute Human Systemic Toxicity. In: Proceedings of the World Congress on Alternatives and Animal Use in the Life Sciences: Education, Research, Testing. Alternative Methods in Toxicology and the Life Sciences 11: 275-282. Mary Ann Liebert, New York.

4.2 Correlation of acute lethal potency with *in vitro* cytotoxicity. (Fry et al., 1990)

Fry et al. (1990) evaluated the *in vitro* cytotoxicity of 27 compounds believed to act by interference with cell basal functions/structures. The cytotoxic endpoint assessed was growth inhibition in Chinese hamster V79 cells. ID_{50} values were calculated and compared to either oral or intraperitoneal (i.p.) LD_{50} values from mice or rats. Although significant positive correlations were found when either log i.p. or log oral LD50 values were compared to log ID_{50} values, the correlation was 'better' when log i.p. LD_{50} values were used. A further improvement was obtained when data from three compounds (>10%) were excluded for which metabolism is a major determinant of toxicity *in vivo*. Close correlations of log i.p. LD_{50}/log ID_{50} values were obtained with groups of six anti-metabolites and six alkylating agents, although the locations of the regression lines for these two groups were significantly different. Based on these results, the authors concluded that the *in vitro* cytotoxicity of compounds that exert their toxicity by interference with cell basal functions/structures is correlated with their intrinsic lethal potency. However, information on absorption, metabolism, and disposition is required before *in vitro* cytotoxicity data can be used to assess *in vivo* potency. The data also indicated that the precise relation of LD_{50} to ID_{50} values was determined by the mode of toxicity. In this study, there was no assessment of test method reliability, either within or between laboratories.

4.3 Determination of the starting dose for acute oral toxicity (LD50) testing in the up and down procedure (UDP) from cytotoxicity data. (Spielmann et al., 1999)

Additional details of this study are reported in Spielmann et al. (1999), while related information are provided in **Appendix B [Section 7.0 of the *In Vitro* Workshop Report]**.

4.3.1 *General Study Description*

The Spielmann et al. (1999) study was conducted to investigate the feasibility of using the standard regression between mean IC_{50} ($IC_{50}\,\bar{x}$) and acute oral LD_{50} values reported for rats and mice in the Register of Cytotoxicity (Halle and Goeres, 1988) to determine the starting dose for *in vivo* acute toxicity testing. The linear regression line determined using 347 chemicals was used to predict the LD_{50} values for nine chemicals that had been investigated in an evaluation study of the UDP (Lipnick et al., 1995).

4.3.2 *List of Chemicals Tested and Selection Rationale*

Since the focus of the study was to determine if the linear regression extrapolation method could be used to adequately predict starting doses for the UDP, chemicals evaluated in a study considered to be the official evaluation for OECD acceptance of the UDP (Lipnick et al., 1995) were used. Lipnick et al. (1995) investigated 35 materials. Nine of those were excluded from the Spielmann et al. (1999) study because they were mixtures or formulations (e.g., laundry detergent). Of the remaining 26 chemicals, nine (acetonitrile, *p*-aminophenol, caffeine, coumarin, dimethylformamide, mercury (II) chloride, nicotine, phenylthiourea, and resorcinol) were also reported in the Register of Cytotoxicity, and thus were selected for evaluation.

4.3.3 *Summary Conclusions*

The predicted LD_{50} values for seven of the nine chemicals were the same as those calculated from *in vivo* testing. For the two remaining chemicals,

the dose-range differed from *in vivo* test results by one order of magnitude. The authors concluded that this method of predicting starting doses seemed promising, given the results from the limited data set, and that the use of this technique, coupled with the use of the UDP in place of the conventional LD_{50} test, would reduce animal use. However, the use of the IC_{50}/LD_{50} linear regression to estimate *in vivo* acute toxicity from cytotoxicity data assumes that a linear relationship exists between the IC_{50} and the LD_{50} values. This linear relationship could only be expected if all of the reference chemicals were found to be mechanistically similar and if all of the reference chemicals demonstrated similar toxicokinetics.

4.3.4 *Publications Containing Additional Study Information*

Seibert, H., M. Gülden, And J.-U. Voss. 1994b. An *In Vitro* Toxicity Testing Strategy For The Classification And Labelling Of Chemicals According To Their Potential Acute Lethal Potency. Toxicol. *In Vitro* 8: 847-850.

5.0 *In Vitro* Methods for Assessing Acute Toxicity –Toxicokinetic Determinations (Breakout Group 2)

This Breakout Group will evaluate the capabilities of *in vitro* methods for providing toxicokinetic information (absorption, distribution, metabolism, and elimination) that can be used to estimate target organs and dosimetry for acute toxicity testing and to provide recommendations for future research needs to accomplish this goal. The role of QSAR in toxicokinetic determinations will also be explored.

The toxicity of a substance *in vivo* is strongly influenced by the time-dependent processes of intake, uptake (absorption), distribution, biotransformation (metabolism), and elimination (excretion). As a consequence, such information is essential for the accurate prediction of *in vivo* toxicity from *in vitro* cytotoxicity test results. This need has been recognized by a number of investigators (see also **Sections 3** and **6.1**).

One method for estimating toxicokinetic parameters is through physiologically based biokinetic (PBBK) [or physiologically based pharmacokinetic modeling (PBPK)] or modeling. However, the method is complex and requires a great deal of knowledge about *in vivo* target organs and about various *in vivo* toxicokinetic parameters for the chemical under investigation. Whether PBBK modeling can be considered to be a suitable method for assessing a large number of chemicals remains to be determined.

Another approach would be to use a few, carefully selected *in vivo* toxicokinetic parameters, such as the fraction absorbed from the intestine and the apparent volume of distribution in combination with other information (e.g., lipid solubility, pKa) to estimate body doses from *in vitro* concentrations and to estimate organ concentrations from body doses. If such *in vivo* data is not available, the fraction absorbed from the intestine could be estimated from knowledge about the general relationships between physicochemical properties of chemicals and their absorption in the gastrointestinal tract, or from *in vitro* experimental data. One *in vitro* approach is the use of two-compartment systems comprising epithelia-like monolayers of human colon carcinoma cells (e.g., Caco-2 or HT-29 cells).

Additionally, *in vitro* data on specific chemicals and parameters defining the composition/compartmentalization of the *in vivo* model can be used as the basis for converting *in vitro* effective concentrations into equivalent body doses. This requires the following information/tools at a minimum:

- Various physicochemical characteristics of the chemical (e.g., pKa, lipophilicity, or volatility);
- Quantitative estimates of protein binding;
- Basis characteristics of the *in vitro* system (e.g., cell concentration, cell protein concentration, ratio of cell-medium volumes, and medium albumin concentration); and
- A mathematical model that permits the calculation of equivalent body doses, such as one described by Gülden et al. (1994), who derived a formula that allows for the conversion of calculated EC_{50} values to

ED_{50} values, which can then be compared to known LD_{50} values.

5.1 Tests for Metabolic Effects

Because the liver is the primary organ involved in xenobiotic metabolism, liver-derived *in vitro* systems have been used to estimate metabolic activation and the production of toxic metabolites. Test systems commonly used include whole liver homogenates, subcellular fractions (e.g., microsomes), liver slices, freshly isolated hepatocytes in suspension, primary monolayer hepatocyte cultures, metabolically competent hepatocyte or hepatoma cell lines, and cell lines transfected with human or rodent cytochromes. Studies of metabolism require the use of preparations that maintain appropriate and sufficient metabolic competence. Noted limitations of these *in vitro* tests include a lack of Phase II enzymes that are not membrane bound in some tests using liver homogenates and subcellular fractions, and variable stability in the expression of both Phase I and II enzyme activities in tests using freshly isolated hepatocytes or primary hepatocyte cultures. Co-culturing metabolically active hepatocytes with targets cells is one promising approach for assessing the role of metabolism in *in vivo* toxicity. An alternative (but less attractive) approach would be to expose the hepatocytes to the test substance, and then culture the target cells in the resulting conditioned culture medium. The advantages of the former method are that it enables the detection of hepatocyte-specific cytotoxicity, interference with specific functions of hepatocytes, and metabolism-mediated effects on target cells.

5.1.1 *Publications Containing Further Information*

Blaauboer, B.J., A.R. Boobis, J.V. Castell, S. Coecke, G.MM. Groothuis, A. Guillouzo, T.J. Hall, G.M. Hawksworth, G. Lorenzen, H.G. Miltenburger, V. Rogiers, P. Skett, P. Villa, and F.J Wiebel. 1994. The Practical Applicability of Hepatocyte Cultures in Routine Testing. The Report and Recommendations of ECVAM Workshop 1. ATLA 22: 231-241.

Ericsson, A.C., and E. Walum. 1988. Differential Effects of Allyl Alcohol on Hepatocytes and Fibroblasts Demonstrated in Roller Chamber Co-Cultures. ATLA 15: 208-213.

Paillard, F., F. Finot, I. Mouche, A. Prenez, and J. A. Verical. 1999. Use of Primary Cultures of Rat Hepatocytes to Predict Toxicity in the Early Development of New Chemical Entities. Toxicol. *In Vitro* 13: 693-700.

Voss, J.-U., and H. Seibert. 1992. Toxicity of Glycols and Allyl Alcohol Evaluated by Means of Co-Cultures of Microcarrier-Attached Rat Hepatocytes and Balb/c 3T3 Mouse Fibroblasts. ATLA 20: 266-270.

Voss, J.-U., and H. Seibert. 1991. Microcarrier-Attached Rat Hepatocytes as a Xenobiotic-Metabolizing System in Cocultures. Cell Biol. Toxicol. 7(4): 387-397.

6.0 *In Vitro* Methods for Assessing Acute Toxicity - Specific Organ Toxicity and Mechanisms (Breakout Group 3)

This Breakout Group will review *in vitro* methods that can be used to predict specific organ toxicity or toxicity associated with alteration of specific cellular or organ functions, and will develop recommendations for priority research efforts necessary to support the development of methods that can accurately assess target organ toxicity.

While the focus of most *in vitro* cytotoxicity research for predicting *in vivo* acute toxicity has been on an assessment of general cytotoxicity, the accurate prediction of *in vivo* acute toxicity for many substances absolutely requires critical information on the potential for organ-specific toxicity. Selective toxicity occurs when some types of differentiated cells are more sensitive to the effects of a particular toxicant than others, potentially as a result of, for example, biotransformation, binding to specific receptors, or uptake by a cell-type specific mechanism. A number of specific cell type assays (e.g., liver, nervous system, heart, kidney) have been developed for assessing selective toxicity. In the absence of appropriate information on target organ specificity for structurally-related

substances, detection of selective cell toxicity requires the evaluation of toxicity of the same test substance in multiple cell types.

Not specifically considered, but potentially relevant to specific organ toxicity is so-called specific function cytotoxicity. This type of toxicity occurs when the toxicant affects structures or processes that may not be critical for the affected cells themselves, but which are critical for the organism as a whole. For example, such toxicity can involve effects on cell-to-cell communication, via the synthesis, release, binding and degradation of cytokines, hormones and transmitters. No specific studies evaluating this type of toxicity were located.

Studies conducted to evaluate the suitability of *in vitro* organ-specific toxicity methods for predicting *in vivo* toxicity are described briefly; more detailed information can be obtained as indicated.

6.1 Evaluation-Guided Development of *In Vitro* Tests (EDIT)

In recognition that additional *in vitro* tests were needed to enhance the accuracy of the proposed MEIC *in vitro* battery for predicting human acute toxicity, a second multicenter program was initiated by the Cytotoxicology Laboratory, Uppsala (CTLU). The CTLU designed a blueprint for an extended battery and invited interested laboratories to develop the "missing" tests of this battery (i.e., extracellular receptor toxicity, excitatory toxicity, passage across blood-brain barrier, absorption in the gut, blood protein binding, distribution volumes, metabolic activation to more toxic metabolites) within the framework of the EDIT program. More information is available on the Internet (www.ctlu.se). The aim of EDIT is to provide a full replacement of the animal acute toxicity tests. Among the needed developments are assays for the accumulation of chemicals in cells, passage across the intestinal and blood-brain barriers, and biotransformation to more toxic metabolites. Purported advantages of the project are as follows. First, the evaluation-guided test development in EDIT is rational since tests are designed according to specific needs and as tests of single processes that can be integrated into sequential testing models. This is the potential strength of the *in vitro* toxicity testing strategy. Second, the direct testing of chemicals in newly developed *in vitro* assays will lead to a rapid evaluation of the potential value of each assay. Further information is provided in the MEIC Summary prepared by NICEATM (**Appendix A [Appendix E of the *In Vitro* Workshop Report]**)).

6.1.1 *Publications Containing Further Information*

Ekwall, B., C. Clemedson, Ba. Ekwall, P. Ring, And L. Romert. 1999. Edit: A New International Multicentre Programme To Develop And Evaluate Batteries Of *In Vitro* Tests For Acute And Chronic Systemic Toxicity. Atla 27: 339-349.

6.2 European Research Group for Alternatives In Toxicity Testing (ERGATT)/ Swedish National Board for Laboratory Animals (CFN) Integrated Toxicity Testing Scheme (ECITTS)

6.2.1 *General Study Description*

The ECITTS approach was to develop integrated testing schemes by combining sets of test batteries for predicting local and systemic toxicity in ways that would be more efficient than animal-based methods (Seibert et al., 1996). Evaluation of basal cytotoxicity and biokinetic parameters were considered to be essential to the investigation, although further testing would be adapted based on the test chemical; such testing may involve evaluation of developmental toxicity, immunotoxicity, nephrotoxicity, or neurotoxicity, as deemed appropriate. The basal cytotoxicity data were specifically used to interpret specific effects on potential target cells and tissues, while protein binding and biotransformation data were used to evaluate biokinetics.

In an initial pilot study reported by Blaauboer et al. (1994), the neurotoxic properties of five chemicals (acrylamide, lindane, methyl mercury (II) chloride, trethyltin chloride, and *n*-hexane) were studied in combination with biokinetic

analysis, in which blood and brain concentrations were predicted from biokinetic modeling. A follow-up study was conducted by Forsby et al. (1995), in which four of these chemicals (acrylamide, lindane, methyl mercury (II) chloride, and trethyltin chloride) were evaluated for general cytotoxicity and neurite degeneration in human epithelial and neuronal cells.

6.2.2 *Publications Containing Further Study Information*

Forsby, A., F. Pilli, V. Bianchi, And E. Walum. 1995. Determination Of Critical Cellular Neurotoxic Concentrations In Human Neuroblastoma (Sh-Sy5y) Cell Cultures. Atla 23: 800-811.

Walum, E., M. Balls, B. Bianchi, B. Blaauboer, G. Bolcsfoldi, A. Guillouzo, G.A. Moor, L. Odland, C.A. Reinhardt, and H. Spielmann. 1992. ECITTS: An Integrated Approach for the Application of *In Vitro* Test Systems for the Hazard Assessment of Chemicals. ATLA 20: 406-428.

6.3 Institute of Toxicology, University of Kiel

6.3.1 *General Study Description*

The study used a continuous cell line (Balb/c 3T3 cells) and differentiated mammalian cells (primary cultures of rat hepatocytes, rat skeletal muscle cells, and bovine spermatozoa) to assess acute systemic toxicity (Seibert et al., 1996). The importance of comparative cell toxicology and physicochemical data were emphasized. Comparative cell toxicology was investigated using tests with different endpoints, tissues, and species, while tests for effects such as lipophilicity were used to assess physicochemical interactions.

Chemicals evaluated in Seibert et al. (1994a) included 2,4-dinitrophenol, cyclophosphamide, and lidocaine. The paper demonstrated a comparative cell toxicological approach that enabled the detection of various toxic potencies and provided a limited interpretation of the mechanisms behind the toxic actions. Such information could serve as the basis for the assessment of the toxicological characteristics of a new chemical by providing information on which to base decisions on appropriate further testing.

Gülden et al. (1994) used the first 30 chemicals tested in the MEIC battery to evaluate the relevance of *in vitro* test systems for acute toxicity assessment. In order to make an appropriate comparison, the calculated EC_{50} values for inhibition of spontaneous contractility of primary cultured rat skeletal muscle cells were converted to ED_{50} values (i.e., effective model body doses) that were then compared directly to the known LD_{50} values for these chemicals. Although the extrapolation model was based on oversimplifications, the investigators concluded that the approach shows promise and that more complex models should be investigated.

6.3.2 *Publications Containing Further Study Information*

Gülden, M., H. Seibert, and J.-U. Voss. 1994. Inclusion of Physicochemical Data in Quantitative Comparisons of *In Vitro* and *In Vivo* Toxic Potencies. ATLA 22: 185-192.

Gülden, M., H. Seibert, and J.-U. Voss. 1994. The Use of Cultured Skeletal Muscle Cells in Testing for Acute Systemic Toxicity. Toxicol. *In Vitro* 8: 779-782.

Halle, W., and H. Spielmann. 1992. Two Procedures for the Prediction of Acute Toxicity (LD50) from Cytotoxicity Data. ATLA 20: 40-49.

Seibert, H., M. Gülden, And J.-U. Voss. 1994b. An *In Vitro* Toxicity Testing Strategy For The Classification And Labelling Of Chemicals According To Their Potential Acute Lethal Potency. Toxicol. *In Vitro* 8: 847-850.

7.0 Chemical Data Sets for Validation of *In Vitro* Toxicity Tests (Workshop Group 4)

This Breakout Group has the responsibility of defining what chemical data sets are required for validation studies, identifying existing resources,

and recommending approaches for using existing data sets and/or compiling or developing new data sets. Developing a single listing of chemicals that will address all test validation needs is not feasible. Instead, a library of useful chemicals should be developed that could be used when designing test development or validation efforts. Using this library, chemicals can be selected according to the purpose of the test and of the validation study. Developing appropriate criteria for chemical selection is a critical aspect of this process. Examples of selection criteria to be considered include:

- Chemicals that cover a wide range of acute LD50's, corresponding to the dose ranges used in the OECD classification (**Table 1**).
- Different chemical classes (structure; use; activity).
- Chemicals that are directly active and those that require metabolic activation (by internal organs; gut flora).
- General toxins and specific organ toxins.
- Chemicals active by different mechanisms.
- Chemicals that are commercially available in high purity, and relatively inexpensive.
- Gases; insolubles; immiscible liquids; unstable substances; dangerous substances should be avoided.
- Controlled substances (e.g., requiring a license) or those with shipping and handling restrictions should be avoided.

The most important components of the database will be the chemical name, CASRN, Smiles (or other structure-search) code, and biological endpoints. These endpoints could include acute toxicity data (e.g., LD50); organ/tissue specificity (e.g., hepatotoxin; neurotoxin; etc.); and ADME-related information (e.g., metabolism; peak blood levels; organ distribution; membrane permeability; excretion route). At a second level, the database should also include physico-chemical parameters (e.g., pH, volatility, and solubility), and product and use classes.

This database will enable users to pick the endpoint of interest (e.g., LD50; hepatotoxicity) and select the chemicals that can be used to validate the *in vitro* test. The candidate chemicals selected for use in the validation test can then be further grouped by class (e.g., chemical; product; use). If the chemical structure data are appropriately entered, the chemical classes that best correspond to the chemicals showing a specific endpoint can be defined by the database user.

Chemicals selected should be backed with adequate animal data showing acute toxicity, organ specificity, general mechanism of action, metabolic and toxicokinetic requirements, etc.

Where possible, structurally related chemicals with differing toxicities should be used to determine if the *in vitro* system could distinguish among them. It would be helpful to find homologous series of chemicals with differing toxicities.

Databases specific to *in vitro* cytotoxicity tests for use in assessing acute toxicity include the following:

- The Register of Cytotoxicity is a collection of acute oral LD_{50} values from rats and mice, as listed in the NIOSH Registry of Toxic Effects of Chemical Substances (RTECS), and mean cytotoxicity data (IC_{50} *x*) on chemicals and drugs (Halle and Goeres, 1988).
- The MEIC *in vitro* database contains both the methods used in testing (Part I, http://www.ectoxconsulting.a.se/Web/Mei/default.htm) and the results (Part II, http://www.ectoxconsulting.a.se/Web/Res.default.htm) for the 50 chemicals tested in the MEIC study. The associated MEMO database (http://www.ectoxconsulting.a.se/meicinvivo.htm) contains the human lethal blood concentration data used for comparison against the *in vitro* test results.

An *in vivo* acute toxicity database that may be useful is provided in **Appendix C [Appendix F of the *In Vitro* Workshop Report]**. In the United States, regulations regarding packaging, labeling,

and transport of acutely toxic liquids or solids are provided under 49 CFR 173. Materials with oral LD_{50} values less than or equal to 200 mg/kg (for solids) or 500 mg/kg (for liquids), dermal LD_{50} values less than or equal to 1000 mg/kg, or inhalation LC_{50} values less than or equal to 10 mg/L are considered to be poisonous and to pose a hazard to human health during transport. These materials, listed in the regulation as Division 6.1 materials, are further categorized into packing groups based on the level of hazard. Information on packing group designations, materials reported in the DOT regulation as Division 6.1 (49 CFR 172.101) hazardous materials and their packing group designations are provided in **Appendix C [Appendix F of the *In Vitro* Workshop Report]**), along with their packing group designation.

A list of 375 substances tested *in vitro* with comparative *in vivo* data, as reported in five studies (MEIC, Fry et al., 1990; Gülden et al., 1994; Lipnick et al., 1995; Spielmann et al. 1999), as well as in the Register of Cytotoxicity database developed under the direction of W. Halle, has been compiled for this Workshop (**Appendix B [Section 7.0 of the *In Vitro* Workshop Report]**). Detailed information on the cell system/endpoint used to assess cytotoxicity and the IC50 and/or ID50 values, the oral corresponding LD50 for rat and/or mouse, and the average or acute human lethal dose, can be obtained in the appropriate citations.

8.0 Relevant General Databases

Relevant general databases that may include pertinent information for this Workshop include:

- INVITTOX is a searchable database of protocols for *in vitro* toxicity test methods. Its aim is to provide precise and up-to-date technical information on the performance of the *in vitro* techniques currently in use and under development, their applications, advantages, and disadvantages. Sixty-two protocols, as well as information on the number of chemicals tested using the protocols and relevant publications, are available at http://embryo.ib.amwaw.edu.pl/invittox/invittox.htm.
- The German Center for Documentation and Evaluation of Alternative Methods to Animal Experiments (ZEBET) searchable database contains information on 300 alternatives in biomedicine fields and contains about 4,000 bibliographical references. It is available at http://gripsdb.dimdi.de/engl/guieng.html.
- The National Library of Medicine (NLM) maintains a bibliography of publications on alternatives to animal testing. This bibliography is available at http://www.sis.nlm.nih.gov/altanimal.cfm.
- The Akademie für Tierschutz, which is part of the German Animal Welfare Federation, has established a bibliographical database on alternatives. It contains 15,000 references and is available on floppy disk. Requests may be directed to akademie.fuer.tierschutz@muenchen.org.
- The Galileo Databank contains toxicology data from alternative studies, mostly related to cosmetics testing. The databank contains data on over 800 ingredients, over 300 cosmetic formulations, 50 methods, 26 animal models, and over 100 biosystems, with a total of nearly 21,000 individual results. The databank is not currently available online, but printouts may be requested by contacting Gregorio Loprieno, Technical Services SAS, Via Vecchia Lucchese 59, I-56123, Pisa, Italy, 39-50-555-685 (phone), 39-50-555-687 (fax).
- VetBase is a database of literature references to over 12,000 doses for 800 veterinary drugs in 130 species, including farm and laboratory animals, zoo species, fish, birds, amphibians and reptiles. The database is a custom-made MS Windows application, and is available by contacting J.D.Kuiper@cc.ruu.nl.

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10.0 Glossary

[See Section 6.0 of the In Vitro Workshop Report]

APPENDIX E

NICEATM Summary of the Multicenter Evaluation of In Vitro Cytotoxicity (MEIC

APPENDIX E

NICEATM Summary of The Multicenter Evaluation of *In Vitro* Cytotoxicity (MEIC)

This document was provided in the Background Materials and Supplemental Information Notebook for the International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity [Section I, TAB 6].

The following ATLA (Alternatives To Laboratory Animals) excerpts are reprinted with permission from Professor Michael Balls, editor of ATLA.

- Clemedson et al., 1998. MEIC Evaluation of Acute Systemic Toxicity, Part IV. ATLA 26: 131-183. **[Table 1]**
- Ekwall et al., 1998. MEIC Evaluation of Acute Systemic Toxicity, Part V. ATLA 26: 571-616. **[Tables II, III, IV, V, VI, IX]**
- Ekwall et al., 2000. MEIC Evaluation of Acute Systemic Toxicity, Part VIII, ATLA 28 Suppl 1, 201-234. **[Figures 1 and 10]**
- Ekwall et al., 1999. EDIT: A new international multicentre programme to develop and evaluate batteries of in vitro tests for acute chronic systemic toxicity. ATLA 27: 339-349. **[Table 1 and Figure 1]**

The following table was reproduced with permission from Dr. Gary Hook (NIEHS).

- Wallum, E. 1998. Acute Oral Toxicity. EHP 106: 497-503. **[reproduction of Table 1]**

The Multicenter Evaluation of *In Vitro* Cytotoxicity (MEIC)

Summary

September 2000

National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

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1.0 Introduction

The Multicenter Evaluation of *In Vitro* Cytotoxicity (MEIC) program was organized by the Scandinavian Society for Cell Toxicology in 1989. MEIC was started with two goals. The first was to investigate the relevance of results from *in vitro* tests for predicting the acute toxic action of chemicals in humans. The second was to establish batteries of existing *in vitro* toxicity tests as replacements for acute toxicity tests on animals (LD50). Achievement of the second goal, the practical and ethical one, was considered to be entirely dependent on a successful outcome of the first, scientific goal. At the same time, it was recognized that a demonstrated high relevance of *in vitro* toxicity tests for human acute toxicity did not mean that all problems of replacement of animal tests would be solved. MEIC was a voluntary effort involving 96 international laboratories that evaluated the relevance and reliability of *in vitro* cytotoxicity tests originally developed as alternatives to or supplements for animal tests for acute systemic toxicity, chronic systemic toxicity, organ toxicity, skin irritancy, or other forms of general toxicity. In establishing the framework for this program, a minimum of methodological directives was provided in order to maximize protocol diversity among the participating laboratories. The collection of test method data was completed in 1996. The multiple publications originating from these studies are provided in chronological order in Section 12. All *in vitro* toxicity test results collected during MEIC are available on the Cytotoxicology Laboratory, Uppsala (CTLU) website (www.ctlu.se) as a searchable database.

2.0 Test Chemicals

Fifty reference chemicals were selected for testing (**Appendix 1**). Selection was based on the availability of reasonably accurate human data on acute toxicity. Due to the anticipated five-year duration of MEIC, it was recognized that multiple samples (lots) of each chemical would be needed. However, it was decided that the chemicals would not be provided by a central supplier, but rather that each laboratory would purchase each chemical at the highest purity obtainable with the proviso that storage duration would be kept to a minimum. The decision to not have a central supplier was based on the rationale that most reference chemicals are drugs, which presents fewer impurity problems. It is also based on the recognition that the results would be evaluated against human poisonings, which involve chemicals of different origin and purity.

3.0 *In Vitro* Test Assays

By the end of the project in 1996, 39 laboratories had tested the first 30 reference chemicals in 82 *in vitro* assays, while the last 20 chemicals were tested in 67 *in vitro* assays (**Appendix 2**). Slight variants of four of the assays were also used to test some chemicals. The primary 82 assays included:

- Twenty human cell line assays utilizing Chang liver, HeLa, Hep 2, Hep G2, HFL1, HL-60, McCoy, NB-1, SQ-5, and WI-1003 cells;
- Seven human primary culture assays utilizing hepatocytes, keratinocytes, and polymorphonuclear leukocytes;
- Nineteen animal cell line assays utilizing 3T3, 3T3-L1, Balb 3T3, BP8, ELD, Hepa-1c1c7, HTC, L2, LLC-PK1, LS-292, MDBK, PC12h, and V79 cells;
- Eighteen animal primary culture assays utilizing bovine spermatozoa, chicken neurons, mouse erythrocytes, rat hepatocytes, and rat muscle cells; and
- Eighteen ecotoxicological tests utilizing bacteria (*Bacillus subtilis, Escherichia coli* B, *Photobacterium phosphoreum, Vibrio fisheri*), rotifer (*Brachionus calyciflorus*), crustacea (*Artemia salina, Daphnia magna, Streptocephalus proscideus*), plant (*Allium cepa* root, tobacco plant pollen tubes), and fish (trout hepatocytes, trout R1 fibroblast-like cells).

4.0 Assay Endpoints

The analyses conducted by the MEIC management team were based on *in vitro* toxicity data presented as IC50 values (i.e., the dose

estimated to reduce the endpoint in question by 50%) (**Appendix 2**).

These values were generated by the participating laboratories and were not independently verified; original data were not presented in the MEIC publications. Thirty-eight of these assays were based on viability, 29 on growth, and the remaining assays involved more specific endpoints, such as locomotion, contractility, motility, velocity, bioluminescence, and immobilization. The endpoints assessed were based on exposure durations ranging from five minutes to six weeks, and included:

- Cell viability as measured by the metabolism of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H* tetrazolium bromide (MTT), neutral red uptake (NRU), lactate dehydrogenase (LDH) release, cell morphology, adenosine triphosphate (ATP) content or leakage, trypan blue exclusion, viable cell count, tritiated-proline uptake, 86Rb leakage, creatine kinase activity, and glucose consumption;
- Cell growth as measured by protein content, macromolecule content, cell number, pH change, and optical density;
- Colony formation as measured by plating efficiency;
- An organotypic cellular endpoint (i.e., contractility of rat skeletal muscle cells);
- Motility and velocity for bovine sperm;
- Bioluminescence; and
- Mortality in lower eukaryotic organisms.

5.0 Comparative Data

The types of comparative data used to evaluate the predictive accuracy of the *in vitro* IC50 toxicity data for human acute toxicity included:

- Oral rat and mouse LD50 values obtained from Registry of Toxic Effects of Chemical Substances (RTECS) (**Appendix 3**, which contains rat and mouse LD50 data and average human lethal dose data for the 50 MEIC chemicals, ranked in three consecutive tables according to potency for rat, then mouse, and finally human. It also contains an U.S. Environmental Protection Agency (EPA) classification scheme for the acute toxicity of chemicals in humans.);
- Acute oral lethal doses in humans obtained from nine reference handbooks (**Appendix 4**);
- Clinically measured acute lethal serum concentrations in humans obtained from ten reference handbooks (**Appendix 5**);
- Acute lethal blood concentrations in humans measured post-mortem obtained from one forensic handbook and six forensic tabulations (**Appendix 6**);
- Human pharmacokinetics following single doses, including absorption, peak time, distribution/elimination curves, plasma half-life, distribution volume, distribution to organs (notably brain), and blood protein binding (**Appendix 7**);
- Peaks from curves of an ~50% lethal blood/serum concentration over time after ingestion (LC50 curves derived from human acute poisoning case reports) (**Appendix 8**);
- Qualitative human acute toxicity data, including lethal symptoms, main causes of death, average time to death, target organs, presence of histopathological injury in target organs, presence of toxic metabolites, and known or hypothetical mechanisms for the lethal injury (**Appendix 9**).

Early in the MEIC project, the *in vitro* cytotoxicity results were compared with average lethal blood concentrations (LCs) from acute human poisoning. However, these LCs were of limited value because they were averages of data with a wide variation due to different time between exposure and sampling (clinical) or death (forensic medicine). Therefore, a project was started to collect published and unpublished (from poison information centers and medico-legal institutes) case reports from human poisonings for the 50 MEIC reference chemicals that had lethal or sublethal blood concentrations with known time between ingestion and sampling/death. The aim was to compile enough case reports to be able to construct time-related lethal concentration

curves to be compared with the IC50 values for different incubation times *in vitro*. The results from the project were presented and analyzed in a series of 50 MEIC monographs. All monographs with sufficient case reports contain five tables presenting blood concentrations and two figures presenting LC curves. Three tables present (i) clinically measured, time-related sublethal blood concentrations, (ii) clinically measured, time-related lethal blood concentrations, and (iii) post-mortem, time-related blood concentrations. In these tables, blood concentration and the time interval between exposure and sampling for these concentrations are listed, as well as other important information on the cases. One table contains case reports with blood concentrations without a known time after ingestion and one table presents average blood concentrations calculated from the values presented in the other tables. The two figures presented in each of the monographs are scatter plots of sublethal and lethal blood concentrations. Based on these plots, concentration curves over time were drawn for the highest no lethal concentrations (NLC100); the lowest lethal concentrations (LC0); and the median curve between NLC100 and LC0, which is called the approximate LC50 even though it is not equivalent to a 50% mortality.

6.0 Statistical Analyses

The statistical analyses conducted by the MEIC management team involved:

- Principal components analysis (PCA);
- Analysis of Variance (ANOVA) and pairwise comparison of means using Tukey's method;
- Linear regression and ANOVA linear contrast analysis; and
- Multivariable partial least square (PLS) modeling with latent variables.

7.0 Results (based on IC50 response)

The MEIC management team, based on their analyses of the *in vitro* IC50 data, obtained the following results:

- The 1st PCA component described 80% of the variance of all the cytotoxicity data.
- Tukey's ANOVA indicated a similar sensitivity (~80%) for the assays.
- The toxicity of many chemicals increased with exposure time, making it necessary to perform a test at several exposure times to fully characterize the cytotoxicity.
- In general, human cytotoxicity was predicted well by animal cytotoxicity.
- Prediction of human cytotoxicity by ecotoxicological tests was only fairly good.
- One organotypic endpoint (muscle cell contractility) gave different results to those obtained with viability/growth assays.
- Sixteen comparisons of similar test systems involving different cell types and exposure times revealed similar toxicities, regardless of cell type.
- Nine of ten comparisons of test systems with identical cell types and exposure times revealed similar toxicities, regardless of the viability or growth endpoint measurement used.
- Nine comparisons of similar test systems employing different primary cultures and cell lines indicated that they shared similar toxicities.
- A high correlation between an intracellular protein denaturation test and average human cell line toxicity test suggested that denaturation may be a frequently occurring mechanism in basal cytotoxicity.

The following results were based on comparisons between *in vitro* data and *in vivo* data:

- Simple human cell tests were shown to be relevant for human acute lethal action for as many as 43 of the 50 MEIC reference chemicals (86%). The exceptions were atropine, digoxin, malathion, nicotine, cyanide, paracetamol, and paraquat -- all specific receptor-mediated toxicants.
- A battery of three of these human cell line tests (nos. 1, 9, 5/16) was found to be highly predictive (R^2 = 0.77) of the peak human lethal blood concentrations (LC50) of chemicals. The prediction increased markedly (R^2 = 0.83) when a simple

algorithm based on the knowledge of passage across the blood-brain barrier was used to adapt *in vitro* to *in vivo* concentrations (**Appendix 7**). The battery involved four endpoints and two exposure times (protein content/24 hours; ATP content/24 hours; inhibition of elongation of cells/24 hours; pH change/7 days). Prediction was better than the prediction of human lethal doses by rat and mouse LD50-values ($R^2 = 0.65$). The correlation between calculated oral LD50 doses in rats and mice and acute lethal dose in humans is presented graphically in **Appendix 10**, while the correlation between IC50 values and peak lethal blood concentrations in humans is presented graphically in **Appendix 11**.

- In the *in vitro* -- *in vivo* MEIC evaluation of chemicals that do easily not cross the blood-brain barrier, the 24 hour cytotoxic concentrations for rapidly acting chemicals correlated well with the human lethal peak blood concentrations, while the corresponding cytotoxicity for the slow-acting chemicals did not correlate as well with the peak concentrations. The prediction of human toxicity by the tests of slow-acting chemicals was much improved when 48-hour cytotoxic concentrations were compared with 48-hour human lethal blood concentrations. Thus, an *in vitro* test providing a discrimination between a rapid and a slow cytotoxic action would increase the predictive power of a cell test battery on acute toxicity.
- The findings from both the *in vitro-in vitro* comparisons and the *in vitro-in vivo* comparisons strongly supported the basal cytotoxicity concept.

8.0 MEIC Conclusions and Recommendations

Based on the analyses conducted, the MEIC management team made the following conclusions:

- The MEIC 1, 9, 5/16 test battery can be used directly as a surrogate for a LD50 test. However, since the battery predicts lethal blood concentrations, not lethal dosages, it is not a direct counterpart of the animal LD50 test. Thus, the 1, 9, 5/16 battery must be supplemented with data on gut absorption as well as the distribution volumes (Vd) of chemicals. Vd essentially depends on whether chemicals penetrate cells or not, and the degree of accumulation in the cell for chemicals that enter cells. Binding to proteins, lipids, bone and intracellular matrix will also influence Vd. Probably, a simple test of accumulation in cells over time would provide adequate Vd data. There is sufficient *knowledge of kinetics and Vd to enable an evaluation of results from such an assay for most of the 50 MEIC chemicals.
- An ongoing evaluation is being conducted to address the issue of predicting human oral lethal doses rather than human lethal blood concentrations. One MEIC manuscript in preparation will focus on the importance of the kinetic determinants of target organs for basal cytotoxicity. A second MEIC manuscript will describe how human lethal doses may be predicted by cellular tests on basal cytotoxicity (the 1, 9, 5/16 battery) and kinetic data.
- If human lethal doses are shown to be well predicted by the 1, 9, 5/16 battery, when combined with absorption and distribution data, a new but simple *in vitro* test to predict distribution volumes must be developed. An effective *in vitro* test on absorption is stated to already exist. Development of new *in vitro* methods is not addressed by MEIC, which only evaluated existing methods.
- In MEIC, only two of the 50 reference chemicals (ethylene glycol and methanol) were biotransformed to more toxic metabolites, contributing to the acute lethal action. The occurrence of toxic metabolites for the two chemicals did not affect the prediction of human lethal peak concentrations by human cell line inhibitory concentrations, but seemed to interfere with the correlation between *in vitro* delayed effects and the prediction of

later lethal effects of the chemicals. These results confirm the proposed usefulness of an *in vitro* test that could measure the formation and release of a toxic metabolite by metabolically competent cells within the time frame of acute toxicity. One design of such a test would be to use human hepatocytes in co-cultures with a target cell line. Since so few metabolically active chemicals were tested in MEIC, future studies will need to include additional metabolically activated chemicals.

9.0 Evaluation-Guided Development of *In Vitro* Tests (EDIT)

In recognition that additional *in vitro* tests were needed to enhance the accuracy of the proposed *in vitro* battery for predicting human acute toxicity, a second voluntary multicenter program was initiated by the CTLU. The CTLU has designed a blueprint for an extended battery and has invited all interested laboratories to develop the "missing" tests of this battery within the

framework of the EDIT program (**Appendix 12** and **13**). The EDIT research program is published on the Internet (www.ctlu.se). The aim of EDIT is to provide a full replacement of the animal acute toxicity tests. The most urgently needed developments are assays on the accumulation of chemicals in cells (test of Vd), passage across the intestinal and blood-brain barriers, and biotransformation to more toxic metabolites. CTLU will provide interested laboratories with human reference data and will evaluate results as single components of complex models. The Internet version of the general EDIT research program contains additional, regularly updated information on the project. Purported advantages of the project are as follows. First, the evaluation-guided test development in EDIT is rational since tests are designed according to obvious needs and as elementary tests of single events integrated into whole models, which is the potential strength of the *in vitro* toxicity testing strategy. Second, the direct testing of MEIC chemicals in newly developed *in vitro* assays will lead to a rapid evaluation of the potential value of each assay.

10.0 Recommended Integration of MEIC/EDIT into the EPA High Production Volume (HPV) Program

Dr. Ekwall, the principle scientist for the MEIC program, has provided several suggestions for using MEIC results and the forthcoming EDIT results to reduce animal testing in the HPV program. These suggestions include the following:

1. Formal validation by ECVAM/ICCVAM of the existing 3 test MEIC battery. If considered validated, use of the battery to test every chemical in the HPV program would provide inexpensive and useful supplementary data.
2. Evaluate some of the HPV chemicals in a battery of *in vitro* toxicity and toxicokinetic tests on acute toxicity (EDIT and similar models) as follows:
   - Engage poison information experts to select a set of HPV chemicals with sound human acute toxicity data, including time-related lethal blood concentrations.
   - Give priority to standard testing of the same chemicals in the HPV program.
   - Testing of the same chemicals in the newly developed *in vitro* systems (EDIT, etc.), including modeling of acute toxicity by the new assays.
   - Comparison of HPV standard animal data and the *in vitro* data with the human data for the selected set of chemicals.

   If the new *in vitro* models can be shown to predict human acute toxicity better than the HPV animal tests, *in vitro* batteries may totally replace the animal acute toxicity tests in further HPV testing.

11.0 MEIC Evaluation Guidelines Checklist

A complete and formal assessment of the validation status of MEIC in regard to the ICCVAM evaluation guidelines would require the following to be reviewed and evaluated:

ICCVAM Evaluation Guidelines

1.0 Introduction and Rationale of each Test Method
1.1 Scientific basis for each test method
1.1.1 Purpose of each proposed method, including the mechanistic basis
1.1.2 Similarities and differences of modes and mechanisms of action in each test system as compared to the species of interest (e.g., humans for human health-related toxicity testing).
1.2. Intended uses of each proposed test method.
1.2.1 Intended regulatory use(s) and rationale.
1.2.2 Substitute, replace, or complement existing test methods.
1.2.3 Fits into the overall strategy of hazard or safety assessment. If a component of a tiered assessment process, indicate the weight that will be applied relative to other measures.
1.2.4 Intended range of materials amenable to test and/or limits according to chemical class or physico-chemical factors.
2.0 Proposed Each Test Method Protocol(s)
2.1 Detailed protocol for each test method, duration of exposure, know limits of use, and nature of the response assessed, including:
2.1.1 Materials, equipment, and supplies needed
2.1.2 Suggested positive or negative controls.
2.1.3 Detailed procedures for conducting the test
2.1.4 Dose-selection procedures, including the need for any dose range-finding studies or acute toxicity data prior to conducting the test, if applicable;
2.1.5 Endpoint(s) measured
2.1.6 Duration of exposure
2.1.7 Known limits of use
2.1.8 Nature of the response assessed
2.1.9 Appropriate vehicle, positive and negative controls and the basis for their selection
2.1.10 Acceptable range of vehicle, positive and negative control responses
2.1.11 Nature of the data to be collected and the methods used for data collection
2.1.12 Type of media in which data are stored
2.1.13 Measures of variability
2.1.14 Statistical or non-statistical method(s) used to analyze the resulting data (including methods to analyze for a dose response relationship). The method(s) employed should

be justified and described
2.1.15 Decision criteria or the prediction model used to classify a test chemical (e.g., positive, negative, or equivocal), as appropriate
2.1.16 Information that will be included in the test report
2.2 Basis for each test system
2.3 Confidential information
2.4 Basis for the decision criteria established for each test
2.5 Basis for the number of replicate and repeat experiments; provide the rationale if studies are not replicated or repeated
2.6 Basis for any modifications to each proposed protocol that were made based on results from validation studies
3.0 Characterization of Materials Tested
3.1 Rationale for the chemicals/products selected for evaluation. Include information on suitability of chemicals selected for testing, indicating any chemicals that were found to be unsuitable
3.2 Rationale for the number of chemicals that were tested
3.3 The chemicals/products evaluated, including:
3.3.1. Chemical or product name; if a mixture, describe all components.
3.3.2 CAS number(s)
3.3.3 Chemical or product class
3.3.4 Physical/chemical characteristics
3.3.5 Stability of the test material in the test medium
3.3.6 Concentration tested.
3.3.7 Purity; presence and identity of contaminants.
3.3.8 Supplier/source of compound.
3.4 If mixtures were tested, constituents and relative concentrations should be provided whenever possible
3.5 Describe coding used (if any) during validation studies.
4.0 Reference Data Used for Performance Assessment
4.1 Clear description of the protocol for the reference test method. If a specific guideline has been followed, it should also be provided. Any deviation should be indicated, including the rationale for the deviation.
4.2. Provide reference data used to assess the performance of the proposed test method.
4.3 Availability of original datasheets for the reference data
4.4 Quality of the reference test data, including the extent of GLP compliance and any use of coded chemicals.
4.5 Availability and use of relevant toxicity information from the species of interest.
5.0 Test Method Data and Results
5.1 Complete, detailed protocol used to generate each set of data for each proposed test method.

Any deviations should be indicated, including the rationale for the deviation. Any protocol modifications made during the development process and their impact should be clearly stated for each data set.
5.2 Provide all data obtained using each proposed test method. This should include copies of original data from individual animals and/or individual samples, as well as derived data. The laboratory's summary judgement as to the outcome of each test should be indicated. The submission should also include data (and explanations) from unsuccessful, as well as successful, experiments.
5.3 Statistical approach used to evaluate the data from each proposed test method
5.4 Provide a summary, in graphic or tabular form, of the results.
5.5 For each set of data, indicate whether coded chemicals were tested, experiments were conducted blind, and the extent to which experiments followed GLP procedures.
5.6 Indicate the lot-to-lot consistency of the test materials, the time frame of the various studies, and the laboratory in which the study or studies were done. A coded designation for each laboratory is acceptable.
5.7 Any data not submitted should be available for external audit, if requested
6.0 Test Method Performance Assessment
6.1 Describe performance characteristics (e.g., accuracy, sensitivity, specificity, positive and negative predictivity, and false positive and negative rates) of each proposed test method separately and in combination compared with the reference test method currently accepted by regulatory agencies for the endpoint of interest. Explain how discordant results from each proposed test were considered when calculating performance values.
6.2 Results that are discordant with results from the reference method.
6.3 Performance characteristics of each proposed test method compared to data or recognized toxicity from the species of interest (e.g., humans for human health-related toxicity testing), where such data or toxicity classification is available. In instances where the proposed test method was discordant from the reference test method, describe the frequency of correct predictions of each test method compared to recognized toxicity information from the species of interest.
6.4 Strengths and limitations of the method, including those applicable to specific chemical classes or physical/chemical properties
6.5 Salient issues of data interpretation, including why specific parameters were selected for inclusion
7.0 Test Method Reliability (Repeatability/Reproducibility)
7.1 Rationale for the chemicals selected to evaluate intra- and inter-laboratory reproducibility for each test method, and the extent to which they represent the range of possible test outcomes.
7.2 Analyses and conclusions reached regarding inter- and intra-laboratory repeatability and reproducibility for each test method
7.3 Summarize historical positive and negative control data for each test method, including number of trials, measures of central tendency and variability.
8.0 Test Method Data Quality
8.1 Extent of adherence to GLPs

8.2. Results of any data quality audits
8.3 Impact of deviations from GLPs or any non-compliance detected in data quality audits
9.0 Other Scientific Reports and Reviews
9.1 All data from other published or unpublished studies conducted using the proposed test method should be included.
9.2 Comment on and compare the conclusions published in independent peer-reviewed reports or other independent scientific reviews of the test method. The conclusions of such scientific reports and/or reviews should be compared to the conclusions reached in this submission. Any other ongoing evaluations of the method should be mentioned.
10.0 Animal Welfare Considerations (Refinement, Reduction, and Replacement)
10.1 Describe how the proposed test methods will refine (reduce pain or distress), reduce, and/or replace animal use compared to the current methods used.
11.0 Other Considerations
11.1 Aspects of test method transferability. Include an explanation of how this compares to the transferability of the reference test method.
11.1.1 Facilities and major fixed equipment needed to conduct the test.
11.1.2 Required level of training and expertise needed for personnel to conduct the test.
11.1.3 General availability of other necessary equipment and supplies.
11.2 Cost involved in conducting each test. Discuss how this compares to the cost of the reference test method.
11.3 Indicate the amount of time needed to conduct each test and discuss how this compares with the reference test method.
12.0 Supporting Materials
12.1 Provide copies of all relevant publications, including those containing data from the proposed test method or the reference test method.
12.2 Include all available non-transformed original data for both each proposed test method and the reference test method.
12.3 Summarize and provide the results of any peer reviews conducted to date, and summarize any other ongoing or planned reviews.
12.4 Availability of laboratory notebooks or other records for an independent audit. Unpublished data should be supported by laboratory notebooks.

12.0 MEIC Related Publications (in chronological order)

Bernson, V., Bondesson, I., Ekwall, B., Stenberg, K., and Walum, E. (1987) A multicentre evaluation study of in vitro cytotoxicity. ATLA, 14, 144-145.

Bondesson, I., Ekwall, B., Stenberg, K., Romert, L. and Walum, E. (1988) Instruction for participants in the multicentre evaluation study of in vitro cytotoxicity (MEIC). ATLA, 15, 191-193.

Bondesson, I., Ekwall, B., Hellberg, S., Romert, L., Stenberg, K., and Walum, E. (1989) MEIC - A new international multicenter project to evaluate the relevance to human toxicity of in vitro cytotoxicity tests. Cell Biol. Toxicol., 5, 331-347.

Ekwall, B. (1989) Expected effects of the MEIC-study. In Report from The MEIC In Vitro Toxicology Meeting, Stockholm 9/3 1989, (Eds. T. Jansson and L.Romert), pp 6-8, Swedish National Board for Technical Development.

Ekwall, B., Gómez-Lechón, M.J., Hellberg, S., Bondsson, I., Castell, J.V., Jover, R., Högberg, J., Ponsoda, X., Stenberg, K., and Walum, E. (1990) Preliminary results from the Scandinavian multicentre evaluation of in vitro cytotoxicity (MEIC). Toxicol. In Vitro, 4, 688-691.

Hellberg, S., Bondesson, I., Ekwall, B., Gómez-Lechón, M.J., Jover, R., Högberg, J., Ponsoda; X., Romert, L., Stenberg, K., and Walum, E. (1990) Multivariate validation of cell toxicity data: The first ten MEIC chemicals. ATLA, 17, 237-238.

Hellberg, S., Eriksson, L., Jonsson, J., Lindgren, F., Sjöström, M., Wold, S., Ekwall, B., Gómez-Lechón, J.M., Clothier, R., Accomando, N.J., Gimes, G., Barile, F.A., Nordin, M., Tyson, C.A., Dierickx, P., Shrivastava, R.S., Tingsleff-Skaanild, M., Garza-Ocanas, L., and Fiskesjö, G. (1990) Analogy models for prediction of human toxicity. ATLA, 18, 103-116.

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Ekwall, B., Abdulla, E., Barile, F., Bondesson, I., Clemedson, C., Clothier, R.,Curren, R., Dierickx, P., Fiskesjö, G., Garza-Ocanas, L., Gómez-Lechón, M.J., Gülden, M., Imai, K., Janus, J., Kristen, U., Kunimoto,, M., Kärenlampi, S., Lavrijsen, K., Lewan, L.,Malmsten, A., Miura, T., Nakamura, M., Ohno, T., Ono, H., Persoone, G., Rouget, R., Romert, L., Sandberg, M., Sawyer, T., Seibert, H., Shrivastava, R., Stammati, A., Tanaka, N., Walum, E., Wang, X & Zucco, F. (1992) Acute lethal toxicity in man predicted by cytotoxicity in 55 cellular assays and by oral LD50 tests in rodents for the first 30 MEIC chemicals, In Proc. of JSAAE (Japanese Society for Alternatives to Animal Experiments) 6th annual meeting in Tokyo, Dec 17-18, 1992, (Ed. S. Sato), pp 114-115, Tokyo.

Ekwall, B., Abdulla, E., Barile, F.,Chesne, C., Clothier, Cottin, M., Curren, R., Daniel- Szolgay, E., Dierickx, P., Ferro, M., Fiskesjö, G., Garza-Ocanas, L., Gómez-Lechón, M.J., Gülden, M. Isomaa, B., Kahru, A., Kemp, R.B., Kerszman, G., Kristen, U., Kunimoto,, M., Kärenlampi, S., Lavrijsen, K., Lewan, L., Ohno, T., Persoone, G., Pettersson, R., Rouget, R., Romert, L., Sawyer, T., Seibert, H., Shrivastava, R., Sjöström, M., Tanaka, N., Zucco, F., Walum, E., & Clemedson, C. (1994) A comparative cytotoxicity analysis of the results from tests of the first 30 MEIC reference chemicals in 68 different in vitro toxicity systems, pp 117-118 in Alternatives Research - Proceedings of the 8th Annual Meeting of the Japanese Society for Alternatives to Animal Experiments, Nov. 28-29, 1994, Tokyo.

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validation of toxicity test procedures - The report and recommendations of ECVAM Workshop 5. ATLA 23, 129-147.

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Appendix 1
First Fifty Reference Chemicals

Acetaminophen
Aspirin
Ferrous sulfate
Diazepam
Amitriptyline
Digoxin
Ethylene glycol
Methyl alcohol
Ethyl alcohol
Isopropyl alcohol
1,1,1-Trichloroethane
Phenol
Sodium chloride
Sodium fluoride
Malathion
2,4-Dichlorophenoxyacetic acid
Xylene
Nicotine
Potassium cyanide
Lithium sulfate
Theophylline
Dextropropoxyphene HCl
Propranolol HCl
Phenobarbital
Paraquat
Arsenic trioxide
Cupric sulfate
Mercuric chloride
Thioridazine HCl
Thallium sulfate
Warfarin
Lindane
Chloroform
Carbon tetrachloride
Isoniazid
Dichloromethane
Barium nitrate
Hexachlorophene
Pentachlorophenol
Varapamil HCl
Chloroquine phosphate
Orphenadrine HCl
Quinidine sulfate
Diphenylhydantoin
Chloramphenicol
Sodium oxalate
Amphetamine sulfate
Caffeine
Atropine sulfate
Potassium chloride

Appendix II: Descriptions of the Essential Traits of 67 *in vitro* Methods

Table I: Descriptions of the essential traits of 67 *in vitro* methods

Method								
No.	Old No.[a]	Cell type/ test system	Tissue of origin	Species	Endpoint	Incubation time	Testing laboratory[b]	Reference
Human cell lines								
1.	II:1	Hep G2	Hepatoma	Human	Protein content/Lowry	24 hours	Dierickx	3
2.	III:2	Hep G2	Hepatoma	Human	Protein content/ Sulphorhodamine B	24 hours	Hall, Cambridge & James	5
3.	II:2	Hep G2	Hepatoma	Human	MTT	24 hours	Gómez-Lechón, Jover, Ponsoda & Castell[c]	3, 12
4.	II:4	WI-1003/Hep G2[d]	Lung/Hepatoma	Human	Morphology	24 hours	Garza-Ocañas & Torres-Alanis	3
5.	II:3	Chang liver cells	Liver	Human	Morphology	24 hours	Garza-Ocañas & Torres-Alanis	3
6.	II:5	HeLa	Cervical carcinoma	Human	Morphology	24 hours	Ekwall & Malmsten	3
7.	II:6	Hep 2	Epithelial carcinoma of larynx	Human	Protein content/ Coomassie blue staining	24 hours	Stammati, Zucco, Zanetti & De Angelis	3
8.	II:7	Hep 2	Epithelial carcinoma of larynx	Human	LDH release	24 hours	Stammati, Zucco, Zanetti & De Angelis	3
9.	II:8	HL-60	Promyelocytic leukaemia	Human	ATP content	24 hours	Tanaka, Wakuri, Izumi, Sasaki & Ono	3
10.	III:10	HFL1	Fetal lung cells	Human	MTT	24 hours	Barile & Sookhoo[e]	5, 13
11.	III:11A	SQ-5	Lung squamous carcinoma	Human	LDH content[f]	48 hours	Ohno, Wang, Sasaki & Hirano	3, 14
12.	III:12	SQ-5	Lung squamous carcinoma	Human	Killing index[g]	48 hours	Ohno, Wang, Sasaki & Hirano	3, 14
13.	II:10	NB-1	Neuroblastoma	Human	Protein content/ Crystal violet staining	48 hours	Kunimoto, Miura, Aoki & Kunimoto	3
14.	II:11	McCoy	Epithelial cells from synovial fluid	Human	Morphology/Trypan blue exclusion[h]	72 hours	Shrivastava & Chevalier	3
15.	II:13	WI-1003/Hep G2[d]	Lung/Hepatoma	Human	Morphology/pH changes	168 hours	Garza-Ocañas & Torres-Alanis	3

Source: Clemedson et al. 1998. MEIC Evaluation of Acute Systemic Toxicity. Part IV. ATLA 26:131-183. (reprinted with permission from the editor)

16.	II:12	Chang liver	Liver	Human	Morphology/pH changes	168 hours	Garza-Ocañas & Torres-Alanis	3
17.	II:14	HeLa	Cervical carcinoma	Human	pH changes (phenol red)	168 hours	Ekwall & Malmsten	3
18.	II:15	MRC-5 (finite cell line)	Epithelial cells from embryonic lung	Human	Protein content/Lowry	6 weeks	Dierickx	3, 15
Human primary cultures								
19.	III:21	Polymorphonuclear leukocytes[i]	Blood	Human	Viable cell count fluorescein diacetate/ Ethidium bromide	3 hours	Valentino, Monaco, Pieragostini, Amati & Governa	5
20.	III:22	Polymorphonuclear leukocytes[i]	Blood	Human	Locomotion stimulated by chemotactic peptide	3 hours	Valentino, Monaco, Pieragostini, Amati & Governa	5
Animal cell lines								
21.	II:19	ELD	Subline of Ehrlich ascites tumour cells	Mouse	ATP leakage	10 minutes	Lewan & Andersson	3
22.	II:20	ELD	Subline of Ehrlich ascites tumour cells	Mouse	ATP leakage	10 minutes	Lewan & Andersson	3
23.	II:23	HTC	Hepatoma	Rat	Macromolecular content	24 hours	Ferro, Bassi & Canepa[k]	3
24.	II:25	L2	Lung epithelial cells	Rat	[^{3}H]-proline uptake	24 hours	Barile, Borges, Arjun & Hopkinson[l]	3, 16
25.	II:30	3T3	Fibroblasts	Mouse	MTT	24 hours	Gómez-Lechón, Jover, Ponsoda & Castell[f]	3, 42
26.	III:40	LLC-PK1	Kidney	Pig	Protein content/ Sulphorhodamine B	24 hours	Hall, Cambridge & James	5
27.	II:31	BP8	Ascites sarcoma	Mouse	Cell number/ Coulter counter	48 hours	Romert, Jansson & Jenssen	3
28.	II:32	PC12h	Phenchromocytoma	Rat	Protein content	48 hours	Kunimoto, Miura, Aoki & Kunimoto	3
29.	II:33	MDBK	Kidney	Bovine[m]	Morphology/Trypan blue exclusion[n]	72 hours	Shrivastava & Chevalier	3
30.	II:34	Hepa-1c1c7 (Subclone of Hepa-1)	Hepatoma	Mouse	Protein content/ Coomassie blue staining	72 hours	Kärenlampi & Malmivuori	3 *INVITTOX* protocol 112[m]

Table I: continued

Method No.	Old No.[a]	Cell type/ test system	Tissue of origin	Species	Endpoint	Incubation time	Testing laboratory[b]	Reference
31.	II:35	3T3-L1 (Subclone of 3T3)	Embryonal fibroblasts	Swiss mouse	Protein content/Kenacid blue staining	72 hours	Clothier	3
32.	II:36	Balb 3T3 A31-1-1	Whole embryo	Balb/c mouse	Colony formation	168 hours	Tanaka, Wakuri, Izumi, Sasaki & Ono	3
Animal primary cultures								
33.		Muscle cells	Skeletal muscle	Rat	Spontaneous contractility	1 hour	Gülden, Seibert & Voss	3, *INVITTOX* protocol 93[m]
34.	II:45A	Neurons	Embryonal forebrain	Chicken	Neutral red uptake	20 hours	Sawyer & Weiss	3
35.	II:46A	Neurons	Embryonal forebrain	Chicken	MTT	21 hours	Sawyer & Weiss	3
36.	II:50	Hepatocytes[n]	Liver	Male rat	MTT	24 hours	Gómez-Lechón, Jover, Ponsoda & Castell[c]	3, 12
37.	II:51	Hepatocytes[n]	Liver	Male rat	Morphology/Trypan blue exclusion/LDH release[h]	24 hours	Shrivastava & Chevalier	3
38.	II:52	Erythrocytes	Peripheral blood of 9-week males	Balb/c mouse	ATP content	24 hours	Tanaka, Wakuri, Izumi, Sasaki & Ono	3
39.		Muscle cells	Skeletal muscle	Rat	Intracellular creatine kinase activity	24 hours	Gülden, Seibert & Voss	3, *INVITTOX* protocol 93[m]
40.		Muscle cells	Skeletal muscle	Rat	Glucose consumption	24 hours	Gülden, Seibert & Voss	3, *INVITTOX* protocol 93[m]
41.		Muscle cells	Skeletal muscle	Rat	Spontaneous contractility	24 hours	Gülden, Seibert & Voss	3, *INVITTOX* protocol 93[m]

Appendix III:Oral LD50 Doses for Rat and Mouse and Mean Oral Lethal Doses for Humans and Toxicity Categories

Oral LD50 Doses for Rat and Mouse and Mean Oral Lethal Doses for Humans

Chemical Number	Chemical	Rat LD50		Mouse LD50		Ave. Human Dose	
		mg/kg	umol/kg	mg/kg	umol/kg	mg/kg	umol/kg
28	Mercuric chloride	1	4	6	22	25.7	94.7
31	Warfarin	2	5	3	10	107.1	347.4
18	Potassium cyanide	5	77	9	131	2.9	43.9
26	Arsenic trioxide	15	74	31	159	4.1	20.9
30	Thallium sulfate	16	32	24	47	14.0	27.7
39	Pentachlorophenol	27	101	28	105	28.6	107.3
6	Digoxin	28	36	18	23	0.1	0.17
17	Nicotine	50	308	3	21	0.7	4.4
13	Sodium fluoride	52	1238	57	1357	92.8	2210.9
47	Amphetamine sulfate	55	149	24	65	20.0	54.3
38	Hexachlorophene	56	138	67	165	214.3	526.6
32	Lindane	76	261	44	151	242.9	835.1
21	Propoxyphene HCL	84	223	255	678	24.6	65.4
25	Paraquat	100	537	120	644	40.0	214.7
40	Verapamil HCL	108	220	163	331	122.3	249.1
23	Penobarbital	162	697	137	590	111.4	479.7
48	Caffeine	192	989	127	654	135.7	698.8
2	Acetylsalicylic acid	200	1110	232	1287	385.7	2140.5
20	Theophylline	244	1354	235	1304	157.1	872.1
42	Orphenadrine HCL	255	834	100	327	50.0	163.4
43	Quinidine sulfate	258	610	286	676	79.2	187.4
14	Malathion	290	878	190	575	742.8	2248.4
11	Phenol	317	3369	270	2869	157.2	1670.0
3	Ferrous sulfate	319	2100	680	4477	392.1	2581.0
5	Amitriptyline	320	1154	140	505	37.1	133.8
4	Diazepam	352	1236	45	159	71.4	250.8
37	Barium nitrate	355	1358	266	1016	37.1	142.1
15	2,4-Dichlorophenoxy-acetic acid	375	1697	347	1570	385.8	1745.3
22	Propamolol HCL	466	1575	320	1082	71.5	241.7
27	Cupric sulfate	469	1880	502	2012	290.6	1163.6
19	Lithium sulfate	492	4478	1190	10,828	1065.5	9691.8
49	Atropine sulfate	585	864	456	674	1.7	2.5
41	Chloroquine phosphate	623	1208	500	969	84.3	163.4
33	Chloroform	908	7605	36	302	999.8	8375.2
29	Thioridazine HCL	995	2445	385	946	68.6	1684
35	Isoniazid	1250	9117	133	970	171.5	1250.4
36	Dichloromethane	1601	18,846	873	10,280	1386.2	16,321.7
44	Diphenylhydantoin	1635	6480	150	595	300.0	1189.1
34	Carbon tetrachloride	2350	15,280	8264	53,726	1314.4	8545.4
1	Paracetamol	2404	15,899	338	2235	271.4	1795.2
45	Chloramphenicol	2500	7735	1500	4641	285.7	884.0
50	Potassium chloride	2598	34,853	1499	20,107	285.5	3830.0
12	Sodium chloride	3002	51,370	4003	68,493	2287.3	39,138.9

Oral LD50 Doses for Rat and Mouse and Mean Oral Lethal Doses for Humans

16	Xylene	4299	40,490	2119	19,953	899.8	8474.6
7	Ethylene glycol	4698	75,684	5498	88,567	1570.9	25,304.8
8	Methanol	5619	175,327	7289	227,414	1569.0	48,954.2
9	Ethanol	7057	153,145	3448	74,837	4712.2	102,262.2
46	Sodium oxalate	11160	83,284	5095	38,019	357.1	2665.3
10	1,1,1-Trichloroethane	11196	83,927	7989	59,884	5707.6	42,785.8

Source: E. Walum. 1998. Acute oral toxicity. EHP 106:497-503. (reprinted with permission from the editor)

Oral LD50 Doses for Rat and Mouse and Mean Oral Lethal Doses for Humans

Chemical Number	Chemical	Rat LD50		Mouse LD50		Ave. Human Dose	
		mg/kg	umol/kg	mg/kg	umol/kg	mg/kg	umol/kg
31	Warfarin	2	5	3	10	107.1	347.4
17	Nicotine	50	308	3	21	0.7	4.4
28	Mercuric chloride	1	4	6	22	25.7	94.7
18	Potassium cyanide	5	77	9	131	2.9	43.9
6	Digoxin	28	36	18	23	0.1	0.2
30	Thallium sulfate	16	32	24	47	14.0	27.7
47	Amphetamine sulfate	55	149	24	65	20.0	54.3
39	Pentachlorophenol	27	101	28	105	28.6	107.3
26	Arsenic trioxide	15	74	31	159	4.1	20.9
33	Chloroform	908	7605	36	302	999.8	8375.2
32	Lindane	76	261	44	151	242.9	835.1
4	Diazepam	352	1236	45	159	71.4	250.8
13	Sodium fluoride	52	1238	57	1357	92.8	2210.9
38	Hexachlorophene	56	138	67	165	214.3	526.6
42	Orphenadrine HCL	255	834	100	327	50.00	163.4
25	Paraquat	100	537	120	644	40.00	214.7
48	Caffeine	192	989	127	654	135.7	698.8
35	Isoniazid	1250	9117	133	970	171.5	1250.4
23	Penobarbital	162	697	137	590	111.4	479.7
5	Amitriptyline	320	1154	140	505	37.1	133.8
44	Diphenylhydantoin	1635	6480	150	595	300.0	1189.1
40	Varapamil HCL	108	220	163	331	122.3	249.1
14	Malathion	290	878	190	575	742.8	2248.4
2	Acetylsalicylic acid	200	1110	232	1287	385.7	2140.5
20	Theophylline	244	1354	235	1304	157.1	872.1
21	Propoxyphene HCL	84	223	255	678	24.6	65.4
37	Barium nitrate	355	1358	266	1016	37.1	142.1
11	Phenol	317	3369	270	2869	157.2	1670.0
43	Quinidine sulfate	258	610	286	676	79.2	187.4
22	Propamolol HCL	466	1575	320	1082	71.5	241.7
1	Paracetamol	2404	15,899	338	2235	271.4	1795.2
15	2,4-Dichlorophenoxy-acetic	375	1697	347	1570	385.8	1745.3
29	Thioridazine HCL	995	2445	385	946	68.6	168.5
49	Altropine sulfate	585	864	456	674	1.7	2.5
41	Chloroquine phosphate	623	1208	500	969	84.3	163.4
27	Cupric sulfate	469	1880	502	2012	290.6	1163.6
3	Ferrous sulfate	319	2100	680	4477	392.1	2581.0
36	Dichloromethane	1601	18,846	873	10,280	1386.2	16,321.7
19	Lithium sulfate	492	4478	1190	10,828	1065.5	9691.8
50	Potassium chloride	2598	34,853	1499	20,107	285.5	3830.0
45	Chloramphenicol	2500	7735	1500	4641	285.7	884.0
16	Xylene	4299	40,490	2119	19,953	899.8	8474.6
9	Ethanol	7057	153,145	3448	74,837	4712.2	102,262.2
12	Sodium chloride	3002	51,370	4003	68,493	2287.3	39,138.9
46	Sodium oxalate	11160	83,284	5095	38,019	357.1	2665.3
7	Ethylene glycol	4698	75,684	5498	88,567	1570.9	25,304.8
8	Methanol	5619	175,327	7289	227,414	1569.0	48,954.2
10	1,1,1-Trichloroethane	11196	83,927	7989	59,884	5707.6	42,785.8
34	Carbon tetrachloride	2350	15,280	8264	53,726	1314.4	8545.4

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Oral LD50 Doses for Rat and Mouse and Mean Oral Lethal Doses for Humans

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17	Nicotine	50	308	3	21	0.7	4.4
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26	Arsenic trioxide	15	74	31	159	4.1	20.9
30	Thallium sulfate	16	32	24	47	14.0	27.7
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5	Amitriptyline	320	1154	140	505	37.1	133.8
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3	Ferrous sulfate	319	2100	680	4477	392.1	2581.0
14	Malathion	290	878	190	575	742.8	2248.4
16	Xylene	4299	40,490	2119	19,953	899.8	8474.6
33	Chloroform	908	7605	36	302	999.8	8375.2
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9	Ethanol	7057	153,145	3448	74,837	4712.2	102,262.2
10	1,1,1-Trichloroethane	11196	83,927	7989	59,884	5707.6	42,786.8

Source: E. Walum. 1998. Acute oral toxicity. EHP 106:497-503. (reprinted with permission from the editor)

Toxicity Categories

Category	Signal Word	Oral LD_{50} (mg/kg)	Dermal LD_{50} (mg/kg)	Inhalation LD_{50} (mg/L)[1]	Oral Lethal Dose	Eye Irritation	Skin Irritation
I - Highly Toxic	DANGER, POISON (skull & crossbones), WARNING	0 to 50	0 to 200	0 to 0.05	A few drops to a teaspoonful	Corrosive (irreversible destruction of ocular tissue) or corneal involvement or irritation persisting for more than 21 days	Corrosive (tissue destruction into the dermis and/or scarring)
II - Moderately Toxic	CAUTION	>50 to 500	>200 to 2,000	> 0.05 to 0.5	Over a teaspoonful to one ounce	Corneal involvement or irritation clearing in 8-21 days	Severe irritation at 72 hours (severe erythema or edema)
III - Slightly Toxic	CAUTION	>500 to 5,000	>2,000 to 20,000	>0.5 to 2	Over one ounce to one pint	Corneal involvement or irritation clearing in 7 days or less	Moderate irritation at 72 hours (moderate erythema)
IV - Relatively Non-toxic	none	>5,000	>20,000	> 2	Over one pint to one pound	Moderate irritation at 72 hours (moderate erythema)	Mild or slight irritation at 72 hours (no irritation or slight erythema)

1 EPA/OPP does not currently use the inhalation toxicity values in 40 CFR 150.10(h). Instead, OPP uses values that are from a 2/1/94 Health Effects Division paper entitled "Interim Policy for Particle Size and Limit Concentration Issues in Inhalation Toxicity Studies".

[2] Four hour exposure.

Sources:

(1) U.S. EPA, Office of Pesticide Programs. Label Review Manual. Chapter 8: Precautionary Labeling. http://www.epa.gov/oppfead1/labeling/lrm/chap-08.htm.
(2) National Ag Safety Database. Toxicity of Pesticides. http://www.cdc.gov/niosh/nasd/docs2/as18700.html.
(3) 40 CFR 156.10(h) – Labeling Requirements for Pesticides and Devices. Warnings and precautionary statements.

Appendix IV: Oral Acute Single Lethal Doses in Humans

Table II: Oral acute single lethal doses in humans

No.	Chemical	LD/MLD	Dose values (g) Reference numbers 10	11	12	13	14	15	16	17	18	19	Other references	Mean doses
1.	Paracetamol	LD	19											19
		MLD	10	10	>10	17.5	22.5	>10	17.5			10		15
2.	Acetylsalicylic acid	LD	33.6	17.5	30			25						27
		MLD	36	17.5			20							22
3A.	Fe^{2+} in iron (II) sulphate	LD	16.8	17.5			6	15.7	11.5	7.7	23.2			14
3B.	Iron (II) sulphate	MLD			2.1	1.5	1.5				4.28			2.3
4.	Diazepam	LD												38
		MLD						>2				2		E5*
5.	Amitriptyline hydrochloride	LD	5					1	1.75					2
		MLD			>2.1	2	2							2.6
6.	Digoxin	LD				0.006		0.015	0.0375					0.0092
		MLD												0.0011
7.	Ethylene glycol	LD			0.001									110
		MLD	111	111	100		111	111	111	111	111	111		110
8.	Methanol	LD	70		123		67	150	111					110
		MLD	23.8	23.8					123	119				110
9.	Ethanol	LD	455	280	278		455	455	455		17.6	59		31
		MLD												330
10.	Isopropanol	LD	132	188	196		196	168	157	188			88 (9, 59)	98
		MLD										196		160
														196
11.	1,1,1-Trichloroethane	LD											193 (60), 802 (61)	600
		MLD												24
12.	Phenol	LD	20	>42	>6.7						8.8			11
		MLD	4.6	4.5										6.6
13.	Sodium chloride	LD	140	140	2	11.5						8	210*	160
		MLD												nr

Source: Ekwall et al. 1998. MEIC Evaluation of Acute Systemic Toxicity. Part V. ATLA 26:571-616. (reprinted with permission from the editor)

14.	Sodium fluoride	LD	7.5				5	5	7.5	7.5		4.5				6.2
		MLD		4.6	1.2			2	1				1			2.9
15.	Malathion	LD	60						17.5		7.5			70 (62)		52
		MLD									60			25 (9, 59)[c]		25
16.	2,4-Dichloro-phenoxyacetic acid	LD		28[d]					24.1		28				27[e]	
		MLD		5.6	6.5									5.6 (9)[c]	5.9	
17.	Xylene	LD		120	245				19.4	53	12.9		21.5		63[e]	
		MLD		0.060			0.040		0.060	0.045	0.05	0.05	0.045		51	
18.	Nicotine	LD		0.050			0.045		0.005						0.05	
		MLD		0.25	0.20	0.14			0.20		0.20	0.25	0.2		0.036	
19.	Potassium cyanide	LD													0.21	
		MLD							9.4[f]						0.20	
20A.	Lithium	LD													9.4	
		MLD													nr	
20B.	Lithium sulphate	LD									42[g]				68	
21.	Theophylline	LD									11[g]				11	
		MLD							4						5.4	
22.	Dextropropoxyphene hydrochloride	LD							0.78[h]			0.64	7	11 (63)	0.71[i]	
		MLD		1.1		0.5	0.75							5.2 (9, 59)[c]	0.86	
23.	Propranolol hydrochloride	LD					E9.6		E5.1	4	0.65	1.2			5	
		MLD				>1									1	
24.	Phenobarbital	LD		8	8			8	7.5	7.5	6	5	5		7.8	
		MLD				1.5				3.1	1.5		1.5		4.8	
25.	Paraquat	LD		4.5	2.1								D.075		2.5	
		MLD				0.28									0.18	
26.	Arsenic trioxide	LD							0.25	0.33				0.3[b]	0.29	
		MLD		0.21	0.23	0.12				0.2	0.2		0.1		0.18	
27.	Copper (II) sulphate	LD							15	15	15	10			14	
		MLD											15	3.5 (59)[c]	9.3	
28.	Mercury (II) chloride	LD			2.1	1			2.5		1	1			1.5	
		MLD			0.5				0.5				0.5		0.5	
29.	Thioridazine hydrochloride	LD		4.8						3.5					4.2	
		MLD								>3					3	
30.	Thallium sulphate	LD		1	0.85	1			1	1	1	1			0.99	
		MLD			0.56								0.8		0.68	

Table II: continued

No.	Chemical	LD/MLD	Dose values (g) Reference numbers 10	11	12	13	14	15	16	17	18	19	Other references	Mean doses
31.	Warfarin	LD							7.5[i]	7.5[i]				7.5
		MLD												nr
32.	Lindane	LD	15					8.75		28				17
		MLD			3.5									3.5
33.	Chloroform	LD						44			96			70
		MLD	44	14.8	14.8					14.8		22		22
34.	Carbon tetrachloride	LD	151					32.8[a,f]						92
		MLD	12		6.4			3.2	6.4			6.4		6.9
35.	Isoniazid	LD	12.5					14	8	12.5[f]	14			12
		MLD	8	8		8		8				10		8.4
36.	Dichloromethane	LD			33.2				146[a]			110[a]		97
		MLD									24			24
37.	Barium nitrate	LD		2	2[a]					3.9[g]				2.6
		MLD										1		1
38.	Hexachlorophene	LD			5					17.6	21			15
		MLD										2.25		2.3
39.	Pentachlorophenol	LD								2			2 (62)	2
		MLD	2		1									1.5
40.	Verapamil hydrochloride	LD							8.6[f]					8.6
		MLD		3					>3	3.8[f]				3.4
41.	Chloroquine phosphate	LD	2.5			7.2	8		5.6		6.4			5.9
		MLD											2.2 (9)[f]	2.2
42.	Orphenadrine hydrochloride	LD	2.8	2.8	5.5[d]	2.8			2.8					3.3
		MLD								1				1
43.	Quinidine sulphate	LD				11.5			8		11.5			10
		MLD	4						>6	8[g]	2			5

44.	Diphenylhydantoin	LD			E21									21
		MLD	E7.5	9.1					8.5[j]			2		6.8
45.	Chloramphenicol	LD	20							20				20
		MLD											10 (62), 28 (9)[e]	19
46.	Sodium oxalate	LD		30	15					23				23
		MLD									15	5	5 (64)	8.3
47.	Amphetamine sulphate	LD							0.5		1.4[d]			0.95
		MLD		0.1	0.1			0.15	0.12			0.25		0.14
48.	Caffeine	LD	7.5		15	7.5	7.5	10	12					9.9
		MLD	E6.5	10							10	10		9.1
49.	Atropine sulphate	LD	E0.10[k]		E0.1[k]	E0.2[k]					0.1[k]	0.075		0.12[k]
		MLD						0.10	0.2	0.050				0.12
50.	Potassium chloride	LD							E45		16.2		24 (65)	28
		MLD								18[h]				18

[a]high variability as well as tolerance makes it difficult to establish human LD.
[b]POISINDEX™, Information Systems (ed. B.H. Rumack & D.G. Spoerke), Micromedex (Denver, CO, USA).
[c]Low LD
[d]Extrapolated from animal dosage.
[e]Geometric mean value, when the quotient between original values (range) is larger than ten.
[f]Two lethal poisonings.
[g]One survivor and one dead.
[h]One death.
[i]12.5mg/kg lethal in 14 days (16),1g lethal in 13 days (17).
[j]Several survivors.
[k]Very variable.
LD = mean lethal dose, MLD = minimal lethal dose; E = extrapolated; nr = not reported.

Appendix V: Clinically Measured Acute Lethal Serum Concentrations in Humans

Table III: Clinically measured acute lethal serum concentrations in humans

No.	Chemical	LC/MLC	Concentrations (mg/l)										Other references	Mean concentration (mg/ml)
			References numbers											
			10	11	12	13	14	15	16	17	18	19		
1.	Paracetamol	LC	300[a]	300[a]		300[a]						400		330
		MLC		160[a]	300[a]		300[a]							250[a]
2.	Salicylic acid	LC	1300[b]									600		950
		MLC		1000	900[b]	800[b]	1000							930
3.	Iron	LC										8		8
		MLC	10[c]	10[c]	5			5	8.1[c]					7.6
4.	Diazepam	LC										20		20
		MLC	15	20						20				18
5.	Amitriptyline	LC		5.								10		7.5
		MLC								2.5[d]				2.5
6.	Digoxin	LC							0.003	0.027		0.01		0.018
		MLC	0.015								0.003			0.007
7.	Ethylene glycol	LC	4370							4370		2000		3600
		MLC												nr
8.	Methanol	LC	1750	1000						1600	1809	800		1400
		MLC						500		400				450
9.	Ethanol	LC		5000			4000	5000				4500		4600
		MLC	5000		5000				5000		3700			4600
10.	Isopropanol	LC	3400	2000				1500	2000			3000		2800
		MLC												1800
11.	1,1,1-Trichloro-ethane	LC											180 (26)[e]	E180
		MLC												nr
12.	Phenol	LC												nr
		MLC	42						50					46[f]

Source: Ekwall et al. 1998. MEIC Evaluation of Acute Systemic Toxicity. Part V. ATLA 26:571-616. (reprinted with permission from the editor)

13.	Sodium in sodium chloride	LC											nr
		MLC	10800	10800			10800						11000[f]
14.	Fluoride	LC			3			14.2	14[d,g]		3		8.6
		MLC											nr
15.	Malathion	LC										4.4 (26)[a]	E4.4
		MLC							0.35[d,g]				E0.35
16.	2,4-Dichlorophenoxy acetic acid	LC	415						600[d]				510
		MLC											nr
17.	Xylene	LC						E50				43 (66)	47
		MLC										11[h,i]	11
18.	Nicotine	LC									5		5
		MLC		10			10						10[f]
19.	Cyanide	LC										5	5
		MLC	2.5				3		3.1[d]				2.9
20.	Lithium	LC					69[j]		77[e]				72[k]
		MLC		24		24							24[k]
21.	Theophylline	LC		183[l]				136	130[m]	150			150
		MLC	100[n]				100	64			50		79
22.	Dextropropoxy-phene	LC	6								10		8
		MLC			2			2	1.8[d]				1.9
23.	Propranolol	LC	16	3.3[h]				3.3[h]	3[e]				6.4
		MLC		4.7			4[d]						3.9
24.	Phenobarbital	LC	115						117	110	200		136
		MLC		80				120					100
25.	Paraquat	LC	2										2[f]
		MLC		0.2					0.2	0.1			0.17
26.	Arsenic	LC			3.9[d]					1.5	2[o]		2.5
		MLC											nr
27.	Copper	LC								4.5	6		5.5
		MLC											nr

Table III: continued

No.	Chemical	LC/MLC	Concentrations (mg/l)										Other references	Mean concentration (mg/ml)
			References numbers											
			10	11	12	13	14	15	16	17	18	19		
28.	Mercury	LC								0.65[e]		2	14.3 (67)[d,k]	2.6[h]
		MLC	0.22		>0.1									0.22
29.	Thioridazine	LC								7.1[d]		20		14
		MLC												nr
30.	Thallium	LC								1.5[d,h]				1.5[h]
		MLC	0.3											0.3
31.	Warfarin	LC											110 (26)[d,k]	E110[p]
		MLC												107
32.	Lindane	LC												E0.92
		MLC							0.5	0.92[e]	107[d]			0.5
33.	Chloroform	LC										400		400
		MLC								165[g]		200[g]		180
34.	Carbon tetrachloride	LC								20[d,k]		1		4.5[h]
		MLC								1.8[d,e]				1.8
35.	Isoniazid	LC								77[e]				E77
		MLC		10										10
36.	Dichloromethane	LC										300[f]		300
		MLC												nr
37.	Barium	LC											97 (26)[d]	E97
		MLC								5.6[d]				5.6
38.	Hexachlorophene	LC	35.6[e]							52[e]				44
		MLC												nr
39.	Pentachlorophenol	LC								74[e]				74
		MLC	40											40

40.	Verapamil	LC	3[d,x]					4.1	4[d,x]				3.7
		MLC											nr
41.	Chloroquine	LC	10[d]			8		.9	22[d]		4		11
		MLC											nr
42.	Orphenadrine	LC		6					3.6[e]				4.8
		MLC	6										6[f]
43.	Quinidine	LC		16.8[d]					14.6[d]		40		24
		MLC	14		10		9						11
44.	Diphenylhydantoin	LC	95						98		80		91
		MLC	60	50									55
45.	Chloramphenicol	LC							190[e]				E190
		MLC				75[t]		68[t]	68[t]				70
46.	Oxalate	LC							20[e]		20	20 (26)[e]	20
		MLC											nr
47.	Amphetamine	LC								4	2		3
		MLC											nr
48.	Caffeine	LC	150							160[d,g]	150		150
		MLC				160			135[d]				140
49.	Atropine	LC							0.13[d,x]				E0.13[r]
		MLC											nr
50.	Potassium	LC	397						364[d]	352			370
		MLC	293						313				300

[a]After 4 hours. [b]After 6 hours. [c]After 3 hours. [d]As judged from high survived concentrations. [e]SD analysis. [f]This value will substitute for the presented LC value in calculations based on LC values. [g]Based on one case only. [h]Geometrical mean value from a range of values with a quotient larger than ten. [i]TOMES® Information Services (ed. B.H. Rumack & D.G. Spoerke), Micromedex (Denver, CO, USA). [j]Also 69mg/l as judged from high survived concentrations in reference 16. [k]May include acute chronic dosage. [l]Peak concentration. [m]S/D: 90/170 =130 mg/l (17). [n]Acute dosage. [o]In blood. [p]Represents acute on chronic dosage: no reports on single-dose lethal poisonings. [q]Plane 4 anaesthesia. [r]Value probably originating from forensic medicine data. [s]Reported value of 90mg/l, which seems too high. [t]Grey baby syndrome.

***E** = estimated/extrapolated; **LC** = mean lethal serum concentration; **MLC** = minimal lethal serum concentration; **S/D** = high survived and lethal concentrations from case reports, with a resulting mean value; **nr** = not reported.*

Appendix VI: Post-Mortem Acute Lethal Concentrations in Humans

Table IV: Post-mortem acute lethal concentrations in humans

No.	Chemical	LC/MLC	Concentrations (mg/l) Reference numbers 17	20	21	22	23	24	25	Other references	Mean concentration (mg/ml)
1.	Paracetamol	LC	248		250	280[a]			160		230
		MLC		160	160		150	250			180
2.	Salicylic acid	LC	661	500	500	772			700		620
		MLC					450	450			450
3.	Iron	LC	9.0[b]	35							22
		MLC									nr
4.	Diazepam	LC	18							10 (68)	14
		MLC		20	5	50				50 (69)	31
5.	Amitriptyline	LC	3.7	6.32[a]	3.3[c]	5.58[a]			2		4.2
		MLC			0.55[d]		1.5	1.75			1.3
6.	Digoxin	LC	0.025	0.015	0.0103[c]				0.015		0.016
		MLC			0.0015[d]		0.005	0.005			0.0038
7.	Ethylene glycol	LC	2400	3000	2400						2600
		MLC			300						300
8.	Methanol	LC	1900		1900						1900
		MLC		890	200				900		660
9.	Ethanol	LC	5500		4000[c]				5000		4800
		MLC		3500	2250[d]	4000					3300
10.	Isopropanol	LC	1500								1500
		MLC			1000						1000
11.	1,1,1-Trichloroethane	LC	126							316[e]	170
		MLC			60[c]						15
12.	Phenol	LC	49	90	15[d]				90		76
		MLC									nr
13.	Sodium in sodium chloride	LC								13000 (26)[e]	13000
		MLC									nr

Source: Ekwall et al. 1998. MEIC Evaluation of Acute Systemic Toxicity. Part V. ATLA 26:571-616. (reprinted with permission from the editor)

14.	Fluoride	LC	15	2	3			2		5.5
		MLC								nr
15.	Malathion	LC	281							280
		MLC								nr
16.	2,4-Dichlorophenoxy-acetic acid	LC	464			669				570
		MLC								nr
17.	Xylene	LC	43	10.9	13.4[a,b]			10.9		20
		MLC								nr
18.	Nicotine	LC	29	16[c]	25	17.7[a]				22
		MLC			5					9.3
19.	Cyanide	LC	24.7	3.7		7.6[c]		13.6		9.9[f]
		MLC			5					5[f]
20.	Lithium sulphate	LC	31.9[c]	34				3.7		34[d]
		MLC			13.9			14		14[d]
21.	Theophylline	LC	150	150			50	50	150	150
		MLC			50					50
22.	Dextropropoxyphene	LC	4.7	4.1[a]	15	7.7[a]				7.9
		MLC			2			1.5		1.8
23.	Propranolol	LC	14	10	9	16	7	7	7	11
		MLC			4					6
24.	Phenobarbital	LC	97		45[c]	210	7	7	125	120
		MLC			4.3[d]					36[e]
25.	Paraquat	LC	1.2[a,b]	80	1.2		80	55	2	3.2[a]
		MLC		35						nr
26.	Arsenic	LC	3.3	12	15	2.36[a]				8.2
		MLC								nr
27.	Copper	LC	36	12.5[a]						24
		MLC								nr
28.	Mercury	LC	4.2[c]			0.58			0.6	2.4
		MLC								0.6
29.	Thioridazine	LC	5.1	4.24[a]	5	7				4.9
		MLC			2		10	11.5		6.5
30.	Thallium	LC	4.0[d]	0.5					3.3 (27)[c]	2.3
		MLC							2.4 (27)[d]	nr

Table IV: continued

No.	Chemical	LC/MLC	Concentrations (mg/l): Reference numbers 17	20	21	22	23	24	25	Other references	Mean concentration (mg/ml)
31.	Warfarin	LC								100 (28)	100
		MLC			>10		> 10	> 10	> 11		> 10
32.	Lindane	LC									nr
		MLC	0.02[e,k]								0.02
33.	Chloroform	LC	64	390	30[c]	29			390		97[b]
		MLC			7[d]						7
34.	Carbon tetrachloride	LC	274[h]		260				150		230
		MLC									nr
35.	Isoniazid	LC	117[h]		150[c]						130
		MLC					100	100			100
36.	Dichloromethane	LC	364	280	395[h]	496			280		360
		MLC									nr
37.	Barium	LC									nr
		MLC	1.9[e,l]							< 20[e,l,m]	1.9
38.	Hexachlorophene	LC	35[f]	35					35		35
		MLC									nr
39.	Pentachlorophenol	LC	107		99						100
		MLC		46	46				45		46
40.	Verapamil	LC	11	6.4					6		7.8
		MLC						2.5			2.5
41.	Chloroquine	LC	30.5	17.2*	10	11.2*			3		14
		MLC			3		4.5	3			3.5
42.	Orphenadrine	LC	20.6	6	9	16.7			6		12
		MLC			4		7	3.6			4.9
43.	Quinidine	LC	45[f]	40	55	40			40		44
		MLC			30		15				23
44.	Diphenylhydantoin	LC	54[e,n]		94				100		83
		MLC		100	70		50	50			68

Appendix VII: Human Kinetic Data

Table V: Human kinetic data[a]

No.	Chemical	Absorption in the gut[b]	Time to peak (ingestion)	Kinetics	T½	Vd l/kg	Passage of blood–brain barrier	Accumulation in vital organs	Blood protein binding	References[d]
1.	Paracetamol	Good	0.5–> 4 hours[e]	First-order[e]	> 12 hours[e]	0.9	Free?	Liver,[f] kidney[f]	20–50%[e]	[c]
2A	Acetylsalicylic acid	Good		Zero-order	0.27 hours	0.2	Restricted	None	50–90%	
2B	Salicylic acid	"	12–24 hours[e]	Zero-order	27 hours[e]	0.17	Restricted	None	< 80%	30
3.	Iron (II) sulphate	Good[e]	2–4 hours[e]	Biphasic	nr	nr[f]	Restricted	Blood, liver	100%	16, 30
4.	Diazepam	Complete	1–7 hours	Biphasic	96 hours[g]	1.1	Free	CNS, liver,[f] kidney[g]	99%	
5.	Amitriptyline hydrochloride	Good[f]	20 hours[g]	Biphasic	8 and 27 hours[e]	15	Free	Liver,[f] kidney, lung, heart, CNS[g]	95%	
6.	Dioxin	Moderate	2–5 hours[e]	Biphasic	48 hours[e]	5	Restricted	Heart, kidney, liver[f,m]	29%	
7.	Ethylene glycol	Complete	1–4 hours	First-order?	3.4 hours[e]	0.65	Free	Liver,[f] kidney[f]	None	
8.	Methanol	Good	0.5–1.5 hours	Zero-order	27 hours[e]	0.65	Free	Kidney, liver[f]	None	"
9.	Ethanol	Good	0.5–> 3 hours[e]	Zero-order	4 hours[e]	0.6	Free	None	None	
10.	Isopropanol	Complete	1 hour	First-order	5.4 hours[e]	0.6	Free	None	nr	31
11.	1,1,1-Trichloroethane	Complete	1 hour?	Triphasic	0.7, 6 and 53 hours	> 1[e]	Free	CNS[h]	30–70%	32, 33
12.	Phenol	Complete	20.5 hours[e]	Biphasic?	2.8 hours	nr	Free	CNS	30–70%?	34, 35
13.	Sodium chloride	Complete	5 hours[e]	Zero-order	nr	0.64	Restricted	None	None	
14.	Sodium fluoride	Complete	> 1 hours[e]	Biphasic	5.5 hours	0.6	Restricted	None (bone only)	None	36
15.	Malathion	Good	1–6 hours[e]	Multiphasic	nr[n]	nr[f]	Free	Kidney, liver,[f] CNS[m]	nr[f]	37–39
16.	2,4-Dichlorophenoxy-acetic acid	Complete	7–24 hours[e]	First-order	58 hours[e,p]	0.2[e]	Restricted	Liver, kidney	High	
17.	Xylene	Good		Biphasic	1 and 25 hours	nr[f]	Free	Lipid-rich organs[h]	High	15
18	Nicotine	Complete[f]	1.5 hours	Biphasic	10 minutes and 2.2 hours	2	Free	CNS, liver,[f] kidney[f,h]	High	
19	Potassium cyanide	Complete	> 0.5 hours?	Biphasic	1 and 6–66 hours[e]	1	Free	Erythrocytes[h]	5%	15
20.	Lithium sulphate	Complete	< 1 hour[e]	Biphasic	3–12 and 8–65 hours[e]	0.9	Restricted	Liver, kidney[h]	None	16

Source: Ekwall et al. 1998. MEIC Evaluation of Acute Systemic Toxicity. Part V.. ATLA 26:571-616. (reprinted with permission from the editor)

21.	Theophylline	Complete	2–8 hours[a]	Biphasic[a]	17 minutes and 6 hours[a]	0.5	Free	None	56%	63
22.	Dextropropoxyphene hydrochloride	Complete[j]	1–2 hours	Biphasic[a]	5 and 15 hours[a]	16	Free	CNS, liver,[f] lung, kidney[k]	78%	[s]
23.	Propranolol hydrochloride	Complete[j]	1–2 hours	Biphasic?	3.9 and 16 hours[a]?	4.3	Free	CNS, liver,[f] kidney[k]	80–95%	28
24.	Phenobarbital	Complete	nr	First-order	100 hours[a]	0.6	Free	Liver[f]	50%	15, 30
25.	Paraquat	Moderate[u]	< 4 hours[a]	Biphasic	5 and 84 hours[a]	1.4[a]	Free?	Lung, liver, kidney	nr	40
26.	Arsenic trioxide	Good	1 hour	Biphasic[a]	1–2 and 30 hours[a]	0.27	Restricted	Liver, kidney, heart, GIT[m]	nr	
27.	Copper (II) sulphate	Poor	nr	Biphasic	2–3 hours and 26 days[a]	2	Restricted	Blood, liver[f]	95%	16, 41
28.	Mercury (II) chloride	Moderate[j]	nr	Biphasic	2 and 24–50 days[a]	> 1	Restricted	Blood, kidney, liver, heart	nr[a]	67
29.	Thioridazine hydrochloride	Good[j]	2–4 hours	Multiphasic	26 hours	18	Free	CNS, lung, liver,[f] kidney[k]	98–99%	16, 30, 42
30.	Thallium sulphate	Good	2–4 hours	Biphasic[a]	48 and 96 hours?[a]	4.6	Restricted	Kidney, heart, liver, CNS[m]	None	16, 30, 43
31.	Warfarin	Good	3–9 hours	First-order	22–96 hours[a]	0.11[a]	Restricted	none	99%	16, 16, 44
32.	Lindane	Good[a]	6 hours	Biphasic[a]	21 hours and 10 days[a]?	nr[f]	Free	CNS, liver, kidney, (fat)	nr	16, 28
33.	Chloroform	Complete	1 hour	First-order?	1.5 hours	2.8	Free	CNS, liver, kidney, (fat)[k]	nr	16
34.	Carbon tetrachloride	Good	nr	Biphasic[a]	11 and 43 hours[a]	nr[f]	Free	Liver,[f] kidney,[f] (fat)	nr	16, 45
35.	Isoniazid	Complete	1.5–3 hours[a]	First-order	2.4 and 6 hours[a,l]	0.6	Free	Liver,[f] kidney, lung, skin	10%	16
36.	Dichloromethane	Complete	2 hours	First-order?	40 minutes	0.62	Free	None	nr	16
37.	Barium nitrate	Good	> 2 hours[a]	Triphasic	3.6, 34 and 1033 days[a,n]	nr	Restricted	Muscle, lung, (bone)	54%	15, 26
38.	Hexachlorophene	Good	3–6 hours?	Biphasic?[a]	24 hours[a]	nr	Restricted	Liver,[f] kidney	92%[a]	46, 47
39.	Pentachlorophenol	Good	4 hours	First-order?	13 hours to 16 days	0.35[a]	Restricted	Liver,[f] kidney	> 96%	16, 48
40.	Verapamil hydrochloride	Good[j]	2 hours	Biphasic	23 minutes and 5 hours	5	Restricted?	Liver[f,k]	90%	

Table V: continued

No.	Chemical	Absorption in the gut[b]	Time to peak (ingestion)	Kinetics	T½[c]	Vd l/kg	Passage of blood–brain barrier	Accumulation in vital organs	Blood protein binding	References[d]
41.	Chloroquine phosphate	Good	1–3 hours[e]	Triphasic	2, 7 and 45 days[e,v]	94	Free	Heart, liver, kidney, lung, erythrocytes[f]	55–61%	16, 49
42.	Orphenadrine hydrochloride	Good[l]	3 hours	First-order Biphasic?[e]	15 hours 6 and 15 hours[e]	6	Free	CNS, liver, lung[g]	20–95%	16, 50, 51
43.	Quinidine sulphate	Good[l]	> 2 hours[e]	First-order?	> 7.8 hours[e]	2.7[e]	Restricted	Liver,[f] kidney, heart[h]	60–90%	15, 16
44.	Diphenyl-hydantoin	Poor/good	30–120 hours[e]	Zero-order and first-order[e]	24–200 hours[e,v]	0.6[e]	Free	Liver, kidney, CNS	60%[e]	52
45.	Chloramphenicol	Good	2–3 hours	First-order	2.5 hours	1.2	Free	Liver,[f] kidney	55%	
46.	Sodium oxalate	Poor	6 hours?	First-order?	4 hours?[e]	[e]0.4[e]	Restricted	Kidney, liver	nr	25, 64
47.	Amphetamine sulphate	Complete[i]	1–4 hours[e]	First-order?	7–34 hours[e]	3–6.1	Free	Liver, kidney	16%	15, 16
48.	Caffeine	Complete	1 hour	First-order?	9–16 hours[e]	0.6	Free	None (liver 2x)	35–60%	53, 54
49.	Atropine sulphate	Good	> 2 hours[e]	First-order?	3.5 hours	3	Free	Kidney, liver	50%	
50.	Potassium chloride	Complete	0.5 hours	Multiphasic	nr	nr	Free?	None	None	65[j]

[a]Data for the overdose situation are indicated by an asterisk. [b]Absorption: complete = 100% and rapid, good = 80%, moderate = 20–80%, and poor = 0–20%. [c]One value indicates T½ of the elimination phase. Successive values represent separate phases (alpha, beta, etc.). [d]Other than references 10, 11, 13, 14 and 17. [e]Non-linear in overdose? [f]Also a biotransforming organ. [g]POISINDEX®, Information Systems (ed. B.H. Rumack & D.G. Spoerke), Micromedex (Denver, CO, USA). [h]Absorbed as acetylsalicylic acid. [i]Due to corrosivity. [j]Probably large, i.e. around 5l/kg. [k]Early accumulation. [l]Documented first therapeutic doses, i.e. bioavailability is decreased by rapid binding in the liver of a large fraction of the absorbed dose (25–85%). For most such chemicals, passage of the intestinal mucosa is probably complete. However, the term "good" is often used in this table based on literature reports on the total absorption (the sum of intestinal passage and first pass reduction of bioavailability). [m]Slow accumulation. [n]Alpha phase: 2.9 hours. [o]Probably large Vd and protein binding. [p]pH-dependent. [q]Dependent on formulation. [r]Biphasic up to 160 hours. [s]TOMES®, Information Systems (ed. B.H. Rumack & D.G. Spoerke), Micromedex (Denver, CO, USA). [t]Varies between rapid and slow acetylators. [u]Alpha-phase: 3 hours in overdose. [v]Dose-dependent.*

nr = not reported; CNS = central nervous system (brain); GIT = gastrointestinal tract (gut); T½ = plasma half-life; Vd = distribution volume.

Appendix VIII: Peaks from Approximate 50% Lethal Concentration (LC50) Curves

Table VI: Peaks from approximate 50% lethal concentration (LC50) curves[a]

No.	Chemical	Time to peak (hours)	Peak conc. mg/l	Type of curve	Case reports: Sub-lethal	Lethal (clinical)	Lethal (post-mortem)	Total
1.	Paracetamol	4	358	LC50	81	62	0	143
2.	Salicylic acid	20	1070	LC50	31	46	1	78
3.	Iron	4	43.5	LC50	15	12	0	27
4.	Diazepam	2	19.9	LC100	4	0	0	4
5.	Amitriptyline	6	1.69	LC50	8	6	10	24
6.	Digoxin	3	0.071	LC50	15	9	1	25
7.	Ethylene glycol	2.5	1550	LC50	28	12	9	49
8.	Methanol	2	3790	LC50	76	37	7	120
9.	Ethanol	1	8440	LC50	20	1	143	164
10.	Isopropanol	1	4960	LC50	13	2	2	17
11.	1,1,1-Trichloro-ethane	1	231	LC50	3	0	2	5
12.	Phenol	0.5	80	LC50	3	0	4	7
13.	Sodium in sodium chloride	5	11700	LC50	3	9	1	13
14.	Fluoride	3	19.4	LC0	3	3	7	13
15.	Malathion	5	1.88	LC0	2	1	11	14
16.	2,4-Dichloro-phenoxyacetic acid	14	1125	LC50	7	1	4	12
17.	Xylene	1	110	LC0	3	0	1	4
18.	Nicotine	0.5	13.5	LC0	1	1	3	5
19.	Cyanide	0.5	16.4	LC50	12	9	10	31
20.	Lithium	3	97.2	LC100	4[b]	0	0	4[b]
21.	Theophylline	12	180	LC50	57	18	1	76
22.	Dextropropoxy-phene	2	8	LC0	2[b]	1	6	9
23.	Propranolol	4	3.11	LC50	6	2	1	9
24.	Phenobarbital	15	230	LC50	20	1	0	21
25.	Paraquat	2.5	12.6	LC50	23	66	16	105
26.	Arsenic	4	1.65	LC50	10	8	3	21
27.	Copper	11	15.9	LC50	10	5	1	16
28.	Mercury	12	40.1	LC50	12	2	4	18
29.	Thioridazine	4	4.08	LC50	1	1	4	6
30.	Thallium	24	7.35	LC50	25	5	2	32

[a] *From reference 26.*
[b] *Documented single-dose cases (not overdose on previous medication).*

Source: Ekwall et al. 1998. MEIC Evaluation of Acute Systemic Toxicity. Part V. ATLA 26:571-616. (reprinted with permission from the editor)

Table VI: continued

No.	Chemical	Time to peak (hours)	Peak conc. mg/l	Type of curve	Case reports: Sub-lethal	Lethal (clinical)	Lethal (post-mortem)	Total
31.	Warfarin	6	200	LC0	3	0	0	3
32.	Lindane	6	1.3	LC0	5	2	1	8
33.	Chloroform	2	490	LC50	2	0	5	7
34.	Carbon tetrachloride	6	5.8	LC50	5	1	1	7
35.	Isoniazid	3	167	LC50	24	3	4	31
36.	Dichloromethane	3	344	LC0	0	0	9	9
37.	Barium	2	305	LC100	9	0	0	9
38.	Hexachlorophene	5	116	LC50	2	1	1	4
39.	Pentachlorophenol	10	79.1	LC50	1	0	3	4
40.	Verapamil	2	13.2	LC50	10	9	4	23
41.	Chloroquine	2	9.41	LC50	4	1	9	14
42.	Orphenadrine	2	11.3	LC50	6	1	8	15
43.	Quinidine	6	26	LC50	4	2	0	6
44.	Diphenylhydantoin	34	202	LC50	13	1	0	14
45.	Chloramphenicol	6	180	LC0	5	4	0	9
46.	Oxalate	6	110	LC0	1	1	0	2
47.	Amphetamine	2	15.5	LC50	1	1	5	7
48.	Caffeine	3	179	LC50	6	0	4	10
49.	Atropine	3	4.05	LC100	2	0	0	2
50.	Potassium	1	375	LC0	4	3	1	8

[b] *Documented single-dose cases (not overdose on previous medication).*

a few organs are routinely screened for chemicals, such as blood, heart, liver, kidney, brain and lung. Thus, the information on body distribution is often limited to these organs.

The qualitative human toxicity data

The human toxicity data presented in Table IX are the result of a study of references 10–17, in a few instances supplemented by data from other sources. In the same way as the kinetic data in Table V, the toxicity data represent the sum of the information from all the handbooks consulted. The classification of lethal symptoms into main causes and other causes of death, as well as the classification of lethal action into known, unknown and hypothetical mechanisms, represent judgements by the handbook authors. However, the lists of lethal symptoms in various handbooks have been extensively edited to provide uniform terminology. The handbook authors have used a plethora of terms for essentially the same type of event. To mention only one example, circulatory failure in Table IX stands for vascular collapse, vasomotor collapse, shock, circulatory shock, hypovolaemic shock, hypotensive shock, and so on.

Potentially the most controversial data in Table IX are those that are based on mecha-

Appendix IX: Human Acute, Single-Dose Toxicity Data

Table IX: Human acute, single-dose toxicity data

No.	Chemical	Lethal symptoms*	Mean time to death	Danger over	Target organs	Toxic metab-olites†	Lethal mechanisms	Refer-ences‡
1.	Paracetamol	Hypoglycaemic coma M Liver failure M Kidney failure	3–5 days	nr	Liver P Kidney P (CNS)	More toxic intracellular metabolites	Known: Covalent NAPQI binding and lipid peroxidation	
2.	Acetylsalicylic acid	Metabolic acidosis M Cerebral bleedings Pulmonary oedema Cardiovascular failure	48 hours	nr	Kidney P Liver P CNS P Lung P GIT P	Salicylic acid is the reactive metabolite of the parent compound	Known: General cell poison. Uncoupling of oxidative phosphorylation, inhibition of Kreb's cycle dehydrogenases	
3.	Iron (II) sulphate	Haematemesis GIT perforation Pulmonary oedema CNS excitation/depression Circulatory failure Liver and kidney failure	6 or 48 hours	72 hours	GIT P Liver P Kidney CNS CVS Lung P	tp	Known: General cell poison. Inhibition of oxidative phosphorylation and ATP; lipid peroxidation	
4.	Diazepam	CNS depression M	2 hours	3 hours	CNS	(Nordiazepam)	Unknown	
5.	Amitriptyline hydrochloride	CNS excitation/ depression Heart arrythmias/arrest M	< 12 hours	6 days	CNS Heart	(Nortriptyline)	Hypothetical: Blocks noradrenaline, 5-HT and dopamine presynaptic uptake; prevents reuptake of heart noradrenaline	
6.	Digoxin	Heart arrythmias/ arrest M Hyperkalaemia	7 hours	20 hours	Heart GIT CNS	(Metabolites)	Known: Impairing ion transport and increasing sarcoplasmic Ca by binding to Na/K ATPase, increasing automaticity of cells	

Source: Ekwall et al. 1998. MEIC Evaluation of Acute Systemic Toxicity. Part V. ATLA 26:571-616. (reprinted with permission from the editor)

7.	Ethylene glycol	1–12 hours: CNS excitation/depression M 12–24 hours: heart failure 24–72 hours: kidney failure	17 hours	72 hours	CNS P Heart P Kidney P	Glycolate Glycolate Oxalate	Hypothetical: Metabolites inhibit mitochondria, leading to metabolic acidosis. Oxalate decreases S-Ca			
8.	Methanol	CNS depression M Metabolic acidosis Cardiovascular failure	32 hours[d] 173 hours[f]	nr	CNS pr Pancreas P Liver P Kidney P Heart P	Formaldehyde Formic acid	Hypothetical: Accumulation of formic acid leads to metabolic acidosis. Lactate inhibits mitochondrial respiration			
9.	Ethanol	CNS depression M Cardiovascular failure	6 hours[d]	12 hours	CNS CVS	(Acetaldehyde)	Hypothetical: Interference with cell membrane fluidity, perturbing proteins, such as ion channels. Depression of postsynaptic potentials in CNS			
10.	Isopropanol	CNS depression M Cardiovascular failure Pneumonia	3 hours	48 hours	CNS CVS Lung P	tp	Unknown	-	60[e]	
11.	1,1,1-Tri-chloroethane	CNS depression M Heart arrythmias Cardiovascular failure Pneumonia	3 hours	4 hours	CNS P CVS Lung P	tp	Unknown			
12.	Phenol	CNS excitation/depression M Heart arrest/pulmonary oedema Liver and kidney failure	1 hour	24 hours	CNS Heart Liver Kidney GIT P	tp	Known: General protoplasmic poison that denaturates proteins			18, 34
13.	Sodium chloride	CNS excitation/depression M Cerebral bleedings Cardiovascular failure Pulmonary oedema Vasculitis	20 hours	25 hours	CNS P Lungs Kidney VS P	tp	Known: Acute dehydration of brain cells caused by osmotic shift of water to the outside of the blood–brain barrier			

Table IX: continued

No.	Chemical	Lethal symptoms[a]	Mean time to death	Danger over	Target organs	Toxic metabolites[b]	Lethal mechanisms	References[c]
14.	Sodium fluoride	Cardiovascular failure CNS excitation/depression	2-4 hours	20 hours	Heart[h] CNS[i] Liver Kidney	tp	Hypothetical: Protoplasmic poison interfering with many enzymes. May lower S-Ca and induce potassium efflux from cells	
15.	Malathion	Early: Cholinergic crisis/ respiratory failure M Later: Heart failure Heart arrythmias/arrest	0.5-6 hours	24 hours	CNS Muscles Heart P	Malaxon	Known: Inhibition of acetylcholine esterase resulting in acetylcholine accumulation in CNS and effector organs	
16.	2,4-Dichlorophenoxyacetic acid	Hyperthermia/myotonia CNS excitation/depression Metabolic acidosis Heart failure Liver failure	8-96 hours	48 hours	CNS P Liver P Kidney P Heart	tp	Hypothetical: Hypermetabolism due to uncoupling of oxidative phosphorylation. Direct toxin to striated muscle	
17.	Xylene	CNS depression M Heart arrythmias/arrest Heart failure Pulmonary oedema	1-2 hours?	72 hours	CNS P Heart Lung P Liver P	tp	Unknown: Heart failure caused by sensitisation of myocardium to endogenous catecholamines?	
18.	Nicotine	CNS excitation/depression M Cardiovascular failure	minutes -1 hour	4 hours	CNS PNS	tp	Known: Cholinergic block causing polarisation of CNS and PNS synapses	
19.	Potassium cyanide	CNS excitation/depression M Metabolic acidosis Circulatory failure	0.5-1 hour	4 hours	CNS P Heart VS	tp	Known: General enzyme inhibition. High affinity for ferric ion. Inhibits cytochrome oxidase and thereby cell respiration	

20. Lithium sulphate	CNS depression Circulatory failure Kidney failure	1–7 days	7 days	CNS Heart Kidney	tp	Unknown: Partial substitution for normal cations of cells may disturb energy processes?
21. Theophylline	CNS excitation M Metabolic acidosis Heart arrythmias Electrolyte disturbances GIT bleedings	1–5 days	nr	CNS Heart (GIT)	tp	Unknown: Inhibits prostaglandins and cGMP metabolism. Adenosine receptor antagonist.
22. Dextropropoxy-phene hydrochloride	CNS excitation/depression Heart arrythmias/arrest Cardiovascular failure	0.5–2 hours	24 hours	CNS Heart	(Norpropoxyphene)	Hypothetical: Binds to morphine receptors. Stabilises cell membranes. Norpropoxyphene is a primary cardiotoxin
23. Propranolol hydrochloride	CNS excitation/depression Cardiovascular failure Bronchospasm	0.5–2 hours	4–20 hours	CNS Heart VS	tp?	Unknown: Beta-adrenergic blockade?
24. Phenobarbital	CNS depression M Circulatory failure	5 hours–7 days	10 days	CNS Heart	tp	Hypothetical: CNS depression through inhibition of GABA synapses? Inhibits hepatic NADH cytochrome oxidoreductase
25. Paraquat	Early (24 hours): CNS excitation Pulmonary oedema Heart failure Kidney failure M Liver failure Later (48 hours–6 days): Pulmonary fibrosis M	3 hours–4 weeks	nr	Lung P Kidney P Heart P Liver P CNS P	tp	Hypothetical: Multisystem failure due to depletion of superoxide dispulase, formation of free-radicals, and lipid peroxidation. Lung fibrosis due to accumulation of paraquat in this oxygen-rich organ

Table IX: continued

No.	Chemical	Lethal symptoms[a]	Mean time to death	Danger over	Target organs	Toxic metabolites[b]	Lethal mechanisms	References[c]
26.	Arsenic trioxide	Gastroenteritis Circulatory failure Heart failure Pulmonary oedema Intravascular haemolysis Kidney failure Liver failure CNS excitation/depression	1 hour–4 days	4 days	Kidney P Heart Liver P VS P CNS P GIT P	tp	Known: Cellular poison. Multisystem failure due to uncoupling of oxidative phosphorylation and inhibition of pyruvate and succinate oxidative pathways	
27.	Copper (II) sulphate	Liver failure Kidney failure Intravascular haemolysis Circulatory failure CNS excitation/depression	1 hours–7 days	4 days	Liver P Kidney VS	tp	Hypothetical: Cupric cuprus is reduced to cuprous form by thiol groups in cell membranes. Superoxide is formed by reoxidation of cuprous copper, which induces lipid peroxidation	18
28.	Mercury (II) chloride	Gastroenteritis Circulatory failure Kidney failure	3 hours–14 days	14 days	Kidney P VS GIT p	tp	Hypothetical: Changes membrane potentials and blocks enzyme reactions in cells by targeting the sulphydryl part of active sites of some enzymes	
29.	Thioridazine hydrochloride	CNS depression Heart arrythmia/arrest M	2–10 hours	nr	CNS Heart	(Mesoridazine?)	Unknown	
30.	Thallium sulphate	Gastroenteritis Cardiovascular failure M Respiratory failure Kidney failure Liver failure CNS excitation/depression	24 hours–3 weeks	4–5 weeks	Heart P VS Kidney P Liver P CNS P PNS	tp	Hypothetical: Enzyme inhibition by binding to sulphydryl groups of mitochondrial membranes. Interference with oxidative phosphorylation by inhibition Na/K ATPase	18

31.	Warfarin	Bleeding M[f]	36-48 hours	nr	Liver VS	(Metabolites?)	Known: Inhibition of liver synthesis of vitamin K-requiring clotting factors, notably prothrombin. Direct action on capillaries?
32.	Lindane	CNS excitation/depression M Pulmonary oedema Metabolic acidosis	1 hour–8 days	8 days	CNS Heart VS P Kidney P Muscle P	tp?	Unknown: CNS depression through inhibition of TBPS binding to the GABA receptor linked chloride channel, leading to blockade of chloride influx into neurons?
33.	Chloroform	CNS depression M Heart arrythmias/arrest Liver failure Kidney failure	10 minutes–6 days	5 days	CNS Heart P Liver P Kidney P	More toxic intracellular metabolites?	Hypothetical: Liver and/or kidney injury through covalent binding of toxic metabolites, for example, phosgene, to cell proteins and lipids
34.	Carbon tetrachloride	CNS depression[h] Kidney failure[i] Liver failure[i] Heart arrythmias/arrest	24 hours–7 days[i]	7 days	CNS P Heart Kidney P Liver P Pancreas	More toxic intracellular metabolites?	Hypothetical: Covalent binding of toxic intracellular metabolites (see above). Free-radicals inducing lipid peroxidation?
35	Isoniazid	CNS excitation M Metabolic acidosis Circulatory failure CNS depression Liver failure	14 hours–3 days	nr	CNS Liver P	(Intracellular metabolites)	Hypothetical: Interference with metabolism of vitamin B6 reduces GABA and seizure threshold. Conversion of acetylhydrazine (ICM) to alkylating agent
36.	Dichloromethane	CNS depression M Heart arrythmias Pulmonary oedema Metabolic acidosis	2 hours	3 hours	CNS Heart	(Carbon monoxide?)	Unknown: Carbon monoxide–haemoglobin complex formation?

Table IX: continued

No.	Chemical	Lethal symptoms[a]	Mean time to death	Danger over	Target organs	Toxic metabolites[b]	Lethal mechanisms	Reference[c]
37	Barium nitrate	Muscle paralysis/ respiratory failure Heart arrythmias/arrest High blood pressure Convulsions	2–3 hours or 2–3 days	24 hours	Muscle[m] Heart (Kidney)	tp	Hypothetical: Neuromuscular depolarization. Potassium is forced into cells by an action on Na/K ATPase?	19
38.	Hexachlorophene	Early: Gastroenteritis Hyperthermia Circulatory failure 12–18 hours: CNS excitation/depression 48–60 hours: Heart arrythmias/arrest	4–60 hours	3 days	GIT VS Heart CNS[n]	tp	Hypothetical: Uncoupling of oxidative phosphorylation in cells Binding to proteins in cytoplasma membrane and cell organelles	47
39.	Pentachlorophenol	Hyperthermia CNS excitation/depression Circulatory failure Myotonia Metabolic acidosis	4–24 hours	24 hours	Heart P VS CNS Liver P Kidney P	tp	Hypothetical: Uncoupling of oxidative phosphorylation. Protein binding, including selective enzyme inhibition (liver/kidney P450)	
40.	Verapamil hydrochloride	Circulatory failure Heart arrythmias/arrest Metabolic acidosis CNS depression Hyperglycaemia	24 hours	36 hours	VS Heart	(Metabolites)	Known: Inhibition of transmembrane Ca flux in excitatory tissues. Also alpha-adrenergic blocking	
41.	Chloroquine phosphate	Cardiovascular failure Cardiac arrythmias/arrest M CNS excitation/depression Hypokalaemia	1–24 hours	24 hours	Heart VS CNS	tp	Hypothetical: Stabilisation of cell membranes leading to reduction of excitation and conduction in heart. Interference with mitochondria	

42.	Orphenadrine hydrochloride	CNS excitation/depression (max. 2-5 hours) M Heart arrythmias (max. 12-18 hours) Heart failure Liver failure	1-48 hours	24 hours	CNS Heart Liver P	tp?	Unknown
43.	Quinidine sulphate	Early: Heart failure Heart arrythmias/arrest M Later: CNS excitation/depression Kidney failure	6 hours?	nr	Heart VS CNS Kidney	tp?	Unknown: Decreased electrolyte permeability of cell membranes leading to depression of heart excitability, conduction velocity and contractility.
44.	Diphenyl-hydantoin	(Nystagmus/ataxia) CNS excitation/depression M Heart arrythmias/arrest"	30 hours-14 days	14 days	CNS (Cerebellum) Heart	tp	Unknown: Binds to specific receptors in neuronal cell membranes. Inhibits voltage-dependent sodium channels
45.	Chloramphenicol	Cardiovascular failure CNS excitation/depression Metabolic acidosis (Liver and kidney failure)	5 hours-2 days	nr	Heart VS CNS Liver Kidney	tp	Hypothetical: Binds to mitochondrial ribosomes and inhibits enzyme synthesis, for example, enzymes necessary for oxidative phosphorylation
46.	Sodium oxalate	Initially (minutes): Gastroenteritis Circulatory failure Later (hours): CNS excitation/depression Heart arrythmias/arrest Later (2 days): Kidney failure	3 hours	nr	GIT CNS Heart Kidney	tp	Hypothetical: Calcium-complexing action, depressing the level of ionized calcium in body fluids. The direct action on GIT, VS and kidney cannot explained that way. Corrosivity is not caused by acidity.
47.	Amphetamine sulphate	(Hyperthermia) Cardiac arrythmias/arrest CNS excitation/depression M Metabolic acidosis	2-4 hours	nr	CNS PP Heart P Liver P Kidney	tp	Hypothetical: Release of biogenic amines (dopamine, norepinephrine) from nerve terminal stores. Direct action as false transmitter

Table IX: continued

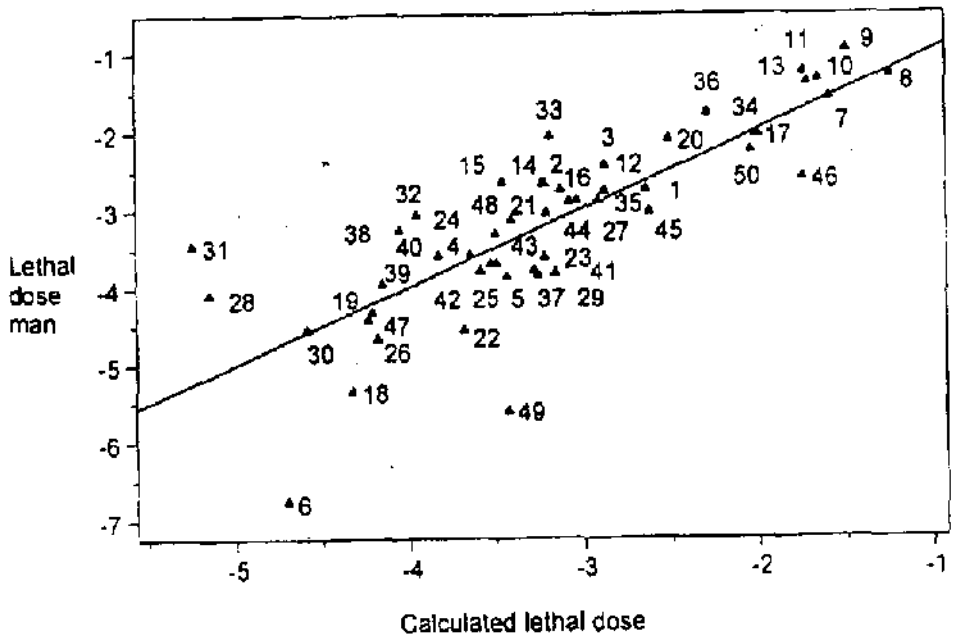
No.	Chemical	Lethal symptoms[a]	Mean time to death	Danger over	Target organs	Toxic metabolites[b]	Lethal mechanisms	References[c]
48.	Caffeine	Initially (3 hours): Heart arrythmias/arrest Pulmonary oedema Later (3 hours–3 days): CNS excitation/depression	3 hours–3 days	nr	Heart CNS	tp	Hypothetical: Inhibition of phosphodiesterase leading to AMP accumulation. Translocation of intracellular calcium? Adenosine receptor antagonism?	
49.	Atropine sulphate	(Psychosis/hyperthermia) CNS excitation/depression Heart arrythmias/arrest M	16 hours	24–48 hours	CNS Heart PNS	tp	Known: Antimuscarinic, anticholinergic action. Competitive antagonism of acetylcholine at cardiac and CNS receptor sites	19
50.	Potassium chloride	CNS excitation/depression Paralysis Heart arrythmias/arrest M	2 hours	nr	Heart CNS (Muscle)	tp	Known: Essential cellular electrolyte maintains normal trans-membrane potential, necessary for heart conduction	-

"Arranged in order of appearance, when possible. Characteristic but non-lethal symptoms have generally been omitted. CNS excitation stands for seizures, and CNS depression stands for all phases of coma including final respiratory arrest. For chemicals with multisystem failure or a very rapid action, it is difficult to indicate the main cause of death. [b]Metabolites with higher toxicity than the parent compound. Metabolites with the same toxicity as the parent compound are bracketed. TP indicates toxicity from the parent compound, only. [c]Other than references 10–17. [d]Post-mortem cases. [e]Clinical cases. [f]POISINDEX®, Information Systems (ed. B.H. Rumack & D.G. Spoerke), Micromedex (Denver, CO, USA). [g]Targets of a decreased blood calcium level? [h]TOMES®, Information Systems (ed. B.H. Rumack & D.G. Spoerke), Micromedex (Denver, CO, USA). [i]Cerebral bleeding is most life-threatening. [j]Inhalation. [k]Ingestion. [l]Motor end-plates of muscles. [m]Repeated dermal exposure. [n]Intravenous administration. [o]Vasculitis, haemorrhages.

***M** = main causes of death; **P** = histopathological organ lesions; **CNS** = central nervous system (brain); **CVS** = cardiovascular system; **VS** = vascular system (blood vessels/capillaries); **GIT** = gastrointestinal tract (gut); **PNS** = peripheral nervous system; **tp** = toxicity of parent compound only; **nr** = not reported.*

Appendix X: Plot of Acute Lethal Dosage in Humans Against Values Calculated by a PLS Model Based on Rat Oral LD50 and Mouse Oral LD50

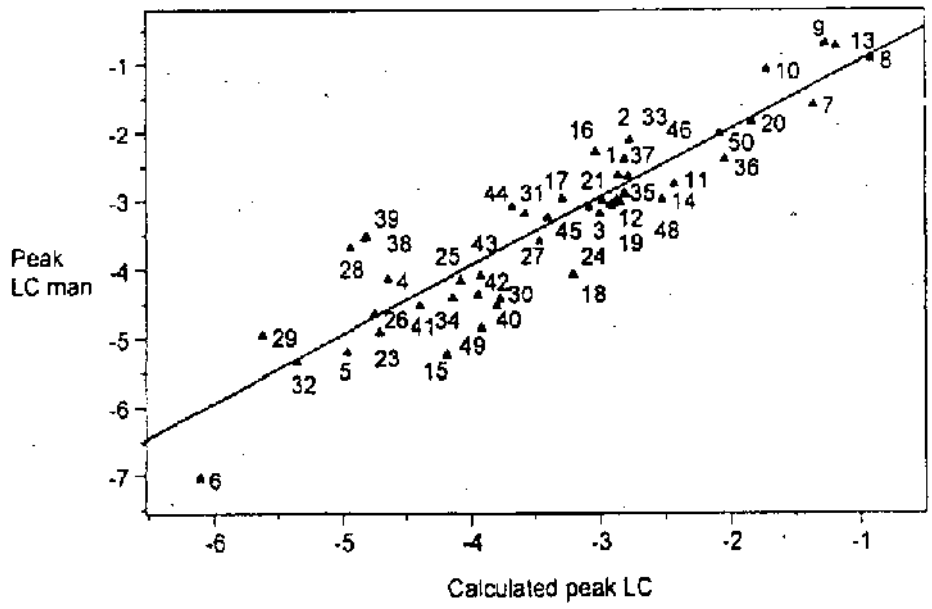
Figure 1: Plot of acute lethal dosage in humans against values calculated by a PLS model based on rat oral LD50 and mouse oral LD50.



Source: Ekwall et al. 1999. MEIC Evaluation of Acute Systemic Toxicity. Part VIII. (reprinted with permission from the editor)

Appendix XI: Plot of Peak Lethal Blood Concentrations in Man Against IC50 Values

Figure 10: Plot of peak lethal blood concentrations in man against IC-50 values calculated by a PLS model based on peak lethal blood concentrations in man, all 50 chemicals, and "blood-brain barrier compensated results" from assays 1, 5, 9 and 16.



Source: Ekwall et al. 1999. MEIC Evaluation of Acute Systemic Toxicity. Part VIII.
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Appendix XII: Priority Areas for Development and Evaluation of New *In Vitro* Tests

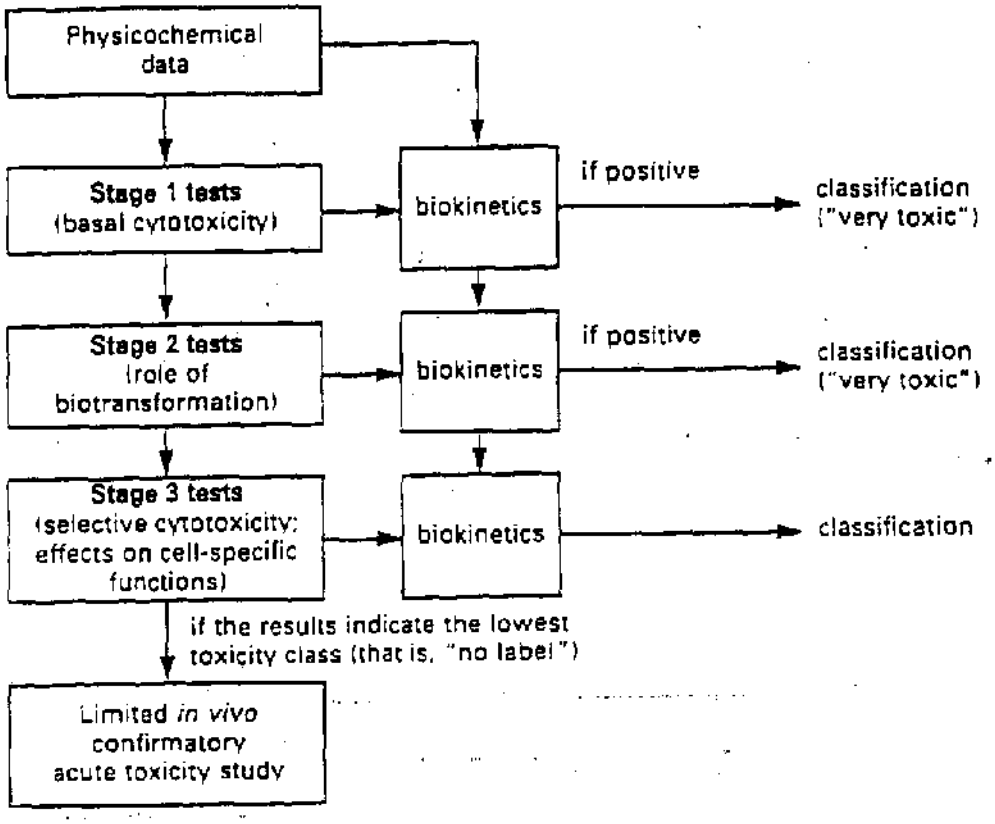
Table I: Priority areas for development and evaluation of new *in vitro* tests on systemic toxicity

No.	Subproject
1.	Repeat dose toxicity
2.	Mechanism studies:
	a) protein denaturation
	b) morphology of injury to cell lines
	c) differential cytotoxicity 30 minutes/24 hours
	d) toxicity to aerobic cells
	e) time-frames for cytotoxic effects
3.	Extracellular receptor toxicity
4.	Excitatory toxicity
5.	Reversibility of cytotoxicity
6.	Passage across blood-brain barrier
7.	Absorption in the gut
8.	Blood protein binding
9.	Distribution volumes (Vd)
10.	More-toxic metabolites

Source: Ekwall et al. 1999. EDIT: A new international multicentre programme to develop and evaluate batteries of *in vitro* tests for acute chronic systemic toxicity. ATLA 27:339-349. (reprinted with permission from the editor)

Appendix XIII: Proposed Testing Scheme for the Classification and Labelling of Chemicals

Figure 1: Proposed testing scheme for the classification and labelling of chemicals according to their potential acute toxicities



Source: Ekwall et al. 1999. EDIT: A new international multicentre programme to develop and evaluate batteries of *in vitro* tests for acute chronic systemic toxicity. ATLA 27:339-349. (reprinted with permission from the editor).

APPENDIX F

Federal Regulations on Acute Toxicity

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APPENDIX F.1

Federal Regulations on Acute Toxicity
U.S. Environmental Protection Agency (EPA)

U.S. EPA Classification and Labeling Regulations

40 CFR Ch.I 156.10: General Provisions – Labeling Requirements
40 CFR Ch.I 159.165: Toxicological and Ecological Studies

Subpart A—General Provisions

§ 156.10 Labeling requirements.

(a) *General*—(1) *Contents of the label.* Every pesticide products shall bear a label containing the information specified by the Act and the regulations in this part. The contents of a label must show clearly and prominently the following:

(i) The name, brand, or trademark under which the product is sold as prescribed in paragraph (b) of this section;

(ii) The name and address of the producer, registrant, or person for whom produced as prescribed in paragraph (c) of this section;

(iii) The net contents as prescribed in paragraph (d) of this section;

(iv) The product registration number as prescribed in paragraph (e) of this section;

(v) The producing establishment number as prescribed in paragraph (f) of this section;

(vi) An ingredient statement as prescribed in paragraph (g) of this section;

(vii) Warning or precautionary statements as prescribed in paragraph (h) of this section;

(viii) The directions for use as prescribed in paragraph (i) of this section; and

(ix) The use classification(s) as prescribed in paragraph (j) of this section.

(2) *Prominence and legibility.* (i) All words, statements, graphic representations, designs or other information required on the labeling by the Act or the regulations in this part must be clearly legible to a person with normal vision, and must be placed with such conspicuousness (as compared with other words, statements, designs, or graphic matter on the labeling) and expressed in such terms as to render it likely to be read and understood by the ordinary individual under customary conditions of purchase and use.

(ii) All required label text must:

(A) Be set in 6-point or larger type;

(B) Appear on a clear contrasting background; and

(C) Not be obscured or crowded.

(3) *Language to be used.* All required label or labeling text shall appear in the English language. However, the Agency may require or the applicant may propose additional text in other languages as is considered necessary to protect the public. When additional text in another language is necessary, all labeling requirements will be applied equally to both the English and other-language versions of the labeling.

(4) *Placement of Label*—(i) *General.* The label shall appear on or be securely attached to the immediate container of the pesticide product. For purposes of this section, and the misbranding provisions of the Act, "securely attached" shall mean that a label can reasonably be expected to remain affixed during the foreseeable conditions and period of use. If the immediate container is enclosed within a wrapper or outside container through which the label cannot be clearly read, the label must also be securely attached to such outside wrapper or container, if it is a part of the package as customarily distributed or sold.

(ii) *Tank cars and other bulk containers*—(A) *Transportation.* While a pesticide product is in transit, the appropriate provisions of 49 CFR parts 170–189, concerning the transportation of hazardous materials, and specifically those provisions concerning the labeling, marking and placarding of hazardous materials and the vehicles carrying them, define the basic Federal requirements. In addition, when any registered pesticide product is transported in a tank car, tank truck or other mobile or portable bulk container, a copy of the accepted label must be attached to the shipping papers, and left with the consignee at the time of delivery.

(B) *Storage.* When pesticide products are stored in bulk containers, whether mobile or stationary, which remain in the custody of the user, a copy of the label of labeling, including all appropriate directions for use, shall be securely attached to the container in the immediate vicinity of the discharge control valve.

(5) *False or misleading statements.* Pursuant to section 2(q)(1)(A) of the Act, a pesticide or a device declared subject to the Act pursuant to § 152.500, is misbranded if its labeling is false or misleading in any particular including both pesticidal and non-pesticidal

claims. Examples of statements or representations in the labeling which constitute misbranding include:

(i) A false or misleading statement concerning the composition of the product;

(ii) A false or misleading statement concerning the effectiveness of the product as a pesticide or device;

(iii) A false or misleading statement about the value of the product for purposes other than as a pesticide or device;

(iv) A false or misleading comparison with other pesticides or devices;

(v) Any statement directly or indirectly implying that the pesticide or device is recommended or endorsed by any agency of the Federal Government;

(vi) The name of a pesticide which contains two or more principal active ingredients if the name suggests one or more but not all such principal active ingredients even though the names of the other ingredients are stated elsewhere in the labeling;

(vii) A true statement used in such a way as to give a false or misleading impression to the purchaser;

(viii) Label disclaimers which negate or detract from labeling statements required under the Act and these regulations;

(ix) Claims as to the safety of the pesticide or its ingredients, including statements such as "safe," "nonpoisonous," "noninjurious," "harmless" or "nontoxic to humans and pets" with or without such a qualifying phrase as "when used as directed"; and

(x) Non-numerical and/or comparative statements on the safety of the product, including but not limited to:

(A) "Contains all natural ingredients";

(B) "Among the least toxic chemicals known"

(C) "Pollution approved"

(6) *Final printed labeling.* (i) Except as provided in paragraph (a)(6)(ii) of this section, final printed labeling must be submitted and accepted prior to registration. However, final printed labeling need not be submitted until draft label texts have been provisionally accepted by the Agency.

(ii) Clearly legible reproductions or photo reductions will be accepted for unusual labels such as those silk-screened directly onto glass or metal containers or large bag or drum labels. Such reproductions must be of microfilm reproduction quality.

(b) *Name, brand, or trademark.* (1) The name, brand, or trademark under which the pesticide product is sold shall appear on the front panel of the label.

(2) No name, brand, or trademark may appear on the label which:

(i) Is false or misleading, or

(ii) Has not been approved by the Administrator through registration or supplemental registration as an additional name pursuant to §152.132.

(c) Name and address of producer, registrant, or person for whom produced. An unqualified name and address given on the label shall be considered as the name and address of the producer. If the registrant's name appears on the label and the registrant is not the producer, or if the name of the person for whom the pesticide was produced appears on the label, it must be qualified by appropriate wording such as "Packed for * * *," "Distributed by * * *," or "Sold by * * *" to show that the name is not that of the producer.

(d) *Net weight or measure of contents.* (1) The net weight or measure of content shall be exclusive of wrappers or other materials and shall be the average content unless explicitly stated as a minimum quantity.

(2) If the pesticide is a liquid, the net content statement shall be in terms of liquid measure at 68 °F (20 °C) and shall be expressed in conventional American units of fluid ounces, pints, quarts, and gallons.

(3) If the pesticide is solid or semi-solid, viscous or pressurized, or is a mixture of liquid and solid, the net content statement shall be in terms of weight expressed as avoirdupois pounds and ounces.

(4) In all cases, net content shall be stated in terms of the largest suitable units, i.e., "1 pound 10 ounces" rather than "26 ounces."

(5) In addition to the required units specified, net content may be expressed in metric units.

(6) Variation above minimum content or around an average is permissible only to the extent that it represents

deviation unavoidable in good manufacturing practice. Variation below a stated minimum is not permitted. In no case shall the average content of the packages in a shipment fall below the stated average content.

(e) *Product registration number.* The registration number assigned to the pesticide product at the time of registration shall appear on the label, preceded by the phrase "EPA Registration No.," or the phrase "EPA Reg. No." The registration number shall be set in type of a size and style similar to other print on that part of the label on which it appears and shall run parallel to it. The registration number and the required identifying phrase shall not appear in such a manner as to suggest or imply recommendation or endorsement of the product by the Agency.

(f) *Producing establishments registration number.* The producing establishment registration number preceded by the phrase "EPA Est.", of the final establishment at which the product was produced may appear in any suitable location on the label or immediate container. It must appear on the wrapper or outside container of the package if the EPA establishment registration number on the immediate container cannot be clearly read through such wrapper or container.

(g) *Ingredient statement*—(1) *General.* The label of each pesticide product must bear a statement which contains the name and percentage by weight of each active ingredient, the total percentage by weight of all inert ingredients; and if the pesticide contains arsenic in any form, a statement of the percentages of total and water-soluble arsenic calculated as elemental arsenic. The active ingredients must be designated by the term "active ingredients" and the inert ingredients by the term "inert ingredients," or the singular forms of these terms when appropriate. Both terms shall be in the same type size, be aligned to the same margin and be equally prominent. The statement "Inert Ingredients, none" is not required for pesticides which contain 100 percent active ingredients. Unless the ingredient statement is a complete analysis of the pesticide, the term "analysis" shall not be used as a heading for the ingredient statement.

(2) *Position of ingredient statement.* (i) The ingredient statement is normally required on the front panel of the label. If there is an outside container or wrapper through which the ingredient statement cannot be clearly read, the ingredient statement must also appear on such outside container or wrapper. If the size or form of the package makes it impracticable to place the ingredient statement on the front panel of the label, permission may be granted for the ingredient statement to appear elsewhere.

(ii) The text of the ingredient statement must run parallel with other text on the panel on which it appears, and must be clearly distinguishable from and must not be placed in the body of other text.

(3) *Names to be used in ingredient statement.* The name used for each ingredient shall be the accepted common name, if there is one, followed by the chemical name. The common name may be used alone only if it is well known. If no common name has been established, the chemical name alone shall be used. In no case will the use of a trademark or proprietary name be permitted unless such name has been accepted as a common name by the Administrator under the authority of section 25(c)(6).

(4) *Statements of percentages.* The percentages of ingredients shall be stated in terms of weight-to-weight. The sum of percentages of the active and the inert ingredients shall be 100. Percentages shall not be expressed by a range of values such as "22–25%." If the uses of the pesticide product are expressed as weight of active ingredient per unit area, a statement of the weight of active ingredient per unit volume of the pesticide formulation shall also appear in the ingredient statement.

(5) *Accuracy of stated percentages.* The percentages given shall be as precise as possible reflecting good manufacturing practice. If there may be unavoidable variation between manufacturing batches, the value stated for each active ingredient shall be the lowest percentage which may be present.

(6) *Deterioration.* Pesticides which change in chemical composition significantly must meet the following labeling requirements:

(i) In cases where it is determined that a pesticide formulation changes chemical composition significantly, the product must bear the following statement in a prominent position on the label: "Not for sale or use after [date]."

(ii) The product must meet all label claims up to the expiration time indicated on the label.

(7) *Inert ingredients.* The Administrator may require the name of any inert ingredient(s) to be listed in the ingredient statement if he determines that such ingredient(s) may pose a hazard to man or the environment.

(h) *Warnings and precautionary statements.* Required warnings and precautionary statements concerning the general areas of toxicological hazard including hazard to children, environmental hazard, and physical or chemical hazard fall into two groups: those required on the front panel of the labeling and those which may appear elsewhere. Specific requirements concerning content, placement, type size, and prominence are given below.

(1) *Required front panel statements.* With the exception of the child hazard warning statement, the text required on the front panel of the label is determined by the Toxicity Category of the pesticide. The category is assigned on the basis of the highest hazard shown by any of the indicators in the table below:

Hazard indicators	Toxicity categories			
	I	II	III	IV
Oral LD_{50}	Up to and including 50 mg/kg.	From 50 thru 500 mg/kg	From 500 thru 5000 mg/kg.	Greater than 5000 mg/kg.
Inhalation LC_{50}	Up to and including .2 mg/liter.	From .2 thru 2 mg/liter	From 2 thru 20 mg/liter	Greater than 20 mg/liter.
Dermal LD_{50}	Up to and including 200 mg/kg.	From 200 thru 2000	From 2,000 thru 20,000	Greater than 20,000.
Eye effects	Corrosive; corneal opacity not reversible within 7 days.	Corneal opacity reversible within 7 days; Irritation persisting for 7 days.	No corneal opacity; irritation reversible within 7 days.	No irritation.
Skin effects	Corrosive	Severe irritation at 72 hours.	Moderate irritation at 72 hours.	Mild or slight irritation at 72 hours.

(i) *Human hazard signal word*—(A) *Toxicity Category I.* All pesticide products meeting the criteria of Toxicity Category I shall bear on the front panel the signal word "Danger." In addition if the product was assigned to Toxicity Category I on the basis of its oral, inhalation or dermal toxicity (as distinct from skin and eye local effects) the word "Poison" shall appear in red on a background of distinctly contrasting color and the skull and crossbones shall appear in immediate proximity to the word "poison."

(B) *Toxicity Category II.* All pesticide products meeting the criteria of Toxicity Category II shall bear on the front panel the signal word "Warning."

(C) *Toxicity Category III.* All pesticide products meeting the criteria of Toxicity Category III shall bear on the front panel the signal word "Caution."

(D) *Toxicity Category IV.* All pesticide products meeting the criteria of Toxicity Category IV shall bear on the front panel the signal word "Caution."

(E) *Use of signal words.* Use of any signal word(s) associated with a higher Toxicity Category is not permitted except when the Agency determines that such labeling is necessary to prevent unreasonable adverse effects on man or the environment. In no case shall more than one human hazard signal word appear on the front panel of a label.

(ii) *Child hazard warning.* Every pesticide product label shall bear on the front panel the statement "keep out of reach of children." Only in cases where the likelihood of contact with children during distribution, marketing, storage or use is demonstrated by the applicant to be extremely remote, or if the nature of the pesticide is such that it is approved for use on infants or small children, may the Administrator waive this requirement.

(iii) *Statement of practical treatment*—(A) *Toxicity Category I.* A statement of

practical treatment (first aid or other) shall appear on the front panel of the label of all pesticides falling into Toxicity Category I on the basis of oral, inhalation or dermal toxicity. The Agency may, however, permit reasonable variations in the placement of the statement of practical treatment is some reference such as "See statement of practical treatment on back panel" appears on the front panel near the word "Poison" and the skull and crossbones.

(B) *Other toxicity categories.* The statement of practical treatment is not required on the front panel except as described in paragraph (h)(1)(iii)(A) of this section. The applicant may, however, include such a front panel statement at his option. Statements of practical treatment are, however, required elsewhere on the label in accord with paragraph (h)(2) of this section if they do not appear on the front panel.

(iv) *Placement and prominence.* All the require front panel warning statements shall be grouped together on the label, and shall appear with sufficient prominence relative to other front panel text and graphic material to make them unlikely to be overlooked under customary conditions of purchase and use. The following table shows the minimum type size requirements for the front panel warning statements on various sizes of labels:

Size of label front panel in square inches	Points	
	Required signal word, all capitals	"Keep out of reach of children"
5 and under	6	6
Above 5 to 10	10	8
Above 10 to 15	12	8
Above 15 to 30	14	10
Over 30	18	12

(2) *Other required warnings and precautionary statements.* The warnings and precautionary statements as required below shall appear together on the label under the general heading "Precautionary Statements" and under appropriate subheadings of "Hazard to Humans and Domestic Animals," "Environmental Hazard" and "Physical or Chemical Hazard."

(i) *Hazard to humans and domestic animals.* (A) Where a hazard exists to humans or domestic animals, precautionary statements are required indicating the particular hazard, the route(s) of exposure and the precautions to be taken to avoid accident, injury or damage. The precautionary paragraph shall be immediately preceded by the appropriate hazard signal word.

(B) The following table depicts typical precautionary statements. These statements must be modified or expanded to reflect specific hazards.

Toxicity category	Precautionary statements by toxicity category	
	Oral, inhalation, or dermal toxicity	Skin and eye local effects
I	Fatal (poisonous) if swallowed [inhaled or absorbed through skin]. Do not breathe vapor [dust or spray mist]. Do not get in eyes, on skin, or on clothing [Front panel statement of practical treatment required.].	Corrosive, causes eye and skin damage [or skin irritation]. Do not get in eyes, on skin, or on clothing. Wear goggles or face shield and rubber gloves when handling. Harmful or fatal if swallowed. [Appropriate first aid statement required.]
II	May be fatal if swallowed [inhaled or absorbed through the skin]. Do not breathe vapors [dust or spray mist]. Do not get in eyes, on skin, or on clothing. [Appropriate first aid statements required.].	Causes eye [and skin] irritation. Do not get in eyes, on skin, or on clothing. Harmful if swallowed. [Appropriate first aid statement required.]
III	Harmful if swallowed [inhaled or absorbed through the skin]. Avoid breathing vapors [dust or spray mist]. Avoid contact with skin [eyes or clothing]. [Appropriate first aid statement required.].	Avoid contact with skin, eyes or clothing. In case of contact immediately flush eyes or skin with plenty of water. Get medical attention if irritation persists.
IV	[No precautionary statements required.]	[No precautionary statements required.]

(ii) *Environmental hazards.* Where a hazard exists to non target organisms excluding humans and domestic animals, precautionary statements are required stating the nature of the hazard and the appropriate precautions to avoid potential accident, injury or damage. Examples of the hazard statements and the circumstances under which they are required follow:

(A) If a pesticide intended for outdoor use contains an active ingredient with

a mammalian acute oral LD_{50} of 100 or less, the statement "This Pesticide is Toxic to Wildlife" is required.

(B) If a pesticide intended for outdoor use contains an active ingredient with a fish acute LC_{50} of 1 ppm or less, the statement "This Pesticide is Toxic to Fish" is required.

(C) If a pesticide intended for outdoor use contains an active ingredient with an avian acute oral LD_{50} of 100 mg/kg or less, or a subacute dietary LC_{50} of 500 ppm or less, the statement "This Pesticide is Toxic to Wildlife" is required.

(D) If either accident history or field studies demonstrate that use of the pesticide may result in fatality to birds, fish or mammals, the statement "This pesticide is extremely toxic to wildlife (fish)" is required.

(E) For uses involving foliar application to agricultural crops, forests, or shade trees, or for mosquito abatement treatments, pesticides toxic to pollinating insects must bear appropriate label cautions.

(F) For all outdoor uses other than aquatic applications the label must bear the caution "Keep out of lakes, ponds or streams. Do not contaminate water by cleaning of equipment or disposal of wastes."

(iii) *Physical or chemical hazards.* (A) Warning statements on the flammability or explosive characteristics of all pesticides are required as set out in Table 1 and Table 2 of this paragraph as follows:

TABLE 1—PRESSURIZED CONTAINERS

Flash Point	Required Text
Flash point at or below 20 °F; if there is a flashback at any valve opening	Extremely flammable. Contents under pressure. Keep away from fire, sparks, and heated surfaces. Do not puncture or incinerate container. Exposure to temperatures above 130 °F may cause bursting
Flash point above 20 °F and not over 80 °F or if the flame extension is more than 18 inches long at a distance of 6 inches from the flame	Flammable. Contents under pressure. Keep away from heat, sparks, and open flame. Do not puncture or incinerate container. Exposure to temperatures above 130 °F may cause bursting
All other pressurized containers	Contents under pressure. Do not use or store near heat or open flame. Do not puncture or incinerate container. Exposure to temperatures above 130 °F may cause bursting.

TABLE 2—NONPRESSURIZED CONTAINERS

Flash Point	Required Text
At or below 20 °F	Extremely flammable. Keep away from fire, sparks, and heated surfaces.
Above 20 °F and not over 80 °F	Flammable. Keep away from heat and open flame.
Above 80 °F and not over 150 °F	Do not use or store near heat or open flame.

(B) A "total release fogger" is defined as a pesticide product in a pressurized container designed to automatically release the total contents in one operation, for the purpose of creating a permeating fog within a confined space to deliver the pesticide throughout the space.

(C) *(1)* If the pesticide product is a total release fogger containing a propellant with a flash point at or below 20 °F, then the following special instructions must be added to the "Physical and Chemical Hazards" warning statement:

This product contains a highly flammable ingredient. It may cause a fire or explosion if not used properly. Follow the "Directions for Use" on this label very carefully.

(2) A graphic symbol depicting fire such as illustrated in this paragraph or an equivalent symbol, must be displayed along with the required language adjoining the "Physical and Chemical Hazards" warning statement. The graphic symbol must be no smaller than twice the size of the first character of the human hazard signal word.

Highly Flammable Ingredient
Ingrediente Altamente Inflamable

(i) *Directions for Use*—(1) *General requirements*—(i) *Adequacy and clarity of directions.* Directions for use must be stated in terms which can be easily read and understood by the average person likely to use or to supervise the use of the pesticide. When followed, directions must be adequate to protect the public from fraud and from personal injury and to prevent unreasonable adverse effects on the environment.

(ii) *Placement of directions for use.* Directions may appear on any portion of the label provided that they are conspicuous enough to be easily read by the user of the pesticide product. Directions for use may appear on printed or graphic matter which accompanies the pesticide provided that:

(A) If required by the Agency, such printed or graphic matter is securely attached to each package of the pesticide, or placed within the outside wrapper or bag;

(B) The label bears a reference to the directions for use in accompanying leaflets or circulars, such as "See directions in the enclosed circular;" and

(C) The Administrator determines that it is not necessary for such directions to appear on the label.

(iii) *Exceptions to requirement for direction for use.* (A) Detailed directions for use may be omitted from labeling of pesticides which are intended for use only by manufacturers of products other than pesticide products in their regular manufacturing processes, provided that:

(*1*) The label clearly shows that the product is intended for use only in manufacturing processes and specifies the type(s) of products involved.

(*2*) Adequate information such as technical data sheets or bulletins, is available to the trade specifying the type of product involved and its proper use in manufacturing processes;

(*3*) The product will not come into the hands of the general public except after incorporation into finished products; and

(*4*) The Administrator determines that such directions are not necessary to prevent unreasonable adverse effects on man or the environment.

(B) Detailed directions for use may be omitted from the labeling of pesticide products for which sale is limited to physicians, veterinarians, or druggists, provided that:

(*1*) The label clearly states that the product is for use only by physicians or veterinarians;

(*2*) The Administrator determines that such directions are not necessary to prevent unreasonable adverse effects on man or the environment; and

(*3*) The product is also a drug and regulated under the provisions of the Federal Food, Drug and Cosmetic Act.

(C) Detailed directions for use may be omitted from the labeling of pesticide products which are intended for use only by formulators in preparing pesticides for sale to the public, provided that:

(*1*) There is information readily available to the formulators on the composition, toxicity, methods of use, applicable restrictions or limitations, and effectiveness of the product for pesticide purposes;

(*2*) The label clearly states that the product is intended for use only in manufacturing, formulating, mixing, or repacking for use as a pesticide and specifies the type(s) of pesticide products involved;

(*3*) The product as finally manufactured, formulated, mixed, or repackaged is registered; and

(*4*) The Administrator determines that such directions are not necessary to prevent unreasonable adverse effects on man or the environment.

(2) *Contents of Directions for Use.* The directions for use shall include the following, under the headings "Directions for Use":

(i) The statement of use classification as prescribed in paragraph (j) of this section immediately under the heading "Directions for Use."

(ii) Immediately below the statement of use classification, the statement "It is a violation of Federal law to use this

product in a manner inconsistent with its labeling."

(iii) The site(s) of application, as for example the crops, animals, areas, or objects to be treated.

(iv) The target pest(s) associated with each site.

(v) The dosage rate associated with each site and pest.

(vi) The method of application, including instructions for dilution, if required, and type(s) of application apparatus or equipment required.

(vii) The frequency and timing of applications necessary to obtain effective results without causing unreasonable adverse effects on the environment.

(viii) Worker protection statements meeting the requirements of subpart K of this part.

(ix) Specific directions concerning the storage and disposal of the pesticide and its container, meeting the requirements of 40 CFR part 165. These instructions shall be grouped and appear under the heading "Storage and Disposal." This heading must be set in type of the same minimum sizes as required for the child hazard warning. (See table in § 162.10(h)(1)(iv))

(x) Any limitations or restrictions on use required to prevent unreasonable adverse effects, such as:

(A) Required intervals between application and harvest of food or feed crops.

(B) Rotational crop restrictions.

(C) Warnings as required against use on certain crops, animals, objects, or in or adjacent to certain areas.

(D) For total release foggers as defined in paragraph (h)(2)(iii)(B) of this section, the following statements must be included in the "Directions for Use":

DO NOT use more than one fogger per room. DO NOT use in small, enclosed spaces such as closets, cabinets, or under counters or tables. Do not use in a room 5 ft. x 5 ft. or smaller; instead, allow fog to enter from other rooms. Turn off ALL ignition sources such as pilot lights (shut off gas valves), other open flames, or running electrical appliances that cycle off and on (i.e., refrigerators, thermostats, etc.). Call your gas utility or management company if you need assistance with your pilot lights."

(E) For restricted use pesticides, a statement that the pesticide may be applied under the direct supervision of a certified applicator who is not physically present at the site of application but nonetheless available to the person applying the pesticide, unless the Agency has determined that the pesticide may only be applied under the direct supervision of a certified applicator who is physically present.

(F) Other pertinent information which the Administrator determines to be necessary for the protection of man and the environment.

(j) *Statement of Use Classification.* By October 22, 1976, all pesticide products must bear on their labels a statement of use classification as described in paragraphs (j) (1) and (2) of this section. Any pesticide product for which some uses are classified for general use and others for restricted use shall be separately labeled according to the labeling standards set forth in this subsection, and shall be marketed as separate products with different registration numbers, one bearing directions only for general use(s) and the other bearing directions for restricted use(s) except that, if a product has both restricted use(s) and general use(s), both of these uses may appear on a product labeled for restricted use. Such products shall be subject to the provisions of paragraph (j)(2) of this section.

(1) *General Use Classification.* Pesticide products bearing directions for use(s) classified general shall be labeled with the exact words "General Classification" immediately below the heading "Directions for Use." And reference to the general classification that suggests or implies that the general utility of the pesticide extends beyond those purposes and uses contained in the Directions for Use will be considered a false or misleading statement under the statutory definitions of misbranding.

(2) *Restricted Use Classification.* Pesticide products bearing direction for use(s) classified restricted shall bear statements of restricted use classification on the front panel as described below:

(i) *Front panel statement of restricted use classification.* (A) At the top of the front panel of the label, set in type of the same minimum sizes as required for human hazard signal words (see

table in paragraph (h)(1)(iv) of this section), and appearing with sufficient prominence relative to other text and graphic material on the front panel to make it unlikely to be overlooked under customary conditions of purchase and use, the statement "Restricted Use Pesticide" shall appear.

(B) Directly below this statement on the front panel, a summary statement of the terms of restriction imposed as a precondition to registration shall appear. If use is restricted to certified applicators, the following statement is required: "For retail sale to and use only by Certified Applicators or persons under their direct supervision and only for those uses covered by the Certified Applicator's certification." If, however, other regulatory restrictions are imposed, the Administrator will define the appropriate wording for the terms of restriction by regulation.

[40 FR 28268, July 3, 1975; 40 FR 32329, Aug. 1, 1975; 40 FR 36571, Aug. 21, 1975, as amended at 43 FR 5786, Feb. 9, 1978. Redesignated and amended at 53 FR 15991, 15999, May 4, 1988; 57 FR 38146, Aug. 21, 1992; 60 FR 32096, June 19, 1995; 63 FR 9082, Feb. 23, 1998]

Subparts B–J [Reserved]

Subpart K—Worker Protection Statements

SOURCE: 57 FR 38146, Aug. 21, 1992, unless otherwise noted.

§ 156.200 Scope and applicability.

(a) *Scope.* (1) This subpart prescribes statements that must be placed on the pesticide label and in pesticide labeling. These statements incorporate by reference the Worker Protection Standard, part 170 of this chapter. The requirements addressed in these statements are designed to reduce the risk of illness or injury resulting from workers' and pesticide handlers' occupational exposures to pesticides used in the production of agricultural plants on agricultural establishments as defined in §170.3 of this chapter. These statements refer to specific workplace practices designed to reduce or eliminate exposure and to respond to emergencies that may arise from the exposures that may occur.

(2) This subpart prescribes interim requirements that must be placed on the pesticide label and in pesticide labeling. These interim requirements pertain to restricted-entry intervals, personal protective equipment, and notification. On a case-by-case basis, these interim requirements will be reviewed and may be revised during reregistration or other agency review processes.

(b) *Applicability.* (1) The requirements of this subpart apply to each pesticide product that bears directions for use in the production of any agricultural plant on any agricultural establishment as defined in §170.3 of this chapter, or whose labeling reasonably permits such use.

(2) The requirements of this subpart do not apply to a product that bears directions solely for uses excepted by §170.202(b) of this chapter.

(c) *Effective dates.* (1) The effective date of this subpart is October 20, 1992.

(2) No pesticide product bearing labeling amended and revised as required by this subpart shall be distributed or sold by a registrant prior to April 21, 1993.

(3) No product to which this subpart applies shall be distributed or sold without amended labeling by any registrant after April 21, 1994.

(4) No product to which this subpart applies shall be distributed or sold without amended labeling by any person after October 23, 1995.

§ 156.203 Definitions.

Terms in this subpart have the same meanings as they do in the Federal Insecticide, Fungicide, and Rodenticide Act, as amended. In addition, the following terms, as used in this subpart, shall have the meanings stated below:

Fumigant means any pesticide product that is a vapor or gas or forms a vapor or gas on application and whose method of pesticidal action is through the gaseous state.

Restricted-entry interval means the time after the end of a pesticide application during which entry to the treated area is restricted.

products which arises in the course of litigation concerning the effects of such products, regardless of when the information is first acquired, provided that neither of the provisions of paragraphs (b)(3) or (b)(4) of this section are met. Such information shall be submitted in the same manner and according to the same schedules as it would have to be submitted by a current registrant of a pesticide product to which the information pertained.

[62 FR 49388, Sept. 19, 1997; 63 FR 33582, June 19, 1998]

§ 159.165 Toxicological and ecological studies.

Adverse effects information must be submitted as follows:

(a) *Toxicological studies.* (1) The results of a study of the toxicity of a pesticide to humans or other non-target domestic organisms if, relative to all previously submitted studies, they show an adverse effect under any of the following conditions:

(i) That is in a different organ or tissue of the test organism.

(ii) At a lower dosage, or after a shorter exposure period, or after a shorter latency period.

(iii) At a higher incidence or frequency.

(iv) In a different species, strain, sex, or generation of test organism.

(v) By a different route of exposure.

(2) Acute oral, acute dermal, acute inhalation or skin and eye irritation studies in which the only change in toxicity is a numerical decrease in the median lethal dose (LD_{50}), median lethal concentration (LC_{50}) or irritation indices, are not reportable under this part unless the results indicate a more restrictive toxicity category for labeling under the criteria of 40 CFR 156.10(h).

(b) *Ecological studies.* The results of a study of the toxicity of a pesticide to terrestrial or aquatic wildlife or plants if, relative to all previously submitted studies, they show an adverse effect under any of the following conditions:

(1) At levels 50 percent or more lower than previous acute toxicity studies with similar species, including determinations of the median lethal dose (LD_{50}), median lethal concentration (LC_{50}), or median effective concentration (EC_{50}).

(2) At lower levels in a chronic study than previous studies with similar species.

(3) In a study with a previously untested species the results indicate the chronic no observed effect level (NOEL) is 10 percent or less of the lowest LC_{50} or LD_{50} for a similar species.

(4) For plants when tested at the maximum label application rate or less, if either of the following conditions is met:

(i) More than 25 percent of terrestrial plants show adverse effects on plant life cycle functions and growth such as germination, emergence, plant vigor, reproduction and yields.

(ii) More than 50 percent of aquatic plants show adverse effects on plant life cycle functions and growth such as germination, emergence, plant vigor, reproduction and yields.

(c) Results from a study that demonstrates any toxic effect (even if corroborative of information already known to the Agency), must be submitted if the pesticide is or has been the subject of a Formal Review based on that effect within 5 years of the time the results are received. Within 30 calendar days of the publication of a Notice of Commencement of a Formal Review in the FEDERAL REGISTER, all information which has become reportable due to the commencement of the Formal Review must be submitted.

(d) *Incomplete studies.* Information from an incomplete study of the toxicity to any organism of a registered pesticide product or any of its ingredients, impurities, metabolites, or degradation products which would otherwise be reportable under paragraphs (a), (b) or (c) of this section must be submitted if the information meets any one of the folowing three sets of criteria:

(1) *Short-term studies.* A study using a test regimine lasting 90 calendar days or less, and all of the following conditions are met:

(i) All testing has been completed.

(ii) A preliminary data analysis or gross pathological analysis has been conducted.

(iii) Final analysis has not been completed.

(iv) A reasonable period for completion of the final analysis not longer than 90 calendar days following completion of testing has elapsed.

(v) Comparable information concerning the results of a completed study would be reportable.

(2) *Long-term studies.* A study using a test regimine lasting 90 calendar days or less, and all of the following conditions are met:

(i) All testing has been completed.

(ii) A preliminary data analysis or gross pathological analysis has been conducted.

(iii) Final analysis has not been completed.

(iv) A reasonable period of completion of final analysis (not longer that 1 year following completion of testing) has elapsed.

(v) Comparable information concerning the results of a completed study would be reportable.

(3) *Serious adverse effects.* Any study in which testing or analysis of results is not yet complete but in which serious adverse effects have already been observed which may reasonably be attributed to exposure to the substances tested, because the effects observed in exposed organisms differ from effects observed in control organisms, are atypical in view of historical experience with the organism tested, or otherwise support a reasonable inference of causation, and 30 days have passed from the date the registrant first has the information.

[62 FR 49388, Sept. 19, 1997; 63 FR 33582, June 19, 1998]

§ 159.167 Discontinued studies.

The fact that a study has been discontinued before the planned termination must be reported to EPA, with the reason for termination, if submission of information concerning the study is, or would have been, required under this part.

§ 159.170 Human epidemiological and exposure studies.

Information must be submitted which concerns any study that a person described in § 159.158(a) has concluded, or might reasonably conclude, shows that a correlation may exist between exposure to a pesticide and observed adverse effects in humans. Information must also be submitted which concerns exposure monitoring studies that indicate higher levels of risk or exposure than would be expected based on previously available reports, data, or exposure estimates. Such information must be submitted regardless of whether the registrant considers any observed correlation or association to be significant.

§ 159.178 Information on pesticides in or on food, feed or water.

(a) *Food and feed.* Information must be submitted if it shows that the pesticide is present in or on food or feed at a level in excess of established levels, except that information on excess residues resulting solely from studies conducted under authority of FIFRA section 5 or under other controlled research studies conducted to test a pesticide product need not be submitted, provided that the treated crop is not marketed as a food or feed commodity. The information to be submitted is the same as that required in § 159.184(c)(1), (2), (3), and (4)(iv)(E), (F), (G), and (H).

(b) *Water.* (1) Information must be submitted if it shows that a pesticide is present above the water reference level in any of the following instances:

(i) Waters of the United States, as defined in § 122.2 of this chapter, except paragraph (d) of § 122.2.

(ii) Ground water.

(iii) Finished drinking water.

(2) If the lowest detectable amount of the pesticide is reported, the detection limit must also be reported.

(3) Information need not be submitted regarding the detection of a pesticide in waters of the United States or finished drinking water if the pesticide is registered for use in finished drinking water or surface water and the amount detected does not exceed the amounts reported by a registrant in its application for registration, as resulting in those waters from legal applications of the pesticide.

(4) Information need not be submitted concerning detections of pesticides in waters of the United States, ground water or finished drinking water if the substance detected is an inert ingredient, or a metabolite, degradate, contaminant or impurity of

APPENDIX F.2

Federal Regulations on Acute Toxicity
Consumer Products Safety Commission (CPSC)

CPSC Regulations

16 CFR Ch.II 1500.1 – 1500.5: CPSC Regulations Submitted Pursuant to and for the implementation of the Federal Hazardous Substances Act

16 CFR Ch.II 1500.40 – 1500.42: Method of Testing Toxic Substances and Irritant Substances

AUTHORITY: 15 U.S.C. 1261–1278.

SOURCE: 38 FR 27012, Sept. 27, 1973, unless otherwise noted.

§ 1500.1 Scope of subchapter.

Set forth in this subchapter C are the regulations of the Consumer Product Safety Commission issued pursuant to and for the implementation of the Federal Hazardous Substances Act as amended (see § 1500.3(a)(1)).

§ 1500.2 Authority.

Authority under the Federal Hazardous Substances Act is vested in the Consumer Product Safety Commission by section 30(a) of the Consumer Product Safety Act (15 U.S.C. 2079(a)).

§ 1500.3 Definitions.

(a) *Certain terms used in this part.* As used in this part:

(1) *Act* means the Federal Hazardous Substances Act (Pub. L. 86–613, 74 Stat. 372–81 (15 U.S.C. 1261–74)), as amended by:

(i) The Child Protection Act of 1966 (Pub. L. 89–756, 80 Stat. 1303–05).

(ii) The Child Protection and Toy Safety Act of 1969 (Pub. L. 91–113, 83 Stat. 187–90).

(iii) The Poison Prevention Packaging Act of 1970 (Pub. L. 91–601, 84 Stat. 1670–74).

(2) *Commission* means the Consumer Product Safety Commission established May 14, 1973, pursuant to provisions of the Consumer Product Safety Act (Pub. L. 92–573, 86 Stat. 1207–33 (15 U.S.C. 2051–81)).

(b) *Statutory definitions.* Except for the definitions given in section 2 (c) and (d) of the act, which are obsolete, the definitions set forth in section 2 of the act are applicable to this part and are repeated for convenience as follows (some of these statutory definitions are interpreted, supplemented, or provided with alternatives in paragraph (c) of this section):

(1) *Territory* means any territory or possession of the United States, including the District of Columbia and the Commonwealth of Puerto Rico but excluding the Canal Zone.

(2) *Interstate commerce* means (i) commerce between any State or territory and any place outside thereof and (ii) commerce within the District of Columbia or within any territory not organized with a legislative body.

(3) *Person* includes an individual, partnership, corporation, and association.

(4)(i) *Hazardous substance* means:

(A) Any substance or mixture of substances which is toxic, corrosive, an irritant, a strong sensitizer, flammable or combustible, or generates pressure through decomposition, heat, or other means, if such substance or mixture of substances may cause substantial personal injury or substantial illness during or as a proximate result of any customary or reasonably foreseeable handling or use, including reasonably foreseeable ingestion by children.

(B) Any substance which the Commission by regulation finds, pursuant to the provisions of section 3(a) of the act, meet the requirements of section 2(f)(1)(A) of the act (restated in (A) above).

(C) Any radioactive substance if, with respect to such substance as used in a particular class of article or as packaged, the Commission determines by regulation that the substance is sufficiently hazardous to require labeling in accordance with the act in order to protect the public health.

(D) Any toy or other article intended for use by children which the Commission by regulation determines, in accordance with section 3(e) of the act, presents an electrical, mechanical, or thermal hazard.

(ii) *Hazardous substance* shall not apply to pesticides subject to the Federal Insecticide, Fungicide, and Rodenticide Act, to foods, drugs, and cosmetics subject to the Federal Food, Drug, and Cosmetic Act, nor to substances intended for use as fuels when stored in containers and used in the heating, cooking, or refrigeration system of a house. "Hazardous substance" shall apply, however, to any article which is not itself a pesticide within the meaning of the Federal Insecticide, Fungicide, and Rodenticide Act but which is a hazardous substance within the meaning of section 2(f)(1) of the Federal Hazardous Substances Act (restated in paragraph (b)(4)(i) of this section) by reason of bearing or containing such a pesticide.

(iii) *Hazardous substance* shall not include any source material, special nuclear material, or byproduct material as defined in the Atomic Energy Act of 1954, as amended, and regulations issued pursuant thereto by the Atomic Energy Commission.

(5) *Toxic* shall apply to any substance (other than a radioactive substance) which has the capacity to produce personal injury or illness to man through ingestion, inhalation, or absorption through any body surface.

(6)(i) *Highly toxic* means any substance which falls within any of the following categories:

(A) Produces death within 14 days in half or more than half of a group of 10 or more laboratory white rats each weighing between 200 and 300 grams, at a single dose of 50 milligrams or less per kilogram of body weight, when orally administered; or

(B) Produces death within 14 days in half or more than half of a group of 10 or more laboratory white rats each weighing between 200 and 300 grams, when inhaled continuously for a period of 1 hour or less at an atmospheric concentration of 200 parts per million by volume or less of gas or vapor or 2 milligrams per liter by volume or less of mist or dust, provided such concentration is likely to be encountered by man when the substance is used in any reasonably foreseeable manner; or

(C) Produces death within 14 days in half or more than half of a group of 10 or more rabbits tested in a dosage of 200 milligrams or less per kilogram of body weight, when administered by continuous contact with the bare skin for 24 hours or less.

(ii) If the Commission finds that available data on human experience with any substance indicate results different from those obtained on animals in the dosages and concentrations specified in paragraph (b)(6)(i) of this section, the human data shall take precedence.

(7) *Corrosive* means any substance which in contact with living tissue will cause destruction of tissue by chemical action, but shall not refer to action on inanimate surfaces.

(8) *Irritant* means any substance not corrosive within the meaning of section 2(i) of the act (restated in paragraph (b)(7) of this section) which on immediate, prolonged, or repeated contact with normal living tissue will induce a local inflammatory reaction.

(9) *Strong sensitizer* means a substance which will cause on normal living tissue through an allergic or photodynamic process a hypersensitivity which becomes evident on reapplication of the same substance and which is designated as such by the Commission. Before designating any substance as a strong sensitizer, the Commission, upon consideration of the frequency of occurrence and severity of the reaction, shall find that the substance has a significant potential for causing hypersensitivity.

(10) The terms *extremely flammable*, *flammable*, and *combustible* as they apply to any substances, liquid, solid, or the contents of any self-pressurized container, are defined by regulations issued by the Commission and published at § 1500.3(c)(6).

(11) *Radioactive substance* means a substance which emits ionizing radiation.

(12) *Label* means a display of written, printed, or graphic matter upon the immediate container of any substance or, in the cases of an article which is unpackaged or is not packaged in an immediate container intended or suitable for delivery to the ultimate consumer, a display of such matter directly upon the article involved or upon a tag or other suitable material affixed thereto. A requirement made by or under authority of the act that any word, statement, or other information appear on the label shall not be considered to be complied with unless such word, statement, or other information also appears (i) on the outside container or wrapper, if any there be, unless it is easily legible through the outside container or wrapper and (ii) on all accompanying literature where there are directions for use, written or otherwise.

(13) *Immediate container* does not include package liners.

(14) *Misbranded hazardous substance* means a hazardous substance (including a toy, or other article intended for use by children, which is a hazardous substance, or which bears or contains a hazardous substance in such manner as

to be susceptible of access by a child to whom such toy or other article is entrusted) intended, or packaged in a form suitable, for use in the household or by children, if the packaging or labeling of such substance is in violation of an applicable regulation issued pursuant to section 3 or 4 of the Poison Prevention Packaging Act of 1970 or if such substance, except as otherwise provided by or pursuant to section 3 of the act (Federal Hazardous Substances Act), fails to bear a label:

(i) Which states conspicuously:

(A) The name and place of business of the manufacturer, packer, distributor, or seller;

(B) The common or usual name or the chemical name (if there be no common or usual name) of the hazardous substance or of each component which contributes substantially to its hazard, unless the Commission by regulation permits or requires the use of a recognized generic name;

(C) The signal word "DANGER" on substances which are extremely flammable, corrosive, or highly toxic;

(D) The signal word "WARNING" or "CAUTION" on all other hazardous substances;

(E) An affirmative statement of the principal hazard or hazards, such as "Flammable," "Combustible," "Vapor Harmful," "Causes Burns," "Absorbed Through Skin," or similar wording descriptive of the hazard;

(F) Precautionary measures describing the action to be followed or avoided, except when modified by regulation of the Commission pursuant to section 3 of the act;

(G) Instruction, when necessary or appropriate, for first-aid treatment;

(H) The word *Poison* for any hazardous substance which is defined as "highly toxic" by section 2(h) of the act (restated in paragraph (b)(6) of this section);

(I) Instructions for handling and storage of packages which require special care in handling or storage; and

(J) The statement (*1*) "Keep out of the reach of children" or its practical equivalent, or, (*2*) if the article is intended for use by children and is not a banned hazardous substance, adequate directions for the protection of children from the hazard; and

(ii) On which any statements required under section 2(p)(1) of the act (restated in paragraph (b)(14)(i) of this section) are located prominently and are in the English language in conspicuous and legible type in contrast by typography, layout, or color with other printed matter on the label.

Misbranded hazardous substance also means a household substance as defined in section 2(2)(D) of the Poison Prevention Packaging Act of 1970 if it is a substance described in section 2(f)(1) of the Federal Hazardous Substances Act (restated in paragraph (b)(4)(i)(A) of this section) and its packaging or labeling is in violation of an applicable regulation issued pursuant to section 3 or 4 of the Poison Prevention Packaging Act of 1970.

(15)(i) *Banned hazardous substance* means:

(A) Any toy, or other article intended for use by children, which is a hazardous substance, or which bears or contains a hazardous substance in such manner as to be susceptible of access by a child to whom such toy or other article is entrusted; or

(B) Any hazardous substance intended, or packaged in a form suitable, for use in the household, which the Commission by regulation classifies as a "banned hazardous substance" on the basis of a finding that, notwithstanding such cautionary labeling as is or may be required under the act for that substance, the degree or nature of the hazard involved in the presence or use of such substance in households is such that the objective of the protection of the public health and safety can be adequately served only by keeping such substance, when so intended or packaged, out of the channels of interstate commerce; *Provided*, That the Commission by regulation (*1*) shall exempt from section 2(q)(1)(A) of the act (restated in paragraph (b)(15)(i)(A) of this section) articles, such as chemistry sets, which by reason of their functional purpose require the inclusion of the hazardous substance involved, or necessarily present an electrical, mechanical, or thermal hazard, and which bear labeling giving adequate directions and warnings for safe

use and are intended for use by children who have attained sufficient maturity, and may reasonably be expected, to read and heed such directions and warnings, and (*2*) shall exempt from section 2(q)(1)(A) of the act (restated in paragraph (b)(15)(i)(A) of this section), and provide for the labeling of, common fireworks (including toy paper caps, cone fountains, cylinder fountains, whistles without report, and sparklers) to the extent that the Commission determines that such articles can be adequately labeled to protect the purchasers and users thereof.

(ii) Proceedings for the issuance, amendment, or repeal of regulations pursuant to section 2(q)(1)(B) of the act (restated in paragraph (b)(15)(i)(B) of this section) shall be governed by the provisions of section 701 (e), (f), and (g) of the Federal Food, Drug, and Cosmetic Act: *Provided,* That if the Commission finds that the distribution for household use of the hazardous substance involved presents an imminent hazard to the public health, the Commission may by order published in the FEDERAL REGISTER give notice of such finding, and thereupon such substance when intended or offered for household use, or when so packaged as to be suitable for such use, shall be deemed to be a "banned hazardous substance" pending the completion of proceedings relating to the issuance of such regulations.

(16) "Electrical hazard"—an article may be determined to present an electrical hazard if, in normal use or when subjected to reasonably foreseeable damage or abuse, its design or manufacture may cause personal injury or illness by electric shock.

(17) "Mechanical hazard"—an article may be determined to present a mechanical hazard if, in normal use or when subjected to reasonably foreseeable damage or abuse, its design or manufacture presents an unreasonable risk of personal injury or illness:

(i) From fracture, fragmentation, or disassembly of the article;

(ii) From propulsion of the article (or any part or accessory thereof);

(iii) From points or other protrusions, surfaces, edges, openings, or closures;

(iv) From moving parts;

(v) From lack or insufficiency of controls to reduce or stop motion;

(vi) As a result of self-adhering characteristics of the article;

(vii) Because the article (or any part or accessory thereof) may be aspirated or ingested;

(viii) Because of instability; or

(ix) Because of any other aspect of the article's design or manufacture.

(18) "Thermal hazard"—an article may be determined to present a thermal hazard if, in normal use or when subjected to reasonably foreseeable damage or abuse, its design or manufacture presents an unreasonable risk of personal injury or illness because of heat as from heated parts, substances, or surfaces.

(c) *Certain statutory definitions interpreted, supplemented, or provided with alternatives.* The following items interpret, supplement, or provide alternatives to definitions set forth in section 2 of the act (and restated in paragraph (b) of this section):

(1) To provide flexibility as to the number of animals tested, the following is an alternative to the definition of "highly toxic" in section 2(h) of the act (and paragraph (b)(6) of this section): *Highly toxic* means:

(i) A substance determined by the Commission to be highly toxic on the basis of human experience; and/or

(ii) A substance that produces death within 14 days in half or more than half of a group of:

(A) White rats (each weighing between 200 and 300 grams) when a single dose of 50 milligrams or less per kilogram of body weight is administered orally;

(B) White rats (each weighing between 200 and 300 grams) when a concentration of 200 parts per million by volume or less of gas or vapor, or 2 milligrams per liter by volume or less of mist or dust, is inhaled continuously for 1 hour or less, if such concentration is likely to be encountered by man when the substance is used in any reasonably foreseeable manner; and/or

(C) Rabbits (each weighing between 2.3 and 3.0 kilograms) when a dosage of 200 milligrams or less per kilogram of body weight is administered by continuous contact with the bare skin for 24

hours or less by the method described in § 1500.40.

The number of animals tested shall be sufficient to give a statistically significant result and shall be in conformity with good pharmacological practices.

(2) To give specificity to the definition of "toxic" in section 2(g) of the act (and restated in paragraph (b)(5) of this section), the following supplements that definition. The following categories are not intended to be inclusive.

(i) *Acute toxicity. Toxic* means any substance that produces death within 14 days in half or more than half of a group of:

(A) White rats (each weighing between 200 and 300 grams) when a single dose of from 50 milligrams to 5 grams per kilogram of body weight is administered orally. Substances falling in the toxicity range between 500 milligrams and 5 grams per kilogram of body weight will be considered for exemption from some or all of the labeling requirements of the act, under § 1500.82, upon a showing that such labeling is not needed because of the physical form of the substances (solid, a thick plastic, emulsion, etc.), the size or closure of the container, human experience with the article, or any other relevant factors;

(B) White rats (each weighing between 200 and 300 grams) when an atmospheric concentration of more than 200 parts per million but not more than 20,000 parts per million by volume of gas or vapor, or more than 2 but not more than 200 milligrams per liter by volume of mist or dust, is inhaled continuously for 1 hour or less, if such concentration is likely to be encountered by man when the substance is used in any reasonably foreseeable manner; and/or

(C) Rabbits (each weighing between 2.3 and 3.0 kilograms) when a dosage of more than 200 milligrams but not more than 2 grams per kilogram of body weight is administered by continuous contact with the bare skin for 24 hours by the method described in § 1500.40.

The number of animals tested shall be sufficient to give a statistically significant result and shall be in conformity with good pharmacological practices. "Toxic" also applies to any substance that is "toxic" (but not "highly toxic") on the basis of human experience.

(ii) *Chronic toxicity.* A substance is toxic because it presents a chronic hazard if it falls into one of the following categories. (For additional information see the chronic toxicity guidelines at 16 CFR 1500.135.)

(A) *For Carcinogens.* A substance is toxic if it is or contains a known or probable human carcinogen.

(B) *For Neurotoxicological Toxicants.* A substance is toxic if it is or contains a known or probable human neurotoxin.

(C) *For Developmental or Reproductive Toxicants.* A substance is toxic if it is or contains a known or probable human developmental or reproductive toxicant.

(3) The definition of *corrosive* in section 2(i) of the act (restated in paragraph (b)(7) of this section) is interpreted to also mean the following: *Corrosive* means a substance that causes visible destruction or irreversible alterations in the tissue at the site of contact. A test for a corrosive substance is whether, by human experience, such tissue destruction occurs at the site of application. A substance would be considered corrosive to the skin if, when tested on the intact skin of the albino rabbit by the technique described in § 1500.41, the structure of the tissue at the site of contact is destroyed or changed irreversibly in 24 hours or less. Other appropriate tests should be applied when contact of the substance with other than skin tissue is being considered.

(4) The definition of *irritant* in section 2(j) of the act (restated in paragraph (b)(8) of this section) is supplemented by the following: *Irritant* includes "primary irritant to the skin" as well as substances irritant to the eye or to mucous membranes. *Primary irritant* means a substance that is not corrosive and that human experience data indicate is a primary irritant and/or means a substance that results in an empirical score of five or more when tested by the method described in § 1500.41. *Eye irritant* means a substance that human experience data indicate is an irritant to the eye and/or means a substance for which a positive test is obtained when tested by the method described in § 1500.42.

(5) The definition of *strong sensitizer* in section 2(k) of the Federal Hazardous Substances Act (restated in 16 CFR 1500.3(b)(9)) is supplemented by the following definitions:

(i) *Sensitizer.* A *sensitizer* is a substance that will induce an immunologically-mediated (allergic) response, including allergic photosensitivity. This allergic reaction will become evident upon reexposure to the same substance. Occasionally, a sensitizer will induce and elicit an allergic response on first exposure by virtue of active sensitization.

(ii) *Strong.* In determining that a substance is a "strong" sensitizer, the Commission shall consider the available data for a number of factors. These factors should include any or all of the following (if available): Quantitative or qualitative risk assessment, frequency of occurrence and range of severity of reactions in healthy or susceptible populations, the result of experimental assays in animals or humans (considering dose-response factors), with human data taking precedence over animal data, other data on potency or bioavailability of sensitizers, data on reactions to a cross-reacting substance or to a chemical that metabolizes or degrades to form the same or a cross-reacting substance, the threshold of human sensitivity, epidemiological studies, case histories, occupational studies, and other appropriate *in vivo* and *in vitro* test studies.

(iii) *Severity of reaction.* The minimal severity of reaction for the purpose of designating a material as a "strong sensitizer" is a clinically important allergic reaction. For example, strong sensitizers may produce substantial illness, including any or all of the following: physical discomfort, distress, hardship, and functional or structural impairment. These may, but not necessarily, require medical treatment or produce loss of functional activities.

(iv) *Significant potential for causing hypersensitivity.* "Significant potential for causing hypersensitivity" is a relative determination that must be made separately for each substance. It may be based upon the chemical or functional properties of the substance, documented medical evidence of allergic reactions obtained from epidemiological surveys or individual case reports, controlled *in vitro* or *in vivo* experimental assays, or susceptibility profiles in normal or allergic subjects.

(v) *Normal living tissue.* The allergic hypersensitivity reaction occurs in normal living tissues, including the skin and other organ systems, such as the respiratory or gastrointestinal tract, either singularly or in combination, following sensitization by contact, ingestion, or inhalation.

(6) The Consumer Product Safety Commission, by the regulations published in this section, defines the terms *extremely flammable, flammable,* and *combustible,* appearing in section 2(l) of the Federal Hazardous Substances Act, as follows:

(i) The term *extremely flammable* shall apply to any substance which has a flashpoint at or below 20 °F (−6.7 °C) as determined by the test method described at § 1500.43a, except that, any mixture having one component or more with a flashpoint higher than 20 °F (−6.7 °C) which comprises at least 99 percent of the total volume of the mixture is not considered to be an extremely flammable substance.

(ii) The term *flammable* shall apply to any substance having a flashpoint above 20 °F (−6.7 °C) and below 100 °F (37.8 °C), as determined by the method described at § 1500.43a, except that:

(A) Any mixture having one component or more with a flashpoint at or above 100 °F (37.8 °C) which comprises at least 99 percent of the total volume of the mixture is not considered to be a flammable substance; and

(B) Any mixture containing 24 percent or less of water miscible alcohols, by volume, in aqueous solution is not considered to be flammable if the mixture does not present a significant flammability hazard when used by consumers.

(iii) The term *combustible* shall apply to any substance having a flashpoint at or above 100 °F (37.8 °C) to and including 150 °F (65.6 °C) as determined by the test method described at § 1500.43a, except that:

(A) Any mixture having one component or more with a flashpoint higher than 150 °F (65.6 °C) which comprises at least 99 percent of the total volume of

the mixture is not considered to be a combustible hazardous substance; and

(B) Any mixture containing 24 percent or less of water miscible alcohols, by volume, in aqueous solution is not considered to be combustible if the mixture does not present a significant flammability hazard when used by consumers.

(iv) To determine flashpoint temperatures for purposes of enforcing and administering requirements of the Federal Hazardous Substances Act applicable to "extremely flammable," "flammable," and "combustible" hazardous substances, the Commission will follow the procedures set forth in §1500.43a. However, the Commission will allow manufacturers and labelers of substances and products subject to those requirements to rely on properly conducted tests using the Tagliabue open-cup method which was in effect prior to the issuance of §1500.43a (as published at 38 FR 27012, September 27, 1973, and set forth below), and the definitions of the terms "extremely flammable," "flammable," and "combustible" in this section before its amendment (as published at 38 FR 27012, September 27, 1983, and amended 38 FR 30105, November 1, 1973, set forth in the note following this section) if all of the following conditions are met:

(A) The substance or product was subject to and complied with the requirements of the Federal Hazardous Substances Act for "extremely flammable," "flammable," or "combustible" hazardous substances before the effective date of §1500.43a; and

(B) No change has been made to the formulation or labeling of such substance or product after the effective date of §1500.43a, prescribing a closed-cup test apparatus and procedure.

(v) *Extremely flammable solid* means a solid substance that ignites and burns at an ambient temperature of 80 °F or less when subjected to friction, percussion, or electrical spark.

(vi) *Flammable solid* means a solid substance that, when tested by the method described in §1500.44, ignites and burns with a self-sustained flame at a rate greater than one-tenth of an inch per second along its major axis.

(vii) *Extremely flammable contents of self-pressurized container* means contents of a self-pressurized container that, when tested by the method described in §1500.45, a flashback (a flame extending back to the dispenser) is obtained at any degree of valve opening and the flashpoint, when tested by the method described in §1500.43a is less than 20 °F (−6.7 °C).

(viii) *Flammable contents of self-pressurized container* means contents of a self-pressurized container that, when tested by the method described in §1500.45, a flame projection exceeding 18 inches is obtained at full valve opening, or flashback (a flame extending back to the dispenser) is obtained at any degree of valve opening.

(7) The definition of *hazardous substance* in section 2(f)(1)(A) of the act (restated in paragraph (b)(4)(i)(A) of this section) is supplemented by the following definitions or interpretations or terms used therein:

(i) A substance or mixture of substances that "generates pressure through decomposition, heat, or other means" is a hazardous substance:

(A) If it explodes when subjected to an electrical spark, percussion, or the flame of a burning paraffin candle for 5 seconds or less.

(B) If it expels the closure of its container, or bursts its container, when held at or below 130 °F. for 2 days or less.

(C) If it erupts from its opened container at a temperature of 130 °F. or less after having been held in the closed container at 130 °F. for 2 days.

(D) If it comprises the contents of a self-pressurized container.

(ii) *Substantial personal injury or illness* means any injury or illness of a significant nature. It need not be severe or serious. What is excluded by the word "substantial" is a wholly insignificant or negligible injury or illness.

(iii) *Proximate result* means a result that follows in the course of events without an unforeseeable, intervening, independent cause.

(iv) *Reasonably foreseeable handling or use* includes the reasonably foreseeable accidental handling or use, not only by the purchaser or intended user of the product, but by all others in a household, especially children.

(8) The definition of "radioactive substance" in section 2(m) of the act (restated in paragraph (b)(11) of this section) is supplemented by the following: *Radioactive substance* means a substance which, because of nuclear instability, emits electromagnetic and/or particulate radiation capable of producing ions in its passage through matter. Source materials, special nuclear material, and byproduct materials described in section 2(f)(3) of the act are exempt.

(9) In the definition of "label" in section 2(n) of the act (restated in paragraph (b)(12) of this section), a provision stipulates that words, statements, or other information required to be on the label must also appear on all accompanying literature where there are directions for use, written or otherwise. To make this provision more specific, "accompanying literature" is interpreted to mean any placard, pamphlet, booklet, book, sign, or other written, printed, or graphic matter or visual device that provides directions for use, written or otherwise, and that is used in connection with the display, sale, demonstration, or merchandising of a hazardous substance intended for or packaged in a form suitable for use in the household or by children.

(10) The definition of "misbranded hazardous substance" in section 2(p) of this act (restated in paragraph (b)(14) of this section) is supplemented by the following definitions or interpretations of terms used therein:

(i) *Hazardous substances intended, or packaged in a form suitable, for use in the household* means any hazardous substance, whether or not packaged, that under any customary or reasonably foreseeable condition of purchase, storage, or use may be brought into or around a house, apartment, or other place where people dwell, or in or around any related building or shed including, but not limited to, a garage, carport, barn, or storage shed. The term includes articles, such as polishes or cleaners, designed primarily for professional use but which are available in retail stores, such as hobby shops, for nonprofessional use. Also included are items, such as antifreeze and radiator cleaners, that although principally for car use may be stored in or around dwelling places. The term does not include industrial supplies that might be taken into a home by a serviceman. An article labeled as, and marketed solely for, industrial use does not become subject to this act because of the possibility that an industrial worker may take a supply for his own use. Size of unit or container is not the only index of whether the article is suitable for use in or around the household; the test shall be whether under any reasonably foreseeable condition of purchase, storage, or use the article may be found in or around a dwelling.

(ii) *Conspicuously* in section 2(p)(1) of the act and *prominently* and *conspicuous* in section 2(p)(2) of the act mean that, under customary conditions of purchase, storage, and use, the required information shall be visible, noticeable, and in clear and legible English. Some factors affecting a warning's prominence and conspicuousness are: Location, size of type, and contrast of printing against background. Also bearing on the effectiveness of a warning might be the effect of the package contents if spilled on the label.

NOTE: The definitions of *extremely flammable*, *flammable*, and *combustible* hazardous substances set forth above in paragraphs (b)(10) and (c)(6) are effective August 10, 1987. The definitions remaining in effect until August 10, 1987, as published at 38 FR 27012, Sept. 27, 1973, and amended at 38 FR 30105, Nov. 1, 1973, are set forth below. Manufacturers and labelers of products subject to the Federal Hazardous Substances Act may continue to use these definitions for labeling of those products under the conditions set forth in § 1500.3(c)(6)(iv), as amended.

(b)(10) *Extremely flammable* shall apply to any substance which has a flashpoint at or below 20 °F. as determined by the Tagliabue Open Cup Tester; *flammable* shall apply to any substance which has a flashpoint of above 20 °F., to and including 80 °F., as determined by the Tagliabue Open Cup Tester; and *combustible* shall apply to any substance which has a flashpoint above 80 °F. to and including 150 °F., as determined by the Tagliabue Open Cup Tester; except that the flammability or combustibility of solids and of the contents of self-pressurized containers shall be determined by methods found by the Commission to be generally applicable to such materials or containers, respectively, and established by regulations issued by the

Commission, which regulations shall also define the terms *flammable*, *combustible*, and *extremely flammable* in accord with such methods.

* * * * *

(c)(6)(i) *Extremely flammable* means any substance that has a flashpoint at or below 20 °F. as determined by the method described in § 1500.43.

(ii) *Flammable* means any substance that has a flashpoint of above 20 °F., to and including 80 °F., as determined by the method described in § 1500.43.

[38 FR 27012, Sept. 27, 1973, as amended at 38 FR 30105, Nov. 1, 1973; 49 FR 22465, May 30, 1984; 51 FR 28736, Aug. 8, 1986; 51 FR 29096, Aug. 14, 1986; 51 FR 30209, Aug. 25, 1986; 57 FR 46689, Oct. 9, 1992]

§ 1500.4 Human experience with hazardous substances.

(a) Reliable data on human experience with any substance should be taken into account in determining whether an article is a "hazardous substance" within the meaning of the act. When such data give reliable results different from results with animal data, the human experience takes precedence.

(b) Experience may show that an article is more or less toxic, irritant, or corrosive to man than to test animals. It may show other factors that are important in determining the degree of hazard to humans represented by the substance. For example, experience shows that radiator antifreeze is likely to be stored in the household or garage and likely to be ingested in significant quantities by some persons. It also shows that a particular substance in liquid form is more likely to be ingested than the same substance in a paste or a solid and that an aerosol is more likely to get into the eyes and the nasal passages than a liquid.

§ 1500.5 Hazardous mixtures.

For a mixture of substances, the determination of whether the mixture is a "hazardous substance" as defined by section 2(f) of the act (repeated in § 1500.3(b)(4)) should be based on the physical, chemical, and pharmacological characteristics of the mixture. A mixture of substances may therefore be less hazardous or more hazardous than its components because of synergistic or antagonistic reactions. It may not be possible to reach a fully satisfactory decision concerning the toxic, irritant, corrosive, flammable, sensitizing, or pressure-generating properties of a substance from what is known about its components or ingredients. The mixture itself should be tested.

§ 1500.12 Products declared to be hazardous substances under section 3(a) of the act.

(a) The Commission finds that the following articles are hazardous substances within the meaning of the act because they are capable of causing substantial personal injury or substantial illness during or as a proximate result of any customary or reasonably foreseeable handling or use:

(1) Charcoal briquettes and other forms of charcoal in containers for retail sale and intended for cooking or heating.

(2) [Reserved]

(b) [Reserved]

§ 1500.13 Listing of "strong sensitizer" substances.

On the basis of frequency of occurrence and severity of reaction information, the Commission finds that the following substances have a significant potential for causing hypersensitivity and therefore meet the definition for "strong sensitizer" in section 2(k) of the act (repeated in § 1500.3(b)(9)):

(a) Paraphenylenediamine and products containing it.

(b) Powdered orris root and products containing it.

(c) Epoxy resins systems containing in any concentration ethylenediamine, diethylenetriamine, and diglycidyl ethers of molecular weight of less than 200.

(d) Formaldehyde and products containing 1 percent or more of formaldehyde.

(e) Oil of bergamot and products containing 2 percent or more of oil of bergamot.

§ 1500.14 Products requiring special labeling under section 3(b) of the act.

(a) Human experience, as reported in the scientific literature and to the Poison Control Centers and the National

1995, if the non-identical requirement was in effect on October 2, 1993.

[60 FR 10752, Feb. 27, 1995, as amended at 60 FR 41802, Aug. 14, 1995]

§ 1500.40 Method of testing toxic substances.

The method of testing the toxic substances referred to in § 1500.3(c)(1)(ii)(C) and (2)(iii) is as follows:

(a) *Acute dermal toxicity (single exposure).* In the acute exposures, the agent is held in contact with the skin by means of a sleeve for periods varying up to 24 hours. The sleeve, made of rubber dam or other impervious material, is so constructed that the ends are reinforced with additional strips and should fit snugly around the trunk of the animal. The ends of the sleeve are tucked, permitting the central portion to "balloon" and furnish a reservoir for the dose. The reservoir must have sufficient capacity to contain the dose without pressure. In the following table are given the dimensions of sleeves and the approximate body surface exposed to the test substance. The sleeves may vary in size to accommodate smaller or larger subjects. In the testing of unctuous materials that adhere readily to the skin, mesh wire screen may be employed instead of the sleeve. The screen is padded and raised approximately 2 centimeters from the exposed skin. In the case of dry powder preparations, the skin and substance are moistened with physiological saline prior to exposure. The sleeve or screen is then slipped over the gauze that holds the dose applied to the skin. In the case of finely divided powders, the measured dose is evenly distributed on cotton gauze which is then secured to the area of exposure.

DIMENSIONS OF SLEEVES FOR ACUTE DERMAL TOXICITY TEST

[Test animal—Rabbits]

Measurements in centimeters		Range of weight of animals (grams)	Average area of exposure (square centimeters)	Average percentage of total body surface
Diameter at ends	Overall length			
7.0	12.5	2,500–3,500	240	10.7

(b) *Preparation of test animal.* The animals are prepared by clipping the skin of the trunk free of hair. Approximately one-half of the animals are further prepared by making epidermal abrasions every 2 or 3 centimeters longitudinally over the area of exposure. The abrasions are sufficiently deep to penetrate the stratum corneum (horny layer of the epidermis) but not to distrub the derma; that is, not to obtain bleeding.

(c) *Procedures for testing.* The sleeve is slipped onto the animal which is then placed in a comfortable but immobilized position in a multiple animal holder. Selected doses of liquids and solutions are introduced under the sleeve. If there is slight leakage from the sleeve, which may occur during the first few hours of exposure, it is collected and reapplied. Dosage levels are adjusted in subsequent exposures (if necessary) to enable a calculation of a dose that would be fatal to 50 percent of the animals. This can be determined from mortality ratios obtained at various doses employed. At the end of 24 hours the sleeves or screens are removed, the volume of unabsorbed material (if any) is measured, and the skin reactions are noted. The subjects are cleaned by thorough wiping, observed for gross symptoms of poisoning, and then observed for 2 weeks.

§ 1500.41 Method of testing primary irritant substances.

Primary irritation to the skin is measured by a patch-test technique on the abraded and intact skin of the albino rabbit, clipped free of hair. A minimum of six subjects are used in abraded and intact skin tests. Introduce under a square patch, such as surgical gauze measuring 1 inch by 1 inch and two single layers thick, 0.5 milliliter (in the case of liquids) or 0.5 gram (in the case of solids and semisolids) of the

test substance. Dissolve solids in an appropriate solvent and apply the solution as for liquids. The animals are immobilized with patches secured in place by adhesive tape. The entire trunk of the animal is then wrapped with an impervious material, such as rubberized cloth, for the 24-hour period of exposure. This material aids in maintaining the test patches in position and retards the evaporation of volatile substances. After 24 hours of exposure, the patches are removed and the resulting reactions are evaluated on the basis of the designated values in the following table:

Skin reaction	Value[1]
Erythema and eschar formation:	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formations (injuries in depth)	4
Edema formation:	
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well defined by definite raising)	2
Moderate edema (raised approximately 1 millimeter)	3
Severe edema (raised more than 1 millimeter and extending beyond the area of exposure)	4

[1] The "value" recorded for each reading is the average value of the six or more animals subject to the test.

Readings are again made at the end of a total of 72 hours (48 hours after the first reading). An equal number of exposures are made on areas of skin that have been previously abraded. The abrasions are minor incisions through the stratum corneum, but not sufficiently deep to disturb the derma or to produce bleeding. Evaluate the reactions of the abraded skin at 24 hours and 72 hours, as described in this paragraph. Add the values for erythema and eschar formation at 24 hours and at 72 hours for intact skin to the values on abraded skin at 24 hours and at 72 hours (four values). Similarly, add the values for edema formation at 24 hours and at 72 hours for intact and abraded skin (four values). The total of the eight values is divided by four to give the primary irritation score; for example:

Skin reaction	Exposure time (hours)	Evaluation value
Erythema and eschar formation:		
Intact skin	24	2
Do	72	1
Abraded skin	24	3
Do	72	2
Subtotal		8
Edema formation:		
Intact skin	24	0
Do	72	1
Abraded skin	24	1
Do	72	2
Subtotal		4
Total		12

Thus, the primary irritation score is 12÷4=3.

§ 1500.42 Test for eye irritants.

(a)(1) Six albino rabbits are used for each test substance. Animal facilities for such procedures shall be so designed and maintained as to exclude sawdust, wood chips, or other extraneous materials that might produce eye irritation. Both eyes of each animal in the test group shall be examined before testing, and only those animals without eye defects or irritation shall be used. The animal is held firmly but gently until quiet. The test material is placed in one eye of each animal by gently pulling the lower lid away from the eyeball to form a cup into which the test substance is dropped. The lids are then gently held together for one second and the animal is released. The other eye, remaining untreated, serves as a control. For testing liquids, 0.1 milliliter is used. For solids or pastes, 100 milligrams of the test substance is used, except that for substances in flake, granule, powder, or other particulate form the amount that has a volume of 0.1 milliliter (after compacting as much as possible without crushing or altering the individual particles, such as by tapping the measuring container) shall be used whenever this volume weighs less than 100 milligrams. In such a case, the weight of the 0.1 milliliter test dose should be recorded. The eyes are not washed following instillation of test material except as noted below.

(2) The eyes are examined and the grade of ocular reaction is recorded at

24, 48, and 72 hours. Reading of reactions is facilitated by use of a binocular loupe, hand slit-lamp, or other expert means. After the recording of observations at 24 hours, any or all eyes may be further examined after applying fluorescein. For this optional test, one drop of fluorescein sodium ophthalmic solution U.S.P. or equivalent is dropped directly on the cornea. After flushing out the excess fluorescein with sodium chloride solution U.S.P. or equivalent, injured areas of the cornea appear yellow; this is best visualized in a darkened room under ultraviolet illumination. Any or all eyes may be washed with sodium chloride solution U.S.P. or equivalent after the 24-hour reading.

(b)(1) An animal shall be considered as exhibiting a positive reaction if the test substance produces at any of the readings ulceration of the cornea (other than a fine stippling), or opacity of the cornea (other than a slight dulling of the normal luster), or inflammation of the iris (other than a slight deepening of the folds (or rugae) or a slight circumcorneal injection of the blood vessels), or if such substance produces in the conjunctivae (excluding the cornea and iris) an obvious swelling with partial eversion of the lids or a diffuse crimson-red with individual vessels not easily discernible.

(2) The test shall be considered positive if four or more of the animals in the test group exhibit a positive reaction. If only one animal exhibits a positive reaction, the test shall be regarded as negative. If two or three animals a positive reaction, the test is repeated using a different group of six animals. The second test shall be considered positive if three or more of the animals exhibit a positive reaction. If only one or two animals in the second test exhibit a positive reaction, the test shall be repeated with a different group of six animals. Should a third test be needed, the substance will be regarded as an irritant if any animal exhibits a positive response.

(c) To assist testing laboratories and other interested persons in interpreting the results obtained when a substance is tested in accordance with the method described in paragraph (a) of this section, an "Illustrated Guide for Grading Eye Irritation by Hazardous Substances" will be sold by the Superintendent of Documents, U.S. Government Printing Office, Washington, D.C. 20402.[1] The guide will contain color plates depicting responses of varying intensity to specific test solutions. The grade of response and the substance used to produce the response will be indicated.

[38 FR 27012, Sept. 27, 1973; 38 FR 30105, Nov. 1, 1973; 62 FR 46667, Sept. 4, 1997]

§ 1500.43 Method of test for flashpoint of volatile flammable materials by Tagliabue open-cup apparatus.

SCOPE

1. (a) This method describes a test procedure for the determination of open-cup flashpoints of volatile flammable materials having flashpoints below 175 °F

(b) This method, when applied to paints and resin solutions which tend to skin over or which are very viscous, gives less reproducible results than when applied to solvents.

OUTLINE OF METHOD

2. The sample is placed in the cup of a Tag Open Tester, and heated at a slow but constant rate. A small test flame is passed at a uniform rate across the cup at specified intervals. The flashpoint is taken as the lowest temperature at which application of the test flame causes the vapor at the surface of the liquid to flash, that is, ignite but not continue to burn.

APPARATUS

3. The Tag open-cup tester is illustrated in Fig. 1. It consists of the following parts, which must conform to the dimensions shown, and have the additional characteristics as noted:

[1] The Illustrated Guide is out of print and, as of January 1, 1981, no longer available. However, information about the test method, and black and white photocopies may be obtained by writing to the Directorate for Epidemiology and Health Sciences, CPSC, Washington, D.C. 20207, (301) 504-0957.

APPENDIX F.3

Federal Regulations on Acute Toxicity
Department of Transportation (DOT)

Appendix F.3.1

49 CFR 173.132: Class 6, Division 6.1 – Definitions

49 CFR 173.133: Assignment of Packing Group and Hazard Zones for Division 6.1 Materials

Appendix F.3.2

Materials Assigned as Division 6.1 Materials

(6) *Type F.* Organic peroxide type F is an organic peroxide which will not detonate in a cavitated state, does not deflagrate, shows only a low, or no, effect if heated when confined, and has low, or no, explosive power.

(7) *Type G.* Organic peroxide type G is an organic peroxide which will not detonate in a cavitated state, will not deflagrate at all, shows no effect when heated under confinement, and shows no explosive power. A type G organic peroxide is not subject to the requirements of this subchapter for organic peroxides of Division 5.2 provided that it is thermally stable (self-accelerating decomposition temperature is 50 °C (122 °F) or higher for a 50 kg (110 pounds) package). An organic peroxide meeting all characteristics of type G except thermal stability and requiring temperature control is classed as a type F, temperature control organic peroxide.

(c) *Procedure for assigning an organic peroxide to a generic type.* An organic peroxide shall be assigned to a generic type based on—

(1) Its physical state (i.e., liquid or solid), in accordance with the definitions for liquid and solid in §171.8 of this subchapter;

(2) A determination as to its control temperature and emergency temperature, if any, under the provisions of §173.21(f); and

(3) Performance of the organic peroxide under the test procedures specified in the UN Manual of Tests and Criteria, and the provisions of paragraph (d) of this section.

(d) *Approvals.* (1) An organic peroxide must be approved, in writing, by the Associate Administrator for Hazardous Materials Safety, before being offered for transportation or transported, including assignment of a generic type and shipping description, except for—

(i) An organic peroxide which is identified by technical name in the Organic Peroxides Table in §173.225(b);

(ii) A mixture of organic peroxides prepared according to §173.225(c)(5); or

(iii) An organic peroxide which may be shipped as a sample under the provisions of §173.225(c).

(2) A person applying for an approval must submit all relevant data concerning physical state, temperature controls, and tests results or an approval issued for the organic peroxide by the competent authority of a foreign government.

(e) *Tests.* The generic type for an organic peroxide shall be determined using the testing protocol from Figure 20.1(a) (Classification and Flow Chart Scheme for Organic Peroxides) from the UN Manual of Tests and Criteria.

[Amdt. 173-224, 55 FR 52634, Dec. 21, 1990, as amended at 56 FR 66268, Dec. 20, 1991; Amdt. 173-234, 58 FR 51532, Oct. 1, 1993; Amdt. 173-241, 59 FR 67508, Dec. 29, 1994; Amdt. 173-261, 62 FR 24732, May 6, 1997]

§173.129 Class 5, Division 5.2—Assignment of packing group.

All Division 5.2 materials are assigned to Packing Group II in column 5 of the §172.101 table.

§173.132 Class 6, Division 6.1—Definitions.

(a) For the purpose of this subchapter, *poisonous material* (Division 6.1) means a material, other than a gas, which is known to be so toxic to humans as to afford a hazard to health during transportation, or which, in the absence of adequate data on human toxicity:

(1) Is presumed to be toxic to humans because it falls within any one of the following categories when tested on laboratory animals (whenever possible, animal test data that has been reported in the chemical literature should be used):

(i) *Oral Toxicity.* A liquid with an LD_{50} for acute oral toxicity of not more than 500 mg/kg or a solid with an LD_{50} for acute oral toxicity of not more than 200 mg/kg.

(ii) *Dermal Toxicity.* A material with an LD_{50} for acute dermal toxicity of not more than 1000 mg/kg.

(iii) *Inhalation Toxicity.* (A) A dust or mist with an LC_{50} for acute toxicity on inhalation of not more than 10 mg/L; or

(B) A material with a saturated vapor concentration in air at 20 °C (68 °F) of more than one-fifth of the LC_{50} for acute toxicity on inhalation of vapors and with an LC_{50} for acute toxicity on inhalation of vapors of not more than 5000 ml/m³; or

(2) Is an irritating material, with properties similar to tear gas, which

causes extreme irritation, especially in confined spaces.

(b) For the purposes of this subchapter—

(1) LD_{50} for acute oral toxicity means that dose of the material administered to both male and female young adult albino rats which causes death within 14 days in half the animals tested. The number of animals tested must be sufficient to give statistically valid results and be in conformity with good pharmacological practices. The result is expressed in mg/kg body mass.

(2) LD_{50} for acute dermal toxicity means that dose of the material which, administered by continuous contact for 24 hours with the shaved intact skin (avoiding abrading) of an albino rabbit, causes death within 14 days in half of the animals tested. The number of animals tested must be sufficient to give statistically valid results and be in conformity with good pharmacological practices. The result is expressed in mg/kg body mass.

(3) LC_{50} for acute toxicity on inhalation means that concentration of vapor, mist, or dust which, administered by continuous inhalation for one hour to both male and female young adult albino rats, causes death within 14 days in half of the animals tested. If the material is administered to the animals as a dust or mist, more than 90 percent of the particles available for inhalation in the test must have a diameter of 10 microns or less if it is reasonably foreseeable that such concentrations could be encountered by a human during transport. The result is expressed in mg/L of air for dusts and mists or in mL/m^3 of air (parts per million) for vapors. See §173.133(b) for LC_{50} determination for mixtures and for limit tests.

(i) When provisions of this subchapter require the use of the LC_{50} for acute toxicity on inhalation of dusts and mists based on a one-hour exposure and such data is not available, the LC_{50} for acute toxicity on inhalation based on a four-hour exposure may be multiplied by four and the product substituted for the one-hour LC_{50} for acute toxicity on inhalation.

(ii) When the provisions of this subchapter require the use of the LC_{50} for acute toxicity on inhalation of vapors based on a one-hour exposure and such data is not available, the LC_{50} for acute toxicity on inhalation based on a four-hour exposure may be multiplied by two and the product substituted for the one-hour LC_{50} for acute toxicity on inhalation.

(iii) A solid substance should be tested if at least 10 percent of its total mass is likely to be dust in a respirable range, e.g. the aerodynamic diameter of that particle-fraction is 10 microns or less. A liquid substance should be tested if a mist is likely to be generated in a leakage of the transport containment. In carrying out the test both for solid and liquid substances, more than 90% (by mass) of a specimen prepared for inhalation toxicity testing must be in the respirable range as defined in this paragraph (b)(3)(iii).

(c) For purposes of classifying and assigning packing groups to mixtures possessing oral or dermal toxicity hazards according to the criteria in §173.133(a)(1), it is necessary to determine the acute LD_{50} of the mixture. If a mixture contains more than one active constituent, one of the following methods may be used to determine the oral or dermal LD_{50} of the mixture:

(1) Obtain reliable acute oral and dermal toxicity data on the actual mixture to be transported;

(2) If reliable, accurate data is not available, classify the formulation according to the most hazardous constituent of the mixture as if that constituent were present in the same concentration as the total concentration of all active constituents; or

(3) If reliable, accurate data is not available, apply the formula:

$$\frac{C_A}{T_A} = \frac{C_B}{T_B} + \frac{C_Z}{T_Z} = \frac{100}{T_M}$$

where:

C = the % concentration of constituent A, B ... Z in the mixture;

T = the oral LD_{50} values of constituent A, B ... Z;

T_M = the oral LD_{50} value of the mixture.

NOTE TO FORMULA IN PARAGRAPH (C)(3): This formula also may be used for dermal toxicities provided that this information is available on the same species for all constituents. The use of this formula does not take into account any potentiation or protective phenomena.

(d) The foregoing categories shall not apply if the Associate Administrator for Hazardous Materials Safety has determined that the physical characteristics of the material or its probable hazards to humans as shown by documented experience indicate that the material will not cause serious sickness or death.

[Amdt. 173–224, 55 FR 52634, Dec. 21, 1990, as amended at 56 FR 66268, Dec. 20, 1991; Amdt. 173–234, 58 FR 51532, Oct. 1, 1993; Amdt. 173–261, 62 FR 24732, May 6, 1997; 62 FR 45702, August 28, 1997]

§ 173.133 Assignment of packing group and hazard zones for Division 6.1 materials.

(a) The packing group of Division 6.1 materials shall be as assigned in column 5 of the §172.101 table. When the §172.101 table provides more than one packing group or hazard zone for a hazardous material, the packing group and hazard zone shall be determined by applying the following criteria:

(1) The packing group assignment for routes of administration other than inhalation of vapors shall be in accordance with the following table:

Packing Group	Oral toxicity LC_{50} (mg/kg)	Dermal toxicity LD_{50} (mg/kg)	Inhalation toxicity by dusts and mists LC_{50} (mg/L)
I	≤ 5	≤ 40	≤ 0.5
II	> 5, ≤ 50	> 40, ≤ 200	> 0.5, ≤2
III	solids: > 50, ≤200; liquids: > 50, ≤ 500	> 200, ≤ 1000	> 2, ≤ 10

(2)(i) The packing group and hazard zone assignments for liquids (see §173.115(c) of this subpart for gases) based on inhalation of vapors shall be in accordance with the following table:

Packing Group	Vapor concentration and toxicity
I (Hazard Zone A)	V ≥ 500 LC_{50} and LC_{50} ≤ 200 mL/M³.
I (Hazard Zone B)	V ≥ 10 LC_{50}; LC_{50} < 1000 mL/m³; and the criteria for Packing Group I, Hazard Zone A are not met.
II	V ≥ LC_{50}; LC_{50} ≤ 3000 mL/m³; and the criteria for Packing Group I, are not met.
III	V ≥ .2 LC_{50}; LC_{50} ≤ 5000 mL/m³; and the criteria for Packing Groups I and II, are not met.

Note 1: V is the saturated vapor concentration in air of the material in mL/m³ at 20C° and standard atmospheric pressure.
Note 2: A liquid in Division 6.1 meeting criteria for Packing Group I, Hazard Zones A or B stated in paragraph (a)(2) of this section is a material poisonous by inhalation subject to the additional hazard communication requirements in §§ 172.203(m)(3), 172.313 and table 1 of §172.504(e) of this subchapter.

(ii) These criteria are represented graphically in Figure 1:

(d) The foregoing categories shall not apply if the Associate Administrator for Hazardous Materials Safety has determined that the physical characteristics of the material or its probable hazards to humans as shown by documented experience indicate that the material will not cause serious sickness or death.

[Amdt. 173–224, 55 FR 52634, Dec. 21, 1990, as amended at 56 FR 66268, Dec. 20, 1991; Amdt. 173–234, 58 FR 51532, Oct. 1, 1993; Amdt. 173–261, 62 FR 24732, May 6, 1997; 62 FR 45702, August 28, 1997]

§ 173.133 Assignment of packing group and hazard zones for Division 6.1 materials.

(a) The packing group of Division 6.1 materials shall be as assigned in column 5 of the § 172.101 table. When the § 172.101 table provides more than one packing group or hazard zone for a hazardous material, the packing group and hazard zone shall be determined by applying the following criteria:

(1) The packing group assignment for routes of administration other than inhalation of vapors shall be in accordance with the following table:

Packing Group	Oral toxicity LD_{50} (mg/kg)	Dermal toxicity LD_{50} (mg/kg)	Inhalation toxicity by dusts and mists LC_{50} (mg/L)
I	≤ 5	≤ 40	≤ 0.5
II	> 5, ≤ 50	> 40, ≤ 200	> 0.5, ≤2
III	solids: > 50, ≤ 200; liquids: > 50, ≤ 500	> 200, ≤ 1000	> 2, ≤ 10

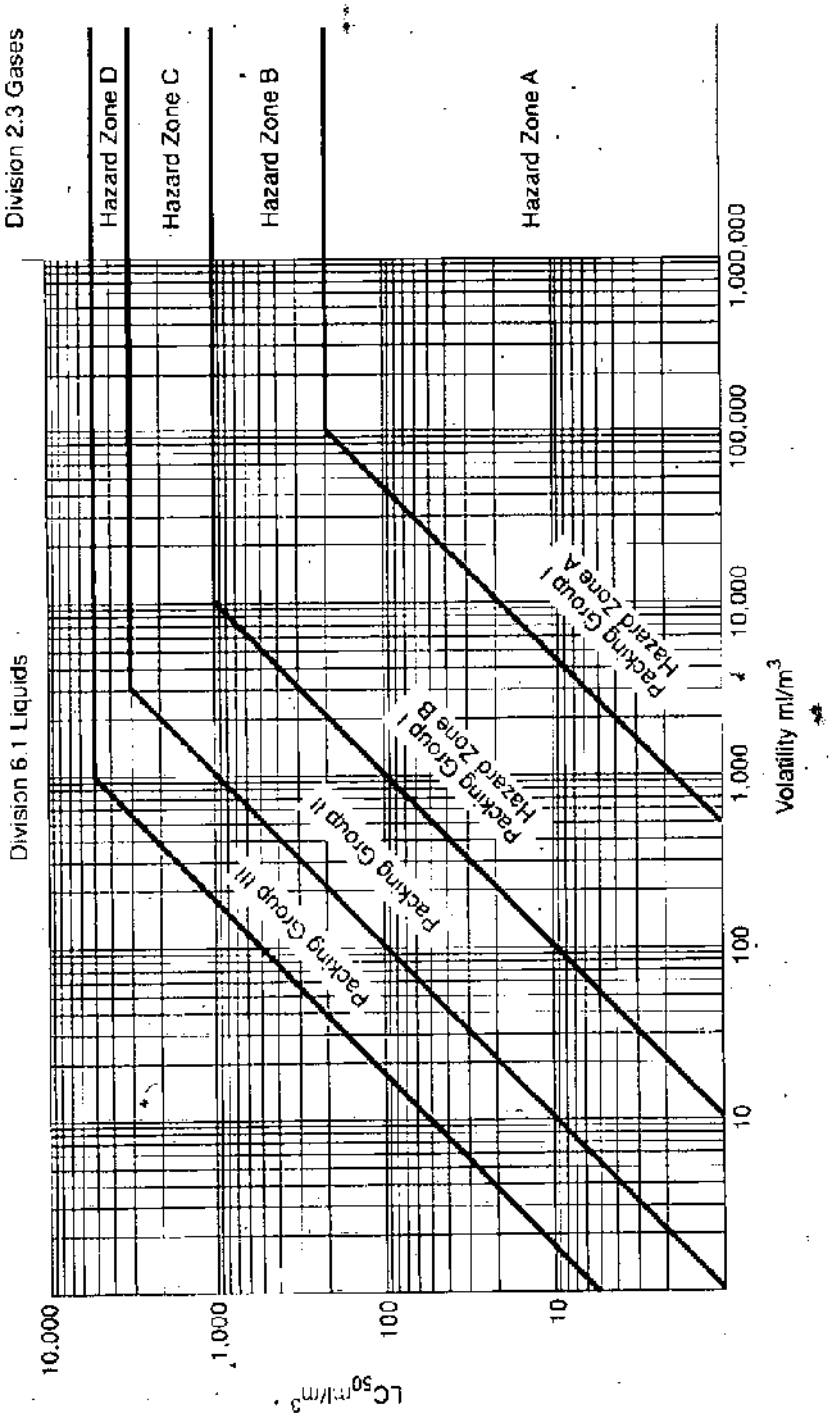
(2)(i) The packing group and hazard zone assignments for liquids (see § 173.115(c) of this subpart for gases) based on inhalation of vapors shall be in accordance with the following table:

Packing Group	Vapor concentration and toxicity
I (Hazard Zone A)	V ≥ 500 LC_{50} and LC_{50} ≤ 200 mL/M³.
I (Hazard Zone B)	V ≥ 10 LC_{50}; LC_{50} ≤ 1000 mL/m³; and the criteria for Packing Group I, Hazard Zone A are not met.
II	V ≥ LC_{50}; LC_{50} ≤ 3000 mL/m³; and the criteria for Packing Group I, are not met.
III	V ≥ .2 LC_{50}; LC_{50} ≤ 6000 mL/m³; and the criteria for Packing Groups I and II, are not met.

Note 1: V is the saturated vapor concentration in air of the material in mL/m³ at 20C° and standard atmospheric pressure.
Note 2: A liquid in Division 6.1 meeting criteria for Packing Group I, Hazard Zones A or B stated in paragraph (a)(2) of this section is a material poisonous by inhalation subject to the additional hazard communication requirements in §§ 172.203(m)(3), 172.313 and table 1 of § 172.504(e) of this subchapter.

(ii) These criteria are represented graphically in Figure 1:

Figure 1
Inhalation Toxicity: Packing Group and Hazard Zone Borderlines



(3) When the packing group determined by applying these criteria is different for two or more (oral, dermal or inhalation) routes of administration, the packing group assigned to the material shall be that indicated for the highest degree of toxicity for any of the routes of administration.

(4) Notwithstanding the provisions of this paragraph, the packing group and hazard zone of a tear gas substance is as assigned in column 5 of the §172.101 table.

(b) The packing group and hazard zone for Division 6.1 mixtures that are poisonous (toxic) by inhalation may be determined by one of the following methods:

(1) Where LC_{50} data is available on each of the poisonous (toxic) substances comprising the mixture—

(i) The LC_{50} of the mixture is estimated using the formula:

$$LC50\ (\text{mixture}) = \frac{1}{\sum_{i=1}^{n} \frac{f_i}{LC50_i}}$$

where

f_i = mole fraction of the i^{th} component substance of the liquid.

$LC50_i$ = mean lethal concentration of the i^{th} component substance in ml/m³

(ii) The volatility of each component substance is estimated using the formula:

$$V_i = P_i \times \frac{10^6}{101.3}\ ml/m^3$$

where:

P_i = partial pressure of the *ith* component substance in kPa at 20 °C and one atmospheric pressure. Pi may be calculated according to Raoult's Law using appropriate activity coefficients. Where activity coefficients are not available, the coefficient may be assumed to be 1.0.

(iii) The ratio of the volatility to the LC_{50} is calculated using the formula:

$$R = \sum_{i=1}^{n} \frac{V_i}{L_{c50i}}$$

(iv) Using the calculated values LC50 (mixture) and R, the packing group for the mixture is determined as follows:

Packaging group (hazard zone)	Ratio of volatility and LC_{50}
I (Hazard Zone A)	R ≥ 500 and LC_{50} (mixture) ≤ 200 ml/m³.
I (Hazard Zone B)	R ≥ 10 and LC_{50} (mixture) ≤ 1000 ml/m³; and the criteria for Packing Group I, Hazard Zone A, are not met.
II	R ≥ 1 and LC_{50} (mixture) ≤ 3000 ml/m³; and the criteria for Packing Group I, Hazard Zones A and B are not met.
III	R ≥ 1/6 and LC_{50} (mixture) ≤ 5000 ml/m³; and the criteria for Packing Group I, Hazard Zones A and B, and Packing Group II are not met.

(2) In the absence of LC50 data on the poisonous (toxic) constituent substances, the mixture may be assigned a packing group and hazard zone based on the following simplified threshold toxicity tests. When these threshold tests are used, the most restrictive packing group and hazard zone must be determined and used for the transportation of the mixture.

(i) A mixture is assigned to Packing Group I, Hazard Zone A only if both the following criteria are met:

(A) A sample of the liquid mixture is vaporized and diluted with air to create a test atmosphere of 200 ml/m³ vaporized mixture in air. Ten albino rats (five male and five female) are exposed to the test atmosphere as determined by an analytical method appropriate for the material being classified for one hour and observed for fourteen days. If five or more of the animals die within the fourteen-day observation period, the mixture is presumed to have an LC50 equal to or less than 200 ml/m³.

(B) A sample of the vapor in equilibrium with the liquid mixture is diluted with 499 equal volumes of air to form a test atmosphere. Ten albino rats (five male and five female) are exposed to the test atmosphere for one hour and observed for fourteen days. If five or more of the animals die within the fourteen-day observation period, the mixture is presumed to have a volatility equal to or greater than 500 times the mixture LC50.

(ii) A mixture is assigned to Packing Group I, Hazard Zone B only if both the following criteria are met, and the mixture does not meet the criteria for Packing Group I, Hazard Zone A:

(A) A sample of the liquid mixture is vaporized and diluted with air to create a test atmosphere of 1000 ml/m³ vaporized mixture in air. Ten albino rats (five male and five female) are exposed to the test atmosphere for one hour and observed for fourteen days. If five

or more of the animals die within the fourteen-day observation period, the mixture is presumed to have an LC50 equal to or less than 1000 ml/m³.

(B) A sample of the vapor in equilibrium with the liquid mixture is diluted with 9 equal volumes of air to form a test atmosphere. Ten albino rats (five male and five female) are exposed to the test atmosphere for one hour and observed for fourteen days. If five or more of the animals die within the fourteen-day observation period, the mixture is presumed to have a volatility equal to or greater than 10 times the mixture LC50.

(iii) A mixture is assigned to Packing Group II only if both the following criteria are met, and the mixture does not meet the criteria for Packing Group I (Hazard Zones A or B):

(A) A sample of the liquid mixture is vaporized and diluted with air to create a test atmosphere of 3000 ml/m³ vaporized mixture in air. Ten albino rats (five male and five female) are exposed to the test atmosphere for one hour and observed for fourteen days. If five or more of the animals die within the fourteen-day observation period, the mixture is presumed to have an LC50 equal to or less than 3000 ml/m³.

(B) A sample of the vapor in equilibrium with the liquid mixture is used to form a test atmosphere. Ten albino rats (five male and five female) are exposed to the test atmosphere for one hour and observed for fourteen days. If five or more of the animals die within the fourteen-day observation period, the mixture is presumed to have a volatility equal to or greater than the mixture LC50.

(iv) A mixture is assigned to Packing Group III only if both the following criteria are met, and the mixture does not meet the criteria for Packing Groups I (Hazard Zones A or B) or Packing Group II (Hazard Zone C):

(A) A sample of the liquid mixture is vaporized and diluted with air to create a test atmosphere of 5000 ml/m³ vaporized mixture in air. Ten albino rats (five male and five female) are exposed to the test atmosphere for one hour and observed for fourteen days. If five or more of the animals die within the fourteen-day observation period, the mixture is presumed to have an LC50 equal to or less than 5000 ml/m³.

(B) The vapor pressure of the liquid mixture is measured and if the vapor concentration is equal to or greater than 1000 ml/m³, the mixture is presumed to have a volatility equal to or greater than ⅕ the mixture LC50.

[Amdt. 173-224, 55 FR 52634, Dec. 21, 1990, as amended at 56 FR 66268-66270, Dec. 20, 1991; 57 FR 45461-45463, Oct. 1, 1992; Amdt. 173-234, 58 FR 51532, Oct. 1, 1993; Amdt. 173-138, 59 FR 49133, Sept. 26, 1994; Amdt. 173-255, 61 FR 50626, Sept. 26, 1996]

§ 173.134 Class 6, Division 6.2—Definitions, exceptions and packing group assignments.

(a) *Definitions.* For the purposes of this subchapter, the categories of materials that constitute Division 6.2 are defined as follows:

(1) An *infectious substance* means a viable microorganism, or its toxin, that causes or may cause disease in humans or animals, and includes those agents listed in 42 CFR 72.3 of the regulations of the Department of Health and Human Services and any other agent that causes or may cause severe, disabling or fatal disease. The terms *infectious substance* and *etiologic agent* are synonymous.

(2) A *diagnostic specimen* means any human or animal material including, but not limited to, excreta, secreta, blood, blood components, tissue, and tissue fluids, being shipped for purposes of diagnosis.

(3) A *biological product* means a material that is prepared and manufactured in accordance with the provisions of 9 CFR part 102 (Licenses for biological products), 9 CFR part 103 (Experimental products, distribution, and evaluation of biological products prior to licensing), 9 CFR part 104 (Permits for biological products), 21 CFR part 312 (Investigational new drug application), or 21 CFR parts 600 to 680 (Biologics).

(4) A *regulated medical waste* means a waste or reusable material, other than a culture or stock of an infectious substance, that contains an infectious substance and is generated in—

(i) The diagnosis, treatment or immunization of human beings or animals;

Department of Transportation List of Division 6.1 Materials

SYMBOL	PS_NAME1	HAZ CLASS	UN NUMBER	PACK GROUP	LABEL	SPEC_PROV	EXCEPTIONS	NONBULK	BULK	PASS_AIR	CARGO_AIR	VESSEL	VESSELSP
	2-Chloroethanal	6.1	UN2232	I	6.1	2, B9, B14, B32, B74, T38, T43, T45	None	227	244	Forbidden	Forbidden	D	40
	2-Methyl-2-heptanethiol	6.1	UN3023	I	6.1, 3	2, B9, B14, B32, B74, T38, T43, T45	None	227	244	Forbidden	Forbidden	D	40, 102
D	3,5-Dichloro-2,4,6-trifluoropyridine	6.1	NA9264	I	6.1	2, B9, B14, B32, B74, T38, T43, T45	None	227	244	Forbidden	Forbidden	A	40, 95
	Acetone cyanohydrin, stabilized	6.1	UN1541	I	6.1	2, A3, B5, B14, B32, B75, B77, N34, T38, T43, T45	None	227	244	Forbidden	30 L	D	95, 40, 49
	Acrolein, inhibited	6.1	UN1092	I	6.1, 3	1, B9, B14, B30, B42, B72, B77, T38, T43, T44	None	226	244	Forbidden	Forbidden	D	40
G	Alkaloids, liquid, n.o.s., or Alkaloid salts, liquid, n.o.s.	6.1	UN3140	I	6.1	A4, T42	None	201	243	1 L	30 L	A	
G	Alkaloids, solid, n.o.s. or Alkaloid salts, solid, n.o.s. poisonous	6.1	UN1544	I	6.1		None	211	242	5 kg	50 kg	A	
	Allyl alcohol	6.1	UN1098	I	6.1, 3	2, B9, B14, B32, B74, B77, T38, T43, T45	None	227	244	Forbidden	Forbidden	D	40
	Allyl chloroformate	6.1	UN1722	I	6.1, 3, 8	2, B9, B14, B32, B74, N41, T38, T43, T45	None	227	244	Forbidden	Forbidden	D	40
	Allylamine	6.1	UN2334	I	6.1, 3	2, B9, B14, B32, B74, T38, T43, T45	None	227	244	Forbidden	Forbidden	D	40
	Aluminum phosphide pesticides	6.1	UN3048	I	6.1	A8	None	211	242	Forbidden	15 kg	E	40, 85
	Arsenic acid, liquid	6.1	UN1553	I	6.1	T18, T27	None	201	243	1 L	30 L	B	40
	Arsenic compounds, liquid, n.o.s. inorganic, including arsenates n.o.s.; arsenites, n.o.s.; arsenic sulfides, n.o.s., and organic compounds of arsenic, n.o.s.	6.1	UN1556	I	6.1		None	201	243	1 L	30 L	B	40
	Arsenic compounds, solid, n.o.s. inorganic, including arsenates, n.o.s.; arsenites, n.o.s.; arsenic sulfides, n.o.s.; and organic compounds of arsenic, n.o.s.	6.1	UN1557	I	6.1		None	211	242	5 kg	50 kg	A	
	Arsenic trioxide	6.1	UN1561	I	6.1	2, B9, B14, B32, B74, T38, T43, T45	None	227	244	Forbidden	Forbidden	B	40
	Arsenical pesticides, liquid, toxic	6.1	UN2994	I	6.1	T42	None	201	243	1 L	30 L	B	40
	Arsenical pesticides, liquid, toxic, flammable flashpoint not less than 23 degrees C	6.1	UN2993	I	6.1, 3	T42	None	201	243	1 L	30 L	B	40
	Arsenical pesticides, solid, toxic	6.1	UN2759	I	6.1		None	211	242	5 kg	50 kg	A	40
	Barium cyanide	6.1	UN1565	I	6.1	N74, N75	None	211	242	5 kg	50 kg	A	26, 40
	Bipyridilium pesticides, liquid, toxic	6.1	UN3016	I	6.1	T42	None	201	243	1 L	30 L	B	40
	Bipyridilium pesticides, liquid, toxic, flammable, flashpoint not less than 23 degrees C	6.1	UN3015	I	6.1, 3	T42	None	201	243	1 L	30 L	B	21, 40
	Bipyridilium pesticides, solid, toxic	6.1	UN2781	I	6.1		None	211	242	5 kg	50 kg	A	40
	Bromobenzyl cyanides, liquid	6.1	UN1694	I	6.1	T18	None	201	243	Forbidden	30 L	D	12, 40
	Bromobenzyl cyanides, solid	6.1	UN1694	I	6.1	T18	None	211	242	Forbidden	50 kg	D	12, 40
	Brucine	6.1	UN1570	I	6.1		None	211	242	5 kg	50 kg	A	
	Cadmium compounds	6.1	UN2570	I	6.1		None	211	242	5 kg	50 kg	A	
	Calcium cyanide	6.1	UN1575	I	6.1	N79, N80	None	211	242	5 kg	50 kg	A	26, 40
	Carbamate pesticides, liquid, toxic	6.1	UN2992	I	6.1	T42	None	201	243	1 L	30 L	B	40
	Carbamate pesticides, liquid, toxic, flammable, flash point not less than 23 degrees C	6.1	UN2991	I	6.1, 3	T42	None	201	243	1 L	30 L	B	40
	Carbamate pesticides, solid, toxic	6.1	UN2757	I	6.1		None	211	242	5 kg	50 kg	A	40
	Chloroacetone, stabilized	6.1	UN1695	I	6.1, 3, 8	2, B9, B14, B32, B74, N12, N32, N34, T38, T43, T45	None	227	244	Forbidden	Forbidden	D	20, 40, 95
	Chloroacetyl chloride	6.1	UN1752	I	6.1, 8	2, A3, A6, A7, B3, B8, B9, B14, B32, B74, B77, N34, N43, T38, T43, T45	None	227	244	Forbidden	Forbidden	D	40
+	Chloropicrin	6.1	UN1580	I	6.1	2, B7, B9, B14, B32, B46, B74, T38, T43, T45	None	227	244	Forbidden	Forbidden	D	40
	Chloropicrin mixtures, n.o.s.	6.1	UN1583	I	6.1	5	None	201	243	Forbidden	Forbidden	C	40
D	Chloropivaloyl chloride	6.1	NA9263	I	6.1, 8	2, B9, B14, B32, B74, T38, T43, T45	None	227	244	Forbidden	Forbidden	B	40
D, G	Compounds, tree killing, liquid or Compounds, weed killing, liquid	6.1	NA2810	I	6.1		None	201	243	1 L	30 L	B	40
	Copper based pesticides, liquid, toxic	6.1	UN3010	I	6.1	T42	None	201	243	1 L	30 L	B	40
	Copper based pesticides, liquid, toxic, flammable flashpoint not less than 23 degrees C	6.1	UN3009	I	6.1, 3	T42	None	201	243	1 L	30 L	B	40

Department of Transportation List of Division 6.1 Materials

SYMBOL	PS_NAME1	HAZ CLASS	UN NUMBER	PACK GROUP	LABEL	SPEC_PROV	EXCEPTIONS	NONBULK	BULK	PASS_AIR	CARGO_AIR	VESSEL	VESSELSP
	Copper based pesticides, solid, toxic	6.1	UN2775	I	6.1		None	211	242	5 kg	50 kg	A	40
	Coumarin derivative pesticides, liquid, toxic	6.1	UN3026	I	6.1		None	201	243	1 L	30 L	B	40
	Coumarin derivative pesticides, liquid, toxic, flammable flashpoint not less than 23 degrees C	6.1	UN3025	I	6.1, 3		None	201	243	1 L	30 L	B	40
	Coumarin derivative pesticides, solid, toxic	6.1	UN3027	I	6.1		None	211	242	5 kg	50 kg	A	40
	Crotonaldehyde, stabilized	6.1	UN1143	I	6.1, 3	2, B9, B14, B32, B74, B77, T38, T43, T45	None	227	244	Forbidden	30 L	B	40
	Cyanide solutions, n.o.s.	6.1	UN1935	I	6.1	B37, T18, T26	None	201	243	1 L	30 L	B	40, 52
	Cyanides, inorganic, solid, n.o.s.	6.1	UN1588	I	6.1	N74, N75	None	211	242	5 kg	50 kg	A	52
	Cyanogen bromide	6.1	UN1889	I	6.1, 8	A6, A9	None	211	242	1 kg	15 kg	D	40
	Cyclohexyl isocyanate	6.1	UN2488	I	6.1, 3	2, B9, B14, B32, B74, B77, T38, T43, T45	None	227	244	Forbidden	Forbidden	D	20, 40, 95
	Dichlorodimethyl ether, symmetrical	6.1	UN2249	I	6.1	T25	None	201	243	Forbidden	Forbidden	D	40
	Diketene, inhibited	6.1	UN2521	I	6.1, 3	2, B9, B14, B32, B74, T38, T43, T45	None	227	244	Forbidden	Forbidden	D	40, 49
	Dimethyl sulfate	6.1	UN1595	I	6.1, 8	2, B9, B14, B32, B74, B77, T38, T43, T45	None	227	244	Forbidden	Forbidden	D	40
	Dimethylhydrazine, symmetrical	6.1	UN2382	I	6.1, 3	2, B9, B14, B32, B74, B77, T38, T43, T45	None	227	244	Forbidden	Forbidden	D	40
	Dimethylhydrazine, unsymmetrical	6.1	UN1163	I	6.1, 3, 8	2, B7, B9, B14, B32, B74, T38, T43, T45	None	227	244	Forbidden	Forbidden	D	21, 38, 40, 100
	Diphenylamine chloroarsine	6.1	UN1698	I	6.1		None	201	None	Forbidden	Forbidden	D	40
	Diphenylchloroarsine, liquid	6.1	UN1699	I	6.1	A8, B14, B32, N33, N34	None	201	243	Forbidden	30 L	D	40
	Diphenylchloroarsine, solid	6.1	UN1699	I	6.1	A8, B14, B32, N33, N34	None	211	242	Forbidden	15 kg	D	40
G	Disinfectants, liquid, toxic, n.o.s	6.1	UN3142	I	6.1	A4, T42	None	201	243	1 L	30 L	A	40
G	Dyes, solid, toxic, n.o.s. or Dye intermediates, solid, toxic, n.o.s.	6.1	UN3143	I	6.1	A5	None	211	242	5 kg	50 kg	A	
	Epibromohydrin	6.1	UN2558	I	6.1, 3	T18, T26	None	201	243	Forbidden	Forbidden	D	40
	Ethyl chloroformate	6.1	UN1182	I	6.1, 3, 8	2, A3, A6, A7, B9, B14, B32, B74, N34, T38, T43, T45	None	227	244	Forbidden	Forbidden	D	21, 40, 100
D	Ethyl phosphonothioic dichloride, anhydrous	6.1	NA2927	I	6.1, 8	2, B9, B14, B32, B74, T38, T43, T45	None	227	244	Forbidden	Forbidden	D	20, 40, 95
D	Ethyl phosphorodichloridate	6.1	NA2927	I	6.1, 8	2, B9, B14, B32, B74, T38, T43, T45	None	227	244	Forbidden	Forbidden	D	20, 40, 95
D	Ethyl phosphorus dichloride, anhydrous pyrophoric liquid	6.1	NA2845	I	6.1, 4.2	2, B9, B14, B32, B74, T38, T43, T45	None	227	244	Forbidden	Forbidden	D	18
	Ethyldichloroarsine	6.1	UN1892	I	6.1	2, B9, B14, B32, B74, T38, T43, T45	None	227	244	Forbidden	Forbidden	D	40
	Ethylene chlorohydrin	6.1	UN1135	I	6.1, 3	2, B9, B14, B32, B74, T38, T43, T45	None	227	244	Forbidden	Forbidden	D	40
	Ethylene dibromide	6.1	UN1605	I	6.1	2, B9, B14, B32, B74, B77, T38, T43, T45	None	227	244	Forbidden	Forbidden	D	40
	Ethyleneimine, inhibited	6.1	UN1185	I	6.1, 3	1, B9, B14, B30, B72, B77, N25, N32, T38, T43, T44	None	226	244	Forbidden	Forbidden	D	40
	Fluoroacetic acid	6.1	UN2642	I	6.1	B100	None	211	242	1 kg	15 kg	E	
	Hexachlorocyclopentadiene	6.1	UN2646	I	6.1	2, B9, B14, B32, B74, B77, T38, T43, T45	None	227	244	Forbidden	Forbidden	D	40
	Hydrocyanic acid, aqueous solutions or Hydrogen cyanide, aqueous solutions with not more than 20 percent hydrogen cyanide	6.1	UN1613	I	6.1	2, B61, B65, B77, B82	None	195	244	Forbidden	Forbidden	D	40
	Hydrogen cyanide, solution in alcohol with not more than 45 percent hydrogen cyanide	6.1	UN3294	I	6.1, 3	2, 25, B9, B14, B32, B74, T38, T43, T45	None	227	244	Forbidden	Forbidden	D	40
	Hydrogen cyanide, stabilized with less than 3 percent water	6.1	UN1051	I	6.1, 3	1, B35, B61, B65, B77, B82	None	195	244	Forbidden	Forbidden	D	40
	Hydrogen cyanide, stabilized, with less than 3 percent water and absorbed in a porous inert material	6.1	UN1614	I	6.1	5	None	195	None	Forbidden	Forbidden	D	25, 40
	Iron pentacarbonyl	6.1	UN1994	I	6.1, 3	1, B9, B14, B30, B72, B77, T38, T43, T44	None	192	244	Forbidden	Forbidden	D	40
D	Isobutyl chloroformate	6.1	NA2742	I	6.1, 3, 8	2, B9, B14, B32, B74, T38, T43, T45	None	227	244	1 L	30 L	A	12, 13, 22, 25, 40, 48, 100
	Isopropyl chloroformate	6.1	UN2407	I	6.1, 3, 8	2, B9, B14, B32, B74, B77, T38, T43, T45	None	227	244	Forbidden	Forbidden	B	40
+	Mercuric potassium cyanide	6.1	UN1626	I	6.1	N74, N75	None	211	242	5 kg	50 kg	A	26
	Mercury based pesticides, liquid, toxic	6.1	UN3012	I	6.1	T42	None	201	243	1 L	30 L	B	40

Department of Transportation List of Division 6.1 Materials

SYMBOL	PS_NAME1	HAZ CLASS	UN NUMBER	PACK GROUP	LABEL	SPEC_PROV	EXCEPTIONS	NONBULK	BULK	PASS_AIR	CARGO_AIR	VESSEL	VESSELSP
	Mercury based pesticides, liquid, toxic, flammable, flashpoint not less than 23 degrees C	6.1	UN3011	I	6.1, 3	T42	None	201	243	1 L	30 L	B	40
	Mercury based pesticides, solid, toxic	6.1	UN2777	I	6.1		None	211	242	5 kg	50 kg	A	40
	Mercury compounds, liquid, n.o.s.	6.1	UN2024	I	6.1		None	201	243	1 L	30 L	B	40
	Mercury compounds, solid, n.o.s.	6.1	UN2025	I	6.1		None	211	242	5 kg	50 kg	A	
	Metal carbonyls, n.o.s.	6.1	UN3281	I	6.1	5	None	201	243	1 L	30 L	B	40
	Methanesulfonyl chloride	6.1	UN3246	I	6.1, 8	2, 25, B9, B14, B32, B74, T38, T43, T45	None	227	244	Forbidden	Forbidden	D	40
	Methyl bromide and ethylene dibromide mixtures, liquid	6.1	UN1647	I	6.1	2, B9, B14, B32, B74, N65, T38, T43, T45	None	227	244	Forbidden	30 L	C	40
	Methyl chloroacetate	6.1	UN2295	I	6.1, 3	T42	None	201	243	1 L	30 L	D	
	Methyl chloroformate	6.1	UN1238	I	6.1, 3, 8	1, B9, B14, B30, B72, N34, T38, T43, T44	None	226	244	Forbidden	Forbidden	D	21, 40, 100
	Methyl chloromethyl ether	6.1	UN1239	I	6.1, 3	1, B9, B14, B30, B72, T38, T43, T44	None	226	244	Forbidden	Forbidden	D	40
	Methyl iodide	6.1	UN2644	I	6.1	2, B9, B14, B32, B74, T38, T43, T45	None	227	244	Forbidden	Forbidden	A	12, 40
	Methyl isocyanate	6.1	UN2480	I	6.1, 3	1, B9, B14, B30, B72, T38, T43, T44	None	226	244	Forbidden	Forbidden	D	26, 40
	Methyl isothiocyanate	6.1	UN2477	I	6.1, 3	2, B9, B14, B32, B74, T38, T43, T45	None	227	244	Forbidden	Forbidden	A	
	Methyl orthosilicate	6.1	UN2606	I	6.1, 3	2, B9, B14, B32, B74, T38, T43, T45	None	227	244	Forbidden	30 L	E	40
D	Methyl phosphonic dichloride	6.1	NA9206	I	6.1, 8	2, A3, B9, B14, B32, B74, N34, N43, T38, T43, T45	None	227	244	Forbidden	Forbidden	C	
D	Methyl phosphorus dichloride, pyrophoric liquid	6.1	NA2845	I	6.1, 4.2	2, B9, B14, B16, B32, B74, T38, T43, T45	None	227	244	Forbidden	Forbidden	D	18
	Methyl vinyl ketone, stabilized	6.1	UN1251	I	6.1, 3, 8	1, 25, B9, B14, B30, B72, T38, T43, T44	None	226	244	Forbidden	Forbidden	B	40
D	Methyldichloroarsine	6.1	NA1556	I	6.1	2	None	192	None	Forbidden	Forbidden	D	40, 95
	Methylhydrazine	6.1	UN1244	I	6.1, 3, 8	1, B7, B9, B14, B30, B72, B77, N34, T38, T43, T44	None	226	244	Forbidden	Forbidden	D	21, 40, 49, 100
+	Motor fuel anti-knock mixtures	6.1	UN1649	I	6.1, 3	14, B9, B90, T26, T39	None	201	244	Forbidden	30 L	D	25, 40
	n-Butyl chloroformate	6.1	UN2743	I	6.1, 8, 3	2, B9, B14, B32, B74, T38, T43, T45	None	227	244	1 L	30 L	A	12, 13, 21, 25, 40, 100
	n-Butyl isocyanate	6.1	UN2485	I	6.1, 3	2, A7, B9, B14, B32, B74, B77, T38, T43, T45	None	227	244	Forbidden	30 L	D	40
	Nickel carbonyl	6.1	UN1259	I	6.1, 3	1	None	198	None	Forbidden	Forbidden	D	18, 40
	Nicotine compounds, liquid, n.o.s. or Nicotine preparations, liquid, n.o.s.	6.1	UN3144	I	6.1	A4, T42	None	201	243	1 L	30 L	B	40
	Nicotine compounds, solid, n.o.s. or Nicotine preparations, solid, n.o.s.	6.1	UN1655	I	6.1		None	211	242	5 kg	50 kg	B	
G	Nitriles, toxic, flammable, n.o.s.	6.1	UN3275	I	6.1, 3	5	None	201	243	1 L	30 L	B	40
G	Nitriles, toxic, n.o.s.	6.1	UN3276	I	6.1	6	None	201	243	1 L	30 L	B	
	n-Propyl chloroformate	6.1	UN2740	I	6.1, 3, 8	2, A3, A6, A7, B9, B14, B32, B74, B77, N34, T38, T43, T45	None	227	244	Forbidden	2.5 L	B	21, 40, 100
	n-Propyl isocyanate	6.1	UN2482	I	6.1, 3	1, B9, B14, B30, B72, T38, T43, T44	None	226	244	Forbidden	Forbidden	D	40
	Organoarsenic compound, n.o.s.	6.1	UN3280	I	6.1	5	None	211	242	5 kg	50 kg	B	
	Organochlorine pesticides, liquid, toxic	6.1	UN2996	I	6.1	T42	None	201	243	1 L	30 L	B	40
	Organochlorine pesticides, liquid, toxic, flammable, flashpoint not less than 23 degrees C	6.1	UN2995	I	6.1, 3	T42	None	201	243	1 L	30 L	B	40
	Organochlorine pesticides, solid toxic	6.1	UN2761	I	6.1		None	211	242	5 kg	50 kg	A	40
G	Organometallic compound, toxic n.o.s.	6.1	UN3282	I	6.1	B106	None	211	242	5 kg	50 kg	B	
	Organophosphorus compound, toxic n.o.s.	6.1	UN3278	I	6.1	5	None	201	243	1 L	30 L	B	
	Organophosphorus compound, toxic, flammable, n.o.s.	6.1	UN3279	I	6.1, 3	5	None	201	243	1 L	30 L	B	40
	Organophosphorus pesticides, liquid, toxic	6.1	UN3018	I	6.1	N76, T42	None	201	243	1 L	30 L	B	40
	Organophosphorus pesticides, liquid, toxic, flammable, flashpoint not less than 23 degrees C	6.1	UN3017	I	6.1, 3	N76, T42	None	201	243	1 L	30 L	B	40
	Organophosphorus pesticides, solid, toxic	6.1	UN2783	I	6.1	N77	None	211	242	5 kg	50 kg	A	40
	Organotin compounds, liquid, n.o.s.	6.1	UN2788	I	6.1	A3, N33, N34, T42	None	201	243	1 L	30 L	B	40
	Organotin compounds, solid, n.o.s.	6.1	UN3146	I	6.1	A5	None	211	242	5 kg	50 kg	B	40
	Organotin pesticides, liquid, toxic	6.1	UN3020	I	6.1	T42	None	201	243	1 L	30 L	B	40

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SYMBOL	PS_NAME1	HAZ CLASS	UN NUMBER	PACK GROUP	LABEL	SPEC_PROV	EXCEPTIONS	NONBULK	BULK	PASS_AIR	CARGO_AIR	VESSELSP
	Organotin pesticides, liquid, toxic, flammable, flashpoint not less than 23 degrees C	6.1	UN3019	I	6.1, 3	T42	None	201	243	1 L	30 L	B40
	Organotin pesticides, solid, toxic	6.1	UN2786	I	6.1		None	211	242	5 kg	50 kg	A40
	Osmium tetroxide	6.1	UN2471	I	6.1	A8, B100, N33, N34	None	211	242	5 kg	50 kg	B40
D	Parathion	6.1	NA2783	I	6.1	T42	None	201	243	Forbidden	1 L	A40
	Perchloromethyl mercaptan	6.1	UN1670	I	6.1	2, A3, A7, B9, B14, B32, B74, N34, T38, T43, T45	None	227	244	Forbidden	Forbidden	D40
G	Pesticides, liquid, toxic, flammable, n.o.s. flashpoint not less than 23 degrees C	6.1	UN2903	I	6.1, 3	T42	None	201	243	1 L	30 L	B40
G	Pesticides, liquid, toxic, n.o.s.	6.1	UN2902	I	6.1	T42	None	201	243	1 L	30 L	B40
G	Pesticides, solid, toxic, n.o.s.	6.1	UN2588	I	6.1		None	211	242	5 kg	50 kg	A40
	Phenoxyacetic acid derivative pesticide, liquid, toxic, flammable, flashpoint not less than 23°C	6.1	UN3347	I	6.1, 3	T24, T26	None	201	243	1 L	30 L	B40
	Phenoxyacetic acid derivative pesticide, liquid, toxic	6.1	UN3348	I	6.1	T24, T26	None	201	243	1 L	30 L	B40
	Phenoxyacetic acid derivative pesticide, solid, toxic	6.1	UN3345	I	6.1		None	211	242	5 kg	50 kg	A40
	Phenyl isocyanate	6.1	UN2487	I	6.1, 3	2, B9, B14, B32, B74, B77, N33, N34, T38, T43, T45	None	227	244	Forbidden	Forbidden	D20, 40, 96
	Phenylmercaptan	6.1	UN2337	I	6.1, 3	2, B9, B14, B32, B74, B77, T38, T43, T45	None	227	244	Forbidden	Forbidden	B26, 40
	Phenyl urea pesticides, liquid, toxic	6.1	UN3002	I	6.1	T42	None	201	243	1 L	30 L	B40
	Phenylcarbylamine chloride	6.1	UN1672	I	6.1	2, B9, B14, B32, B74, T38, T43, T45	None	227	244	Forbidden	Forbidden	D40
	Phenylmercuric compounds, n.o.s.	6.1	UN2026	I	6.1		None	211	242	5 kg	50 kg	A
	Phosphorus trichloride	6.1	UN1809	I	6.1, 8	2, B9, B14, B15, B32, B74, B77, N34, T38, T43, T45	None	227	244	Forbidden	Forbidden	C40
	Potassium cyanide	6.1	UN1680	I	6.1	B69, B77, N74, N75, T18, T26	None	211	242	5 kg	50 kg	B52
	Potassium fluoroacetate	6.1	UN2628	I	6.1		None	211	242	5 kg	50 kg	E
	Pyrethroid pesticide, liquid toxic	6.1	UN3352	I	6.1		None	211	242	1 L	30 L	A40
	Pyrethroid pesticide, liquid, flammable, toxic, flashpoint not less than 23°C	6.1	UN3351	I	6.1, 3	T24, T26	None	201	243	1 L	30 L	B40
	Pyrethroid pesticide, solid, toxic	6.1	UN3349	I	6.1		None	211	242	5 kg	50 kg	A40
D	sec-Butyl chloroformate	6.1	NA2742	I	6.1, 3, 8	2, B9, B14, B32, B74, T38, T43, T45	None	227	244	1 L	30 L	A12, 13, 22, 25, 40, 48, 100
	Selenates or Selenites	6.1	UN2630	I	6.1		None	211	242	5 kg	50 kg	E
	Selenium compound, n.o.s.	6.1	UN3283	I	6.1		None	211	242	5 kg	50 kg	B
D	Selenium oxide	6.1	NA2811	I	6.1		None	211	242	5 kg	50 kg	B
	Sodium cuprocyanice, solid	6.1	UN2316	I	6.1		None	211	242	5 kg	50 kg	A26
	Sodium cuprocyanide, solution	6.1	UN2317	I	6.1	T8, T26	None	201	243	1 L	30 L	B26, 40
	Sodium cyanide	6.1	UN1689	I	6.1	B69, B77, N74, N75, T42	None	211	242	5 kg	50 kg	B52
	Sodium fluoroacetate	6.1	UN2629	I	6.1		None	211	242	5 kg	50 kg	E
	Strychnine or Strychnine salts	6.1	UN1692	I	6.1		None	211	242	5 kg	50 kg	A40
	Substituted nitrophenol pesticides, liquid, toxic	6.1	UN3014	I	6.1	T42	None	201	243	1 L	30 L	B40
	Substituted nitrophenol pesticides, liquid, toxic, flammable flashpoint not less than 23 degrees C	6.1	UN3013	I	6.1, 3	T42	None	201	243	1 L	30 L	B40
	Substituted nitrophenol pesticides, solid, toxic	6.1	UN2779	I	6.1		None	211	242	5 kg	50 kg	A40
D	Tear gas devices with more than 2 percent tear gas substances, by mass	6.1	NA1693	I	6.1		None	340	None	Forbidden	Forbidden	D40
G	Tear gas substances, liquid, n.o.s.	6.1	UN1693	I	6.1		None	201	None	Forbidden	Forbidden	D40
G	Tear gas substances, solid, n.o.s	6.1	UN1693	I	6.1		None	211	None	Forbidden	Forbidden	D40
	Tellurium compound, n.o.s.	6.1	UN3284	I	6.1		None	211	242	5 kg	50 kg	B
	tert-Butyl isocyanate	6.1	UN2484	I	6.1, 3	1, A7, B9, B14, B30, B72, T38, T43, T44	None	226	244	Forbidden	Forbidden	D40
D	Tetraethyl lead, liquid	6.1	NA1649	I	6.1, 3		None	201	None	Forbidden	Forbidden	B40
D	Tetraethyl pyrophosphate solid	6.1	NA2783	I	6.1	N77	None	211	242	Forbidden	50 kg	A40
D	Tetraethyl pyrophosphate, liquid	6.1	NA3018	I	6.1		None	201	243	Forbidden	1 L	A40
	Thiocarbamate pesticides, liquid, flammable, toxic, flash point not less than 23 degrees C	6.1	UN3005	I	6.1, 3	T42	None	201	243	1 L	30 L	B40
	Thiocarbamate pesticides, liquid, toxic	6.1	UN3006	I	6.1	T42	None	201	243	1 L	30 L	B40
	Thiocarbamate pesticides, solid, toxic	6.1	UN2771	I	6.1		None	211	242	5 kg	50 kg	A40
G	Toxic liquid, corrosive, inorganic, n.o.s.	6.1	UN3289	I	6.1, 8	T42	None	201	243	0.5 L	2.5 L	A
G	Toxic liquid, corrosive, inorganic, n.o.s. Inhalation Hazard, Packing Group I, Zone A	6.1	UN3289	I	6.1, 8	1, B9, B14, B30, B72, T38, T43, T44	None	226	244	Forbidden	Forbidden	B40

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SYMBOL	PS_NAME1	HAZ CLASS	UN NUMBER	PACK GROUP	LABEL	SPEC_PROV	EXCEPTIONS	NONBULK	BULK	PASS_AIR	CARGO_AIR	VESSEL	VESSELSP
G	Toxic liquid, corrosive, inorganic, n.o.s. Inhalation hazard, Packing Group I, Zone B	6.1	UN3289	I	6.1, 8	2, B9, B14, B32, B74, T38, T43, T45	None	227	244	Forbidden	Forbidden	B	40
G	Toxic liquid, inorganic, n.o.s.	6.1	UN3287	I	6.1	T42	None	201	243	1 L	30 L	A	
	Toxic liquid, inorganic, n.o.s. Inhalation Hazard, Packing Group I, Zone A	6.1	UN3287	I	6.1	1, B9, B14, B30, B72, T38, T43, T44	None	226	244	Forbidden	Forbidden	B	40
	Toxic liquid, inorganic, n.o.s. Inhalation Hazard, Packing Group I, Zone B	6.1	UN3287	I	6.1	2, B9, B14, B32, B74, T38, T43, T45	None	227	244	Forbidden	Forbidden	B	40
G	Toxic liquids, corrosive, organic, n.o.s.	6.1	UN2927	I	6.1, 8	T42	None	201	243	0.5 L	2.5 L	B	40
G	Toxic liquids, corrosive, organic, n.o.s., Inhalation hazard, Packing Group I, Zone A	6.1	UN2927	I	6.1, 8	1, B9, B14, B30, B72, T38, T43, T44	None	226	244	Forbidden	Forbidden	D	20, 40, 95
G	Toxic liquids, corrosive, organic, n.o.s., Inhalation hazard, Packing Group I, Zone B	6.1	UN2927	I	6.1, 8	2, B9, B14, B32, B74, T38, T43, T45	None	227	244	Forbidden	Forbidden	D	20, 40, 95
G	Toxic liquids, flammable, organic, n.o.s.	6.1	UN2929	I	6.1, 3	T42	None	201	243	1 L	30 L	B	40
G	Toxic liquids, flammable, organic, n.o.s., Inhalation hazard, Packing Group I, Zone A	6.1	UN2929	I	6.1, 3	1, B9, B14, B30, B72, T38, T43, T44	None	226	244	Forbidden	Forbidden	D	20, 40, 95
G	Toxic liquids, flammable, organic, n.o.s., Inhalation hazard, Packing Group I, Zone B	6.1	UN2929	I	6.1, 3	2, B9, B14, B32, B74, T38, T43, T45	None	227	244	Forbidden	Forbidden	D	20, 40, 95
G	Toxic liquids, oxidizing, n.o.s.	6.1	UN3122	I	6.1, 5.1	A4	None	201	243	Forbidden	2.5 L	C	
G	Toxic liquids, oxidizing, n.o.s. Inhalation hazard, Packing Group I, Zone A	6.1	UN3122	I	6.1, 5.1	1, B9, B14, B30, B72, T38, T43, T44	None	226	244	Forbidden	Forbidden	C	
G	Toxic liquids, oxidizing, n.o.s. Inhalation Hazard, Packing Group I, Zone B	6.1	UN3122	I	6.1, 5.1	2, B9, B14, B32, T38, T43, T45	None	227	244	Forbidden	Forbidden	C	
	Toxic liquids, water-reactive, n.o.s.	6.1	UN3123	I	6.1, 4.3	A4	None	201	243	Forbidden	1 L	B	40
G	Toxic liquids, water-reactive, n.o.s. Inhalation hazard, packing group I, Zone A	6.1	UN3123	I	6.1, 4.3	1, B9, B14, B30, B72, T38, T43, T44	None	226	244	Forbidden	Forbidden	B	40
G	Toxic liquids, water-reactive, n.o.s. Inhalation hazard, packing group I, Zone B	6.1	UN3123	I	6.1, 4.3	2, B9, B14, B32, B74, T38, T43, T45	None	227	244	Forbidden	Forbidden	B	40
	Toxic solid, corrosive, inorganic, n.o.s.	6.1	UN3290	I	6.1, 8		None	211	242	1 kg	25 kg	A	
G	Toxic solid, inorganic, n.o.s.	6.1	UN3288	I	6.1		None	211	242	5 kg	50 kg	A	
G	Toxic solids, corrosive, organic, n.o.s.	6.1	UN2928	I	6.1, 8		None	211	242	1 kg	25 kg	B	40
G	Toxic solids, flammable, organic, n.o.s.	6.1	UN2930	I	6.1, 4.1	B106	None	211	242	1 kg	15 kg	B	
G	Toxic solids, organic, n.o.s.	6.1	UN2811	I	6.1		None	211	242	5 kg	50 kg	B	
G	Toxic solids, oxidizing, n.o.s.	6.1	UN3086	I	6.1, 5.1		None	211	242	1 kg	15 kg	C	
G	Toxic solids, self-heating, n.o.s.	6.1	UN3124	I	6.1, 4.2	A5, B100	None	211	242	5 kg	15 kg	D	40
G	Toxic solids, water-reactive, n.o.s.	6.1	UN3125	I	6.1, 4.3	A5, B100	None	211	242	5 kg	15 kg	D	40
G	Toxic, liquids, organic, n.o.s.	6.1	UN2810	I	6.1	T42	None	201	243	1 L	30 L	B	40
G	Toxic, liquids, organic, n.o.s. Inhalation hazard, Packing Group I, Zone A	6.1	UN2810	I	6.1	1, B9, B14, B30, B72, T38, T43, T44	None	226	244	Forbidden	Forbidden	D	20, 40, 95
G	Toxic, liquids, organic, n.o.s. Inhalation hazard, Packing Group I, Zone B	6.1	UN2810	I	6.1	2, B9, B14, B32, B74, T38, T43, T45	None	227	244	Forbidden	Forbidden	D	20, 40, 95
	Triazine pesticides, liquid, toxic	6.1	UN2998	I	6.1	T42	None	201	243	1 L	30 L	B	40
	Triazine pesticides, liquid, toxic, flammable, flashpoint not less than 23 degrees C	6.1	UN2997	I	6.1, 3	T42	None	201	243	1 L	30 L	B	40
	Triazine pesticides, solid, toxic	6.1	UN2763	I	6.1		None	211	242	5 kg	50 kg	A	40
D	Trimethoxysilane	6.1	NA9269	I	6.1, 3	2, B9, B14, B32, B74, T38, T43, T45	None	227	244	Forbidden	Forbidden	E	40
	Trimethylacetyl chloride	6.1	UN2438	I	6.1, 8, 3	2, A3, A6, A7, B3, B9, B14, B32, B74, N34, T38, T43, T45	None	227	244	Forbidden	Forbidden	D	25, 40
	Vanadium compound, n.o.s.	6.1	UN3285	I	6.1		None	211	242	5 kg	50 kg	B	
	Zinc cyanide	6.1	UN1713	I	6.1		None	211	242	5 kg	50 kg	A	26
	1,1-Dichloro-1-nitroethane	6.1	UN2650	II	6.1	T8	None	202	243	5 L	60 L	A	12, 40
	1,2-Dibromobutan-3-one	6.1	UN2648	II	6.1		None	202	243	5 L	60 L	B	40
	1,3-Dichloroacetone	6.1	UN2649	II	6.1		None	212	242	25 kg	100 kg	B	12, 40
	1,3-Dichloropropanol-2	6.1	UN2750	II	6.1	T8	None	202	243	5 L	60 L	A	12, 40
	2,2'-Dichlorodiethyl ether	6.1	UN1916	II	6.1, 3	N33, N34, T8	None	202	243	5 L	60 L	A	
	2-Amino-4-chlorophenol	6.1	UN2673	II	6.1		None	212	242	25 kg	100 kg	A	
	2-Chloropyridine	6.1	UN2822	II	6.1	T14	None	202	243	5 L	60 L	A	40
	2-Dimethylaminoethyl acrylate	6.1	UN3302	II	6.1	T8	None	202	243	5 L	60 L	D	25
	2-Dimethylaminoethyl methacrylate	6.1	UN2522	II	6.1	T8	None	202	243	5 L	60 L	B	40
	2-Ethylhexyl chloroformate	6.1	UN2748	II	6.1, 8	T12	None	202	243	1 L	30 L	A	12, 13, 21, 25, 40, 100
	3-Chloro-4-methylphenyl isocyanate	6.1	UN2236	II	6.1		None	202	243	5 L	60 L	B	40
	3-Nitro-4-chlorobenzotrifluoride	6.1	UN2307	II	6.1	T8	None	202	243	5 L	60 L	A	40
	3-Trifluoromethylaniline	6.1	UN2948	II	6.1	T14	None	202	243	5 L	60 L	A	40

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SYM BOL	PS_NAME1	HAZ CLASS	UN NUMBER	PACK GROUP	LABEL	SPEC_PROV	EXCEPTIONS	NONBULK	BULK	PASS_AIR	CARGO_AIR	VESSEL	VESSELSP
	Aldol	6.1	UN2839	II	6.1	T8	None	202	243	5 L	60 L	A	12
D	Aldrin, liquid	6.1	NA2762	II	6.1		None	202	243	5 L	60 L	B	
D	Aldrin, solid	6.1	NA2761	II	6.1		None	212	242	25 kg	100 kg	A	40
G	Alkaloids, liquid, n.o.s., or Alkaloid salts, liquid, n.o.s.	6.1	UN3140	II	6.1	T14	None	202	243	5 L	60 L	A	
G	Alkaloids, solid, n.o.s. or Alkaloid salts, solid, n.o.s. poisonous	6.1	UN1544	II	6.1		None	212	242	25 kg	100 kg	A	
	Allyl isothiocyanate, stabilized	6.1	UN1545	II	6.1, 3	A3, A7	None	202	243	Forbidden	60 L	D	40
	Aminopyridines (o-; m-; p-)	6.1	UN2671	II	6.1	T7	None	212	242	25 kg	100 kg	B	12, 40
	Ammonium arsenate	6.1	UN1546	II	6.1		None	212	242	25 kg	100 kg	A	
	Ammonium dinitro-o-cresolate	6.1	UN1843	II	6.1	T8	None	212	242	25 kg	100 kg	B	36, 65, 66, 77
	Ammonium metavanadate	6.1	UN2859	II	6.1		None	212	242	25 kg	100 kg	A	
	Ammonium polyvanadate	6.1	UN2861	II	6.1		None	212	242	25 kg	100 kg	A	
	Ammunition, tear-producing, non-explosive, without burster or expelling charge, non-fuzed	6.1	UN2017	II	6.1, 8		None	212	None	Forbidden	50 kg	E	13, 40
	Ammunition, toxic, non-explosive, without burster or expelling charge, non-fuzed	6.1	UN2016	II	6.1		None	212	None	Forbidden	100 kg	E	13, 40
+	Aniline	6.1	UN1547	II	6.1	T8	None	202	243	5 L	60 L	A	40
	Arsenic	6.1	UN1558	II	6.1		None	212	242	25 kg	100 kg	A	
	Arsenic acid, solid	6.1	UN1554	II	6.1		None	212	242	25 kg	100 kg	A	
	Arsenic bromide	6.1	UN1555	II	6.1		None	212	242	25 kg	100 kg	A	12, 40
	Arsenic compounds, liquid, n.o.s. inorganic, including arsenates n.o.s.; arsenites, n.o.s.; arsenic sulfides, n.o.s.; and organic compounds of arsenic, n.o.s.	6.1	UN1556	II	6.1		None	202	243	5 L	60 L	B	40
	Arsenic compounds, solid, n.o.s. inorganic, including arsenates, n.o.s.; arsenites, n.o.s.; arsenic sulfides, n.o.s.; and organic compounds of arsenic, n.o.s.	6.1	UN1557	II	6.1		None	212	242	25 kg	100 kg	A	
	Arsenic pentoxide	6.1	UN1559	II	6.1		None	212	242	25 kg	100 kg	A	
D	Arsenic sulfide	6.1	NA1557	II	6.1		None	212	242	25 kg	100 kg	A	
	Arsenic trioxide	6.1	UN1561	II	6.1		None	212	242	25 kg	100 kg	A	
D	Arsenic trisulfide	6.1	NA1557	II	6.1		None	212	242	25 kg	100 kg	A	
	Arsenical dust	6.1	UN1562	II	6.1		None	212	242	25 kg	100 kg	A	
	Arsenical pesticides, liquid, toxic	6.1	UN2994	II	6.1	T14	None	202	243	5 L	60 L	B	40
	Arsenical pesticides, liquid, toxic, flammable flashpoint not less than 23 degrees C	6.1	UN2993	II	6.1, 3	T14	None	202	243	5 L	60 L	B	40
	Arsenical pesticides, solid, toxic	6.1	UN2759	II	6.1		None	212	242	25 kg	100 kg	A	40
	Barium compounds, n.o.s.	6.1	UN1564	II	6.1		None	212	242	25 kg	100 kg	A	
	Benzidine	6.1	UN1885	II	6.1		None	212	242	25 kg	100 kg	A	
	Benzonitrile	6.1	UN2224	II	6.1	T14	None	202	243	5 L	60 L	A	28, 40
	Benzoquinone	6.1	UN2587	II	6.1		None	212	242	25 kg	100 kg	A	
	Benzyl bromide	6.1	UN1737	II	6.1, 8	A3, A7, N33, N34, T12, T26	None	202	243	1 L	30 L	D	13, 40
	Benzyl chloride	6.1	UN1738	II	6.1, 8	A3, A7, B70, N33, N42, T12, T26	None	202	243	1 L	30 L	D	13, 40
	Benzyl chloride unstabilized	6.1	UN1738	II	6.1, 8	A3, A7, B8, B11, N33, N34, N43, 112, T26	None	202	243	1 L	30 L	D	13, 40
	Benzyl iodide	6.1	UN2653	II	6.1	T8	None	202	243	5 L	60 L	B	12, 40
	Benzyldene chloride	6.1	UN1886	II	6.1	T8	None	202	243	5 L	60 L	D	40
	Beryllium compounds, n.o.s.	6.1	UN1566	II	6.1		None	212	242	25 kg	100 kg	A	
	Beryllium, powder	6.1	UN1567	II	6.1, 4.1		None	212	242	15 kg	50 kg	A	
	beta-Naphthylamine	6.1	UN1650	II	6.1	T12, T26	None	212	242	25 kg	100 kg	A	
	Bipyridilium pesticides, liquid, toxic	6.1	UN3016	II	6.1	T14	None	202	243	5 L	60 L	B	40
	Bipyridilium pesticides, liquid, toxic, flammable, flashpoint not less than 23 degrees C	6.1	UN3015	II	6.1, 3	T14	None	202	243	5 L	60 L	B	21, 40
	Bipyridilium pesticides, solid, toxic	6.1	UN2781	II	6.1		None	212	242	25 kg	100 kg	A	40
+	Bromoacetone	6.1	UN1569	II	6.1, 3	2	None	150	245	Forbidden	Forbidden	D	40
	Cacodylic acid	6.1	UN1572	II	6.1		None	212	242	25 kg	100 kg	E	28
	Cadmium compounds	6.1	UN2570	II	6.1		None	212	242	25 kg	100 kg	A	
	Calcium arsenate	6.1	UN1573	II	6.1		None	212	242	25 kg	100 kg	A	
	Calcium arsenate and calcium arsenite, mixtures, solid	6.1	UN1574	II	6.1		None	212	242	25 kg	100 kg	A	
D	Calcium arsenite, solid	6.1	NA1574	II	6.1		None	212	242	25 kg	100 kg	A	
	Carbamate pesticides, liquid, toxic	6.1	UN2992	II	6.1	T14	None	202	243	5 L	60 L	B	40

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SYMBOL	PS_NAME1	HAZ CLASS	UN NUMBER	PACK GROUP	LABEL	SPEC_PROV	EXCEPTIONS	NONBULK	BULK	PASS_AIR	CARGO_AIR	VESSEL	VESSELSP
	Carbamate pesticides, liquid, toxic, flammable, flash point not less than 23 degrees C	6.1	UN2991	II	6.1, 3	T14	None	202	243	5 L	60 L		B40
	Carbamate pesticides, solid, toxic	6.1	UN2757	I	6.1		None	212	242	25 kg	100 kg		A40
	Carbon tetrachloride	6.1	UN1846	I	6.1	N36, T8	None	202	243	5 L	60 L		A40
	Chloral, anhydrous, inhibited	6.1	UN2075	I	6.1	B101, T14	None	202	243	5 L	60 L		D40
	Chloroacetic acid, molten	6.1	UN3250	II	6.1, 8	T9	None	202	243	Forbidden	Forbidden		C40
	Chloroacetic acid, solid	6.1	UN1751	I	6.1, 8	A3, A7, N34	None	212	242	15 kg	50 kg		A40
	Chloroacetic acid, solution	6.1	UN1750	II	6.1, 8	A7, N34, T8, T27	None	202	243	1 L	30 L		C40
+	Chloroacetonitrile	6.1	UN2668	II	6.1, 3	2, B9, B14, B32, B74, T38, T43, T45	None	227	244	Forbidden	60 L		A12, 28, 40
	Chloroacetophenone (CN), liquid	6.1	UN1697	II	6.1	A8, N12, N32, N33	None	202	243	Forbidden	60 L		D12, 40
	Chloroacetophenone (CN), solid	6.1	UN1697	II	6.1	A8, N12, N32, N33, N34	None	212	None	Forbidden	100 kg		D12, 40
	Chloroanilines, liquid	6.1	UN2019	II	6.1	T14	None	202	243	5 L	60 L		A
	Chloroanilines, solid	6.1	UN2018	II	6.1	T14, T38	None	212	242	25 kg	100 kg		A
	Chlorocresols, liquid	6.1	UN2669	II	6.1	T8	None	202	243	5 L	60 L		A12
	Chlorocresols, solid	6.1	UN2669	II	6.1		None	212	242	25 kg	100 kg		A12
+	Chlorodinitrobenzenes	6.1	UN1577	II	6.1	T14	None	212	242	25 kg	100 kg		A91
G	Chloroformates, toxic, corrosive, flammable, n.o.s.	6.1	UN2742	II	6.1, 8, 3	B	None	202	243	1 L	30 L		A12, 13, 21, 25, 40, 100
G	Chloroformates, toxic, corrosive, n.o.s.	6.1	UN3277	II	6.1, 8	T12, T26	None	202	243	1 L	30 L		A12, 13, 25, 40
	Chloromethyl chloroformate	6.1	UN2745	II	6.1, 8	T18	None	202	243	1 L	30 L		A12, 13, 21, 25, 40, 100
+	Chloronitrobenzene, ortho, liquid	6.1	UN1578	II	6.1	T14	None	202	243	5 L	60 L		A
+	Chloronitrobenzenes meta or para, solid	6.1	UN1578	II	6.1	T14	None	212	242	25 kg	100 kg		A
	Chloropicrin mixtures, n.o.s.	6.1	UN1583	II	6.1		None	202	243	Forbidden	Forbidden		C40
D, G	Compounds, tree killing, liquid or Compounds, weed killing, liquid	6.1	NA2810	II	6.1		None	202	243	5 L	60 L		B40
	Copper acetoarsenite	6.1	UN1585	II	6.1		None	212	242	25 kg	100 kg		A
	Copper arsenite	6.1	UN1586	II	6.1		None	212	242	25 kg	100 kg		A
	Copper based pesticides, liquid, toxic	6.1	UN3010	II	6.1	T14	None	202	243	5 L	60 L		B40
	Copper based pesticides, liquid, toxic, flammable flashpoint not less than 23 degrees C	6.1	UN3009	II	6.1, 3	T14	None	202	243	5 L	60 L		B40
	Copper based pesticides, solid, toxic	6.1	UN2775	II	6.1		None	212	242	25 kg	100 kg		A40
	Copper cyanide	6.1	UN1587	II	6.1		None	204	242	25 kg	100 kg		A26
	Coumarin derivative pesticides, liquid, toxic	6.1	UN3026	II	6.1		None	202	243	5 L	60 L		B40
	Coumarin derivative pesticides, liquid, toxic, flammable flashpoint not less than 23 degrees C	6.1	UN3025	II	6.1, 3		None	202	243	5 L	60 L		B40
	Coumarin derivative pesticides, solid, toxic	6.1	UN3027	II	6.1		None	212	242	25 kg	100 kg		A40
	Cresols	6.1	UN2076	II	6.1, 8	B110, T8	None	202	243	1 L	30 L		B
	Cresylic acid	6.1	UN2022	II	6.1, 8	B110, T8	None	202	243	1 L	30 L		B
	Cyanide solutions, n.o.s.	6.1	UN1935	II	6.1	T18, T26	None	202	243	5 L	60 L		A40, 52
	Cyanides, inorganic, solid, n.o.s.	6.1	UN1588	II	6.1	N74, N75	None	212	242	25 kg	100 kg		A52
	Cyclobutyl chloroformate	6.1	UN2744	II	6.1, 8, 3	T18	None	202	243	1 L	30 L		A12, 13, 21, 25, 40, 100
+	Dichloroanilines, liquid	6.1	UN1590	II	6.1	T14	None	202	243	5 L	60 L		A40
+	Dichloroanilines, solid	6.1	UN1590	II	6.1	T14	None	212	242	25 kg	100 kg		A40
	Dichloroisopropyl ether	6.1	UN2490	II	6.1	T8	None	202	243	5 L	60 L		B
	Dichlorophenyl isocyanates	6.1	UN2250	II	6.1		None	212	242	25 kg	100 kg		B25, 40, 48
D	Dieldrin	6.1	NA2761	II	6.1		None	212	242	0.5 kg	5 kg		A40
	Diethyl sulfate	6.1	UN1594	II	6.1	B101, T14	None	202	243	5 L	60 L		C
	Dimethyl thiophosphoryl chloride	6.1	UN2267	II	6.1, 8	T7	None	202	243	1 L	30 L		B25
	Dinitroanilines	6.1	UN1596	II	6.1	T14	None	212	242	25 kg	100 kg		A91
	Dinitrobenzenes, liquid	6.1	UN1597	II	6.1	11, T14	None	202	243	5 L	60 L		A91
	Dinitrobenzenes, solid	6.1	UN1597	II	6.1	11	None	212	242	25 kg	100 kg		A91
	Dinitro-o-cresol, solid	6.1	UN1598	II	6.1	T14	None	212	242	25 kg	100 kg		A
	Dinitro-o-cresol, solution	6.1	UN1598	II	6.1	T14	None	202	243	5 L	60 L		A
	Dinitrophenol solutions	6.1	UN1599	II	6.1	T8	None	202	243	5 L	60 L		A36
	Dinitrotoluenes, liquid	6.1	UN2038	II	6.1	T8	None	202	243	5 L	60 L		A
	Dinitrotoluenes, molten	6.1	UN1600	II	6.1	B100, T14	None	202	243	Forbidden	Forbidden		C
	Dinitrotoluenes, solid	6.1	UN2038	II	6.1	T8	None	212	242	25 kg	100 kg		A
G	Disinfectants, liquid, toxic, n.o.s.	6.1	UN3142	II	6.1	T14	None	202	243	5 L	60 L		A40
G	Disinfectants, solid, toxic, n.o.s.	6.1	UN1601	II	6.1		None	212	242	25 kg	100 kg		A40

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SYMBOL	PS_NAME1	HAZ CLASS	UN NUMBER	PACK GROUP	LABEL	SPEC_PROV	EXCEPTIONS	NONBULK	BULK	PASS_AIR	CARGO_AIR	VESSEL	VESSELSP
G	Dyes, liquid, toxic, n.o.s or Dye intermediates, liquid, toxic, n.o.s.	6.1	UN1602	II	6.1		None	202	243	5 L	60 L	A	
G	Dyes, solid, toxic, n.o.s. or Dye intermediates, solid, toxic, n.o.s.	6.1	UN3143	II	6.1		None	212	242	25 kg	100 kg	A	
+	Epichlorhydrin	6.1	UN2023	I	6.1, 3	T14	None	202	243	5 L	60 L	A	40
	Ethyl bromide	6.1	UN1891	I	6.1	B100, T17	None	202	243	5 L	60 L	B	40, 85
	Ethyl bromoacetate	6.1	UN1603	II	6.1, 3	T14	None	202	243	Forbidden	Forbidden	D	40
	Ethyl chloroacetate	6.1	UN1181	II	6.1, 3	T14	None	202	243	5 L	60 L	A	
	Ferric arsenate	6.1	UN1606	II	6.1		None	212	242	25 kg	100 kg	A	
	Ferric arsenite	6.1	UN1607	II	6.1		None	212	242	25 kg	100 kg	A	
	Ferrous arsenate	6.1	UN1608	II	6.1		None	212	242	25 kg	100 kg	A	
	Furaldehydes	6.1	UN1199	II	6.1, 3	T15	None	202	243	5 L	60 L	A	
	Hexaethyl tetraphosphate liquid	6.1	UN1611	II	6.1	N76	None	202	243	5 L	60 L	D	40
-	Hexaethyl tetraphosphate, solid	6.1	UN1611	II	6.1	N76	None	212	242	25 kg	100 kg	B	40
	Hexafluoroacetone hydrate	6.1	UN2552	II	6.1	T14	None	202	243	5 L	60 L	B	40
	Hexamethylene diisocyanate	6.1	UN2281	II	6.1	B101, T14	None	202	243	5 L	60 L	C	13, 40
D	Hydrocyanic acid, aqueous solutions with less than 5 percent hydrogen cyanide	6.1	NA1613	II	6.1	T18; T25	None	195	243	Forbidden	5 L	D	40
G	Isocyanates, toxic, flammable, n.o.s. or Isocyanate solutions, toxic, flammable, n.o.s., flash point not less than 23 degrees C but not more than 61 degrees C and boiling point less than 300 degrees C	6.1	UN3080	II	6.1, 3	T16	None	202	243	5 L	60 L	B	25, 40, 48
G	Isocyanates, toxic, n.o.s. or Isocyanate, solutions, toxic, n.o.s., flash point more than 61 degrees C and boiling point less than 300 degrees C	6.1	UN2206	II	6.1	T15	None	202	243	5 L	60 L	B	25, 40, 48
	Isocyanatobenzotrifluorides	6.1	UN2285	II	6.1, 3	5, B101, T14	None	202	243	5 L	60 L	D	25, 40, 48
	Lead arsenates	6.1	UN1617	II	6.1		None	212	242	25 kg	100 kg	A	
	Lead arsenites	6.1	UN1618	II	6.1		None	212	242	25 kg	100 kg	A	
	Lead cyanide	6.1	UN1620	II	6.1		None	212	242	25 kg	100 kg	A	26
	London purple	6.1	UN1621	II	6.1		None	212	242	25 kg	100 kg	A	
+	Magnesium arsenate	6.1	UN1622	II	6.1		None	212	242	25 kg	100 kg	A	
	Malononitrile	6.1	UN2647	II	6.1		None	212	242	25 kg	100 kg	A	12
	Medicine, liquid, toxic, n.o.s.	6.1	UN1851	II	6.1		153	202	243	5 L	5 L	C	40
	Medicine, solid, toxic, n.o.s.	6.1	UN3249	II	6.1	36	153	212	None	5 kg	5 kg	C	40
	Mercaptans, liquid, toxic, flammable, n.o.s. or Mercaptan mixtures, liquid, toxic, flammable, n.o.s., flash point not less than 23 degrees C	6.1	UN3071	II	6.1, 3	T14	None	202	243	5 L	60 L	C	40, 121
	Mercuric arsenate	6.1	UN1623	II	6.1		None	212	242	25 kg	100 kg	A	
	Mercuric chloride	6.1	UN1624	II	6.1		None	212	242	25 kg	100 kg	A	
	Mercuric nitrate	6.1	UN1625	II	6.1	N73	None	212	242	25 kg	100 kg	A	
	Mercurous nitrate	6.1	UN1627	II	6.1		None	212	242	25 kg	100 kg	A	
	Mercury acetate	6.1	UN1629	II	6.1		None	212	242	25 kg	100 kg	A	
	Mercury ammonium chloride	6.1	UN1630	II	6.1		None	212	242	25 kg	100 kg	A	
	Mercury based pesticides, liquid, toxic	6.1	UN3012	II	6.1	T14	None	202	243	5 L	60 L	B	40
	Mercury based pesticides, liquid, toxic, flammable, flashpoint not less than 23 degrees C	6.1	UN3011	II	6.1, 3	T14	None	202	243	5 L	60 L	B	40
	Mercury based pesticides, solid, toxic	6.1	UN2777	II	6.1		None	212	242	25 kg	100 kg	A	40
	Mercury benzoate	6.1	UN1631	II	6.1		None	212	242	25 kg	100 kg	A	
	Mercury bromides	6.1	UN1634	II	6.1		None	212	242	25 kg	100 kg	A	
	Mercury compounds, liquid, n.o.s.	6.1	UN2024	II	6.1		None	202	243	5 L	60 L	B	40
	Mercury compounds, solid, n.o.s.	6.1	UN2025	II	6.1		None	212	242	25 kg	100 kg	A	
	Mercury cyanide	6.1	UN1636	II	6.1	N74, N75	None	212	242	25 kg	100 kg	A	26
	Mercury gluconate	6.1	UN1637	II	6.1		None	212	242	25 kg	100 kg	A	
	Mercury iodide, solid	6.1	UN1638	II	6.1		None	212	242	25 kg	100 kg	A	
	Mercury iodide, solution	6.1	UN1638	II	6.1		None	202	243	5 L	60 L	A	
	Mercury nucleate	6.1	UN1639	II	6.1		None	212	242	25 kg	100 kg	A	
	Mercury oleate	6.1	UN1640	II	6.1		None	212	242	25 kg	100 kg	A	
	Mercury oxide	6.1	UN1641	II	6.1		None	212	242	25 kg	100 kg	A	
	Mercury oxycyanide, desensitized	6.1	UN1642	II	6.1		None	212	242	25 kg	100 kg	A	26, 91
	Mercury potassium iodide	6.1	UN1643	II	6.1		None	212	242	25 kg	100 kg	A	
	Mercury salicylate	6.1	UN1644	II	6.1		None	212	242	25 kg	100 kg	A	

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SYMBOL	PS_NAME1	HAZ CLASS	UN NUMBER	PACK GROUP	LABEL	SPEC_PROV	EXCEPTIONS	NONBULK	BULK	PASS_AIR	CARGO_AIR	VESSEL VESSELSP
+	Mercury sulfates	6.1	UN1645	II	6.1		None	212	242	25 kg	100 kg	A
	Mercury thiocyanate	6.1	UN1646	II	6.1		None	212	242	25 kg	100 kg	A
	Metal carbonyls, n.o.s.	6.1	UN3281	II	6.1	T14	None	202	243	5 L	60 L	B40
	Methyl bromoacetate	6.1	UN2643	II	6.1	B100, T8	None	202	243	5 L	60 L	B40
D	Methyl parathion liquid	6.1	NA3018	II	6.1	N75, T14	None	202	243	Forbidden	1 L	A40
D	Methyl parathion solid	6.1	NA2783	II	6.1	N77	None	212	242	25 kg	100 kg	A40
	N,N-Dimethylaniline	6.1	UN2253	II	6.1	T8	None	202	243	5 L	60 L	A
	Naphthylthiourea	6.1	UN1651	II	6.1		None	212	242	25 kg	100 kg	A
	Naphthylurea	6.1	UN1652	II	6.1		None	212	242	25 kg	100 kg	A
	N-Butylaniline	6.1	UN2738	II	6.1	T8	None	202	243	5 L	60 L	A
	N-Ethyltoluidines	6.1	UN2754	II	6.1	T14	None	202	243	5 L	60 L	A
	Nickel cyanide	6.1	UN1653	II	6.1	N74, N75	None	212	242	25 kg	100 kg	A28
	Nicotine	6.1	UN1654	II	6.1		None	202	243	5 L	60 L	A
	Nicotine compounds, liquid, n.o.s. or Nicotine preparations, liquid, n.o.s.	6.1	UN3144	II	6.1	T14	None	202	243	5 L	60 L	B40
	Nicotine compounds, solid, n.o.s. or Nicotine preparations, solid, n.o.s.	6.1	UN1655	II	6.1		None	212	242	25 kg	100 kg	A
	Nicotine hydrochloride or Nicotine hydrochloride solution	6.1	UN1656	II	6.1		None	202	243	5 L	60 L	A
	Nicotine salicylate	6.1	UN1657	II	6.1		None	212	242	25 kg	100 kg	A
	Nicotine sulfate, solid	6.1	UN1658	II	6.1		None	212	242	25 kg	100 kg	A
	Nicotine sulfate, solution	6.1	UN1658	II	6.1	T14	None	202	243	5 L	60 L	A
	Nicotine tartrate	6.1	UN1659	II	6.1		None	212	242	25 kg	100 kg	A
G	Nitriles, toxic, flammable, n.o.s.	6.1	UN3275	II	6.1, 3	T14	None	202	243	5 L	60 L	B40
G	Nitriles, toxic, n.o.s.	6.1	UN3276	II	6.1	T14	None	202	243	5 L	60 L	B
+	Nitroanilines (o-; m-; p-)	6.1	UN1661	II	6.1	T14	None	212	242	25 kg	100 kg	A
+	Nitrobenzene	6.1	UN1662	II	6.1	T14	None	202	243	5 L	60 L	A40
	Nitrobenzotrifluorides	6.1	UN2306	II	6.1	T8	None	202	243	5 L	60 L	A40
	Nitrotoluenes, liquid o-; m-; p-	6.1	UN1664	II	6.1	T14	None	202	243	5 L	60 L	A
	Nitrotoluenes, solid m- or p-	6.1	UN1664	II	6.1	T14	None	212	242	25 kg	100 kg	A
	Nitroxylenes, (o-; m-; p-)	6.1	UN1665	II	6.1	T14	None	202	243	5 L	60 L	A
	N-n-Butyl imidazole	6.1	UN2690	II	6.1	T8	None	202	243	5 L	60 L	A
	Organoarsenic compound, n.o.s.	6.1	UN3280	II	6.1	T14	None	212	242	25 kg	100 kg	B
	Organochlorine pesticides, liquid, toxic	6.1	UN2996	II	6.1	T14	None	202	243	5 L	60 L	B40
	Organochlorine pesticides, liquid, toxic, flammable, flashpoint not less than 23 degrees C	6.1	UN2995	II	6.1, 3	T14	None	202	243	5 L	60 L	B40
	Organochlorine pesticides, solid toxic	6.1	UN2761	II	6.1		None	212	242	25 kg	100 kg	A40
C	Organomercury compound, toxic n.o.s.	6.1	UN3282	II	6.1	T14	None	212	242	25 kg	100 kg	B
	Organophosphorus compound, toxic n.o.s.	6.1	UN3278	II	6.1	T14	None	202	243	5 L	60 L	B
	Organophosphorus compound, toxic, flammable, n.o.s.	6.1	UN3279	II	6.1, 3	T14	None	202	243	5 L	60 L	B40
	Organophosphorus pesticides, liquid, toxic	6.1	UN3018	II	6.1	N75, T14	None	202	243	5 L	60 L	B40
	Organophosphorus pesticides, liquid, toxic, flammable, flashpoint not less than 23 degrees C	6.1	UN3017	II	6.1, 3	N75, T14	None	202	243	5 L	60 L	B40
	Organophosphorus pesticides, solid, toxic	6.1	UN2783	II	6.1	N77	None	212	242	25 kg	100 kg	A40
	Organotin compounds, liquid, n.o.s.	6.1	UN2788	II	6.1	A3, N33, N34, T14	None	202	243	5 L	60 L	A40
	Organotin compounds, solid, n.o.s.	6.1	UN3146	II	6.1		None	212	242	25 kg	100 kg	A40
	Organotin pesticides, liquid, toxic	6.1	UN3020	II	6.1	T14	None	202	243	5 L	60 L	B40
	Organotin pesticides, liquid, toxic, flammable, flashpoint not less than 23 degrees C	6.1	UN3019	II	6.1, 3	T14	None	202	243	5 L	60 L	B40
	Organotin pesticides, solid, toxic	6.1	UN2786	II	6.1		None	212	242	25 kg	100 kg	A40
D	Parathion	6.1	NA2783	II	6.1	T14	None	202	243	Forbidden	5 L	A40
	Pentachloroethane	6.1	UN1669	II	6.1	T14	None	202	243	5 L	60 L	A40
	Pentachlorophenol	6.1	UN3155	II	6.1		None	212	242	25 kg	100 kg	A
G	Pesticides, liquid, toxic, flammable, n.o.s. flashpoint not less than 23 degrees C	6.1	UN2903	II	6.1, 3	T14	None	202	243	5 L	60 L	B40
G	Pesticides, liquid, toxic, n.o.s.	6.1	UN2902	II	6.1	T14	None	202	243	5 L	60 L	B40
G	Pesticides, solid, toxic, n.o.s.	6.1	UN2588	II	6.1		None	212	242	25 kg	100 kg	A40
	Phenacyl bromide	6.1	UN2645	II	6.1	B136	None	212	242	25 kg	100 kg	B40
	Phenol solutions	6.1	UN2821	II	6.1	T14	None	202	243	5 L	60 L	A
	Phenol, molten	6.1	UN2312	II	6.1	B14, B100, T8	None	202	243	Forbidden	Forbidden	B40
+	Phenol, solid	6.1	UN1671	II	6.1	N76, T14	None	212	242	25 kg	100 kg	A

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SYMBOL	PS_NAME1	HAZ CLASS	UN NUMBER	PACK GROUP	LABEL	SPEC_PROV	EXCEPTIONS	NONBULK	BULK	PASS_AIR	CARGO_AIR	VESSEL	VESSELSP
	Phenoxyacetic acid derivative pesticide, liquid, toxic, flammable, flashpoint not less than 23°C	6.1	UN3347	II	6.1, 3	T14	153	202	243	5 L	60 L	B	40
	Phenoxyacetic acid derivative pesticide, liquid, toxic	6.1	UN3348	II	6.1	T14	153	202	243	5 L	60 L	B	40
	Phenoxyacetic acid derivative pesticide, solid, toxic	6.1	UN3345	II	6.1		153	212	242	25 kg	100 kg	A	40
	Phenyl chloroformate	6.1	UN2746	II	6.1, 8	T12	None	202	243	1 L	30 L	A	12, 13, 21, 29, 40, 100
	Phenyl urea pesticides, liquid, toxic	6.1	UN3002	II	6.1	T14	None	202	243	5 L	60 L	B	40
	Phenylhydrazine	6.1	UN2572	II	6.1	T8	None	202	243	5 L	60 L	A	40
	Phenylmercuric acetate	6.1	UN1674	II	6.1		None	212	242	25 kg	100 kg	A	
	Phenylmercuric compounds, n.o.s.	6.1	UN2026	II	6.1		None	212	242	25 kg	100 kg	A	
	Phenylmercuric hydroxide	6.1	UN1894	II	6.1		None	212	242	25 kg	100 kg	A	
	Phenylmercuric nitrate	6.1	UN1895	II	6.1		None	212	242	25 kg	100 kg	A	
	Potassium arsenate	6.1	UN1677	II	6.1		None	212	242	25 kg	100 kg	A	
	Potassium arsenite	6.1	UN1678	II	6.1		None	212	242	25 kg	100 kg	A	
	Potassium cuprocyanide	6.1	UN1679	II	6.1		None	212	242	25 kg	100 kg	A	26
	Potassium metavanadate	6.1	UN2864	II	6.1		None	212	242	25 kg	100 kg	A	
	Propylene chlorohydrin	6.1	UN2611	II	6.1, 3	T9	None	202	243	5 L	60 L	A	12, 40, 48
	Pyrethroid pesticide, liquid toxic	6.1	UN3352	II	6.1		153	212	242	5 L	60 L	A	40
	Pyrethroic pesticide, liquid, flammable, toxic, flashpoint not less than 23°C	6.1	UN3351	II	6.1, 3	T14	153	202	243	5 L	60 L	B	40
	Pyrethroid pesticide, solid, toxic	6.1	UN3349	II	6.1		153	212	242	25 kg	100 kg	A	40
	Selenium compound, n.o.s.	6.1	UN3283	II	6.1	T14	None	212	242	25 kg	100 kg	D	
	Selenium disulfide	6.1	UN2657	II	6.1		None	212	242	25 kg	100 kg	A	
	Silver arsenite	6.1	UN1683	II	6.1		None	212	242	25 kg	100 kg	A	
	Silver cyanide	6.1	UN1684	II	6.1		None	212	242	25 kg	100 kg	A	26, 40
	Sodium ammonium vanadate	6.1	UN2863	II	6.1		None	212	242	25 kg	100 kg	A	
	Sodium arsenate	6.1	UN1685	II	6.1		None	212	242	25 kg	100 kg	A	
	Sodium arsenite, aqueous solutions	6.1	UN1686	II	6.1	T15	None	202	243	5 L	60 L	A	
	Sodium arsenite, solid	6.1	UN2027	II	6.1		None	212	242	25 kg	100 kg	A	
	Sodium azide	6.1	UN1687	II	6.1	B28	None	212	242	25 kg	100 kg	A	36, 52, 91
	Sodium cacodylate	6.1	UN1688	II	6.1		None	212	242	25 kg	100 kg	A	26
	Sodium pentachlorophenate	6.1	UN2567	II	6.1		None	212	242	25 kg	100 kg	A	
D	Sodium selenite	6.1	NA2630	II	6.1		None	212	242	25 kg	100 kg	E	
G	Solids containing toxic liquid, n.o.s.	6.1	UN3243	II	6.1	48	None	212	240	25 kg	100 kg	B	40
	Strontium arsenite	6.1	UN1691	II	6.1		None	212	242	25 kg	100 kg	A	
	Substituted nitrophenol pesticides, liquid, toxic	6.1	UN3014	II	6.1	T14	None	202	243	5 L	60 L	B	40
	Substituted nitrophenol pesticides, liquid, toxic, flammable flashpoint not less than 23 degrees C	6.1	UN3013	II	6.1, 3	T14	None	202	243	5 L	60 L	B	40
	Substituted nitrophenol pesticides, solid, toxic	6.1	UN2779	II	6.1		None	212	242	25 kg	100 kg	A	40
	Tear gas candles	6.1	UN1700	II	6.1, 4.1		None	340	None	Forbidden	50 kg	D	40
D	Tear gas devices with more than 2 percent tear gas substances, by mass	6.1	NA1693	II	6.1		None	340	None	Forbidden	Forbidden	D	40
G	Tear gas substances, liquid, n.o.s.	6.1	UN1693	II	6.1		None	202	None	Forbidden	5 L	D	40
G	Tear gas substances, solid, n.o.s.	6.1	UN1693	II	6.1		None	212	None	Forbidden	25 kg	D	40
	Tellurium compound, n.o.s.	6.1	UN3284	II	6.1	T14	None	212	242	25 kg	100 kg	B	
	Tetrachloroethane	6.1	UN1702	II	6.1	N36, T14	None	202	243	5 L	60 L	A	40
	Tetraethyl dithiopyrophosphate	6.1	UN1704	II	6.1		None	212	242	25 kg	100 kg	D	40
	Thallium compounds, n.o.s.	6.1	UN1707	II	6.1		None	212	242	25 kg	100 kg	A	
	Thallium nitrate	6.1	UN2727	II	6.1, 5.1		None	212	242	5 kg	25 kg	A	
D	Thallium sulfate, solid	6.1	NA1707	II	6.1		None	212	242	5 kg	50 kg	A	
	Thiocarbamate pesticides, liquid, flammable, toxic, flash point not less than 23 degrees C	6.1	UN3005	II	6.1, 3	T14	None	202	243	5 L	60 L	B	40
	Thiocarbamate pesticides, liquid, toxic	6.1	UN3006	II	6.1	T14	None	202	243	5 L	60 L	B	40
	Thiocarbamate pesticides, solid, toxic	6.1	UN2771	II	6.1		None	212	242	25 kg	100 kg	A	40
	Thioglycol	6.1	UN2966	II	6.1	T8	None	202	243	5 L	60 L	A	
	Thiolactic acid	6.1	UN2936	II	6.1	T8	None	212	242	25 kg	100 kg	A	
+	Thiophosgene	6.1	UN2474	II	6.1	2, A7, B9, B14, B32, B74, N33, N34, T38, T43, T45	None	227	244	Forbidden	60 L	B	26, 40
+	Toluene diisocyanate	6.1	UN2078	II	6.1	B110, T14	None	202	243	5 L	60 L	D	25, 40
+	Toluidines, liquid	6.1	UN1708	II	6.1	T14	None	202	243	5 L	60 L	A	

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SYMBOL	PS_NAME1	HAZ CLASS	UN NUMBER	PACK GROUP	LABEL	SPEC_PROV	EXCEPTIONS	NONBULK	BULK	PASS_AIR	CARGO_AIR	VESSELSP
+	Toluidines solid	6.1	UN1708	II	6.1		None	212	242	25 kg	100 kg	A
G	Toxic liquid, corrosive, inorganic, n.o.s.	6.1	UN3289	II	6.1, 8	T14	None	202	243	1 L	30 L	A
	Toxic liquid, inorganic, n.o.s.	6.1	UN3287	II	6.1	B110, T14	None	202	243	5 L	60 L	A
G	Toxic liquids, corrosive, organic, n.o.s.	6.1	UN2927	II	6.1, 8	T42	None	202	243	1 L	30 L	B40
G	Toxic liquids, flammable, organic, n.o.s.	6.1	UN2929	II	6.1, 3	T15	None	202	243	5 L	60 L	B40
G	Toxic liquids, oxidizing, n.o.s.	6.1	UN3122	II	6.1, 5.1		None	202	243	1 L	5 L	C
	Toxic liquids, water-reactive, n.o.s.	6.1	UN3123	II	6.1, 4.3		None	202	243	1 L	5 L	E40
	Toxic solid, corrosive, inorganic, n.o.s.	6.1	UN3290	II	6.1, 8		None	212	242	15 kg	50 kg	A
G	Toxic solid, inorganic, n.o.s.	6.1	UN3288	II	6.1		None	212	242	25 kg	100 kg	A
G	Toxic solids, corrosive, organic, n.o.s.	6.1	UN2928	II	6.1, 8		None	212	242	15 kg	50 kg	D40
G	Toxic solids, flammable, organic, n.o.s.	6.1	UN2930	II	6.1, 4.1	B106	None	212	242	15 kg	50 kg	B
G	Toxic solids, organic, n.o.s.	6.1	UN2811	II	6.1		None	212	242	25 kg	100 kg	B
G	Toxic solids, oxidizing, n.o.s.	6.1	UN3086	II	6.1, 5.1		None	212	242	15 kg	50 kg	C
G	Toxic solids, self-heating, n.o.s.	6.1	UN3124	II	6.1, 4.2		None	212	242	15 kg	50 kg	D40
G	Toxic solids, water-reactive, n.o.s.	6.1	UN3125	II	6.1, 4.3	B101	None	212	242	15 kg	50 kg	D40
G	Toxic, liquids, organic, n.o.s.	6.1	UN2810	II	6.1	B110, T14	None	202	243	5 L	60 L	B40
	Triazine pesticides, liquid, toxic	6.1	UN2998	II	6.1	T14	None	202	243	5 L	60 L	B40
	Triazine pesticides, liquid, toxic, flammable, flashpoint not less than 23 degrees C	6.1	UN2997	II	6.1, 3	T14	None	202	243	5 L	60 L	B40
	Triazine pesticides, solid, toxic	6.1	UN2763	II	6.1		None	212	242	25 kg	100 kg	A40
	Tributylamine	6.1	UN2542	II	6.1	B110, T14	None	202	243	5 L	60 L	A
	Trichlorobutene	6.1	UN2322	II	6.1	T8	None	202	243	5 L	60 L	A25, 40
	Tricresyl phosphate with more than 3 percent ortho isomer	6.1	UN2574	II	6.1	A3, N33, N34, T8	None	202	243	5 L	60 L	A
	Tris-(1-aziridinyl)phosphine oxide, solution	6.1	UN2501	II	6.1	T8	None	202	243	5 L	60 L	A
	Vanadium compound, n.o.s.	6.1	UN3285	II	6.1	T14	None	212	242	25 kg	100 kg	B
	Vanadyl sulfate	6.1	UN2931	II	6.1		None	212	242	25 kg	100 kg	A
	Vinyl chloroacetate	6.1	UN2589	II	6.1, 3	T14	None	202	243	5 L	60 L	A
	Vinylpyridines, inhibited	6.1	UN3073	II	6.1, 3, 8	B120, T8	None	202	243	1 L	30 L	B40
	Xylenols	6.1	UN2261	II	6.1	T8	None	212	242	25 kg	100 kg	A
	Xylidines, solid	6.1	UN1711	II	6.1	T14	None	212	242	25 kg	100 kg	A
	Xylidines, solution	6.1	UN1711	II	6.1	T14	None	202	243	5 L	60 L	A
	Xylyl bromide	6.1	UN1701	II	6.1	A3, A6, A7, N33	None	340	None	Forbidden	60 L	D40
	Zinc arsenate or Zinc arsenite or Zinc arsenate and zinc arsenite mixtures	6.1	UN1712	II	6.1		None	212	242	25 kg	100 kg	A
	1,1,1-Trichloroethane	6.1	UN2831	III	6.1	N36, T7	153	203	241	60 L	220 L	A40
	1,4-Butynediol	6.1	UN2716	III	6.1	A1	None	213	240	100 kg	200 kg	A61, 70
	1,5,9-Cyclododecatriene	6.1	UN2518	III	6.1	T7	153	203	241	60 L	220 L	A40
	1-Chloro-3-bromopropane	6.1	UN2688	III	6.1	T2	153	203	241	60 L	220 L	A
	2,4-Toluylenediamine or 2,4-Toluenediamine	6.1	UN1709	III	6.1	T7	153	213	240	100 kg	200 kg	A
	2-Amino-6-diethylaminopentane	6.1	UN2946	III	6.1	T1	153	203	241	60 L	220 L	A
	2-Ethylaniline	6.1	UN2273	III	6.1	T2	153	203	241	60 L	220 L	A
	2-Methyl-5-ethylpyridine	6.1	UN2300	III	6.1	T7	153	203	241	60 L	220 L	A
	2-Trifluoromethylaniline	6.1	UN2942	III	6.1		153	203	241	60 L	220 L	A
	3-Chloropropanol-1	6.1	UN2849	III	6.1	T8	153	203	241	60 L	220 L	A
	4,4'-Diaminodiphenyl methane	6.1	UN2651	III	6.1		153	213	240	100 kg	200 kg	A
	4-Chloro-o-toluidine hydrochloride	6.1	UN1579	III	6.1		153	213	240	100 kg	200 kg	A
	4-Thiapentanal	6.1	UN2785	III	6.1	T8	153	203	241	60 L	220 L	D25, 49
	Acridine	6.1	UN2713	III	6.1		153	213	240	100 kg	200 kg	A
	Acrylamide	6.1	UN2074	III	6.1	T8	153	213	240	100 kg	200 kg	A12
	Adiponitrile	6.1	UN2205	III	6.1	T1	153	203	241	60 L	220 L	A
G	Alkaloids, liquid, n.o.s., or Alkaloid salts, liquid, n.o.s.	6.1	UN3140	III	6.1	T7	153	203	241	60 L	220 L	A
G	Alkaloids, solid, n.o.s. or Alkaloid salts, solid, n.o.s. poisonous	6.1	UN1544	III	6.1		153	213	240	100 kg	200 kg	A
	alpha-Methylbenzyl alcohol	6.1	UN2937	III	6.1	T1	153	203	241	60 L	220 L	A
	alpha-Naphthylamine	6.1	UN2077	III	6.1	T7	153	213	240	100 kg	200 kg	A
+	Aminophenols (o-; m-; p-)	6.1	UN2512	III	6.1	T1	153	213	240	100 kg	200 kg	A
	Ammonium fluoride	6.1	UN2505	III	6.1		153	213	240	100 kg	200 kg	A26
	Ammonium fluorosilicate	6.1	UN2854	III	6.1		153	213	240	100 kg	200 kg	A26
	Aniline hydrochloride	6.1	UN1548	III	6.1		153	213	240	100 kg	200 kg	A
	Anisidines	6.1	UN2431	III	6.1	T1	153	203	241	60 L	220 L	A

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SYMBOL	PS_NAME1	HAZ CLASS	UN NUMBER	PACK GROUP	LABEL	SPEC_PROV	EXCEPTIONS	NONBULK	BULK	PASS_AIR	CARGO_AIR	VESSEL	VESSELSP
	Antimony compounds, inorganic, liquid, n.o.s.	6.1	UN3141	III	6.1	35, T7	153	203	241	60 L	220 L	A	
	Antimony compounds, inorganic, solid, n.o.s.	6.1	UN1549	III	6.1	35	153	213	240	100 kg	200 kg	A	
	Antimony lactate	6.1	UN1550	III	6.1		153	213	240	100 kg	200 kg	A	
	Antimony potassium tartrate	6.1	UN1551	III	6.1		153	213	240	100 kg	200 kg	A	
	Antimony powder	6.1	UN2871	III	6.1		153	213	240	100 kg	200 kg	A	
	Arsenic compounds, liquid, n.o.s. inorganic, including arsenates n.o.s.; arsenites n.o.s.; arsenic sulfides, n.o.s.; and organic compounds of arsenic, n.o.s.	6.1	UN1556	III	6.1		153	203	241	60 L	220 L	B	40
	Arsenic compounds, solid, n.o.s. inorganic, including arsenates, n.o.s.; arsenites, n.o.s.; arsenic sulfides, n.o.s.; and organic compounds of arsenic, n.o.s.	6.1	UN1557	III	6.1		153	213	240	100 kg	200 kg	A	
	Arsenical pesticides, liquid, toxic	6.1	UN2994	III	6.1	T14	153	203	241	60 L	220 L		A40
	Arsenical pesticides, liquid, toxic, flammable flashpoint not less than 23 degrees C	6.1	UN2993	III	6.1, 3	B1, T14	153	203	242	60 L	220 L		A40
	Arsenical pesticides, solid, toxic	6.1	UN2759	III	6.1		153	213	240	100 kg	200 kg		A40
	Barium compounds, n.o.s.	6.1	UN1564	III	6.1		153	213	240	100 kg	200 kg		A
	Barium oxide	6.1	UN1884	III	6.1		153	213	240	100 kg	200 kg		A
	Beryllium compounds, n.o.s.	6.1	UN1566	III	6.1		153	213	240	100 kg	200 kg		A
	Bipyridilium pesticides, liquid, toxic	6.1	UN3016	III	6.1	T14	153	203	241	60 L	220 L		A40
	Bipyridilium pesticides, liquid, toxic, flammable, flashpoint not less than 23 degrees C	6.1	UN3015	III	6.1, 3	B1, T14	153	203	242	60 L	220 L		A21, 40
	Bipyridilium pesticides, solid, toxic	6.1	UN2781	III	6.1		153	213	240	100 kg	200 kg		A40
	Bromochloromethane	6.1	UN1887	III	6.1	T7	153	203	241	60 L	220 L		A
	Bromoform	6.1	UN2515	III	6.1	T7	153	203	241	60 L	220 L		A12, 40
	Butyltoluenes	6.1	UN2667	III	6.1	T2	153	203	241	60 L	220 L		A
	Cadmium compounds	6.1	UN2570	III	6.1		153	213	240	100 kg	200 kg		A
	Carbamate pesticides, liquid, toxic	6.1	UN2992	III	6.1	T14	153	203	241	60 L	220 L		A40
	Carbamate pesticides, liquid, toxic, flammable, flash point not less than 23 degrees C	6.1	UN2991	III	6.1, 3	B1, T14	153	203	242	60 L	220 L		A40
	Carbamate pesticides, solid, toxic	6.1	UN2757	III	6.1		153	213	240	100 kg	200 kg		A40
	Carbon tetrabromide	6.1	UN2516	III	6.1		153	213	240	100 kg	200 kg		A25
	Chloroanisidines	6.1	UN2233	III	6.1		153	213	240	100 kg	200 kg	A	
	Chlorobenzyl chlorides	6.1	UN2235	II	6.1	T8	153	203	241	60 L	220 L	A	
	Chloroform	6.1	UN1888	II	6.1	N36, T14	153	203	241	60 L	60 L		A40
	Chloronitroanilines	6.1	UN2237	II	6.1		153	213	240	100 kg	200 kg	A	
	Chloronitrotoluenes liquid	6.1	UN2433	III	6.1		153	203	241	60 L	220 L	A	
	Chloronitrotoluenes, solid	6.1	UN2433	III	6.1		153	213	240	100 kg	200 kg	A	
	Chlorophenols, liquid	6.1	UN2021	III	6.1	T7	153	203	241	60 L	220 L	A	
	Chlorophenols, solid	6.1	UN2020	III	6.1	T7	153	213	240	100 kg	200 kg	A	
	Chloropicrin mixtures, n.o.s.	6.1	UN1583	III	6.1		153	203	241	Forbidden	Forbidden	C	40
	Chlorotoluidines liquid	6.1	UN2238	III	6.1	T7	153	203	241	60 L	220 L	A	
	Chlorotoluidines solid	6.1	UN2239	III	6.1		153	213	240	100 kg	200 kg	A	
D, G	Compounds, tree killing, liquid or Compounds, weed killing, liquid	6.1	NA2810	III	6.1		153	203	241	60 L	220 L		A40
	Copper based pesticides, liquid, toxic	6.1	UN3010	III	6.1	T14	153	203	241	60 L	220 L		A40
	Copper based pesticides, liquid, toxic, flammable flashpoint not less than 23 degrees C	6.1	UN3009	III	6.1, 3	B1, T14	153	203	242	60 L	220 L		A40
	Copper based pesticides, solid, toxic	6.1	UN2775	III	6.1		153	213	240	100 kg	200 kg		A40
	Coumarin derivative pesticides, liquid, toxic	6.1	UN3026	III	6.1		153	203	241	60 L	220 L		A40
	Coumarin derivative pesticides, liquid, toxic, flammable flashpoint not less than 23 degrees C	6.1	UN3025	III	6.1, 3	B1	153	203	242	60 L	220 L		A40
	Coumarin derivative pesticides, solid, toxic	6.1	UN3027	III	6.1		153	213	240	100 kg	200 kg		A40
	Cyanide solutions, n.o.s.	6.1	UN1935	III	6.1	T18; T26	153	203	241	60 L	220 L		A40, 52
	Cyanides, inorganic, solid, n.o.s.	6.1	UN1588	III	6.1	N74, N75	153	213	240	100 kg	200 kg		A52
	Dibromochloropropane	6.1	UN2872	III	6.1	T7	153	203	241	60 L	220 L		A
	Dibromomethane	6.1	UN2664	III	6.1	T7	153	203	241	60 L	220 L		A
	Dibutylaminoethanol	6.1	UN2873	III	6.1	T1	153	203	241	60 L	220 L		A
	Dichloromethane	6.1	UN1593	III	6.1	N36, T13	153	203	241	60 L	220 L		A
	Dinitrophenol solutions	6.1	UN1599	II	6.1	T7	153	203	241	60 L	220 L		A36
G	Disinfectants, liquid, toxic, n.o.s.	6.1	UN3142	III	6.1	T7	153	203	241	60 L	220 L		A40

Department of Transportation List of Division 6.1 Materials

SYMBOL	PS_NAME1	HAZ CLASS	UN NUMBER	PACK GROUP	LABEL	SPEC_PROV	EXCEPTIONS	NONBULK	BULK	PASS_AIR	CARGO_AIR	VESSEL	VESSELSP
C	Disinfectants, solid, toxic, n.o.s.	6.1	UN1601	III	6.1		153	213	240	100 kg	200 kg		A40
G	Dyes, liquid, toxic, n.o.s or Dye intermediates, liquid, toxic, n.o.s.	6.1	UN1602	III	6.1		153	203	241	60 L	220 L		A
G	Dyes, solid, toxic, n.o.s. or Dye intermediates, solid, toxic, n.o.s.	6.1	UN3143	III	6.1		153	213	240	100 kg	200 kg		A
	Ethyl oxalate	6.1	UN2525	III	6.1	T1	153	203	241	60 L	220 L		A
	Fluoroanilines	6.1	UN2941	III	6.1	T8	153	203	241	60 L	220 L		A
	Fluorosilicates, n.o.s.	6.1	UN2856	III	6.1		153	213	240	100 kg	200 kg		A26
	Furfuryl alcohol	6.1	UN2874	III	6.1	T2	153	203	241	60 L	220 L		A26, 74
	Glycerol alpha monochlorohydrin	6.1	UN2689	III	6.1	T2	153	203	241	60 L	220 L		A
	Hexachloroacetone	6.1	UN2661	III	6.1	T8	153	203	241	60 L	220 L		B12, 40
	Hexachlorobenzene	6.1	UN2729	III	6.1		153	203	241	60 L	220 L		A
	Hexachlorobutadiene	6.1	UN2279	III	6.1	T7	153	203	241	60 L	220 L		A
	Hexachlorophene	6.1	UN2875	III	6.1		153	213	240	100 kg	200 kg		A
	Hydrazine, aqueous solution with not more than 37 percent hydrazine, by mass	6.1	UN3293	III	6.1	T7	153	203	241	60 L	220 L		A
	Hydroquinone	6.1	UN2662	III	6.1		153	213	240	100 kg	200 kg		A
G	Isocyanates, toxic, n.o.s. or Isocyanate solutions, toxic, n.o.s., flash point more than 61 degrees C and boiling point less than 300 degrees C	6.1	UN2206	III	6.1	T8	153	203	241	60 L	220 L		B25, 40, 48
	Isophorone diisocyanate	6.1	UN2290	III	6.1	T7	153	203	241	60 L	220 L	B	40
	Lead acetate	6.1	UN1616	III	6.1		153	213	240	100 kg	200 kg	A	
	Lead compounds, soluble, n.o.s.	6.1	UN2291	III	6.1	T38	153	213	240	100 kg	200 kg	A	
	Magnesium fluorosilicate	6.1	UN2853	III	6.1		153	213	240	100 kg	200 kg	A	26
	Medicine, liquid, toxic, n.o.s.	6.1	UN1851	III	6.1		153	203	241	5 L	5 L	C	40
	Medicine, solid, toxic, n.o.s	6.1	UN3249	III	6.1	36	153	213	None	5 kg	5 kg	C	40
	Mercury based pesticides, liquid, toxic	6.1	UN3012	III	6.1	T14	153	203	241	60 L	220 L	A	40
	Mercury based pesticides, liquid, toxic, flammable, flashpoint not less than 23 degrees C	6.1	UN3011	III	6.1, 3	T14	153	203	242	50 L	220 L	A	40
	Mercury based pesticides, solid, toxic	6.1	UN2777	III	6.1		153	213	240	100 kg	200 kg	A	40
	Mercury compounds, liquid, n.o.s.	6.1	UN2024	III	6.1		153	203	241	60 L	220 L	B	40
	Mercury compounds, solid, n.o.s.	6.1	UN2025	III	6.1		153	213	240	100 kg	200 kg	A	
	Metal carbonyls, n.o.s.	6.1	UN3281	III	6.1	T7	153	203	241	60 L	220 L	A	40
	Methyl dichloroacetate	6.1	UN2299	III	6.1	T1	153	203	241	60 L	220 L	A	
	Methyl trichloroacetate	6.1	UN2533	III	6.1	T1	153	203	241	60 L	220 L	A	
+	N,N-Diethylaniline	6.1	UN2432	III	6.1	T2	153	203	241	60 L	220 L	A	
	N-Ethylaniline	6.1	UN2272	III	6.1	T2	153	203	241	60 L	220 L	A	
	N-Ethylbenzyltoluidines liquid	6.1	UN2753	III	6.1	T14	153	203	241	60 L	220 L	A	
	N-Ethylbenzyltoluidines solid	6.1	UN2753	III	6.1		153	213	240	100 kg	200 kg	A	
	N-Ethyl-N-benzylaniline	6.1	UN2274	III	6.1	T2	153	203	241	60 L	220 L	A	
	Nicotine compounds, liquid, n.o.s or Nicotine preparations, liquid, n.o.s.	6.1	UN3144	III	6.1	T7	153	203	241	60 L	220 L	B	40
	Nicotine compounds, solid, n.o.s. or Nicotine preparations, solid, n.o.s.	6.1	UN1655	III	6.1		153	213	240	100 kg	200 kg	A	
G	Nitriles, toxic, n.o.s.	6.1	UN3276	III	6.1	T7	153	203	241	60 L	220 L	A	
+	Nitroanisole	6.1	UN2730	III	6.1	T8	153	213	240	100 kg	200 kg	A	
	Nitrobromobenzenes liquid	6.1	UN2732	III	6.1	T8, T38	153	203	241	60 L	220 L	A	
	Nitrobromobenzenes solid	6.1	UN2732	III	6.1		153	213	240	100 kg	200 kg	A	
	Nitrocresols	6.1	UN2446	III	6.1		153	213	240	100 kg	200 kg	A	
+	Nitrophenols (o-; m-; p-)	6.1	UN1663	III	6.1	T8, T38	153	213	240	100 kg	200 kg	A	
	Nitrotoluidines (mono)	6.1	UN2660	III	6.1		153	213	240	100 kg	200 kg	A	
	N-Methylaniline	6.1	UN2294	III	6.1	T7	153	203	241	60 L	220 L	A	
+	o-Dichlorobenzene	6.1	UN1591	III	6.1	T7	153	203	241	60 L	220 L	A	
	Organoarsenic compound, n.o.s.	6.1	UN3280	III	6.1	T7	153	213	240	100 kg	200 kg	A	
	Organoarsenic compound, n.o.s	6.1	UN3280	III	6.1	T7	153	213	240	100 kg	200 kg	A	
	Organochlorine pesticides, liquid, toxic	6.1	UN2996	III	6.1	T14	153	203	241	60 L	220 L	A	40
	Organochlorine pesticides, liquid, toxic, flammable, flashpoint not less than 23 degrees C	6.1	UN2995	III	6.1, 3	B1, T14	153	203	242	60 L	220 L	A	40
	Organochlorine pesticides, solid toxic	6.1	UN2761	III	6.1		153	213	240	100 kg	200 kg	A	40
G	Organometallic compound, toxic n.o.s	6.1	UN3282	III	6.1	T7	153	213	240	100 kg	200 kg	A	
	Organophosphorus compound, toxic n.o.s.	6.1	UN3278	III	6.1	T7	153	203	241	60 L	220 L	A	

Department of Transportation List of Division 6.1 Materials

SYMBOL	PS_NAME1	HAZ CLASS	UN NUMBER	PACK GROUP	LABEL	SPEC_PROV	EXCEPTIONS	NONBULK	BULK	PASS_AIR	CARGO_AIR	VESSEL	VESSELS*
	Organophosphorus pesticides, liquid, toxic	6.1	UN3018	III	6.1	N76, T14	153	203	241	60 L	220 L	A	40
	Organophosphorus pesticides, liquid, toxic, flammable, flashpoint not less than 23 degrees C	6.1	UN3017	III	6.1, 3	B1, N76, T14	153	203	242	60 L	220 L	A	40
	Organophosphorus pesticides, solid, toxic	6.1	UN2783	III	6.1	N77	153	213	240	100 kg	200 kg	A	40
	Organotin compounds, liquid, n.o.s.	6.1	UN2788	III	6.1	T14	153	203	241	60 L	220 L	A	40
	Organotin compounds, solid, n.o.s.	6.1	UN3146	III	6.1		153	213	240	100 kg	200 kg	A	40
	Organotin pesticides, liquid, toxic	6.1	UN3020	III	6.1	T14	153	203	241	60 L	220 L	A	40
	Organotin pesticides, liquid, toxic, flammable, flashpoint not less than 23 degrees C	6.1	UN3019	III	6.1, 3	B1, T14	153	203	242	60 L	220 L	A	40
	Organotin pesticides, solid, toxic	6.1	UN2786	III	6.1		153	213	240	100 kg	200 kg	A	40
G	Pesticides, liquid, toxic, flammable, n.o.s. flashpoint not less than 23 deg C	6.1	UN2903	III	6.1, 3	B1, T14	153	203	242	60 L	220 L	A	40
G	Pesticides, liquid, toxic, n.o.s.	6.1	UN2902	III	6.1	T14	153	203	241	60 L	220 L	A	40
G	Pesticides, solid, toxic, n.o.s.	6.1	UN2588	III	6.1		153	213	240	100 kg	200 kg	A	40
+	Phenetidines	6.1	UN2311	III	6.1	T7	153	203	241	60 L	220 L	A	
	Phenol solutions	6.1	UN2821	III	6.1	T7	153	200	241	60 L	220 L	A	
	Phenoxyacetic acid derivative pesticide, liquid, toxic, flammable, flashpoint not less than 23°C.	6.1	UN3347	III	6.1, 3	T14	153	203	241	60 L	220 L	A	40
	Phenoxyacetic acid derivative pesticide, liquid, toxic.	6.1	UN3348	III	6.1	T14	153	203	241	60 L	220 L	A	40
	Phenoxyacetic acid derivative pesticide, solid, toxic	6.1	UN3345	III	6.1		153	213	240	100 kg	200 kg	A	40
	Phenylurea pesticides, liquid, toxic	6.1	UN3002	III	6.1	T14	153	203	241	60 L	220 L	A	40
	Phenylacetonitrile, liquid	6.1	UN2470	III	6.1	T8	153	203	241	60 L	220 L	A	26
+	Phenylenediamines (o-; m-; p-)	6.1	UN1673	III	6.1		153	213	240	100 kg	200 kg	A	
	Phenylmercuric compounds, n.o.s.	6.1	UN2026	III	6.1		153	213	240	100 kg	200 kg	A	
	Potassium fluoride	6.1	UN1812	III	6.1	T8	153	213	240	100 kg	200 kg	A	26
	Potassium fluorosilicate	6.1	UN2655	III	6.1		153	213	240	100 kg	200 kg	A	26
	Pyrethroid pesticide, liquid toxic.	6.1	UN3352	III	6.1		153	213	240	60 L	220 L	A	40
	Pyrethroid pesticide, liquid, flammable, toxic, flashpoint not less than 23°C.	6.1	UN3351	III	6.1, 3	T14	153	203	241	60 L	220 L	B	40
	Pyrethroid pesticide, solid, toxic.	6.1	UN3349	III	6.1		153	213	240	100 kg	200 kg	A	40
	Quinoline	6.1	UN2656	III	6.1	T8	153	203	241	60 L	220 L	A	12
	Resorcinol	6.1	UN2876	III	6.1		153	213	240	100 kg	200 kg	A	
	Selenium compound, n.o.s.	6.1	UN3283	III	6.1	T7	153	213	240	100 kg	200 kg	A	
	Sodium arsanilate	6.1	UN2473	III	6.1		153	213	240	100 kg	200 kg	A	
	Sodium arsenite, aqueous solutions	6.1	UN1686	III	6.1	T15	153	203	241	60 L	220 L	A	
	Sodium chloroacetate	6.1	UN2659	III	6.1		153	213	240	100 kg	200 kg	A	
	Sodium fluoride	6.1	UN1690	III	6.1	T8	153	213	240	100 kg	200 kg	A	26
	Sodium fluorosilicate	6.1	UN2674	III	6.1		153	213	240	100 kg	200 kg	A	26
	Substituted nitrophenol pesticides, liquid, toxic	6.1	UN3014	III	6.1	T14	153	203	241	60 L	220 L	A	40
	Substituted nitrophenol pesticides, liquid, toxic, flammable flashpoint not less than 23 degrees C	6.1	UN3013	III	6.1, 3	B1, T14	153	203	242	60 L	220 L	A	40
	Substituted nitrophenol pesticides, solid, toxic	6.1	UN2779	III	6.1		153	213	240	100 kg	200 kg	A	40
	Tellurium compound, n.o.s.	6.1	UN3284	III	6.1	T7	153	213	240	100 kg	200 kg	A	
	tert-Butylcyclohexylchloroformate	6.1	UN2747	III	6.1	T8	153	203	241	60 L	220 L	A	12, 13, 25
	Tetrabromoethane	6.1	UN2504	III	6.1	T7	153	203	241	60 L	220 L	A	
	Tetrachloroethylene	6.1	UN1897	III	6.1	N36, T1	153	203	241	60 L	220 L	A	40
	Thiocarbamate pesticides, liquid, flammable, toxic, flash point not less than 23 degrees C.	6.1	UN3005	III	6.1, 3	T14	153	203	242	60 L	220 L	A	40
	Thiocarbamate pesticides, liquid, toxic	6.1	UN3006	III	6.1	T14	153	203	241	60 L	220 L	A	40
	Thiocarbamate pesticides, solid, toxic.	6.1	UN2771	III	6.1		153	213	240	100 kg	200 kg	A	40
G	Toxic liquid, inorganic, n.o.s.	6.1	UN3287	III	6.1	T7	153	203	241	60 L	220 L	A	
G	Toxic solid, inorganic, n.o.s.	6.1	UN3288	III	6.1		153	213	240	100 kg	200 kg	A	
G	Toxic solids, organic, n.o.s.	6.1	UN2811	III	6.1		153	213	240	100 kg	200 kg	A	
G	Toxic, liquids, organic, n.o.s.	6.1	UN2810	III	6.1	T7	153	203	241	60 L	220 L	A	40
	Triallyl borate	6.1	UN2609	III	6.1		153	203	241	60 L	220 L	A	13
	Triazine pesticides, liquid, toxic	6.1	UN2998	III	6.1	T14	153	203	241	60 L	220 L	A	40
	Triazine pesticides, liquid, toxic, flammable, flashpoint not less than 23 degrees C	6.1	UN2997	III	6.1, 3	T14	153	203	242	60 L	220 L	A	40
	Triazine pesticides, solid, toxic	6.1	UN2763	III	6.1		153	213	240	100 kg	200 kg	A	40
	Trichlorobenzenes, liquid	6.1	UN2321	III	6.1	T7	153	203	241	60 L	220 L	A	

Department of Transportation List of Division 6.1 Materials

SYMBOL	PS_NAME1	HAZ CLASS	UN NUMBER	PACK GROUP	LABEL	SPEC_PROV	EXCEPTIONS	NONBULK	BULK	PASS_AIR	CARGO_AIR	VESSEL	VESSELSP
	Trichloroethylene	6.1	UN1710	III	6.1	N36, T1	153	203	241	60 L	220 L	A	40
	Trimethylhexamethylene diisocyanate	6.1	UN2328	III	6.1	T8	163	203	241	60 L	220 L	B	
	Tris-(1-aziridinyl)phosphine oxide, solution	6.1	UN2501	III	6.1	T7	153	203	241	60 L	220 L	A	
	Vanadium compound, n.o.s.	6.1	UN3285	III	6.1	T7	153	213	240	100 kg	200 kg	A	
	Vanadium pentoxide, non-fused form	6.1	UN2862	III	6.1		153	213 212	240	100 KG	200 kg 100 kg	A	40
	Zinc fluorosilicate	6.1	UN2855	III	6.1		153	213	240	100 kg	200 kg	A	26

APPENDIX F.4

Department of Labor
Ocupational Health and Safety Administration

OSHA Regulations

29 CFR 1910.1200. Chapter XVII. Subpart Z: Toxic And Hazardous Substances. Section 1200: Hazard Communication.

(ii) If a chemical manufacturer, importer, or employer demonstrates to OSHA that the execution of a confidentiality agreement would not provide sufficient protection against the potential harm from the unauthorized disclosure of a trade secret specific chemical identity, the Assistant Secretary may issue such orders or impose such additional limitations or conditions upon the disclosure of the requested chemical information as may be appropriate to assure that the occupational health services are provided without an undue risk of harm to the chemical manufacturer, importer, or employer.

(11) If a citation for a failure to release specific chemical identity information is contested by the chemical manufacturer, importer, or employer, the matter will be adjudicated before the Occupational Safety and Health Review Commission in accordance with the Act's enforcement scheme and the applicable Commission rules of procedure. In accordance with the Commission rules, when a chemical manufacturer, importer, or employer continues to withhold the information during the contest, the Administrative Law Judge may review the citation and supporting documentation *in camera* or issue appropriate orders to protect the confidentiality of such matters.

(12) Notwithstanding the existence of a trade secret claim, a chemical manufacturer, importer, or employer shall, upon request, disclose to the Assistant Secretary any information which this section requires the chemical manufacturer, importer, or employer to make available. Where there is a trade secret claim, such claim shall be made no later than at the time the information is provided to the Assistant Secretary so that suitable determinations of trade secret status can be made and the necessary protections can be implemented.

(13) Nothing in this paragraph shall be construed as requiring the disclosure under any circumstances of process or percentage of mixture information which is a trade secret.

(j) *Effective dates.* Chemical manufacturers, importers, distributors, and employers shall be in compliance with all provisions of this section by March 11, 1994.

NOTE: The effective date of the clarification that the exemption of wood and wood products from the Hazard Communication standard in paragraph (b)(6)(iv) only applies to wood and wood products including lumber which will not be processed, where the manufacturer or importer can establish that the only hazard they pose to employees is the potential for flammability or combustibility, and that the exemption does not apply to wood or wood products which have been treated with a hazardous chemical covered by this standard, and wood which may be subsequently sawed or cut generating dust has been stayed from March 11, 1994 to August 11, 1994.

APPENDIX A TO § 1910.1200—HEALTH HAZARD DEFINITIONS (MANDATORY)

Although safety hazards related to the physical characteristics of a chemical can be objectively defined in terms of testing requirements (e.g. flammability), health hazard definitions are less precise and more subjective. Health hazards may cause measurable changes in the body such as decreased pulmonary function. These changes are generally indicated by the occurrence of signs and symptoms in the exposed employees such as shortness of breath, a non-measurable, subjective feeling. Employees exposed to such hazards must be apprised of both the change in body function and the signs and symptoms that may occur to signal that change.

The determination of occupational health hazards is complicated by the fact that many of the effects or signs and symptoms occur commonly in non-occupationally exposed populations, so that effects of exposure are difficult to separate from normally occurring illnesses. Occasionally, a substance causes an effect that is rarely seen in the population at large, such as angiosarcomas caused by vinyl chloride exposure, thus making it easier to ascertain that the occupational exposure was the primary causative factor. More often, however, the effects are common, such as lung cancer. The situation is further complicated by the fact that most chemicals have not been adequately tested to determine their health hazard potential, and data do not exist to substantiate these effects.

There have been many attempts to categorize effects and to define them in various ways. Generally, the terms "acute" and "chronic" are used to delineate between effects on the basis of severity or duration. "Acute" effects usually occur rapidly as a result of short-term exposures, and are of short duration. "Chronic" effects generally occur as a result of long-term exposure, and are of long duration.

The acute effects referred to most frequently are those defined by the American

National Standards Institute (ANSI) standard for Precautionary Labeling of Hazardous Industrial Chemicals (Z129.1–1988)—irritation, corrosivity, sensitization and lethal dose. Although these are important health effects, they do not adequately cover the considerable range of acute effects which may occur as a result of occupational exposure, such as, for example, narcosis.

Similarly, the term chronic effect is often used to cover only carcinogenicity, teratogenicity, and mutagenicity. These effects are obviously a concern in the workplace, but again, do not adequately cover the area of chronic effects, excluding, for example, blood dyscrasias (such as anemia), chronic bronchitis and liver atrophy.

The goal of defining precisely, in measurable terms, every possible health effect that may occur in the workplace as a result of chemical exposures cannot realistically be accomplished. This does not negate the need for employees to be informed of such effects and protected from them. Appendix B, which is also mandatory, outlines the principles and procedures of hazard assessment.

For purposes of this section, any chemicals which meet any of the following definitions, as determined by the criteria set forth in Appendix B are health hazards. However, this is not intended to be an exclusive categorization scheme. If there are available scientific data that involve other animal species or test methods, they must also be evaluated to determine the applicability of the HCS.7

1. *Carcinogen:* A chemical is considered to be a carcinogen if:

(a) It has been evaluated by the International Agency for Research on Cancer (IARC), and found to be a carcinogen or potential carcinogen; or

(b) It is listed as a carcinogen or potential carcinogen in the Annual Report on Carcinogens published by the National Toxicology Program (NTP) (latest edition); or,

(c) It is regulated by OSHA as a carcinogen.

2. *Corrosive:* A chemical that causes visible destruction of, or irreversible alterations in, living tissue by chemical action at the site of contact. For example, a chemical is considered to be corrosive if, when tested on the intact skin of albino rabbits by the method described by the U.S. Department of Transportation in appendix A to 49 CFR part 173, it destroys or changes irreversibly the structure of the tissue at the site of contact following an exposure period of four hours. This term shall not refer to action on inanimate surfaces.

3. *Highly toxic:* A chemical falling within any of the following categories:

(a) A chemical that has a median lethal dose (LD_{50}) of 50 milligrams or less per kilogram of body weight when administered orally to albino rats weighing between 200 and 300 grams each.

(b) A chemical that has a median lethal dose (LD_{50}) of 200 milligrams or less per kilogram of body weight when administered by continuous contact for 24 hours (or less if death occurs within 24 hours) with the bare skin of albino rabbits weighing between two and three kilograms each.

(c) A chemical that has a median lethal concentration (LC_{50}) in air of 200 parts per million by volume or less of gas or vapor, or 2 milligrams per liter or less of mist, fume, or dust, when administered by continuous inhalation for one hour (or less if death occurs within one hour) to albino rats weighing between 200 and 300 grams each.

4. *Irritant:* A chemical, which is not corrosive, but which causes a reversible inflammatory effect on living tissue by chemical action at the site of contact. A chemical is a skin irritant if, when tested on the intact skin of albino rabbits by the methods of 16 CFR 1500.41 for four hours exposure or by other appropriate techniques, it results in an empirical score of five or more. A chemical is an eye irritant if so determined under the procedure listed in 16 CFR 1500.42 or other appropriate techniques.

5. *Sensitizer:* A chemical that causes a substantial proportion of exposed people or animals to develop an allergic reaction in normal tissue after repeated exposure to the chemical.

6. *Toxic.* A chemical falling within any of the following categories:

(a) A chemical that has a median lethal dose (LD_{50}) of more than 50 milligrams per kilogram but not more than 500 milligrams per kilogram of body weight when administered orally to albino rats weighing between 200 and 300 grams each.

(b) A chemical that has a median lethal dose (LD_{50}) of more than 200 milligrams per kilogram but not more than 1,000 milligrams per kilogram of body weight when administered by continuous contact for 24 hours (or less if death occurs within 24 hours) with the bare skin of albino rabbits weighing between two and three kilograms each.

(c) A chemical that has a median lethal concentration (LC_{50}) in air of more than 200 parts per million but not more than 2,000 parts per million by volume of gas or vapor, or more than two milligrams per liter but not more than 20 milligrams per liter of mist, fume, or dust, when administered by continuous inhalation for one hour (or less if death occurs within one hour) to albino rats weighing between 200 and 300 grams each.

7. *Target organ effects.*

The following is a target organ categorization of effects which may occur, including examples of signs and symptoms and chemicals which have been found to cause such effects. These examples are presented to illustrate the range and diversity of effects and hazards found in the workplace, and the

broad scope employers must consider in this area, but are not intended to be all-inclusive.

a. Hepatotoxins: Chemicals which produce liver damage
   Signs & Symptoms: Jaundice; liver enlargement
   Chemicals: Carbon tetrachloride; nitrosamines
b. Nephrotoxins: Chemicals which produce kidney damage
   Signs & Symptoms: Edema; proteinuria
   Chemicals: Halogenated hydrocarbons; uranium
c. Neurotoxins: Chemicals which produce their primary toxic effects on the nervous system
   Signs & Symptoms: Narcosis; behavioral changes; decrease in motor functions
   Chemicals: Mercury; carbon disulfide
d. Agents which act on the blood or hematopoietic system: Decrease hemoglobin function; deprive the body tissues of oxygen
   Signs & Symptoms: Cyanosis; loss of consciousness
   Chemicals: Carbon monoxide; cyanides
e. Agents which damage the lung: Chemicals which irritate or damage pulmonary tissue
   Signs & Symptoms: Cough; tightness in chest; shortness of breath
   Chemicals: Silica; asbestos
f. Reproductive toxins: Chemicals which affect the reproductive capabilities including chromosomal damage (mutations) and effects on fetuses (teratogenesis)
   Signs & Symptoms: Birth defects; sterility
   Chemicals: Lead; DBCP
g. Cutaneous hazards: Chemicals which affect the dermal layer of the body
   Signs & Symptoms: Defatting of the skin; rashes; irritation
   Chemicals: Ketones; chlorinated compounds
h. Eye hazards: Chemicals which affect the eye or visual capacity
   Signs & Symptoms: Conjunctivitis; corneal damage
   Chemicals: Organic solvents; acids

APPENDIX B TO § 1910.1200—HAZARD DETERMINATION (*Mandatory*)

The quality of a hazard communication program is largely dependent upon the adequacy and accuracy of the hazard determination. The hazard determination requirement of this standard is performance-oriented. Chemical manufacturers, importers, and employers evaluating chemicals are not required to follow any specific methods for determining hazards, but they must be able to demonstrate that they have adequately ascertained the hazards of the chemicals produced or imported in accordance with the criteria set forth in this Appendix.

Hazard evaluation is a process which relies heavily on the professional judgment of the evaluator, particularly in the area of chronic hazards. The performance-orientation of the hazard determination does not diminish the duty of the chemical manufacturer, importer or employer to conduct a thorough evaluation, examining all relevant data and producing a scientifically defensible evaluation. For purposes of this standard, the following criteria shall be used in making hazard determinations that meet the requirements of this standard.

1. *Carcinogenicity:* As described in paragraph (d)(4) of this section and Appendix A of this section, a determination by the National Toxicology Program, the International Agency for Research on Cancer, or OSHA that a chemical is a carcinogen or potential carcinogen will be considered conclusive evidence for purposes of this section. In addition, however, all available scientific data on carcinogenicity must be evaluated in accordance with the provisions of this Appendix and the requirements of the rule.

2. *Human data:* Where available, epidemiological studies and case reports of adverse health effects shall be considered in the evaluation.

3. *Animal data:* Human evidence of health effects in exposed populations is generally not available for the majority of chemicals produced or used in the workplace. Therefore, the available results of toxicological testing in animal populations shall be used to predict the health effects that may be experienced by exposed workers. In particular, the definitions of certain acute hazards refer to specific animal testing results (see Appendix A).

4. *Adequacy and reporting of data.* The results of any studies which are designed and conducted according to established scientific principles, and which report statistically significant conclusions regarding the health effects of a chemical, shall be a sufficient basis for a hazard determination and reported on any material safety data sheet. *In vitro* studies alone generally do not form the basis for a definitive finding of hazard under the HCS since they have a positive or negative result rather than a statistically significant finding.

The chemical manufacturer, importer, or employer may also report the results of other scientifically valid studies which tend to refute the findings of hazard.

APPENDIX C TO § 1910.1200—[RESERVED]

APPENDIX D TO § 1910.1200—DEFINITION OF "TRADE SECRET" (MANDATORY)

The following is a reprint of the *Restatement of Torts* section 757, comment *b* (1939):

APPENDIX G

In Vitro Workshop Participants and Attendees

Appendix G
In Vitro Workshop Participants and Attendees

Fuad Amsyari
Environment Research Center Airlangga University Mulyorejo Camps-Surabaya Indonesia

Jim Antonini
National Institute for Occupational Safety & Health

Angela Auletta
U.S. EPA/OPPTS

Drew Badger
Procter & Gamble

Dan Bagley
Colgate-Palmolive Company

Michael Balls
ECVAM JRC Environment Institute Ispra (Va) Italy

Martin Barratt
Martin Consultancy United Kingdom

Bas Blaauboer
Utrecht University Research Institute of Toxicology, Utrecht, The Netherlands

Meta Bonner
U.S. FDA

Nancy Bordelon
NIEHS

June Bradlaw
International Foundation for Ethical Research

Ron Brown
FDA/DHHS/CDRH/OS T/DLS

Nancy Brown-Kobil
Animal Legal Defense Fund

Peter Bullock
Purdue Pharma LP

Leon Bruner
The Gillette Company

Jennifer Burpee
American Society of Clinical Pathology

Nicole Cardello
Physicians Committee for Responsible Medicine

Kathleen Cater
The Dial Corporation

Jerry Chandler
George Mason University

Rajendra Chhabra
NIEHS

Larry Claxton
U.S. EPA

Harvey Clewell
KS Crump Group/ICF Kaiser

Richard Clothier
University of Nottingham School of Biomedical Sciences United Kingdom

Catherine Cohen
L'Oreal Aulnay-sousbois, France

Tom Collins
FDA/CFSAN

Robert Combes
FRAME United Kingdom

James Cone
CA Dept. of Health Services

Charles Crespi
Gentest Corp.

Mike Cunningham
NIEHS

Rodger Curren
Institute of Vitro Sciences Inc.

George Cushmac
Dept. of Transportation

Carol Eisenmann
Cosmetic, Toiletry and Fragrance Association

Joe Elder
U.S. EPA

Gene Elmore
University of California,Irvine

Julia Fentem
SEAC Unilever Research United Kingdom

Oliver Flint
Bristol-Meyers Squibb Company

Anna Forsby
Stockholm University Dept. of Neurochemistry & Neurotoxicology Stockholm, Sweden

John Frazier
U.S. Air Force DOD

Steve Galson
U.S. EPA

Roger Gardner
BPPD U.S. EPA

Elke Genschow
ZEBET at the BG VV Berlin, Germany

Benjamin Gerson
University Services, BIEX Clinical Laboratory Boston Clinical Laboratories

David Giampocaro
U.S. EPA

Martin Gilman
Celsis Laboratory Group

Alan Goldberg
Johns Hopkins University School of Hygiene & Public Health

Janet Gould
National Starch & Chemical Co.,

Sidney Green
Howard University College of Medicine

Stan Gross
US EPA

Karen Hamernik
U.S. EPA OPP

John Harbell
Institute of Vitro Sciences, Inc.

Masseh Hashim
U.S EPA

Kenneth Hastings
FDA/CDER

Gabrielle Hawksworth
University of Aberdeen
dept. of Medicine,
United Kingdom

A. Wallace Hayes
The Gillette Company

Jerry Heindel
NIEHS

Oscar Hernandez
U.S. EPA OPPTS

Richard Hill
U.S. EPA OPPTS

Stephen Hundley
FDA/CDER

Kim In Suk
USDA

Michael Ioannou
U.S. EPA OPP

Brian Jones
Avon Products

Peter Jurs
Pennsylvania State
University

Gregory Kedderis
Chemical Industry
Institute of Toxicolgy

Leonard Keifer
U.S. EPA

Abdullah Khasawinah
U.S. EPA OPP

Giles Klopman
Case Western Reserve
University

David Lester
FDA-
DHHS/CDER/OPS/
DAPR

Rich Leukroth
U.S. EPA

Marianne Lewis
U.S. EPA

Manfred Liebsch
ZEBET at the BG VV
Berlin, Germany

Elizabeth Margosches
U.S. EPA
RAD/OPPT/OPPTS

Irving Mauer
U.S. EPA

Eugenia McAndrew
U.S. EPA OPP/RD/TRB

Jill Merrill
Institute for In Vitro
Sciences, Inc.

David Monroe
U.S. EPA
OPPT/RAD/ECAB

Hari Mukhoty
U.S. EPA
PRB/SRRD/OPP

Tina Nelson
American
Antivivisection Society

David Nuber
L'Oreal

Alan Nugent
Midwest Research
Institute

Michael Pelekis
Exxon Mobile
Biomedical Sciences

Pat Phibbs
BNA's Chemical
Regulation Reporter

Kathleen Plotzke
Dow Corning
Corporation Health of
Environmental Sciences

Alberto Protzel
U.S. EPA OPP

John Redden
U.S. EPA

Mike Rexrode
U.S. EPA

Lennart Romert
Swedish National
Chemicals inspectorate,
Solna, Sweden

Karl Rozman
Kansas University
Medical Center

Harry Salem
USA Edgewood CB
Center Department of
Defense

Jessica Sandler
People for the Ethical
Treatment of Animals

Dalj Sawhney
Annandale, VA

Philip Sayre
U.S. EPA OPPT

Louis Scarano
U.S. EPA

Leonard Schechtman
FDA/CDER

Karen Schweikart
National Cancer
Institute

Troy Seidle
Canadian Federation of
Humane Societies

Suhair Shallal
U.S. EPA OPP

Valerie Stanley
Animal Legal Defense
Fund

Martin Stephens
Humane Society of the
U.S.

Kathy Stitzel
Proctor & Gamble

William Stokes
NIEHS

Mary Beth Sweetland
People for the Ethical
Treatment of Animals

Noriho Tanaka
Hatano Research
Institute - Jabagawa,
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Regina Tihan
Institute for In Vitro
Sciences, Inc.

Joe Tomszewski
National Cancer
Institute

Charles Tyson
SRI International

Mary Ann Vasbinder
GlaxoSmith Kline

Bellina Veronesi
U.S. EPA

Erik Walum
Pharmacia & Upjohn
AB Biopharmaceuticals
Stockholm, Sweden

Jay Wang
NIH
DHHS/NIH/CC/CPD

Cal Willhite
State of California Dept.
of Toxic Substances
Control

Megan Wilson
EPA/OSCP

Marilyn Wind
U.S. CPSC

Anne Wolven
A.M. Wolven Inc.

Errol Zeiger
NIEHS

APPENDIX H

Federal Register Notices for International *In Vitro* Workshop

signed Confidential Disclosure Agreement will be required to receive a copy of any pending patent applications.

SUPPLEMENTARY INFORMATION: Gaucher Disease is a rare inborn error of metabolism which affects between 10,000 and 20,000 people worldwide, 40% in the United States. Gaucher Disease is the most common lipid storage disease. The symptoms associated with Gaucher Disease result from the accumulation of a lipid called glucocerebroside. This lipid is a byproduct of the normal recycling of red blood cells. When the gene with the instructions for producing an enzyme to break down this byproduct is defective, the lipid accumulates. The lipid is found in many places in the body, but most commonly in the macrophages in the bone marrow. There it interferes with normal bone marrow functions, such as production of platelets (leading to bleeding and bruising) and red blood cells (leading to anemia) and potentially death. The presence of glucocerebroside seems to also trigger the loss of minerals in the bones, causing the bones to weaken, and can interfere with the bone's blood supply.

The field of use is directed to the development of therapies for remedying enzyme deficiencies in the treatment of Gaucher Disease.

The prospective exclusive license will be royalty-bearing and will comply with the terms and conditions of 35 U.S.C. 209 and 37 CFR 404.7. The prospective exclusive license may be granted unless, within ninety (90) days from the date of this published notice, NIH receives written evidence and argument that establishes that the grant of the license would not be consistent with the requirements of 35 U.S.C. 209 and 37 CFR 404.7.

Applications for a license filed in response to this notice will be treated as objections to the grant of the contemplated license. Comments and objections submitted in response to this notice will not be made available for public inspection, and, to the extent permitted by law, will not be released under the Freedom of Information Act, 5 U.S.C. 552.

Dated: September 11, 2000.

Jack Spiegel,

Director, Division of Technology Development and Transfer, Office of Technology Transfer.

[FR Doc. 00–24241 Filed 9–20–00; 8:45 am]

BILLING CODE 4140–01–M

DEPARTMENT OF HEALTH AND HUMAN SERVICES

Public Health Service

National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health (NIH), National Toxicology Program (NTP); Notice of an International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity, co-sponsored by NIEHS, NTP and the U.S. Environmental Protection Agency (EPA): Workshop Agenda and Registration Information

SUMMARY: Pursuant to Public Law 103–43, notice is hereby given of a public meeting sponsored by NIEHS, the NTP, and the EPA, and coordinated by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM). The agenda topic is a scientific workshop to assess the current status of *in vitro* test methods for evaluating the acute systemic toxicity potential of chemicals and to develop recommendations for future research, development, and validation studies. The workshop will take place on October 17–20, 2000, at the Hyatt Regency Crystal City Hotel, 2799 Jefferson Davis Highway, Arlington, VA, 22202. The meeting will be open to the public.

In a previous **Federal Register** notice (Vol. 65, No. 115, pp. 37400–37403), ICCVAM requested information and data that should be considered at the Workshop and nominations of expert scientists to participate in the Workshop. A preliminary list of relevant studies to be considered for the Workshop was also provided. As a result of this request, an ICCVAM Interagency Workshop Organizing Committee has selected an international group of scientific experts to participate in this Workshop. NICEATM, in collaboration with ICCVAM, has developed a background summary of data and performance characteristics for available *in vitro* methods. This summary will be made available to invited expert scientists and the public before the Workshop. Requests for the summary can be made to the address given below. This notice provides an agenda, registration information, and updated details about the Workshop.

Workshop Background and Scope

A. Background

Acute toxicity testing is conducted to determine the hazards of various chemicals and products. This information is used to properly classify and label materials as to their lethality in accordance with an internationally harmonized system (OECD, 1998). Non-lethal endpoints may also be evaluated to identify potential target organ toxicity, toxicokinetic parameters, and dose-response relationships. While animals are currently used to evaluate acute toxicity, recent studies suggest that *in vitro* methods may also be helpful in predicting acute toxicity.

Studies by Spielmann *et al.* (1999) suggest that *in vitro* cytotoxicity methods may be useful in predicting a starting dose for *in vivo* studies, and thus may potentially reduce the number of animals necessary for such determinations. Other studies (*e.g.*, Ekwall *et al.*, 2000) have indicated an association between chemical concentrations leading to *in vitro* cytotoxicity and human lethal blood concentrations. A program to assess toxicokinetics and target organ toxicity utilizing *in vitro* methods has been proposed that may provide enhanced predictions of toxicity and potentially reduce or replace animal use for some tests (Ekwall *et al.*, 1999). However, many of the necessary *in vitro* methods for this program have not yet been developed. Other methods have not been evaluated in validation studies to determine their usefulness and limitations for generating information to meet regulatory requirements for acute toxicity testing. Development and validation of *in vitro* methods which can establish accurate dose-response relationships will be necessary before such methods can be considered for the reduction or replacement of animal use for acute toxicity determinations.

This workshop will examine the status of available *in vitro* methods for assessing acute toxicity. This includes screening methods for acute toxicity, such as methods that may be used to predict the starting dose for *in vivo* animal studies, and methods for generating information on toxicokinetics, target organ toxicity, and mechanisms of toxicity. The workshop will develop recommendations for validation efforts necessary to characterize the usefulness and limitations of these methods. Recommendations will also be developed for future mechanism-based research and development efforts that might further improve *in vitro* assessments of acute systemic lethal and non-lethal toxicity.

B. Objectives of the Workshop

Four major topics will be addressed:

- *In Vitro* Screening Methods for Assessing Acute Toxicity;

- *In Vitro* Methods for Toxicokinetic Determinations;
- *In Vitro* Methods for Predicting Organ Specific Toxicity; and
- Chemical Data Sets for Validation of *In Vitro* Acute Toxicity Test Methods.

The objectives of the meeting are to:

1. Review the status of *in vitro* methods for assessing acute systemic toxicity:

a. Review the validation status of available *in vitro* screening methods for their usefulness in estimating *in vivo* acute toxicity,

b. Review *in vitro* methods for predicting toxicokinetic parameters important to acute toxicity (*i.e.*, absorption, distribution, metabolism, elimination), and

c. Review *in vitro* methods for predicting specific target organ toxicity;

2. Recommend candidate methods for further evaluation in prevalidation and validation studies;

3. Recommend validation study designs that can be used to characterize adequately the usefulness and limitations of proposed *in vitro* methods;

4. Identify reference chemicals that can be used for development and validation of *in vitro* methods for assessing *in vivo* acute toxicity; and

5. Identify priority research efforts necessary to support the development of mechanism-based *in vitro* methods to assess acute systemic toxicity. Such efforts might include incorporation and evaluation of new technologies, such as gene microarrays, and development of methods necessary to generate dose response information.

Workshop Information

A. Workshop Agenda

Tuesday, October 17, 2000

8:30 a.m.—Opening Plenary Session
- Workshop Introduction
- Welcome from the National Toxicology Program (NTP)
- Overview of ICCVAM and NICEATM
- Acute Toxicity: Historical and Current Regulatory Perspectives
- Acute Toxicity Data: A Clinical Perspective

10:30 a.m. *In Vitro* Approaches to Estimate the Acute Toxicity Potential of Chemicals
- Estimating Starting Doses for *In Vivo* Studies using *In Vitro* Data
- An Integrated Approach for Predicting Systemic Toxicity
- Opportunities for Future Progress

Public Comment
Breakout Groups' Charges
12:30 p.m.—Lunch Break
1:45 p.m.—Breakout Groups: Identifying What Is Needed from *In Vitro* Methods
- Screening Methods;
- Toxicokinetic Determinations;
- Predicting Organ Specific Toxicity and Mechanisms; and
- Chemical Data Sets for Validation

5:30 p.m.—Adjourn for the Day

Wednesday, October 18, 2000

8:00 a.m.—Plenary Session—Status Reports by Breakout Group Co-Chairs
9:00 a.m.—Breakout Groups: Current Status of *In Vitro* Methods for Acute Toxicity
- Screening Methods;
- Toxicokinetic Determinations;
- Predicting Organ Specific Toxicity and Mechanisms; and
- Chemical Data Sets for Validation

12:00 p.m.—Lunch Break
1:30 p.m.—Breakout Groups: Current Status of *In Vitro* Methods for Acute Toxicity (Cont'd)
5:30 p.m.—Adjourn for the Day

Thursday, October 19, 2000

8:00 a.m.—Plenary Session—Status Reports by Breakout Group Co-Chairs
9:00 a.m.—Breakout Groups: Future Directions for *In Vitro* Methods for Acute Toxicity
- Screening Methods;
- Toxicokinetic Determinations;
- Predicting Organ Specific Toxicity and Mechanisms; and
- Chemical Data Sets for Validation

12:00 p.m.—Lunch Break
1:30 p.m.—Breakout Groups: Future Directions for *In Vitro* Methods for Acute Toxicity (Cont'd)
5:30 p.m.—Adjourn for the Day

Friday, October 20, 2000

8:00 a.m.—Closing Plenary Session—Reports by Breakout Group Co-Chairs
- Screening Methods;
- Toxicokinetic Determinations;
- Predicting Organ Specific Toxicity and Mechanisms; and
- Chemical Data Sets for Validation

Public Comment
Closing Comments
12:15 p.m.—Adjourn

B. Workshop Registration

The Workshop meeting will be open to the public, limited only by the space available. Due to space limitations, advance registration is requested by October 13, 2000. Registration forms can be obtained by contacting NICEATM at the address given below or by accessing the on-line registration form at: *http://iccvam.niehs.nih.gov/invi_reg.htm*. Other relevant Workshop information (*i.e.*, accommodations, transportation, etc.) is also provided at this website.

C. Public Comment

The Public is invited to attend the Workshop and the number of observers will be limited only by the space available. Two formal public comment sessions on Tuesday, October 17th and Friday, October 20th will provide an opportunity for interested persons or groups to present their views and comments to the Workshop participants (please limit to one speaker per group). Additionally, time will be allotted during each of the Breakout Group sessions for general discussion and comments from observers and other participants. The Public is invited to present oral comments or to submit comments in writing for distribution to the Breakout Groups to NICEATM at the address given below by October 13, 2000. Oral presentations will be limited to seven minutes per speaker to allow for a maximum number of presentations. Individuals presenting oral comments are asked to provide a hard copy of their statement at registration. For planning purposes, persons wishing to give oral comments are asked to check the box provided on the Registration Form, although requests for oral presentations will also be accepted on-site (subject to availability of time). Persons registering for oral comments or submitting written remarks are asked to include their contact information (name, address, affiliation, telephone, fax, and e-mail).

Guidelines for Requesting Registration Form and Submission of Public Comment

Requests for registration information and submission of public comments should be directed to the NTP Interagency Center for the Evaluation of Alternative Toxicological Methods, Environmental Toxicology Program, NIEHS/NTP, MD EC–17, PO Box 12233, Research Triangle Park, NC 27709; 919–541–3398 (phone); 919–541–0947 (fax); *iccvam@niehs.nih.gov* (e-mail). Public comments should be accompanied by complete contact information including name, (affiliation, if applicable), address, telephone number, and e-mail address.

References

- OECD (Organisation for Economic Cooperation and Development). (1998). Harmonized integrated hazard classification system for human health and environmental effects of chemical substances. OECD, Paris. (website: *http://www.oecd.org//ehs/Class/HCL6.HTM*)
- Spielmann, H., Genschow, E., Leibsch, M., and Halle, W. (1999) Determination of the starting dose for

acute oral toxicity (LD50) testing in the up and down procedure (UDP) from cytotoxicity data. ATLA, 27(6), 957–966.

• Ekwall, B., Ekwall, B., and Sjorstrom, M. (2000) MEIC evaluation of acute systemic toxicity: Part VIII. Multivariate partial least squares evaluation, including the selection of a battery of cell line tests with a good prediction of human acute lethal peak blood concentrations for 50 chemicals. ATLA, 28, Suppl. 1, 201–234.

• Ekwall, B., Clemedson, C., Ekwall, B., Ring, P., and Romert, L. (1999) EDIT: A new international multicentre programme to develop and evaluate batteries of *in vitro* tests for acute and chronic systemic toxicity. ATLA 27, 339–349.

Dated: September 12, 2000.

Samuel H. Wilson,

Deputy Director, National Institute of Environmental Health Sciences.

[FR Doc. 00–24244 Filed 9–20–00; 8:45 am]

BILLING CODE 4140–01–P

DEPARTMENT OF HOUSING AND URBAN DEVELOPMENT

[Docket No. FR–4463–N–04]

Notice of FHA Debenture Call

AGENCY: Office of the Assistant Secretary for Housing-Federal Housing Commissioner, HUD.

ACTION: Notice.

SUMMARY: This Notice announces a debenture recall of certain Federal Housing Administration debentures, in accordance with authority provided in the National Housing Act.

FOR FURTHER INFORMATION CONTACT: Richard Keyser, Room 3119P, L'Enfant Plaza, Department of Housing and Urban Development, 451 Seventh Street, SW., Washington, DC 20410, telephone (202) 755–7510 x137. This is not a toll-free number.

SUPPLEMENTARY INFORMATION: Pursuant to Sections 204(c) and 207(j) of the National Housing Act, 12 U.S.C. 1710(c), 1713(j), and in accordance with HUD's regulation at 24 CFR 203.409 and § 207.259(e)(3), the Federal Housing Commissioner, with approval of the Secretary of the Treasury, announces the call of all Federal Housing Administration debentures, with a coupon rate of 6.625 percent or above, except for those debentures subject to "debenture lock agreements", that have been registered on the books of the Federal Reserve Bank of Philadelphia, and are, therefore, "outstanding" as of September 30, 2000. The date of the call is January 1, 2001.

The debentures will be redeemed at par plus accrued interest. Interest will cease to accrue on the debentures as of the call date. Final interest on any called debentures will be paid with the principal at redemption.

During the period from the date of this notice to the call date, debentures that are subject to the call may not be used by the mortgagee for a special redemption purchase in payment of a mortgage insurance premium.

No transfer of debentures covered by the foregoing call will be made on the books maintained by the Treasury Department on or after October 1, 2000. This does not affect the right of the holder of a debenture to sell or assign the debenture on or after this date. Payment of final principal and interest due on January 1, 2001, will be made automatically to the registered holder.

Dated: September 15, 2000.

William C. Apgar,

Assistant Secretary for Housing-Federal Housing Commissioner.

[FR Doc. 00–24288 Filed 9–20–00; 8:45 am]

BILLING CODE 4210–27–M

DEPARTMENT OF THE INTERIOR

Fish and Wildlife Service

Notice of Receipt of Applications for Permit

Endangered Species

The following applicants have applied for a permit to conduct certain activities with endangered species. This notice is provided pursuant to Section 10(c) of the Endangered Species Act of 1973, *as amended* (16 U.S.C. 1531, *et seq.*):

PRT–841026

Applicant: Thane Wibbels, University of Alabama at Birmingham, Birmingham, AL

The applicant requests a permit to import up to 1000 blood samples and up to 500 tissue samples taken from Kemp's Ridley sea turtles (*Lepidochelys kempii*) in Mexico for enhancement of the species through scientific research. This notification covers activities conducted by the applicant over a five year period.

PRT–032758

Applicant: Exotic Feline Breeding Compound, Inc., Rosamond, CA

The applicant requests a permit to import 1 captive-born male Amur leopard (*Panthera pardus orientalis*) from the Novosibirsk Zoo, Russia for the purpose of propagation for the enhancement of the survival of the species.

PRT–032757

Applicant: Omaha's Henry Doorly Zoo, Omaha, NE

The applicant requests a permit to import 1 captive-born female Sumatran tiger (*Panthera tigris sumatrae*) from the Surabaya Zoo, Indonesia for the purpose of propagation for the enhancement of the survival of the species.

PRT–031061

Applicant: Susan E. Aronoff, Tampa, FL. 33624

The applicant requests a permit to import 1 captive-born male cheetah (*Acinonyx jubatus*) from the Endangered Animal Foundation, Driftweg, the Netherlands to enhance the survival of the species through conservation education.

PRT–830414

Applicant: Duke University Primate Center, Durham, NC

The applicant requests re-issuance of a permit to import two male and three female wild-caught golden-crowned sifakas (*Propithecus tattersalli*) from Dariana, Madagascar for the purpose of propagation for the enhancement of the survival of the species. This notification covers requests for re-issuances of the permit by the applicant over a five year period.

PRT–808256

Applicant: Duke University Primate Center, Durham, NC

The applicant requests re-issuance of a permit to import one male and two female wild-caught diademed sifakas (*Propithecus diadema*) from the Department of Water and Forest, Maramize, Madagascar for the purpose of propagation for the enhancement of the survival of the species. This notification covers requests for re-issuances of the permit by the applicant over a five year period.

PRT–031796

Applicant: Larry Edward Johnson, Boerne, TX

The applicant requests a permit to export two male and two female captive-born ring-tailed lemurs (*Catta lemur*) to Munchi's Zoo, Buenos Aires, Argentina to enhance the survival of the species through conservation education and captive propagation.

PRT–026102

Applicant: Elizabeth C. Stone/University of Georgia, Athens, GA

The applicant requests a permit to import salvaged specimens, non-viable eggs, and biological samples from Thick-billed parrots (*Rhynchopsitta pachyrhyncha*) collected in the wild in Mexico, for scientific research. This

is hereby given of the following meeting.

The meeting will be closed to the public in accordance with the provisions set forth in sections 552b(c)(4) and 552b(c)(6), Title 5 U.S.C., as amended. The grant applications and the discussions could disclose confidential trade secrets or commercial property such as patentable material, and personal information concerning individuals associated with the grant applications, the disclosure of which would constitute a clearly unwarranted invasion of personal privacy.

Name of Committee: National Institute of Diabetes and Digestive and Kidney Diseases Special Emphasis Panel, ZDK1 GRB 4 (01).

Date: June 16, 2000.

Time: 8:00 am to 2:00 pm.

Agenda: To review and evaluate grant applications.

Place: Embassy Suites Hotel, 1300 Concourse Drive, Linthicum, Maryland 21090.

Contact Person: William E. Elzinga,, Scientific Review Administrator, Review Branch, DEA, NIDDK, Room 647, 6707 Democracy Boulevard, National Institutes of Health, Bethesda, MD 20892–6600, (301) 594–8895.

This notice is being published less than 15 days prior to the meeting due to the timing limitations imposed by the review and funding cycle.

(Catalogue of Federal Domestic Assistance Program Nos. 93.047, Diabetes, Endocrinology and Metabolic Research; 93.848, Digestive Diseases and Nutrition Research; 93.849, Kidney Diseases, Urology and Hematology Research, National Institutes of Health, HHS)

Dated: June 8, 2000.

LaVerne Y. Stringfield,

Director, Office of Federal Advisory Committee Policy.

[FR Doc. 00–14960 Filed 6–13–00; 8:45 am]

BILLING CODE 4140–01–M

DEPARTMENT OF HEALTH AND HUMAN SERVICES

National Institute of Health

National Institute of Nursing Research; Notice of Closed Meeting

Pursuant to section 10(d) of the Federal Advisory Committee Act, as amended (5 U.S.C. Appendix 2), notice is hereby given of the following meeting.

The meeting will be closed to the public in accordance with the provisions set forth in sections 552b(c)(4) and 552b(c)(6), Title 5 U.S.C., as amended. The grant applications and the discussions could disclose confidential trade secrets or commercial property such as patentable material, and personal information concerning individuals associated with the grant applications, the disclosure of which would constitute a clearly unwarranted invasion of personal privacy.

Name of Committee: National Institute of Nursing Research Special Emphasis Panel, NINR Career Transitional Award Applications (K22s).

Date: June 21, 2000.

Time: 3:00 PM to 5:00 PM.

Agenda: To review and evaluate grant applications.

Place: Bethesda Holiday Inn, 8120 Wisconsin Avenue, Bethesda, MD 20852.

Contact Person: Mary J. Stephens-Frazier, Scientific Review Administrator, National Institute of Nursing Research, National Institutes of Health, Natcher Building, Room 3AN32, (301) 594–5971.

This notice is being published less than 15 days prior to the meeting due to the timing limitations imposed by the review and funding cycle.

(Catalogue of Federal Domestic Assistance Program Nos. 93.361, Nursing Research, National Institutes of Health, HHS)

Dated: June 8, 2000.

LaVerne Y. Stringfield,

Director, Office of Federal Advisory Committee Policy

[FR Doc. 00–14963 Filed 6–13–00; 8:45 am]

BILLING CODE 4140–01–M

DEPARTMENT OF HEALTH AND HUMAN SERVICES

National Institutes of Health

National Institute of Nursing Research; Notice of Closed Meeting

Pursuant to section 10(d) of the Federal Advisory Committee Act, as amended (5 U.S.C. Appendix 2), notice is hereby given of the following meeting.

The meeting will be closed to the public in accordance with the provisions set forth in sections 552b(c)(4) and 552b(c)(6), Title 5 U.S.C., as amended. The grant applications and the discussions could disclose confidential trade secrets or commercial property such as patentable material, and personal information concerning individuals associated with the grant applications, the disclosure of which would constitute a clearly unwarranted invasion of personal privacy.

Name of Committee: National Institute of Nursing Research Special Emphasis Panel, NINR/ORMH Mentored Research Scientist Development Award for Minority Investigators (KO1s).

Date: June 21, 2000.

Time: 8:30 a.m. to 2 p.m.

Agenda: To review and evaluate grant applications.

Place: Bethesda Holiday Inn, 8120 Wisconsin Avenue, Bethesda, MD 20814.

Contact Person: Mary J. Stephens-Frazier, Scientific Review Administrator, National Institute of Nursing Research, National Institutes of Health, Natcher Building, Room 3AN32, Bethesda, MD 20892, (301) 594–5971.

This notice is being published less than 15 days prior to the meeting due to the timing limitations imposed by the review and funding cycle.

(Catalogue of Federal Domestic Assistance Program Nos. 93.361, Nursing Research, National Institutes of Health, HHS)

Dated: June 8, 2000.

LaVerne Y. Stringfield,

Director, Office of Federal Advisory Committee Policy.

[FR Doc. 00–14964 Filed 6–13–00; 8:45 am]

BILLING CODE 4140–01–M

DEPARTMENT OF HEALTH AND HUMAN SERVICES

Public Health Service

National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health (NIH), National Toxicology Program (NTP); Notice of an International Workshop on In Vitro Methods for Assessing Acute Systemic Toxicity, co-sponsored by NIEHS, NTP and the U.S. Environmental Protection Agency (EPA): Request for Data and Suggested Expert Scientists

SUMMARY: Pursuant to Public Law 103–43, notice is hereby given of a public meeting sponsored by NIEHS, the NTP, and the EPA, and coordinated by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM). The agenda topic is a scientific workshop to assess the current status of in vitro test methods for evaluating the acute systemic toxicity potential of chemicals, and to develop recommendations for future development and validation studies. The workshop will take place on October 17–20, 2000 at the Hyatt Regency Crystal City Hotel, 2799 Jefferson Davis Highway, Arlington, VA, 22202. The meeting will be open to the public.

In preparing for this Workshop, ICCVAM is requesting: (1) Information and data that should be considered at the Workshop, including relevant data on currently available in vitro methods for assessing acute systemic toxicity; and (2) nominations of expert scientists to participate in the Workshop. An agenda, registration information, and other details will be provided in a subsequent **Federal Register** notice.

Background

ICCVAM, with participation by 14 Federal regulatory and research agencies and programs, was established in 1997 to coordinate issues relating to the development, validation, acceptance, and national/international harmonization of toxicological test methods. ICCVAM seeks to promote the scientific validation and regulatory acceptance of new and improved test methods applicable to Federal agencies, including methods that may reduce or replace animal use, or that refine protocols to lessen animal pain and distress. The Committee's functions include the coordination of interagency reviews of toxicological test methods and communication with stakeholders throughout the process of test method development and validation. The following Federal regulatory and research agencies participate:

Consumer Product Safety Commission
Department of Defense
Department of Energy
Department of Health and Human Services
  Agency for Toxic Substances and Disease Registry
  Food and Drug Administration
  National Institute for Occupational Safety and Health/CDC
  National Institutes of Health
  National Cancer Institute
  National Institute of Environmental Health Sciences
  National Library of Medicine
Department of the Interior
Department of Labor
  Occupational Safety and Health Administration
Department of Transportation
  Research and Special Programs Administration
Environmental Protection Agency

NICEATM was established in 1998 and provides operational support for the ICCVAM. NICEATM and ICCVAM collaborate to carry out activities associated with the development, validation, and regulatory acceptance of proposed new and improved test methods. These activities may include:

• Test Method Workshops, which are convened as needed to evaluate the adequacy of current methods for assessing specific toxicities, to identify areas in need of improved or new testing methods, to identify research efforts that may be needed to develop new test methods, and to identify appropriate development and validation activities for proposed new methods.

• Expert Panel Meetings, which are typically convened to evaluate the validation status of a method following the completion of initial development and pre-validation studies. Expert Panels are asked to recommend additional validation studies that might be helpful in further characterizing the usefulness of a method, and to identify any additional research and development efforts that might enhance the effectiveness of a method.

• Independent Peer Review Panel Meetings, which are typically convened following the completion of comprehensive validations studies on a test method. Peer Review Panels are asked to develop scientific consensus on the usefulness and limitations of test methods to generate information for specific human health and/or ecological risk assessment purposes. Following the independent peer review of a test method, ICCVAM forwards recommendations on its usefulness to agencies for their consideration. Federal agencies then determine the regulatory acceptability of a method according to their mandates.

Additional information about ICCVAM and NICEATM can be found at the website: *http://iccvam.niehs.nih.gov*.

Workshop Background and Scope

A. Background

Federal regulatory agencies require toxicity testing to determine the safety or hazard of various chemicals and products prior to human exposure. Agencies use this information to properly classify and label products as to their hazard potential. Acute oral toxicity determinations are currently made using animals. However, recent studies (e.g., Spielmann et al., 1999) suggest that in vitro cytotoxicity methods may be useful in predicting a starting dose for in vivo studies, and thus may potentially reduce the number of animals necessary for such determinations.

Other studies (e.g., Ekwall et al., 2000) have indicated an association between in vitro cytotoxicity and human lethal blood concentrations. However, these in vitro methods have not yet been evaluated in validation studies to determine their usefulness and limitations for generating acute toxicity testing information necessary to meet regulatory testing requirements. Additionally, other in vitro methods would likely be necessary to establish accurate dose-response relationships before such methods could substantially reduce or replace animal use for acute toxicity determinations.

This workshop will examine the status of available in vitro methods and develop recommendations for validation efforts necessary to characterize the usefulness and limitations of existing methods. Recommendations for future research and development efforts that might further enhance the usefulness of in vitro assessments of acute systemic lethal toxicity will also be developed.

B. Objectives of the Workshop

Four major topics will be addressed:

1. General cytotoxicity methods predictive of acute lethal toxicity;
2. Toxicokinetic and organ specific toxicity methods;
3. Reference chemicals for validation of the above methods; and
4. The use of quantitative structure activity relationships (QSAR) and chemical/physical properties for predicting acute lethal toxicity.

The objectives of the meeting are to:

1 a. Identify and review the status of in vitro general cytotoxicity screening methods that may reduce animal use for assessing acute systemic toxicity;

b. Identify information from in vitro methods necessary to predict acute systemic toxicity and review the status of relevant methods (e.g., in vitro methods to assess gut absorption, metabolism, blood-brain barrier penetration, volume distribution to critical target organs, and specific target organ toxicity);

2. Identify candidate methods for further evaluation in prevalidation and validation studies;

3. Identify reference chemicals useful for development and validation of in vitro methods for assessing acute systemic toxicity;

4. Identify validation study designs needed to adequately characterize the proposed methods in 2.; and

5. Identify priority research efforts necessary to support the development of in vitro methods to adequately assess acute systemic toxicity. Such efforts might include incorporation and evaluation of new technologies such as gene microarrays, and development of methods necessary to generate dose response information.

C. Methods for Consideration

Given the breadth of the workshop topics, many methods are likely to be considered relevant to the discussion. Methods will include but are not limited to those proposed in the Multicentre Evaluation of In Vitro Cytotoxicity (MEIC) battery (*http://www.ctlu.se*). A background document summarizing the data and performance characteristics for available methods is being prepared by NICEATM in collaboration with the ICCVAM interagency organizing committee. Information received as a result of this **Federal Register** notice will be

considered for inclusion in the background document. In formulating its recommendations, the Workshop participants will evaluate information in the background document and relevant information from other sources.

D. Test Method Data and Information Sought

Data are sought from completed, ongoing, or planned studies that provide comparative performance data for in vitro methods compared to currently accepted in vivo methods for determining acute lethal toxicity and hazard classification. Data from test methods that provide toxicokinetic and specific target organ toxicity information are also sought. Submissions should describe the extent to which established criteria for validation and regulatory acceptance have been addressed. These criteria are provided in "Validation and Regulatory Acceptance of Toxicological Test Methods: A Report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods," NIH publication 97–3981 (*http://ntp-server.niehs.nih.gov/htdocs/ICCVAM/iccvam.html*). Where possible, submitted data and information should adhere to the guidance provided in the document, "Evaluation of the Validation Status of Toxicological Methods: General Guidelines for Submissions to ICCVAM," NIH Publication 99–4496, (*http://iccvam.niehs.nih.gov/doc1.htm*). Both publications are also available on request from NICEATM at the address provided below. Relevant information submitted in response to this request will be incorporated into the background material provided to Workshop participants. A preliminary list of relevant studies is provided at the end of this announcement, and public comment and suggestions for additions are invited.

NICEATM and the ICCVAM interagency workshop organizing committee will compile information on the studies to be considered at the Workshop. All data should be submitted by July 15, 2000 in order to ensure full consideration.

E. Request for Nomination of Expert Scientists for the Test Method Workshop

NICEATM is soliciting nominations for expert scientists to participate in the Workshop. (See Guidelines for Submission of Comments below). Types of expertise likely to be relevant include acute toxicity testing in animals, evaluation and treatment of acute toxicity in humans, development and use of in vitro methodologies, statistical data analysis, knowledge of chemical data sets useful for validation of acute toxicity studies, and hazard classification of chemicals and products. Expertise need not be limited to these areas, nor will these areas necessarily be included on the Panel. An appropriate breadth of expertise will be sought. If other areas of scientific expertise are recommended, the rationale should be provided.

Nominations should be accompanied by complete contact information including name, address, institutional affiliation, telephone number, and e-mail address. The rationale for nomination should be provided. If possible, a biosketch or a curriculum vitae should be included. To avoid the potential for candidates being contacted by a large number of nominators, candidates need not be contacted prior to nomination.

Workshop experts will be selected by an ICCVAM interagency workshop organizing committee after considering all nominations received from the public as well as nominations developed internally. All nominees will be contacted for interest and availability, and curricula vitae will be solicited from the nominees. Candidates will be required to disclose potential conflicts of interest.

Schedule for the Workshop

The Workshop will take place on October 17–20, 2000 at the Hyatt Regency Crystal City Hotel, 2799 Jefferson Davis Highway, Arlington, VA 22202. The Workshop meeting will be open to the public, limited only by space available.

Submitted methods and supporting data will be reviewed during the July to August 2000 timeframe and a background review document will be prepared by NICEATM in collaboration with the ICCVAM interagency organizing committee. The background information will be made available to Workshop experts for discussion at the meeting and will be available to the Public in advance of the Workshop.

Public Input Invited

As described above, ICCVAM invites comments on the scope and process for the review; comments on the ICCVAM preliminary list of studies for consideration; the submission of other test methods for consideration; and the nomination of experts to participate in the Workshop. Nominations must be submitted within 30 days of the publication date of this notice, and other information should be submitted by July 15, 2000.

Guidelines for Submission of Public Comment

Correspondence should be directed to Dr. William S. Stokes, NTP Interagency Center for the Evaluation of Alternative Toxicological Methods, Environmental Toxicology Program, NIEHS/NTP, MD EC–17, PO Box 12233, Research Triangle Park, NC 27709; 919–541–3398 (phone); 919–541–0947 (fax); *iccvam@niehs.nih.gov* (e-mail). Public comments should be accompanied by complete contact information including name, (affiliation, if applicable), address, telephone number, and e-mail address.

Preliminary List of Studies to be Considered for the Workshop on In Vitro Methods for Assessing Acute Systemic Toxicity

ICCVAM has compiled a preliminary list of relevant studies. The public is invited to comment on this list, and suggestions for additions may be submitted. (See Section of this **Federal Register** announcement on Guidelines for Submission of Public Comments).

Studies that may be completed but not published are not included here. This list provides examples of studies and information that may be appropriate for consideration by the Workshop experts.

Balls, M., Blaauboer, B.J., Fentem, J.H., Bruner, L., Combes, R.D., Ekwall, B., Fielder, R.J., Guillouzo, A., Lewis, R.W., Lovell, D.P., Reinhardt, C.A., Repetto, G., Sladowski, D., Spielmann, H., and Zucco, F. (1995) Practical aspects of the validation of toxicity test procedures—The report and recommendations of ECVAM Workshop 5. ATLA 23, 129–147.

Bernson, V., Bondesson, I., Ekwall, B., Stenberg, K., and Walum, E. (1987) A multicenter evaluation study of *in vitro* cytotoxicity. ATLA, 14, 144–145.

Bondesson, I., Ekwall, B., Stenberg, K., Romert, L., and Walum, E. (1988) Instruction for participants in the multicenter evaluation study of *in vitro* cytotoxicity (MEIC). ATLA, 15, 191–193.

Bondesson, I., Ekwall, B., Hellberg, S., Romert, L., Stenberg, K., and Walum, E. (1989) MEIC—A new international multicenter project to evaluate the relevance to human toxicity of *in vitro* cytotoxicity tests. Cell Biol. Toxicol., 5, 331–347.

Clemedson, C., and Ekwall, B. (1999) Overview of the final MEIC results: I. The *in vitro-in vivo* evaluation. Toxicology In vitro, 13, 657–663.

Clemedson, C., McFarlane-Abdulla, E., Andersson, M., Barile, F.A., Calleja, M.C., Chesnes, C., Clothier, R., Cottin, M., Curren, R., Daniel-Szolgay, E., Dierickx, P., Ferro, M., Fiskesjö, G., Garza-Ocanas, L., Goamez-Lechoan, M.J., Gualden, M., Isomaa, B., Janus, J., Judge, P., Kahru, A., Kemp, R.B., Kerszman, G., Kristen, U., Kunimoto, M., Karenlampi, S., Lavrijsen, K., Lewan L., Lilius, H., Ohno, T., Persoone, C., Roguet, R.,

Romert, L., Sawyer, T., Seibert, H., Shrivastava, R., Stammati, A., Tanaka, N., Torres Alanis, O., Voss, J–U., Wakuri, S., Walum, E., Wang, X., Zucco, F., and Ekwall, B. (1998) MEIC evaluation of acute systemic toxicity. Part I. Methodology of 68 *in vitro* toxicity assays used to test the first 30 reference chemicals. ATLA, 24, Suppl. 1, 249–272.

Clemedson, C., McFarlane-Abdulla, E., Andersson, M., Barile, F.A., Calleja, M.C., Chesne, C., Clothier, R., Cottin, M., Curren, R., Dierickx, P., Ferro, M., Fiskesjø, G., Garza-Ocanas, L., Gomez-Lechon, M.J., Gulden, M., Isomaa, B., Janus, J., Judge, P., Kahru, A., Kemp, R.B., Kerszman, G., Kristen, U., Kunimoto, M., Karenlampi, S., Lavrijsen, K., Lewan L., Lilius, H., Malmsten, A., Ohno, T., Persoone, G., Pettersson, R., Roguet, R., Romert, L., Sandberg, M., Sawyer, T., Seibert, H., Shrivastava, R., Sjostrom, M., Stammati, A., Tanaka, N., Torres Alanis, O., Voss, J–U., Wakuri, S., Walum, E., Wang, X., Zucco, F. and, Ekwall, B. (1996) MEIC evaluation of acute systemic toxicity. Part II. *In vitro* results from 68 toxicity assays used to test the first 30 reference chemicals and a comparative cytotoxicity analysis. ATLA, 24, Suppl. 1, 273–311.

Clemedson, C., Barile, F.A., Ekwall, B., Gomez-Lechon, M.J., Hall, T., Imai, K., Kahru, A., Logemann, P., Monaco, F., Ohno, T., Segner, H., Sjostrom, M., Valentino, M., Walum, E., Wang, X., and Ekwall, B. (1998). MEIC evaluation of acute systemic toxicity: Part III. *in vitro* results from 16 additional methods used to test the first 30 reference chemicals and a comparative cytotoxicity analysis. ATLA 26, Suppl. 1, 91–129.

Clemedson, C., Aoki, Y., Andersson, M., Barile, F.A., Bassi, A M., Calleja, M.C., Castano, A., Clothier, R.H., Dierickx, P., Ekwall, B., Ferro, M., Fiskeso, G., Garza-Ocanas, L. Gomez-Lechoan, M.J., Gulden, M., Hall, T., Imai, K., Isomaa, B., Kahru, A., Kerszman, G., Kjellstrand, P., Kristen, U., Kunimoto, M., Karenlampi, S., Lewan, L., Lilius, H., Lonkianov, A., Monaco, F., Ohno, T., Persoone, C., Romert, L., Sawyer, T.W., Shrivastava, R., Segner, H., Seibert, H., Sjostrom, M., Stammati, A., Tanaka, N., Thuvander, A., Torres-Alanis, O., Valentino, M., Wakuri, S., Walum, E., Wieslander, A., Wang, X., Zucco, F., and Ekwall, B. (1998). MEIC evaluation of acute systemic toxicity. Part IV. *In vitro* results from 67 toxicity assays used to test reference chemicals 31–50 and a comparative cytotoxicity analysis. ATLA 26, Suppl. 1, 131–183.

Clemedson, C., Barile, F.A., Chesne, C., Cottin, M., Curren, R., Ekwall, B., Ferro, M., Gomez-Lechon, M.J., Imai, K., Janus, J., Kemp, R.B., Kerszman, G., Kjellstrand, P., Lavrijsen, K., Logemann, P., McFarlane-Abdulla, E., Roguet, R., Segner, H., Seibert, H., Thuvander, A., Walum, E., and Ekwall, Bj. (2000) MEIC evaluation of acute systemic toxicity: Part VII. Prediction of human toxicity by results from testing of the first 30 reference chemicals with 27 further *in vitro* assays. ATLA 28, Suppl. 1, 161–200.

Ekwall, B. (1995) The basal cytotoxicity concept, pp 721–725. In Proceedings of the World Congress on Alternatives and Animal Use in the Life Sciences: Education, Research, Testing. Alternative Methods in Toxicology and the Life Sciences, Vol. 11. Mary Ann Liebert, New York, 1995.

Ekwall, B. (1999) Overview of the Final MEIC Results: II. The *In vitro/In vivo* evaluation, including the selection of a practical battery of cell tests for prediction of acute lethal blood concentrations in humans. Toxicol. *In vitro*, 13, 665–673.

Ekwall, B., Gomez-Lechon, M.J., Hellberg, S., Bondsson, I., Castell, J.V., Jover, R., Hogberg, J., Ponsoda, X., Stenberg, K., and Walum, E. (1990) Preliminary results from the Scandinavian multicentre evaluation of *in vitro* cytotoxicity (MEIC). Toxicol. *In vitro*, 4, 688–691.

Ekwall, B., Clemedson, C., Crafoord, B., Ekwall, Ba., Hallander, S., Walum E., and Bondesson, I. (1998) MEIC evaluation of acute systemic toxicity. Part V. Rodent and human toxicity data for the 50 reference chemicals. ATLA 26, Suppl. 2, 569–615.

Ekwall, B., Barile., F.A., Castano, A., Clemedson, C., Clothier, R.H., Dierickx, P., Ekwall, B., Ferro, M., Fiskesjø, G., Garza-Ocanas, L., Gomez-Lechon, M-J., Gulden, M., Hall, T., Isomaa, B., Kahru, A, Kerszman, G., Kristen, U., Kunimoto, M., Karenlampi, S., Lewan, L, Loukianov, A., Ohno, T., Persoone, G., Romert, L., Sawyer, T.W., Segner, H., Shrivastava, R., Stammati, A., Tanaka, N., Valentino, M., Walum, E., and Zucco, F. (1998) MEIC evaluation of acute systemic toxicity. Part VI. Prediction of human toxicity by rodent LD50 values and results from 61 *in vitro* tests. ATLA 26, Suppl. 2, 617–658.

Ekwall, B., Clemedson, C., Ekwall, B., Ring, P., and Romert, L. (1999) EDIT: A new international multicentre programme to develop and evaluate batteries of *in vitro* tests for acute and chronic systemic toxicity. ATLA 27, 339–349.

Ekwall, B., Ekwall, B., and Sjostrom, M. (2000) MEIC evaluation of acute systemic toxicity: Part VIII. Multivariate partial least squares evaluation, including the selection of a battery cell line tests with a good prediction of human acute lethal peak blood concentrations for 50 chemicals. ATLA 28, Suppl. 1, 201–234.

Hellberg, S., Bondesson, I., Ekwall, B., Gomez-Lechon, M.J., Jover, R., Hogberg, J., Ponsoda, X., Romert, L., Stenberg, K., and Walum, E. (1990) Multivariate validation of cell toxicity data: The first ten MEIC chemicals. ATLA, 17, 237–230.

Hellberg, S., Eriksson, L., Jonsson, J., Lindgren, F., Sjostrom, M., Wold, S., Ekwall, B., Gomez-Lechon, J.M., Clothier, R., Accomando, N.J., Gimes, G., Barile, F.A., Nordin, M., Tyson, C.A., Dierickx, P., Shrivastava, R.S., Tingsleff-Skaanild, M., Garza-Ocanas, L., and Fiskesjo;, C. (1990) Analogy models for prediction of human toxicity. ATLA, 18, 103–116.

Shrivastava, R., Delomenie, C., Chevalier, A., John, G., Ekwall, B., Walum, E., and Massingham, R. (1992) Comparison of *in vivo* acute lethal potency and *in vitro* cytotoxicity of 46 chemicals. Cell Biol. Toxicol., 8(2), 157–170.

Spielmann, H., Genschow, E., Liebsch, M., and Halle, W. (1999) Determination of the starting dose for acute oral toxicity (LD50) testing in the up and down procedure (UDP) from cytotoxicity data. ATLA, 27(5), 957–966.

Walum, E, Nilsson, M, Clemedson, C. and Ekwall, B. (1995) The MEIC program and its implications for the prediction of acute human systemic toxicity, pp 275–282 In Proceedings of the World Congress on Alternatives and Animal Use in the Life Sciences: Education, Research, Testing. Alternative Methods in Toxicology and the Life Sciences, Vol. 11. Mary Ann Liebert, New York, 1995.

Dated: June 6, 2000.

Samuel H. Wilson,

Deputy Director, National Institute of Environmental Health Sciences.

[FR Doc. 00–14968 Filed 6–13–00; 8:45 am]

BILLING CODE 4140–01–P

DEPARTMENT OF HOUSING AND URBAN DEVELOPMENT

[Docket No. FR–4564–N–03]

Notice of Proposed Information Collection: Lead Hazard Control Grant Program Data Collection—Progress Reporting

AGENCY: Office of Lead Hazard Control.

ACTION: Notice.

SUMMARY: The revised information collection requirement described below will be submitted to the Office of Management and Budget (OMB) for review, as required by the Paperwork Reduction Act. The Department is soliciting public comments on the subject proposal.

DATES: Comments Due Date: August 14, 2000.

ADDRESSES: Interested persons are invited to submit comments regarding this proposal. Comments should refer to the proposal by name and/or OMB Control Number and should be sent to: Gail Ward, Reports Liaison Officer, Department of Housing and Urban Development, 451 7th Street, SW, Room P–3206, Washington, DC 20410.

FOR FURTHER INFORMATION CONTACT: Matthew Ammon at (202) 755–1785, ext. 158 (this is not a toll-free number) for copies of the proposed forms and other available documents.

SUPPLEMENTARY INFORMATION: The Department is submitting the revised information collection to OMB for review, as required by the Paperwork Reduction Act of 1995 (44 U.S.C. Chapter 35, as amended).

This Notice is soliciting comments from members of the public and affected agencies concerning the proposed collection of information to: (1) Evaluate whether the revised collection of information is necessary for the proper performance of the functions of the agency, including whether the

APPENDIX I

ICCVAM Recommendations

ICCVAM Recommendations on *In Vitro* Methods for Assessing Acute Systemic Toxicity

An International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity was convened in Arlington, VA, on October 17-20, 2000. The Workshop was organized by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), and was co-sponsored by the U.S. Environmental Protection Agency (EPA), the National Institute of Environmental Health Sciences (NIEHS), and the National Toxicology Program (NTP). The Workshop focused on reviewing the validation status and possible current uses of *in vitro* methods to assess acute oral lethality potential of chemicals. Workshop participants also recommended research, development, and validation efforts that would further advance the usefulness of *in vitro* methods. For a complete account of Workshop discussions and recommendations, please refer to the *Report of the International Workshop on In Vitro Methods for Assessing Acute Systemic Toxicity* (NIH Publication 01-4499). Based on a review of the Workshop Report, ICCVAM developed the following recommendations to forward to Federal agencies with the Report and Guidance Document.

Current Uses for *In Vitro* Methods

Workshop participants considered the merit of using *in vitro* cytotoxicity tests for predicting the acute oral lethality of chemicals in humans and animals, as suggested by previous studies (e.g., Clemedson and Ekwall, 1999; Halle and Goeres, 1988). They concluded that the available *in vitro* assays would require further development to accurately predict acute lethality (i.e., LD50). Workshop participants recommended that *in vitro* cytotoxicity data be included as one of the factors used to identify appropriate starting doses for *in vivo* acute lethality studies as described by Spielmann et al. (1999). In the approach developed by Spielmann, *in vitro* cytotoxicity tests are used to predict starting doses for acute *in vivo* lethality assays.

ICCVAM agrees with the Workshop Report that data from *in vitro* cytotoxicity assays can be useful as one of the tools (e.g., SAR or bridging from similar compounds or mixtures) in setting a starting dose for the *in vivo* assessment of acute oral toxicity.. The attached *Guidance Document on Using In Vitro Data to Estimate In Vivo Starting Doses for Acute Toxicity* (NIH Publication 01-4500) describes one method, the murine BALB/c 3T3 neutral red uptake assay, for which data for a number of chemicals supports its potential utility for estimating the starting dose. Starting doses are calculated using a regression formula based on an *in vitro-in vivo* correlation for 347 chemicals. Preliminary information suggests that use of this *in vitro* approach could reduce the number of animals currently used in *in vivo* acute toxicity tests. Additionally, new OECD Guidelines for *in vivo* acute toxicity testing recommend a starting dose below the estimated LD50 to minimize the number of animals that receive lethal doses and to avoid underestimating the hazard. ICCVAM recommends that Federal agencies consider making information about this *in vitro* approach available as one of the tools that can be used to select an appropriate starting dose for acute oral toxicity tests.

Research Directions

Workshop participants identified several areas for research and development activities to advance the use of *in vitro* methods for predicting acute oral toxicity in animals and humans. ICCVAM recognizes that there are many directions that such future research and testing might take. These include both near-term and long-term research activities.

➢ **Near-Term Research**

ICCVAM concurs with the Workshop recommendation that near-term validation studies should focus on two standard cytotoxicity assays: one using a human cell system and one using a rodent cell system. Since the murine BALB/c 3T3 cytotoxicity assay has been evaluated for only a limited number of chemical classes, there is

merit in determining its usefulness with a broader array of chemical classes. Cell lines established from the rat rather than the mouse might also be considered, as most acute oral toxicity testing is conducted in this species. Human cell lines should also be considered since one of the aims of toxicity testing is to make predictions of potential toxicity in humans. Future validation studies should therefore compare rodent and human *in vitro* data with one another, with rodent *in vivo* data, and with human *in vivo* data. Correlations between *in vitro* and *in vivo* data might help in selecting cytotoxicity assays for further evaluation.

The U.S. EPA and NIEHS are collaborating to further characterize the usefulness of *in vitro* methods for acute toxicity testing. ICCVAM recognizes that these activities may yield important information on the near-term and long-term application of *in vitro* tests. ICCVAM recommends the establishment of an interagency expert group under ICCVAM to advise on near-term activities such as assay selection, study design, and chemical selection.

➢ **Long-Term Research**

Longer-term research activities should be directed at improving *in vitro* systems that provide information on biokinetics, metabolism, and organ-specific toxicity. *In vitro* methodologies for gathering biokinetic and target organ specific effects data are needed to facilitate reasonably accurate predictions of LD50s, signs and symptoms associated with toxicity, and pathophysiological effects. Research efforts that might increase the predictive capability of *in vitro* assays include:

- Developing the use of quantitative structure-activity relationship (QSAR)/quantitative structure-property relationship (QSPR) models that predict kinetic parameters such as gut absorption and passage across the brain, kidney, and skin barrier systems.

- Developing efficient *in vitro* systems that provide accurate metabolic and biokinetic data.

- Developing accurate physiologically-based biokinetic models.

- Developing *in vitro* systems that accurately predict organ-specific toxicity.

- Investigating the mechanistic basis for "outlier" chemicals in *in vitro-in vivo* correlations and developing "exclusion" rules for identifying chemicals that cannot be accurately evaluated using *in vitro* methods.

- Investigating the utility of toxicogenomics/proteomics for the assessment of acute toxicity, especially the prediction of NOAELs/LOAELs for acute exposure.

ICCVAM appreciates that most of these long-term research activities will yield further improvements in the usefulness of *in vitro* methods for predicting acute systemic toxicity, but that significant resources would be required. ICCVAM concludes that such activities will warrant consideration along with other potential research efforts in establishing priorities.

Adopted by ICCVAM
April 23, 2001

The effect of phenylephrine on pain and flare intensity in eyes with uveitis

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ABSTRACT.

Purpose: **To investigate the influence of protein concentration in the anterior chamber, measured by laser flare meter, on pain sensation after phenylephrine instillation in patients with iridocyclitis.**

Methods: **Twenty-five consecutive patients with iridocyclitis were included. Patients with cataract, exfoliation syndrome, diabetes mellitus, glaucoma or any other previous ocular diseases or ocular surgery were excluded. Patients were divided into two groups: Group 1 – without fibrinoid reaction (FR) in the anterior chamber (18 patients), and Group 2 – with FR (7 patients). Protein concentration in the anterior chamber was measured with laser flare meter (FC 500, Kowa Co., Japan). Pupil size was measured by Alcon Tilo Scale, and pain sensation was estimated by Visual Analogue Scale (VAS, Kabi Pharmacia). All measurements were done before and 1 hour after topical instillation of 10% phenylephrine hydrochloride into the subconjunctival sac of the inflammed eyes.**

Results: **Eyes with iridocyclitis and fibrinoid reaction (FR) have a higher flare intensity compared to those without FR ($p<0.05$). Pupil size was significantly increased after phenylephrine instillation in both study groups (Wilcoxon test, $p<0.05$). The VAS pain and flare intensity were significantly decreased in group without FR after phenylephrine instillation (Group 1) compared to values before treatment (Wilcoxon test, $p<0.05$). In eyes with FR (Group 2), no significant influence of phenylephrine instillation was found on VAS pain and flare intensity.**

Conclusions: **After phenylephrine instillation, flare intensity and pain were significantly decreased only in eyes with iridocyclitis and without FR. The decreasing level of flare intensity, and paralysis of the pupil after phenylephrine instillation seem to alleviate pain in those eyes.**

Key words: iridocyclitis – phenylephrine – flare intensity – pain – pupil size.

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Photophobia, tearing and decreased vision constitute the subjective signs of iridocyclitis. Pain is frequently associated with inflammation in the anterior part of the eye, but the origin of this pain is not exactly known.

Generally, pain during any inflammation is defined as a complex response involving the interaction of multiple inflammatory mediators that are released at the sites of inflammation (Zimmerman 1981). Biomicroscopic examinations of eyes with iridocyclitis confirm the existence of typical inflammatory changes in the anterior chamber. The diminished translucence of the aqueous humor, as well as cells and fibrin formation, are regularly observed during inflammation. Increased protein content in the anterior chamber is a manifestation of a breakdown of the blood-ocular barrier. A technique to quantitatively measure the flare and cells in the aqueous in "vivo" in the anterior chamber was designed by Sawa (1990). In this method, flare is defined as the amount of light scattering, proportional to the concentration of protein in the anterior chamber (Shah et al. 1992). Many studies have found that laser flare meter provides valuable information on the severity and the activity of inflammation in eyes with uveitis (Oshika et al. 1989; Gucx-Crosier et al. 1994).

Phenylephrine is widely used in ophthalmology as one of the most efficient mydriatic agents producing maximal, prompt and transient mydriasis. This drug is not only used for visualisation of the retina, but also therapeutically to avoid synechiae formation during inflammation. Phenylephrine is also found to decrease the flare intensity values (Oshika et al. 1989).

We investigated the influence of the protein concentration in the anterior chamber, measured by laser flare meter, on pain sensation after phenylephrine instillation in patients with iridocyclitis.

Material and Methods

This prospective study included 25 consecutive patients with anterior uveitis, selected from the outpatient unit at St. Erik's Hospital, Stockholm. Only one eye from each patient was included in the study. All patients came to the emergency department with the complaint of eye pain and other symptoms of anterior uveitis. Some patients had their first or recurrent attack of iridocyclitis. Patients with cataract, exfoliation syndrome, diabetes mellitus, glaucoma, and with any eyedrops instillation or any other previous ocular diseases and ocular surgery, were excluded.

All patients were examined in a slit lamp before and after phenylephrine instil-

Table 1. Patient characteristics.

Group	No. of eyes	Age (median) (range) (yrs)	Male/Female
Group 1 – without fibrinoid reaction	18	41.0 (24–69)	11/7
Group 2 – with fibrinoid reaction	7	44.0 (39–84)	7/0

lation. They were divided into two groups: Group 1 - without fibrinoid reaction (FR) in the anterior chamber (18 eyes), and Group 2 – with FR (7 eyes). Patient characteristics are shown in Table 1.

After entry into the study, patients received the same standardized questionnaire using a visual analogue scale (VAS) (Kabi Pharmacia) to grade his or her pain. The patients were allowed to only see the patient portion of the scale, and not the chart for examiner use. They scored their pain by marking along a continuum ranging from "I do not have any pain" (score 0) to "My pain could not be worse" (score 10) before and after receiving phenylephrine. The position of pain which was marked by patients on their scale was then immediately transferred onto a scale compiled by the examiner.

Pupil size and flare intensity were measured immediately after VAS scoring. Pupil size was estimated according to the Alcon Thilo Scale with a range of pupil size from 2.0 mm to 9.0 mm. Laser flare meter (FC500, Kowa Co., Japan) was used to evaluate the protein concentration in the anterior chamber. Each patient then received 10 µl of 10% phenylephrine (Metaoxedrine, Chauvin Pharmaceuticals Ltd.) in the lower conjunctival sac using a pipette. The second instillation of 10 µl of phenylephrine was given 15 minutes after the first. One hour after the first instillation, patients again assessed the magnitude of their pain using VAS, and pupil size with flare intensity were again measured.

The distribution of the variables was evaluated employing histograms and the Kolmogorov-Smirnov test. The variables of flare intensity, pupil size and pain were not normally distributed, therefore the data were analyzed using nonparametric statistical methods. The Wilcoxon Matched Pairs test was used to estimate the significant influence of phenylephrine treatment on the level of flare, pain and pupil size. The Mann-Whitney test was used to compare variables between the two study groups. The significance level was 5%. The analyses were performed using computerized statistical software (Statistica® 5.0, StatSoft).

Results

There was no significant difference in age between the two study groups (Mann-Whitney test, $p<0.05$) (Table 1). Before and after phenylephrine treatment, eyes with fibrinoid reaction (FR) had significantly higher flare intensity than eyes without FR (Mann-Whitney test, $p<0.05$).

Group 1 – without fibrinoid reaction (FR)

The initial pain score measured before phenylephrine instillation was 2.5 (Median; range: 1.0–7.0) on the VAS. One hour after phenylephrine the VAS pain was significantly reduced to 1.5 (1.0–4.0) (Wilcoxon Matched Pairs test, $p<0.05$) (Fig. 1). Also, flare intensity and pupil size were significantly changed after phenylephrine instillation compared to values before the phenylephrine treatment (Wilcoxon Matched Pairs test, $p<0.05$) (Fig. 1).

Group 2 with fibrinoid reaction (FR)

The VAS was not significantly different after topical phenylephrine instillation (Median: 2.0; range: 1.0–8.0) compared to the initial values (2.0; 1.0–7.0) (Wilcoxon Matched Pairs test, $p>0.05$) (Fig. 2). Flare intensity values were not changed after phenylephrine instillation compared to values before treatment ($p>0.05$) (Wilcoxon Matched Pairs test, $p<0.05$) (Fig. 2). Only pupil size was significantly increased after phenylephrine treatment in those eyes (Wilcoxon Matched Pairs test, $p<0.05$) (Fig. 2).

Discussion

This study shows that uveitic eyes with fibrinoid reaction have a higher flare intensity compared to those without fibrinoid reaction. Moreover, significant pain relief in response to phenylephrine instillation was found in the group without FR, while laser flare intensity was also significantly diminished. We believe that the reason for this significantly diminished pain after phenylephrine treatment is due to the decreased level of protein and other endogenous chemical substances in the anterior chamber in response to the phenylephrine instillation. However, pupil paralysis following phenylephrine instillation also seems to be involved in alleviation of pain in those eyes.

Eyes with fibrinoid reaction did not response in pain or flare reduction to phenylephrine instillation. Eyes with fibrinoid reaction are generally assumed to have worse uveitis and our results with flare confirm this. It seems that these eyes need

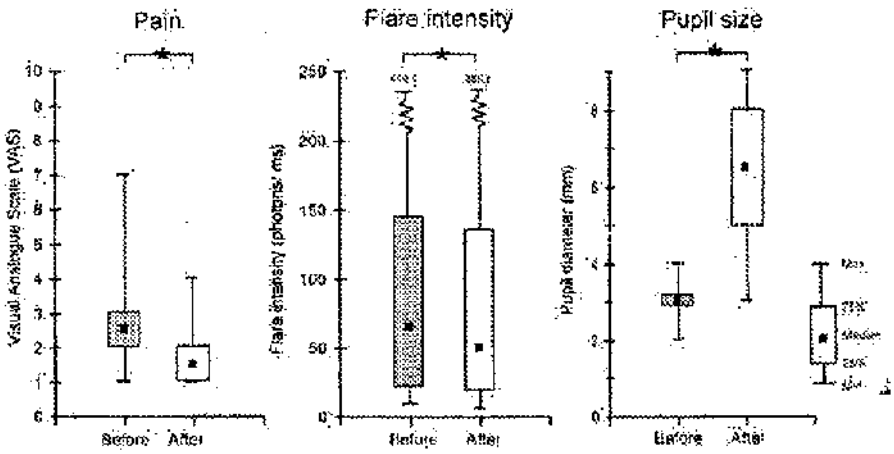

Fig. 1. Pain, flare intensity and pupil size before and after phenylephrine treatment in patients with iridocyclitis without fibrinoid reaction in the anterior chamber. *Significant differences (Wilcoxon test, $p<0.05$).

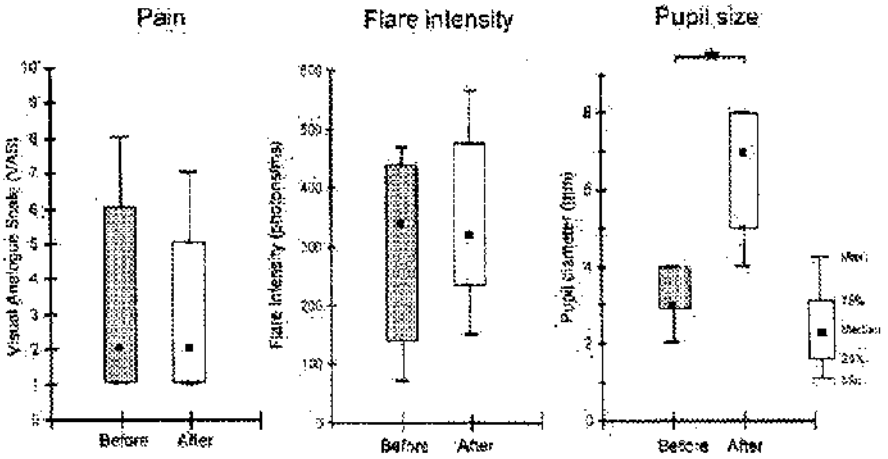

Fig. 2. Pain, flare intensity and pupil size before and after phenylephrine treatment in patients with iridocyclitis with fibrinoid reaction in the anterior chamber. *Significant difference (Wilcoxon test, $p<0.05$).

more time to respond to phenylephrine treatment.

It was suggested that the pupillary reaction to light is responsible for the pain during iridocyclitis, and this pain is decreased after paralyzing the pupil with mydriatics (Yue-Kong & Henkend 1981). Generally, it is proposed that endogenous substances that are released at the site of inflammation play a causal role in the origin of pain during the inflammation phenomena (Zimmerman 1981; Terenius 1981). Pain is usually caused by the chemical stimulation of receptors, called nociceptors, or their afferent nerve fibers by algesic substances (Zimmerman 1981). It seems that prostaglandins and many other inflammatory mediators, released in response to any inflammation, can excite nociceptors, or make them more sensitive to other stimuli, in all organs. At the same time they can also be responsible for other phenomena of inflammation. For example, the role of prostaglandins in inflammation and pain sensation following a mechanical or chemical injury has been shown (Ferreira & Vane 1967).

Therefore, the sensation of pain during anterior uveitis may result from direct stimulation of the pain nerve endings, originating from the ophthalmic division of the Vth cranial nerve. Under normal conditions the composition of the aqueous humor is regulated by the restricted permeability of the blood-aqueous barrier (Caprioli 1987). During anterior uveitis, breakdown of blood-aqueous barrier leads to the entry of protein and other inflammatory particles into the naqueous humor, resulting in increased aqueous flare intensity (Shah et al. 1992). It is possible that a high concentration of endogenous chemicals stimulate more chemoreceptors responsible for the pain during iridicyclitis, and, therefore, may induce severe pain. Our results shows that the pain sensation was decreased after phenylephrine treatment, when at the same time, flare intensity was decreased in those eyes. There have been a few studies concerning the pharmacological effects of phenylephrine on aqueous humor dynamics. Pupillary dilation has been postulated to influence flare intensity by a mechanical effect (Oshika & Kato 1989). However, it has been reported that the reduction in flare is not related to the change in pupillary dilation. Rather, it is a pharmacologic effect of the mydriatic drops on the aqueous flow rate or vascular permeability in the anterior uvea (Araie 1983).

The results of our study indicate that proteins or other endogenous substances that are released during inflammation in the anterior chamber may play a significant role in stimulation of pain in eyes with anterior uveitis. Decreasing the level of flare intensity, as well as paralyzing the pupil after phenylephrine instillation, alleviates pain in eyes with iridocyclitis.

Acknowledgements

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United States Patent [19]

Valle

[11] **4,260,600**

[45] **Apr. 7, 1981**

[54] **METHOD OF TREATING DEPRESSION**

[76] Inventor: **Ronald Valle**, 36 Fitch St., Carteret, N.J. 07008

[21] Appl. No.: **87,470**

[22] Filed: **Oct. 22, 1979**

[51] **Int. Cl.**[3] **A61K 31/135**; A61K 31/165; A61K 33/02

[52] **U.S. Cl.** **424/166**; 424/324; 424/330

[58] **Field of Search** 424/330, 324, 166

Primary Examiner—Stanley J. Friedman

[57] **ABSTRACT**

A method of treating depression by administering several active compounds throughout the day to a patient in need of such treatment. Also included are pharmaceutical composition claims incorporating these compounds.

3 Claims, No Drawings

METHOD OF TREATING DEPRESSION

BACKGROUND OF INVENTION

This invention relates to a method of treating depression by administering to a patient in need thereof several active compounds forming a novel combination. The invention also relates to pharmaceutical composition claims which incorporate the novel combination of active compounds. The compounds which form this novel combination are all known and are known for their adrenergic, antihistamine, analgesic or antipyretic uses, such as for example to relieve the symptons of a common cold.

I have found that by using a particular combination of these compounds, in a particular dosage range an antidepressive yet tranquilizing effect results. As such using my combination for this use avoids the use of other well known, costly, prescription only tranquilizers or antidepression drugs with their resultant potentially dangerous and in many cases unknown side effects and habit forming tendencies. The compounds which make up my combination are readily available, are quite safe for their uses, and are not as costly as the prescription tranquilizers and antidepressants.

The compounds to be administered are as follows:

(1) Phenylephrine hydrochloride which is (R)-3-hydroxy-α-[(methylamino)methyl]benzenemethanol hydrochloride. This compound alone has a therapeutic use as an adrenergic.

(2) Phenylpropanolamine hydrochloride which is chemically known as α-(1-aminoethyl)benzenemethanol hydrochloride. This compound when administered alone or in its hydrochloride salt form is an adrenergic (vasoconstrictor) agent also.

(3) Phenyltoloxamine which is N,N-dimethyl-2-[2-(phenylmethyl)phenoxy]ethanamine. This compound when administered as the dihydrogen citrate salt is useful as an antihistamine.

(4) Acetaminophen which is N-(4-hydroxyphenyl)-acetamide. This compound when administered alone is useful as an analgesic or antipyretic.

(5) Ammonium Chloride. This compound when administered alone has a therapeutic use in the pharmaceutical field as a systemic acidifier.

All the above compounds and their listed therapeutic uses in the pharmaceutical field are known from the prior art, i.e., all are disclosed in the Merck Index, Ninth Edition.

I have found, however, that these compounds or combinations thereof when administered to a patient in dosage ranges listed below have an anti-depressive yet tranquilizing effect. They are useful in treating schizophrenia. This combination of drugs (compounds as described more fully below) when administered in the proportions listed below provide a patient with normal sleep and make the patient less temperamental. In effect, the combination of compounds of this invention brings a patient to a normal mental state, makes him or her physically and emotionally more stable and decreases nervousness and tremors.

The combination of compounds of this invention can be administered in capsule form or they may be compressed into tablets using conventional pharmaceutical excipients, binders and lubricants and with or without other adjuncts. In fact, several of the active ingredients listed above come in commercially available cold and cough tablets and several examples of typical tablets are

described. The compounds are preferably administered orally in daily amounts as follows:

Acetaminophen	100–1000 mg/day
Phenylephrine hydrochloride	1–10 mg/day
Phenylpropanolamine	5–30 mg/day
Phenyltoloxamine dihydrogen citrate	5–30 mg/day
Ammonium chloride	50–1000 mg/day

A daily specific dose of the following compounds administered to a patient suffering from depression is exemplified below:

Acetaminophen	520 mg/day
Phenylephrine hydrochloride	5 mg/day
Phenylpropanolamine	20 mg/day
Phenyltoloxamine dihydrogen citrate	25 mg/day
Ammonium Chloride	320–480 mg/day

A typical commercially available cough tablet contains the following ingredients:

Acetaminophen	260 mg.
Phenylephrine HCl	2.5 mg.
Phenylpropanolamine hydrochloride	10 mg.
Phenyltoloxamine	12.5 mg.
Vitamin C	20 mg.

and a glyceryl guaiacolate base.

Typical contents of another commercially available cough tablet:

Ammonium chloride	80 mg.

and a citric acid in a sugar base.

In order to obtain the specific dosage listed above, a patient suffering from depression can take two tablets during a 24 hour period of the first listed commercially available cough tablet and 4–6 tablets every 24 hours of the second commercially available cough tablet.

The compounds described in this invention are advantageously administered at a dosage range as described or a somewhat higher or lower dosage as the conditions warrant. It will be realized by those skilled in the art that the dosage range for any particular patient or human will depend on the severity of the depression or condition being treated, the weight of the patient and any other condition which a physician or other person skilled in the art will take account of.

What is claimed is:

1. A method of treating depression which comprises administering to a patient in need of such treatment a pharmaceutically effective combination of the following compounds in the following daily dosages

Acetaminophen	100–1,000 mg/day
Phenylephrine hydrochloride	1–10 mg/day
Phenylpropanolamine	5–30 mg/day
Phenyltoloxamine dihydrogen citrate	5–30 mg/day
Ammonium chloride	50–1,000 mg/day

2. A method of treating depression which comprises administering to a patient in need of such treatment a pharmaceutically effective combination of the following compounds in the following daily dosages

Phenylephrine hydrochloride	1–10 mg/day
Phenylpropanolamine	5–30 mg/day
Phenyltoloxamine dihydrogen citrate	5–30 mg/day
Ammonium chloride	50–1,000 mg/day

3. A method of treating depression which comprises administering to a patient in need of such treatment a pharmaceutically effective combination of the following compounds in the following daily dosages

Phenylephrine hydrochloride	1–10 mg/day
Phenylpropanolamine	5–30 mg/day
Ammonium chloride	50–1,000 mg/day

* * * * *

US008859623B1

(12) **United States Patent**
Witham et al.

(10) **Patent No.: US 8,859,623 B1**
(45) **Date of Patent: Oct. 14, 2014**

(54) **METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS**

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(72) Inventors: **Patrick H. Witham**, Eugene, OR (US); **Sailaja Machiraju**, Beaverton, OR (US); **Lauren Mackensie-Clark Bluett**, Milwaukie, OR (US)

(73) Assignee: **Paragon Bioteck, Inc.**, Portland, OR (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **14/080,771**

(22) Filed: **Nov. 14, 2013**

(51) **Int. Cl.**
A61K 31/137 (2006.01)

(52) **U.S. Cl.**
CPC *A61K 31/137* (2013.01)
USPC **514/653**

(58) **Field of Classification Search**
None
See application file for complete search history.

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Primary Examiner David J Blanchard
Assistant Examiner Barbara Frazier
(74) *Attorney, Agent, or Firm* — Wilson Sonsini; Goodrich & Rosati

(57) **ABSTRACT**

The invention is directed to methods and compositions of stabilizing phenylephrine formations. The composition has good time-dependent stability at low temperature and has no change in its outward appearance even after having been stored at least 6 months.

13 Claims, 4 Drawing Sheets

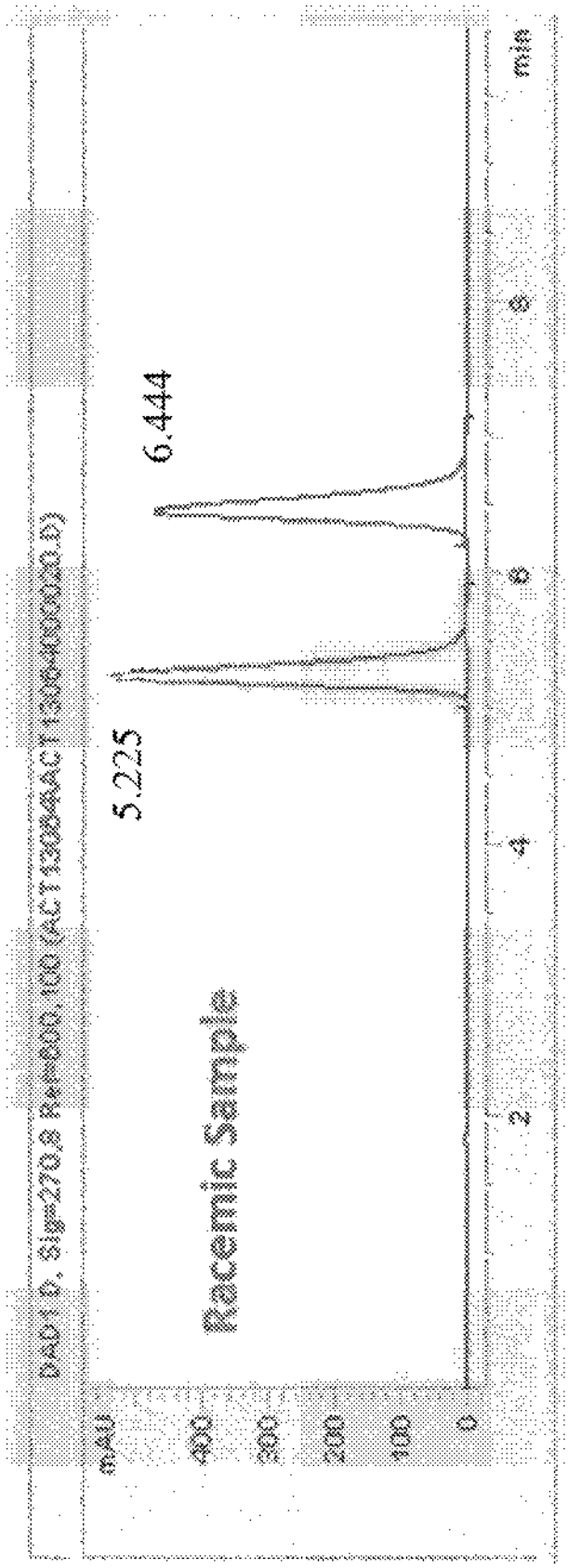
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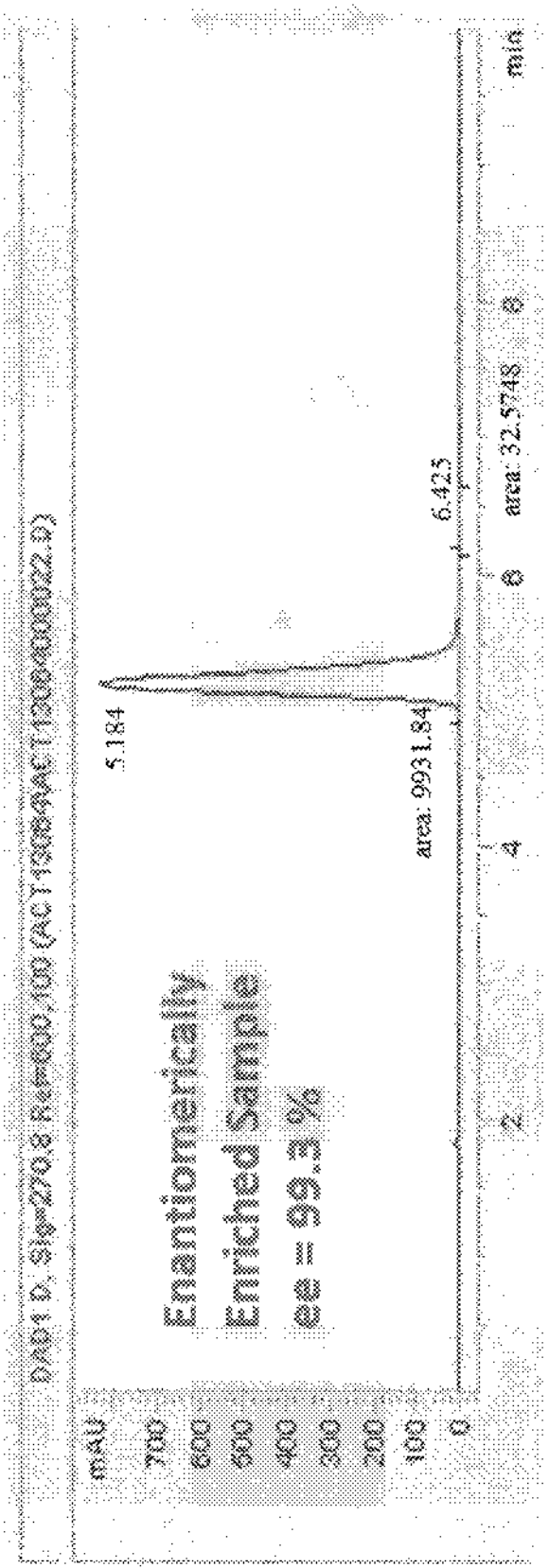
Figure 1

Figure 2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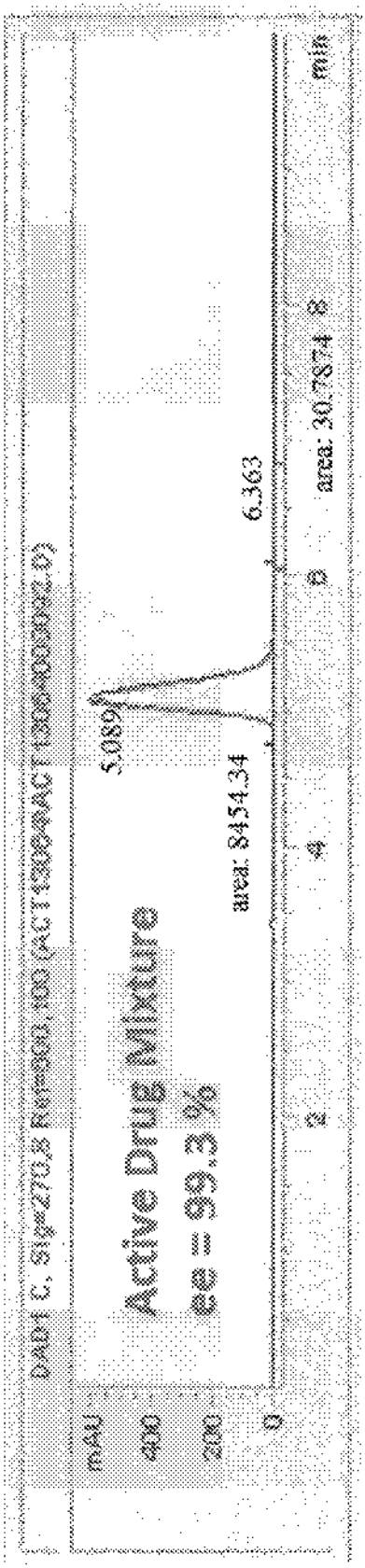
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Figure 3

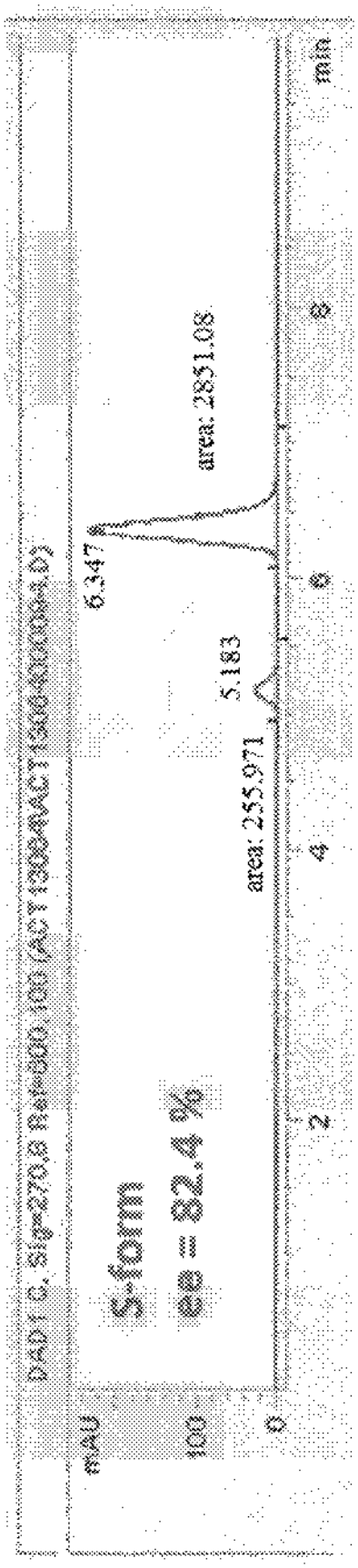
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Figure 4

METHODS AND COMPOSITIONS OF STABLE PHENYLEPHRINE FORMULATIONS

BACKGROUND OF THE INVENTION

Phenylephrine is a selective α1-adrenergic receptor agonist used primarily as a decongestant, as an agent to dilate the pupil, and to increase blood pressure. Phenylephrine is marketed as a substitute for the decongestant pseudoephedrine, though clinical studies differ regarding phenylephrine's effectiveness in this role.

SUMMARY OF THE INVENTION

In accordance with the present invention, the present invention provide a composition comprising at least 95% R-phenylephrine hydrochloride and an aqueous buffer, wherein the composition substantially maintains an initial chiral purity of R-phenylephrine hydrochloride for at least 6 months stored between −10 to 10 degree Celsius.

In another aspect, provided herein are methods of stabilizing a phenylephrine hydrochloride composition comprising storing a solution of aqueous R-phenylephrine hydrochloride at less than 10 degree Celsius, wherein the composition substantially maintains the initial chiral purity of R-phenylephrine hydrochloride for at least 6 months.

In one aspect, provided herein are methods of assaying chiral purity of R-phenylephrine hydrochloride, wherein the chiral purity is determined by chiral column chromatography, optical rotation, capillary electrophoresis, circular dichroism, or Nuclear Magnetic Resonance.

In another aspect provides compositions comprising R-phenylephrine hydrochloride, wherein the composition substantially maintains the initial chiral purity of R-phenylephrine hydrochloride for at least 6 months.

In another aspect provides methods of dilating the pupil comprising administering a composition comprising R-phenylephrine hydrochloride topically to a mammal, wherein the composition substantially maintains the initial chiral purity of R-phenylephrine hydrochloride for at least 6 months.

In another aspect provides methods of treating Uveitis in a subject comprising administering a composition comprising R-phenylephrine hydrochloride to said subject, wherein the composition substantially maintains the initial chiral purity of R-phenylephrine hydrochloride for at least 6 months

In another aspect provides methods of performing certain ocular testing such as ultrasonography, provocative closed angle glaucoma test, Retinoscopy, compromised circulation (i.e., blanching test), Refraction, fundus examination comprising administering a composition comprising R-phenylephrine hydrochloride, wherein the composition substantially maintains the initial chiral purity of R-phenylephrine hydrochloride for at least 6 months.

In another aspect provides methods of aiding surgical procedures requiring visualization of the posterior chamber comprising administering a composition comprising R-phenylephrine hydrochloride to a subject, wherein the composition substantially maintains the initial chiral purity of R-phenylephrine hydrochloride for at least 6 months.

INCORPORATION BY REFERENCE

All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

FIG. 1 shows a HPLC chromatogram of racemic R-phenylephrine hydrochloride by a chiral column purification (OJ-RH (150x4.6) mm). Two peaks at the retention time 5.225 minutes and 6.444 minutes are shown.

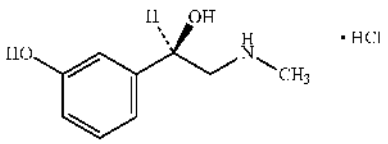
FIG. 2 shows a HPLC chromatogram of the exemplary R-Phenylephrine Hydrochloride Opthalmic Solution (10%) before storage. The chiral purity was determined to be 99.3% ee based on the peaks at 5.184 minutes (area: 9931.84) and at 6.425 minutes (area: 32.5748).

FIG. 3 shows a HPLC chromatogram of the exemplary R-Phenylephrine Hydrochloride Opthalmic Solution (10%) stored at 2 to 8° C. after 6 months. The chiral purity was determined to be 99.3% ee based on the peaks at 5.089 minutes (area: 8454.34) and at 6.363 minutes (area: 30.7874).

FIG. 4 shows a HPLC chromatogram of the purified "impurity" which is a S-Phenylephrine Hydrochloride. The chiral purity was determined to be 82.4% ee based on the peaks at 5.183 minutes (area: 255.971) and at 6.347 minutes (area: 2851.08).

DETAILED DESCRIPTION OF THE INVENTION

Phenylephrine differs chemically from epinephrine only in lacking one hydroxyl group (OH) in the four position on the benzene ring. It is a bitter-tasting crystalline material soluble in water and alcohols, with a melting point of 140°-145° C. Chemically it is Benzenemethanol, 3-hydroxy-α-[(methylamino)methyl]-, hydrochloride or (R)-(−)-m-hydroxy-α-[methylamino)methyl]benzyl alcohol hydrochloride with the following chemical structure.



It is known in the art that a Phenylephrine Hydrochloride solution should be stored protected from light. The benzylic hydrogen is acidic and can be deprotonated easily. The hydroxyl group may be oxidized to form a carbonyl moiety conjugated with phenyl group, especially with help of the adjacent basic amino group. Thus, it is known in the art that a Phenylephrine Hydrochloride solution should be stored protected from light. For example, an insert from a commercially available Phenylephrine Hydrochloride Ophthalmic Solution provides that the solution should be stored at 20° to 25° C. (USP controlled room temperature) and keep container tightly closed. Do not use if solution is brown or contains precipitate. (AKORN Package Insert)

However, a solution under such condition often turns brown over time despite of carefully keeping container tightly

closed at 20° to 25° C. (USP controlled room temperature). Those packages containing the brown solution cannot be used and thus create waste.

The present invention provides the improvement to overcome such instability problem.

In some embodiments, there are provided a composition comprising at least 95% R-phenylephrine hydrochloride and an aqueous buffer for substantially maintaining chiral purity of R-phenylephrine hydrochloride for at least 6 months, the improvement comprising storing the composition between −10 to 10 degree Celsius. In certain embodiments, the composition is stored between 2 to 8 degree Celsius. In certain embodiments, the composition comprises at least 99% or 99.3%, R-phenylephrine hydrochloride. In certain embodiments, the chiral purity of R-phenylephrine hydrochloride is at least 95%, 97%, 99%, or 99.5% of the initial chiral purity after 6 months. In certain embodiments, the composition comprises 2.5% w/v or 10% w/v R-phenylephrine hydrochloride by weight. In certain embodiments, the composition further comprises a preservative such as benzalkonium chloride, stearalkonium chloride, polyaminopropyl biguanide, or the like. In some embodiments, the composition is in a 1-15 ml plastic or glass bottle. In some embodiments, the composition is in a glass or plastic bottle of about 2 ml, about 3 ml, about 5 ml, about 10 ml or about 15 ml. In certain embodiments, the plastic or glass bottle is opaque.

In some embodiments provide methods of stabilizing a phenylephrine hydrochloride composition such as a solution of aqueous R-phenylephrine hydrochloride at less than 10 degree Celsius wherein the composition substantially maintains the initial chiral purity of R-phenylephrine hydrochloride for at least 6 months.

In some embodiments provide herein compositions comprising R-phenylephrine hydrochloride, wherein the composition substantially maintains the initial chiral purity of R-phenylephrine hydrochloride for at least 6 months.

In some embodiments, the composition is stored at −10 to 10 degree Celsius. In certain embodiments, the composition is stored at −5 to 10 degree Celsius. In certain embodiments, the composition is stored at 0 to 10 degree Celsius. In certain embodiments, the composition is stored at 2 to 8 degree Celsius.

The term "substantial" or "substantially maintains" described herein refers to not more than 15% deviation of the initial purity. In some embodiments, the chiral purity of the composition is at least 85%, 90%, 95%, 97%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8% or 99.9% of the initial chiral purity.

In some embodiments provide herein methods of assaying chiral purity of R-phenylephrine hydrochloride, wherein the chiral purity is determined by chiral column chromatography, optical rotation, capillary electrophoresis, circular dichroism, or Nuclear Magnetic Resonance.

In certain embodiments, the chiral purity is determined by chiral column chromatography.

Chiral Column Chromatography

Chiral column chromatography is a variant of column chromatography in which the stationary phase contains a single enantiomer of a chiral compound rather than being achiral. The two enantiomers of the same analyte compound differ in affinity to the single-enantiomer stationary phase and therefore they exit the column at different times.

The chiral stationary phase can be prepared by attaching a suitable chiral compound to the surface of an achiral support such as silica gel, which creates a Chiral Stationary Phase (CSP). Many common chiral stationary phases are based on oligosaccharides such as cellulose or cyclodextrin (in particular with β-cyclodextrin, a seven sugar ring molecule). As with all chromatographic methods, various stationary phases are particularly suited to specific types of analytes.

The packing material of the chiral column may be amylose tris(3,5-dimethylphenylcarbamate), β-cyclodextrin, cellobiohydrolase, selector R-(−)-N-(3,5-dinitrobenzoyl)-phenylglycine, cellulose tris(3,5-dimethylphenylcarbamate), cellulose tris(3,5-dichlorophenylcarbamate), or combinations thereof. In some embodiments, the chiral column for analytical purpose is packed with amylose tris(3,5-dichlorophenylcarbamate). The column may have a packing particle of a size of about 3 μm to about 50 μm. In some embodiments, the column has a packing particle a size of about 3 μm, 5 μm, 10 μm, 20 μm, 30 μm, 40 μm, or 50 μm. In certain embodiments, the column has a packing particle a size of about 3 μm. In some embodiments, when using a chiral column system, the first mobile phase is non-polar solvent such as n-hexane, n-pentane, and the like, and the second mobile phase is polar solvent such as isopropanol, ethanol, methanol, or the like. In certainly embodiments, the mobile phase comprises small amount of amine such as ethylenediamine. The first mobile phase may be present in an amount of about 75% to about 95% by volume and the second mobile phase is present in an amount of about 5% to about 25% by volume. In some embodiments, the first mobile phase is present in an amount of about 85% by volume and the second mobile phase is present in an amount of about 15% by volume with or without ethylenediamine.

Other Chiral Compound Analysis Methods

There are several chiral compound purification and analysis methods available besides chiral column chromatography. For example, it is known in the art chiral purity can be determined by optical rotation. In some embodiments, the chiral purity of R-phenylephrine hydrochloride in the stabilized compositions and methods thereof can be determined by comparison of optical rotation of pure R-phenylephrine hydrochloride.

Optical Purity Measured by Optical Rotation

Molecules with chrial centers cause the rotation of plane polarised light and are said to be "optically active" (hence the term optical isomers). Enantiomeric molecules rotate the plane in opposite directions but with the same magnitude. This provides a means of measuring the "optical purity" or "enantiomeric excess (ee)" of a sample of a mixture of enantiomers.

Specific rotation is a physical property like boiling point and can be looked up in references. It is defined according to the following equation based on the experimental measurements: Specific rotation $[\alpha]D$ α_{obs}/cl where "α_{obs}" is the experimentally observed rotation, "c" is the concentration in g/ml and "l" is the path length of the cell used expressed in dm (10 cm).

A non-racemic mixture of two enantiomers will have a net optical rotation. It is possible to determine the specific rotation of the mixture and, with knowledge of the specific rotation of the pure enantiomer, the optical purity can be determined.

$$\%\ \text{Optical purity of sample} = 100 * (\text{specific rotation of sample}) / (\text{specific rotation of a pure enantiomer})$$

In some embodiments, there are provided methods of assaying chiral purity of R-phenylephrine hydrochloride, wherein the chiral purity is determined by optical rotation. In certain embodiments, the optical rotation is determined by comparison of optical rotation of pure R-phenylephrine hydrochloride.

Capillary Electrophoresis

Capillary electrophoresis (CE), also known as capillary zone electrophoresis (CZE), can be used to separate ionic species by their charge and frictional forces and hydrodynamic radius.

Capillary electrophoresis (CE) in general offers highly efficient separations. To achieve chiral separation, the capillary is filled with a separation buffer containing a chiral additive. Although many chiral selectors have been used successfully, the most comprehensive separation strategies have been achieved with highly sulfated cyclodextrins. In some embodiments, the chiral purity of the compositions provided herein is determined by capillary electrophoresis. In certain embodiments, the capillary electrophoresis uses cyclodextrin or its derivatives (such as sulfated cyclodextrins).

Chiral Purity Measured by Circular Dichroism

Circular dichroism (CD) refers to the differential absorption of left and right circularly polarized light. This phenomenon is exhibited in the absorption bands of optically active chiral molecules. CD spectroscopy has a wide range of applications in many different fields. For example, vibrational circular dichroism, which uses light from the infrared energy region, is used for structural studies of small organic molecules, and most recently proteins and DNA. In general, this phenomenon will be exhibited in absorption bands of any optically active molecule. As a consequence, circular dichroism is exhibited by biological molecules, because of their dextrorotary and levorotary components. Even more important is that a secondary structure will also impart a distinct CD to its respective molecules.

Optical rotation and circular dichroism stem from the same quantum mechanical phenomena and one can be derived mathematically from the other if all spectral information is provided. In some embodiments, the chiral purity is determined by circular dichroism. In certain embodiments, the chiral purity is determined by Fourier transform infrared vibrational circular dichroism (FTIR-VCD). A skilled person in the art can readily apply the general knowledge and procedure to determine chirality of the compositions provided herein.

NMR Spectroscopy of Stereoisomers

It is known in the art that NMR spectroscopy techniques can determine the absolute configuration of stereoisomers such as cis or trans alkenes, R or S enantiomers, and R,R or R,S diastereomers. In a mixture of enantiomers, these methods can help quantify the optical purity by integrating the area under the NMR peak corresponding to each stereoisomer. Accuracy of integration can be improved by inserting a chiral derivatizing agent with a nucleus other than hydrogen or carbon, then reading the heteronuclear NMR spectrum: for example fluorine-19 NMR or phosphorus-31 NMR. Mosher's acid contains a CF_3 group, so if the adduct has no other fluorine atoms, the 19F NMR of a racemic mixture shows just two peaks, one for each stereoisomer. In some embodiments, the chiral purity of the compositions provided herein is determined by Nuclear Magnetic Resonance Spectroscopy (NMR). In certain embodiments, a chirally pure complexing reagent (i.e., a chiral derivatizing agent) is used in measuring NMR. A skilled person in the art can readily utilize NMR and any suitable chiral complexing agent to determine the chirality of the compositions provided herein.

Dosage Forms and Strengths

In some embodiments, the stabilized compositions provided herein comprise a solution of 2.5% w/v or 10% w/v R-phenylephrine hydrochloride by weight. In certain embodiments, the compositions further comprise sodium phosphate monobasic, sodium phosphate dibasic, boric acid and benzalkonium chloride. The followings are non-limited exemplary compositions:

Phenylephrine Hydrochloride Ophthalmic Solution, 2.5% is a clear, colorless to yellowish, sterile topical ophthalmic solution containing phenylephrine hydrochloride 2.5%.

Phenylephrine Hydrochloride Ophthalmic Solution, 10% is a clear, colorless to yellowish, sterile topical ophthalmic solution containing phenylephrine hydrochloride 10%.

Application of the Stabilized Compositions Comprising R-Phenylephrine Hydrochloride

It has been established that Phenylephrine Hydrochloride Ophthalmic Solution is recommended as a vasoconstrictor, decongestant, and mydriatic in a variety of ophthalmic conditions and procedures. Some of its uses are for pupillary dilation in uveitis (to prevent or aid in the disruption of posterior synechia formation), for many ophthalmic surgical procedures and for refraction without cycloplegia. Phenylephrine Hydrochloride Ophthalmic Solution may also be used for funduscopy and other diagnostic procedures.

For example, R-Phenylephrine is used to dilate the iris through α-adrenergic stimulation of the iris dilator muscle. Sympathetic stimulation of the ciliary muscle is believed to be inhibitory, decreasing accommodative amplitude. R-Phenylephrine is formulated in an eye drop to dilate the pupil in order to facilitate visualization of the retina. It is often used in combination with tropicamide as a synergist when tropicamide alone is not sufficient. Surprisingly it was found that S-Phenylephrine dilated the eye only slightly more than that was untreated. Thus it is important that an eye drop containing Phenylephrine Hydrochloride used for dilation of the pupil contains predominantly the R-isomer in order to maintain maximum efficacy of the ophthalmic solution.

Sympathetic innervation leads to pupillary dilation. It is innervated by the sympathetic system, which acts by releasing noradrenaline, which acts on α1-receptors causing dilation.

The alpha-1 (α_1) adrenergic receptor is a G protein-coupled receptor (GPCR) associated with the G_q heterotrimeric G-protein. It consists of three highly homologous subtypes, including α_{1A}-, α_{1B}-, and α_{1D}-adrenergic. Catecholamines like norepinephrine (noradrenaline) and epinephrine (adrenaline) signal through the α_1-adrenergic receptor in the central and peripheral nervous systems.

Phenylephrine is a selective α_1-adrenergic receptor agonist used primarily as a decongestant, as an agent to dilate the pupil, and to increase blood pressure. Dilation is controlled by the dilator pupillae, a group of muscles in the peripheral ⅔ of the iris. Sympathetic innervation begins at the cortex with the first synapse at the cilliospinal center (also known as Budge's center after German physiologist Julius Ludwig Budge). Post synaptic neurons travel down all the way through the brain stem and finally exit through the cervical sympathetic chain and the superior cervical ganglion. They synapse at the superior cervical ganglion where third-order neurons travel through the carotid plexus and enter into the orbit through the first division of the trigeminal nerve.

In the anesthetized rats, infusion of large amount of (+)-epinephrine, (+)-norepinephrine, epinine, and (−)- or (+)-phenylephrine induces tachyphylaxis to vasopressor effect of (−)-epinephrine, (−)-norepinephrine, and tetraethylammonium. The tachyphylactic potency of the amines was (−)-phenylephrine (R-phenylephrine)>epinine>(+)-norepinephrine (+)-epinephrine>(+)-phenylephrine.

Two ophthalmic formulations, formulated 10% Phenylephrine hydrochloride (S-isomer) and the exemplary invention composition, 10% Phenylephrine hydrochloride (R-isomer)

were tested for their ocular activity in NZW rabbits. It was observed that formulated S-isomer showed minimal dilation, responded to light exposure and constricted slightly more slowly than the untreated eye, where as the exemplary invention composition, 10% Phenylephrine hydrochloride showed maximal dilation with in 15 min of dosing and the pupil did not respond to light and remained dilated for 4 hrs.

According to the above study it could be postulated that, when an ophthalmic solution of phenylephrine hydrochloride. (R-isomer) containing S-isomer as an impurity is used for dilation of pupil, the s-isomer may cause the saturation of the a-adrenergic receptors resulting in the decrease in the response of the drug after its administration (tachyphylasis). Furthermore, the presence of S-isomer in the ophthalmic solution may lead to poor/delayed dilation of the pupil.

In some embodiments provide methods of dilating the pupil comprising administering a composition comprising R-phenylephrine hydrochloride topically to a mammal, wherein the composition substantially maintains the initial chiral purity of R-phenylephrine hydrochloride for at least 6 months. It is evident from the literature that the pharmacological evaluation of both R & S-Phenylephrine hydrochloride is not same. R-Phenylephrine is referenced as useful synthetic adrenergic drug.

Uveitis

Uveitis is. broadly, inflammation of the uvea. The uvea consists of the middle, pigmented. vascular structures of the eye and includes the iris, ciliary body, and choroid. Uveitis requires an urgent referral and thorough examination by an ophthalmologist or Optometrist and urgent treatment to control the inflammation. Anterior uveitis (iritis) affects the front portion of the eye, intermediate uveitis (cyclitis) affects the ciliary body, and posterior uveitis (choroiditis) affects the back portion of the uvea. Diffuse uveitis affects all portions of the uvea. Anterior uveitis commonly occurs in conjunction with juvenile rheumatoid arthritis, but does not manifest in all juvenile arthritis patients. Uveitis is most likely to be present in juvenile arthritis patients with pauciarticular disease (fewer than five joints involved), a positive anti-nuclear antibody test. and a negative rheumatoid factor test. It has been demonstrated that after phenylephrine hydrochloride ophthalmic solution instillation, flare intensity and pain were significantly decreased only in eyes with iridocyclitis and without fibrinoid reaction (FR). The decreasing level of flare intensity, and paralysis of the pupil after phenylephrine instillation seem to alleviate pain in those eyes. See e.g., Zaczek. et. al., Acta Ophthalmol Scand. 2000 October; 78(5):516-8.

In some embodiments provide methods of treating Uveitis in a subject comprising administering a composition comprising R-phenylephrine hydrochloride to said subject, wherein the composition substantially maintains the initial chiral purity of R-phenylephrine hydrochloride for at least 6 months.

In some embodiments provide methods of performing certain ocular testing such as ultrasonography. provocative closed angle glaucoma test, Retinoscopy, compromised circulation (i.e., blanching test), Refraction, fundus examination comprising administering a composition comprising R-phenylephrine hydrochloride, wherein the composition substantially maintains the initial chiral purity of R-phenylephrine hydrochloride for at least 6 months.

In some embodiments provide methods of aiding surgical procedures requiring visualization of the posterior chamber comprising administering a composition comprising R-phenylephrine hydrochloride, wherein the composition substantially maintains the initial chiral purity of R-phenylephrine hydrochloride for at least 6 months.

After presentation of R-phenylephrine hydrochloride ophthalmic solution 2.5% or 10% to the ocular surface, a broad variation in the delay of onset of dilation is widely reported, varying between 20-to-30 minutes and as much as up to 60 minutes. While a number of contributors to this delay of onset have been theorized, the absence of phenylephrine hydrochloride's pharmacologic activity in the eye due to the presence of S-phenylephrine may in fact be the explanation for such delay.

Dropper Bottle or Storage Bottle

Conventional dropper bottles for administering ophthalmic fluid are well known in the prior art. The basic commercial design of such dropper bottles has remained fairly unchanged over the last several decades: a squeezable container is provided with a tapered dispenser that terminates in a discharge aperture. To administer ophthalmic fluid, the discharge aperture is aligned above a target eye and the bottle is squeezed to urge out a drop or dose of the fluid.

Alternatively, liquid dispensers have been developed in which the formulation is supplied from a storage bottle through a dropper, for example (dropper bottles or EDO-Ophthiols). The aqueous formulation usually flows out of the dropper opening as a result of manual pressure being applied to the compressible storage bottle.

In some embodiments, the composition described herein is stored in a plastic or glass bottle. In certain embodiments. the plastic bottle is a low-density polyethylene bottle. In certain embodiments, the composition described herein is stored in a glass bottle with or without a liquid dispenser. In certain embodiments. the plastic or glass bottle is opaque.

Additionally. the compositions described herein are either packaged for single use or for multiple uses with or without a preservative.

Certain Pharmaceutical and Medical Terminology

The term "acceptable" with respect to a formulation, composition or ingredient, as used herein, means having no persistent detrimental effect on the general health of the subject being treated.

The term "carrier," as used herein, refers to relatively nontoxic chemical compounds or agents that facilitate the incorporation of a compound into cells or tissues.

The terms "co-administration" or the like, as used herein, are meant to encompass administration of the selected therapeutic agents to a single patient, and are intended to include treatment regimens in which the agents are administered by the same or different route of administration or at the same or different time.

The term "diluent" refers to chemical compounds that are used to dilute the compound of interest prior to delivery. Diluents can also be used to stabilize compounds because they can provide a more stable environment. Salts dissolved in buffered solutions (which also can provide pH control or maintenance) are utilized as diluents in the art. including. but not limited to a phosphate buffered saline solution.

The terms "effective amount" or "therapeutically effective amount," as used herein, refer to a sufficient amount of an agent or a compound being administered which will relieve to some extent one or more of the symptoms of the disease or condition being treated. The result can be reduction and/or alleviation of the signs, symptoms, or causes of a disease, or any other desired alteration of a biological system. For example. an "effective amount" for therapeutic uses is the amount of the composition comprising a compound as disclosed herein required to provide a clinically significant decrease in disease symptoms. An appropriate "effective" amount in any individual case may be determined using techniques. such as a dose escalation study.

The terms "enhance" or "enhancing," as used herein, means to increase or prolong either in potency or duration a desired effect. Thus, in regard to enhancing the effect of therapeutic agents, the term "enhancing" refers to the ability to increase or prolong, either in potency or duration. the effect of other therapeutic agents on a system. An "enhancing-effective amount." as used herein, refers to an amount adequate to enhance the effect of another therapeutic agent in a desired system.

The term "subject" or "patient" encompasses mammals. Examples of mammals include, but are not limited to, any member of the Mammalian class: humans, non-human primates such as chimpanzees. and other apes and monkey species; farm animals such as cattle, horses, sheep. goats. swine; domestic animals such as rabbits. dogs. and cats; laboratory animals including rodents, such as rats, mice and guinea pigs, and the like. In one embodiment, the mammal is a human.

The terms "treat," "treating" or "treatment," as used herein, include alleviating, abating or ameliorating at least one symptom of a disease or condition, preventing additional symptoms, inhibiting the disease or condition. e.g., arresting the development of the disease or condition. relieving the disease or condition, causing regression of the disease or condition, relieving a condition caused by the disease or condition, or stopping the symptoms of the disease or condition either prophylactically and/or therapeutically.

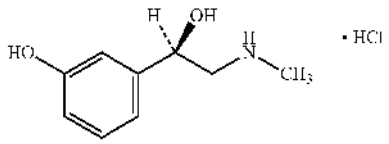
All of the various embodiments or options described herein can be combined in any and all variations. The following Examples serve only to illustrate the invention and are not to be construed in anyway to limit the invention.

EXAMPLES

Example 1

Exemplary Phenylephrine HCl Ophthalmic Formulation

R-Phenylephrine Hydrochloride Ophthalmic Solution, USP 2.5% or 10%, is a sterile, clear, colorless to light yellow, topical mydriatic agent for ophthalmic use. The chemical name is (R)-3-hydroxy-α-[(methylamino)methyl]benzenemethanol hydrochloride. R-Phenylephrine hydrochloride is represented by the following structural formula:



Phenylephrine hydrochloride has a molecular weight of 203.67 and an empirical formula of $C_9H_{13}NO_2$-HCl.

Each mL of R-Phenylephrine Hydrochloride Ophthalmic Solution, 2.5% contains: ACTIVE: phenylephrine hydrochloride 25 mg (2.5%); INACTIVES: sodium phosphate monobasic. sodium phosphate dibasic; boric acid. water for injection. Hydrochloric acid and/or sodium hydroxide may be added to adjust pH (6.0 to 6.4). The solution has a tonicity of 500 mOsm/kg: PRESERVATIVE: benzalkonium chloride 0.01%.

Each mL of R-Phenylephrine Hydrochloride Ophthalmic Solution, 10% contains: ACTIVE: R-phenylephrine hydrochloride 100 mg (10%); INACTIVES: sodium phosphate monobasic. sodium phosphate dibasic: water for injection. Hydrochloric acid and/or sodium hydroxide may be added to adjust pH (6.3 to 6.7). The solution has a tonicity of 1000 mOsm/kg; PRESERVATIVE: benzalkonium chloride 0.01%.

The composition of Phenylephrine HCl Ophthalmic Solution. 2.5% and 10% is listed in Table 1.

Table 1: Phenylephrine HCl Ophthalmic Solution, 2.5% and 10% Quantitative Composition

Component	2.5% Formulation Quantity (% w/v)	10% Formulation Quantity (% w/v)	Function	Quality Standard
R-Phenylephrine HCl	2.5%	10%	Active	USP
Sodium Phosphate Monobasic.	0.5%	0.5%	Buffer	USP
Sodium Phosphate Dibasic. Anhydrous	0.3%	0.3%	Buffer	USP
Boric Acid	1.0%		Buffer	USP
Benzalkonium Chloride	0.01%	0.01%	Antimicrobial preservative	USP
Sodium Hydroxide	As needed	As needed	pH adjustment	USP
Hydrochloric Acid	As needed	As needed	pH adjustment	USP

Example 2

Stability (Impurity) Test and Results

Stability studies of 2.5% and 10% Phenylephrine HCL solutions prepared as in Example 2 were conducted at 2 to 8° C. for 12 months.

While the testing performed during the historical stability analysis is limited, those parameters evaluated show excellent results. For the 3 batches of 2.5% formulation evaluated. the initial assay averaged 101.2% of label claim (range 99.8%-102.9%). and after 12 months of storage at the labeled storage condition (2-8° C.) the average potency was 99.7% of label claim (range 97.0%-103.4%). All other parameters evaluated (appearance, preservative effectiveness, sterility) conformed to specifications.

For the 3 batches of 10% formulation evaluated, the initial assay averaged 100.4% of label claim (range 99.8%-101.6%). and after 12 months of storage at the labeled storage condition (2-8° C.) the average potency was 99.8% of label claim (range 98.8%-101.0%). All other parameters evaluated (appearance, preservative effectiveness, sterility) conformed to specifications.

Example 3

Chiral HPLC Analysis

The following are non-limited exemplary chiral columns and relevant mobile phases in the methods for analyzing chiral purity of R-phenylephrine.

Column-OJ-RH (150×4.6) mm. 5 μm, Flow: 1 mL min-1, Mobile Phase: Methanol, Column Temp: 25° C., Detection wavelength: 270 nm.

Column-OJ-RH (150×4.6) mm, 5 μm, Flow: 0.8 mL min-1, Mobile Phase: 0.05% Ethylenediamine in Methanol, Column Temp: 25° C., Detection wavelength: 270 nm.

Column-OJ-RH (150×4.6) mm, Flow: 1 ml min-1, Mobile Phase: 0.05% Ethylenediamine in Methanol. Column Temp: Ambient, Detection wavelength: 270 nm.

Column-OJ-RH (150×4.6) mm, 5 μm, Flow: 1 ml min-1, Mobile Phase: 0.05% Ethylenediamine in Methanol. Column Temp: 25° C., Detection wavelength: 270 nm.

Column-OJ-RH (150×4.6) mm, 5 μm, Flow: 1 ml min-1, Mobile Phase: 0.05 Ethylenediamine in Methanol. Column Temp: 25° C., Detection wavelength: 270 nm.

Column-OJ-RH (150×4.6) mm. 5 μm, Flow: 1 ml min-1, Mobile Phase: 0.05% Ethylenediamine in Water (05%): Methanol (95), Column Temp: 25° C., Detection wavelength: 270 nm.

Column-OJ-RH (150×4.6) mm, 5 μm, Flow: 1 ml min-1, Mobile Phase: 0.05% Ethylenediamine in Methanol, Column Temp: 25° C., Detection wavelength: 270 nm.

Column-OJ-RH (150×4.6) mm. 5 μm, Flow: 1 ml min-1, Mobile Phase: 0.05% Ethylenediamine in Methanol. Column Temp: 25° C., Detection wavelength: 270 nm.

Column-OJ-RH (150×4.6) mm, 5 μm, Flow: 0.5 ml min-1, Mobile Phase: Acetonitrile: 0.05% Ethylenediamine in water (30:70) Column Temp.: 25° C., Detection wavelength: 270 nm.

Column-OJ-RH (150×4.6) mm, 5 μm, Flow: 0.5 ml min-1; Mobile Phase: Acetonitrile: 0.05% Ethylenediamine in water (40:60) Column Temp.: 25, Detection wavelength: 270 nm.

Column-Chiralpak IC-3 (150×4.6) mm, 3 μm, Flow: 1.0 ml min-1, Mobile Phase: 0.1% Ethylenediamine in n-Hexane (85%): Ethanol (15%), Column Temp: 25° C., Detection wavelength: 270 nm; ref 600 nm.

Column-Chiralpak IC-3 (150×4.6) mm, 3 μm, Flow: 1.2 ml min-1, Mobile Phase: 0.1% Ethylenediamine in n-Hexane (50%): IPA (50%), Column Temp: 25° C., Detection wavelength: 270 nm.

Column-OJ-RH (150×4.6) mm, Flow: 0.6 ml min-1, Mobile Phase: 0.05% Ethylenediamine in Methanol, Column Temp: 25° C.; Detection wavelength: 270 nm. 4.0 mg sample in 1 mL ethanol was analyzed. The injection volume to HPLC is 3.0 μL. The HPLC chromatogram is shown in FIG. **1**.

The HPLC chromatogram clearly show separation of racemic sample. Chiral HPLC method was thus established to analyze Phenylephrine.

Example 4

Determination of Chiral Purity after 6 Months Storage at Low Temperature

R-Phenylephrine Hydrochloride Opthalmic Solution, 2.5% and 10% prepared as in Example 1 were stored at 2 to 8° C. The chiral purity of Sample 1 (10% solution) was assessed before low temperature stability test. The HPLC chromatogram is shown in FIG. **2**.

The chiral purity of R-Phenylephrine Hydrochloride was determined by the method and conditions as shown in Example 3. The result showed 99.3% ee.

After 6 months of low temperature storage (i.e., 2 to 8° C.), the chiral purity of R-Phenylephrine Hydrochloride in the solution was determined to be 99.3% ee. The HPLC chromatogram is shown in FIG. **3**.

To confirm the "impurity" shown in the chromatogram, the "impurity" was purified and determined by the same method. The "impurity" (i.e., S-Phenylephrine Hydrochloride) was determined to possess 82.4% ee of S-form. The HPLC chromatogram is shown in FIG. **4**.

Thus, it is clearly shown that the solution remain substantially maintains the initial chiral purity of R-phenylephrine hydrochloride for at least 6 months.

Example 5

Dilation Assay of S Form Phenylephrine Solution

Both R and S form solutions (10% solution prepared as in Example 1) were test for dilation on rabbits. The first test rabbit received 3 drops of the S form formulation and the second test rabbit received 3 drops of the R form solution.

The results were as follows:

Test Rabbit No. 1: Minimal Dilation, within 15 minutes of dilation the pupil was only slightly more dilated than the untreated eye. The treated eye responded to light exposure and constricted slowly. The control eye constricted rapidly as was expected.

Test Rabbit No 2: Maximal dilation within 15 minutes of dosing. The pupil did not respond to light exposure and remained fully dilated for 4 hours then regressed.

These results clearly show that an ophthalmic solution of phenylephrine containing S-isomer does not dilate the rabbit pupil as it is achieved with an ophthalmic solution of phenylephrine containing R isomer. Thus it is evident that maintaining the chiral purity of the ophthalmic solution is crucial to keep drug potency.

While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

What is claimed is:

1. A method of using an ophthalmic composition for pupil dilation, the composition comprising R-phenylephrine hydrochloride having an initial chiral purity of at least 95% and an aqueous buffer, wherein the chiral purity of R-phenylephrine hydrochloride is at least 95% of the initial chiral purity after 6 months, the method comprising:

administering the composition into an eye of an individual in need thereof, wherein the composition is stored between −10 to 10 degree Celsius prior to administration, and wherein the composition comprises R-phenylephrine hydrochloride having a chiral purity of at least 95% when administered after storage.

2. The method of claim **1**, wherein the composition is allowed to be stored between 2 to 8 degree Celsius.

3. The method of claim **1**, wherein the composition comprises R-phenylephrine hydrochloride having an initial chiral purity of at least 99%.

4. The method of claim **1**, wherein the composition comprises R-phenylephrine hydrochloride having an initial chiral purity of at least 99.3%.

5. The method of claim **1**, wherein the chiral purity of R-phenylephrine hydrochloride is at least 97% of the initial chiral purity after 6 months.

6. The method of claim **1**, wherein the chiral purity of R-phenylephrine hydrochloride is at least 99% of the initial chiral purity after 6 months.

7. The method of claim **1**, wherein the chiral purity of R-phenylephrine hydrochloride is at least 99.5% of the initial chiral purity after 6 months.

8. The method of claim 1, wherein the composition comprises 2.5% w/v or 10% w/v R-phenylephrine hydrochloride by weight.

9. The method of claim 1, wherein the composition is packaged in a 1-15 ml plastic or glass bottle.

10. The method of claim 9, wherein the package identifies storing the composition at a temperature between −10 to 10 C.

11. The method of claim 10, wherein the package identifies storing the composition at a temperature between 2 to 8 C.

12. The method of claim 9, wherein the composition is in a plastic or glass bottle of about 2 ml, about 3 ml, about 5 ml, about 10 ml or about 15 ml.

13. The method of claim 9, wherein the plastic or glass bottle is opaque.

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