of the amount added and which is not exceeded. An example of such a label statement is "____ (unit) added as preservative." [NOTE—"____ (unit)" would be a number followed by the unit of measurement, e.g., 0.015 mg per mL or 0.1%.]

The most commonly used agents include the two mercurials, phenylmercuric nitrate and thimerosal, the four homologous esters of *p*-hydroxybenzoic acid, phenol, benzyl alcohol, and chlorobutanol. The methods for the first two named are polarographic, while quantitative gas chromatography is employed in the determination of the other agents.

GENERAL GAS CHROMATOGRAPHIC METHOD

The general procedure set forth in the following paragraphs is applicable to the quantitative determination of benzyl alcohol, chlorobutanol, phenol, and the methyl, ethyl, propyl, and butyl esters of *p*-hydroxybenzoic acid, the latter being treated as a group, the individual members of which, if present, are capable of separate determination. Prepare the *Internal Standard Solution* and the *Standard Preparation* for each agent as directed individually below. Unless otherwise directed below, prepare the *Test Preparation* from accurately measured portions of the *Internal Standard Solution* and the sample under test, of such size that the concentration of the agent and the composition of the solvent correspond closely to the concentration and composition of the *Standard Preparation*. Suggested operating parameters of the gas chromatograph apparatus are given in the accompanying table, the carrier gas being helium or nitrogen, and the detector being the flame-ionization type.

Benzyl Alcohol

Internal Standard Solution—Dissolve about 380 mg of phenol in 10 mL of methanol contained in a 200-mL volumetric flask. Add water to volume, and mix.

Standard Preparation—Dissolve about 180 mg of benzyl alcohol, accurately weighed, in 20.0 mL of methanol contained in a 100-mL volumetric flask. Add *Internal Standard Solution* to volume, and mix.

Procedure—Using 5-µL portions of the *Standard Preparation* and the *Test Preparation*, record their gas chromatograms with the apparatus adjusted to the parameters set forth in the accompanying table. Measure the areas under the peaks for benzyl alcohol and phenol of the chromatogram for the *Standard Preparation*, designating them P_1 and P_2, respectively. Similarly, determine the corresponding values p_1 and p_2 for the *Test Preparation*. Calculate the content, in mg per mL, of benzyl alcohol (C_7H_8O) in the specimen taken by the formula:

$$100(C/V)(p_1/p_2)(P_2/P_1),$$

in which C is the concentration, in mg per mL, of benzyl alcohol in the *Standard Preparation*, and V is the volume, in mL, of the specimen under test used in preparing each 100 mL of the *Test Preparation*.

Chlorobutanol

Internal Standard Solution—Dissolve about 130 mg of benzaldehyde in 5 mL of methanol contained in a 100-mL volumetric flask. Add water to volume, and mix.

Standard Preparation—Dissolve about 500 mg of anhydrous chlorobutanol, accurately weighed, in 5 mL of methanol contained in a 100-mL volumetric flask. Add water to volume, and mix. Pipet 2 mL of this solution and 2 mL of the *Internal Standard Solution* into a 50-mL volumetric flask, add dilute methanol (1 in 20) to volume, and mix.

Procedure—Using 5-µL portions of the *Standard Preparation* and the *Test Preparation*, record their gas chromatograms with the apparatus adjusted to the parameters set forth in the accompanying table. Measure the areas under the peaks for chlorobutanol and benzaldehyde of the chromatogram for the *Standard Preparation*, designating them P_1 and P_2, respectively. Similarly, determine the corresponding values p_1 and p_2 for the *Test Preparation*. Calculate the content, in mg per mL, of chlorobutanol ($C_4H_7Cl_3O$) in the specimen taken by the formula:

$$100(C/V)(p_1/p_2)(P_2/P_1),$$

in which C is the concentration, in mg per mL, of chlorobutanol in the *Standard Preparation*, and V is the volume, in mL, of the specimen under test used in preparing each 100 mL of the *Test Preparation*.

Suggested Operating Parameters of Gas Chromatograph Apparatus

Agent	Column Size: Length	Column Size: ID	Column Packing Phases and Support	Flow Rate, mL per min.	Column Temperature
Benzyl Alcohol	1.8 m	3 mm	5 percent G16/S1	50	140°
Chlorobutanol	1.2 m	3 mm	5 percent G16/S1	40	110°
Phenol	1.2 m	3 mm	5 percent G16/S1	50	145°
Parabens	1.8 m	2 mm	5 percent G2/S1	20	150°

Phenol

Internal Standard Solution—Pipet 1 mL of benzyl alcohol into a 500-mL volumetric flask, add methanol to volume, and mix.

Standard Preparation—Dissolve about 75 mg of phenol, accurately weighed, in 7.5 mL of methanol contained in a 100-mL volumetric flask. Add 20.0 mL of *Internal Standard Solution*, then add water to volume, and mix.

Procedure—Using 3-µL portions of the *Standard Preparation* and the *Test Preparation*, record their gas chromatograms with the apparatus adjusted to the parameters set forth in the accompanying table. Measure the areas under the peaks for phenol and benzyl alcohol of the chromatogram for the *Standard Preparation*, designating them P_1 and P_2, respectively. Similarly, determine the corresponding values p_1 and p_2 for the *Test Preparation*. Calculate the content, in mg per mL, of phenol (C_6H_6O) in each mL of the specimen taken by the formula:

$$100(C/V)(p_1/p_2)(P_2/P_1),$$

in which C is the concentration, in mg per mL, of phenol in the *Standard Preparation*, and V is the volume, in mL, of the specimen under test used in preparing each 100 mL of the *Test Preparation*.

Methylparaben and Propylparaben

Internal Standard Solution—Place about 200 mg of benzophenone in a 250-mL volumetric flask, add ether to volume, and mix.

Standard Preparation—Place 100 mg of methylparaben and 10 mg of propylparaben, each accurately weighed, in a 200-mL volumetric flask, add *Internal Standard Solution* to volume, and mix. Place 10 mL of this solution in a 25-mL conical flask, and proceed as directed under *Test Preparation*, beginning with "Add 3 mL of pyridine."

Test Preparation—Pipet 10 mL of the specimen under test and 10 mL of the *Internal Standard Solution* into a small separator. Shake vigorously, allow the layers to separate, draw off the aqueous layer into a second separator, and transfer the ether layer into a small flask through a funnel containing anhydrous sodium sulfate. Extract the aqueous layer with two 10-mL portions of ether, also filtering the extracts through the anhydrous sodium sulfate. Evaporate the combined extracts under a stream of dry air until the volume is reduced to about 10 mL, then transfer the residue to a 25-mL conical flask. Add 3 mL of pyridine, complete the evaporation of the ether, and boil on a hot plate until the volume is reduced to about 1 mL. Cool, and add 1.0 mL of a suitable silylation agent, such as hexamethyldisilazane to which has been added trimethylchlorosilane, bis(trimethylsilyl)acetamide, or bis(trimethylsilyl)trifluoroacetamide. Mix, and allow to stand for not less than 15 minutes.

Procedure—Using a 2-μL portion of the silanized solution from the *Standard Preparation*, record the gas chromatogram with the apparatus adjusted to the parameters set forth in the accompanying table. Measure the areas under the peaks for methylparaben, propylparaben, and benzophenone, designating them P_1, P_2, and P_3, respectively. Similarly, measure the corresponding areas for the silanized solution from the *Test Preparation*, designating them p_1, p_2, and p_3, respectively. Calculate the content, in μg per mL, of methylparaben ($C_8H_8O_3$) in the sample under test by the formula:

$$10(C_M/V)(p_1/p_3)(P_3/P_1),$$

in which C_M is the concentration, in μg per mL, of methylparaben in the *Standard Preparation*, and V is the volume, in mL, of the specimen taken. Similarly, calculate the content, in μg per mL, of propylparaben ($C_{10}H_{12}O_3$) in the specimen under test by the formula:

$$10(C_P/V)(p_2/p_3)(P_3/P_2),$$

in which C_P is the concentration, in μg per mL, of propylparaben in the *Standard Preparation*.

Ethylparaben and Butylparaben may be determined in a similar manner.

POLAROGRAPHIC METHOD

Phenylmercuric Nitrate

Standard Preparation—Dissolve about 100 mg of phenylmercuric nitrate, accurately weighed, in sodium hydroxide solution (1 in 250) contained in a 1000-mL volumetric flask, warming if necessary to effect solution, add the sodium hydroxide solution to volume, and mix. Pipet 10 mL of this solution into a 25-mL volumetric flask, and proceed as directed under *Test Preparation*, beginning with "add 2 mL of potassium nitrate solution (1 in 100)."

Test Preparation—Pipet 10 mL of the specimen under test into a 25-mL volumetric flask, add 2 mL of potassium nitrate solution (1 in 100) and 10 mL of pH 9.2 alkaline borate buffer (see under *Buffer Solutions* in the section, *Reagents, Indicators, and Solutions*), and adjust to a pH of 9.2, if necessary, by the addition of 2 *N* nitric acid. Add 1.5 mL of freshly prepared gelatin solution (1 in 1000), then add the pH 9.2 alkaline borate buffer to volume, and mix.

Procedure—Pipet a portion of the *Test Preparation* into the polarographic cell, and deaerate by bubbling nitrogen through the solution for 15 minutes. Insert the dropping mercury electrode of a suitable polarograph (see *Polarography* ⟨801⟩), and record the polarogram from −0.6 to −1.5 volts versus the saturated calomel electrode. Determine the diffusion current of the *Test Preparation*, $(i_d)_U$, as the difference between the residual current and the limiting current. Similarly and concomitantly determine the diffusion current, $(i_d)_S$, of the *Standard Preparation*. Calculate the quantity, in μg, of phenylmercuric nitrate ($C_6H_5HgNO_3$) in each mL of the specimen taken by the formula:

$$2.5C[(i_d)_U/(i_d)_S],$$

in which C is the concentration, in μg per mL, of phenylmercuric nitrate in the *Standard Preparation*.

Thimerosal

Standard Preparation—On the day of use, place about 25 mg of thimerosal, accurately weighed, in a 250-mL volumetric flask, add water to volume, and mix. Protect from light. Pipet 15 mL of this solution into a 25-mL volumetric flask, add 1.5 mL of gelatin solution (1 in 1000), then add potassium nitrate solution (1 in 100) to volume, and mix.

Test Preparation—Pipet 15 mL of the test specimen into a 25-mL volumetric flask, add 1.5 mL of gelatin solution (1 in 1000), add potassium nitrate solution (1 in 100) to volume, and mix.

Procedure—Transfer a portion of the *Test Preparation* to a polarographic cell, and deaerate by bubbling nitrogen through the solution for 15 minutes. Insert the dropping mercury electrode of a suitable polarograph (see *Polarography* ⟨801⟩), and record the polarogram from −0.2 to −1.4 volts versus the saturated calomel electrode. Determine the diffusion current, $(i_d)_U$, as the difference between the residual current and the limiting current. Similarly and concomitantly determine the diffusion current, $(i_d)_S$, of the *Standard Preparation*. Calculate the quantity, in μg, of thimerosal ($C_9H_9HgNaO_2S$) in each mL of the test specimen taken by the formula:

$$1.667C[(i_d)_U/(i_d)_S],$$

in which C is the concentration, in μg per mL, of thimerosal in the *Standard Preparation*.

⟨351⟩ ASSAY FOR STEROIDS

The following procedure is applicable for determination of those Pharmacopeial steroids that possess reducing functional groups such as α-ketols.

Standard Preparation—Dissolve in alcohol a suitable quantity of the USP Reference Standard specified in the individual monograph, previously dried under the conditions specified in the individual monograph and accurately weighed, and dilute quantitatively and stepwise with alcohol to obtain a solution having a concentration of about 10 μg per mL. Pipet 20 mL of this solution into a glass-stoppered, 50-mL conical flask.

Assay Preparation—Prepare as directed in the individual monograph.

Procedure—To each of the two flasks containing the *Assay Preparation* and the *Standard Preparation*, respectively, and to a similar flask containing 20.0 mL of alcohol to serve as the blank, add 2.0 mL of a solution prepared by dissolving 50 mg of blue tetrazolium in 10 mL of methanol, and mix. Then to each flask add 2.0 mL of a mixture of alcohol and tetramethylammonium hydroxide TS (9:1), mix, and allow to stand in the dark for 90 minutes. Without delay, concomitantly determine the absorbances of the solutions from the *Assay Preparation* and the *Standard Preparation* at about 525 nm, with a suitable spectrophotometer, against the blank. Calculate the result by the formula given in the individual monograph, in which C is the concentration, in μg per mL, of the Reference Standard in the *Standard Preparation*, and A_U and A_S are the absorbances of the solutions from the *Assay Preparation* and the *Standard Preparation*, respectively.

⟨361⟩ BARBITURATE ASSAY

Internal Standard, *Internal Standard Solution*, *Standard Preparation*, and *Assay Preparation*—Prepare as directed in the individual monograph.

Chromatographic System—Under typical conditions, the gas chromatograph is equipped with a flame-ionization detector and contains a 0.9-m × 4-mm glass column packed with 3 percent liquid phase G10 on support 80- to 100-mesh S1A. The column is maintained at a temperature of 200 ± 10°, and the injection port and detector are maintained at about 225°, the column temperature being varied within the designated tolerance, as necessary, to meet *System Suitability* specifications and provide suitable retention times. Use a suitable carrier gas, such as dry nitrogen, at an appropriate flow rate, such as 60 to 80 mL per minute. Use on-column injection. [NOTE—If the instrument is not equipped for on-column injection, use an injection port lined with glass that has been washed successively with chromic acid cleansing solution, water, methanol, chloroform, a 1 in 10 solution of trimethylchlorosilane in chloroform, and chloroform.]

System Suitability (see *Chromatography* ⟨621⟩)—Chromatograph five replicate injections of the *Standard Preparation*, and record peak responses as directed under *Procedure*. The relative standard deviation for the ratio R_S is not more than 1.5%. In a suitable chromatogram, the resolution, R, between the barbituric acid and the *Internal Standard* is not less than the value given in the individual monograph, and the tailing factor, T, for each of the two peaks is not more than 2.0.

Procedure—Inject a suitable portion (about 5 μL) of the *Standard Preparation* into a suitable gas chromatograph, and record the chromatogram. Similarly inject a suitable portion of the *Assay Preparation*, and record the chromatogram. Calculate the

content of the barbiturate or barbituric acid in the assay specimen by the formula given in the individual monograph, in which R_U is the ratio of the peak response of the barbituric acid to that of the *Internal Standard* obtained for the *Assay Preparation*, Q_S is the ratio of the weight of the barbituric acid to that of the *Internal Standard* in the *Standard Preparation*, C_i is the concentration, in mg per mL, of *Internal Standard* in the *Internal Standard Solution*, and R_S is the ratio of the peak response of the barbituric acid to that of the *Internal Standard* in the *Standard Preparation*.

⟨371⟩ COBALAMIN RADIOTRACER ASSAY

All radioactive determinations required by this method should be made with a suitable counting assembly over a period of time optimal for the particular counting assembly used. All procedures should be performed in replicate to obtain the greatest accuracy.

Reference Standard—*USP Cyanocobalamin Reference Standard*—Dry over silica gel for 4 hours before using.

Cyanocobalamin Tracer Reagent—Dilute an accurately measured volume of a solution of radioactive cyanocobalamin* with water to yield a solution having a radioactivity between 500 and 5000 counts per minute per mL. Add 1 drop of cresol per liter of solution prepared, and store in a refrigerator.

Standardization—Prepare a solution of a weighed quantity of USP Cyanocobalamin RS in water to contain 20 to 50 µg per mL. Perform the entire assay on a 10.0-mL portion of this solution, proceeding as directed under *Assay Preparation*, beginning with "Add water to make a measured volume."

Cresol–Carbon Tetrachloride Solution—Mix equal volumes of carbon tetrachloride and freshly distilled cresol.

Phosphate-Cyanide Solution—Dissolve 100 mg of potassium cyanide in 1000 mL of a saturated solution of dibasic sodium phosphate, and mix.

Butanol–Benzalkonium Chloride Solution—Dilute benzalkonium chloride solution (17 in 100) with water (3:1), and mix with 36 volumes of butyl alcohol.

Alumina-Resin Column—Place a pledget of glass wool in the bottom of a constricted glass tube such as a 50-mL buret. With the tube held in an upright position, add a volume of a slurry of ion-exchange resin (see in the section, *Reagents, Indicators, and Solutions*), in water, sufficient to give a column of settled resin 7 cm in height. When the solid has settled somewhat, allow the water to drain so that there is only 1 cm of liquid above the resin column, and tamp the resin lightly. Then add a volume of a slurry of anhydrous alumina (not acid-washed) in water sufficient to increase the height of the settled column to 10 cm, and allow the water to drain to about 1 cm from the top of the alumina. Add a pledget of glass wool, and wash the column, using a total of 50 mL of water, and again drain to within 1 cm of the top of the column. Prepare a fresh column for each determination.

Assay Preparation—Transfer to a beaker a weighed quantity or measured volume of the preparation to be assayed, equivalent in vitamin B_{12} activity to that of 200 to 500 µg of cyanocobalamin. Add water to make a measured volume of not less than 25 mL, then add 5.0 mL of *Cyanocobalamin Tracer Reagent*. Add, while working *under a hood*, 5 mg of sodium nitrite and 2 mg of potassium cyanide for each mL of the resulting solution. Adjust the solution with diluted hydrochloric acid to a pH of approximately 4, and heat on a steam bath for 15 minutes. Cool, and adjust the solution with 1 *N* sodium hydroxide to a pH between 7.6 and 8.0. Centrifuge or filter to remove any undissolved solids.

Procedure—Transfer the *Assay Preparation* to a 250-mL centrifuge bottle, add 10 mL of *Cresol–Carbon Tetrachloride Solution*, suitably close the bottle with a glass, polyethylene, or foil-wrapped rubber stopper, shake vigorously for 2 to 5 minutes, and centrifuge. Remove and save the lower, solvent layer. Repeat the extraction using a 5-mL portion of *Cresol–Carbon Tetrachloride Solution*, and combine the lower, solvent-layer extracts in a centrifuge bottle or separator of 50- to 100-mL capacity.

Wash the combined extracts with successive 10-mL portions of 5 *N* sulfuric acid until the last washing is practically colorless (two washings usually suffice). During each washing, shake for 2 to 5 minutes, allow the layers to separate, centrifuge, if necessary, and discard the acid layer. Wash further with two successive 10-mL portions of *Phosphate-Cyanide Solution*. Finally, wash with 10 mL of water. Discard all of the washings.

To the washed extract add 30 mL of a mixture of *Butanol–Benzalkonium Chloride Solution* and carbon tetrachloride (2:1). Extract with two 5-mL portions of water, each time shaking vigorously for 1 minute, centrifuging, and removing and saving the upper, aqueous layer.

Pass the combined aqueous extracts through the *Alumina-Resin Column* at a rate of about 1 mL per minute, maintaining a 1-cm layer of liquid on the head of the column by adding water as needed. Discard as much of the forerun as is colorless (usually about 5 mL), and collect the colored eluate (usually about 10 mL) in a 50-mL centrifuge tube or separator containing 500 µL of diluted acetic acid. Extract the eluate by shaking for 2 to 5 minutes with 5 mL of *Cresol–Carbon Tetrachloride Solution*, and discard the upper, aqueous layer. To the extract add 5.0 mL of water, 5 mL of carbon tetrachloride, and 10 mL of butyl alcohol. Shake, allow to separate until the upper layer is clear, and remove the upper, aqueous layer.

Determine the absorbances of the aqueous extract, in a 1-cm cell, at 361 nm and 550 nm, with a suitable spectrophotometer, using a tungsten light source. Make the 361-nm reading using a filter capable of reducing stray light. Calculate the ratio A_{361}/A_{550}: the purity of the aqueous extract is acceptable if the ratio is between 3.10 and 3.40. If a ratio outside this range is observed, purify the aqueous extract by repeating the extraction cycle, proceeding as directed in the foregoing paragraph.

If an acceptable absorbance ratio is observed in the aqueous extract, determine the radioactivity, in counts per minute, using a suitable counter over a period optimal for the particular counting assembly used. Average the results, and correct the average for the observed background radioactivity determined over two or more 30-minute periods.

Calculation—Calculate the cobalamin content, expressed in µg of cyanocobalamin, of the portion taken for assay by the formula:

$$R(C_S/C_U)(A_U/A_S),$$

in which R is the quantity, in µg, of cyanocobalamin in the portion of the standard solution taken, C_S and C_U are the corrected average radioactivity values, expressed in counts per minute per mL, of the standard and assay solutions, respectively, and A_U and A_S are the absorbances determined at 361 nm of the assay and standard solutions, respectively.

⟨381⟩ ELASTOMERIC CLOSURES FOR INJECTIONS

An elastomeric closure may be of synthetic or natural origin. It is generally a complex mixture of many ingredients. These include the basic polymer, fillers, accelerators, vulcanizing agents, and pigments. The properties of the elastomeric closure are dependent not only upon these ingredients, but also on the processing procedure, such as mixing, milling, dusting agents used, molding, and curing.

Factors such as cleansing procedures, contacting media, and conditions of storage may also affect the suitability of an elastomeric closure for a specific use. Evaluation of such factors should be made by appropriate additional specific tests to determine the suitability of an elastomeric closure for its intended use. Criteria for the selection of an elastomeric closure should also include a careful review of all the ingredients to assure that no known or suspected carcinogens, or other toxic substances are added.

Definition—An *elastomeric closure* is a packaging component that is, or may be, in direct contact with the drug.

Biological Test Procedures

Two stages of testing are indicated. The first stage is the performance of *in-vitro* tests according to the procedures set forth

* A solution of cyanocobalamin made radioactive by the incorporation of ^{60}Co is available from Merck and Co., Inc., Rahway, NJ 07065.

in chapter ⟨87⟩, *Biological Reactivity Tests, In-vitro*. Materials that meet the requirements of the *in-vitro* tests are not required to undergo further testing. Materials that do not meet the requirements of the *in-vitro* tests are subjected to the second stage of testing which is the performance of *in-vivo* tests, i.e., the *Systemic Injection Test* and *Intracutaneous Test*, according to the procedures set forth in chapter ⟨88⟩, *Biological Reactivity Tests, In-vivo*.

Physicochemical Test Procedures

The following tests are designed to determine pertinent physicochemical extraction characteristics of elastomeric closures. Since the tests are based on the extraction of the elastomer, it is essential that the designated amount of surface area of sample be available. In each case, the specified surface area is available for extraction at the designated temperature. The test methods are devised to detect the majority of expected variations.

Extraction Solvents—

A: Purified water.

B: Drug product vehicle (where applicable).

C: Isopropyl alcohol.

Apparatus—

Autoclave—Use an autoclave capable of maintaining a temperature of 121 ± 2°, equipped with a thermometer, a pressure gauge, and a rack adequate to accommodate the test containers above the water level.

Oven—Use an oven, preferably a forced-draft model, that will maintain an operating temperature of 105° ± 2°.

Reflux Apparatus—Use a suitable reflux apparatus having a capacity of about 500 mL.

Procedure—

Preparation of Sample—Place in a suitable extraction container a sufficient number of elastomeric closures to provide 100 cm^2 of exposed surface area. Add 300 mL of purified water to each container, cover with a suitable inverted beaker, and autoclave at 121 ± 0.5° for 30 minutes. [NOTE—Adjust so that the temperature rises rapidly, preferably within 2 to 5 minutes.] Decant, using a stainless steel screen to hold the closures in the containers. Rinse with 100 mL of purified water, gently swirl, and discard the rinsings. Repeat with a second 100-mL portion of purified water. Treat all *blank* containers in a similar manner.

Extracts (with use of *Extraction Solvent A*)—Place a properly prepared *sample*, having an exposed surface area of 100 cm^2, in a suitable container, and add 200 mL of purified water. Cover with a suitable inverted beaker, and extract by heating in an autoclave at 121° for 2 hours, allowing adequate time for the liquid within the container to reach the extraction temperature. Allow the autoclave to cool rapidly, and cool to room temperature. Treat the *blank* container in a similar manner.

Extracts (with use of *Extraction Solvent B* or *C*)—Place a properly prepared sample, having an exposed surface area of 100 cm^2, in a suitable *Reflux Apparatus* containing 200 mL of *Extraction Solvent*, and reflux for 30 minutes. Treat the *blank* in a similar manner.

Turbidity—[NOTE—Use *Extracts* prepared with *Extraction Solvent A, B,* or *C*.] Agitate the container, and transfer a sufficient quantity of *Extract*, diluted with *Extraction Solvent*, if necessary, to a cell. Measure the turbidity in a suitable nephelometer (see *Spectrophotometry and Light-scattering* ⟨851⟩), against fixed reproducible standards.* The turbidity is the difference between the values obtained for the blank and the sample expressed in Nephelos units, an arbitrary linear numerical scale expressing a haze range from absolute clarity to the zone of turbidity.

Reducing Agents—[NOTE—Use *Extracts* prepared with *Extraction Solvent A*.] Agitate the container, transfer 50 mL of *sample* extract to a suitable container, and titrate with 0.01 *N* iodine VS, using 3 mL of starch TS as the indicator. Treat the *blank* extract in a similar manner. The difference between the *blank* and the *sample* titration is expressed in mL of 0.01 *N* iodine.

Heavy Metals ⟨231⟩—[NOTE—Use *Extracts* prepared with *Extraction Solvent A* or *B*.] Transfer 20 mL of the *blank* and the *sample* extracts to separate color-comparison tubes. Transfer 2, 6, and 10 mL of *Standard Lead Solution* into separate color-comparison tubes, add 2 mL of 1 *N* acetic acid to each tube, and adjust the volume to 25 mL with purified water. Add 10 mL of freshly prepared hydrogen sulfide TS to each tube, mix, allow to stand for 5 minutes, and view downward over a white surface. Determine the amount of heavy metals in the *blank* and in the *sample*. The heavy metals content is the difference between the *blank* and the *sample*.

pH Change—[NOTE—Use *Extracts* prepared with *Extraction Solvent A* or *B*, adding to *extracts* obtained with *Solvent A* sufficient potassium chloride to provide a concentration of 0.1%.] Determine the pH of *sample* extracts *A* and *B* potentiometrically, performing blank determinations with *blank* extracts *A* and *B*, and making any necessary corrections. The pH change is the difference between the *blank* and the *sample*.

Total Extractables—[NOTE—Use *Extracts* prepared with *Extraction Solvent A, B,* or *C*.] Agitate the containers, and transfer 100-mL aliquots of the *blank* and the *sample* to separate, tared evaporating dishes. Evaporate on a steam bath to dryness (*Extracts* prepared with *Extraction Solvent C*) or in an oven at 100°, dry at 105° for 1 hour, cool in a desiccator, and weigh. Calculate the total extractables, in mg, by the formula:

$$2(W_U - W_B),$$

in which W_U is the weight, in mg, of residue found in the sample extract aliquot, and W_B is the weight, in mg, of residue found in the blank solution aliquot.

⟨391⟩ EPINEPHRINE ASSAY

Reference Standard—*USP Epinephrine Bitartrate Reference Standard*—Keep container tightly closed and protected from light. Dry in vacuum over silica gel for 18 hours before using.

Ferro-citrate Solution—On the day needed, dissolve 1.5 g of ferrous sulfate in 200 mL of water to which have been added 1.0 mL of dilute hydrochloric acid (1 in 12) and 1.0 g of sodium bisulfite. Dissolve 500 mg of sodium citrate in 10 mL of this solution, and mix.

Buffer Solution—In a 50-mL volumetric flask mix 4.2 g of sodium bicarbonate, 5.0 g of potassium bicarbonate, and 18 mL of water (not all of the solids will dissolve at this stage). To another 18 mL of water add 3.75 g of aminoacetic acid and 1.7 mL of 6 *N* ammonium hydroxide, mix to dissolve, and transfer this solution to the 50-mL volumetric flask containing the other mixture. Dilute with water to volume, and mix until solution is complete.

Standard Preparation—Transfer about 18 mg of USP Epinephrine Bitartrate RS, accurately weighed, to a 100-mL volumetric flask with the aid of 20 mL of sodium bisulfite solution (1 in 50), dilute with water to volume, and mix. Transfer 5.0 mL of this solution to a 50-mL volumetric flask, dilute with sodium bisulfite solution (1 in 500) to volume, and mix. [NOTE—Make the final dilution when the assay is carried out.] The concentration of USP Epinephrine Bitartrate RS in the *Standard Preparation* is about 18 μg per mL.

Assay Preparation—Transfer to a 50-mL volumetric flask an accurately measured volume of the Injection under assay, equivalent to about 500 μg of epinephrine, dilute with sodium bisulfite solution (1 in 500) to volume, if necessary, and mix. [NOTE—The final concentration of sodium bisulfite is in the range of 1 to 3 mg per mL, any bisulfite present in the Injection under assay being taken into consideration.]

Procedure—Into three 50-mL glass-stoppered conical flasks transfer, separately, 20.0-mL aliquots of the *Standard Preparation*, the *Assay Preparation*, and sodium bisulfite solution (1 in 500) to provide the blank. To each flask add 200 μL of *Ferro-citrate Solution* and 2.0 mL of *Buffer Solution*, mix, and allow the solutions to stand for 30 minutes. Determine the absorbances of the solutions in 5-cm cells at the wavelength of maximum absorbance at about 530 nm, with a suitable spectrophotometer, using the blank to set the instrument. Calculate the quantity, in mg, of epinephrine ($C_9H_{13}NO_3$) in each mL of the Injection taken by the formula:

$$(183.21/333.29)(0.05C/V)(A_U/A_S),$$

* A suitable Nephelos Standard is available from Coleman Instruments, Inc., Maywood, IL 60153.

in which 183.21 and 333.29 are the molecular weights of epinephrine and epinephrine bitartrate, respectively, C is the concentration, in µg per mL, of USP Epinephrine Bitartrate RS in the *Standard Preparation*, and V is the volume, in mL, of Injection taken.

⟨401⟩ FATS AND FIXED OILS

The following definitions and general procedures apply to fats, fixed oils, waxes, resins, balsams, and similar substances.

Preparation of Specimen

If a specimen of oil shows turbidity owing to separated stearin, warm the container in a water bath at 50° until the oil is clear, or if the oil does not become clear on warming, filter it through dry filter paper in a funnel contained in a hot-water jacket. Thoroughly mix, and weigh at one time as many portions as are needed for the various determinations, using preferably a bottle having a pipet dropper, or a weighing buret. Keep the specimen melted, if solid at room temperature, until the desired portions of specimen are withdrawn.

Specific Gravity

Determine the specific gravity of a fat or oil as directed under *Specific Gravity* ⟨841⟩.

Melting Temperature

Determine the melting temperature as directed for substances of *Class II* (see *Melting Range or Temperature* ⟨741⟩).

Solidification Temperature of Fatty Acids

Preparation of the Fatty Acids—Heat 75 mL of glycerin–potassium hydroxide solution (made by dissolving 25 g of potassium hydroxide in 100 mL of glycerin) in an 800-mL beaker to 150°, and add 50 mL of the clarified fat, melted if necessary. Heat the mixture for 15 minutes with frequent stirring, but do not allow the temperature to rise above 150°. Saponification is complete when the mixture is homogeneous, with no particles clinging to the beaker at the meniscus. Pour the contents of the beaker into 500 mL of nearly boiling water in an 800-mL beaker or casserole, add slowly 50 mL of dilute sulfuric acid (made by adding water and sulfuric acid (3:1)), and heat the solution, with frequent stirring, until the fatty acids separate cleanly as a transparent layer. Wash the acids with boiling water until free from sulfuric acid, collect them in a small beaker, place on a steam bath until the water has settled and the fatty acids are clear, filter into a dry beaker while hot, and dry at 105° for 20 minutes. Place the warm fatty acids in a suitable container, and cool in an ice bath until they congeal.

Test for Complete Saponification—Place 3 mL of the dry acids in a test tube, and add 15 mL of alcohol. Heat the solution to boiling, and add an equal volume of 6 N ammonium hydroxide. A clear solution results.

Procedure—Using an apparatus similar to the "Congealing Temperature Apparatus" specified therein, proceed as directed for *Procedure* under *Congealing Temperature* ⟨651⟩, reading "solidification temperature" for "congealing point" (the terms are synonymous). The average of not less than four consecutive readings of the highest point to which the temperature rises is the solidification temperature of the fatty acids.

Acid Value (Free Fatty Acids)

The acidity of fats and fixed oils in this Pharmacopeia may be expressed as the number of mL of 0.1 N alkali required to neutralize the free acids in 10.0 g of substance. Acidity is frequently expressed as the *Acid Value*, which is the number of mg of potassium hydroxide required to neutralize the free acids in 1.0 g of the substance.

Procedure—Unless otherwise directed, dissolve about 10.0 g of the substance, accurately weighed, in 50 mL of a mixture of equal volumes of alcohol and ether (which has been neutralized to phenolphthalein with 0.1 N sodium hydroxide) contained in a flask. If the test specimen does not dissolve in the cold solvent, connect the flask with a suitable condenser and warm slowly, with frequent shaking, until the specimen dissolves. Add 1 mL of phenolphthalein TS, and titrate with 0.1 N sodium hydroxide VS until the solution remains faintly pink after shaking for 30 seconds. Calculate either the *Acid Value* or the volume of 0.1 N alkali required to neutralize 10.0 g of specimen (free fatty acids), whichever is appropriate.

If the volume of 0.1 N sodium hydroxide VS required for the titration is less than 2 mL, a more dilute titrant may be used, or the sample size may be adjusted accordingly. The results may be expressed in terms of the volume of titrant used or in terms of the equivalent volume of 0.1 N sodium hydroxide.

If the oil has been saturated with carbon dioxide for the purpose of preservation, gently reflux the alcohol-ether solution for 10 minutes before titration. The oil may be freed from carbon dioxide also by exposing it in a shallow dish in a vacuum desiccator for 24 hours before weighing the test specimens.

Ester Value

The Ester Value is the number of mg of potassium hydroxide required to saponify the esters in 1.0 g of the substance. If the *Saponification Value* and the *Acid Value* have been determined, the difference between these two represents the Ester Value.

Procedure—Place 1.5 g to 2 g of the substance in a tared, 250-mL flask, weigh accurately, add 20 mL to 30 mL of neutralized alcohol, and shake. Add 1 mL of phenolphthalein TS, and titrate with 0.5 N alcoholic potassium hydroxide VS until the free acid is neutralized. Add 25.0 mL of 0.5 N alcoholic potassium hydroxide VS, and proceed as directed under *Saponification Value*, beginning with "Heat the flask" and omitting the further addition of phenolphthalein TS. The difference between the volumes, in mL, of 0.5 N hydrochloric acid consumed in the actual test and in the blank test, multiplied by 28.05 and divided by the weight in g of the specimen taken, is the Ester Value.

Hydroxyl Value

The Hydroxyl Value is the number of mg of potassium hydroxide equivalent to the hydroxyl content of 1.0 g of the substance.

Pyridine–Acetic Anhydride Reagent—Just before use, mix 3 volumes of freshly distilled pyridine with 1 volume of freshly distilled acetic anhydride.

Procedure—Transfer a quantity of the substance, determined by reference to the accompanying table and accurately weighed, to a glass-stoppered, 250-mL conical flask, and add 5.0 mL of *Pyridine–Acetic Anhydride Reagent*. Transfer 5.0 mL of *Pyridine–Acetic Anhydride Reagent* to a second glass-stoppered, 250-mL conical flask to provide the reagent blank. Fit both flasks with suitable glass-jointed reflux condensers, heat on a steam bath for 1 hour, add 10 mL of water through each condenser, and heat on the steam bath for 10 minutes more. Cool, and to each add 25 mL of butyl alcohol, previously neutralized to phenolphthalein TS with 0.5 N alcoholic potassium hydroxide, by pouring 15 mL through each condenser and, after removing the condensers, washing the sides of both flasks with the remaining 10-mL portions. To each flask add 1 mL of phenolphthalein TS, and titrate with 0.5 N alcoholic potassium hydroxide VS, recording the volume, in mL, consumed by the residual acid in the test solution as T and that consumed by the blank as B. In a 125-mL conical flask, mix about 10 g of the substance, accurately weighed, with 10 mL of freshly distilled pyridine, previously neutralized to phenolphthalein TS, add 1 mL of phenolphthalein TS, and titrate with 0.5 N alcoholic potassium hydroxide VS, recording the volume, in mL, consumed by the free acid in the test specimen as

Hydroxyl Value Range	Weight of Test Specimen, g
0 to 20	10
20 to 50	5
50 to 100	3
100 to 150	2
150 to 200	1.5
200 to 250	1.25
250 to 300	1.0
300 to 350	0.75

A, or use the Acid Value to obtain A. Calculate the Hydroxyl Value by the formula:

$$(56.11N/W)[B + (WA/C) - T],$$

in which W and C are the weights, in g, of the substances taken for the acetylation and for the free acid determination, respectively, N is the exact normality of the alcoholic potassium hydroxide, and 56.11 is the molecular weight of potassium hydroxide.

Iodine Value

The Iodine Value represents the number of g of iodine absorbed, under the prescribed conditions, by 100 g of the substance. Unless otherwise specified in the individual monograph, determine the Iodine Value by *Method I*.

METHOD I (HANUS METHOD)

Procedure—Introduce about 800 mg of a solid fat or about 200 mg of an oil, accurately weighed, into a 250-mL iodine flask, dissolve it in 10 mL of chloroform, add 25.0 mL of iodobromide TS, insert the stopper in the vessel securely, and allow it to stand for 30 minutes protected from light, with occasional shaking. Then add, in the order named, 30 mL of potassium iodide TS and 100 mL of water, and titrate the liberated iodine with 0.1 N sodium thiosulfate VS, shaking thoroughly after each addition of thiosulfate. When the iodine color becomes quite pale, add 3 mL of starch TS, and continue the titration with 0.1 N sodium thiosulfate VS until the blue color is discharged. Perform a blank test at the same time with the same quantities of the same reagents and in the same manner (see *Residual Titrations* ⟨541⟩). The difference between the volumes, in mL, of 0.1 N sodium thiosulfate VS consumed by the blank test and the actual test, multiplied by 1.269 and divided by the weight in g of the substance taken for test, is the Iodine Value.

NOTE—If more than half of the iodobromide TS is absorbed by the portion of the substance taken, repeat the determination, using a smaller portion of the substance under examination.

METHOD II (WIJS METHOD)

To a 500-mL iodine flask transfer an accurately weighed quantity, in g, of the substance to be tested, about equal to that calculated by the formula $25/I$, in which I is the iodine value, except that, for substances having iodine values not greater than 2.5, take about 10 g, accurately weighed, for the test.

Procedure—Dissolve it in 20 mL of carbon tetrachloride, add 25.0 mL of iodochloride TS, insert the stopper securely in the vessel, and allow it to stand at 25 ± 5° for 30 minutes, protected from light, with occasional shaking. Then add, in the order named, 20 mL of potassium iodide TS and 100 mL of recently boiled and cooled water, and titrate the liberated iodine with 0.1 N sodium thiosulfate VS, shaking thoroughly after each addition of thiosulfate. When the iodine color becomes quite pale, add 3 mL of starch TS, and continue the titration with 0.1 N sodium thiosulfate VS until the blue color is discharged. Perform a blank test at the same time with the same quantities of the same reagents and in the same manner (see *Residual Titrations* ⟨541⟩). The difference between the volumes, in mL, of 0.1 N sodium thiosulfate consumed by the blank test and the actual test, multiplied by 1.269 and divided by the weight in g of the sample taken, is the Iodine Value.

Saponification Value

The Saponification Value is the number of mg of potassium hydroxide required to neutralize the free acids and saponify the esters contained in 1.0 g of the substance.

Procedure—Place 1.5 g to 2 g of the substance in a tared, 250-mL flask, weigh accurately, and add to it 25.0 mL of 0.5 N alcoholic potassium hydroxide VS. Heat the flask on a steam bath, under a suitable condenser to maintain reflux for 30 minutes, frequently rotating the contents. Then add 1 mL of phenolphthalein TS, and titrate the excess potassium hydroxide with 0.5 N hydrochloric acid VS. Perform a blank determination at the same time, using the same amount of 0.5 N alcoholic potassium hydroxide VS (see *Residual Titrations* ⟨541⟩). The difference between the volumes, in mL, of 0.5 N hydrochloric acid consumed in the actual test and in the blank test, multiplied by 28.05 and divided by the weight in g of specimen taken, is the Saponification Value.

If the oil has been saturated with carbon dioxide for the purpose of preservation, expose it in a shallow dish in a vacuum desiccator for 24 hours before weighing the test specimens.

Unsaponifiable Matter

The term, Unsaponifiable Matter, in oils or fats, refers to those substances that are not saponifiable by alkali hydroxides but are soluble in the ordinary fat solvents, and to products of saponification that are soluble in such solvents.

Procedure—Weigh 5.0 g of the oil or fat into a 250-mL conical flask, add a solution of 2 g of potassium hydroxide in 40 mL of alcohol, and heat the flask on a steam bath under a suitable condenser to maintain reflux for 2 hours. Evaporate the alcohol on a steam bath, dissolve the residue in 50 mL of hot water, and transfer the solution to a separator having a polytetrafluoroethylene stopcock, rinsing the flask with two 25-mL portions of hot water that are added to the separator (do not use grease on stopcock). Cool to room temperature, add a few drops of alcohol to facilitate the separation of the two liquids, and extract with two 50-mL portions of ether, combining the ether extracts in another separator. Wash the combined extracts first with 20 mL of 0.1 N sodium hydroxide, then with 20 mL of 0.2 N sodium hydroxide, and finally with 15-mL portions of water until the last washing is not reddened by the addition of 2 drops of phenolphthalein TS. Transfer the ether extract to a tared beaker, and rinse the separator with 10 mL of ether, adding the rinsings to the beaker. Evaporate the ether on a steam bath just to dryness, and dry the residue at 100° for 30 minutes. Cool the beaker in a desiccator for 30 minutes, and weigh the residue of Unsaponifiable Matter.

Water and Sediment in Fixed Oils

Apparatus—The preferred centrifuge has a diameter of swing (d = distance from tip to tip of whirling tubes) of 38 to 43 cm and is operated at a speed of about 1500 rpm. If a centrifuge of different dimensions is used, calculate the desired rate of revolution by the formula:

$$\text{rpm} = 1500\sqrt{40.6/d}.$$

The centrifuge tubes are pear-shaped, and are shaped to accept closures. The total capacity of each tube is about 125 mL. The graduations are clear and distinct, reading upward from the bottom of the tube according to the scale shown in the accompanying table.

Volume (mL)	Scale Division (mL)
0 to 3	0.1
3 to 5	0.5
5 to 10	1.0
10 to 25	5.0
25 to 50	25.0
50 to 100	50.0

Procedure—Place 50.0 mL of benzene in each of two centrifuge tubes, and to each tube add 50.0 mL of the oil, warmed if necessary to re-incorporate separated stearin, and thoroughly mixed at 25°. Tightly stopper the tubes, and shake them vigorously until the contents are thoroughly mixed, then immerse the tubes in a water bath at 50° for 10 minutes. Centrifuge for 10 minutes. Read the combined volume of water and sediment at the bottom of each tube. Centrifuge repeatedly for 10-minute periods until the combined volume of water and sediment remains constant for 3 consecutive readings. The sum of the volumes of combined water and sediment in the two tubes represents the percentage, by volume, of water and sediment in the oil.

⟨411⟩ FOLIC ACID ASSAY

The following procedure is provided for the estimation of folic acid as an ingredient of Pharmacopeial preparations containing other active constituents.

Reference Standard—*USP Folic Acid Reference Standard*—Do not dry; determine the water content at the time of use.

Mobile Phase—Place 2.0 g of monobasic potassium phosphate in a 1-liter volumetric flask, and dissolve in about 650 mL of water. Add 12.0 mL of a 1 in 4 solution of tetrabutylammonium hydroxide in methanol, 7.0 mL of 3 *N* phosphoric acid, and 240 mL of methanol. Cool to room temperature, adjust with either 3 *N* phosphoric acid or 6 *N* ammonium hydroxide to a pH of 7.0, dilute with water to volume, and mix. Filter through a 0.45-μm filter, and recheck the pH before use. [NOTE—The methanol-to-water ratio may be varied by up to 3 percent and the pH may be increased up to 7.15 to achieve better separation.]

Diluting Solvent—Prepare as directed under *Mobile Phase*. Adjust to a pH of 7.0, and bubble nitrogen through the solution for 30 minutes before use.

Internal Standard Solution—Dissolve about 25 mg of methylparaben in 2.0 mL of methanol, dilute with *Diluting Solvent* to 50 mL, and mix.

Standard Folic Acid Solution—Transfer about 12 mg of USP Folic Acid RS, accurately weighed, to a low-actinic, 50-mL volumetric flask, dissolve in 2 mL of ammonium hydroxide, dilute with *Diluting Solvent* to volume, and mix.

Standard Preparation—Transfer 2.0 mL of *Standard Folic Acid Solution* to a low-actinic, 25-mL volumetric flask, add 2.0 mL of *Internal Standard Solution*, add *Diluting Solvent* to volume, and mix.

Assay Preparation—Transfer an accurately weighed or measured portion of the preparation to be assayed, containing about 1 mg of folic acid, to a low-actinic, 50-mL volumetric flask, add 4.0 mL of *Internal Standard Solution*, add *Diluting Solvent* to volume, and mix.

Chromatographic System (see *Chromatography* ⟨621⟩)—The liquid chromatograph is equipped with a 280-nm detector and a 15-cm × 3.9-mm column that contains packing L1. The flow rate is about 1.0 mL per minute. Chromatograph the *Standard Preparation*, and record the peak response as directed under *Procedure:* there is baseline separation of folic acid and methylparaben.

Procedure—Separately inject equal volumes (about 10 μL) of *Standard Preparation* and *Assay Preparation* into the chromatograph, record the chromatograms, and measure the responses for the major peaks. The relative retention times are about 0.8 for folic acid and 1.0 for methylparaben. Calculate the quantity, in μg, of $C_{19}H_{19}N_7O_6$ in the portion of the preparation taken by the formula:

$$50C(R_U/R_S),$$

in which C is the concentration, in μg per mL, of USP Folic Acid RS in the *Standard Preparation*, and R_U and R_S are the ratios of the response of the folic acid peak to that of the methylparaben peak obtained from the *Assay Preparation* and the *Standard Preparation*, respectively.

⟨421⟩ HYDROXYPROPOXY DETERMINATION

Reference Standard—*USP Methylcellulose Reference Standard*—Dry at 105° for 2 hours before using. Keep container tightly closed.

Apparatus—The apparatus for hydroxypropoxy group determinations is shown diagrammatically in Figure 1. The boiling or reaction flask, *D*, consisting of a 25-mL conical-bottom micro boiling flask modified to provide a side-arm outlet, is fitted with an aluminum foil–jacketed Vigreaux column, *E*, 95 mm long and with an adapter bleeder tube, *C*, having a 0.25- to 1.25-mm capillary tip through the neck and to the bottom of the flask for the introduction of steam and nitrogen. A steam generator, *B*, consisting of a 25- × 150-mm test tube and a gas inlet tube with a 0.25- to 1.25-mm capillary tip is attached to the bleeder tube, *C*, while a microcondenser with a 100-mm jacket, *F*, is attached to the Vigreaux column, *E*. The reaction flask and the steam generator are immersed in an oil bath, *A*, equipped with an electric heater capable of heating the bath at the desired rate and maintaining the temperature at 155°. The distillate is collected in a 125-mL graduated conical flask, *G*, fitted with a glass stopper.

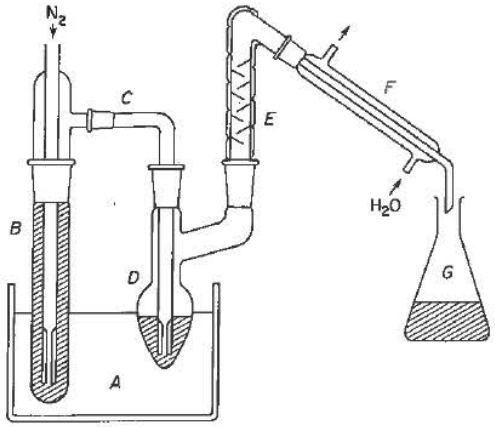


Fig. 1. Apparatus for Hydroxypropoxy Determination.

Procedure—Transfer about 100 mg of Hydroxypropyl Methylcellulose, previously dried at 105° for 2 hours and accurately weighed, into flask *D*, and add 10 mL of chromium trioxide solution (60 g in 140 mL). Fill the steam generator, *B*, with water to the bottom of the standard-taper joint, then assemble the apparatus as shown in the diagram. Immerse the steam generator and sample flask in the oil bath to the level of the chromium trioxide solution. Start the condenser cooling water, and pass nitrogen gas through the flask at a rate of 1 bubble per second. Raise the temperature of the oil bath to 155° during a 30-minute period, and maintain it at this temperature throughout the determination. [NOTE—Too rapid an initial rise in temperature results in high blanks.] Distil until 50 mL of the distillate has been collected. Detach the condenser, *F*, from the Vigreaux column, *E*, and wash with water, collecting the washings in the graduated conical flask containing the distillate. Titrate the solution with 0.02 *N* sodium hydroxide VS to a pH of 7.0 ± 0.1, using an expanded-scale pH meter equipped with glass and calomel electrodes. Record the volume, *V*, of the 0.02 *N* sodium hydroxide used, then add 500 mg of sodium bicarbonate and 10 mL of 2 *N* sulfuric acid. After evolution of carbon dioxide has ceased, add 1 g of potassium iodide, insert the stopper in the flask, shake the mixture, and allow the solution to stand in the dark for 5 minutes. Titrate the liberated iodine with 0.02 *N* sodium thiosulfate VS to the sharp disappearance of the yellow iodine color, adding a few drops of starch TS to confirm the endpoint, and record the volume, *Y*, required. This titration, *Y* mL, multiplied by the empirical factor, *K*, appropriate to the particular apparatus and reagents in use, gives the acid equivalent not caused by acetic acid. The acetic acid equivalent is $(V - KY)$ mL of 0.02 *N* sodium hydroxide.

Empirical Factor, K—Obtain the empirical factor, *K*, for each apparatus by performing a blank determination in which the cellulose ether is omitted. The acidity of the blank for a given apparatus and given reagents is in a fixed ratio to the oxidizing equivalent of the distillate in terms of sodium thiosulfate:

$$K \text{ factor} = (V_b \times N_1)/(Y_b \times N_2), \text{ in which}$$

V_b = mL of 0.02 *N* sodium hydroxide required in blank run,
N_1 = normality of the 0.02 *N* sodium hydroxide,
Y_b = mL of 0.02 *N* sodium thiosulfate required in blank run, and
N_2 = normality of the 0.02 *N* sodium thiosulfate.

Methylcellulose blank—Conduct several determinations using USP Methylcellulose Reference Standard as directed above in the *Procedure*. Calculate the percentage of uncorrected hydroxypropoxy group as follows:

$$OCH_2CHOHCH_3 \text{ percent (uncorrected)} = ([(V_aN_1 - KY_aN_2) \times 0.075]/W) \times 100, \text{ in which}$$

V_a = mL of 0.02 *N* sodium hydroxide required for titration of the sample,

N_1 = normality of the 0.02 *N* sodium hydroxide,
K = empirical factor,
Y_a = mL of 0.02 *N* sodium thiosulfate required for titration of the sample,
N_2 = normality of the 0.02 *N* sodium thiosulfate, and
W = g of sample used.

Calculate the corrected percentage of hydroxypropoxy group by subtracting the percentage of $OCH_2CHOHCH_3$ obtained in the *Methylcellulose blank* determination from the percentage of the uncorrected hydroxypropoxy group calculated above.

The results obtained as percentage of hydroxypropoxy content may be converted to terms of average molecular substitution of glucose units by means of the accompanying graph (Figure 2).

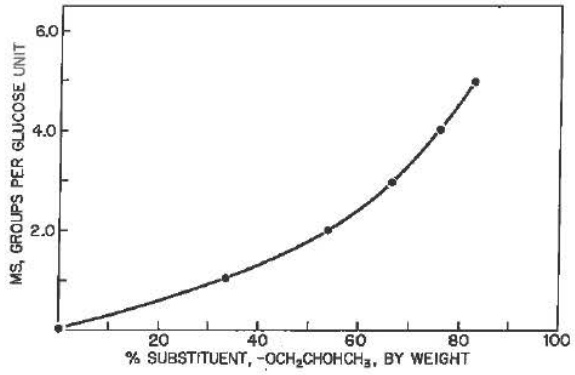


Fig. 2. Graph for Converting Percentage of Substitution, by Weight, of Hydroxypropoxy Groups to Molecular Substitution per Glucose Unit.

⟨425⟩ IODOMETRIC ASSAY—ANTIBIOTICS

The following method is provided for the assay of most of the Pharmacopeial penicillin antibiotic drugs and their dosage forms, for which iodometric titration is particularly suitable.

Standard Preparation—Dissolve in the solvent specified in the table of *Solvents and Final Concentrations* a suitable quantity of the USP Reference Standard specified in the individual monograph, previously dried under the conditions specified in the individual monograph and accurately weighed, and dilute quantitatively and stepwise with the same solvent to obtain a solution having a known concentration of about that specified in the table. Pipet 2.0 mL of this solution into each of two 125-mL glass-stoppered conical flasks.

Solvents and Final Concentrations

Antibiotic	Solvent*	Final concentration
Amoxicillin	Water	1.0 mg per mL
Ampicillin	Water	1.25 mg per mL
Ampicillin Sodium	*Buffer No. 1*	1.25 mg per mL
Cloxacillin Sodium	Water	1.25 mg per mL
Cyclacillin	Water	1.0 mg per mL
Dicloxacillin Sodium	*Buffer No. 1*	1.25 mg per mL
Methicillin Sodium	*Buffer No. 1*	1.25 mg per mL
Nafcillin Sodium	*Buffer No. 1*	1.25 mg per mL
Oxacillin Sodium	*Buffer No. 1*	1.25 mg per mL
Penicillin G Potassium	*Buffer No. 1*	2,000 units per mL
Penicillin G Sodium	*Buffer No. 1*	2,000 units per mL
Penicillin V Potassium	*Buffer No. 1*	2,000 units per mL
Phenethicillin Potassium	*Buffer No. 1*	2,000 units per mL

* Unless otherwise noted, the *Buffers* are the potassium phosphate buffers defined in the section *Media and Diluents* under *Antibiotics—Microbial Assays* ⟨81⟩, except that sterilization is not required before use.

Assay Preparation—Unless otherwise specified in the individual monograph, dissolve in the solvent specified in the table of *Solvents and Final Concentrations* a suitable quantity, accurately weighed, of the specimen under test, and dilute quantitatively with the same solvent to obtain a solution having a known final concentration of about that specified in the table. Pipet 2 mL of this solution into each of two 125-mL glass-stoppered conical flasks.

Procedure—

Inactivation and titration—To 2.0 mL of the *Standard Preparation* and of the *Assay Preparation*, in respective flasks, add 2.0 mL of 1.0 *N* sodium hydroxide, mix by swirling, and allow to stand for 15 minutes. To each flask add 2.0 mL of 1.2 *N* hydrochloric acid, add 10.0 mL of 0.01 *N* iodine VS, immediately insert the stopper, and allow to stand for 15 minutes. Titrate with 0.01 *N* sodium thiosulfate VS. As the end-point is approached, add 1 drop of starch iodide paste TS, and continue the titration to the discharge of the blue color.

Blank determination—To a flask containing 2.0 mL of the *Standard Preparation* add 10.0 mL of 0.01 *N* iodine VS. If the *Standard Preparation* contains amoxicillin or ampicillin, immediately add 0.1 mL of 1.2 *N* hydrochloric acid. Immediately titrate with 0.01 *N* sodium thiosulfate VS. As the end-point is approached, add 1 drop of starch iodide paste TS, and continue the titration to the discharge of the blue color. Similarly treat a flask containing 2.0 mL of the *Assay Preparation*.

Calculations—Calculate the microgram (or unit) equivalent (F) of each mL of 0.01 *N* sodium thiosulfate consumed by the *Standard Preparation* by the formula:

$$(2CP)/(B - I),$$

in which C is the concentration, in mg per mL, of Reference Standard in the *Standard Preparation*, P is the potency, in µg (or units) per mg, of the Reference Standard, B is the volume, in mL, of 0.01 *N* sodium thiosulfate consumed in the *Blank determination*, and I is the volume, in mL, of 0.01 *N* sodium thiosulfate consumed in the *Inactivation and titration*. Calculate the potency of the specimen under test by the formula given in the individual monograph.

⟨431⟩ METHOXY DETERMINATION

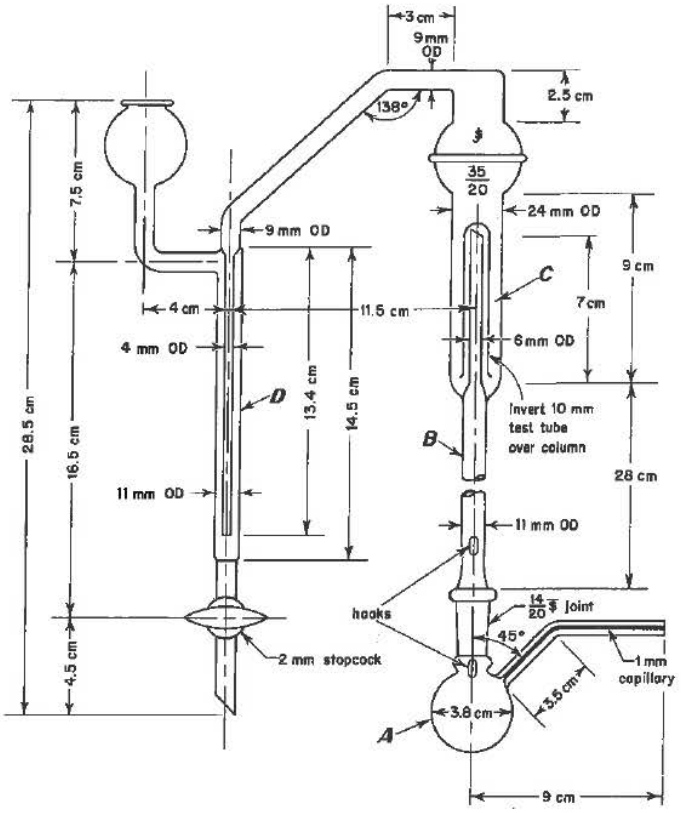
Apparatus—The apparatus for methoxy determination is shown diagrammatically in the accompanying figure. The boiling flask, *A*, is fitted with a capillary side-arm for the introduction of carbon dioxide or nitrogen and is connected to a column, *B*, which serves to separate aqueous hydriodic acid from the more volatile methyl iodide. The methyl iodide passes through water in a scrubber trap, *C*, and is finally absorbed in the bromine–acetic acid solution in absorption tube *D*. The carbon dioxide or nitrogen is introduced through a pressure-regulating device and connected to the apparatus by a small capillary containing a small cotton pledget. [NOTE—Avoid the use of organic solvents in cleaning this apparatus, since traces remaining may interfere with the determination. This test is used also for ethoxy determination with an 80-minute reaction time and a titrant equivalent of 0.751 mg of (OC_2H_5).]

For greater convenience in use and cleaning, a ground-glass ball joint connects the two upright columns of the apparatus. The top of the scrubber *C* consists of a 35/20 ball joint, the upper half of which is connected to the side-arm leading into tube *D*. This permits taking the apparatus apart and facilitates adding the water to the trap. Also, it allows access to the loose inverted (10-mm) test tube that serves as the trap over the inner tube of the scrubber *C*.

Reagents—

BROMINE–ACETIC ACID SOLUTION—Dissolve 100 g of potassium acetate in 1000 mL of a solution consisting of 900 mL of glacial acetic acid and 100 mL of acetic anhydride. On the day of use, to 145 mL of this solution add 5 mL of bromine.

HYDRIODIC ACID—A colorless, or nearly colorless, constant-boiling reagent solution, prepared for this purpose, is available commercially. If not obtained commercially, it may be prepared



Apparatus for Methoxy Determination

by distilling hydriodic acid over red phosphorus, passing carbon dioxide or nitrogen through the apparatus during the distillation. Use the constant-boiling mixture (between 55% and 58% of HI) distilling between 126° and 127°, which is colorless or nearly colorless. (*Caution—Exercise safety precautions when distilling hydriodic acid.*) Place the acid in small, amber, glass-stoppered bottles previously flushed with carbon dioxide, or nitrogen, seal with paraffin, and store in a cool, dark place.

Procedure—Prepare the apparatus by disconnecting the ball joint and pouring water into trap *C* until it is half-full. Connect the two parts, using a minimal amount of a suitable silicone grease to seal the ball joint. Add 7 mL of *Bromine–Acetic Acid Solution* to absorption tube *D*. Weigh the sample in a tared gelatin capsule, and add it to the boiling flask along with a few boiling chips or pieces of porous plate. Finally add 6 mL of *Hydriodic Acid* and attach the flask to the column, using a minimal amount of a suitable silicone grease to seal the junction. Bubble the carbon dioxide or nitrogen through the apparatus at the rate of 2 bubbles per second, place the boiling flask in an oil bath or heating mantle heated to 150°, and continue the reaction for 40 minutes for methoxy determination, or 80 minutes for ethoxy determination. Drain the contents of the absorption tube into a 500-mL conical flask containing 10 mL of sodium acetate solution (1 in 4). Rinse the tube with water, adding the rinsings to the flask, and finally dilute with water to about 125 mL. Add formic acid, dropwise, with swirling, until the reddish brown color of the bromine is discharged, then add 3 additional drops. A total of 12 to 15 drops usually is required. Allow to stand for 3 minutes, and add 15 mL of diluted sulfuric acid and 3 g of potassium iodide, and titrate immediately with 0.1 *N* sodium thiosulfate VS, using 3 mL of starch TS as the indicator. Perform a blank determination, including also a gelatin capsule, and make any necessary correction. Each mL of 0.1 *N* sodium thiosulfate is equivalent to 0.517 mg of (OCH_3).

⟨441⟩ NIACIN OR NIACINAMIDE ASSAY

Reference Standards—*USP Niacin Reference Standard*—Dry at 105° for 1 hour before using. *USP Niacinamide Reference Standard*—Dry over silica gel for 4 hours before using. [NOTE—The previously dried Reference Standards may be stored in a desiccator over silica gel, protected from light.]

Chemical Method

NOTE—Determine from the labeling if the vitamin in the assay specimen is niacin or niacinamide, and use the corresponding standard preparation (either *Standard Niacin Preparation* or *Standard Niacinamide Preparation*) as directed in the *Procedure*.

Cyanogen Bromide Solution—Dissolve 5 g of cyanogen bromide in water to make 50 mL. (*Caution—Prepare this solution under a hood, as cyanogen bromide volatilizes at room temperature, and the vapor is highly irritating and poisonous.*)

Sulfanilic Acid Solution—To 2.5 g of sulfanilic acid add 15 mL of water and 3 mL of 6 *N* ammonium hydroxide. Mix, add, with stirring, more 6 *N* ammonium hydroxide, if necessary, until the acid dissolves, adjust the solution with 3 *N* hydrochloric acid to a pH of about 4.5, using bromocresol green TS as an external indicator, and dilute with water to 25 mL.

Standard Niacin Stock Solution—Transfer 25.0 mg of USP Niacin RS to a 500-mL volumetric flask, dissolve in alcohol solution (1 in 4), dilute with alcohol solution (1 in 4) to volume, and mix. Store in a refrigerator. Each mL of this solution contains 50 µg of USP Niacin RS.

Standard Niacin Preparation—Transfer 10.0 mL of *Standard*

Reaction Mixtures for Niacin or Niacinamide Assay—Chemical Method

Constituent	Tube 1, mL	Tube 2, mL	Tube 3, mL	Tube 4, mL
Standard Preparation	1.0	1.0	—	—
Assay Preparation	—	—	1.0	1.0
Ammonia Dilution (ammonium hydroxide, diluted to 1 in 50)	0.5	0.5	0.5	0.5
Water	6.5	1.5	6.5	1.5
Cyanogen Bromide Solution	—	5.0	—	5.0
Sulfanilic Acid Solution	2.0	2.0	2.0	2.0
Hydrochloric Acid	1 drop	—	1 drop	—

Niacin Stock Solution to a 100-mL volumetric flask, dilute with water to volume, and mix. Each mL of this solution contains 5 µg of USP Niacin RS.

Standard Niacinamide Stock Solution—Transfer 50.0 mg of USP Niacinamide RS to a 500-mL volumetric flask, dissolve in alcohol solution (1 in 4), dilute with alcohol solution (1 in 4) to volume, and mix. Store in a refrigerator. Each mL of this solution contains 100 µg of USP Niacinamide RS.

Standard Niacinamide Preparation—Transfer 10.0 mL of *Standard Niacinamide Stock Solution* to a 100-mL volumetric flask, dilute with water to volume, and mix. Each mL of this solution contains 10 µg of USP Niacinamide RS.

Assay Preparation—Prepare as directed in the individual monograph.

Procedure—Pipet into four marked tubes the quantities of the appropriate *Standard Preparation*, the *Assay Preparation*, the ammonia dilution, and water indicated in the accompanying table. Then add the other constituents, respectively, as listed in the table, according to the directions given herein.

To Tube 1 add the *Sulfanilic Acid Solution*, shake well, add the hydrochloric acid, mix, place in a suitable spectrophotometer, and adjust to zero absorbance at 450 nm. To Tube 2 add the *Cyanogen Bromide Solution*, mix, and 30 seconds, accurately timed, after completion of the addition of the cyanogen bromide add the *Sulfanilic Acid Solution*, with swirling. Close the tube, place it in the spectrophotometer, and after 2 minutes measure its absorbance at 450 nm against Tube 1 as a blank, designating the absorbance as A_S. Repeat the procedure with Tubes 3 (as blank) and 4, designating the absorbance of Tube 4 as A_U. Calculate the quantity of niacin or niacinamide in the sample as directed in the individual monograph.

Microbiological Method

Test Solution of Material to be Assayed—Place the prescribed amount of the material to be assayed in a flask of suitable size, and proceed by one of the methods given below. The concentrations of the sulfuric acid and sodium hydroxide solutions used are not stated in each instance because these concentrations may be varied depending upon the amount of material taken for assay, volume of test solution, and buffering effect of material.

(*a*) *For Dry or Semidry Materials that Contain No Appreciable Amount of Basic Substances*—Add a volume of dilute sulfuric acid (1 in 35) equal, in mL, to not less than 10 times the dry weight of the material, in g, but the resulting solution shall contain not more than 5.0 mg of niacin in each mL. If the material is not readily soluble, comminute it so that it may be evenly dispersed in the liquid, then agitate vigorously, and wash down the sides of the flask with dilute sulfuric acid (1 in 35).

Heat the mixture in an autoclave at 121° to 123° for 30 minutes, and cool. If lumping occurs, agitate the mixture until the particles are evenly dispersed. Adjust the mixture with sodium hydroxide solution to a pH of 6.8, dilute with water to make a final measured volume that has a concentration of niacin equivalent to that of *Standard Niacin Solution*, and filter.

(*b*) *For Dry or Semidry Materials that Contain Appreciable Amounts of Basic Substances*—Add sufficient sulfuric acid solution to bring the pH of the mixture to between 5.0 and 6.0. Add such an amount of water that the total volume of liquid shall be equal in mL to not less than ten times the dry weight of the assay specimen, in g, but the resulting solution shall contain not more than 5.0 mg of niacin in each mL. Then add the equivalent of 10 mL of dilute sulfuric acid (2 in 7) for each 100 mL of liquid, and proceed as directed under (*a*), beginning with the second paragraph.

(*c*) *For Liquid Materials*—Adjust the material with either sulfuric acid solution or sodium hydroxide solution to a pH of 5.0 to 6.0. Add such an amount of water that the total volume of liquid shall be equal, in mL, to not less than 10 times the volume of the specimen, in mL, but the resulting solution shall contain not more than 5.0 mg of niacin in each mL. Then add the equivalent of 10 mL of dilute sulfuric acid (2 in 7) for each mL of liquid, and proceed as directed under (*a*), beginning with the second paragraph.

Standard Niacin Stock Solution I—Transfer 50.0 mg of USP Niacin RS to a 500-mL volumetric flask, dissolve in alcohol, dilute with alcohol to volume, and mix. Store in a refrigerator. Each mL of this solution contains 100 µg of USP Niacin RS.

Standard Niacin Stock Solution II—To 100.0 mL of *Niacin Stock Solution I* add water to make 1000.0 mL. Store under toluene in a refrigerator. Each mL of this solution contains 10 µg of USP Niacin RS.

Standard Niacin Solution—Dilute a suitable volume of *Niacin Stock Solution II* with water to such a measured volume so that after incubation as described in the *Assay Procedure* the transmittance of the 5.0-mL level of *Standard Niacin Solution* is equivalent to that of a dried cell weight of not less than 1.25 mg, when the inoculated blank is set at 100 percent transmittance. This concentration is usually between 10 ng and 40 ng of niacin per mL. Prepare a fresh *Standard Niacin Solution* for each assay.

Basal Medium Stock Solution—

Acid-hydrolyzed Casein Solution	25 mL
Cystine-Tryptophan Solution	25 mL
Dextrose Anhydrous	10 g
Sodium Acetate Anhydrous	5 g
Adenine-Guanine-Uracil Solution	5 mL
Riboflavin–Thiamine Hydrochloride–Biotin Solution	5 mL
Aminobenzoic Acid–Calcium Pantothenate–Pyridoxine Hydrochloride Solution	5 mL
Salt Solution A	5 mL
Salt Solution B	5 mL

Dissolve the anhydrous dextrose and sodium acetate in the solutions previously mixed, and adjust with 1 *N* sodium hydroxide to a pH of 6.8. Finally, add water to make 250 mL.

Acid-Hydrolyzed Casein Solution—Mix 100 g of vitamin-free casein with 500 mL of constant-boiling hydrochloric acid [approximately 20 percent (w/w) HCl], and reflux the mixture for 24 hours. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 *N* sodium hydroxide to a pH of 3.5 (±0.1), and add water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 hour, and filter. Repeat the treatment with activated charcoal if the filtrate does not appear straw-colored to colorless. Store under toluene in a refrigerator. Filter the solution if a precipitate forms upon storage.

Cystine-Tryptophan Solution—Suspend 4.0 g of *l*-cystine and 1.0 g of *l*-tryptophan (or 2.0 g of *dl*-tryptophan) in 700 to 800 mL of water, heat to 70° to 80°, and add the 20 percent (w/w) hydrochloric acid, dropwise, with stirring, until the solids are dissolved. Cool, and add water to make 1000 mL. Store under toluene in a refrigerator at a temperature not below 10°.

Adenine-Guanine-Uracil Solution—Dissolve 100 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 5.0 mL of the 20 percent (w/w) hydrochloric acid, cool, and add water to make 100 mL. Store under toluene in a refrigerator.

Riboflavin–Thiamine Hydrochloride–Biotin Solution—Prepare a solution containing, in each mL, 20 µg of riboflavin, 10 µg of thiamine hydrochloride, and 0.04 µg of biotin by dissolving crystalline riboflavin, crystalline thiamine hydrochloride, and crystalline biotin (free acid) in dilute glacial acetic acid (1 in 850). Store, protected from light, under toluene in a refrigerator.

Aminobenzoic Acid–Calcium Pantothenate–Pyridoxine Hydrochloride Solution—Prepare a solution of neutral 25 percent alcohol having a concentration of 10 µg of aminobenzoic acid, 20 µg of calcium pantothenate, and 40 µg of pyridoxine hydrochloride per mL. Store in a refrigerator.

Salt Solution A—Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid, and store under toluene.

Salt Solution B—Dissolve 10 g of magnesium sulfate, 500 mg of sodium chloride, 500 mg of ferrous sulfate, and 500 mg of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid, and store under toluene.

Stock Culture of *Lactobacillus plantarum*—Dissolve 2.0 g of water-soluble yeast extract in 100 mL of water, add 500 mg of anhydrous dextrose, 500 mg of anhydrous sodium acetate, and 1.5 g of agar, and heat the mixture with stirring, on a steam bath, until the agar dissolves. Add approximately 10-mL portions of the hot solution to test tubes, plug the tubes with cotton, sterilize for 15 minutes in an autoclave at 121° to 123°, and allow the tubes to cool in an upright position. Prepare stab cultures in three or more of the tubes, using a pure culture of *Lactobacillus plantarum*,* incubating for 16 to 24 hours at any selected temperature between 30° and 37°, but held constant to within ±0.5°, and finally store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for inoculum if the culture is more than 1 week old.

Culture Medium—To each of a series of test tubes containing 5.0 mL of the *Basal Medium Stock Solution* add 5.0 mL of water containing 1.0 µg of niacin. Plug the tubes with cotton, sterilize for 15 minutes in an autoclave at 121° to 123°, and cool.

Inoculum—Make a transfer of cells from the stock culture of *Lactobacillus plantarum* to a sterile tube containing 10 mL of culture medium. Incubate this culture for 16 to 24 hours at any selected temperature between 30° and 37°, but held constant to within ±0.5°. The cell suspension so obtained is the inoculum.

Calibration of Spectrophotometer—Add aseptically 1 mL of *Inoculum* to approximately 300 mL of *Culture Medium* containing 1 mL of *Standard Niacin Solution*. Incubate the inoculated medium for the same period and at the same temperature to be employed in the *Assay Procedure*.

Following the incubation period, centrifuge and wash the cells three times with approximately 50-mL portions of saline TS, and then resuspend the cells in about 25 mL of the saline solution.

Dry to constant weight a 10-mL portion, accurately measured, using a steam bath and completing the drying in vacuum at 100°, and calculate the dry weight of the cells, in mg per mL, corrected for the amount of sodium chloride present.

Dilute a second portion, accurately measured, of the saline cell suspension with the saline solution so that each mL contains a known quantity of cells equivalent to 500 µg on a dried basis. To test tubes add, in triplicate, 0.5 mL, 1.0 mL, 1.5 mL, 2.0 mL, 2.5 mL, 3.0 mL, 4.0 mL, and 5.0 mL, respectively, of this diluted cell suspension and 5.0 mL of *Basal Medium Stock Solution*, and make the volume in each tube to 10.0 mL with saline solution. Using as the blanks three similar tubes containing no cell suspension, measure the light transmittance of each tube under the same conditions to be employed in the assay. Plot the observations as the ordinate on cross-section paper against the cell content, expressed as mg of dry weight, as the abscissa.

Repeat this procedure at least twice for the spectrophotometer to be used in the assay. Draw the composite curve best representing the three or more individual curves relating transmittance to cell density for the spectrophotometer under the conditions of the assay.

Assay Procedure—Prepare standard niacin tubes as follows: To test tubes add, in duplicate, 0.0 mL, 0.5 mL, 1.0 mL, 1.5 mL, 2.0 mL, 2.5 mL, 3.0 mL, 3.5 mL, 4.0 mL, 4.5 mL, and 5.0 mL, respectively, of *Standard Niacin Solution*. To each tube add 5.0 mL of *Basal Medium Stock Solution* and water to make 10.0 mL.

Prepare tubes containing the material to be assayed as follows: To test tubes add, in duplicate, 1.0 mL, 2.0 mL, 3.0 mL, and 4.0 mL, respectively, of the test solution of the material to be assayed. To each tube add 5.0 mL of *Basal Medium Stock Solution* and water to make 10.0 mL. After mixing, plug the tubes with cotton or cover with caps, and sterilize in an autoclave at 121° to 123°. (Overheating the assay tubes may produce unsatisfactory results.) Cool, aseptically inoculate each tube with 1 drop of *Inoculum*, and incubate for 16 to 24 hours at any selected temperature between 30° and 37°, but held constant to within ±0.5°. Contamination of the assay tubes with any foreign organism invalidates the assay.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, to which 1 drop of a suitable antifoam agent solution may be added, and transfer to an optical container. After agitating its contents, place the container in a spectrophotometer that has been set at a specific wavelength between 540 nm and 660 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the reading remains constant for 30 seconds or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. If this transmittance reading corresponds to a dried cell weight greater than 600 µg per tube, or if there is evidence of contamination with a foreign microorganism, disregard the results of the assay.

Then with the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. Disregard the results of the assay if the difference between the transmittance observed at the highest level of the standard and that of the inoculated blank is less than the difference corresponding to a dried cell weight of 1.25 mg per tube.

Calculation—Prepare a standard curve of the niacin standard transmittances for each level of *Standard Niacin Solution* plotted against µg of niacin contained in the respective tubes. From this standard curve, determine by interpolation the niacin content of the test solution in each tube. Disregard transmittance values equivalent to less than 0.5 mL or more than 4.5 mL of *Standard Niacin Solution*. The niacin content of the test material is calculated from the average values obtained from not less than six tubes that do not vary by more than ±10 percent from the average. If the transmittance values of less than six tubes containing the test solution are within the range of the 0.5- to 4.5-mL levels of the niacin standard tubes, the data are insufficient to permit calculation of the concentration of niacin in the test material. Transmittance values of inoculated blank exceeding readings corresponding to dried cell weights of more than 600 µg per tube indicate the presence of an excessive amount of niacin in the *Basal Medium Stock Solution* and invalidate the assay.

Multiply the values obtained by 0.992 if the results are to be expressed as niacinamide.

* Pure cultures of *Lactobacillus plantarum* may be obtained, as number 8014, from the the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852.

⟨451⟩ NITRITE TITRATION

The following general method is provided for the determination of most of the Pharmacopeial sulfonamide drugs and their dosage forms, as well as of other Pharmacopeial drugs for which nitrite titration is particularly suitable.

Reference Standard—*USP Sulfanilamide Reference Standard*—Dry at 105° for 3 hours before using. Keep container tightly closed and protected from light.

Procedure—Weigh accurately about 500 mg in the case of a sulfonamide, or otherwise the quantity specified in the individual monograph, and transfer to a suitable open vessel. Add 20 mL of hydrochloric acid and 50 mL of water, stir until dissolved, cool to about 15°, and slowly titrate with 0.1 *M* sodium nitrite VS that previously has been standardized against USP Sulfanilamide RS.

Determine the end-point electrometrically, using suitable electrodes (platinum-calomel or platinum-platinum). Place the buret tip below the surface of the solution to eliminate air oxidation of

the sodium nitrite, and stir the solution gently, using a magnetic stirrer, without pulling a vortex of air under the surface, maintaining the temperature at about 15°. The titration may be carried out manually, or by means of an automatic titrator. In performing it manually, add the titrant until the titration is within 1 mL of the end-point, and then add it in 0.1-mL portions, allowing not less than 1 minute between additions. (The instrument needle deflects and then returns to approximately its original position until the end-point is reached.)

The weight, in mg, of the substance to which each mL of 0.1 *M* sodium nitrite VS is equivalent is as stated in the individual monograph.

For the assay of Tablets of the sulfonamides or other drugs, reduce not less than 20 tablets to a fine powder, weigh accurately a portion of the powder, equivalent to about 500 mg if a sulfonamide, or the quantity of drug specified in the individual monograph, and proceed as directed in the foregoing, beginning with "transfer to a suitable open vessel."

For the assay of Injections and other liquid forms where the nitrite titration is specified, pipet a portion, equivalent to about 500 mg if a sulfonamide, or the quantity of drug specified in the individual monograph, into a suitable open vessel, and proceed as directed in the foregoing, beginning with "Add 20 mL of hydrochloric acid."

⟨461⟩ NITROGEN DETERMINATION

Some alkaloids and other nitrogen-containing organic compounds fail to yield all of their nitrogen upon digestion with sulfuric acid; therefore these methods cannot be used for the determination of nitrogen in all organic compounds.

Method I

Nitrates and Nitrites Absent—Place about 1 g of the substance, accurately weighed, in a 500-mL Kjeldahl flask of hard borosilicate glass. The material to be tested, if solid or semisolid, may be wrapped in a sheet of nitrogen-free filter paper for convenience in transferring it to the flask. Add 10 g of powdered potassium sulfate or anhydrous sodium sulfate, 500 mg of powdered cupric sulfate, and 20 mL of sulfuric acid. Incline the flask at an angle of about 45°, and gently heat the mixture, keeping the temperature below the boiling point until frothing has ceased. Increase the heat until the acid boils briskly, and continue the heating until the solution has been clear green in color or almost colorless for 30 minutes. Allow to cool, add 150 mL of water, mix the contents of the flask, and again cool. Add cautiously 100 mL of sodium hydroxide solution (2 in 5), in such manner as to cause the solution to flow down the inner side of the flask to form a layer under the acid solution. Immediately add a few pieces of granulated zinc, and without delay connect the flask to a Kjeldahl connecting bulb (trap), previously attached to a condenser, the delivery tube from which dips beneath the surface of 100 mL of boric acid solution (1 in 25) contained in a conical flask or a wide-mouth bottle of about 500-mL capacity. Mix the contents of the Kjeldahl flask by gentle rotation, and distil until about four-fifths of the contents of the flask has distilled over. Add not less than 3 drops of methyl red–methylene blue TS to the contents of the receiving vessel, and determine the ammonia by titration with 0.5 *N* sulfuric acid VS. Perform a blank determination, and make any necessary correction. Each mL of 0.5 *N* sulfuric acid VS is equivalent to 7.003 mg of nitrogen.

When the nitrogen content of the substance is known to be low, the 0.5 *N* sulfuric acid VS may be replaced by 0.1 *N* sulfuric acid VS. Each mL of 0.1 *N* sulfuric acid VS is equivalent to 1.401 mg of nitrogen.

Nitrates and Nitrites Present—Place a quantity of the substance, accurately weighed, corresponding to about 150 mg of nitrogen, in a 500-mL Kjeldahl flask of hard borosilicate glass, and add 25 mL of sulfuric acid in which 1 g of salicylic acid previously has been dissolved. Mix the contents of the flask, and allow the mixture to stand for 30 minutes with frequent shaking. To the mixture add 5 g of powdered sodium thiosulfate, again mix, then add 500 mg of powdered cupric sulfate, and proceed as directed under *Nitrates and Nitrites Absent*, beginning with "Incline the flask at an angle of about 45°."

When the nitrogen content of the substance is known to exceed 10%, 500 mg to 1 g of benzoic acid may be added, prior to digestion, to facilitate the decomposition of the substance.

Method II

Apparatus—Select a unit of the general type known as a semimicro Kjeldahl apparatus, by which the nitrogen is first liberated by acid digestion and then transferred quantitatively to the titration vessel by steam distillation.

Procedure—Place an accurately weighed or measured quantity of the material, equivalent to 2 to 3 mg of nitrogen, in the digestion flask of the apparatus. Add 1 g of a powdered mixture of potassium sulfate and cupric sulfate (10:1), and wash down any adhering material from the neck of the flask with a fine jet of water. Add 7 mL of sulfuric acid, allowing it to rinse down the wall of the flask, then, while swirling the flask, add 1 mL of 30 percent hydrogen peroxide cautiously down the side of the flask. (Do not add hydrogen peroxide during the digestion.)

Heat the flask over a free flame or an electric heater until the solution has a clear blue color and the sides of the flask are free from carbonaceous material. Cautiously add to the digestion mixture 20 mL of water, cool the solution, and arrange for steam distillation. Add through a funnel 30 mL of sodium hydroxide solution (2 in 5), rinse the funnel with 10 mL of water, tightly close the apparatus, and begin the distillation with steam immediately. Receive the distillate in 15 mL of boric acid solution (1 in 25), to which has been added 3 drops of methyl red–methylene blue TS and sufficient water to cover the end of the condensing tube. Continue the distillation until the distillate measures 80 to 100 mL. Remove the absorption flask, rinse the end of the condensing tube with a small quantity of water, and titrate the distillate with 0.01 *N* sulfuric acid VS. Perform a blank determination, and make any necessary correction. Each mL of 0.01 *N* acid VS is equivalent to 140.1 μg of nitrogen.

When a quantity of material containing more than 2 to 3 mg of nitrogen is taken, 0.02 *N* or 0.1 *N* sulfuric acid may be employed, provided that at least 15 mL is required for the titration. If the total dry weight of material taken is greater than 100 mg, increase proportionately the quantities of sulfuric acid and sodium hydroxide.

⟨466⟩ ORDINARY IMPURITIES

This test, where called for in the individual monograph, is provided to evaluate the impurity profile of an article. See *Chromatography* ⟨621⟩ for a general discussion of the thin-layer chromatographic technique. Unless otherwise specified in the individual monograph, use the following method.

Test Solution—Prepare, in the solvent specified in the monograph, a solution of the substance under test having an accurately known final concentration of about 10 mg per mL. [NOTE—Heat or sonication may be used to dissolve the drug substance where use of such does not adversely affect the compound.]

Standard Solutions—Prepare, in the solvent specified in the monograph, solutions of the USP Reference Standard or designated substance having accurately known concentrations of 0.01 mg per mL, 0.05 mg per mL, 0.1 mg per mL, and 0.2 mg per mL. [NOTE—Heat or sonication may be used to dissolve the drug substance where use of such does not adversely affect the compound.]

Procedure—Use a thin-layer chromatographic plate coated with a 0.25-mm layer of chromatographic silica gel mixture, and the *Eluant* specified in the monograph. Apply equal volumes (20 μL) of the *Test Solution* and *Standard Solutions* to the plate, using a stream of nitrogen to dry the spots.

Allow the chromatogram to develop in a pre-equilibrated chamber until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the chamber, and air-dry. View the plate using the visualization technique(s) specified. Locate any spots other than the principal spot, in the chromatogram of the *Test Solution*, and determine their relative intensities by comparison with the chromatograms of the appropriate *Standard Solutions*. The total of any ordinary impurities observed does not exceed 2.0%, unless otherwise specified in the individual monograph.

KEY FOR VISUALIZATION TECHNIQUES

(1) Use ultraviolet light at 254 nm and 366 nm.
(2) Use Iodoplatinate TS.
(3) Solution A—Mix 850 mg of bismuth subnitrate with 40 mL of water and 10 mL of glacial acetic acid.

Solution B—Dissolve 8 g of potassium iodide in 20 mL of water. Mix A and B together to obtain a Stock Solution which can be stored for several months in a dark bottle. Mix 10 mL of the Stock Solution with 20 mL of glacial acetic acid, and dilute with water to make 100 mL, to prepare the spray reagent.

(4) *Ninhydrin Spray*—Dissolve 200 mg of ninhydrin in 100 mL of alcohol. Heat the plate after spraying.
(5) *Acid Spray*—In an ice bath, add slowly and cautiously, with stirring, 10 mL of sulfuric acid to 90 mL of alcohol. Spray the plate, and heat until charred.
(6) *Acid–Dichromate Spray*—Add sufficient potassium dichromate to 100 mL of sulfuric acid to make a saturated solution. Spray the plate, and heat until charred.
(7) *Vanillin*—Dissolve 1 g of vanillin in 100 mL of sulfuric acid.
(8) *Chloramine T–Trichloroacetic Acid*—Mix 10 mL of a 3% aqueous solution of chloramine T with 40 mL of a 25% alcoholic solution of trichloroacetic acid. Prepare immediately before use.
(9) *Folin-C*—Add 10 g of sodium tungstate and 2.5 g of sodium molybdate to 70 mL of water, add 5 mL of 85% phosphoric acid and 10 mL of 36% hydrochloric acid, and reflux this solution for 10 hours.
(10) *$KMnO_4$*—Dissolve 100 mg of Potassium Permanganate in 100 mL of water.
(11) *DAB*—Mix 1 g of *p*-dimethylaminobenzaldehyde in 100 mL of 0.6 *N* hydrochloric acid.
(12) *DAC*—Mix 100 mg of *p*-dimethylaminocinnamaldehyde in 100 mL of 1 *N* hydrochloric acid.
(13) *Ferricyanide*—Mix equal volumes of a 1% ferric chloride solution and a 1% potassium ferricyanide solution. Use immediately.
(14) *Fast Blue B*—Reagent A—Dissolve 500 mg of Fast Blue B Salt in 100 mL of water.

Reagent B—0.1 *N* sodium hydroxide.

Spray first with A, then with B.

(15) *Alkaline Ferric Cyanide*—Dilute 1.5 mL of a 1% potassium ferricyanide solution with water to 20 mL, and add 10 mL of 15% sodium hydroxide solution.
(16) *Iodine Spray*—Prepare a 0.5% solution of iodine in chloroform.
(17) Expose the plate for 10 minutes to iodine vapors in a pre-equilibrated closed chamber, on the bottom of which there are iodine crystals.
(18) Solution A—Dissolve 0.5 g of potassium iodide in 50 mL of water.

Solution B—Prepare a solution of 0.5 g of soluble starch in 50 mL of hot water.

Just prior to use, mix equal volumes of Solution A and Solution B.

(19) *PTSS*—Dissolve 20 g of *p*-toluenesulfonic acid in 100 mL of alcohol, spray the plate, dry for 15 minutes at 110°, and view under ultraviolet light at 366 nm.
(20) *o-Tolidine Spray*—Dissolve 160 mg of *o*-tolidine in 30 mL of glacial acetic acid, dilute with water to make 500 mL, add 1 g of potassium iodide, and mix until the potassium iodide has dissolved.
(21) Mix 3 mL of chloroplatinic acid solution (1 in 10) with 97 mL of water, followed by the addition of 100 mL of potassium iodide solution (6 in 100) to prepare the spray reagent.
(22) *Iodine-Methanol Spray*—Prepare a mixture of iodine TS and methanol (1:1).

⟨468⟩ OXYGEN DETERMINATION

For the measurement of oxygen concentrations in air or mixtures of oxygen with air or inert gas diluents, employ an instrument utilizing the variations of electric current produced by the interaction of oxygen with an electrochemical cell to display the oxygen strength of a confined sample or an in-line flow of the gas. This current generates a signal proportional to the oxygen concentration which is displayed on a meter.

The instrument is basically maintenance-free but is to be periodically calibrated. When the monitor can no longer be calibrated with the sensor adjustment, replace or regenerate the cell.

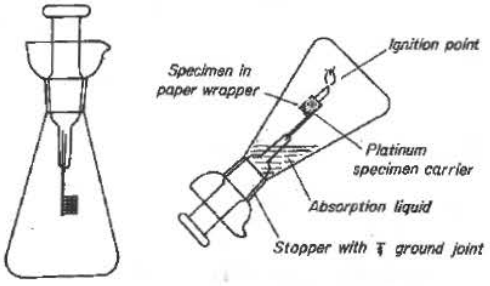
⟨471⟩ OXYGEN FLASK COMBUSTION

The oxygen flask combustion procedure is provided as the preparatory step in the determination of bromine, chlorine, iodine, selenium, and sulfur in some Pharmacopeial articles. Combustion of the material under test (usually organic) yields water-soluble inorganic products, which are analyzed for specific elements as directed in the individual monograph or general chapter.

The caution statement given under *Procedure* covers minimum safety precautions only, and serves to emphasize the need for exceptional care throughout.

Apparatus—The apparatus[1] consists of a heavy-walled conical, deeply lipped or cupped 500-mL flask (unless a larger flask is specified), fitted with a ground-glass stopper to which is fused a test specimen carrier consisting of heavy-gauge platinum wire and a piece of welded platinum gauze measuring about 1.5 × 2 cm.

Procedure—[CAUTION—The analyst should wear safety glasses and use a suitable safety shield between himself and the apparatus. Exercise care to ensure that the flask is scrupulously clean and free from even traces of organic solvents.] Weigh the substance, if a solid, on a piece of halide-free filter paper measuring about 4 cm square, and fold the paper to enclose it. Liquid substances are weighed in tared capsules, cellulose acetate capsules[2] being used for liquids in volumes not exceeding 200 µL, and gelatin capsules being satisfactory for use for larger volumes. [NOTE—Gelatin capsules may contain significant amounts of combined halide or sulfur. If such capsules are used, perform a blank determination, and make any necessary correction.] Place the specimen, together with a filter paper fuse-strip, in the platinum gauze specimen holder. Place the absorbing liquid specified in the individual monograph or general chapter in the flask, moisten the joint of the stopper with water, and flush the air from the flask with a stream of rapidly flowing oxygen, swirling the liquid to favor its taking up oxygen. [NOTE—Saturation of the liquid with oxygen is essential for the successful performance of the combustion procedure.] Ignite the fuse-strip by suitable means. If the strip is ignited outside the flask, immediately plunge the specimen holder into the flask, invert the flask so that the absorption solution makes a seal around the stopper, and hold the stopper firmly in place. If the ignition is carried out in a closed system, the inversion of the flask may be omitted. After combustion is complete, shake the flask vigor-



Apparatus for Oxygen Flask Combustion

[1] A suitable apparatus [Catalog Nos. 6513-C20 (500-mL capacity) and 6513-C30 (1000-mL capacity)] and suitable capsules [Catalog Nos. 6513-C80 (100 capsules) and 6513-C82 (1000 capsules)] are obtainable from Arthur H. Thomas Co., P. O. Box 779, Philadelphia, PA 19105.

[2] A suitable apparatus [Catalog Nos. 6513-C20 (500-mL capacity) and 6513-C30 (1000-mL capacity)] and suitable capsules [Catalog Nos. 6513-C80 (100 capsules) and 6513-C82 (1000 capsules)] are obtainable from Arthur H. Thomas Co., P. O. Box 779, Philadelphia, PA 19105.

ously, and allow to stand for not less than 10 minutes with intermittent shaking. Then proceed as directed in the individual monograph or general chapter.

⟨475⟩ PENICILLIN G DETERMINATION

The following procedure is used to determine the content of penicillin G moiety in an antibiotic drug substance when such a requirement is specified in the individual monograph.

0.05 *M* Phosphate Buffer, pH 6—Dissolve 6.8 g of monobasic potassium phosphate in 900 mL of water, adjust with 1 *N* sodium hydroxide to a pH of 6.00, dilute with water to 1000 mL, and mix.

Mobile Phase—Prepare a mixture of *0.05 M Phosphate Buffer, pH 6* and acetonitrile (4:1), filter through a membrane filter of 5-μm or finer porosity, and degas.

Standard Preparation—Transfer about 80 mg of USP Penicillin G Potassium RS, accurately weighed, to a 100-mL volumetric flask, add about 50 mL of *Mobile Phase*, swirl to dissolve, dilute with *Mobile Phase* to volume, and mix.

Test Preparation—Unless otherwise directed in the individual monograph, proceed with the specimen under test as directed under *Standard Preparation.*

System Suitability Preparation—Prepare a solution of penicillin V potassium in *Mobile Phase* containing about 1 mg per mL. Mix equal volumes of this solution and the *Standard Preparation.*

Chromatographic System (see *Chromatography* ⟨621⟩)—The liquid chromatograph is equipped with a 225-nm detector and a 4-mm × 30-cm column that contains 10-μm packing L1. The flow rate is about 2 mL per minute. Chromatograph the *Standard Preparation* and the *System Suitability Preparation,* and record the peak responses as directed under *Procedure:* the column efficiency determined from the analyte peak is not less than 600 theoretical plates, the resolution, *R*, between the penicillin G and penicillin V peaks is not less than 2.0, and the relative standard deviation for replicate injections of the *Standard Preparation* is not more than 1.0%.

Procedure—Separately inject equal volumes (about 10 μL) of the *Standard Preparation*, the *Test Preparation*, and the *System Suitability Preparation* into the chromatograph, record the chromatograms, and measure the responses for the major peaks. The relative retention times are about 0.7 for penicillin G and 1.0 for penicillin V. Calculate the percentage of penicillin G ($C_{16}H_{18}N_2O_4S$) in the specimen under test by the formula:

$$(G_S W_S / W_U)(r_U / r_S),$$

in which G_S is the designated penicillin G content, in percentage, of USP Penicillin G Potassium RS, W_S and W_U are the amounts, in mg, of USP Penicillin G Potassium RS and test specimen taken, respectively, and r_U and r_S are the responses of the *Test Preparation* and the *Standard Preparation* peaks, respectively.

⟨481⟩ RIBOFLAVIN ASSAY

The following procedure is suitable for preparations in which riboflavin is a constituent of a mixture of several ingredients. In employing it, keep the pH of solutions below 7.0, and protect the solutions from direct sunlight at all stages.

Reference Standard—*USP Riboflavin Reference Standard*—Dry at 105° for 2 hours before using.

Standard Riboflavin Stock Solution—To 50.0 mg of USP Riboflavin RS, previously dried and stored protected from light in a desiccator over phosphorus pentoxide, add about 300 mL of 0.02 *N* acetic acid, and heat the mixture on a steam bath, with frequent agitation, until the riboflavin has dissolved. Then cool, add 0.02 *N* acetic acid to make 500 mL, and mix. Store under toluene in a refrigerator.

Dilute an accurately measured portion of this solution, using 0.02 *N* acetic acid, to a concentration of 10.0 μg of the dried USP Riboflavin RS per mL, to obtain the *Standard Riboflavin Stock Solution.* Store under toluene in a refrigerator.

Standard Preparation—Dilute 10.0 mL of *Standard Riboflavin Stock Solution* with water in a 100-mL volumetric flask to volume, and mix. Each mL represents 1.0 μg of USP Riboflavin RS. Prepare fresh *Standard Preparation* for each assay.

Assay Preparation—Place an amount of the material to be assayed in a flask of suitable size, and add a volume of 0.1 *N* hydrochloric acid equal in mL to not less than 10 times the dry weight of the material in g, but the resulting solution shall contain not more than 100 μg of riboflavin per mL. If the material is not readily soluble, comminute it so that it may be evenly dispersed in the liquid. Then agitate vigorously, and wash down the sides of the flask with 0.1 *N* hydrochloric acid.

Heat the mixture in an autoclave at 121° to 123° for 30 minutes, and cool. If clumping occurs, agitate the mixture until the particles are evenly dispersed. Adjust the mixture, with vigorous agitation, to a pH of 6.0 to 6.5 with sodium hydroxide solution,* then add hydrochloric acid solution* immediately until no further precipitation occurs (usually at a pH of approximately 4.5, the isoelectric point of many of the proteins present). Dilute the mixture with water to make a measured volume that contains about 0.11 μg of riboflavin in each mL, and filter through paper known not to adsorb riboflavin. To an aliquot of the filtrate add, with vigorous agitation, sodium hydroxide solution* to produce a pH of 6.6 to 6.8, dilute the solution with water to make a final measured volume that contains approximately 0.1 μg of riboflavin in each mL, and if cloudiness occurs, filter again.

Procedure—To each of four or more tubes (or reaction vessels) add 10.0 mL of the *Assay Preparation.* To each of two or more of these tubes add 1.0 mL of the *Standard Preparation*, and mix, and to each of two or more of the remaining tubes add 1.0 mL of water, and mix. To each tube add 1.0 mL of glacial acetic acid, mix, then add, with mixing, 0.50 mL of potassium permanganate solution (1 in 25), and allow to stand for 2 minutes. To each tube add, with mixing, 0.50 mL of hydrogen peroxide solution, whereupon the permanganate color is destroyed within 10 seconds. Shake the tubes vigorously until excess oxygen is expelled. Remove any gas bubbles remaining on the sides of the tubes after foaming has ceased, by tipping the tubes so that the solution flows slowly from end to end.

In a suitable fluorophotometer, having an input filter of narrow transmittance range with a maximum at about 440 nm and an output filter of narrow transmittance range with a maximum at about 530 nm, measure the fluorescence of all tubes, designating the average reading from the tubes containing only the *Assay Preparation* as I_U and the average from the tubes containing both the *Assay Preparation* and the *Standard Preparation* as I_S. Then to each of one or more tubes of each kind add, with mixing, 20 mg of sodium hydrosulfite, and within 5 seconds again measure the fluorescence, designating the average reading as I_B.

Calculation—Calculate the quantity, in mg, of $C_{17}H_{20}N_4O_6$ in each mL of the *Assay Preparation* taken by the formula:

$$0.0001(I_U - I_B)/(I_S - I_U).$$

Calculate the quantity, in mg, of $C_{17}H_{20}N_4O_6$ in each capsule or tablet.

⟨501⟩ SALTS OF ORGANIC NITROGENOUS BASES

Standard Preparation—Unless otherwise directed, prepare a solution in dilute sulfuric acid (1 in 70) containing, in each mL, about 500 μg of the specified USP Reference Standard, calculated on the anhydrous basis, and accurately weighed.

Assay Preparation—If the dosage form is a tablet, weigh and finely powder not less than 20 tablets, weigh accurately a portion of the powder, equivalent to about 25 mg of the active ingredient, and transfer to a 125-mL separator; or, if the dosage form is a liquid, transfer a volume of it, equivalent to about 25 mg of the active ingredient and accurately measured, to a 125-mL separator. Then to the separator add 20 mL of dilute sulfuric acid (1 in 350), and shake vigorously for 5 minutes. Add 20 mL of

* The concentrations of the hydrochloric acid and sodium hydroxide solutions used are not stated in each instance because these concentrations may be varied depending upon the amount of material taken for assay, volume of test solution, and buffering effect of material.

ether, shake carefully, and filter the acid phase into a second 125-mL separator. Shake the ether phase with two 10-mL portions of dilute sulfuric acid (1 in 350), filter each portion of acid into the second separator, and discard the ether. To the acid extract add 10 mL of sodium hydroxide TS and 50 mL of ether, shake carefully, and transfer the aqueous phase to a third 125-mL separator containing 50 mL of ether. Shake the third separator carefully, and discard the aqueous phase. Wash the two ether solutions, in succession, with a single 20-mL portion of water, and discard the water. Extract each of the two ether solutions with 20-, 20-, and 5-mL portions of dilute sulfuric acid (1 in 70), in the order listed, but each time extract first the ether solution in the third separator and then that in the second separator. Combine the acid extracts in a 50-mL volumetric flask, dilute with the acid to volume, and mix.

NOTE—Hexane or heptane may be substituted for ether if the distribution ratio of the nitrogenous base between water and hexane, or between water and heptane, favors complete extraction by the organic phase.

Procedure—Unless otherwise directed, dilute 5.0 mL each of the *Standard Preparation* and the *Assay Preparation* with dilute sulfuric acid (1 in 70) to 100.0 mL, and determine the absorbance of each solution at the specified wavelength, using dilute sulfuric acid (1 in 70) as the blank. Designate the absorbance of the solution from the *Standard Preparation* as A_S and that from the *Assay Preparation* as A_U, and calculate the result of the assay as directed in the individual monograph.

⟨511⟩ SINGLE-STEROID ASSAY

In the following procedure, the steroid to be assayed is separated from related foreign steroids and excipients by thin-layer chromatography and determined following recovery from the chromatogram.

Preparation of the Plate—Prepare a slurry from 30 g of chromatographic silica gel with a suitable fluorescing substance by the gradual addition, with mixing, of about 65 mL of a mixture of water and alcohol (5:2). Transfer the slurry to a clean, 20- × 20-cm plate, spread to make a uniform layer 250 μm thick, and allow to dry at room temperature for 15 minutes. Heat the plate at 105° for 1 hour, and store in a desiccator.

Solvent A—Mix methylene chloride with methanol (180:16).

Solvent B—Mix chloroform with acetone (4:1).

Standard Preparation—Dissolve in a mixture of equal volumes of chloroform and alcohol a suitable quantity of the USP Reference Standard specified in the individual monograph, previously dried as directed in the individual monograph, and accurately weighed, to obtain a solution having a known concentration of about 2 mg per mL.

Assay Preparation—Prepare as directed in the individual monograph.

Procedure—Divide the area of the chromatographic plate into three equal sections, the left and right sections to be used for the *Assay Preparation* and the *Standard Preparation*, respectively, and the center section for the blank. Apply 200 μL each of the *Assay Preparation* and the *Standard Preparation* as streaks 2.5 cm from the bottom of the appropriate section of the plate. Dry the solution as it is being applied, with the aid of a stream of air. Using the *Solvent* specified in the individual monograph, develop the chromatogram in a suitable chamber, previously equilibrated and lined with absorbent paper, until the solvent front has moved 15 cm above the initial streaks.

Remove the plate, evaporate the solvent, and locate the principal band occupied by the *Standard Preparation* by viewing under ultraviolet light. Mark this band, as well as corresponding bands in the *Assay Preparation* and blank sections of the plate. Remove the silica gel from each band separately, either by scraping onto glazed weighing papers or by using a suitable vacuum collecting device, and transfer it to a glass-stoppered, 50-mL centrifuge tube. To each tube add 25.0 mL of alcohol, and shake for not less than 2 minutes. Centrifuge the tubes for 5 minutes, pipet 20 mL of the supernatant liquid from each tube into a glass-stoppered, 50-mL conical flask, add 2.0 mL of a solution prepared by dissolving 50 mg of blue tetrazolium in 10 mL of methanol, and mix. Proceed as directed for *Procedure* under *Assay for Steroids* ⟨351⟩, beginning with "Then to each flask."

⟨521⟩ SULFONAMIDES

Identification of Individual Sulfonamides in Mixed Sulfonamides

NOTE—The following instructions for preparations and procedure are applicable to all sulfonamides except sulfadiazine. When testing for sulfadiazine proceed in the same manner, except to use sulfadiazine preparations having one-half the designated concentration, and apply twice the designated volumes of sulfadiazine preparations to the chromatographic plates.

Standard Preparation—Transfer a quantity of the pertinent USP Reference Standard to a suitable glass-stoppered, conical flask, dissolve in methanol to obtain a solution having a concentration of about 2 mg per mL, and mix. A separate *Standard Preparation* is required for each sulfonamide present in mixed sulfonamides.

Test Preparation—Transfer a portion of the thoroughly mixed suspension or finely powdered tablets, equivalent to about 100 mg of each sulfonamide, to a 50-mL volumetric flask containing 10 mL of ammonia TS, and swirl. Add methanol to volume, mix, filter, and use the filtrate in the *Procedure*.

Preparation of Chromatographic Plates—Prepare three identical chromatographic plates according to the following directions. On a suitable thin-layer chromatographic plate (see *Chromatography* ⟨621⟩), coated with a 0.25-mm layer of chromatographic silica gel mixture, apply separately, and 2 cm apart along a spotting line 1.5 cm from the bottom of the plate and parallel to it, 2 μL of each *Standard Preparation* and 2 μL of the *Test Preparation*. On another spot, 2 cm along the spotting line from the application of the *Test Preparation*, apply, successively, 2 μL of each *Standard Preparation* to obtain a mixed standard. Dry the spots immediately with the aid of a stream of nitrogen.

Procedure—Prepare a chromatographic chamber lined with filter paper and containing a solvent system consisting of ethyl acetate, methanol, and a 1 in 4 aqueous solution of ammonium hydroxide (17:6:5), and allow to equilibrate for 1 hour. Similarly prepare a second chamber to contain a solvent system consisting of solvent hexane, chloroform, and butyl alcohol (1:1:1), and a third chamber to contain a solvent system consisting of chloroform and methanol (95:5). Place one prepared chromatographic plate in each equilibrated chamber, and develop the chromatograms until the solvent front has moved about three-fourths of the length of each plate. Remove each plate from its developing chamber, mark the solvent front, and allow the solvent to evaporate. Locate the spots on the plates by viewing under short-wavelength ultraviolet light. Spray the plates with a 1 in 100 solution of *p*-dimethylaminobenzaldehyde in dilute hydrochloric acid (1 in 20), and heat at 110° for 5 minutes or until bright yellow spots become visible. The R_f values of the yellow spots obtained from each *Test Preparation* correspond to those obtained from the mixed *Standard Preparations* on the respective plates. The individual sulfonamides may be identified by comparison of the R_f values of the yellow spots obtained from the *Test Preparations* and individual *Standard Preparations* on the respective plates.

Determination of Individual Sulfonamides in Mixed Sulfonamides

Standard Preparation—A separate *Standard Preparation* is required for each sulfonamide being determined. Transfer about 50 mg, accurately weighed, of the pertinent USP Reference Standard to a 50-mL volumetric flask containing 1.5 mL of ammonium hydroxide, add methanol, dissolve in methanol, dilute with methanol to volume, and mix. Transfer 1.0 mL of this solution to a 100-mL volumetric flask, add dilute hydrochloric acid (1 in 100) to volume, and mix. [NOTE—Retain the methanol solutions for the *Mixed Standard Preparation*. The methanol solutions are stable for at least 1 week, and the acid solutions for at least 1 month.]

Mixed Standard Preparation—Transfer 1.0 mL of each methanol solution, prepared as required for each *Standard Preparation*, to a small glass-stoppered flask, and mix. [NOTE—This Standard is used to identify the components of the *Assay Preparation* on the chromatogram.]

Assay Preparation—Prepare as directed in the individual monograph.

Procedure—Prepare the necessary number of chromatographic sheets (Whatman No. 1 filter paper, or equivalent), about 20 × 20 cm in size, by drawing a pencil line parallel to and 2.5 cm from one edge of the paper. Mark the line at points 2.5 and 5 cm from each edge of the paper. Impregnate the paper by dipping it in the immobile solvent (prepared fresh by dissolving 30 mL of redistilled formamide in 70 mL of acetone) for 30 seconds. Remove the paper, drain for 10 seconds, and blot between filter paper. Place the impregnated paper on dry filter paper, and air-dry for 3 to 5 minutes. With a micropipet, and with repeated applications, streak 100 µL of the *Assay Preparation* along the starting line, applying the volume in five streaks of about 20 µL each and evaporating the solvent with a gentle stream of nitrogen between applications. [NOTE—Make the streak as narrow as possible along the starting line, and keep within the 5-cm border.] Rinse the tip of the pipet with a drop of methanol–ammonia TS mixture (9:1), and then streak the rinse along the starting line between the 5- and 2.5-cm points at the right edge. Repeat the rinsing with two additional drops, and then blow out the pipet.

Apply 10 µL of the *Mixed Standard Preparation* at the mark 2.5 cm from the left edge.

Place 50 mL of methylene chloride (mobile solvent) in a tray in a 23- × 23- × 7.5-cm chromatographic chamber arranged for ascending chromatography (see *Chromatography* ⟨621⟩), and allow the chamber to equilibrate for about 15 minutes. Remove the cover, place from 7 to 10 mL of water in a second tray, and without delay, suspend the prepared chromatographic paper sheet so that it dips into the mobile solvent. Cover and seal the chamber, and allow the chromatogram to develop for 1 hour. Remove the paper from the chamber, and allow to air-dry for 5 minutes. Place the chromatogram on a dry sheet of filter paper, and view it under short-wavelength ultraviolet light. [NOTE—Conduct the following identification and marking without delay to avoid excessive exposure of the sulfonamide spots to ultraviolet irradiation.] Identify and mark the respective spots by matching R_f values with those of the spots produced by the *Mixed Standard Preparation*. [NOTE—Sulfadiazine and sulfamerazine are chromatographed with increasing R_f, respectively.]

Cut the marked zones from the paper, cut each zone into five or six pieces, and place the pieces from each spot in separate, glass-stoppered, 50-mL flasks. Add 20.0 mL of dilute hydrochloric acid (1 in 100) to each flask, and allow to stand for about 30 minutes, swirling each flask at least five times during this period. Filter the solutions through dry glass wool into separate test tubes, discarding the first 5 mL of filtrate. Transfer 5.0 mL of the subsequent filtrate from each solution into separate 10-mL volumetric flasks. Transfer 3.0 mL of each required *Standard Preparation* into separate, 10-mL volumetric flasks. To each flask, and to a blank flask containing 5 mL of dilute hydrochloric acid (1 in 100), add 1.0 mL of sodium nitrite solution (1 in 1000) and 0.10 mL of hydrochloric acid, and allow to stand for 5 minutes with frequent swirling. To each flask add 1.0 mL of ammonium sulfamate solution (1 in 200), and allow to stand for 5 minutes, swirling frequently. Finally, to each flask add 1.0 mL of freshly prepared *N*-(1-naphthyl)ethylenediamine dihydrochloride solution (1 in 1000), mix, dilute with water to volume, and mix. Allow each solution to stand between 15 and 60 minutes, and then concomitantly determine the absorbances of the solutions, in 1-cm cells, recording the spectra from 440 to 700 nm, with a suitable spectrophotometer, using the blank to set the instrument. Draw a baseline, and determine the corrected absorbance for each solution at the wavelength of maximum absorbance at about 545 nm.

Calculate the concentration, in mg per mL, of each sulfonamide in the *Assay Preparation* by the formula:

$$0.12C(A_U/A_S),$$

in which C is the concentration, in µg per mL, of the pertinent USP Reference Standard in the *Standard Preparation*, A_U is the corrected absorbance of the *Assay Preparation*, and A_S is the corrected absorbance of the pertinent *Standard Preparation*. From the concentration of the *Assay Preparation* thus determined, and applying appropriate dilution factors, calculate the percentage of sulfonamide in the specimen taken.

⟨531⟩ THIAMINE ASSAY

Reference standard—*USP Thiamine Hydrochloride Reference Standard*—Do not dry; determine the water content titrimetrically at the time of use.

The following procedure is provided for the determination of thiamine as an ingredient of Pharmacopeial preparations containing other active constituents.

Special Solutions and Solvents—

POTASSIUM FERRICYANIDE SOLUTION—Dissolve 1.0 g of potassium ferricyanide in water to make 100 mL. Prepare fresh on the day of use.

OXIDIZING REAGENT—Mix 4.0 mL of *Potassium Ferricyanide Solution* with sufficient 3.5 *N* sodium hydroxide to make 100 mL. Use this solution within 4 hours.

QUININE SULFATE STOCK SOLUTION—Dissolve 10 mg of quinine sulfate in 0.1 *N* sulfuric acid to make 1000 mL. Preserve this solution, protected from light, in a refrigerator.

QUININE SULFATE STANDARD SOLUTION—Dilute 0.1 *N* sulfuric acid with *Quinine Sulfate Stock Solution* (39:1). This solution fluoresces to approximately the same degree as the thiochrome obtained from 1 µg of thiamine hydrochloride and is used to correct the fluorometer at frequent intervals for variation in sensitivity from reading to reading within an assay. Prepare this solution fresh on the day of use.

Standard Thiamine Hydrochloride Stock Solution—Transfer about 25 mg of USP Thiamine Hydrochloride RS, accurately weighed, to a 1000-mL volumetric flask. Dissolve the weighed Standard in about 300 mL of dilute alcohol solution (1 in 5) adjusted with 3 *N* hydrochloric acid to a pH of 4.0, and add the acidified, dilute alcohol to volume. Store in a light-resistant container, in a refrigerator. Prepare this stock solution fresh each month.

Standard Preparation—Dilute a portion of *Standard Thiamine Hydrochloride Stock Solution* quantitatively and stepwise with 0.2 *N* hydrochloric acid to obtain the *Standard Preparation*, each mL of which represents 0.2 µg of USP Thiamine Hydrochloride RS.

Assay Preparation—Place in a suitable volumetric flask sufficient of the material to be assayed, accurately weighed or measured by volume as directed, such that when diluted to volume with 0.2 *N* hydrochloric acid, the resulting solution will contain about 100 µg of thiamine hydrochloride (or mononitrate) per mL. If the sample is difficultly soluble, the solution may be heated on a steam bath, and then cooled and diluted with the acid to volume. Dilute 5 mL of this solution, quantitatively and stepwise, using 0.2 *N* hydrochloric acid, to an estimated concentration of 0.2 µg of thiamine hydrochloride (or mononitrate) per mL.

Procedure—Into each of three or more tubes (or other suitable vessels), of about 40-mL capacity, pipet 5 mL of *Standard Preparation*. To each of two of these tubes add rapidly (within 1 to 2 seconds), with mixing, 3.0 mL of *Oxidizing Reagent*, and within 30 seconds add 20.0 mL of isobutyl alcohol, then mix vigorously for 90 seconds by shaking the capped tubes manually, or by bubbling a stream of air through the mixture. Prepare a blank in the remaining tube of the standard by substituting for the *Oxidizing Reagent* an equal volume of 3.5 *N* sodium hydroxide and proceeding in the same manner.

Into each of three or more similar tubes pipet 5 mL of the *Assay Preparation*. Treat these tubes in the same manner as directed for the tubes containing the *Standard Preparation*.

Into each of the six tubes pipet 2 mL of dehydrated alcohol, swirl for a few seconds, allow the phases to separate, and decant or draw off about 10 mL of the clear, supernatant isobutyl alcohol solution into standardized cells, then measure the fluorescence in a suitable fluorometer, having an input filter of narrow transmittance range with a maximum at about 365 nm and an output filter of narrow transmittance range with a maximum at about 435 nm.

Calculation—The number of µg of $C_{12}H_{17}ClN_4OS.HCl$ in each 5 mL of the *Assay Preparation* is given by the formula:

$$(A - b)/(S - d),$$

in which A and S are the average fluorometer readings of the portions of the *Assay Preparation* and the *Standard Preparation*

treated with *Oxidizing Reagent*, respectively, and *b* and *d* are the readings for the blanks of the *Assay Preparation* and the *Standard Preparation*, respectively.

Calculate the quantity, in mg, of thiamine hydrochloride ($C_{12}H_{17}ClN_4OS.HCl$) in the assay material on the basis of the aliquots taken. Where indicated, the quantity, in mg, of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) may be calculated by multiplying the quantity of $C_{12}H_{17}ClN_4OS.HCl$ found by 0.9706.

⟨541⟩ TITRIMETRY

Direct Titrations—Direct titration is the treatment of a soluble substance, contained in solution in a suitable vessel (the titrate), with an appropriate standardized solution (the titrant), the end-point being determined instrumentally or visually with the aid of a suitable indicator.

The titrant is added from a suitable buret and is so chosen, with respect to its strength (normality), that the volume added is between 30% and 100% of the rated capacity of the buret. [NOTE—Where less than 10 mL of titrant is required, a suitable microburet is to be used.] The end-point is approached directly but cautiously, and finally the titrant is added dropwise from the buret in order that the final drop added will not over run the end-point. The quantity of the substance being titrated may be calculated from the volume and the normality or molarity factor of the titrant and the equivalence factor for the substance given in the individual monograph.

Residual Titrations—Some Pharmacopeial assays require the addition of a measured volume of a volumetric solution, in excess of the amount actually needed to react with the substance being assayed, the excess of this solution then being titrated with a second volumetric solution. This constitutes a residual titration and is known also as a "back titration." The quantity of the substance being titrated may be calculated from the difference between the volume of the volumetric solution originally added and that consumed by the titrant in the back titration, due allowance being made for the respective normality or molarity factors of the two solutions, and the equivalence factor for the substance given in the individual monograph.

Chelometric Titrations—Simple, direct titrations of some polyvalent cations are possible by the use of reagents with which the cations form complexes. The titration of the calcium ion by this means is particularly advantageous, in comparison to the oxalate precipitation method previously used for Pharmacopeial purposes. The success of complexometry depends in large measure upon the indicator chosen. Often the color change of an indicator can be improved by the addition of a screening agent.

Titrations in Nonaqueous Solvents—Acids and bases have long been defined as substances that furnish, when dissolved in water, hydrogen, and hydroxyl ions, respectively. This definition, introduced by Arrhenius, fails to recognize the fact that properties characteristic of acids or bases may be developed also in other solvents. A more generalized definition is that of Brönsted, who defined an acid as a substance that furnishes protons, and a base as a substance that combines with protons. Even broader is the definition of Lewis, who defined an acid as any material that will accept an electron pair, a base as any material that will donate an electron pair, and neutralization as the formation of a coordination bond between an acid and a base.

The apparent strength of an acid or a base is determined by the extent of its reaction with a solvent. In water solution all strong acids appear equally strong because they react with the solvent to undergo almost complete conversion to oxonium ion and the acid anion (leveling effect). In a weakly protophilic solvent such as acetic acid the extent of formation of the acetate acidium ion shows that the order of decreasing strength for acids is perchloric, hydrobromic, sulfuric, hydrochloric, and nitric (differentiating effect).

Acetic acid reacts incompletely with water to form oxonium ion and is, therefore, a weak acid. In contrast, it dissolves in a base such as ethylenediamine, and reacts so completely with the solvent that it behaves as a strong acid. The same holds for perchloric acid.

This leveling effect is observed also for bases. In sulfuric acid almost all bases appear to be of the same strength. As the acid properties of the solvent decrease in the series sulfuric acid, acetic acid, phenol, water, pyridine, and butylamine, the bases become progressively weaker until all but the strongest have lost their basic properties. In order of decreasing strength, the strong bases are sodium 2-aminoethoxide, potassium methoxide, sodium methoxide, and lithium methoxide.

Many water-insoluble compounds acquire enhanced acidic or basic properties when dissolved in organic solvents. Thus the choice of the appropriate solvent permits the determination of a variety of such materials by nonaqueous titration. Furthermore, depending upon which part of a compound is the physiologically active moiety, it is often possible to titrate that part by proper selection of solvent and titrant. Pure compounds can be titrated directly, but it is often necessary to isolate the active ingredient in pharmaceutical preparations from interfering excipients and carriers.

The types of compounds that may be titrated as acids include acid halides, acid anhydrides, carboxylic acids, amino acids, enols such as barbiturates and xanthines, imides, phenols, pyrroles, and sulfonamides. The types of compounds that may be titrated as bases include amines, nitrogen-containing heterocyclic compounds, oxazolines, quaternary ammonium compounds, alkali salts of organic acids, alkali salts of weak inorganic acids, and some salts of amines. Many salts of halogen acids may be titrated in acetic acid or acetic anhydride after the addition of mercuric acetate, which removes halide ion as the un-ionized mercuric halide complex and introduces the acetate ion.

For the titration of a basic compound, a volumetric solution of perchloric acid in glacial acetic acid is preferred, although perchloric acid in dioxane is used in special cases. The calomel-glass electrode system is useful in this case. In acetic acid solvent this electrode system functions as predicted by theory.

For the titration of an acidic compound, two classes of titrant are available: the alkali metal alkoxides and the tetraalkylammonium hydroxides. A volumetric solution of sodium methoxide in a mixture of methanol and toluene is used frequently, although lithium methoxide in methanol-benzene solvent is used for those compounds yielding a gelatinous precipitate on titration with sodium methoxide.

The alkali error limits the use of the glass electrode as an indicating electrode in conjunction with alkali metal alkoxide titrants, particularly in basic solvents. Thus, the antimony-indicating electrode, though somewhat erratic, is used in such titrations. The use of quaternary ammonium hydroxide compounds, e.g., tetra-*n*-butylammonium hydroxide and trimethylhexadecylammonium hydroxide (in benzene-methanol or isopropyl alcohol), has two advantages over the other titrants in that (a) the tetraalkylammonium salt of the titrated acid is soluble in the titration medium, and (b) the convenient and well-behaved calomel-glass electrode may be used to conduct potentiometric titrations.

Because of interference by carbon dioxide, solvents for acidic compounds need to be protected from excessive exposure to the atmosphere by a suitable cover or by an inert atmosphere during the titration. Absorption of carbon dioxide may be determined by performing a blank titration. The blank should not exceed 0.01 mL of 0.1 *N* sodium methoxide VS per mL of solvent.

The end-point may be determined visually by color change, or potentiometrically, as indicated in the individual monograph. If the calomel reference electrode is used, it is advantageous to replace the aqueous potassium chloride salt bridge with 0.1 *N* lithium perchlorate in glacial acetic acid for titrations in acidic solvents or potassium chloride in methanol for titrations in basic solvents.

Where these or other mixtures are specified in individual monographs, the calomel reference electrode is modified by first removing the aqueous potassium chloride solution and residual potassium chloride, if any, by rinsing with water, then eliminating residual water by rinsing with the required nonaqueous solvent, and finally filling the electrode with the designated nonaqueous mixture.

The more useful systems for titration in nonaqueous solvents are listed in Table 1.

Indicator and Potentiometric End-point Detection—The simplest and most convenient method by which the equivalence point, i.e., the point at which the stoichiometric analytical reaction is complete, may be determined is with the use of indicators. These chemical substances, usually colored, respond to changes in solution conditions before and after the equivalence point by exhibiting color changes that may be taken visually as the end-point, a reliable estimate of the equivalence point.

Table 1. Systems for Nonaqueous Titrations.

Type of Solvent	*Acidic* (for titration of bases and their salts)	*Relatively Neutral* (for differential titration of bases)	*Basic* (for titration of acids)	*Relatively Neutral* (for differential titration of acids)
Solvent[1]	Glacial Acetic Acid Acetic Anhydride Formic Acid Propionic Acid Sulfuryl Chloride	Acetonitrile Alcohols Chloroform Benzene Toluene Chlorobenzene Ethyl Acetate Dioxane	Dimethylformamide *n*-Butylamine Pyridine Ethylenediamine Morpholine	Acetone Acetonitrile Methyl Ethyl Ketone Methyl Isobutyl Ketone *tert*-Butyl Alcohol
Indicator	Crystal Violet Quinaldine Red *p*-Naphtholbenzein Alphazurine 2-G Malachite green	Methyl Red Methyl Orange *p*-Naphtholbenzein	Thymol Blue Thymolphthalein Azo Violet *o*-Nitroaniline *p*-Hydroxyazobenzene	Azo Violet Bromothymol Blue *p*-Hydroxyazobenzene Thymol Blue
Electrodes	Glass-calomel Glass-silver–silver chloride Mercury–mercuric acetate	Glass-calomel Calomel-silver–silver chloride	Antimony-calomel Antimony-glass Antimony-antimony[2] Platinum-calomel Glass-calomel	Antimony-calomel Glass-calomel Glass-platinum[2]

[1] Relatively neutral solvents of low dielectric constant such as benzene, toluene, chloroform or dioxane may be used in conjunction with any acidic or basic solvent in order to increase the sensitivity of the titration end-points.
[2] In titrant.

A useful method of end-point determination results from the use of electrochemical measurements. If an indicator electrode, sensitive to the concentration of the species undergoing titrimetric reaction, and a reference electrode, whose potential is insensitive to any dissolved species, are immersed in the titrate to form a galvanic cell, the potential difference between the electrodes may be sensed by a pH meter and used to follow the course of the reaction. Where such a series of measurements is plotted correctly (i.e., for an acid-base titration, pH versus mL of titrant added; for a precipitimetric, complexometric, or oxidation-reduction titration, mV versus mL of titrant added), a sigmoid curve results with a rapidly changing portion (the "break") in the vicinity of the equivalence point. The mid-point of this linear vertical portion or the inflection point may be taken as the end-point. However, it should be noted that in asymmetrical reactions, which are reactions in which the number of anions reacting is not the same as the number of cations reacting, the end-point as defined by the inflection of the titration curve does not occur exactly at the stoichiometric equivalence point. Thus, potentiometric end-point detection by this method is not suitable in the case of asymmetric reactions, examples of which are the precipitation reaction,

$$2Ag^{+} + CrO_4^{-2}$$

and the oxidation-reduction reaction,

$$5Fe^{+2} + MnO_4^{-}.$$

All acid-base reactions, however, are symmetrical. Thus, potentiometric end-point detection may be employed in acid-base titrations and in other titrations involving symmetrical reversible reactions where an indicator is specified, unless otherwise directed in the individual monograph.

Two types of automatic electrometric titrators are available. The first is one that carries out titrant addition automatically and records the electrode potential differences during the course of titration as the expected sigmoid curve. In the second type, titrant addition is performed automatically until a preset potential or pH, representing the end-point, is reached, at which point the titrant addition ceases.

Several acceptable electrode systems for potentiometric titrations are summarized in Table 2.

Blank Corrections—As previously noted, the end-point determined in a titrimetric assay is an estimate of the reaction equivalence point. The validity of this estimate depends upon, among other factors, the nature of the titrate constituents and the concentration of the titrant. An appropriate *blank correction* is em-

Table 2. Potentiometric Titration Electrode Systems.

Titration	Indicating Electrode	Equation[1]	Reference Electrode	Applicability[2]
Acid-base	Glass	$E = k + 0.0591\ \text{pH}$	Calomel or silver–silver chloride	Titration of acids and bases
Precipitimetric (silver)	Silver	$E = E^{\circ} + 0.0591 \log [Ag^{+}]$	Calomel (with potassium nitrate salt bridge)	Titration with or of silver involving halides or thiocyanate
Chelometric	Mercury-mercury(II)	$E = E^{\circ} + 0.0296(\log k' - \text{pM})$	Calomel	Titration of various metals (M), e.g., Mg^{+2}, Ca^{+2}, Al^{+3}, Bi^{+3}, with EDTA
Oxidation-reduction	Platinum	$E = E^{\circ} + \frac{0.0591}{n} \log \frac{[\text{ox}]}{[\text{red}]}$	Calomel or silver–silver chloride	Titrations with arsenite, bromine, cerate, dichromate, hexacyanoferrate(III), iodate, nitrite, permanganate, thiosulfate

[1] Appropriate form of Nernst equation describing the indicating electrode system: k = glass electrode constant; k' = constant derived from Hg-Hg(II)-EDTA equilibrium; M = any metal undergoing EDTA titration; [ox] and [red] from the equation, $\text{ox} + ne \rightleftarrows \text{red}$.
[2] Listing is representative but not exhaustive.

ployed in titrimetric assays to enhance the reliability of the end-point determination. Such a blank correction is usually obtained by means of a *residual blank titration*, wherein the required procedure is repeated in every detail except that the substance being assayed is omitted. In such instances, the actual volume of titrant equivalent to the substance being assayed is the difference between the volume consumed in the residual blank titration and that consumed in the titration with the substance present. The corrected volume so obtained is used in calculating the quantity of the substance being titrated, in the same manner as prescribed under *Residual Titrations*. Where potentiometric end-point detection is employed, the blank correction is usually negligible.

⟨551⟩ ALPHA TOCOPHEROL ASSAY

The following procedure is provided for the determination of tocopherol as an ingredient of *Decavitamin Capsules* and *Decavitamin Tablets*.

Hydrogenator—A suitable device for low-pressure hydrogenation may be assembled as follows: Arrange in a rack or in clamps two 50-mL conical centrifuge tubes, connected in series by means of glass and inert plastic tubing and suitable stoppers of glass, polymer, or cork (avoiding all use of rubber). Use one tube for the blank and the other for the assay specimen. Arrange a gas-dispersion tube so that the hydrogen issues as bubbles at the bottom of each tube. Pass the hydrogen first through the blank tube and then through the specimen tube.

Procedure—Pipet into a suitable vessel 25 mL of the final washed ether solution of the unsaponifiable fraction obtained as directed for *When Tocopherol Is Present* under *Procedure* in the *Vitamin A Assay* ⟨571⟩, and evaporate to about 5 mL. *Without applying heat*, remove the remaining ether in a stream of inert gas or by vacuum. Dissolve the residue in sufficient alcohol to give an expected concentration of about 0.15 mg of alpha tocopherol per mL. Pipet 15 mL into a 50-mL centrifuge tube, add about 200 mg of palladium catalyst, stir with a glass rod, and hydrogenate for 10 minutes in the *Hydrogenator*, using hydrogen that has been passed through alcohol in a blank tube. Add about 300 mg of chromatographic siliceous earth, stir with a glass rod, and immediately centrifuge until the solution is clear.

Test a 1-mL aliquot of the solution by removing the solvent by evaporation, dissolving the residue in 1 mL of chloroform, and adding 10 mL of antimony trichloride TS: no detectable blue color appears. [NOTE—If a blue color appears, repeat the hydrogenation for a longer time period, or with a new lot of catalyst.]

Pipet 2 mL of the supernatant solution into a glass-stoppered, opaque flask, add 1.0 mL of a 1 in 500 solution of ferric chloride in dehydrated alcohol,* and begin timing the reaction, preferably with a stop watch. Add immediately 1.0 mL of a 1 in 200 solution of 2,2′-bipyridine in dehydrated alcohol, mix with swirling, add 21.0 mL of dehydrated alcohol, close the tube, and shake vigorously to ensure complete mixing. When about 9½ minutes have elapsed from the beginning of the reaction, transfer part of the mixture to one of a pair of matched 1-cm spectrophotometer cells. After 10 minutes, accurately timed, following the addition of the ferric chloride–dehydrated alcohol solution, determine the absorbance at 520 nm, with a suitable spectrophotometer, using dehydrated alcohol as the blank. Perform a blank determination with the same quantities of the same reagents and in the same manner, but using 2 mL of dehydrated alcohol in place of the 2 mL of the hydrogenated solution. Subtract the absorbance determined for the blank from that determined for the assay specimen, and designate the difference as A_D.

Calculate the alpha tocopherol content, in mg, in the assay specimen taken by the formula:

$$30.2\ A_D/(LC_D),$$

in which A_D is the corrected absorbance, L is the length, in cm, of the absorption cell, and C_D is the content of the assay specimen in the alcohol solution employed for the measurement of absorbance, expressed as g, capsules, or tablets per 100 mL.

* NOTE—The absorbance of the blank may be reduced, and the precision of the determination thereby improved, by purification of the dehydrated alcohol that is used throughout the assay. Purification may be accomplished by the addition of a few crystals (about 0.02%) of potassium permanganate and of a few pellets of potassium hydroxide to the dehydrated alcohol, and subsequent redistillation.

⟨561⟩ VEGETABLE DRUGS—SAMPLING AND METHODS OF ANALYSIS

Sampling

In order to reduce the effect of sampling bias in qualitative and quantitative results, it is necessary to ensure that the composition of the sample used be representative of the batch of drugs being examined. The following sampling procedures are the minimum considered applicable to vegetable drugs. Some articles, or some tests, may require more rigorous procedures involving more containers being sampled and/or more samples per container.

Gross Sample—Where external examination of containers, markings, and labels indicates that the batch can be considered to be homogeneous, take individual samples from the number of randomly selected containers indicated below. Where the batch cannot be considered to be homogeneous, divide it into sub-batches that are as homogeneous as possible, then sample each one as a homogeneous batch.

No. of Containers in Batch (N)	No. of Containers to be Sampled (n)
1 to 10	all
11 to 19	11
>19	$n = 10 + (N/10)$

(Round calculated "n" to next highest whole number.)

Samples shall be taken from the upper, middle, and lower sections of each container. If the crude material consists of component parts which are 1 cm or less in any dimension, and in the case of all powdered or ground materials, withdraw the sample by means of a sampling device that removes a core from the top to the bottom of the container, not less than two cores being taken in opposite directions. For materials with component parts over 1 cm in any dimension, withdraw samples by hand. In the case of large bales or packs, samples should be taken from a depth of 10 cm because the moisture content of the surface layer may be different from that of the inner layers.

Prepare the gross sample by combining and mixing the individual samples taken from each opened container, taking care not to increase the degree of fragmentation or significantly affect the moisture content.

Laboratory Sample—Prepare the laboratory sample by repeated quartering of the gross sample. (NOTE—Quartering consists of placing the sample, adequately mixed, as an even and square-shaped heap and dividing it diagonally into four equal parts. The two opposite parts are then taken and carefully mixed. The process is repeated as necessary until the required quantity is obtained.)

The laboratory sample should be of a size sufficient for performing all the necessary tests.

Test Sample—Unless otherwise directed in the individual monograph or test procedure below, prepare the test sample as follows:

Decrease the size of the laboratory sample by quartering, taking care that each withdrawn portion remains representative. In the case of unground or unpowdered drugs, grind the withdrawn sample so that it will pass through a No. 20 standard-mesh sieve, and mix the resulting powder well. If the material cannot be ground, reduce it to as fine a state as possible, mix by rolling it on paper or sampling cloth, spread it out in a thin layer and withdraw the portion for analysis.

Foreign Organic Matter

Test Sample—Unless otherwise specified in the individual monograph, weigh the following quantities of the laboratory sam-

ple, taking care that the withdrawn portion is representative (quartering if necessary):

Roots, rhizomes, bark, and herbs	500 g
Leaves, flowers, seeds, and fruit	250 g
Cut vegetable drugs (average weight of the pieces is less than 0.5 g)	50 g

Spread the sample out in a thin layer, and separate the foreign organic matter by hand as completely as possible. Weigh it, and determine the percentage of foreign organic matter in the weight of drug taken.

Total Ash

Accurately weigh a quantity of the *test sample*, representing 2 to 4 g of the air-dried material, in a tared crucible, and incinerate, gently at first, and gradually increase the temperature to 675 ± 25°, until free from carbon, and determine the weight of the ash. If a carbon-free ash cannot be obtained in this way, extract the charred mass with hot water, collect the insoluble residue on an ashless filter paper, incinerate the residue and filter paper until the ash is white or nearly so, then add the filtrate, evaporate it to dryness, and heat the whole to a temperature of 675 ± 25°. If a carbon-free ash cannot be obtained in this way, cool the crucible, add 15 mL of alcohol, break up the ash with a glass rod, burn off the alcohol, and again heat the whole to a temperature of 675 ± 25°. Cool in a desiccator, weigh the ash, and calculate the percentage of total ash from the weight of the drug taken.

Acid-insoluble Ash

Boil the ash obtained as directed under *Total Ash*, above, with 25 mL of 3 *N* hydrochloric acid for 5 minutes, collect the insoluble matter on a tared filtering crucible or ashless filter, wash with hot water, ignite, and weigh. Determine the percentage of acid-insoluble ash calculated from the weight of drug taken.

Crude Fiber

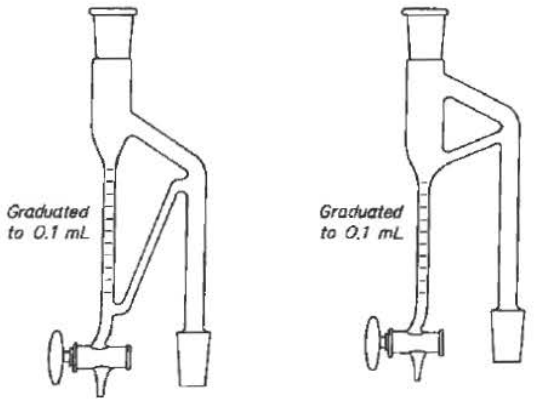
Exhaust a weighed quantity of the *test sample*, representing about 2 g of the drug, with ether. Add 200 mL of boiling dilute sulfuric acid (1 in 78) to the ether-exhausted marc, in a 500-mL flask, and connect the flask to a reflux condenser. Reflux the mixture for 30 minutes, accurately timed, then filter through a linen or hardened-paper filter, and wash the residue on the filter with boiling water until the effluent washing is no longer acid. Rinse the residue back into the flask with 200 mL of boiling sodium hydroxide solution, adjusted to 1.25 percent by titration and free from sodium carbonate. Again reflux the mixture for 30 minutes, accurately timed, then rapidly filter through a tared filter, wash the residue with boiling water until the last washing is neutral, and dry it at 110° to constant weight. Incinerate the dried residue, ignite to constant weight, cool in a desiccator, and weigh the ash: the difference between the weight obtained by drying at 110° and that of the ash represents the weight of the crude fiber.

NOTE—The boiling with acid and alkali should continue for 30 minutes, accurately timed, from the time that the liquid (which is cooled below the boiling point by being added to the cold flask) again boils. After the solution has been brought to boiling, the heat should be turned low enough just to maintain boiling. During the boiling, the flask should be gently rotated from time to time to wash down any particles that may adhere to the walls of the flask. A slow current of air introduced into the flask during the boiling operation aids in preventing excessive frothing.

Volatile Oil Determination

Set up a round-bottom, shortneck, 1-liter flask in a heating mantle set over a magnetic stirrer. Insert an egg-shaped stirring bar magnet in the flask, and attach a cold-finger condenser and an appropriate volatile oil trap of the type illustrated.

Coarsely comminute a sufficient quantity of the drug to yield from 1 to 3 mL of volatile oil. Small seeds, fruits, or broken leaves of herbs ordinarily do not need comminution. Very fine powders are to be avoided. If this is not possible, it may be necessary to mix them with purified sawdust or purified sand. Place a suitable quantity of the drug, accurately weighed, in the flask, and fill it one-half with water. Attach the condenser and the proper separator. Boil the contents of the flask, using a suitable amount of heat to maintain gentle boiling for 2 hours, or until the volatile oil has been completely separated from the drug and no longer collects in the graduated tube of the separator.



Traps for Volatile Oil Apparatus

If a proper quantity of the volatile oil has been obtained in the graduated tube of the separator, it can be read to tenths of 1 mL, and the volume of volatile oil from each 100 g of drug can be calculated from the weight of the drug taken. The graduations on the separator "for oils heavier than water" are so placed that oil remains below the aqueous condensate that automatically flows back into the flask.

Water

For unground or unpowdered drugs, prepare about 10 g of the *laboratory sample* by cutting, granulating, or shredding, so that the parts are about 3 mm in thickness. Seeds or fruits smaller than 3 mm should be cracked. Avoid the use of high-speed mills in preparing the sample, and exercise care that no appreciable amount of moisture is lost during the preparation and that the portion taken is representative of the *laboratory sample*. Determine the water content as directed under *Procedure for Vegetable Drugs* in the test for *Water Determination—Gravimetric Method* ⟨921⟩.

⟨571⟩ VITAMIN A ASSAY

The following procedure is provided for the determination of vitamin A as an ingredient of Pharmacopeial preparations. It conforms to that which was adopted in 1956 for international use by the International Union of Pure and Applied Chemistry.

Complete the assay promptly, and exercise care throughout the procedure to keep to a minimum the exposure to actinic light and to atmospheric oxygen and other oxidizing agents, preferably, by the use of non-actinic glassware and an atmosphere of an inert gas.

Special Reagents—

ETHER—Use ethyl ether, and use it within 24 hours after opening the container.

ISOPROPYL ALCOHOL—Use spectrophotometric-grade isopropyl alcohol (see *Isopropyl Alcohol* under *Reagent Specifications* in the section, *Reagents, Indicators, and Solutions*).

Procedure—Accurately weigh, count, or measure a portion of the test specimen expected to contain the equivalent of not less than 0.15 mg of retinol but containing not more than 1 g of fat. If in the form of capsules, tablets, or other solid, so that it cannot be saponified efficiently by the ensuing instructions, reflux the portion taken in 10 mL of water on a steam bath for about 10 minutes, crush the remaining solid with a blunt glass rod, and warm for about 5 minutes longer.

Transfer to a suitable borosilicate glass flask, and add 30 mL of alcohol, followed by 3 mL of potassium hydroxide solution (9

in 10). Reflux in an all-borosilicate glass apparatus for 30 minutes. Cool the solution, add 30 mL of water, and transfer to a conical separator. Add 4 g of finely powdered sodium sulfate decahydrate. Extract by shaking with one 150-mL portion of ether for 2 minutes, and then, if an emulsion forms, with three 25-mL portions of ether. Combine the ether extracts, if necessary, and wash by swirling gently with 50 mL of water. Repeat the washing more vigorously with three additional 50-mL portions of water. Transfer the washed ether extract to a 250-mL volumetric flask, add ether to volume, and mix.

Evaporate a 25.0-mL portion of the ether extract to about 5 mL. *Without applying heat and with the aid of a stream of inert gas or vacuum,* continue the evaporation to about 3 mL. Dissolve the residue in sufficient isopropyl alcohol to give an expected concentration of the equivalent to 3 μg to 5 μg of vitamin A per mL or such that it will give an absorbance in the range 0.5 to 0.8 at 325 nm. Determine the absorbances of the resulting solution at the wavelengths 310 nm, 325 nm, and 334 nm, with a suitable spectrophotometer fitted with matched quartz cells, using isopropyl alcohol as the blank.

WHEN TOCOPHEROL IS PRESENT—Transfer to a suitable borosilicate glass flask a test specimen, accurately measured, or not less than 5 previously crushed Decavitamin Capsules or Decavitamin Tablets. Reflux in an all-borosilicate glass apparatus with 30 mL of alcohol and 3 mL of potassium hydroxide solution (9 in 10) for 30 minutes. Add through the condenser 2.0 g of citric acid monohydrate, washing the walls of the condenser with 10 mL of water. Cool, and transfer the solution to a conical separator with the aid of 20 mL of water. Add 4 g of finely powdered sodium sulfate decahydrate. Extract with one 150-mL portion of ether and then, if an emulsion forms, with three 25-mL portions of ether. Combine the ether extracts, if necessary, and wash by swirling gently with 50 mL of water. Repeat the washing more vigorously with three additional 50-mL portions of water. Transfer the washed ether extract to a 250-mL volumetric flask, and add ether to volume. Transfer a 100.0-mL aliquot of the resulting ether solution to a conical separator, and wash once with 50 mL of potassium hydroxide solution (1 in 33), using alcohol, if necessary, to break any emulsion that forms. Wash by swirling gently with 50 mL of water. Repeat the washing more vigorously with three additional 50-mL portions of water. Transfer the washed ether extract to a 100-mL volumetric flask, add ether to volume, and mix.

Evaporate a 50.0-mL aliquot of the ether solution of the unsaponifiable extract to about 5 mL. *Without applying heat and with the aid of a stream of inert gas or vacuum,* remove the residual ether. Dissolve the residue in 50.0 mL of isopropyl alcohol.

Hydrogenated portion—Pipet 15.0 mL of the isopropyl alcohol solution into a 50-mL centrifuge tube, add approximately 200 mg of palladium catalyst, stir with a glass rod, and hydrogenate for 10 minutes in a *Hydrogenator* such as is described in the *Alpha Tocopherol Assay* ⟨551⟩, using isopropyl alcohol in the blank tube. Add about 300 mg of chromatographic siliceous earth, stir with a glass rod, and immediately centrifuge until the solution is clear.

Test a 1-mL aliquot of the solution by removing the solvent by evaporation, dissolving the residue in 1 mL of chloroform, and adding 10 mL of phosphomolybdic acid TS: no detectable blue-green color appears. [NOTE—If a blue-green color appears, repeat the hydrogenation for a longer time period, or with a new lot of catalyst.]

Into two separate flasks pipet equal volumes of the *Hydrogenated portion* and the untreated isopropyl alcohol solution, respectively, and add sufficient isopropyl alcohol to give an expected concentration of vitamin A equivalent to 3 μg to 5 μg per mL. Determine the absorbances of the untreated solution against the solution from the *Hydrogenated portion* as a blank, at the wavelengths 310 nm, 325 nm, and 334 nm, with a suitable spectrophotometer fitted with matched quartz cells.

Calculation—Calculate the vitamin A content as follows:

$$\text{Content (in mg)} = 0.549A_{325}/LC,$$

in which A_{325} is the observed absorbance at 325 nm, L is the length, in cm, of the absorption cell, and C is the amount of test specimen expressed as g, capsule, or tablet in each 100 mL of the final isopropyl alcohol solution, provided that A_{325} has a value not less than $[A_{325}]/1.030$ and not more than $[A_{325}]/0.970$, where $[A_{325}]$ is the *corrected* absorbance at 325 nm and is given by the equation:

$$[A_{325}] = 6.815A_{325} - 2.555A_{310} - 4.260A_{334},$$

in which A designates the absorbance at the wavelength indicated by the subscript.

Where $[A_{325}]$ has a value less than $A_{325}/1.030$, apply the following equation:

$$\text{Content (in mg)} = 0.549[A_{325}]/LC,$$

in which the values are as defined herein. Each mg of vitamin A (alcohol) represents 3333 USP Units of vitamin A.

Confidence Interval—The range of the limits of error, indicating the extent of discrepancy to be expected in the results of different laboratories at $P = 0.05$, is approximately ±8%.

⟨581⟩ VITAMIN D ASSAY

Chromatographic Method

The following pressurized liquid chromatographic procedure is provided for the determination of vitamin D, as cholecalciferol or as ergocalciferol, as an ingredient of Pharmacopeial multiple-vitamin preparations.

Throughout this assay, protect solutions containing, and derived from, the test specimen and the Reference Standard from the atmosphere and light, preferably by the use of a blanket of inert gas and low-actinic glassware.

Reference Standard—[NOTE—Use USP Ergocalciferol RS, or USP Cholecalciferol RS, for assaying pharmaceutical dosage forms that are labeled to contain vitamin D as ergocalciferol, or as cholecalciferol, respectively.] *USP Ergocalciferol Reference Standard*—Store in a cold place, protected from light. Allow it to attain room temperature before opening ampul. Use the material promptly, and discard the unused portion.

USP Cholecalciferol Reference Standard—Store in a cold place, protected from light. Allow it to attain room temperature before opening ampul. Use the material promptly, and discard the unused portion.

USP Vitamin D Assay System Suitability Reference Standard—Store in a cool place, protected from light. Allow it to attain room temperature before opening ampul. Do not dry. Transfer unused contents of ampul to a tightly closed container, and store under nitrogen, in the dark, in a cool place.

USP Δ4,6-Cholestadienol Reference Standard—Store in a cool place, protected from light. Allow it to attain room temperature before opening ampul. Do not dry. Transfer unused contents of ampul to a tightly closed container, and store under nitrogen, in the dark, in a cool place.

Special Reagents and Solutions—

ETHER—Use ethyl ether. Use within 24 hours after opening container.

DEHYDRATED HEXANE—Prepare a chromatographic column by packing a chromatographic tube, 60 cm × 8 cm in diameter, with 500 g of 50- to 250-μm chromatographic siliceous earth, activated by drying at 150° for 4 hours (see *Column adsorption chromatography* under *Chromatography* ⟨621⟩). Pass 500 mL of hexanes through the column, and collect the eluate in a glass-stoppered flask.

BUTYLATED HYDROXYTOLUENE SOLUTION—Dissolve a quantity of butylated hydroxytoluene in chromatographic hexane to obtain a solution containing 10 mg per mL.

AQUEOUS POTASSIUM HYDROXIDE SOLUTION—Dissolve 500 g of potassium hydroxide in 500 mL of freshly boiled water, mix, and cool. Prepare this solution fresh daily.

ALCOHOLIC POTASSIUM HYDROXIDE SOLUTION—Dissolve 3 g of potassium hydroxide in 50 mL of freshly boiled water, add 10 mL of alcohol, dilute with freshly boiled water to 100 mL, and mix. Prepare this solution fresh daily.

SODIUM ASCORBATE SOLUTION—Dissolve 3.5 g of ascorbic acid in 20 mL of 1 *N* sodium hydroxide. Prepare this solution fresh daily.

SODIUM SULFIDE SOLUTION—Dissolve 12 g of sodium sulfide in 20 mL of water, dilute with glycerin to 100 mL, and mix.

Mobile Phase A—Prepare a mixture of acetonitrile, methanol, and water (25:25:1). The amount of water and the flow rate may be varied to meet system suitability requirements.

Mobile Phase B—Prepare a 3 in 1000 mixture of *n*-amyl alcohol in *Dehydrated hexane*. The ratio of components and the flow rate may be varied to meet system suitability requirements.

Internal Standard Solution—Transfer 15 mg of USP Δ4,6-Cholestadienol RS, accurately weighed, to a 200-mL volumetric flask, add a 1 in 10 mixture of toluene and *Mobile Phase B* to volume, and mix.

Standard Preparation—Transfer about 25 mg of USP Ergocalciferol RS or Cholecalciferol RS, accurately weighed, to a 50-mL volumetric flask, dissolve without heat in toluene, add toluene to volume, and mix. Pipet 10 mL of this stock solution into a 100-mL volumetric flask, dilute with toluene to volume, and mix. Prepare stock solution fresh daily.

Assay Preparation—

For oily solutions—Accurately weigh a portion of the specimen to be assayed, preferably more than 0.5 g and equivalent to about 125 μg of cholecalciferol or ergocalciferol (5000 USP Units). Add 1 mL of *Sodium Ascorbate Solution*, 25 mL of alcohol, and 2 mL of *Aqueous Potassium Hydroxide Solution*, and mix.

For capsules or tablets—Reflux not less than 10 capsules or tablets with a mixture of 10 mL of *Sodium Ascorbate Solution* and 2 drops of *Sodium Sulfide Solution* on a steam bath for 10 minutes, crush any remaining solids with a blunt glass rod, and continue heating for 5 minutes. Cool, add 25 mL of alcohol and 3 mL of *Aqueous Potassium Hydroxide Solution*, and mix.

For dry preparations and aqueous dispersions—Accurately weigh a portion of the specimen to be assayed, preferably more than 0.5 g and equivalent to about 125 μg of cholecalciferol or ergocalciferol (5000 USP Units). Add, in small quantities and with gentle swirling, 25 mL of alcohol, 5 mL of *Sodium Ascorbate Solution*, and 3 mL of *Aqueous Potassium Hydroxide Solution*.

SAPONIFICATION AND EXTRACTION—Reflux the mixture prepared from the specimen to be assayed on a steam bath for 30 minutes. Cool rapidly under running water, and transfer the saponified mixture to a conical separator, rinsing the saponification flask with two 15-mL portions of water, 10 mL of alcohol, and two 50-mL portions of ether. Shake the combined saponified mixture and rinsings vigorously for 30 seconds, and allow to stand until both layers are clear. Transfer the aqueous phase to a second conical separator, add a mixture of 10 mL of alcohol and 50 mL of solvent hexane, and shake vigorously. Allow to separate, transfer the aqueous phase to a third conical separator, and transfer the hexane phase to the first separator, rinsing the second separator with two 10-mL portions of solvent hexane, adding the rinsings to the first separator. Shake the aqueous phase in the third separator with 50 mL of solvent hexane, and add the hexane phase to the first separator. Wash the combined ether-hexane extracts by shaking vigorously with three 50-mL portions of *Alcoholic Potassium Hydroxide Solution*, and wash with 50-mL portions of water vigorously until the last washing is neutral to phenolphthalein. Drain any remaining drops of water from the combined ether-hexane extracts, add 2 sheets of 9-cm filter paper, in strips, to the separator, and shake. Transfer the washed ether-hexane extracts to a round-bottom flask, rinsing the separator and paper with solvent hexane. Combine the hexane rinsings with the ether-hexane extracts, add 5.0 mL of *Internal Standard Solution* and 100 μL of *Butylated Hydroxytoluene Solution*, and mix. Evaporate to dryness in vacuum by swirling in a water bath maintained at a temperature not higher than 40°. Cool under running water, and introduce nitrogen sufficient to restore atmospheric pressure. Without delay, dissolve the residue in 5.0 mL of a mixture of equal volumes of acetonitrile and methanol, or in a measured portion of the acetonitrile-methanol mixture until the concentration of vitamin D is about 25 μg per mL, to obtain the *Assay Preparation*.

Chromatographic System—Use a chromatograph, operated at room temperature, fitted with an ultraviolet detector that monitors absorption at 254 nm, a 30-cm × 4.6-mm stainless steel cleanup column packed with column packing L7 and using *Mobile Phase A*, and a 25-cm × 4.6-mm stainless steel analytical column packed with column packing L3 and using *Mobile Phase B*.

CLEANUP COLUMN SYSTEM SUITABILITY TEST—Pipet 5 mL of the *Standard Preparation* into a round-bottom flask fitted with a reflux condenser, and add 2 or 3 crystals of butylated hydroxytoluene. Displace the air with nitrogen, and heat in a water bath maintained at a temperature of 90° in subdued light under an atmosphere of nitrogen for 45 minutes, to obtain a solution containing vitamin D and pre-vitamin D. Cool, add 10.0 mL of *Internal Standard Solution*, mix, and evaporate in vacuum to dryness by swirling in a water bath maintained at a temperature not higher than 40°. Cool under running water, and introduce nitrogen sufficient to restore atmospheric pressure. Without delay, dissolve the residue in 10.0 mL of a mixture of equal volumes of acetonitrile and methanol, and mix. Inject 500 μL of this solution into the cleanup column, and record the chromatogram as directed under *Procedure*. The chromatogram exhibits a peak exhibiting a retention time between 5 and 9 minutes, corresponding to the separation under a single peak of the mixture of vitamin D, pre-vitamin D, and Δ4,6-cholestadienol from other substances. Adjust the water content or other operating parameters, if necessary (see *Mobile Phase A*).

ANALYTICAL COLUMN SYSTEM SUITABILITY TEST—Transfer about 100 mg of USP Vitamin D Assay System Suitability RS to a 100-mL volumetric flask, add a 1 in 20 mixture of toluene and *Mobile Phase B* to volume, and mix. Heat a portion of this solution, under reflux, at 90° for 45 minutes, and cool. Chromatograph five injections of the resulting solution, and measure the peak responses as directed under *Procedure*. The relative standard deviation for the cholecalciferol peak response does not exceed 2.0%, and the resolution between *trans*-cholecalciferol and pre-cholecalciferol is not less than 1.0. [NOTE—Chromatograms obtained as directed for this test exhibit relative retention times of approximately 0.4 for pre-cholecalciferol, 0.5 for *trans*-cholecalciferol, and 1.0 for cholecalciferol.]

Calibration—

VITAMIN D RESPONSE FACTOR—Transfer 4.0 mL of the *Standard Preparation* and 10.0 mL of *Internal Standard Solution* to a 100-mL volumetric flask, dilute with *Mobile Phase B* to volume, and mix to obtain the *Working Standard Preparation*. Store this *Working Standard Preparation* at a temperature not above 0°, retaining the unused portion for the *Procedure*. Inject 200 μL of the *Working Standard Preparation* into the analytical column, and measure the peak responses for vitamin D and for Δ4,6-cholestadienol. The relative retention time of Δ4,6-cholestadienol is about 1.3. Calculate the response factor, F_D, by the formula:

$$C_s/(R_s C_r),$$

in which C_s and C_r are the concentrations, in μg per mL, of vitamin D and Δ4,6-cholestadienol, respectively, in the *Working Standard Preparation*, and R_s is the ratio of the peak response of vitamin D to that of Δ4,6-cholestadienol.

PRE-VITAMIN D RESPONSE FACTOR—Pipet 4 mL of the *Standard Preparation* into a round-bottom flask fitted with a reflux condenser, and add 2 or 3 crystals of butylated hydroxytoluene. Displace the air with nitrogen, and heat in a water bath maintained at a temperature of 90° in subdued light under a nitrogen atmosphere for 45 minutes, to obtain a solution containing vitamin D and pre-vitamin D. Cool, transfer with the aid of several portions of *Mobile Phase B* to a 100-mL volumetric flask containing 10.0 mL of *Internal Standard Solution*, dilute with *Mobile Phase B* to volume, and mix to obtain the *Working Mixture*. Inject 200 μL of this *Working Mixture* into the analytical column, and measure the peak responses for vitamin D, pre-vitamin D, and Δ4,6-cholestadienol. Calculate the concentration, C'_s, in μg per mL, of vitamin D in the (heated) *Working Mixture* by the formula:

$$F_D C_r R'_s,$$

in which C_r is the concentration, in μg per mL, of Δ4,6-cholestadienol, and R'_s is the ratio of the peak response for vitamin D to that for Δ4,6-cholestadienol. Calculate the concentration, C'_{pre}, in μg per mL, of pre-vitamin D, in the *Working Mixture* by the formula:

$$C'_{pre} = C_s - C'_s.$$

Calculate the response factor, F_{pre}, for pre-vitamin D by the formula:

$$(F_D R'_s C'_{pre})/(R'_{pre} C'_s),$$

in which R'_{pre} is the ratio of the peak response of pre-vitamin D to that of Δ4,6-cholestadienol. [NOTE—Value of F_{pre} determined in duplicate, on different days, can be used during the whole procedure.]

Procedure—Inject 500 µL of the *Assay Preparation* into the cleanup column, and collect the fraction representing 0.7 to 1.3 relative to the retention time of the mixed vitamin D peak (see *Cleanup column system suitability test*) in a round-bottom flask. Add 50 µL of *Butylated Hydroxytoluene Solution*, mix, and evaporate in vacuum to dryness by swirling in a water bath maintained at a temperature not higher than 40°. Cool under running water, and introduce nitrogen sufficient to restore atmospheric pressure. Without delay, dissolve the residue in 5.0 mL of a 1 in 20 mixture of toluene and *Mobile Phase B*, and mix. Inject 200 µL of this solution into the analytical column, and measure the peak responses for vitamin D, pre-vitamin D, and Δ4,6-cholestadienol. Calculate the concentration, in µg per mL, of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the *Assay Preparation* by the formula:

$$(R''_D F_D + R''_{pre} F_{pre}) C''_r,$$

in which R''_D is the ratio of the peak response of vitamin D to that of Δ4,6-cholestadienol, R''_{pre} is the ratio of the peak response of pre-vitamin D to that of Δ4,6-cholestadienol, and C''_r is the concentration, in µg per mL, of Δ4,6-cholestadienol in the *Assay Preparation*.

Chemical Method

The following procedure is provided for the determination of vitamin D as an ingredient of Pharmacopeial preparations.

Complete the assay promptly, and exercise care throughout the procedure to keep to a minimum the exposure to air and to actinic light, preferably by the use of a blanket of inert gas and low-actinic glassware.

Reference Standard—[NOTE—Use USP Ergocalciferol RS, or USP Cholecalciferol RS, for assaying pharmaceutical dosage forms that are labeled to contain vitamin D as ergocalciferol, or as cholecalciferol, respectively.] *USP Ergocalciferol Reference Standard*—Store in a cold place, protected from light. Allow it to attain room temperature before opening ampul. Use the material promptly, and discard the unused portion. *USP Cholecalciferol Reference Standard*—Store in a cold place, protected from light. Allow it to attain room temperature before opening ampul. Use the material promptly, and discard the unused portion.

Special Reagents and Solutions—

CHROMATOGRAPHIC FULLER'S EARTH—Use chromatographic fuller's earth having a water content corresponding to between 8.5% and 9.0% of loss on drying.

SOLVENT HEXANE—Use solvent hexane (see under *Reagents*), redistilling if necessary so that it meets the following additional specification:

Spectral purity—Measure in a 1-cm cell at 300 nm, with a suitable spectrophotometer, against air as the blank: the absorbance is not more than 0.070.

ETHYLENE DICHLORIDE—Purify by passage through a column of granular (20 to 200 mesh) silica gel.

POTASSIUM HYDROXIDE SOLUTION—Dissolve 500 g of potassium hydroxide in water to make 1000 mL.

BUTYLATED HYDROXYTOLUENE SOLUTION—Dissolve 10 mg of butylated hydroxytoluene in 100 mL of alcohol. Prepare this solution fresh daily.

ETHER—Use freshly distilled ether, discarding the first and last 10% portions of the distillate.

COLOR REAGENT—Prepare two stock solutions as follows.

Solution A—Empty, without weighing, the entire contents of a previously unopened 113-g bottle of dry, crystalline antimony trichloride into a flask containing about 400 mL of *Ethylene Dichloride*. Add about 2 g of anhydrous alumina, mix, and filter through filter paper into a clear-glass, glass-stoppered container calibrated at 500 mL. Add *Ethylene Dichloride* to make 500 mL, and mix: the absorbance of the solution, measured in a 20-mm cell at 500 nm, with a suitable spectrophotometer, against *Ethylene Dichloride*, does not exceed 0.070.

Solution B—Mix, under a hood, 100 mL of acetyl chloride and 400 mL of *Ethylene Dichloride*.

Mix 45 mL of *Solution A* and 5 mL of *Solution B* to obtain the *Color Reagent*. Store in a tight container, and use within 7 days, but discard any reagent in which a color develops.

Chromatographic Tubes—

FIRST COLUMN—Arrange for descending column chromatography a tube of 2.5-cm (inside) diameter, about 25 cm long, and constricted to 8-mm diameter for a distance of 5 cm at the lower end, by inserting at the point of constriction a coarse-porosity, sintered-glass disk or a small plug of glass wool. The constricted portion may be fitted with an inert, plastic stopcock.

SECOND COLUMN—Select a tube that is made up of three sections: (1) a flared top section, 18 mm in (inside) diameter and approximately 14 cm long, (2) a middle section, 6 mm in (inside) diameter and approximately 25 cm long, and (3) a tapered, constricted lower exit tube approximately 5 cm long. Insert a small plug of glass wool in the upper 1-cm portion of the constricted section.

Chromatographic Columns—

FIRST COLUMN—To about 125 mL of isooctane contained in a screw-capped, wide-mouth bottle add 25 g of chromatographic siliceous earth, and shake until a slurry is formed. Add, dropwise and with vigorous mixing, 10 mL of polyethylene glycol 600. Replace the bottle cover, and shake vigorously for 2 minutes. Pour about half of the resulting slurry into the chromatographic tube, and allow it to settle by gravity. Then apply gentle suction, and add the remainder of the slurry in small portions, packing each portion with a 20-mm disk plunger. When a solid surface has formed, remove the vacuum, and add about 2 mL of isooctane.

SECOND COLUMN—Pack the midsection of the tube with 3 g of moderately coarse *Chromatographic Fuller's Earth* with the aid of gentle suction (about 125 mm of mercury).

Standard Preparation—Dissolve about 25 mg of *Reference Standard*, accurately weighed, in isooctane to give a known concentration of about 250 µg per mL. Store in a refrigerator.

On the day of assay, pipet 1 mL of the standard solution into a 50-mL volumetric flask, remove the solvent with a stream of nitrogen, and dissolve the residue in, and dilute to volume with, *Ethylene Dichloride*, and mix.

Sample Preparation—Accurately weigh or measure a portion of the sample to be assayed, equivalent to not less than 125 µg but preferably about 250 µg of ergocalciferol (10,000 USP Units). If little or no vitamin A is present in the sample, add about 1.5 mg (the equivalent of 3000 USP Units) of vitamin A acetate to provide the needed pilot bands in the subsequent chromatography.

For capsules or tablets, reflux not less than 10 of them in 10 mL of water on a steam bath for about 10 minutes, crush the remaining solid with a blunt glass rod, and warm for 5 minutes longer.

Add a volume of *Potassium Hydroxide Solution* representing 2.5 mL for each g of the total weight of the sample, but not less than a total of 3.0 mL. Add 10 mL of *Butylated Hydroxytoluene Solution* and 20 mL of alcohol. Reflux vigorously on a steam bath for 30 minutes. Cool, and transfer the saponified mixture to a conical separator, rinsing the saponification flask with three 10-mL portions of water and three 50-mL portions of *Ether*, adding each rinse to the separator. Add about 4 g of sodium sulfate decahydrate to the separator, and extract by shaking for 2 minutes. If an emulsion forms, extract with three 25-mL portions of *Ether*. Combine the ether extracts, if necessary, and wash by swirling gently with 50 mL of water. Repeat the washing more vigorously with additional 50-mL portions of water until the last portion shows no pink color on the addition of phenolphthalein TS. Transfer the washed ether extract to a 250-mL volumetric flask, add *Ether* to volume, and mix. Transfer the entire sample or an accurately measured aliquot containing about 250 µg to a tall-form, 400-mL beaker containing about 5 g of anhydrous sodium sulfate. Stir for 2 minutes, then decant the solution into a second 400-mL beaker. Rinse the sodium sulfate with three 25-mL portions of *Ether*, adding each rinse to the main portion. Reduce the total volume to about 30 mL by evaporation on a steam bath, and transfer the concentrate to a small, round-bottom evaporation flask. Rinse the beaker with three 10-mL portions

of *Ether*, adding the rinsings to the flask. With the aid of vacuum in a water bath at a temperature not exceeding 40°, or with a stream of nitrogen at room temperature, remove the remaining solvent completely. Dissolve the residue in a small amount of *Solvent Hexane*, transfer to a 10-mL volumetric flask, dilute with *Solvent Hexane* to volume, and mix, to obtain the *Sample Preparation.*

Procedure—

FIRST COLUMN CHROMATOGRAPHY—Just as the 2 mL of isooctane disappears into the surface of the prepared *First Column*, pipet 2 mL of the *Sample Preparation* onto the column. As the meniscus of the *Sample Preparation* reaches the column surface, add the first of three 2-mL portions of *Solvent Hexane*, adding each succeeding portion as the preceding portion disappears into the column. Continue adding *Solvent Hexane* in portions of 5 to 10 mL until 100 mL has been added. If necessary, adjust the flow rate to between 3 and 6 mL per minute, by application of gentle pressure at the top of the chromatographic tube.

Discard the first 20 mL of effluent, and collect the remainder. Examine the column under ultraviolet light at intervals during the chromatography, and stop the flow when the front of the fluorescent band representing vitamin A is about 5 mm from the bottom of the column. (The ultraviolet lamp should provide *weak* radiation in the 300-nm region. It is frequently necessary to use a narrow aperture or screen with commercial lamps to reduce the amount of radiation to the minimum required to visualize the vitamin A on the column.)

Transfer the eluate to a suitable evaporation flask, and remove the solvent hexane completely under vacuum at a temperature not above 40° or with a stream of nitrogen at room temperature. Dissolve the residue in about 10 mL of *Solvent Hexane.*

SECOND COLUMN CHROMATOGRAPHY—Add the solvent hexane solution obtained as directed under *First Column Chromatography* onto the *Second Column.* Rinse the evaporation flask with a total of 10 mL of *Solvent Hexane* in small portions, adding each portion to the *Second Column* and allowing it to flow through the column, and discard the effluent. When about 1 mL of the hexane remains above the surface of the column, add 75 mL of benzene, and elute with the aid of gentle suction (about 125 mm of mercury), collecting the eluate. Evaporate the benzene under vacuum at a temperature not above 40°, or with a stream of nitrogen at room temperature.

ASSAY PREPARATION—Dissolve the residue obtained as directed under *Second Column Chromatography* in a small amount of *Ethylene Dichloride*, transfer to a 10-mL volumetric flask, dilute with *Ethylene Dichloride* to volume, and mix, to obtain the *Assay Preparation.*

COLOR DEVELOPMENT—Into each of three suitable, matched colorimeter tubes of about 20-mm (inside) diameter, and designated *1*, *2*, and *3*, respectively, pipet 1 mL of the *Assay Preparation.* Into tube *1* pipet 1 mL of the *Standard Preparation*, into tube *2*, 1 mL of *Ethylene Dichloride*, and into tube *3*, 1 mL of a mixture of equal volumes of acetic anhydride and *Ethylene Dichloride.* To each tube add quickly, and preferably from an automatic pipet, 5.0 mL of *Color Reagent*, and mix. After 45 seconds, accurately timed, following the addition of the color reagent, determine the absorbances of the three solutions at 500 nm, with a suitable spectrophotometer, using *Ethylene Dichloride* as the blank. Similarly, 45 seconds after making the first reading on each solution, determine the absorbances of the solutions in tubes *2* and *3* at 550 nm, in a similar manner. Designate the absorbances as A^1_{500}, A^2_{500}, A^3_{500}, A^2_{550}, and A^3_{550}, respectively, in which the superscript indicates the number of the tube and the subscript the wavelength.

Calculation—Calculate the quantity, in µg, of vitamin D in the portion of the sample taken by the formula:

$$(C_S/C)(A_U/A_S),$$

in which C_S is the concentration of vitamin D, in µg per mL, of the *Standard Preparation*, C is the concentration of the sample (as g, capsules, tablets, etc.) in each mL of the final solution, A_U has the value of $(A^2_{500} - A^3_{500}) - 0.67(A^2_{550} - A^3_{550})$ determined from the absorbances observed on the solution from the *Assay Preparation*, and A_S has the value of $A^1_{500} - A^2_{500}$ determined on the solutions from the *Standard Preparation.*

Biological Method

The biological assay of vitamin D comprises the recording and interpretation of observations on groups of rats maintained on specified dietary regimens throughout specified periods of their lives whereby the biological response to the preparation under assay is compared with the response to USP Vitamin D Capsules RS.

Reference Standard—*USP Cholecalciferol Reference Standard*—Store in a cold place, protected from light. Allow it to attain room temperature before opening ampul. Use the material promptly, and discard the unused portion.

Preliminary Period—Throughout the preliminary period in the life of a rat, which is not longer than 30 days and extends from birth to the first day of the depletion period, maintain litters of rats under the immediate supervision of, or according to the directions of, the individual responsible for the assay. During the preliminary period, use a dietary regimen that provides for normal development but is limited in its content of vitamin D, so that when placed upon the *Rachitogenic Diet* in the depletion period the rats develop rickets. At the end of the preliminary period, reject any rat that weighs less than 44 g or more than 60 g, or that shows evidence of injury, disease, or anatomical abnormality.

Depletion Period—Through the depletion period, which extends from the end of the preliminary period to the first day of the assay period, provide each rat ad libitum with the *Rachitogenic Diet* and water, and allow access to no other food or dietary supplement.

Rachitogenic Diet—The *Rachitogenic Diet* consists of a uniform mixture of the following ingredients in the proportions shown in the accompanying table.

Rachitogenic Diet

Ingredient	Parts by weight
Whole yellow corn, ground	76
Wheat gluten, ground	20
Calcium carbonate	3
Sodium chloride	1

When a chemical analysis of the entire ration shows a Ca:P ratio of less than 4:1 or more than 5:1, the proportion of calcium carbonate may be varied to bring the adjusted ratio to a uniform level within this range.

Assigning Rats to Groups for Assay Period—Consider a litter suitable for the assay period when individual rats in the litter show evidence of rickets such as enlarged joints and a distinctive wobbly, rachitic gait, provided that the depletion period is not less than 19 or more than 25 days. The presence of rickets may be established also from the width of the rachitic metaphysis upon X-ray examination or by applying the *Line Test* (described below) to a leg bone of one member of each litter.

Record the weight of each rat, and assign it to a group, in which each rat will be fed a specified dose of the Reference Standard or of an assay sample that is under examination for its vitamin D potency. For each assay sample provide one or more assay groups and not less than two standard groups. The two standard groups may be used for the concurrent assay of more than one assay sample. Within an interval not exceeding 30 days, complete the assignment of rats to groups according to a design that divides litters among the groups, to achieve a complete balance.

For complete balance, whereby each litter is represented equally in every group, use 7 or more litters containing at least as many depleted rats as there are groups. From a given litter, assign one rat, selected at random, to each group on the same day. If a litter contains twice as many rats as there are groups, assign a second series of rats similarly. The last one or two litters to be assigned may be allotted to groups so that at the start of the assay period the average body weight of any completed groups will not differ by more than 8 g from that of any other group.

Assay Doses—Select two dosage levels of the USP Cholecalciferol RS, spaced so that the ratio of the larger to the smaller dose is not less than 1.5 or more than 2.5. Select one or two dosage levels based upon a single assumed potency for each sample. The dosage levels of the sample are equivalent to those of

the standard or to a mid-level equal to the square root of the product of the two dosage levels of the standard.

Select dosage levels such that, when fed to rachitic rats, they are expected to produce degrees of calcification within the range specified under the test of data acceptability. Before feeding, the Reference Standard and/or sample may be diluted with cottonseed oil, provided that not more than 0.2 mL is fed on any one day. Store the oil solutions in well-closed bottles, protected from light, at a temperature not exceeding 10°, and use within 5 weeks.

Assign one group of rats to each dosage level of the standard and of the one or more samples.

Assay Period—During the assay period, which extends from the end of the depletion period for a fixed interval of 7 to 10 days, cage each rat individually and provide it ad libitum with the *Rachitogenic Diet* and water. Supply a *Rachitogenic Diet* prepared from the same lots of ingredients to all rats. On the first and on the third (or fourth) day of the assay period, feed each rat one-half of its total assigned dose.

Throughout the assay period, maintain as uniform environmental conditions as possible for all rats, and exclude exposure to antirachitic radiations. At the end of a fixed period of 7 to 10 days, weigh and kill each rat. From those rats that do not weigh less at the end than at the start of the assay period and that have consumed each assigned dose within 24 hours of the time it was fed, dissect out one or more leg bones for examination by the *Line Test*.

Line Test—Remove the proximal end of a tibia or the distal end of a radius, and clean adhering tissue from it, in any one assay using the same bone from all animals. With a clean, sharp blade cut a median, longitudinal section through the juncture of the epiphysis and diaphysis at the same place on each bone. Rinse both sections in purified water, immerse immediately in silver nitrate solution (1 in 50) for 1 minute, and rinse again in purified water. Expose the cut surface of bone, in water, to daylight or another source of actinic light until the calcified areas develop a clearly defined stain without marked discoloration of the uncalcified areas. The staining procedure may be modified to differentiate more clearly between calcified and uncalcified areas.

Score the degree of calcification of the rachitic metaphysis in each rat, according to a scale that allows the average response to be plotted as a straight line against the logarithm of the dose.

Acceptability—Observations are acceptable for use in calculation of the potency only from those groups in which two-thirds or more but not less than 7 rats show calcification at least as great as the lowest level and not greater than the highest level illustrated in the figure. If the average score of the standard group on the high dosage level is not greater than the average score of the standard group on the low dosage level, discard the results; and repeat the assay. If an assay sample is represented solely by assay groups that are not acceptable for measuring vitamin D potency and in each of which the average score is less than the average score of the standard group on the low dosage level or more than the average score of the standard group on the high dosage level, its assayed content of vitamin D is respectively less than that represented by the low dose or more than that represented by the high dose of the Reference Standard.

Calculation—Tabulate the scores (y), listing each litter in a separate row with treatment groups in columns. Omit any groups that do not meet the test for *Acceptability*. Equalize the number of observations in the acceptable groups by disregarding the results on all litters not equally represented in the groups or by other suitable means (see *Design and Analysis of Biological Assays* ⟨111⟩). Total the f scores for each of the treatment groups, where f is the number of litters, and designate each total as T with subscripts 1 and 2 for the low and high dosage levels, respectively. Compute the slope b from the sums of T_1, i.e., ΣT_1, and of T_2, i.e., ΣT_2, for the standard and sample, provided the latter is represented at both dosage levels, from the equation:

$$b = (\Sigma T_2 - \Sigma T_1)/ifh',$$

in which i is the logarithm of the ratio of the high dose to the low dose and is the same for each preparation, and h' is the number of preparations represented by two dosage levels and included in the calculation of the value of b.

Compute the logarithm of the relative potency of each specimen under assay from the equation:

$$\begin{aligned}\log\ (\text{relative potency}) &= M' \\ &= (\bar{y}_U - \bar{y}_S)/b \\ &= ih'T_a/2\Sigma T_b,\end{aligned}$$

in which each mean score, $\bar{y}_U$ for the assay sample and $\bar{y}_S$ for the Reference Standard, is the average of the individual scores for an intermediate dosage level or of the two means for the high and the low dosage levels and where $T_b = \Sigma T_2 - \Sigma T_1$ and T_a is as defined (see *Design and Analysis of Biological Assays* ⟨111⟩). Convert each observed M' to its antilogarithm to obtain the relative potency of the sample. Multiply the relative potency by the assumed potency of the assay oil in Units per g, adopted at the start of the assay, to obtain its assayed content of vitamin D in USP Units per g.

⟨591⟩ ZINC DETERMINATION

The need for a quantitative determination of zinc in the Pharmacopeial insulin preparations reflects the fact that the element is an essential component of zinc-insulin crystals. In common with lead, zinc may be determined either by the dithizone method or by atomic absorption.

Dithizone Method

Select all reagents for this test to have as low a content of heavy metals as practicable. If necessary, distil water and other solvents into hard or borosilicate glass apparatus. Rinse thoroughly all glassware with warm dilute nitric acid (1 in 2) followed by water. Avoid using on the separator any lubricants that dissolve in chloroform.

Special Solutions and Solvents—

ALKALINE AMMONIUM CITRATE SOLUTION—Dissolve 50 g of dibasic ammonium citrate in water to make 100 mL. Add 100 mL of ammonium hydroxide. Remove any heavy metals that may be present by extracting the solution with 20-mL portions of *Dithizone Extraction Solution* (see *Lead* ⟨251⟩) until the dithizone solution retains a clear green color, then extract any dithizone remaining in the citrate solution by shaking with chloroform.

CHLOROFORM—Distil chloroform in hard or borosilicate glass apparatus, receiving the distillate in sufficient dehydrated alcohol to make the final concentration 1 mL of alcohol for each 100 mL of distillate.

DITHIZONE SOLUTION—Use *Standard Dithizone Solution* (see *Lead* ⟨251⟩), prepared with the distilled *Chloroform*.

STANDARD ZINC SOLUTION—Dissolve 625 mg of zinc oxide, accurately weighed, and previously gently ignited to constant weight, in 10 mL of nitric acid, and add water to make 500.0 mL. This solution contains 1.0 mg of zinc per mL.

DILUTED STANDARD ZINC SOLUTION—Dilute 1 mL of *Standard Zinc Solution*, accurately measured, with 2 drops of nitric acid and sufficient water to make 100.0 mL. This solution contains 10 µg of zinc per mL. Use this solution within 2 weeks.

TRICHLOROACETIC ACID SOLUTION—Dissolve 100 g of trichloroacetic acid in water to make 1000 mL.

Procedure—Transfer 1 to 5 mL of the preparation to be tested, accurately measured, to a centrifuge tube graduated at 40 mL. If necessary, add 0.25 *N* hydrochloric acid, dropwise, to obtain a clear solution. Add 5 mL of *Trichloroacetic Acid Solution* and sufficient water to make 40.0 mL. Mix, and centrifuge.

Transfer to a hard-glass separator an accurately measured volume of the supernatant liquid believed to contain from 5 to 20 µg of zinc, and add water to make about 20 mL. Add 1.5 mL of *Alkaline Ammonium Citrate Solution* and 35 mL of *Dithizone Solution*. Shake vigorously 100 times. Allow the chloroform phase to separate. Insert a cotton plug in the stem of the separator to remove any water emulsified with the chloroform. Collect the chloroform extract (discarding the first portion that comes through) in a test tube, and determine the absorbance at 530 nm, with a suitable spectrophotometer.

Calculate the amount of zinc present by reference to a standard absorbance-concentration curve obtained by using 0.5 mL, 1.0 mL, 1.5 mL, and, if the zinc content of the sample extracted exceeds 15 µg, 2.0 mL of the *Diluted Standard Zinc Solution*, corrected as indicated by a blank determination run concomitantly, using all of the reagents but no added zinc.

Physical Tests and Determinations

⟨601⟩ AEROSOLS

Delivery Rate—Select not less than four aerosol containers, shake, if the label includes this directive, remove the caps and covers, and actuate each valve for 2 to 3 seconds. Weigh each container accurately, and immerse in a constant-temperature bath until the internal pressure is equilibrated at a temperature of 25 ± 1° as determined by constancy of internal pressure as directed under *Pressure Testing*. Remove the containers from the bath, remove the excess moisture by blotting with a paper towel, shake, if the label includes this directive, actuate each valve for 5.0 seconds (accurately timed by use of a stopwatch), and weigh each container again. Return the containers to the constant-temperature bath, and repeat the foregoing procedure three times for each container. Calculate the average delivery rate, in g per second, for each container.

Leak Testing—Select 12 aerosol containers, and record the date and time to the nearest half-hour. Weigh each container to the nearest mg, and record the weight, in mg, of each as W_1. Allow the containers to stand in an upright position at room temperature for not less than 3 days, and again weigh each container, recording the weight, in mg, of each as W_2 and recording the date and time to the nearest half-hour. Determine the time, T, in hours, during which the containers were under test. Calculate the leakage rate, in mg per year, of each container by the formula:

$$(365)(24/T)(W_1 - W_2).$$

Where plastic-coated glass aerosol containers are tested, dry the containers in a desiccator for 12 to 18 hours, and allow them to stand in a constant-humidity environment for 24 hours prior to determining the initial weight as indicated above. Conduct the test under the same humidity conditions.

Empty the contents of each container tested by employing any safe technique; e.g., chill to reduce the internal pressure, remove the valve, and pour. Remove any residual contents by rinsing with suitable solvents, then rinse with a few portions of methanol. Retain as a unit the container, the valve, and all associated parts, and heat them at 100° for 5 minutes. Cool, weigh, record the weight as W_3, and determine the net fill weight ($W_1 - W_3$) for each container tested.

The requirements are met if the average leakage rate of the 12 containers is not more than 3.5% of the net fill weight per year, and none of the containers leaks more than 5.0% of the net fill weight per year. If 1 container leaks more than 5.0% per year, and if none of the containers leaks more than 7.0% per year, determine the leakage rate of an additional 24 containers as directed herein. Not more than 2 of the 36 containers leak more than 5.0% of the net fill weight per year, and none of the 36 containers leaks more than 7.0% of the net fill weight per year.

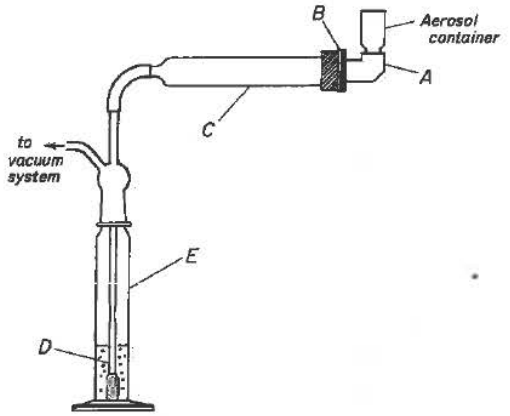
Where the net fill weight is less than 15 g and the label bears an expiration date, the requirements are met if the average leakage rate of the 12 containers is not more than 525 mg per year, and none of the containers leaks more than 750 mg per year. If 1 container leaks more than 750 mg per year, but not more than 1.1 g per year, determine the leakage rate of an additional 24 containers as directed herein. Not more than 2 of the 36 containers leak more than 750 mg per year, and none of the 36 containers leaks more than 1.1 g per year.

This test is in addition to the customary in-line leak testing of each container.

Pressure Testing—Select not less than four aerosol containers, remove the caps and covers, and immerse in a constant-temperature bath until the internal pressure is constant at a temperature of 25 ± 1°. Remove the containers from the bath, shake well, and remove the actuator and water, if any, from the valve stem. Place each container in an upright position, and determine the pressure in each container by placing a pre-pressurized gauge on the valve stem, holding firmly, and actuating the valve so that it is fully open. The gauge is of a calibration approximating the expected pressure and is fitted with an adapter appropriate for the particular valve stem dimensions. Read the pressure direct from the gauge.

Unit Spray Sampling Apparatus—The apparatus described herein is employed, where indicated in the individual monograph, to obtain a test specimen from metered-dose aerosols through the inhalation actuators provided.

The apparatus consists of an intake system comprising the inhalation actuator (*A*), intake adapter (*B*), and intake tube (*C*, approximately 5 cm × 15 cm, drawn to 8 mm at one end); a delivery tube to which is attached a coarse-porosity, sintered-glass dispersion bubbler (*D*); a collection chamber (*E*, gas-washing bottle) that contains an absorbing solution; and a vacuum system comprising a vacuum source, a flow-regulator, and a flowmeter. The intake adapter is constructed to provide the necessary coupling to the inhalation actuator provided with the aerosol under test. To avoid loss of the drug into the atmosphere when the aerosol is discharged, air is drawn continuously at the rate of 12 ± 1 liters per minute through the intake system into the collection chamber and absorbing solution by means of a suitable vacuum system. Alternatively, an apparatus embodying the principle of the assembly described and illustrated may be used.



Apparatus for Metered-dose Aerosols

Propellants—

Caution—Hydrocarbon propellants are highly flammable and explosive. Observe precautions and perform sampling and analytical operations in a well-ventilated fume hood.

General Sampling Procedure—This procedure is used to obtain test specimens for those propellants that occur as gases at about 25° and that are stored in pressurized cylinders. Use a stainless steel specimen cylinder, equipped with a stainless steel valve, having a capacity of not less than 200 mL and a pressure rating of 240 psi or more. Dry the cylinder with the valve open at 110° for 2 hours, and evacuate the hot cylinder to less than 1 mm of mercury. Close the valve, cool, and weigh. Connect one end of a charging line tightly to the propellant container and the other end loosely to the specimen cylinder. Carefully open the propellant container, and allow the propellant to flush out the charging line through the loose connection. Avoid excessive flushing that causes moisture to freeze in the charging line and connections. Tighten the fitting on the specimen cylinder, and open the specimen cylinder valve, allowing the propellant to flow into the evacuated cylinder. Continue sampling until the desired amount of specimen is obtained, then close the propellant container valve, and finally close the specimen cylinder valve. [*Caution—Do not overload the specimen cylinder.*] Again weigh the charged specimen cylinder, and calculate the specimen weight.

Approximate Boiling Temperature—Transfer a 100-mL specimen to a tared, pear-shaped, 100-mL centrifuge tube containing a few boiling stones, and weigh. Suspend a suitable thermometer in the liquid, and place the tube in a medium maintained at a temperature 32 ± 1° above the expected boiling temperature. When the thermometer reading becomes constant, record as the boiling temperature the thermometer reading after 5% of the

specimen has distilled. Retain the remainder of the specimen for the determination of *High-boiling Residues*.

High-boiling Residues, Method I—Allow 85 mL of the specimen to distil as directed in the test for *Approximate Boiling Temperature*, and transfer the centrifuge tube containing the remaining 15 mL of specimen to a medium maintained at a temperature 10 ± 1° above the boiling temperature. After 30 minutes, remove the tube from the water bath, blot dry, and weigh. Calculate the weight of the residue.

High-boiling Residue, Method II—Prepare a cooling coil from copper tubing (about 6 mm outside diameter × about 6.1 m long) to fit into a suitable vacuum-jacketed flask. Immerse the cooling coil in a mixture of dry ice and acetone in a vacuum-jacketed flask, and connect one end of the tubing to the propellant specimen cylinder. Carefully open the specimen cylinder valve, flush the cooling coil with about 50 mL of the propellant, and discard this portion of liquefied propellant. Continue delivering liquefied propellant from the cooling coil, and collect it in a previously chilled 1000-mL sedimentation cone until the cone is filled to the 1000-mL mark. Allow the propellant to evaporate, using a warm water bath maintained at about 40° to reduce evaporating time. When all of the liquid has evaporated, rinse the sedimentation cone with two 50-mL portions of pentane, and combine the rinsings in a tared 150-mL evaporating dish. Transfer 100 mL of the pentane solvent to a second tared 150-mL evaporating dish, place both evaporating dishes on a water bath, evaporate to dryness, and heat the dishes in an oven at 100° for 60 minutes. Cool the dishes in a desiccator, and weigh. Repeat the heating for 15-minute periods until successive weighings are within 0.1 mg, and calculate the weight of the residue obtained from the propellant as the difference between the weights of the residues in the two evaporating dishes.

Water Content—Proceed as directed under *Water Determination* ⟨921⟩, with the following modifications: (a) Provide the closed-system titrating vessel with an opening through which passes a coarse-porosity gas dispersion tube connected to a sampling cylinder. (b) Dilute the *Reagent* with anhydrous methanol to give a water equivalence factor of between 0.2 and 1.0 mg per mL; age this diluted solution not less than 16 hours before standardization. (c) Obtain a 100-g specimen as directed under *General Sampling Procedure*, and introduce the specimen into the titration vessel through the gas dispersion tube at a rate of about 100 mL of gas per minute; if necessary, heat the specimen cylinder gently to maintain this flow rate.

⟨611⟩ ALCOHOL DETERMINATION

Method I—Distillation Method

Method I is to be used for the determination of alcohol, unless otherwise specified in the individual monograph. It is suitable for examining most fluidextracts and tinctures, provided the capacity of the distilling flask is sufficient (commonly two to four times the volume of the liquid to be heated) and the rate of distillation is such that clear distillates are produced. Cloudy distillates may be clarified by agitation with talc, or with precipitated calcium carbonate, and filtered, after which the temperature of the filtrate is adjusted and the alcohol content determined from the specific gravity. During all manipulations, take precautions to minimize the loss of alcohol by evaporation.

FROTHING—Treat liquids that froth to a troublesome extent during distillation by rendering them strongly acid with phosphoric, sulfuric, or tannic acid, or treat with a slight excess of calcium chloride solution, or with a small amount of paraffin or silicone oil before starting the distillation.

BUMPING—Prevent bumping during distillation by adding porous chips of insoluble material such as silicon carbide, or beads.

For liquids presumed to contain 30% of alcohol or less—By means of a pipet, transfer to a suitable distilling apparatus not less than 25 mL of the liquid in which the alcohol is to be determined, and note the temperature at which the volume was measured. Add an equal volume of water, distil, and collect a volume of distillate about 2 mL less than the volume taken of the original test liquid, adjust to the temperature at which the original test liquid was measured, add sufficient water to measure exactly the original volume of the test liquid, and mix. The distillate is clear or not more than slightly cloudy, and does not contain more than traces of volatile substances other than alcohol and water. Determine the specific gravity of the liquid at 25°, as directed under *Specific Gravity* ⟨841⟩, using this result to ascertain the percentage, by volume, of C_2H_5OH contained in the liquid examined by reference to the *Alcoholometric Table* in the section, *Reference Tables*.

For liquids presumed to contain more than 30% of alcohol—Proceed as directed in the foregoing paragraph, except: dilute the specimen with about twice its volume of water, and collect a volume of distillate about 2 mL less than twice the volume of the original test liquid, bring to the temperature at which the original liquid was measured, add sufficient water to measure exactly twice the original volume of the test liquid, mix, and determine its specific gravity. The proportion of C_2H_5OH, by volume, in this distillate, as ascertained from its specific gravity, equals one-half that in the liquid examined.

Special Treatment—

VOLATILE ACIDS AND BASES—Render preparations containing volatile bases slightly acidic with diluted sulfuric acid before distilling. If volatile acids are present, render the preparation slightly alkaline with sodium hydroxide TS.

GLYCERIN—To liquids that contain glycerin add sufficient water so that the residue, after distillation, contains not less than 50% of water.

IODINE—Treat all solutions containing free iodine with powdered zinc before the distillation, or decolorize with just sufficient sodium thiosulfate solution (1 in 10), followed by a few drops of sodium hydroxide TS.

OTHER VOLATILE SUBSTANCES—Spirits, elixirs, tinctures, and similar preparations that contain appreciable proportions of volatile materials other than alcohol and water, such as volatile oils, chloroform, ether, camphor, etc., require special treatment, as follows:

For liquids presumed to contain 50% of alcohol or less—Mix 25 mL of the specimen under examination, accurately measured, with about an equal volume of water in a separator. Saturate this mixture with sodium chloride, then add 25 mL of solvent hexane, and shake the mixture to extract the interfering volatile ingredients. Draw off the separated, lower layer into a second separator, and repeat the extraction twice with two further 25-mL portions of solvent hexane. Extract the combined solvent hexane solutions with three 10-mL portions of a saturated solution of sodium chloride. Combine the saline solutions, and distil in the usual manner, collecting a volume of distillate having a simple ratio to the volume of the original specimen.

For liquids presumed to contain more than 50% of alcohol—Adjust the specimen under examination to a concentration of approximately 25% of alcohol by diluting it with water, then proceed as directed in the preceding paragraph, beginning with "Saturate this mixture with sodium chloride."

In preparing *Collodion* or *Flexible Collodion* for distillation, use water in place of the saturated solution of sodium chloride directed above.

If volatile oils are present in small proportions only, and a cloudy distillate is obtained, the solvent hexane treatment not having been employed, the distillate may be clarified and rendered suitable for the specific gravity determination by shaking it with about one-fifth its volume of solvent hexane, or by filtering it through a thin layer of talc.

Method II—Gas-liquid Chromatographic Method

Method II is to be used where specified in the individual monograph. For a discussion of the principles upon which it is based, see *Gas Chromatography* under *Chromatography* ⟨621⟩.

Apparatus—Under typical conditions, use a gas chromatograph equipped with a flame-ionization detector and a 1.8-m × 4-mm (ID) glass column packed with 100- to 120-mesh chromatographic column packing No. S3, using nitrogen or helium as the carrier. Prior to use, condition the column overnight at 235° with a slow flow of carrier gas. Adjust the carrier flow and temper-

ature (about 120°) so that acetonitrile, the internal standard, elutes in 5 to 10 minutes.

Solutions—

Standard Solution—Dilute 5.0 mL of dehydrated alcohol with water to 250 mL.

Internal Standard Solution—Dilute 5.0 mL of acetonitrile with water to 250 mL.

Test Solution—Dilute the specimen under examination stepwise with water to obtain a solution containing approximately 2% (v/v) of alcohol.

Test Preparation—Pipet 10 mL each of the *Test Solution* and the *Internal Standard Solution* into a 100-mL volumetric flask, and dilute with water to volume.

Standard Preparation—Pipet 10 mL each of the *Standard Solution* and the *Internal Standard Solution* into a 100-mL volumetric flask, dilute with water to volume, and mix.

Procedure—Inject about 5 µL each of *Test Preparation* and *Standard Preparation*, in duplicate, into the gas chromatograph, record the chromatograms, and determine the peak response ratios. Calculate the percentage of alcohol (v/v) in the specimen under test according to the formula:

$$2R_U/R_SD,$$

in which D is the dilution factor, expressed as a fraction, used in preparing the *Test Solution*, and R_U and R_S are the peak response ratios obtained for the *Test Preparation* and the *Standard Preparation*, respectively.

System Suitability Test—In a suitable chromatogram, the resolution factor, R, is not less than 2, and six replicate injections of the *Standard Preparation* show a relative standard deviation of not more than 4.0% in the ratio of the peak of alcohol to the peak of the internal standard, and the tailing factor of the alcohol peak is not greater than 1.5.

⟨621⟩ CHROMATOGRAPHY

This chapter defines the terms and procedures used in chromatography and provides general information. Specific requirements for chromatographic tests and assays of drug substances and dosage forms, including adsorbant and developing solvents, are given in the individual monographs.

Chromatography is defined as a procedure by which solutes are separated by a dynamic differential migration process in a system consisting of two or more phases, one of which moves continuously in a given direction and in which the individual substances exhibit different mobilities by reason of differences in adsorption, partition, solubility, vapor pressure, molecular size, or ionic charge density. The individual substances thus obtained can be identified or determined by analytical methods.

The general chromatographic technique requires that a solute undergo distribution between two phases, one of them fixed (stationary phase), the other moving (mobile phase). It is the mobile phase that transfers the solute through the medium until it eventually emerges separated from other solutes that are eluted earlier or later. Generally, the solute is transported through the separation medium by means of a flowing stream of a liquid or a gaseous solvent known as the "eluant." The stationary phase may act through adsorption, as in the case of adsorbants such as activated alumina, silica gel, and ion-exchange resins, or it may act by dissolving the solute, thus partitioning the latter between the stationary and mobile phases. In the latter process, a liquid coating held on an inert support serves as the stationary phase. Partitioning is the predominant mechanism of separation in gas-liquid chromatography, paper chromatography, and forms of column chromatography designated as liquid-liquid chromatography. In practice, separations frequently result from a combination of adsorption and partitioning effects.

The types of chromatography useful in qualitative and quantitative analysis that are employed in the USP assays and tests are Column, Gas, Paper, Thin-layer, and Pressurized Liquid Chromatography (commonly called high-pressure or high-performance liquid chromatography). Paper and thin-layer chromatography are ordinarily more useful for purposes of identification, because of their convenience and simplicity. Column chromatography offers a wider choice of stationary phases and is useful for the separation of individual compounds, in quantity, from mixtures. Both gas chromatography and pressurized liquid chromatography require more elaborate apparatus and usually provide high-resolution methods that will identify and quantitate very small amounts of material.

Use of Reference Substances in Identity Tests—In paper and thin-layer chromatography, the ratio of the distance (this distance being measured to the point of maximum intensity of the spot) traveled on the medium by a given compound to the distance traveled by the front of the mobile phase, from the point of application of the test substance, is designated as the R_f value of the compound. The ratio between the distances traveled by a given compound and a reference substance is the R_r value. R_f values vary with the experimental conditions, and thus identification is best accomplished where an authentic specimen of the compound in question is used as a reference substance on the same chromatogram.

For this purpose, chromatograms are prepared by applying on the thin-layer adsorbant or on the paper in a straight line, parallel to the edge of the chromatographic plate or paper, solutions of the substance to be identified, the authentic specimen, and a mixture of nearly equal amounts of the substance to be identified and the authentic specimen. Each sample application contains approximately the same quantity by weight of material to be chromatographed. If the substance to be identified and the authentic specimen are identical, all chromatograms agree in color and R_f value and the mixed chromatogram yields a single spot; i.e., R_r is 1.0.

Location of Components—The spots produced by paper or thin-layer chromatography may be located by: (1) direct inspection if the compounds are visible under white or either short- (254 nm) or long-wavelength (360 nm) ultraviolet light, (2) inspection in white or ultraviolet light after treatment with reagents that will make the spots visible (reagents are most conveniently applied with an atomizer), (3) use of a Geiger-Müller counter or autoradiographic techniques in the case of the presence of radioactive substances, or (4) evidence resulting from stimulation or inhibition of bacterial growth by the placing of removed portions of the adsorbant and substance on inoculated media.

In open-column chromatography, in pressurized liquid chromatography performed under conditions of constant flow rate, and in gas chromatography, the retention time, t, defined as the time elapsed between sample injection and appearance of the peak concentration of the eluted sample zone, may be used as a parameter of identification. Solutions of the substance to be identified or derivatives thereof, of the reference compound, and of a mixture of equal amounts of these two are chromatographed successively on the same column under the same chromatographic conditions. Only one peak should be observed for the mixture. The ratio of the retention times of the test substance, the reference compound, and a mixture of these, to the retention time of an internal standard is called the relative retention time R_r and is also used frequently as a parameter of identification.

The deviations of R_r, R_f, or t values measured for the test substance from the values obtained for the reference compound and mixture should not exceed the reliability estimates determined statistically from replicate assays of the reference compound.

Chromatographic identification by these methods under given conditions strongly indicates identity but does not constitute definitive identification. Coincidence of identity parameters under 3 to 6 different sets of chromatographic conditions (temperatures, column packings, adsorbants, eluants, developing solvents, various chemical derivatives, etc.) increases the probability that the test and reference substances are identical. However, many isomeric compounds cannot be separated. Specific and pertinent chemical, spectroscopic, or physicochemical identification of the eluted component combined with chromatographic identity is the most valid criterion of identification. For this purpose, the individual components separated by chromatography may be collected for further identification.

Paper Chromatography

In paper chromatography the adsorbant is a sheet of paper of suitable texture and thickness. Chromatographic separation may proceed through the action of a single liquid phase in a process analogous to adsorption chromatography in columns. Since the

natural water content of the paper, or selective imbibition of a hydrophilic component of the liquid phase by the paper fibers, may be regarded as a stationary phase, a partitioning mechanism may contribute significantly to the separation.

Alternatively, a two-phase system may be used. The paper is impregnated with one of the phases, which then remains stationary (usually the more polar phase in the case of unmodified paper). The chromatogram is developed by slow passage of the other, mobile, phase over the sheet. Development may be ascending, in which case the solvent is carried up the paper by capillary forces, or descending, in which case the solvent flow is also assisted by gravitational force.

Differences in the value of R_f have been reported where chromatograms developed in the direction of the paper grain (machine direction) are compared with others developed at right angles to the grain. Therefore, the orientation of paper grain with respect to solvent flow should be maintained constant in a series of chromatograms. (The machine direction is usually designated by the manufacturer on packages of chromatography paper.)

DESCENDING CHROMATOGRAPHY

In descending chromatography, the mobile phase flows downward on the chromatographic sheet.

Apparatus—The essential equipment for descending chromatography consists of the following.

A *vapor-tight chamber* provided with inlets for addition of solvent or for releasing internal pressure. The chamber is constructed preferably of glass, stainless steel, or porcelain and is so designed as to permit observation of the progress of the chromatographic run without opening of the chamber. Tall glass cylinders are convenient if they are made vapor-tight with suitable covers and a sealing compound.

A *rack of corrosion-resistant material* about 5 cm shorter than the inside height of the chamber. The rack serves as a support for solvent troughs and for antisiphoning rods which, in turn, hold up the chromatographic sheets.

One or more *glass troughs* capable of holding a volume of solvent greater than that needed for one chromatographic run. The troughs must also be longer than the width of the chromatographic sheets.

Heavy glass anti-siphoning rods to be supported by the rack and running outside of, parallel to, and slightly above the edge of the glass trough.

Chromatographic sheets of special filter paper at least 2.5 cm wide and not wider than the length of the troughs are cut to a length approximately equal to the height of the chamber. A fine pencil line is drawn horizontally across the filter paper at a distance from one end such that, when the sheet is suspended from the anti-siphoning rods with the upper end of the paper resting in the trough and the lower portion hanging free into the chamber, the line is located a few centimeters below the rods. Care is necessary to avoid contaminating the filter paper by excessive handling or by contact with dirty surfaces.

Procedure—The substance or substances to be analyzed are dissolved in a suitable solvent. Convenient volumes, delivered from suitable micropipets, of the resulting solution, normally containing 1 to 20 μg of the compound, are placed in 6- to 10-mm spots along the pencil line not less than 3 cm apart. If the total volume to be applied would produce spots of a diameter greater than 6 to 10 mm, it is applied in separate portions to the same spot, each portion being allowed to dry before the next is added.

The spotted chromatographic sheet is suspended in the chamber by use of the anti-siphoning rod, which holds the upper end of the sheet in the solvent trough. The bottom of the chamber is covered with the prescribed solvent system. Saturation of the chamber with solvent vapor is facilitated by lining the inside walls with paper that is wetted with the prescribed solvent system. It is important to ensure that the portion of the sheet hanging below the rods is freely suspended in the chamber without touching the rack or the chamber walls or the fluid in the chamber. The chamber is sealed to allow equilibration (saturation) of the chamber and the paper with the solvent vapor. Any excess pressure is released as necessary. For large chambers, equilibration overnight may be necessary.

A volume of the mobile phase in excess of the volume required for complete development of the chromatogram is saturated with the immobile phase by shaking. After equilibration of the chamber, the prepared mobile solvent is introduced into the trough through the inlet. The inlet is closed and the mobile solvent phase is allowed to travel down the paper the desired distance. Precautions must be taken against allowing the solvent to run down the sheet when opening the chamber and removing the chromatogram. The location of the solvent front is quickly marked, and the sheets are dried.

The chromatogram is observed and measured directly or after suitable development to reveal the location of the spots of the isolated drug or drugs. The paper section(s) predetermined to contain the isolated drug(s) may be cut out and eluted by an appropriate solvent, and the solutions may be made up to a known volume and quantitatively analyzed by appropriate chemical or instrumental techniques. Similar procedures should be conducted with various amounts of similarly spotted reference standard on the same paper in the concentration range appropriate to prepare a valid calibration curve.

ASCENDING CHROMATOGRAPHY

In ascending chromatography the lower edge of the sheet (or strip) is dipped into the mobile phase, to permit the mobile phase to rise on the chromatographic sheet by capillary action.

Apparatus—The essential equipment for ascending chromatography is substantially the same as that described under *Descending Chromatography*.

Procedure—The test materials are applied to the chromatographic sheets as directed under *Descending Chromatography*, and above the level to which the paper is dipped into the developing solvent. The bottom of the developing chamber is covered with the developing solvent system. If a two-phase system is used, both phases are added. It is also desirable to line the walls of the chamber with paper and to saturate this lining with the solvent system. Empty solvent troughs are placed on the bottom of the chamber, and the chromatographic sheets are suspended so that the end on which the spots have been added hangs free inside the empty trough.

The chamber is sealed, and equilibration is allowed to proceed as described under *Descending Chromatography*. Then the developing solvent (mobile phase) is added through the inlet to the trough in excess of the solvent required for complete moistening of the chromatographic sheet. The chamber is re-sealed. When the solvent front has reached the desired height, the chamber is opened and the sheet is removed and dried.

Quantitative analyses of the spots may be conducted as described under *Descending Chromatography*.

Thin-layer Chromatography

In thin-layer chromatography, the adsorbant is a relatively thin, uniform layer of dry, finely powdered material applied to a glass, plastic, or metal sheet or plate, glass plates being most commonly employed. The coated plate can be considered an "open chromatographic column" and the separations achieved may be based upon adsorption, partition, or a combination of both effects, depending on the particular type of support, its preparation, and its use with different solvents. Thin-layer chromatography on ion-exchange films can be used for the fractionation of polar compounds. Presumptive identification can be effected by observation of spots of identical R_f value and about equal magnitude obtained, respectively, with an unknown and a reference sample chromatographed on the same plate. A visual comparison of the size of the spots may serve for semiquantitative estimation. Quantitative measurements are possible by means of densitometry, fluorescence, and fluorescence quenching; or, the spots may be carefully removed from the plate, followed by elution with a suitable solvent and spectrophotometric measurement. For two-dimensional thin-layer chromatography, the chromatographed plate is turned at a right angle and again chromatographed, usually in another chamber equilibrated with a different solvent system.

Apparatus—Acceptable apparatus and materials for thin-layer chromatography consist of the following

Flat *glass plates* of convenient size, typically 20 cm × 20 cm.[1]

An *aligning tray* or a flat surface upon which to align and rest the plates during the application of the adsorbant.

[1] Commercially prepared plates may be substituted for plates prepared as directed herein.

A *storage rack* to hold the prepared plates during drying and transportation. The rack holding the plates should be kept in a desiccator or be capable of being sealed in order to protect the plates from the environment after removal from the drying oven.

The *adsorbant* consists of finely divided adsorbent materials, normally 5 μm to 40 μm in diameter, suitable for chromatography. It can be applied directly to the glass plate or can be bonded to the plate by means of plaster of Paris (hydrated calcium sulfate) [at a ratio of 5 to 15%] or with starch paste or other binders. The former will not yield as hard a surface as will the starch, but it is not affected by strongly oxidizing spray reagents. The adsorbant may contain fluorescing material to aid in the visualization of spots that absorb ultraviolet light.

A *spreader*, which, when moved over the glass plate, will apply a uniform layer of adsorbant of desired thickness over the entire surface of the plate.

A *developing chamber* that can accommodate one or more plates and can be properly closed and sealed as described under *Ascending Chromatography*. The chamber is fitted with a *plate-support rack* that supports the plates, back to back, with the lid of the chamber in place.

A *template* (generally made of plastic) to aid in placing the test spots at definite intervals, to mark distances as needed, and to aid in labeling the plates.

A graduated *micropipet* capable of delivering 10-μL quantities. Total quantities of test and standard solutions are specified in the individual monograph.

A *reagent sprayer* that will emit a fine spray and will not itself be attacked by the reagent.

An *ultraviolet light source* suitable for observations with short (254 nm) and long (360 nm) ultraviolet wavelengths.

Procedure—[NOTE—In this procedure, use purified water that is obtained by distillation.] Clean the plates scrupulously, as by immersion in chromic acid cleansing mixture, rinsing them with copious quantities of water until the water runs off the plates without leaving any visible water or oily spots, then dry. It is important that the plates be completely free from lint and dust when the adsorbant is applied.

Arrange the plate or plates on the aligning tray, place a 5- × 20-cm plate adjacent to the front edge of the first square plate and another 5- × 20-cm plate adjacent to the rear edge of the last square, and secure all of the plates so that they will not slip during the application of the adsorbant. Position the spreader on the end plate opposite to the raised end of the aligning tray. Mix 1 part of adsorbant with 2 parts of water (or in the ratio suggested by the supplier) by shaking vigorously for 30 seconds in a glass-stoppered conical flask, and transfer the slurry to the spreader. Usually 30 g of adsorbant and 60 mL of water are sufficient for five 20- × 20-cm plates. Complete the application of adsorbants using plaster of Paris binder within 2 minutes of addition of the water, since thereafter the mixture begins to harden. Draw the spreader smoothly over the plates toward the raised end of the aligning tray, and remove the spreader when it is on the end plate next to the raised end of the aligning tray. (Wash away all traces of adsorbant from the spreader immediately after use.) Allow the plates to remain undisturbed for 5 minutes, then transfer the square plates, layer side up, to the storage rack, and dry at 105° for 30 minutes. Preferably place the rack at an angle in the drying oven to prevent the condensation of moisture on the back side of plates in the rack. When the plates are dry, allow them to cool to room temperature, and inspect the uniformity of the distribution and the texture of the adsorbant layer; transmitted light will show uniformity of distribution, and reflected light will show uniformity of texture. Store the satisfactory plates over silica gel in a suitable chamber.

Place two filter-paper wicks, 18 cm in height and as wide as the length of the developing chamber, into the chamber, add about 100 mL of the solvent (sufficient to have a depth of 5 mm to 10 mm at the bottom of the chamber), seal the cover to the top of the chamber, and allow the system to equilibrate; it is essential that the wicks become completely wet. Alternatively, the chamber may be completely lined with filter paper. In either case, assure that the filter paper dips into the solvent at the bottom of the chamber. Where vapor saturation of the chamber by these methods is undesirable, it is so indicated in the individual monograph.

Apply the *Test solution* and the *Standard solution*, as directed in the individual monograph, at points about 1.5 cm apart and about 2 cm from the lower edge of the plate (the lower edge is the first part over which the spreader moved in the application of the adsorbant layer), and allow to dry. Avoid physical disturbance of the adsorbant during the spotting procedure (by the pipet or other applicator) or when handling the plates. The template will aid in determining the spot points and the 10- to 15-cm distance through which the solvent front should pass.

Place a mark 10 cm to 15 cm above the spot point. Arrange the plate on the supporting rack (test spots toward the bottom), and introduce the rack into the developing chamber. Allow the solvent in the chamber to reach the lower edge of the adsorbant, but do not allow the spot points to be immersed. Put the cover in place, and maintain the system until the solvent ascends to a point 10 cm to 15 cm above the initial spots, this usually requiring about 15 minutes to 1 hour. Remove the plate from the developing chamber, mark the solvent front, air-dry the plates, and observe first under short-wavelength ultraviolet light (254 nm) and then under long-wavelength ultraviolet light (360 nm). Measure and record the distance of each spot from the point of origin, and indicate for each spot the wavelength under which it was observed. Determine the R_f values for the principal spots (see *Glossary of Symbols*). If further directed, spray the spots with the reagent specified, observe, and compare the test chromatogram with the standard chromatogram.

Continuous Development Thin-layer Chromatography

In contrast to conventional thin-layer chromatography, which is carried out in a closed tank, the continuous development or continuous flow technique allows the upper end of the plate to project through a slot in the cover of the developing chamber. When the developing solvent reaches the slot, continuous evaporation occurs, producing a steady flow of solvent over the plate. In conventional thin-layer chromatography, spot migration ceases when the solvent reaches the top of the plate, after which the spots simply enlarge by diffusion. In the continuous flow process, spot migration continues as long as the plate remains in the tank and the developing solvent is not exhausted.

Development may be continued for several hours after the solvent reaches the top of the plate, to provide adequate migration of the spots. Usually spots of a standard solution, a test solution, and a mixture of equal amounts of test and standard solutions, are initially applied at a standard distance from the base of the plate. Identity of the standard and test substances is confirmed by their migrating equal distances from the origin and by the observation that the two substances applied as a mixture show no tendency to separate.

A major advantage of continuous development thin-layer chromatography stems from the greater solvent selectivity for solvents of low solvent strength. Solvent strength refers to the property of a developing solvent that causes solutes to migrate, and it is strongly influenced by the polarity of the solvent. Increasing the solvent strength by adding a more polar solvent causes the R_f value to increase. Solvent selectivity refers to the ability of a solvent system to produce different R_f values for closely related substances. In conventional thin-layer chromatography, a solvent system giving an R_f value in the range of 0.3 to 0.7, but with adequate selectivity to permit separation of the substances being examined is usually selected. It is much easier to find solvent systems producing adequate migration than to find those affording adequate selectivity.

Solvent systems of lower strength generally exhibit higher selectivity, but are difficult to employ in conventional thin-layer chromatography because they result in very little migration before the solvent reaches the top of the plate. Migration may be increased, however, by repeated drying and redevelopment of the plate or, more conveniently, by providing means for evaporation of solvent at the top of the plate, which results in continuous development. Two techniques are used: continuous development and short-bed continuous development thin-layer chromatography.

An R_f value cannot be measured in continuous development thin-layer chromatography. Substances may be compared either by their migration distance over a fixed period of time or by comparison with the migration of a standard substance applied to the plate. The comparison may be expressed as a relative retention, R_r (see *Glossary of Symbols*).

Continuous development—*Continuous development* thin-layer chromatography as required for antibiotics is described in the Appendix to USP XX, *Antibiotic Regulations*.

APPARATUS—Acceptable apparatus and materials for continuous development thin-layer chromatography are the same as those described under conventional *Thin-layer Chromatography*, except as follows:

A *developing chamber* is used that consists of a rectangular tank, approximately 23 cm × 23 cm × 9 cm, equipped with a glass solvent trough and a platform about 3.75 cm high to elevate the solvent trough above the base of the tank. The chamber is fitted with a cover having a 2 cm × 6 cm slot in the front edge.

PROCEDURE—On a line about 2 cm from the base of the plate, apply the *Standard solution*, the *Test solution*, and a mixture of equal amounts of the *Standard* and *Test solutions*. Place the plate in the elevated empty solvent trough with the adsorbant on the underside of the leaning plate. The adsorbant rests against a piece of heavy (about 1 mm thick)[2] filter paper measuring 20 cm × 3 cm, folded lengthwise and placed over the front edge of the tank. Place the developing solvent in the trough; set the cover in place and seal all openings except where the adsorbant contacts the paper wick. The plate extends about 1 cm beyond the top of the tank. After the solvent reaches the top of the plate, allow development to continue for an appropriate time. Then remove and dry the plate, and detect the spots by suitable means.

Short-bed continuous development—A major advantage of the short-bed technique derives from the fact that solvent velocity is inversely related to bed length. Since spot migration depends upon the total amount of solvent passing over the plate, the short-bed permits useful migration to be obtained in a reasonable time with solvent having very low solvent strength. Lower diffusion in solvents of low solvent strength produces smaller and more dense spots, which enhances both detectability and discernment of small differences in migration distance.

APPARATUS—Acceptable apparatus and materials for short-bed continuous development thin-layer chromatography are the same as those described under conventional *Thin-layer Chromatography*, except as follows:

A shallow *developing chamber*[3] approximately 22 cm × 9 cm × 3 cm, equipped with a cover plate and tight-fitting polytef wings that enable the chamber to be sealed against the plate, is used. The inside bottom of the chamber contains ridges that support the plate and allow it to be inserted at different angles, thereby varying the length of the plate contained within the tank.

PROCEDURE—On a line about 2 cm from the base of the plate, apply the *Standard solution*, the *Test solution*, and a mixture of equal parts of the *Standard* and *Test solutions*. Place the plate in the developing chamber (adsorbant side up), and add the developing solvent to the chamber. No paper wick is employed. After the solvent reaches the top of the plate, allow development to continue for an appropriate time. Then remove and dry the plate, and detect the spots by suitable means.

Column Chromatography

Apparatus—The apparatus required for column chromatographic procedures is simple, consisting only of the chromatographic tube itself and a tamping rod which may be needed to pack a pledget of glass wool or cotton, if needed, in the base of the tube and compress the adsorbant or slurry uniformly within the tube. In some cases a porous glass disk is sealed at the base of the tube in order to support the contents. The tube is cylindrical and is made of glass, unless another material is specified in the individual monograph. A smaller-diameter delivery tube is fused or otherwise attached by a leak-proof joint to the lower end of the main tube. Column dimensions are variable; the dimensions of those commonly used in pharmaceutical analysis range from 10 mm to 30 mm in uniform inside diameter and 150 mm to 400 mm in length, exclusive of the delivery tube. The delivery tube, usually 3 mm to 6 mm in inside diameter, may include a stopcock for accurate control of the flow rate of solvents through the column. The tamping rod, a cylindrical ram firmly attached to a shaft, may be constructed of plastic, glass, stainless steel, or aluminum, unless another material is specified in the individual monograph. The shaft of the rod is substantially smaller in diameter than the column and is not less than 5 cm longer than the effective length of the column. The ram has a diameter about 1 mm smaller than the inside diameter of the column.

[2] Whatman No. 3MM filter paper or equivalent.

[3] Suitable equipment is available from Regis Chemical Company, Morton Grove, IL.

COLUMN ADSORPTION CHROMATOGRAPHY

The adsorbant (such as activated alumina or silica gel, calcined diatomaceous silica, or chromatographic purified siliceous earth) as a dry solid or as a slurry is packed into a glass or quartz chromatographic tube. A solution of the drug in a small amount of solvent is added to the top of the column and allowed to flow into the adsorbant. The drug principles are quantitatively removed from the solution and are adsorbed in a narrow transverse band at the top of the column. As additional solvent is allowed to flow through the column, either by gravity or by application of air pressure, each substance progresses down the column at a characteristic rate resulting in a spatial separation to give what is known as the *chromatogram*. The rate of movement for a given substance is affected by several variables, including the adsorptive power of the adsorbant and its particle size and surface area; the nature and polarity of the solvent; the hydrostatic head or applied pressure; and the temperature of the chromatographic system.

If the separated compounds are colored or if they fluoresce under ultraviolet light, the adsorbant column may be extruded and, by transverse cuts, the appropriate segments may then be isolated. The desired compounds are then extracted from each segment with a suitable solvent. If the compounds are colorless, they may be located by means of painting or spraying the extruded column with color-forming reagents. Chromatographed radioactive substances may be located by means of Geiger-Müller detectors or similar sensing and recording instruments. Clear plastic tubing made of a material such as nylon, which is inert to most solvents and transparent to short-wavelength ultraviolet light, may be packed with adsorbant and used as a chromatographic column. Such a column may be sliced with a sharp knife without removing the packing from the tubing. If a fluorescent adsorbant is used, the column may be marked under ultraviolet light in preparation for slicing.

A "flowing" chromatogram, which is extensively used, is obtained by a procedure in which solvents are allowed to flow through the column until the separated drug appears in the effluent solution, known as the "eluate." The drug may be determined in the eluate by titration or by a spectrophotometric or colorimetric method, or the solvent may be evaporated, leaving the drug in more or less pure form. If a second drug principle is involved, it is eluted by continuing the first solvent or by passing a solvent of stronger eluting power through the column. The efficiency of the separation may be checked by obtaining a thin-layer chromatogram on the individual fractions.

A modified procedure for adding the mixture to the column is sometimes employed. The drug, in a solid form, and, as in the case of a powdered tablet, without separation from the excipients, is mixed with some of the adsorbant and added to the top of a column. The subsequent flow of solvent moves the drug down the column in the manner described.

COLUMN PARTITION CHROMATOGRAPHY

In partition chromatography the substances to be separated are partitioned between two immiscible liquids one of which, the immobile phase, is adsorbed on a *Solid Support*, thereby presenting a very large surface area to the flowing solvent or mobile phase. The exceedingly high number of successive liquid-liquid contacts allows an efficiency of separation not achieved in ordinary liquid-liquid extraction.

The *Solid Support* is usually polar, and the adsorbed immobile phase more polar than the mobile phase. The *Solid Support* that is most widely used is chromatographic siliceous earth having a particle size suitable to permit proper flow of eluant.[4] In *Reverse-phase* partition chromatography the adsorbed immobile phase is less polar than the mobile phase and the solid adsorbant is rendered nonpolar by suitable treatment with a silanizing agent, such as dichlorodimethylsilane, to give silanized chromatographic siliceous earth.

[4] A suitable grade is acid-washed Celite 545, available from Johns-Manville Corp., 22 East 40th St., New York, NY 10016.

The sample to be chromatographed is usually introduced into the chromatographic system in one of two ways: (a) a solution of the sample in a small volume of the mobile phase is added to the top of the column; or, (b) a solution of the sample in a small volume of the immobile phase is mixed with the *Solid Support* and transferred to the column as a layer above a bed of a mixture of immobile phase with adsorbant.

Development and elution are accomplished with flowing solvent as before. The mobile solvent usually is saturated with the immobile solvent before use.

In conventional liquid-liquid partition chromatography, the degree of partition of a given compound between the two liquid phases is expressed by its partition or distribution coefficient. In the case of compounds that dissociate, distribution can be controlled by modifying the pH, dielectric constant, ionic strength, and other properties of the two phases. Selective elution of the components of a mixture can be achieved by successively changing the mobile phase to one that provides a more favorable partition coefficient, or by changing the pH of the immobile phase *in situ* with a mobile phase consisting of a solution of an appropriate acid or base in an organic solvent.

Unless otherwise specified in the individual monograph, assays and tests that employ column partition chromatography are performed according to the following general method.

Solid Support—Use purified siliceous earth. Use silanized chromatographic siliceous earth for reverse-phase partition chromatography.

Stationary Phase—Use the solvent or solution specified in the individual monograph. If a mixture of liquids is to be used as the *Stationary Phase*, mix them prior to the introduction of the *Solid Support*.

Mobile Phase—Use the solvent or solution specified in the individual monograph. Equilibrate it with water if the *Stationary Phase* is an aqueous solution; if the *Stationary Phase* is a polar organic fluid, equilibrate with that fluid.

Preparation of Chromatographic Column—Unless otherwise specified in the individual monograph, the chromatographic tube is about 22 mm in inside diameter and 200 mm to 300 mm in length, without porous glass disk, to which is attached a delivery tube, without stopcock, about 4 mm in inside diameter and about 50 mm in length. Pack a pledget of fine glass wool in the base of the tube. Place the specified volume of *Stationary Phase* in a 100- to 250-mL beaker, add the specified amount of *Solid Support*, and mix to produce a homogeneous, fluffy mixture. Transfer this mixture to the chromatographic tube, and tamp, using gentle pressure, to obtain a uniform mass. If the specified amount of *Solid Support* is more than 3 g, transfer the mixture to the column in portions of approximately 2 g, and tamp each portion. If the assay or test requires a multi-segment column, with a different *Stationary Phase* specified for each segment, tamp after the addition of each segment, and add each succeeding segment directly to the previous one.

If a solution of the analyte is incorporated in the *Stationary Phase*, complete the quantitative transfer to the chromatographic tube by scrubbing the beaker used for the preparation of the test mixture with a mixture of about 1 g of *Solid Support* and several drops of the solvent used to prepare the test solution.

Pack a pledget of fine glass wool above the completed column packing. The *Mobile Phase* flows through a properly packed column as a moderate stream or, if reverse-phase chromatography is applied, as a slow trickle.

Procedure—Transfer the *Mobile Phase* to the column space above the column packing, and allow it to flow through the column under the influence of gravity. Rinse the tip of the chromatographic column with about 1 mL of *Mobile Phase* before each change in composition of *Mobile Phase* and after completion of the elution. If the analyte is introduced into the column as a solution in the *Mobile Phase*, allow it to pass completely into the column packing, then add *Mobile Phase* in several small portions, allowing each to drain completely, before adding the bulk of the *Mobile Phase*. Where the assay or test requires the use of multiple chromatographic columns mounted in series and the addition of *Mobile Phase* in divided portions is specified, allow each portion to drain completely through each column, and rinse the tip of each with *Mobile Phase* prior to the addition of each succeeding portion.

Pressurized Liquid Chromatography

Advances in column technology, high-pressure pumping systems, and sensitive detectors have transformed liquid column chromatography into a high-speed, high-efficiency method of separation. This method is sometimes referred to as HPLC, which is alternatively expressed as either high-performance liquid chromatography or high-pressure liquid chromatography. The column technology is based upon the use of small-bore (2- to 5-mm ID) columns and small-particle (3- to 50-μm) packings that allow fast equilibrium between mobile and stationary phases. This small-particle column technology requires high-pressure pumping systems capable of delivering the mobile phase at high pressure, as much as 300 atmospheres, to achieve flow rates of several mL per minute. Since it is often necessary to use small amounts of analyte (usually less than 20 μg) with the column packings, sensitive detectors are needed. With this technology, liquid chromatography can give high-speed separations comparable in many cases to those achieved by gas chromatography, with the advantage that nonvolatile or thermally unstable materials can be chromatographed without decomposition or the necessity for making volatile derivatives.

One type of stationary phase support used in these packings consists of micro-particles 30 μm to 50 μm in diameter, having a solid center and a thin porous crust. Some of these pellicular support materials can be pre-activated to give them adsorptive properties, while others can be covered with a thin film of stationary phase for partition or ion-exchange separations. The stationary phase can be either a liquid or a polymer, either coated or chemically bonded to the surface of the support in a thin film that reduces mass transfer resistances so that fast equilibrium between mobile and stationary phases can be attained. A liquid stationary phase must be largely immiscible in the mobile phase solvent; it is usually necessary to pre-saturate the mobile phase solvent with the stationary phase liquid to prevent stripping of the stationary phase from the column. Polymer stationary phases coated on the support are more durable. Stationary phases that have been chemically bonded to the support provide greater convenience for use with a variety of solvents and at elevated temperatures.

Other, smaller-diameter packing materials of 3- to 10-μm diameter are almost completely porous and give much more efficient separations than the 30- to 50-μm particle packings. The particles can also be made adsorptive or covered with a stationary phase. It is essential that these packings be slurry-packed in order to obtain high-efficiency columns, in contrast to the 30- to 50-μm particles which can be dry-packed.

The three forms of high-performance liquid chromatography most often used are ion-exchange, partition, and adsorption. Ion-exchange chromatography is used mainly for separation of water-soluble ionic or ionizable materials of molecular weight less than 1500. The stationary phases of ion-exchange chromatography are usually synthetic organic resins having different active groups present. Cation-exchange resins contain negatively charged active sites and are used to separate basic substances such as amines, while anion-exchange resins have positively charged active sites, which will attract substances such as those carrying phosphate, sulfonate, or carboxylate groups which are negatively charged. Water-soluble ionic or ionizable compounds are attracted to the resins, and differences in affinity bring about the chromatographic separation. The pH of the mobile phase, temperature, ion type, ionic concentration, and organic modifiers affect the attraction of the solute, and these variables can be adjusted to obtain the desired degree of separation.

In partition chromatography, mobile and stationary phases of different polarity are used. If the mobile phase is polar and the stationary phase nonpolar, referred to as reverse-phase chromatography, then nonpolar, hydrocarbon-soluble compounds of molecular weight less than 1000, such as fat-soluble vitamins and anthraquinones, can be separated by their affinity for the stationary phase. Modification of the polar mobile phase solvent with a less polar solvent causes a decrease in affinity and the retention of the compounds on the column. If the mobile phase is nonpolar and the stationary phase polar, then polar material such as alcohols and amines can be chromatographed. The nonpolar mobile phase can then be modified with a more polar solvent to decrease retention and change the separation.

A wide range of nonionic compounds can be chromatographed by adsorption chromatography with the choice of the proper sta-

tionary and mobile phases. (For further details of both the partition and absorption techniques, see the preceding sections.)

Apparatus—The liquid chromatograph consists basically of a pumping system, analyte injection device, chromatographic column, detector, amplifier, and recorder. The high-pressure pumping system delivers the mobile phase solvent from the solvent reservoir to the column through high-pressure tubing and fittings. Two means for analyte introduction onto the column are injection into a flowing stream and a "stop-flow" injection. These techniques can be used with either a syringe or an injection valve. For the syringe injection technique, a septum may be used where the column head pressures are less than 70 atmospheres (about 1000 psi). At higher pressures, an injection valve may be used to introduce the test specimen. Some valve systems incorporate a calibrated loop that is loaded with a test specimen and then transferred by the valve system to the flowing stream of mobile phase. Other valve systems permit introduction of the analyte into a cavity using a syringe. The loaded cavity is then switched into the high-pressure stream by the valve system. In the "stop-flow" technique, column flow is stopped, and after the injection port pressure drops to zero, the port is opened and the analyte injected with a syringe. The injection port is then closed and pumping is resumed. High pressure is rapidly re-established and little zone-spreading is incurred in the process. The "stop-flow" technique allows better injection reproducibility at higher pressures than does the use of a septum, and the problem of septum deterioration with many solvents is averted.

The columns normally used for analytical separations have small (2- to 4-mm) internal diameters; larger-diameter columns are used for preparative steps. Columns may be heated to give more efficient separations, but only rarely are they used at temperatures above 60° because of potential difficulties resulting from stationary phase degradation or mobile phase volatility at elevated temperatures. Unless otherwise specified in the individual monograph, the column is maintained at ambient temperature.

The detectors commonly used include the ultraviolet photometer, the differential refractometer, and the fluorometer. The low-pressure mercury ultraviolet photometer is a common and stable detector, but its use is limited to the detection of materials that absorb radiation at a wavelength of 254 nm. Its limit of sensitivity to compounds that absorb ultraviolet light strongly may be about 1 ng. Compounds that do not absorb light at 254 nm appreciably may often be converted to suitable derivatives that absorb at this wavelength, thereby increasing the range of applicability of the single-wavelength detector. The introduction of spectrophotometers equipped with micro cells and detectors capable of operating at additional wavelengths has extended the scope of ultraviolet detection.

The differential refractometer detects differences between the refractive indexes of the pure solvent and of a solution of the chromatographed test substance in the solvent. While more generally applicable, it is a less sensitive detector having a lower limit of about 1 μg and is responsive to small changes in solvent composition, flow rate, and temperature, so that a reference column and flow of mobile phase may be required to give a satisfactory baseline.

The fluorometer is a sensitive detector for compounds that are inherently fluorescent or that can be converted to fluorescent derivatives either by chemical transformation of the compound or by coupling with fluorescent reagents at specific functional groups.

With some reagents, derivatization is carried out prior to chromatographic separation of the derivatives. In another approach, the reagent is introduced into the eluent stream and reacts with the analyte *in situ*, and the derivative is then exposed to the detector.

An electrochemical detector employing carbon-paste electrodes mounted in a thin-film cell of very small volume may advantageously be employed to measure very small amounts (1 ng) of easily oxidized compounds, particularly phenols and catechols.

In general, the signal from the detector is amplified before being fed to a suitable automatic recording device, usually a strip-chart potentiometric recorder, where the signal is plotted versus time. The signal may go also to an electronic digital integrator for the automatic measurement of chromatogram peak areas.

The mobile phase composition significantly influences chromatographic performance and should be controlled carefully. The composition can have a far greater effect on capacity factors (k or ratio of amount of time spent in stationary phase to time spent in the mobile phase; see under *Gas Chromatography*) than the temperature.

In partition and adsorption chromatography, the mobile phase may be modified with another solvent, while in ion-exchange chromatography, both pH and ionic strength as well as modification of the solvent can change capacity factors. The technique of continuously changing the solvent composition during the chromatographic run is called gradient elution, or solvent programming, and is sometimes used to chromatograph complex samples having components of greatly differing capacity factors. Detectors that are sensitive to change in solvent composition, such as the differential refractometer, are more difficult to use with the gradient elution technique.

Procedure—The procedures for compound identification and the techniques of calibration and data reduction used in pressurized liquid chromatography are essentially the same as those for gas chromatography (see *Procedure* under *Gas Chromatography*). For accurate quantitative work, it is necessary that the detector have a large linear dynamic range and that the components to be measured be resolved from any interfering substances. The linear dynamic range is defined as the sample size range from the minimum detectable to maximum sample size over which the detector signal response, i.e., the peak responses on the recorder chart, is linearly proportional to the concentration of test substance. For maximum flexibility in quantitative work, this range should be about three orders of magnitude.

Both peak height and area can be related to sample concentration. The term "peak response," designated r_U for sample response and r_S for standard response, has been adopted for use in specifying measurements in chromatography. This encompasses peak areas, peak heights, and other electronic measurements. Peak heights are easy to measure but are greatly influenced by changes in retention time caused by variations in temperature and solvent composition. For these reasons, peak areas are considered to be a more accurate parameter for quantitation. The detector response may be calibrated by relating peak responses to a known concentration of reference standard using either an external or an internal standardization procedure.

One drawback to the method of external calibration, i.e., direct comparison of the peak responses obtained on chromatographing the test specimen and a known concentration of the corresponding Reference Standard, is that the accuracy and precision are dependent upon the reproducibility of analyte injection. Since the reproducibility of injection at high pressure may vary considerably, the better quantitative results usually are obtained when the method for internal calibration is used. An internal standard at known concentration is added both to the test solution and to a solution of the Reference Standard of known concentration, and the ratios of peak responses of drug and internal standard are compared. Because of normal variations in equipment, supplies, and techniques, a system suitability test (see *System Suitability*) may be useful to ensure that a given operating system may be generally applicable.

Gas Chromatography

In gas chromatography, the moving phase is a *gas*. The stationary phase is usually a liquid but may be a solid or a combination of solid and liquid.

In gas-liquid chromatography (GLC), the stationary liquid phase is immobilized as a thin film on a finely divided, inert solid support, such as chromatographic siliceous earth, crushed firebrick, glass beads, or even the inner wall of a small-diameter tube. If the tube is filled with liquid-covered, finely divided solid, it is called a packed column. If the inner wall of a small-diameter tube is coated with the liquid, it is called an open tubular or capillary column. If the inner wall of the open tubular column is treated so as to deposit a porous or irregular support on its surface before coating with the liquid phase, it is called a support coated open tubular (SCOT) column. In gas-solid chromatography (GSC), the identical situation holds except that the liquid phase is absent and the solid is an active adsorbant, such as alumina, silica gel, or carbon. In either case, the mobile phase continuously moves over the stationary phase.

When a vaporized substance is introduced into the gas stream at the head of the column, it is swept into the column and under-

goes distribution between the gas and liquid or solid phases. The distribution process reaches a dynamic equilibration, that is adequately described by an extension of the mathematical treatment of the stepwise process of countercurrent distribution. The behavior of a solute in such a partition process is conveniently defined by a dimensionless partition ratio, k', called the capacity factor, which may be defined alternatively in terms of the relative amounts, or relative residence times, of the substance in the respective phases. The gas phase simply serves to move the substance down the column between excursions into the stationary phase, and *all* substances spend the *same time in the gas phase* in any particular column. The value of the capacity factor, and, therefore, the time in a gas-liquid chromatographic column, depend upon the following considerations: (a) the specific solute; (b) the specific liquid phase; (c) the amount of liquid phase; (d) the temperature; and (e) the gas flow rate. Therefore, a partition ratio exists for each column, solute, and temperature, and in order to reproduce the behavior of a particular solute, every parameter must be carefully reproduced.

Apparatus—The basic apparatus required for gas chromatography is relatively simple. The *carrier gas*, usually available in compressed form in a cylinder fitted with a suitable pressure-reducing valve, is conducted into a flow meter, which is used to reproduce the particular flow found to be satisfactory for the resolution of a particular mixture. Helium, nitrogen, and other inert gases are suitable carriers. The actual carrier gas used is often determined by the characteristics of the detector being used. Since solutes to be chromatographed must be in the vapor phase, the injection port is heated to a temperature high enough to ensure rapid vaporization but not high enough to cause thermal degradation. Most test specimens are injected by syringe through a silicone rubber septum in the injection port. Preferably the test specimen is injected directly into the column packing. Alternatively, the test specimen vapor is mixed with the flowing carrier gas and then swept into the *column*. In pyrolysis gas chromatography, nonvolatile solids are decomposed by heating to several hundred degrees Celsius, and the volatile products produced are passed directly onto the column. It is in the column that the different components of the vaporized test specimen are separated by virtue of their different interactions with the stationary column packing. The tubing that contains the packing usually is made of glass or metal, and is located in a controlled-temperature oven maintained at a selected temperature, which determines the retention time and, to a degree, the resolution and efficiency obtained. Temperature-programmable components allow efficient elution of compounds over a wide range of vapor pressure. As the components emerge individually from the column, they pass through a differential-type *detector*, which indicates the amount of each component leaving the column. The detector temperature is controlled to prevent condensation. The choice of detector is specified in the individual monograph.

Signals from the detector are passed to an *amplifier* or electrometer, which is coupled to an automatic recording device. The resulting record is a signal-time plot, the chromatogram, which is to be used to determine the identities and concentrations of the components. The usual detectors emit a signal proportional to the concentration of the solute in the carrier as it leaves the column, so that the chromatogram for each drug appears as a bell-shaped peak on a time axis. The resulting curves accurately represent the distribution process as it has occurred during the residence time of the solutes in column. Malfunctions in any of these components can degrade the accuracy and precision of measurements.

Detectors—Detectors commonly used for gas chromatography include those that depend upon thermal conductivity, flame-ionization, alkali flame-ionization, electron-capture, and conductivity. For accurate quantitative work, the detector should have a large linear dynamic range. Helium, because of its high thermal conductivity, is the carrier gas of choice for use with a thermal conductivity detector. The thermal conductivity detector is applicable to all organic compounds but has a lower sensitivity and lower dynamic range than some other detectors. Unless otherwise specified in the individual monograph, the use of a flame-ionization detector with either helium or nitrogen carrier gas is assumed. This detector is sensitive to all carbon compounds and has a wide dynamic range. Nitrogen, by virtue of its higher viscosity, reduces zone spreading in the gas phase and may yield higher efficiencies than helium, but the lower viscosity of helium leads to higher carrier gas flow rates at optimum efficiencies and, therefore, to shorter elution times and faster analyses. The alkali flame-ionization detector contains an alkali-metal salt or a glass element containing rubidium or other metal that results in the suppression of the response to carbon, thereby increasing the relative response to nitrogen, sulfur, and phosphorus several fold. It is, therefore, a selective detector which shows little response to hydrocarbons. The electron-capture detector is also selective, showing little response to hydrocarbons and extremely high response to some compounds, such as those containing halogens or some ketones. Depending on the mode of operation, nitrogen or argon containing a small percentage of methane is used as the carrier gas for electron-capture detection. The electron-capture detector is the most sensitive detector available for those compounds to which it responds. The conductivity detection system includes a heated reaction chamber in which compounds are reacted with a reagent gas such as oxygen or hydrogen that converts some compounds to electrically conductive species such as hydrochloric acid or ammonia while simultaneously removing carbon. The conductive species is then trapped in an electrolyte, and the observed change in electrical conductivity is continuously monitored. The conductivity detector can be made selectively responsive to halogens, sulfur, nitrogen, or phosphorus, and provides very high sensitivity.

Combustion of chlorinated solvents, such as chloroform, in the flame-ionization detector produces hydrochloric acid, which in time may damage detector components. Combustion of silicone derivatives produces a deposit of silica within the detector. Frequent inspection and maintenance of the detector is required to obtain optimum performance.

The specified carrier gas flow rate is the flow rate of the gas exiting the column and is usually expressed in mL per minute at atmospheric pressure and room temperature. It is commonly measured, with the column at operating temperature, by the use of a soap-bubble flow-meter attached to the exit of the column. The gas quickly cools and is essentially at room temperature within the flow-meter. It is usually necessary to disconnect the column from the detector to make this measurement. For a given flow rate, the linear flow rate through the column is related to the square of the column diameter. Thus a flow rate of 60 mL per minute for a 4-mm column and a flow rate of 15 mL per minute for a 2-mm column produce comparable linear flow rates in the respective columns and thus give comparable retention times. Unless otherwise specified in the individual monograph, a 30- to 60-mL flow rate is to be used.

Columns—Pharmaceutical analyses usually employ packed columns and, ideally, only the packing influences the relative movement of solutes through the system. Columns should be of glass unless otherwise specified. Columns of various dimensions are used, but normally they are 0.6 m to 1.8 m in length and 2 mm to 4 mm in internal diameter. Low-capacity columns, having about 5% (w/w) or less of liquid phase on the solid support, are preferred for analytical use. High-capacity columns, such as those having 20% (w/w) liquid loadings, may be used for large test specimens and for the determination of low molecular weight compounds such as water. The desired capacity influences the choice of solid supports.

Support materials are available in various mesh-size ranges, with 80- to 100- and 100- to 120-mesh being the most commonly used with 2- to 4-mm diameter columns. The support material should be as inert as possible, particularly for polar drugs being chromatographed on low-capacity, low-polarity liquid phase columns. Reactive supports can result in decomposition, rearrangement, or peak tailing of the solute. Acid-washed, flux-calcined diatomaceous earth is often used for drug analysis. Reactivity of the support is reduced by treatment with a silanizing reagent prior to coating with liquid phase. Supports receiving an additional, alkaline wash should be used with care, since residual alkali decomposes some liquid phases. Polyaromatic porous resins are sometimes specified. They do not require coating with a liquid phase.

Liquid phases are drawn from a wide range of chemical classes, such as polyethylene glycols, high molecular weight esters and amides, hydrocarbons, and silicone gums and fluids (polysiloxanes substituted normally by methyl, phenyl, nitrile, vinyl, or fluoroalkyl groups or mixtures of these). In all cases, batches must be carefully selected for use in gas chromatography. At operating temperatures, even these materials have sufficient vapor pressure

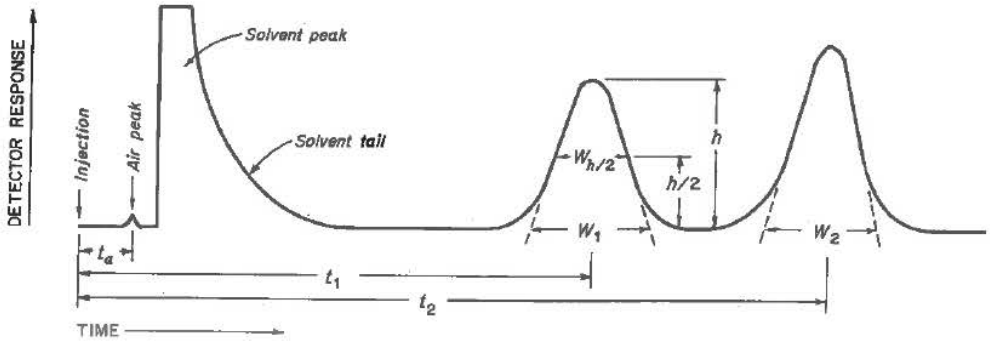


Fig. 1. Chromatographic Separation of Two Substances.

to result in gradual loss of liquid phase by bleeding. Some phases are characterized by quite low bleed rates at operating temperatures and, in such cases, columns may be rejuvenated by repacking the first 10 cm to 15 cm to remove injection residues. Silanized glass wool inserts may be used in the injection ports of some gas chromatographs to trap nonvolatile residues. These should be as small as practicable, to avoid analyte decomposition resulting from the highly active surface.

Figure 1 represents a typical elution chromatographic separation of two substances where t_1 and t_2 are the retention times of substances 1 and 2, h and $h/2$ are the height and half-height of peak 1, $W_{h/2}$ is the width-at-half-height for peak 1, and W_1 and W_2 are the base-widths of peaks 1 and 2, respectively. The air peak is characteristic of gas chromatograms obtained with the thermal conductivity detector and may just precede or even coincide with the front of the solvent peak. A deflection corresponding to the air peak is usually not seen with other gas chromatographic or liquid chromatographic detectors. The interval t_a is the dead time or holdup time and corresponds to the retention time of a nonretarded substance.

Procedure—Since gas chromatography is primarily a separation method, it cannot be used to identify compounds without comparison to a Reference Standard. For qualitative analysis, the retention time for a peak in the chromatogram obtained for a test specimen is "the same as," or "corresponding to," that obtained for a standard preparation under the conditions specified in the individual monograph. The differences in retention times from chromatogram to chromatogram are small, usually less than one-tenth of a minute, as compared with differences between neighboring peaks in the same chromatogram. When a peak appears at the same time or volume under the same experimental conditions, the probability of correct identification is quite high. Alternatively, the individual components may be collected in a cold trap as they emerge from the column for independent analysis by other instrumental or chemical methods, such as mass spectrometry or infrared absorption spectrometry. The retention time or volume for air is an important quantity, since it is used to obtain absolute and relative retention values for characterization of compounds. Drugs may be identified by means of their relative retention, α, determined by the equation

$$\alpha = \frac{t_2 - t_a}{t_1 - t_a}, \qquad (1)$$

in which t_2 is the retention time measured from the point of injection of the desired drug and t_1 is the same for a reference standard material determined with the same column and temperature, and t_a is the retention time for an inert component, such as air, which is not retarded in its passage through the column.

In this and the following expressions written in terms of retention times, the corresponding retention volumes or distances on the chromatogram, both of which are directly proportional to retention time, may be substituted in the equations.

With the flame-ionization detector, which responds to neither air nor water, the retention of a nonretarded compound such as methane, for which natural gas is a convenient source, may be used to estimate t_a. Where t_a is small, α may be estimated from the retentions from the point of injection alone (t_2/t_1).

The capacity factor is related to retention by the equation

$$k'_2 = \frac{t_2}{t_a} - 1. \qquad (2)$$

A measure of the efficiency of a particular column is provided by calculating the number of theoretical plates, n, in the column with the equation

$$n = 16\left(\frac{t}{W}\right)^2, \qquad (3)$$

in which t is the retention time of the substance and W is the width of the base of the peak obtained by extrapolating the relatively straight sides of the peak to the baseline. The value of n is dependent upon the substance being chromatographed as well as the operating conditions such as flow rate and temperature, the quality of the packing, and the uniformity of the packing within the column.

As a measure of efficiency of the separation of two components in a mixture, the resolution, R, is determined by the equation

$$R = \frac{2(t_2 - t_1)}{W_2 + W_1}, \qquad (4)$$

in which t_2 and t_1 are the retention times of the two components and W_2 and W_1 are the corresponding widths of the bases of the peaks, obtained by extrapolating the relatively straight sides of the peaks to the baseline.

Quantitative data can be obtained from the areas under the peaks, determined graphically or by means of an automatic electronic integrator or planimeter. Peak areas are less accurate for small peaks and those having short retention times. The product of peak width at half height and peak height may be substituted for peak areas to minimize graphical error for symmetrical peaks. The chart should be run faster than usual or a comparator should be used to measure the width-at-half-height, so as to minimize error in this measurement. For accurate quantitative work, the components to be measured should be separated from any interfering components. Peak measurements on solvent tails are to be avoided.

Area percentages, $\%A_i$, of species within a chromatogram are used in purity analysis and are equal to 100 times the ratio of the peak areas of the species, A_i, to the sum, ΣA_i, of all of the area of the peaks in the chromatogram. [NOTE—Where used in the individual monograph, the expression "percentage (a/a)" represents 100 times the ratio $(A_i/\Sigma A_i)$.] Where the individual components and response factors are known, measure the area of each peak and convert this to mass of that component by multiplying the peak area by the response factor. Where the identity of other components is known, calibration curves may be based on area percentage alone, such as in water content tests for solvents, provided the detector type is specified.

Assays require quantitative comparison of one chromatogram with another, and lack of control of the specimen size injected is a major source of error. Addition of an internal standard to the test specimen minimizes this error. The ratio of peak response of the species of interest to the internal standard is compared from one chromatogram to another. Where the internal standard is chemically similar to the substance being determined, minor

variations in column and detector parameters are controlled also. In some cases, the internal standard may be carried through the assay procedure prior to gas chromatography to control other quantitative aspects of the procedure.

A quantity of solute may be adsorbed within the system, and this would be reflected in the failure of the calibration curve to pass through zero, terminating instead along the abscissa. This effect may contribute error, particularly for the measurement of small specimens and the use of a single reference point. At high test-specimen concentrations, the solute may overload the liquid phase, leading to relative loss of peak height or symmetry. Before any column is accepted for assay purposes, a calibration curve should be constructed to control these errors prior to the use of the single sample size used in most assays.

Special-grade solvents are available for use especially where extensive evaporative concentration of test specimen is necessary prior to chromatography. Such chromatographic-grade reagents may be specified in the individual monograph. Since most drugs are polar molecules having reactive groups, successful chromatography may require the conversion of the drug to a more volatile or less polar derivative by treatment of reactive groups with appropriate reagents.

Columns should be conditioned by being operated until stable at a temperature higher than that specified for use in the individual monograph. In the case of thermally stable methyl- and phenyl-substituted polysiloxanes, a special sequence increases inertness and efficiency: maintain the column at a temperature of 250° for 1 hour with helium flowing to remove oxygen and solvents, stop the flow of helium, heat at about 340° for 4 hours, then reduce the heating so as to attain a temperature of 250°, and condition with helium flowing until stable. A suitable test for support inertness, which is necessary with low-polarity liquid phases, is the appearance of a single, symmetric peak for injected cholesterol with no evidence of decomposition. A column may occasionally be conditioned by repeated injections of the compound or mixture to be chromatographed.

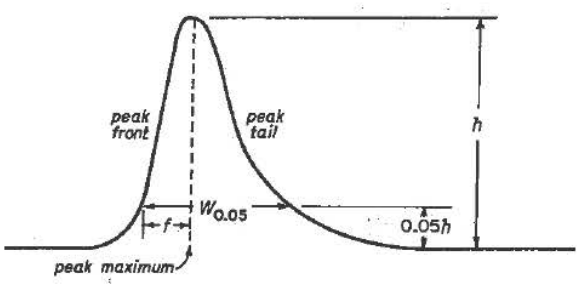


Fig. 2. Asymmetrical Chromatographic Peak.

System Suitability

It is generally desirable to ascertain the suitability and effectiveness of the operating system when employing chromatographic methods such as pressurized liquid chromatography or gas chromatography. It should be noted that the specification of definitive parameters in a monograph does not preclude the use of other suitable operating conditions (see *Procedures* in the *General Notices*). Adjustment of operating conditions to obtain acceptable operation and chromatograms may be required.

To ascertain the effectiveness of the final operating system, it should be subjected to a suitability test prior to use. The essence of such a test is the concept that the electronics, the equipment, the specimens, and the analytical operations constitute a single analytical system, which is amenable to an overall test of system function. Specific data are collected from replicate injections of the assay preparation or standard preparation. These are matched to specified maximum and minimum values, such as efficiency, internal precision, tailing factor, resolution, retention time, nature of the calibration curve, response, and recovery, as specified in the individual monograph.

A useful parameter is the reproducibility of replicate injections of the analytical solution. The reproducibility of replicate injections is best expressed as the relative standard deviation. The calculation is expressed by the equation

$$S_R\,(\%) = \frac{100}{\overline{X}} \left[\frac{\sum_{i=1}^{N}(X_i - \overline{X})^2}{N-1}\right]^{1/2}, \qquad (1)$$

in which S_R is the relative standard deviation in percentage, $\overline{X}$ is the mean of the set of N measurements, and X_i is an individual measurement. The term X_i refers to the measurement of the peak response ratio, R_S (where an internal standard is employed),

$$X_i = R_S = \frac{r_S}{r_i}, \qquad (2)$$

in which r_S is the peak response corresponding to the reference standard, and r_i is the peak response corresponding to the internal standard, or to the peak response, r_S, for an external standard method.

Replicate injections of the *Standard preparations* are usually specified in the individual monograph, and the resulting measurements are compared to ascertain whether requirements for precision are met. Unless otherwise specified in the individual monograph, data from five replicate chromatograms are used for calculation if the stated limit for relative standard deviation is 2.0% or less, and data from six replicate chromatograms are used for calculation if the stated relative standard deviation limit is more than 2.0%.

It is useful also to specify a tailing factor to limit the maximum permissible asymmetry of the peak. For Pharmacopeial purposes, the tailing factor, T, is defined as the ratio of the distance from the leading edge to the trailing edge of the peak, $W_{0.05}$, divided by twice the distance, f, from the peak maximum to the leading edge of the peak, the distances being measured at a point 5% of the peak height from the baseline. For a symmetrical peak, the tailing factor, T, is unity, and the value of T increases as tailing becomes more pronounced.

Resolution, R, is specified to ensure separation of closely eluting components, to establish the general separatory efficiency of the system, or where an internal standard is used.

GLOSSARY OF SYMBOLS

To promote uniformity of interpretation, the following symbols and definitions are employed where applicable in presenting formulas in the individual monographs. [NOTE—Where the terms W and t both appear in the same equation they must be expressed in the same units.]

α relative retention,

$$\alpha = \frac{t_2 - t_a}{t_1 - t_a}.$$

c_r, c_i, c_u concentrations of Reference Standard, internal standard, and analyte in a particular solution.

C_A concentration ratio of analyte and internal standard in test solution or *Assay preparation*,

$$C_A = \frac{q_u}{q_i}.$$

C_S concentration ratio of Reference Standard and internal standard in Standard solution,

$$C_S = \frac{c_r}{c_i}.$$

k' capacity factor,

$$k' = \frac{\text{amount of substance in stationary phase}}{\text{amount of substance in mobile phase}}$$

$$k' = \frac{\text{time spent by substance in stationary phase}}{\text{time spent by substance in mobile phase}} = \frac{t}{t_a} - 1.$$

n — number of theoretical plates in a chromatographic column,

$$n = 16\left(\frac{t}{W}\right)^2.$$

q_r, q_i, q_u — total quantities (weights) of Reference Standard, internal standard, and analyte in a particular solution.

Q_A — quantity ratio of analyte and internal standard in test solution or *Assay preparation*,

$$Q_A = \frac{q_u}{q_i}.$$

Q_S — quantity ratio of Reference Standard and internal standard in Standard solution,

$$Q_S = \frac{q_r}{q_i}.$$

r_S — peak response of the Reference Standard obtained from a chromatogram.

r_U — peak response of the analyte obtained from a chromatogram.

R — resolution between two chromatographic peaks,

$$R = \frac{2(t_2 - t_1)}{W_1 + W_2}.$$

R_f — chromatographic retardation factor equal to the ratio of the distance from the origin to the center of a zone divided by the distance from the origin to the solvent front.

R_r — relative retention

$$R_r = \frac{\text{distance traveled by test substance}}{\text{distance traveled by standard}}.$$

R_S — peak response ratio for *Standard preparation* containing Reference Standard and internal standard,

$$R_S = \frac{r_S}{r_i}.$$

R_U — peak response ratio for *Assay preparation* containing the analyte and internal standard,

$$R_U = \frac{r_U}{r_i}.$$

S_R (%) — relative standard deviation in percentage,

$$S_R\,(\%) = \frac{100}{\bar{X}}\left[\frac{\sum_{i=1}^{N}(X_i - \bar{X})^2}{N-1}\right]^{1/2},$$

where X_i is an individual measurement in a set of N measurements and $\bar{X}$ is the arithmetic mean of the set.

T — tailing factor,

$$T = \frac{W_{0.05}}{2f}.$$

t — retention time measured from time of injection to time of elution of peak maximum.

t_a — retention time of nonretarded component, air with thermal conductivity detection.

W — width of peak measured by extrapolating the relatively straight sides to the baseline.

$W_{h/2}$ — width of peak at half height.

$W_{0.05}$ — width of peak at 5% height.

CHROMATOGRAPHIC REAGENTS

The following list of packings (L), phases (G), and supports (S) is intended to be a convenient reference for the chromatographer. These materials are listed also under *Reagent Specifications* in the section, *Reagents, Indicators, and Solutions*, as *Packings for High-pressure Liquid Chromatography* (L); *Phases for Gas Chromatography* (G); and *Supports for Gas Chromatography* (S). [NOTE—Particle sizes given in this listing are those generally provided. Where other, usually finer, sizes are required, the individual monograph specifies the desired particle size.]

Packings

L1—Octadecyl silane chemically bonded to porous silica or ceramic micro-particles, 5 to 10 μm in diameter.

L2—Octadecyl silane chemically bonded to silica gel of a controlled surface porosity that has been bonded to a solid spherical core, 30 to 50 μm in diameter.

L3—Porous silica particles, 5 to 10 μm in diameter.

L4—Silica gel of controlled surface porosity bonded to a solid spherical core, 30 to 50 μm in diameter.

L5—Alumina of controlled surface porosity bonded to a solid spherical core, 30 to 50 μm in diameter.

L6—Strong cation-exchange packing–sulfonated fluorocarbon polymer coated on a solid spherical core, 30 to 50 μm in diameter.

L7—Octylsilane chemically bonded to totally porous silica particles, 5 to 10 μm in diameter.

L8—An essentially monomolecular layer of aminopropylsilane chemically bonded to totally porous silica gel support, 10 μm in diameter.

L9—10-μm irregular, totally porous silica gel having a chemically bonded, strongly acidic cation-exchange coating.

L10—Nitrile groups chemically bonded to porous silica particles, 5 to 10 μm in diameter.

L11—Phenyl groups chemically bonded to porous silica particles, 5 to 10 μm in diameter.

L12—A strong anion-exchange packing made by chemically bonding a quaternary amine to a solid silica spherical core, 30 to 50 μm in diameter.

L13—Trimethylsilane chemically bonded to porous silica particles, 5 to 10 μm in diameter.

L14—Silica gel 10 μm in diameter having a chemically bonded, strongly basic quaternary ammonium anion-exchange coating.

L15—Hexylsilane chemically bonded to totally porous silica particles, 3 to 10 μm in diameter.

L16—Dimethylsilane chemically bonded to porous silica particles, 5 to 10 μm in diameter.

L17—Strong cation-exchange resin consisting of sulfonated cross-linked styrene-divinylbenzene copolymer in the hydrogen form, 7 to 11 μm in diameter.

L18—Amino and cyano groups chemically bonded to porous silica particles, 5 to 10 μm in diameter.

L19—Strong cation-exchange resin consisting of sulfonated cross-linked styrene-divinylbenzene copolymer in the calcium form, about 9 μm in diameter.

L20—Dihydroxypropane groups chemically bonded to porous silica particles, 5 μm to 10 μm in diameter.

L21—A rigid, spherical styrene-divinylbenzene copolymer, 5 to 10 μm in diameter.

L22—A cation exchange resin made of porous polystyrene gel with sulfonic acid groups, about 10 μm in size.

L23—An anion exchange resin made of porous polymethacrylate or polyacrylate gel with quaternary ammonium groups, about 10 μm in size.

L24—A semi-rigid hydrophilic gel consisting of vinyl polymers with numerous hydroxyl groups on the matrix surface, 32 to 63 μm in diameter.[5]

Phases

G1—Dimethylpolysiloxane oil.

G2—Dimethylpolysiloxane gum.

G3—50% Phenyl–50% methylpolysiloxane.

G4—Diethylene glycol succinate polyester.

G5—3-Cyanopropylpolysiloxane.

[5] Available as Fractogel TSK-HW-40F and distributed by Merck and Co.

G6—Trifluoropropylmethylpolysiloxane.
G7—50% 3-Cyanopropyl–50% phenylmethylsilicone.
G8—90% 3-Cyanopropyl–10% phenylmethylsilicone.
G9—Methylvinylpolysiloxane.
G10—Polyamide.
G11—Bis(2-ethylhexyl) sebacate polyester.
G12—Phenyldiethanolamine succinate polyester.
G13—Sorbitol.
G14—Polyethylene glycol (av. mol. wt. of 950 to 1050).
G15—Polyethylene glycol (av. mol. wt. of 3000 to 3700).
G16—Polyethylene glycol compound (av. mol. wt. about 15,000). A high molecular weight compound of polyethylene glycol and a diepoxide.
G17—75% Phenyl–25% methylpolysiloxane.
G18—Polyalkylene glycol.
G19—25% Phenyl–25% cyanopropyl–50% methylsilicone.
G20—Polyethylene glycol (av. mol. wt. of 380 to 420).
G21—Neopentyl glycol succinate.
G22—Bis(2-ethylhexyl) phthalate.
G23—Polyethylene glycol adipate.
G24—Diisodecyl phthalate.
G25—Polyethylene glycol compound TPA. A high molecular weight compound of a polyethylene glycol and a diepoxide that is esterified with terephthalic acid.
G26—25% 2-Cyanoethyl–75% methylpolysiloxane.
G27—5% Phenyl–95% methylpolysiloxane.
G28—25% Phenyl–75% methylpolysiloxane.
G29—β-β'-Thiodipropionitrile.
G30—Tetraethylene glycol dimethyl ether.
G31—Nonylphenoxypoly(ethyleneoxy)ethanol (av. ethyleneoxy chain length is 30); Nonoxynol 30.
G32—20% Phenylmethyl–80% dimethylpolysiloxane.
G33—20% Carborane–80% methylsilicone.
G34—Diethylene glycol succinate polyester stabilized with phosphoric acid.
G35—A high molecular weight compound of a polyethylene glycol and a diepoxide that is esterified with nitro-terephthalic acid.
G36—1% Vinyl–5% phenylmethylpolysiloxane.
G37—Polyimide.

Supports

[NOTE—Unless otherwise specified, mesh sizes of 80 to 100 or, alternatively, 100 to 120 are intended.]

S1A—Siliceous earth for gas chromatography has been flux-calcined by mixing diatomite with Na_2CO_3 flux and calcining above 900°. The siliceous earth is acid-washed, then water-washed until neutral, but not base-washed. The siliceous earth may be silanized by treating with an agent such as dimethyldichlorosilane[6] to mask surface silanol groups.

S1AB—The siliceous earth as described above is both acid- and base-washed.[6]

S1C—A support prepared from crushed firebrick and calcined or burned with a clay binder above 900° with subsequent acid-wash. It may be silanized.

S1NS—The siliceous earth is untreated.

S2—Styrene-divinylbenzene copolymer having a nominal surface area of less than 50 m^2 per g and an average pore diameter of 0.3 to 0.4 μm.

S3—Copolymer of ethylvinylbenzene and divinylbenzene having a nominal surface area of 500 to 600 m^2 per g and an average pore diameter of 0.0075 μm.

S4—Styrene-divinylbenzene copolymer with aromatic –O and –N groups, having a nominal surface area of 400 to 600 m^2 per g and an average pore diameter of 0.0076 μm.

S5—40- to 60-mesh, high-molecular weight tetrafluorethylene polymer.

S6—Styrene-divinylbenzene copolymer having a nominal surface area of 250 to 350 m^2 per g and an average pore diameter of 0.0091 μm.

S7—Graphitized carbon having a nominal surface area of 12 m^2 per g.

S8—Copolymer of 4-vinyl-pyridine and styrene-divinylbenzene.

S9—A porous polymer based on 2,6-diphenyl-*p*-phenylene oxide.

S10—A highly polar cross-linked copolymer of acrylonitrite and divinylbenzene.

S11—Graphitized carbon having a nominal surface area of 9 m^2 per gram modified with small amounts of petrolatum and polyethylene glycol compound.[7]

[6] Unless otherwise specified in the individual monograph, silanized support is intended.

⟨631⟩ COLOR AND ACHROMICITY

Definition—For the purposes of this chapter, color may be defined as the perception or subjective response by an observer to the objective stimulus of radiant energy in the visible spectrum extending over the range 400 nm to 700 nm in wavelength. Perceived color is a function of three variables: spectral properties of the object, both absorptive and reflective; spectral properties of the source of illumination; and visual characteristics of the observer.

Two objects are said to have a color match for a particular source of illumination when an observer cannot detect a color difference. Where a pair of objects exhibit a color match for one source of illumination and not another, they constitute a metameric pair. Color matches of two objects occur for all sources of illumination if the absorption and reflectance spectra of the two objects are identical.

Achromicity or colorlessness is one extreme of any color scale for transmission of light. It implies the complete absence of color, and therefore the visible spectrum of the object lacks absorbances. For practical purposes, the observer in this case perceives little if any absorption taking place in the visible spectrum.

Color Attributes—Because the sensation of color has both a subjective and an objective part, color cannot be described solely in spectrophotometric terms. The common attributes of color therefore cannot be given a one-to-one correspondence with spectral terminology.

Three attributes are commonly used to identify a color: (1) hue, or the quality by which one color family is distinguished from another, such as red, yellow, blue, green, and intermediate terms; (2) value, or the quality that distinguishes a light color from a dark one; and (3) chroma, or the quality that distinguishes a strong color from a weak one, or the extent to which a color differs from a gray of the same value.

The three attributes of color may be used to define a three-dimensional color space in which any color is located by its coordinates. The color space chosen is a visually uniform one if the geometric distance between two colors in the color space is directly a measure of the color distance between them. Cylindrical coordinates are often conveniently chosen:

Points along the long axis represent value from dark to light or black to white and have indeterminate hue and no chroma. Focusing on a cross-section perpendicular to the value axis, hue is determined by the angle about the long axis and chroma is determined by the distance from the long axis. Red, yellow, green, blue, purple, and intermediate hues are given by different angles. Colors along a radius of a cross-section have the same hue, which become more intense farther out. For example, colorless or achromic water has indeterminate hue, high value, and no chroma. If a colored solute is added, the water takes on a particular hue. As more is added, the color becomes darker, more intense, or deeper; i.e., the chroma generally increases and value decreases. If, however, the solute is a neutral color, i.e., gray, the value decreases, no increase in chroma is observed, and the hue remains indeterminate.

Laboratory spectroscopic measurements can be converted to measurements of the three color attributes. Spectroscopic results for three chosen lights or stimuli are weighted by three distribution functions to yield the tristimulus values, X, Y, Z (see *Color—Instrumental Measurement* ⟨1061⟩). The distribution functions were determined in color matching experiments with human subjects.

The tristimulus values are not coordinates in a visually uniform color space; however, several transformations have been proposed that are close to being uniform, one of which is given in the chapter cited ⟨1061⟩. The value is often a function of only the Y value. Obtaining uniformity in the chroma-hue subspace has

[7] Commercially available as SP1500 on Carbopack C from Supelco.

been less satisfactory. In a practical sense, this means in visual color comparison that if two objects differ significantly in hue, deciding which has a higher chroma becomes difficult. This points out the importance of matching standard to sample color as closely as possible, especially for the attributes of hue and chroma.

Color Determination and Standards—The perception of color and color matches is dependent on conditions of viewing and illumination. Determinations should be made using diffuse, uniform illumination under conditions that reduce shadows and non-spectral reflectance to a minimum. The surface of powders should be smoothed with gentle pressure so that a planar surface free from irregularities is presented. Liquids should be compared in matched color-comparison tubes, against a white background. If results are found to vary with illumination, those obtained in natural or artificial daylight are to be considered correct. Instead of visual determination, a suitable instrumental method may be used.

Colors of standards should be as close as possible to those of test specimens for quantifying color differences. Standards for opaque materials are available as sets of color chips that are arranged in a visually uniform space.* Standards identified by a letter for matching the colors of fluids can be prepared according to the accompanying table. To prepare the matching fluid required, pipet the prescribed volumes of the colorimetric test solutions [see under *Colorimetric Solutions* (CS) in the section, *Reagents, Indicators, and Solutions*] and water into one of the matching containers, and mix the solution in the container. Make the comparison as directed in the individual monograph, under the viewing conditions previously described. The matching fluids, or other combinations of the colorimetric solutions, may be used in very low concentrations to measure deviation from achromicity.

Matching Fluids

Matching Fluid	Parts of Cobaltous Chloride CS	Parts of Ferric Chloride CS	Parts of Cupric Sulfate CS	Parts of Water
A	0.1	0.4	0.1	4.4
B	0.3	0.9	0.3	8.5
C	0.1	0.6	0.1	4.2
D	0.3	0.6	0.4	3.7
E	0.4	1.2	0.3	3.1
F	0.3	1.2	0.0	3.5
G	0.5	1.2	0.2	3.1
H	0.2	1.5	0.0	3.3
I	0.4	2.2	0.1	2.3
J	0.4	3.5	0.1	1.0
K	0.5	4.5	0.0	0.0
L	0.8	3.8	0.1	0.3
M	0.1	2.0	0.1	2.8
N	0.0	4.9	0.1	0.0
O	0.1	4.8	0.1	0.0
P	0.2	0.4	0.1	4.3
Q	0.2	0.3	0.1	4.4
R	0.3	0.4	0.2	4.1
S	0.2	0.1	0.0	4.7
T	0.5	0.5	0.4	3.6

⟨641⟩ COMPLETENESS OF SOLUTION

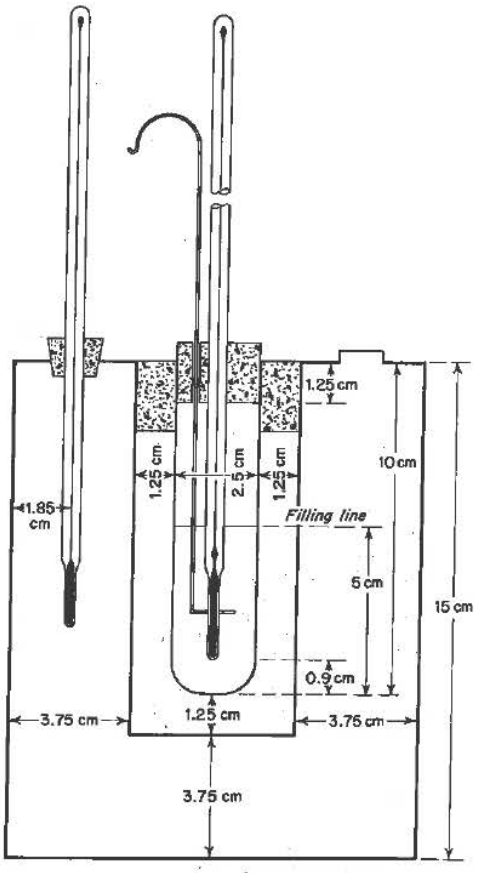
Place the quantity of the substance specified in the individual monograph in a meticulously cleansed, glass-stoppered, 10-mL glass cylinder approximately 13 mm × 125 mm in size. Using the solvent that is specified in the monograph or on the label of the product, fill the cylinder almost to the constriction at the neck. Shake gently to effect solution: the solution is not less clear than an equal volume of the same solvent contained in a similar vessel and examined similarly.

* Collections of color chips, arranged according to hue, value, and chroma in a visually uniform space and suitable for use in color designation of specimens by visual matching are available from Munsell Color, Macbeth Division of Kollmorgen Corp., 2441 N. Calvert St., Baltimore, MD 21218.

⟨651⟩ CONGEALING TEMPERATURE

The temperature at which a substance passes from the liquid to the solid state upon cooling is a useful index to purity if heat is liberated when the solidification takes place, provided that any impurities present dissolve in the liquid only, and not in the solid. Pure substances have a well-defined freezing point, but mixtures generally freeze over a range of temperatures. For many mixtures, the congealing temperature, as determined by strict adherence to the following empirical methods, is a useful index of purity. The method for determining congealing temperatures set forth here is applicable to substances that melt between −20° and 150°, the range of the thermometer used in the bath. The congealing temperature is the maximum point (or lacking a maximum, the point of inflection) in the temperature-time curve.

Apparatus—Assemble an apparatus similar to that illustrated, in which the container for the substance is a 25- × 100-mm test tube. This is provided with a suitable, short-range thermometer suspended in the center, and a wire stirrer, about 30 cm long, bent at its lower end into a horizontal loop around the thermometer.



Congealing Temperature Apparatus

The specimen container is supported, by means of a cork, in a suitable water-tight cylinder about 50 mm in internal diameter and 11 cm in length. The cylinder, in turn, is supported in a suitable bath sufficient to provide not less than a 37-mm layer surrounding the sides and bottom of the cylinder. The outside bath is provided with a suitable thermometer.

Procedure—Use a thermometer having a range not exceeding 30°, graduated in 0.1° divisions, and calibrated for, but not used at, 76-mm immersion. A suitable series of thermometers, covering a range from −20° to +150°, is available as the ASTM

E-1 series 89C through 96C (see *Thermometers* ⟨21⟩). Melt the substance, if a solid, at a temperature not exceeding 20° above its expected congealing point, and pour it into the test tube to a height of 50 mm to 57 mm. Assemble the apparatus with the bulb of the test tube thermometer immersed halfway between the top and bottom of the specimen in the test tube. Fill the bath to about 12 mm from the top of the tube with suitable fluid at a temperature 4° to 5° below the expected congealing point.

In case the substance is a liquid at room temperature, carry out the determination using a bath temperature about 15° below the expected congealing point.

When the test specimen has cooled to about 5° above its expected congealing point, adjust the bath to a temperature 7° to 8° below the expected congealing point. Stir the specimen continuously during the remainder of the test by moving the loop up and down between the top and bottom of the specimen, at a regular rate of 20 complete cycles per minute.

Congelation frequently may be induced by rubbing the inner walls of the test tube with the thermometer, or by introducing a small fragment of the previously congealed substance. Pronounced supercooling may cause deviation from the normal pattern of temperature changes. If the latter occurs, repeat the test, introducing small particles of the material under test in solid form at 1° intervals as the temperature approaches the expected congealing point.

Record the reading of the test tube thermometer every 30 seconds. Continue stirring only so long as the temperature is gradually falling, stopping when the temperature becomes constant or starts to rise slightly. Continue recording the temperature in the test tube every 30 seconds for at least 3 minutes after the temperature again begins to fall after remaining constant.

The average of not less than four consecutive readings that lie within a range of 0.2° constitutes the congealing temperature. These readings lie about a point of inflection or a maximum, in the temperature-time curve, that occurs after the temperature becomes constant or starts to rise and before it again begins to fall. The average to the nearest 0.1° is the congealing temperature.

⟨661⟩ CONTAINERS

Many Pharmacopeial articles are of such nature as to require the greatest attention to the containers in which they are stored or maintained even for short periods of time. While the needs vary widely and some of them are not fully met by the containers available, objective standards are essential. It is the purpose of this chapter to provide such standards as have been developed for the materials of which pharmaceutical containers principally are made, i.e., glass and plastic.

A container intended to provide protection from light or offered as a "light-resistant" container meets the requirements for *Light Transmission*, where such protection or resistance is by virtue of the specific properties of the material of which the container is composed, including any coating applied thereto. A clear and colorless or a translucent container that is made light-resistant by means of an opaque enclosure (see *General Notices*) is exempt from the requirements for *Light Transmission*.

Containers composed of glass meet the requirements for *Chemical Resistance—Glass Containers*, and containers composed of plastic and intended for packaging products prepared for parenteral use meet the requirements under *Biological Tests—Plastics* and *Physicochemical Tests—Plastics*.

Where dry oral dosage forms, not meant for constitution into solution, are intended to be packaged in a container defined in the section *Polyethylene Containers*, the requirements given in that section are to be met.

Guidelines and requirements under *Single-unit Containers and Unit-dose Containers for Nonsterile Solid and Liquid Dosage Forms* apply to official dosage forms that are repackaged into single-unit or unit-dose containers or mnemonic packs for dispensing pursuant to prescription.

LIGHT TRANSMISSION

Apparatus[1]—Use a spectrophotometer of suitable sensitivity and accuracy, adapted for measuring the amount of light transmitted by either transparent or translucent glass or plastic materials used for pharmaceutical containers. For glass containers of nominal capacity up to 5 mL, use a suitable spectrophotometer having an aperture not larger than 2 mm × 1 cm. For containers made of translucent materials other than glass, use a suitable spectrophotometer equipped with an attachment that is capable of measuring and recording light transmitted in diffused as well as parallel rays.

Preparation of Specimen—

GLASS—Break the container or cut it with a circular saw fitted with a wet abrasive wheel, such as a carborundum or a bonded diamond wheel. Select sections to represent the average wall thickness in the case of blown glass containers, and trim them as necessary to give segments of a size convenient for mounting in the spectrophotometer. After cutting, wash and dry each specimen, taking care to avoid scratching the surfaces. If the specimen is too small to cover the opening in the specimen holder, mask the uncovered portion of the opening with opaque paper or masking tape, provided that the length of the specimen is greater than that of the slit in the spectrophotometer. Immediately before mounting in the specimen holder, wipe the specimen with lens tissue. Mount the specimen with the aid of a tacky wax, or by other convenient means, taking care to avoid leaving fingerprints or other marks on the surfaces through which light must pass.

PLASTIC—Cut circular sections from two or more areas of the container, and wash and dry them, taking care to avoid scratching the surfaces. Mount in the apparatus as described for *Glass*.

Procedure—Place the section in the spectrophotometer with its cylindrical axis parallel to the plane of the slit and approximately centered with respect to the slit. When properly placed, the light beam is normal to the surface of the section and reflection losses are at a minimum.

Measure the transmittance of the section with reference to air in the spectral region of interest, continuously with a recording instrument or at intervals of about 20 nm with a manual instrument, in the region of 290 nm to 450 nm.

Limits—The observed light transmission does not exceed the limits given in Table 1 for containers intended for parenteral use.

The observed light transmission for containers of Type NP glass and for plastic containers for products intended for oral or topical administration does not exceed 10% at any wavelength in the range from 290 nm to 450 nm.

Table 1. Limits for Glass Types I, II, and III and Plastic Classes I–VI.

Nominal Size (in mL)	Maximum Percentage of Light Transmission at Any Wavelength Between 290 nm and 450 nm	
	Flame-sealed Containers	Closure-sealed Containers
1	50	25
2	45	20
5	40	15
10	35	13
20	30	12
50	15	10

NOTE—Any container of a size intermediate to those listed above exhibits a transmission not greater than that of the next larger size container listed in the table. For containers larger than 50 mL, the limits for 50 mL apply.

[1] For further detail regarding apparatus and procedures, reference may be made to the following publications of the American Society for Testing and Materials, 1916 Race St., Philadelphia, PA 19103: "Standard Method of Test for Haze and Luminous Transmittance of Transparent Plastics," ASTM Designation D-1003-61; "Tentative Method of Test for Luminous Reflectance, Transmittance, and Color of Materials," ASTM E 308-66.

CHEMICAL RESISTANCE—GLASS CONTAINERS

The following tests are designed to determine the resistance to water attack of new (not previously used) glass containers. The degree of attack is determined by the amount of alkali released from the glass under the influence of the attacking medium under the conditions specified. This quantity of alkali is extremely small in the case of the more resistant glasses, thus calling for particular attention to all details of the tests and the use of apparatus of high quality and precision. The tests should be conducted in an area relatively free from fumes and excessive dust.

Glass Types—Glass containers suitable for packaging Pharmacopeial preparations may be classified as in Table 2 on the basis of the tests set forth in this section. Containers of Type I borosilicate glass are generally used for preparations that are intended for parenteral administration. Containers of Type I glass, or of Type II glass, i.e., soda-lime glass that is suitably de-alkalized, are usually used for packaging acidic and neutral parenteral preparations. Type I glass containers, or Type II glass containers (where stability data demonstrate their suitability), are used for alkaline parenteral preparations. Type III soda-lime glass containers usually are not used for parenteral preparations, except where suitable stability test data indicate that Type III glass is satisfactory for the parenteral preparations that are packaged therein. Containers of Type NP glass are intended for packaging nonparenteral articles; i.e., those intended for oral or topical use.

Table 2. Glass Types and Test Limits.

Type	General Description[a]	Type of Test	Limits: Size,[b] mL	Limits: mL of 0.020 *N* Acid
I	Highly resistant, borosilicate glass	*Powdered Glass*	All	1.0
II	Treated soda-lime glass	*Water Attack*	100 or less	0.7
			Over 100	0.2
III	Soda-lime glass	*Powdered Glass*	All	8.5
NP	General-purpose soda-lime glass	*Powdered Glass*	All	15.0

[a] The description applies to containers of this type of glass usually available.

[b] Size indicates the overflow capacity of the container.

Apparatus—

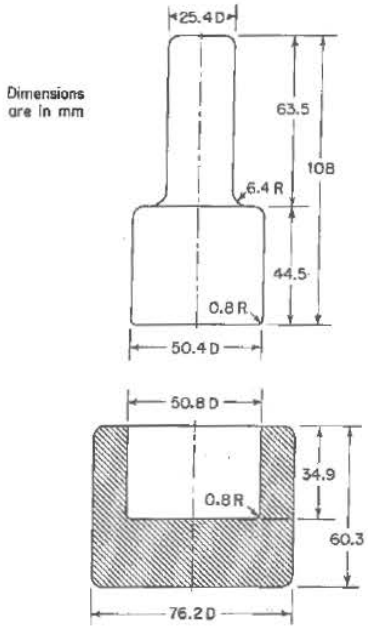
AUTOCLAVE—For these tests, use an autoclave capable of maintaining a temperature of 121 ± 2.0°, equipped with a thermometer, a pressure gauge, a vent cock, and a rack adequate to accommodate at least 12 test containers above the water level.

MORTAR AND PESTLE—Use a hardened-steel mortar and pestle, made according to the specifications in the accompanying illustration.

OTHER EQUIPMENT—Also required are 20.3-cm (8-inch) sieves made of stainless steel including the No. 20, No. 40, and No. 50 sieves along with the pan and cover (see *Openings of Standard Sieves* ⟨811⟩), 250-mL conical flasks made of resistant glass aged as specified, a 900-g (2-lb) hammer, a permanent magnet, a desiccator, and adequate volumetric apparatus.

Reagents—

HIGH-PURITY WATER—The water used in these tests has a conductivity at 25°, as measured in an in-line cell just prior to dispensing, of not greater than 0.15 μmho per cm. It meets the requirements of the test for *Heavy metals* under *Purified Water*, and is free from copper. The water may be prepared by passing distilled water through a deionizer cartridge packed with a mixed bed of nuclear-grade resin, then through a cellulose ester membrane having openings not exceeding 0.45 μm.[3] Do not use copper tubing. Flush the discharge lines before water is dispensed into test vessels. When the low conductivity specification can no longer be met, replace the deionizer cartridge.



Special Mortar and Pestle for Pulverizing Glass[2]

METHYL RED SOLUTION—Dissolve 24 mg of methyl red sodium in purified water to make 100 mL. If necessary, neutralize the solution with 0.02 *N* sodium hydroxide or acidify it with 0.02 *N* sulfuric acid so that the titration of 100 mL of *High-purity Water*, containing 5 drops of indicator, does not require more than 0.020 mL of 0.020 *N* sodium hydroxide to effect the color change of the indicator, which should occur at a pH of 5.6.

Powdered Glass Test

Rinse thoroughly with Purified Water 6 or more containers selected at random, and dry them with a current of clean, dry air. Crush the containers into fragments about 25 mm in size, divide about 100 g of the coarsely crushed glass into three approximately equal portions, and place one of the portions in the special mortar. With the pestle in place, crush the glass further by striking 3 or 4 blows with the hammer. Nest the sieves, and empty the mortar into the No. 20 sieve. Repeat the operation on each of the two remaining portions of glass, emptying the mortar each time into the No. 20 sieve. Shake the sieves for a short time, then remove the glass from the No. 20 and No. 40 sieves, and again crush and sieve as before. Repeat again this crushing and sieving operation. Empty the receiving pan, reassemble the nest of sieves, and shake on a mechanical sieve shaker for 5 minutes or by hand for an equivalent length of time. Transfer the portion retained on the No. 50 sieve, which should weigh

[2] A suitable mortar and pestle is available (catalog No. H-17280) from Humboldt Manufacturing Co., 7300 West Agatite, Norridge, Chicago, IL 60656.

[3] A suitable nuclear-grade resin mixture of the strong acid cation exchanger in the hydrogen form and the strong base anion exchanger in the hydroxide form, with a one-to-one cation to anion equivalence ratio, is available from the Millipore Corp., Bedford, MA 01730; Barnstead Co., 225 Rivermoor St., Boston, MA 02132; Illinois Water Treatment Co., 840 Cedar St., Rockford, IL 61105; and Vaponics, Inc., 200 Cordage Park, Plymouth, MA 02360.

A suitable in-line filter is available from the Millipore Corp.; Gelman Instrument Co., 600 S. Wagner Rd., Ann Arbor, MI 48106; and Schleicher and Schuell, Inc., 540 Washington St., Keene, NH 10003.

in excess of 10 g, to a closed container, and store in a desiccator until used for the test.

Spread the specimen on a piece of glazed paper, and pass a magnet through it to remove particles of iron that may be introduced during the crushing. Transfer the specimen to a 250-mL conical flask of resistant glass, and wash it with six 30-mL portions of acetone, swirling each time for about 30 seconds and carefully decanting the acetone. After washing, the specimen should be free from agglomerations of glass powder, and the surface of the grains should be practically free from adhering fine particles. Dry the flask and contents for 20 minutes at 140°, transfer the grains to a weighing bottle, and cool in a desiccator. Use the test specimen within 48 hours after drying.

Procedure—Transfer 10.00 g of the prepared specimen, accurately weighed, to a 250-mL conical flask that has been digested (aged) previously with *High-purity Water* in a bath at 90° for at least 24 hours or at 121° for 1 hour. Add 50.0 mL of *High-purity Water* to this flask and to one similarly prepared to provide a blank. Cap all flasks with borosilicate glass beakers that previously have been treated as described for the flasks and that are of such size that the bottoms of the beakers fit snugly down on the top rims of the containers. Place the containers in the autoclave, and close it securely, leaving the vent cock open. Heat until steam issues vigorously from the vent cock, and continue heating for 10 minutes. Close the vent cock, and adjust the temperature to 121°, taking 19 to 23 minutes to reach the desired temperature. Hold the temperature at 121 ± 2.0° for 30 minutes, counting from the time this temperature is reached. Reduce the heat so that the autoclave cools and comes to atmospheric pressure in 38 to 46 minutes, being vented as necessary to prevent the formation of a vacuum. Cool the flask at once in running water, decant the water from the flask into a suitably cleansed vessel, and wash the residual powdered glass with four 15-mL portions of *High-purity Water*, adding the decanted washings to the main portion. Add 5 drops of *Methyl Red Solution*, and titrate immediately with 0.020 *N* sulfuric acid. If the volume of titrating solution is expected to be less than 10 mL, use a microburet. Record the volume of 0.020 *N* sulfuric acid used to neutralize the extract from 10 g of the prepared specimen of glass, corrected for a blank. The volume does not exceed that indicated in Table 2 for the type of glass concerned.

Water Attack at 121°

Rinse thoroughly 3 or more containers, selected at random, twice with *High-purity Water*.

Procedure—Fill each container to 90% of its overflow capacity with *High-purity Water*, and proceed as directed for *Procedure* under *Powdered Glass Test*, beginning with "Cap all flasks," except that the time of autoclaving shall be 60 minutes instead of 30 minutes, and ending with "to prevent the formation of a vacuum." Empty the contents from 1 or more containers into a 100-mL graduated cylinder, combining, in the case of smaller containers, the contents of several containers to obtain a volume of 100 mL. Place the pooled specimen in a 250-mL conical flask of resistant glass, add 5 drops of *Methyl Red Solution*, and titrate, while warm, with 0.020 *N* sulfuric acid. Complete the titration within 60 minutes after opening the autoclave. Record the volume of 0.020 *N* sulfuric acid used, corrected for a blank obtained by titrating 100 mL of *High-purity Water* at the same temperature and with the same amount of indicator. The volume does not exceed that indicated in Table 2 for the type of glass concerned.

Arsenic

Arsenic ⟨211⟩—Use as the *Test Preparation* 35 mL of the water from one Type I glass container or, in the case of smaller containers, 35 mL of the combined contents of several Type I glass containers, prepared as directed for *Procedure* under *Water Attack at 121°*: the limit is 0.1 ppm.

BIOLOGICAL TESTS—PLASTICS AND OTHER POLYMERS

Two stages of testing are indicated. The first stage is the performance of *in-vitro* biological tests according to the procedures set forth in chapter ⟨87⟩, *Biological Reactivity Tests, In-vitro*. Materials that meet the requirements of the *in-vitro* tests are not required to undergo further testing. No plastic class designation is assigned to these materials. Materials that do not meet the requirements of the *in-vitro* tests are subjected to the second stage of testing which is the performance of *in-vivo* tests, i.e., the *Systemic Injection Test, Intracutaneous Test*, and *Implantation Test* according to the procedures set forth in chapter ⟨88⟩, *Biological Reactivity Tests, In-vivo*.

PHYSICOCHEMICAL TESTS—PLASTICS

The following tests, designed to determine physical and chemical properties of plastics and their extracts, are based on the extraction of the plastic material, and it is essential that the designated amount of the plastic be used. Also, the specified surface area must be available for extraction at the designated temperature.

Extracting Medium—Unless otherwise directed in a specific test below, use *Purified Water* (see monograph) as the extracting medium, maintained at a temperature of 70° during the extraction of the prepared *Sample*.

Apparatus—Use a water bath and the *Extraction Containers* as described under Chapter ⟨88⟩ *Biological Reactivity Tests, In-vivo*.

Preparation of Apparatus—Proceed as directed in the first paragraph under *Preparation of Apparatus* in Chapter ⟨88⟩ *Biological Reactivity Tests, In-vivo*. [NOTE—The containers and equipment need not be sterile.]

Procedure—

Preparation of Sample—From a homogeneous plastic specimen, use a portion, for each 20.0 mL of extracting medium, equivalent to 120 cm^2 total surface area (both sides combined), and subdivide into strips approximately 3 mm in width and as near to 5 cm in length as is practical. Transfer the subdivided *Sample* to a glass-stoppered, 250-mL graduated cylinder of Type I glass, and add about 150 mL of Purified Water. Agitate for about 30 seconds, drain off and discard the liquid, and repeat with a second washing.

Transfer the prepared *Sample* to a suitable extraction flask, and add the required amount of *Extracting Medium*. Extract by heating in a water bath at the temperature specified for the *Extracting Medium* for 24 hours. Cool, but not below 20°. Pipet 20 mL of the extract of the prepared *Sample* into a suitable container. Use this portion in the test for *Buffering Capacity*. Immediately decant the remaining extract into a suitably cleansed container, and seal.

Blank—Use *Purified Water* where a blank is specified in the following tests.

NONVOLATILE RESIDUE—Transfer, in suitable portions, 50.0 mL of the extract of the prepared *Sample* to a suitable, tared crucible (preferably a fused silica crucible that has been acid-cleaned), and evaporate the volatile matter on a steam bath. Similarly evaporate 50.0 mL of the *Blank* in a second crucible. [NOTE—If an oily residue is expected, inspect the crucible repeatedly during the evaporation and drying period, and reduce the amount of heat if the oil tends to creep along the walls of the crucible.] Dry at 105° for 1 hour: the difference between the amounts obtained from the *Sample* and the *Blank* does not exceed 15 mg.

RESIDUE ON IGNITION ⟨281⟩—Proceed with the *Nonvolatile Residue* obtained from the *Sample* and from the *Blank*, using, if necessary, additional sulfuric acid but adding the same amount of sulfuric acid to each crucible: the difference between the amounts of residue on ignition obtained from the *Sample* and the *Blank* does not exceed 5 mg.

HEAVY METALS—Pipet 20 mL of the extract of the prepared *Sample*, filtered if necessary, into one of two matched 50-mL color-comparison tubes. Adjust with 1 *N* acetic acid or 6 *N* ammonium hydroxide to a pH between 3.0 and 4.0, using short-range pH paper as external indicator, dilute with water to about 35 mL, and mix.

Into the second color-comparison tube pipet 2 mL of *Standard Lead Solution* (see *Heavy Metals* ⟨231⟩), and add 20 mL of the *Blank*. Adjust with 1 *N* acetic acid or 6 *N* ammonium hydroxide to a pH between 3.0 and 4.0, using short-range pH paper as external indicator, dilute with water to about 35 mL, and mix.

Add 10 mL of freshly prepared hydrogen sulfide TS to each tube, dilute with water to 50 mL, and mix: any brown color produced within 10 minutes in the tube containing the extract of the prepared *Sample* does not exceed that in the tube con-

taining the *Standard Lead Solution*, both tubes being viewed downward over a white surface (1 ppm in extract).

BUFFERING CAPACITY—Titrate the previously collected 20-mL portion of the extract of the prepared *Sample* potentiometrically to a pH of 7.0, using either 0.010 *N* hydrochloric acid or 0.010 *N* sodium hydroxide, as required. Treat a 20.0-mL portion of the *Blank* similarly: if the same titrant was required for both *Sample* and *Blank*, the difference between the two volumes is not greater than 10.0 mL; and if acid was required for either the *Sample* or the *Blank* and alkali for the other, the total of the two volumes required is not greater than 10.0 mL.

CONTAINERS FOR OPHTHALMICS—PLASTICS

Plastics for ophthalmics are composed of a mixture of homologous compounds, having a range of molecular weights. Such plastics frequently contain other substances such as residues from the polymerization process, plasticizers, stabilizers, antioxidants, pigments, and lubricants. Factors such as plastic composition, processing and cleaning procedures, contacting media, inks, adhesives, absorption, adsorption and permeability of preservatives, and conditions of storage may also affect the suitability of a plastic for a specific use. Evaluation of such factors should be made by appropriate additional specific tests to determine the suitability of a plastic for use as a container for ophthalmics.

Definition—For the purposes of this chapter, a *container* is that which holds the drug and is or may be in direct contact with the drug.

Biological Tests—Plastics and Other Polymers—Two stages of testing are indicated. The first stage is the performance of *in-vitro* biological tests according to the procedures set forth in chapter ⟨87⟩, *Biological Reactivity Tests, In-vitro*. Materials that meet the requirements of these tests are tested according to the procedures set forth for the *Systemic Injection Test*, *Intracutaneous Test*, and *Eye Irritation Test* in chapter ⟨88⟩, *Biological Reactivity Tests, In-vivo*. Materials that do not meet the requirements of the *in-vitro* tests cannot be used for ophthalmics.

POLYETHYLENE CONTAINERS

The standards and tests provided in this section characterize high-density and low-density polyethylene containers that are interchangeably suitable for packaging dry oral dosage forms not meant for constitution into solution.

Where stability studies have been performed to establish the expiration date of a particular dry oral dosage form not meant for constitution into solution in a container meeting the requirements set forth herein for either high- or low-density polyethylene containers, then any other polyethylene container meeting the same sections of these requirements may be similarly used to package such dosage form, provided that the appropriate stability programs are expanded to include the alternative container, in order to assure that the identity, strength, quality, and purity of the dosage form are maintained throughout the expiration period.

Both high- and low-density polyethylene are long-chain polymers synthesized under controlled conditions of heat and pressure, with the aid of catalysts from not less than 85.0 percent ethylene and not less than 95.0 percent total olefins. The other olefin ingredients most frequently used are butene, hexene, and propylene. The ingredients used to manufacture the polyethylene, and those used in the fabrication of the containers, conform to the requirements in the applicable sections of the Code of Federal Regulations, Title 21.

High-density polyethylene and low-density polyethylene both have an infrared absorption spectrum that is distinctive for polyethylene, and each possesses characteristic thermal properties. High-density polyethylene has a density between 0.941 and 0.965 g per cm^3. Low-density polyethylene has a density between 0.850 and 0.940 g per cm^3. The permeation properties of molded polyethylene containers may be altered when re-ground polymer is incorporated, depending upon the proportion of re-ground material in the final product. Other properties that may affect the suitability of polyethylene used in containers for packaging drugs are: oxygen and moisture permeability, modulus of elasticity, melt index, environmental stress crack resistance, and degree of crystallinity after molding. The requirements in this section are to be met when dry oral dosage forms, not meant for constitution into solution, are intended to be packaged in a container defined by this section.

Multiple Internal Reflectance—

APPARATUS—Use a double-beam infrared spectrophotometer equipped with a multiple internal reflectance accessory and a KRS-5 internal reflection plate.[4] A KRS-5 crystal 2 mm thick having an angle of incidence of 45° provides a sufficient number of reflections.

PREPARATION OF SPECIMEN—Cut 2 flat sections representative of the average wall thickness of the container, and trim them as necessary to obtain segments that are convenient for mounting in the multiple internal reflectance accessory. Taking care to avoid scratching the surfaces, wipe the specimens with dry paper or, if necessary, clean them with a soft cloth dampened with methanol, and permit them to dry. Securely mount the specimens on both sides of the KRS-5 internal reflection plate, ensuring adequate surface contact. Prior to mounting the specimens on the plate, they may be compressed to thin uniform films by exposing them to temperatures of about 177° under high pressures (15,000 psi or more).

PROCEDURE—Place the mounted specimen sections within the multiple internal reflectance accessory, and place the assembly in the specimen beam of the infrared spectrophotometer. Adjust the specimen position and mirrors within the accessory to permit maximum light transmission of the unattenuated reference beam. Upon completing the adjustments in the accessory, attenuate the reference beam to permit full-scale deflection during the scanning of the specimen. Determine the infrared spectrum from 3500 to 600 cm^{-1}: the spectrum of the specimen exhibits major absorption bands only at the same wavelengths as the spectrum of USP High-density Polyethylene RS or USP Low-density Polyethylene RS, similarly determined.

Thermal Analysis—Cut a section weighing about 12 mg, and place it in the test-specimen pan. Determine the thermogram under nitrogen, using equipment capable of performing the determinations described under *Thermal Analysis* ⟨891⟩.

High-density Polyethylene—The thermogram of the specimen is similar to the thermogram of USP High-density Polyethylene RS, similarly determined, and the temperatures of the endotherms and exotherms in the thermogram of the specimen do not differ from those of the standard by more than 6.0°.

Low-density Polyethylene—The thermogram of the specimen is similar to the thermogram of USP Low-density Polyethylene RS, similarly determined, and the temperatures of the endotherms and exotherms in the thermogram of the specimen do not differ from those of the standard by more than 8.0°.

Light Transmission—Polyethylene containers intended to provide protection from light meet the requirements under *Light Transmission*.

Water Vapor Permeation—Fit the containers with impervious seals obtained by heat-sealing the bottles with an aluminum foil–polyethylene laminate or other suitable seal.[5] Test the containers as described under *Containers—Permeation* ⟨671⟩: the high-density polyethylene containers so tested meet the requirements if the moisture permeability exceeds 10 mg per day per liter in not more than 1 of the 10 test containers and exceeds 25 mg per day per liter in none of them. The low-density polyethylene containers so tested meet the requirements if the moisture permeability exceeds 20 mg per day per liter in not more than 1 of the 10 test containers and exceeds 30 mg per day per liter in none of them.

Heavy Metals and Nonvolatile Residue—Prepare extracts of specimens for these tests as directed for *Preparation of Sample* in the *Procedure* under *Physicochemical Tests—Plastics*, except

[4] The multiple internal reflectance accessory and KRS-5 plate are available from several sources, including Beckman Instruments, Inc., 2500 Harbor Blvd., Fullerton, CA 92634, and from Perkin Elmer Corp., Main Ave., Norwalk, CT 06856.

[5] A suitable laminate for sealing has as the container contact layer polyethylene of not less than 0.025 mm (0.001 inch) and a second layer of aluminum foil of not less than 0.018 mm (0.0007 inch), with additional layers of suitable backing materials. A suitable seal can be obtained also by using glass plates and a sealing wax consisting of 60% of refined amorphous wax and 40% of refined crystalline paraffin wax.

that for each 20.0 mL of *Extracting Medium* the portion shall be 60 cm^2, regardless of thickness.

HEAVY METALS—Containers meet the requirements for *Heavy Metals* under *Physicochemical Tests—Plastics*.

NONVOLATILE RESIDUE—Proceed as directed for *Nonvolatile Residue* under *Physicochemical Tests—Plastics*, except that the blank shall be the same solvent used in each of the tests set forth below. The difference between the amounts obtained from the specimen and the blank does not exceed 12.0 mg when water maintained at a temperature of 70° is used as the extracting medium; does not exceed 75.0 mg when alcohol maintained at a temperature of 70° is used as the extracting medium; and does not exceed 100.0 mg for high-density polyethylene and does not exceed 350.0 mg for low-density polyethylene when hexanes maintained at a temperature of 50° are used as the extracting medium. Containers meet these requirements for *Nonvolatile Residue* for all of the above extracting media. [NOTE—Hexanes and alcohol are flammable. When evaporating these solvents, use a stream of air with the water bath; when drying the residue, use an explosion-proof oven.]

SINGLE-UNIT CONTAINERS AND UNIT-DOSE CONTAINERS FOR NON-STERILE SOLID AND LIQUID DOSAGE FORMS

An official dosage form is required to bear on its label an expiration date assigned for the particular formulation and package of the article. This date limits the time during which the product may be dispensed or used. Because the expiration date stated on the manufacturer's or distributor's package has been determined for the drug in that particular package and may not be applicable to the product where it has been repackaged in a different container, repackaged drugs dispensed pursuant to a prescription are exempt from this label requirement. It is necessary, therefore, that other precautions be taken by the dispenser to preserve the strength, quality, and purity of drugs that are repackaged for ultimate distribution or sale to patients.

The following guidelines and requirements are applicable where official dosage forms are repackaged into single-unit or unit-dose containers or mnemonic packs for dispensing pursuant to prescription.

Labeling—It is the responsibility of the dispenser, taking into account the nature of the drug repackaged, the characteristics of the containers, and the storage conditions to which the article may be subjected, to determine a suitable beyond-use date to be placed on the label. Such date is not later than the expiration date of the original package. In the absence of stability data to the contrary, such date should not exceed (1) 25% of the remaining time between the date of repackaging and the expiration date on the original manufacturer's bulk container, or (2) a six-month period from the date the drug is repackaged, whichever is earlier. Each single-unit or unit-dose container bears a separate label, unless the device holding the unit-dose form does not allow for the removal or separation of the intact single-unit or unit-dose container therefrom.

Storage—Store the repackaged article in a humidity-controlled environment and at the temperature specified in the individual monograph or in the product labeling. Where no temperature or humidity is specified in the monograph or in the labeling of the product, controlled room temperature and a relative humidity corresponding to 75% at 23° are not to be exceeded during repackaging or storage.

A refrigerator or freezer shall not be considered to be a humidity-controlled environment, and drugs that are to be stored at a cold temperature in a refrigerator or freezer shall be placed within an outer container that meets the monograph requirements for the drug contained therein.

CUSTOMIZED PATIENT MEDICATION PACKAGES

In lieu of dispensing two or more prescribed drug products in separate containers, a pharmacist may, with the consent of the patient, the patient's caregiver, or a prescriber, provide a customized patient medication package (patient med pak).[6]

A patient med pak is a package prepared by a pharmacist for a specific patient comprising a series of containers and containing two or more prescribed solid oral dosage forms. The patient med pak is so designed or each container is so labeled as to indicate the day and time, or period of time, that the contents within each container are to be taken.

Label—(A) The patient med pak shall bear a label stating:

(1) the name of the patient;

(2) a serial number for the patient med pak itself and a separate identifying serial number for each of the prescription orders for each of the drug products contained therein;

(3) the name, strength, physical description or identification, and total quantity of each drug product contained therein;

(4) the directions for use and cautionary statements, if any, contained in the prescription order for each drug product therein;

(5) any storage instructions or cautionary statements required by the official compendia;

(6) the name of the prescriber of each drug product;

(7) the date of preparation of the patient med pak and the beyond-use date assigned to the patient med pak (such beyond-use date shall be not later than 60 days from the date of preparation);

(8) the name, address, and telephone number of the dispenser and the dispenser's registration number where necessary; and

(9) any other information, statements, or warnings required for any of the drug products contained therein.

(B) If the patient med pak allows for the removal or separation of the intact containers therefrom, each individual container shall bear a label identifying each of the drug products contained therein.

Labeling—The patient med pak shall be accompanied by a patient package insert, in the event that any medication therein is required to be dispensed with such insert as accompanying labeling. Alternatively, such required information may be incorporated into a single, overall educational insert provided by the pharmacist for the total patient med pak.

Packaging—In the absence of more stringent packaging requirements for any of the drug products contained therein, each container of the patient med pak shall comply with the moisture permeation requirements for a Class B single-unit or unit-dose container (see *Containers—Permeation* ⟨671⟩). Each container shall be either not reclosable or so designed as to show evidence of having been opened.

Guidelines—It is the responsibility of the dispenser, when preparing a patient med pak, to take into account any applicable compendial requirements or guidelines and the physical and chemical compatibility of the dosage forms placed within each container, as well as any therapeutic incompatibilities that may attend the simultaneous administration of the medications. In this regard, pharmacists are encouraged to report to USP headquarters any observed or reported incompatibilities.

Record keeping—In addition to any individual prescription filing requirements, a record of each patient med pak shall be made and filed. Each record shall contain, as a minimum:

(1) the name and address of the patient;

(2) the serial number of the prescription order for each drug product contained therein;

(3) the name of the manufacturer or labeler and lot number for each drug product contained therein;

(4) information identifying or describing the design, characteristics, or specifications of the patient med pak sufficient to allow subsequent preparation of an identical patient med pak for the patient;

(5) the date of preparation of the patient med pak and the beyond-use date that was assigned;

(6) any special labeling instructions; and

(7) the name or initials of the pharmacist who prepared the patient med pak.

[6] It should be noted that there is no special exemption for patient med paks from the requirements of the Poison Prevention Packaging Act. Thus the patient med pak, if it does not meet child-resistant standards, shall be placed in an outer package that does comply, or the necessary consent of the purchaser or physician, to dispense in a container not intended to be child-resistant, shall be obtained.

⟨671⟩ CONTAINERS—PERMEATION

This test is provided to determine the moisture permeability of a container utilized for a drug when dispensed on prescription where packaging and storage in a *tight container* or a *well-closed container* is specified in the individual monograph. It is applicable to multiple-unit containers (see *Preservation, Packaging, Storage, and Labeling* under *General Notices*). As used herein, the term "container" refers to the entire system comprising, usually, the container itself, the liner (if used), and the closure. Where the manufacturer's container, previously unopened, is utilized for dispensing the drug, such container is exempt from the requirements of this test.

Desiccant—Place a quantity of 4-mesh, anhydrous calcium chloride[1] in a shallow container, taking care to exclude any fine powder, then dry at 110° for 1 hour, and cool in a desiccator.

Procedure—Select 12 containers of a uniform size and type, clean the sealing surfaces with a lint-free cloth, and close and open each container 30 times. Apply the closure firmly and uniformly each time the container is closed. Close screw-capped containers with a torque that is within the range of tightness specified in the accompanying table. Add *Desiccant* to 10 of the containers, designated *test containers*, filling each to within 13 mm of the closure if the container volume is 20 mL or more, or filling each to two-thirds of capacity if the container volume is less than 20 mL. If the interior of the container is more than 63 mm in depth, an inert filler or spacer may be placed in the bottom to minimize the total weight of the container and *Desiccant;* the layer of *Desiccant* in such a container shall be not less than 5 cm in depth. Close each immediately after adding *Desiccant*, applying the torque designated in the accompanying table when closing screw-capped containers. To each of the remaining 2 containers, designated *controls*, add a sufficient number of glass beads to attain a weight approximately equal to that of each of the *test containers*, and close, applying the torque designated in the accompanying table when closing screw-capped containers. Record the weight of the individual containers so prepared to the nearest 0.1 mg if the container volume is less than 20 mL; to the nearest mg if the container volume is 20 mL or more but less than 200 mL; or to the nearest centigram (10 mg) if the container volume is 200 mL or more; and store at 75 ± 3% relative humidity and a temperature of 20 ± 2°. [NOTE—A saturated system of 35 g of sodium chloride with each 100 mL of water placed in the bottom of a desiccator maintains the specified humidity. Other methods may be employed to maintain these conditions.] After 336 ± 1 hours (14 days), record the weight of the individual containers in the same manner. Completely fill 5 empty containers of the same size and type as the containers under test with water or a non-compressible, free-flowing solid such as well-tamped fine glass beads, to the level indicated by the closure surface when in place. Transfer the contents of each to a graduated cylinder, and determine the average container volume, in mL. Calculate the rate of moisture permeability, in mg per day per liter, by the formula:

$$(1000/14V)[(T_f - T_i) - (C_f - C_i)],$$

in which V is the volume, in mL, of the container, $(T_f - T_i)$ is the difference, in mg, between the final and initial weights of each *test container*, and $(C_f - C_i)$ is the average of the differences, in mg, between the final and initial weights of the 2 *controls*. The containers so tested are *tight containers* if not more than one of the 10 *test containers* exceeds 100 mg per day per liter in moisture permeability, and none exceeds 200 mg per day per liter.

The containers are *well-closed containers* if not more than one of the 10 *test containers* exceeds 2000 mg per day per liter in moisture permeability, and none exceeds 3000 mg per day per liter.

[1] Suitable 4-mesh, anhydrous calcium chloride is available commercially as Item SC11204-5006M from Sargent-Welch Scientific Co., P. O. Box 1026, Skokie, IL 60077.

SINGLE-UNIT CONTAINERS AND UNIT-DOSE CONTAINERS FOR CAPSULES AND TABLETS

To permit an informed judgment regarding the suitability of the packaging for a particular type of product, the following procedure and classification scheme are provided for evaluating the moisture-permeation characteristics of single-unit and unit-dose containers. Inasmuch as equipment and operator performance may affect the moisture permeation of a container formed or closed, the moisture-permeation characteristics of the packaging system being utilized shall be determined.

Desiccant—Dry suitable desiccant pellets[2] at 110° for 1 hour prior to use. Use pellets weighing approximately 400 mg each and having a diameter of approximately 8 mm.

Procedure—

Method I—Seal not less than 10 unit-dose containers with 1 pellet in each, and seal 10 additional, empty unit-dose containers to provide the controls, using finger cots or padded forceps to handle the sealed containers. Number the containers, and record the individual weights[3] to the nearest mg. Weigh the controls as a unit, and divide the total weight by the number of controls to obtain the average. Store all of the containers at 75 ± 3% relative humidity and at a temperature of 20 ± 2°. [NOTE—A saturated system of 35 g of sodium chloride with each 100 mL of water placed in the bottom of a desiccator maintains the specified humidity. Other methods may be employed to maintain these conditions.] After a 24-hour interval, or a multiple thereof (see *Results*), remove the containers from the chamber, and allow them to equilibrate for 15 to 60 minutes in the weighing area. Again record the weight of the individual containers and the combined controls in the same manner. [NOTE—If any indicating pellets turn pink during this procedure, or if the pellet weight increase exceeds 10%, terminate the test, and regard only earlier determinations as valid.] Return the containers to the humidity chamber. Calculate the rate of moisture permeation, in mg per day, of each container by the formula:

$$(1/N)[(W_f - W_i) - (C_f - C_i)],$$

in which N is the number of days expired in the test period, $(W_f - W_i)$ is the difference, in mg, between the final and initial weights of each test container, and $(C_f - C_i)$ is the average of the difference, in mg, between the final and initial weights of the controls, the data being calculated to two significant figures. [NOTE—Where the permeations measured are less than 5 mg per day, and where the controls are observed to reach a steady state in 7 days, the individual permeations may be determined more accurately after an initial 7 days of equilibration by using that weight as W_i, zero time, in the calculation.]

Method II—Use this procedure for packs (e.g., punch-out cards) that incorporate a number of separately sealed unit-dose containers or blisters. Seal a sufficient number of packs, such that not less than 4 packs and a total of not less than 10 unit-dose containers or blisters filled with 1 pellet in each unit are tested. Seal a corresponding number of empty packs, each pack containing the same number of unit-dose containers or blisters as used in the test packs, to provide the controls. Store all of the containers at 75 ± 3% relative humidity and at a temperature of 20 ± 2°. [See *Note* under *Method I*.] After 24 hours, and at multiples thereof (see *Results*), remove the packs from the chamber, and allow them to equilibrate for approximately 45 minutes. Record the weights of the individual packs, and return them to the chamber. Weigh the control packs as a unit, and divide the total weight by the number of control packs to obtain the average empty pack weight. [NOTE—If any indicating pel-

[2] Suitable moisture-indicating desiccant pellets are available commercially from sources such as Medical Packaging, Inc., 525 White Horse Pike, Atco, NJ 08004 [Telephone 800-257-5282; in N. J., 609-767-3604], as Indicating Desiccant Pellets, item No. TK-1002.

[3] Accurate comparisons of *Class A* containers may require test periods in excess of 28 days if weighings are performed on a *Class A* prescription balance (see *Prescription Balances and Volumetric Apparatus* ⟨1176⟩). The use of an analytical balance on which weights can be recorded to 4 or 5 decimal places may permit more precise characterization between containers and/or shorter test periods.

lets turn pink during the procedure, or if the average pellet weight increase in any pack exceeds 10%, terminate the test, and regard only earlier determinations as valid.] Calculate the average rate of moisture permeation, in mg per day, for each unit-dose container or blister in each pack by the formula:

$$(1/NX)[(W_f - W_i) - (C_f - C_i)],$$

in which N is the number of days expired in the test period (beginning after the initial 24 hour equilibration period), X is the number of separately sealed units per pack, $(W_f - W_i)$ is the difference, in mg, between the final and initial weights of each test pack, and $(C_f - C_i)$ is the average of the difference, in mg, between the final and initial weights of the control packs, the rates being calculated to two significant figures.

Results—The individual unit-dose containers as tested in *Method I* are designated *Class A* if not more than 1 of 10 containers tested exceeds 0.5 mg per day in moisture permeation rate and none exceeds 1 mg per day; they are designated *Class B* if not more than 1 of 10 containers tested exceeds 5 mg per day and none exceeds 10 mg per day; they are designated *Class C* if not more than 1 of 10 containers tested exceeds 20 mg per day and none exceeds 40 mg per day; and they are designated *Class D* if the containers tested meet none of the moisture permeation rate requirements.

The packs as tested in *Method II* are designated *Class A* if no pack tested exceeds 0.5 mg per day in average blister moisture permeation rate; they are designated *Class B* if no pack tested exceeds 5 mg per day in average blister moisture permeation rate; they are designated *Class C* if no pack tested exceeds 20 mg per day in average blister moisture permeation rate; and they are designated *Class D* if the packs tested meet none of the above average blister moisture permeation rate requirements.

With the use of the *Desiccant* described herein, suitable test intervals for the final weighings, W_f, are: 24 hours for *Class D;* 48 hours for *Class C;* 7 days for *Class B;* and not less than 28 days for *Class A.*

Torque Applicable to Screw-Type Container

Closure Diameter[1] (mm)	Suggested Tightness Range with Manually Applied Torque[2] (inch-pounds)
15	6 to 9
18	7 to 11
20	8 to 12
22	9 to 13
24	10 to 15
28	11 to 17
33	13 to 20
38	15 to 23
43	17 to 26
48	19 to 29
53	21 to 32
58	23 to 35
63	25 to 38
70	28 to 42
83	34 to 49
86	35 to 51
89	36 to 53
100	40 to 60
110	45 to 65
120	48 to 72
132	53 to 79

[1] The torque designated for the next larger closure diameter is to be applied in testing containers having a closure diameter intermediate to the diameters listed.

[2] A suitable apparatus is available from Owens-Illinois, Toledo, Ohio 43666. (Model 25 torque tester is used for testing between 0 and 25; Model 50 for testing between 0 and 50; and Model 100 for testing between 0 and 100 inch-pounds of torque.) The torque values refer to application, not removal, of the closure. For further detail regarding instructions, reference may be made to "Standard Method of Measuring Application and Removal Torque of Threaded Closures," ASTM Designation D 3198-73, published by the American Society for Testing and Materials, 1916 Race St., Philadelphia, PA 19103.

⟨691⟩ COTTON

Preparatory to the determination of absorbency and of fiber length, remove the Cotton from its wrappings, and condition it for not less than 4 hours in a standard atmosphere of 65 ± 2% relative humidity at 21 ± 1.1° (70 ± 2°F).

Absorbency Test

Procedure—Prepare a test basket, weighing not more than 3 g, from copper wire approximately 0.4 mm in diameter (No. 26 B. & S.) in the form of a cylinder approximately 5 cm in diameter and 8 cm deep, with spaces of about 2 cm between the wires. Take portions of purified cotton weighing 1 ± 0.05 g from five different parts of the package by pulling, not cutting, the specimens, place the combined portions in the basket, and weigh. Hold the basket on its side approximately 12 mm above the surface of water at 25 ± 1°, and drop it into the water. Determine, preferably by use of a stop watch, the time in seconds required for complete submersion.

Remove the basket from the water, allow it to drain for 10 seconds in the same horizontal position, then place it immediately in a tared, covered vessel and weigh, deducting the weight of the test basket and of the purified cotton to find the weight of water absorbed.

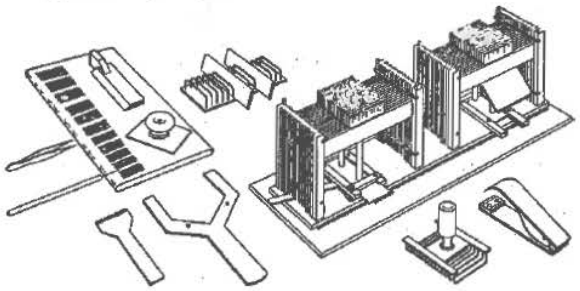
Fiber Length

For the determination of the length and of the length distribution of cotton fibers in purified cotton use the following method:

Carry out all operations associated with the determination of fiber length of purified cotton in an atmosphere maintained at 65 ± 2% relative humidity at 21 ± 1.1° (70 ± 2°F).

These directions describe the mode of procedure that is well adapted to the sorter* most extensively used in the United States at the present time.

Apparatus—The sorter (see illustration) consists of two banks of combs rigidly mounted side by side on a common base. Each bank of combs consists of at least 12 individual combs spaced 3.2 mm apart, one behind the other, and mounted in grooves so that as they are approached during the fractionating process and no longer needed, they may be dropped below the working plane. Each individual comb has a single row of accurately aligned and sharply pointed teeth, 12 mm long, consisting of needles 0.38 mm in diameter. The teeth are spaced 62 to 25 mm over an extent of approximately 50 mm.



Duplex Cotton Fiber Sorter

Accessory equipment consists of fiber-sorter forceps, fiber-depressing grid, fiber-depressing smooth plate, and velvet-covered plates. The sorter forceps consist of two brass pieces approximately 75 mm long, hinged on one end and slightly curved to present a beaked aspect at the gripping end for gripping the protruding fibers close to the surfaces of the combs. Usually, one of the gripping edges has a leather or other fibrous padding. The gripping edge is approximately 19 mm wide.

The fiber-depressing grid consists of a series of brass rods spaced 3.2 mm apart so that they may be placed between the combs to

*NOTE—The method here described is especially adapted to the Suter-Webb Duplex Cotton Fiber sorting apparatus, but with more or less obvious alteration in procedure, may be carried out with two Baer sorters in tandem arrangement, or with a Johannsen or other similar apparatus.

press the fibers down between the teeth. The fiber-depressing smooth plate consists of a polished brass plate approximately 25 × 50 mm, with a knob or handle on the upper surface whereby the plate may be smoothed over the fibers as they are laid on the velvet surface of the array plates. The velvet-covered plates, upon which the fibers may be arrayed, are aluminum sheets approximately 100 mm × 225 mm × 2.4 mm thick, covered on both sides with high-grade velvet, preferably black.

Selection of Cotton—After unrolling the cotton, prepare a representative laboratory test specimen by taking from a package containing from 8 to 16 ounces, 32 pinches (about 75 mg each) well distributed throughout the bulk of the lap, 16 representative pinches being taken from each longitudinal half of the lap. Avoid the cut ends of the lap, and take particular care to secure portions throughout the thickness of the lap. To avoid biased selection of long or short fibers, remove all fibers of the group pinched and do not allow them to slip from between the fingers.

From packages of not more than 4 ounces in weight, take 8 pinches, and from packages weighing more than 4 ounces and not more than 8 ounces, take 16 pinches, all well distributed.

Mix the pinches in pairs promiscuously, and combine each pair by gently drawing and lapping them in the fingers. Then divide each combined pair by splitting longitudinally into two approximately equal parts and utilize one part in the further mixing. (The other part may be discarded or reserved for any further tests or checks.)

Repeat the process described in the preceding paragraph with the successive halves of the bifurcated series until only 1 pinch, the final composite test portion, results. Gently parallel and straighten the fibers of the final composite test portion by drawing and lapping them in the fingers. Take care to retain all of the fibers, including as far as possible those of the neps (specks of entangled fibers) and naps (matted masses of fibers), discarding only motes (immature seed fragments with fibers) and nonfiber foreign material such as stem, leaf, and fragments of seedcoats.

From the final composite portion described in the preceding paragraph, separate longitudinally a test portion of 75 ± 2 mg, accurately weighed. Retain the residue for any check test necessary.

Procedure—With the fiber-depressing grid carefully insert the weighed test portion into one bank of combs of the cotton sorter, so that it extends across the combs at approximately right angles.

With the sorter forceps, grip by the free ends a small portion of the fibers extending through the teeth of the comb nearest to the operator; gently and smoothly draw them forward out of the combs, and transfer them to the tips of the teeth in the second bank of combs, laying them parallel to themselves, straight, and approximately at right angles to the faces of the combs, releasing the gripped ends as near to the face of the front comb as possible. With the depressor grid carefully press the transferred fibers down into the teeth of the combs. Continue the operation until all of the fibers are transferred to the second bank of combs. During this transfer of the fibers, drop the combs of the first bank in succession when and as all of the protruding fibers have been removed.

Turn the machine through 180°, and transfer the cotton fibers back to the *first bank* of combs in the manner described in the preceding paragraph.

Take great care in evening up the ends of the fibers during both of the above transfers, arranging them as closely as possible to the front surface of the proximal comb. Such evening out of the ends of the protruding fibers may involve drawing out straggling fibers from both the front and rear aspects of the banks of combs, and re-depositing them into and over the main bundle in the combs.

Turn the machine again through 180°. Drop successive combs if necessary to expose the ends of the longest fibers. It may be necessary to re-deposit some straggling fibers. With the forceps withdraw the few most protuberant fibers. In this way continue to withdraw successively the remaining protuberant fibers back to the front face of the proximal comb. Drop this comb and repeat the series of operations in the same manner until all of the fibers have been drawn out. In order not to disturb seriously the portion being tested, and thereby vitiate the length fractionation into length groups, make several pulls (as many as 8 to 10) between each pair of combs.

Lay the pulls on the velvet-covered plates alongside each other, as straight as possible, with the ends as clearly defined as possible, and with the distal ends arranged in a straight line, pressing them down gently and smoothly with the fiber-depressing smooth plate before releasing the pull from the forceps. Employ not less than 50 and not more than 100 pulls to fractionate the test portion.

Group together all of the fibers measuring 12.5 mm (about ½ inch) or more in length, and weigh the group to the nearest 0.3 mg. In the same manner, group together all fibers 6.25 mm (about ¼ inch) or less in length, and weigh in the same manner. Finally, group the remaining fibers of intermediate lengths together and weigh. The sum of the three weights does not differ from the initial weight of the test portion by more than 3 mg. Divide the weight of each of the first two groups by the weight of the test portion to obtain the percentage by weight of fiber in the two ranges of length.

⟨695⟩ CRYSTALLINITY

This test is provided to determine compliance with the crystallinity requirement where stated in the individual monograph for a drug substance.

Procedure—Unless otherwise specified in the individual monograph, mount a few particles of the specimen in mineral oil on a clean glass slide. Examine the mixture using a polarizing microscope: the particles show birefringence (interference colors) and extinction positions when the microscope stage is revolved.

⟨701⟩ DISINTEGRATION

This test is provided to determine compliance with the limits on *Disintegration* stated in the individual monographs except where the label states that the tablets or capsules are intended for use as troches, or are to be chewed, or are designed to liberate the drug content gradually over a period of time or to release the drug over two or more separate periods with a distinct time interval between such release periods. Determine the type of units under test from the labeling and from observation, and apply the appropriate procedure to 6 or more dosage units.

For the purposes of this test, disintegration does not imply complete solution of the unit or even of its active constituent. Complete disintegration is defined as that state in which any residue of the unit, except fragments of insoluble coating or capsule shell, remaining on the screen of the test apparatus is a soft mass having no palpably firm core.

Apparatus

The apparatus[1] consists of a basket-rack assembly, a 1000-mL, low-form beaker for the immersion fluid, a thermostatic arrangement for heating the fluid between 35° and 39°, and a device for raising and lowering the basket in the immersion fluid at a constant frequency rate between 29 and 32 cycles per minute through a distance of not less than 5.3 cm and not more than 5.7 cm. The volume of the fluid in the vessel is such that at the highest point of the upward stroke the wire mesh remains at least 2.5 cm below the surface of the fluid and descends to not less than 2.5 cm from the bottom of the vessel on the downward stroke. The time required for the upward stroke is equal to the time required for the downward stroke, and the change in stroke direction is a smooth transition, rather than an abrupt reversal of motion. The basket-rack assembly moves vertically along its axis. There is no appreciable horizontal motion or movement of the axis from the vertical.

Basket-rack Assembly—The basket-rack assembly consists of six open-ended transparent tubes, each 7.75 ± 0.25 cm long and having an inside diameter of approximately 21.5 mm and a wall approximately 2 mm thick; the tubes are held in a vertical position by two plastic plates, each about 9 cm in diameter and 6 mm in thickness, with six holes, each about 24 mm in diameter, equidistant from the center of the plate and equally spaced from one another. Attached to the under surface of the lower plate is 10-mesh No. 23 (0.025-inch) W. and M. gauge woven stainless-steel

[1] A suitable apparatus, meeting these specifications, is available from laboratory supply houses, from Van-Kel Industries, Inc., 36 Meridian Rd., Edison, NJ 08820, or from Hanson Research Corp., P. O. Box 35, Northridge, CA 91324.

wire cloth having a plain square weave. The parts of the apparatus are assembled and rigidly held by means of three bolts passing through the two plastic plates. A suitable means is provided to suspend the basket-rack assembly from the raising and lowering device using a point on its axis.

The design of the basket-rack assembly may be varied somewhat provided the specifications for the glass tubes and the screen mesh size are maintained.

Disks[2]—Each tube is provided with a slotted and perforated cylindrical disk 9.5 ± 0.15 mm thick and 20.7 ± 0.15 mm in diameter. The disk is made of a suitable, transparent plastic material having a specific gravity of between 1.18 and 1.20. Five 2-mm holes extend between the ends of the cylinder, one of the holes being through the cylinder axis and the others parallel with it equally spaced on a 6-mm radius from it. Equally spaced on the sides of the cylinder are four notches that form V-shaped planes that are perpendicular to the ends of the cylinder. The dimensions of each notch are such that the openings on the bottom of the cylinder are 1.60 mm square and those on the top are 9.5 mm wide and 2.55 mm deep. All surfaces of the disk are smooth.

Procedure

Uncoated Tablets—Place 1 tablet in each of the six tubes of the basket, add a disk to each tube, and operate the apparatus, using water maintained at 37 ± 2° as the immersion fluid unless another fluid is specified in the individual monograph. At the end of the time limit specified in the monograph, lift the basket from the fluid, and observe the tablets: all of the tablets have disintegrated completely. If 1 or 2 tablets fail to disintegrate completely, repeat the test on 12 additional tablets: not less than 16 of the total of 18 tablets tested disintegrate completely.

Plain Coated Tablets—Place 1 tablet in each of the six tubes of the basket and, if the tablet has a soluble external coating, immerse the basket in water at room temperature for 5 minutes. Then add a disk to each tube, and operate the apparatus, using simulated gastric fluid TS maintained at 37 ± 2° as the immersion fluid. After 30 minutes of operation in simulated gastric fluid TS, lift the basket from the fluid, and observe the tablets. If the tablets have not disintegrated completely, substitute simulated intestinal fluid TS maintained at 37 ± 2° as the immersion fluid, and continue the test for a total period of time, including previous exposure to water and simulated gastric fluid TS, equal to the time limit specified in the individual monograph plus 30 minutes, lift the basket from the fluid, and observe the tablets: all of the tablets have disintegrated completely. If 1 or 2 tablets fail to disintegrate completely, repeat the test on 12 additional tablets: not less than 16 of the total of 18 tablets tested disintegrate completely.

Enteric-coated Tablets—Place 1 tablet in each of the six tubes of the basket and, if the tablet has a soluble external coating, immerse the basket in water at room temperature for 5 minutes. Then operate the apparatus, without adding the disks, using simulated gastric fluid TS maintained at 37 ± 2° as the immersion fluid. After 1 hour of operation in simulated gastric fluid TS, lift the basket from the fluid, and observe the tablets: the tablets show no evidence of disintegration, cracking, or softening. Then add a disk to each tube, and operate the apparatus, using simulated intestinal fluid TS maintained at 37 ± 2° as the immersion fluid, for a period of time equal to 2 hours plus the time limit specified in the individual monograph, or, where only an enteric-coated tablet is recognized, for only the time limit specified in the monograph. Lift the basket from the fluid, and observe the tablets: all of the tablets disintegrate completely. If 1 or 2 tablets fail to disintegrate completely, repeat the test on 12 additional tablets: not less than 16 of the total of 18 tablets tested disintegrate completely.

Buccal Tablets—Apply the test for *Uncoated Tablets*, but omit the use of the disks. After 4 hours, lift the basket from the fluid, and observe the tablets: all of the tablets have disintegrated. If 1 or 2 tablets fail to disintegrate completely, repeat the test on 12 additional tablets: not less than 16 of the total of 18 tablets tested disintegrate completely.

Sublingual Tablets—Apply the test for *Uncoated Tablets*, but omit the use of the disks. Observe the tablets within the time limit specified in the individual monograph: all of the tablets have disintegrated. If 1 or 2 tablets fail to disintegrate completely, repeat the test on 12 additional tablets: not less than 16 of the total of 18 tablets tested disintegrate completely.

Hard Gelatin Capsules—Apply the test for *Uncoated Tablets*, but omit the use of disks. In place of disks attach a removable 10-mesh wire cloth,[3] as described under *Basket-rack Assembly*, to the surface of the upper plate of the basket-rack assembly. Observe the capsules within the time limit specified in the individual monograph: all of the capsules have disintegrated except for fragments from the capsule shell. If 1 or 2 capsules fail to disintegrate completely, repeat the test on 12 additional capsules: not less than 16 of the total of 18 capsules tested disintegrate completely.

Soft Gelatin Capsules—Proceed as directed under *Hard Gelatin Capsules*.

[2] Disks meeting these specifications are obtainable from Van-Kel Industries, Inc.

[3] A suitable wire cloth cover is available as Van-Kel Industries Part TT-1030.

⟨711⟩ DISSOLUTION

This test is provided to determine compliance with the dissolution requirements where stated in the individual monograph for a tablet or capsule dosage form, except where the label states that the tablets are to be chewed. Requirements for *Dissolution* do not apply to soft gelatin capsules unless specified in the individual monograph. Where the label states that an article is enteric-coated, and a dissolution or disintegration test that does not specifically state that it applied to enteric-coated articles is included in the individual monograph, the test for *Delayed-release Articles* under *Drug Release* ⟨724⟩ is applied unless otherwise specified in the individual monograph. Of the types of apparatus described herein, use the one specified in the individual monograph.

Apparatus 1—The assembly consists of the following: a covered vessel made of glass or other inert, transparent material[1]; a motor; a metallic drive shaft; and a cylindrical basket. The vessel is partially immersed in a suitable water bath of any convenient size that permits holding the temperature inside the vessel at 37 ± 0.5° during the test and keeping the bath fluid in constant, smooth motion. No part of the assembly, including the environment in which the assembly is placed, contributes significant motion, agitation, or vibration beyond that due to the smoothly rotating stirring element. Apparatus that permits observation of the specimen and stirring element during the test is preferable. The vessel is cylindrical, with a hemispherical bottom. It is 160 mm to 175 mm high, its inside diameter is 98 mm to 106 mm, and its nominal capacity is 1000 mL. Its sides are flanged at the top. A fitted cover may be used to retard evaporation.[2] The shaft is positioned so that its axis is not more than 2 mm at any point from the vertical axis of the vessel and rotates smoothly and without significant wobble. A speed-regulating device is used that allows the shaft rotation speed to be selected and maintained at the rate specified in the individual monograph, within ±4%.

Shaft and basket components of the stirring element are fabricated of stainless steel, type 316 or equivalent, to the specifications shown in Figure 1. Unless otherwise specified in the individual monograph, use 40-mesh cloth. A basket having a gold coating 0.0001 inch (2.5 μm) thick may be used. The dosage unit is placed in a dry basket at the beginning of each test. The distance between the inside bottom of the vessel and the basket is maintained at 25 ± 2 mm during the test.

Apparatus 2—Use the assembly from *Apparatus 1*, except that a paddle formed from a blade and a shaft is used as the stirring element. The shaft is positioned so that its axis is not more than 2 mm at any point from the vertical axis of the vessel, and rotates smoothly without significant wobble. The blade passes through the diameter of the shaft so that the bottom of the blade is flush with the bottom of the shaft. The paddle conforms to the specifications shown in Figure 2. The distance of 25 ± 2 mm between the blade and the inside bottom of the vessel is maintained during

[1] The materials should not sorb, react, or interfere with the specimen being tested.

[2] If a cover is used, it provides sufficient openings to allow ready insertion of the thermometer and withdrawal of specimens.

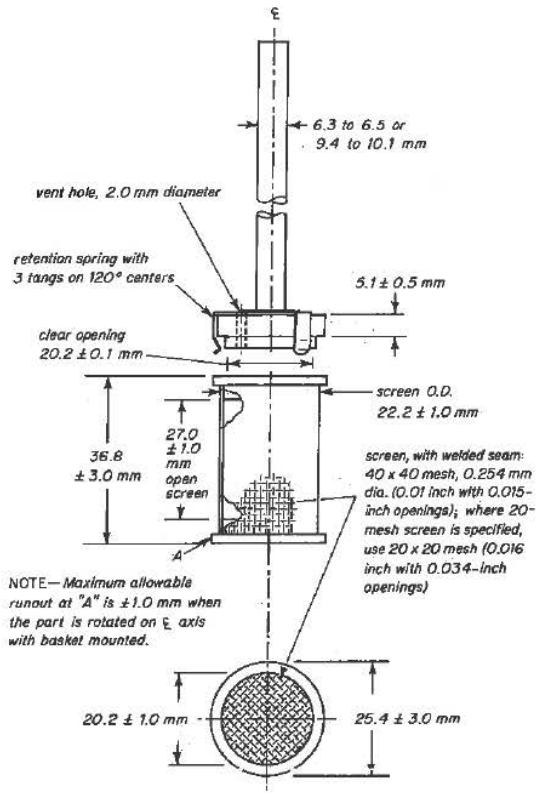


Fig. 1. Basket Stirring Element.

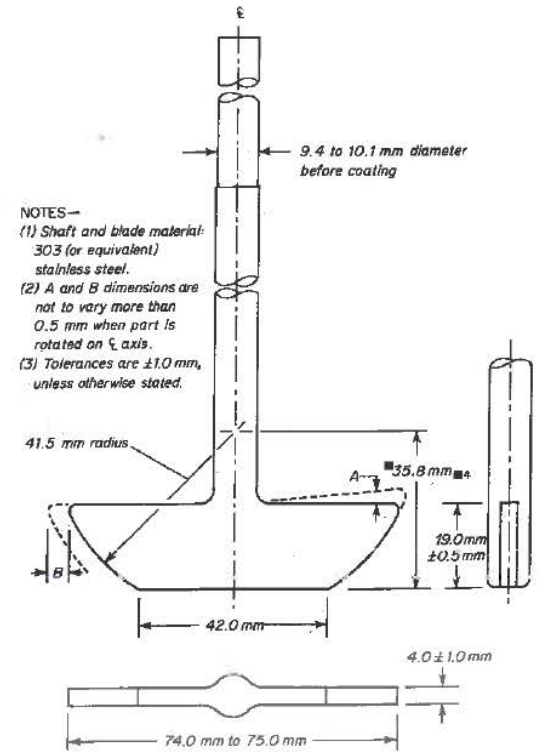


Fig. 2. Paddle Stirring Element.

the test. The metallic blade and shaft comprise a single entity that may be coated with a suitable inert coating. The dosage unit is allowed to sink to the bottom of the vessel before rotation of the blade is started. A small, loose piece of nonreactive material such as not more than a few turns of wire helix may be attached to dosage units that would otherwise float.

Apparatus Suitability Test—Individually test 1 tablet of the *USP Dissolution Calibrator, Disintegrating Type*[3] and 1 tablet of *USP Dissolution Calibrator, Nondisintegrating Type*,[3] according to the operating conditions specified. The apparatus is suitable if the results obtained are within the acceptable range stated in the certificate for that calibrator in the apparatus tested.

Dissolution Medium—Use the solvent specified in the individual monograph. If the *Dissolution Medium* is a buffered solution, adjust the solution so that its pH is within 0.05 unit of the pH specified in the individual monograph. [NOTE—Dissolved gases can cause bubbles to form which may change the results of the test. In such cases, dissolved gases should be removed prior to testing.]

Time—Where a single time specification is given, the test may be concluded in a shorter period if the requirement for minimum amount dissolved is met. If two or more times are specified, specimens are to be withdrawn only at the stated times, within a tolerance of ±2%.

Procedure for Capsules, Uncoated Tablets, and Plain Coated Tablets—Place the stated volume of the *Dissolution Medium* in the vessel of the apparatus specified in the individual monograph, assemble the apparatus, equilibrate the *Dissolution Medium* to 37 ± 0.5°, and remove the thermometer. Place 1 tablet or 1 capsule in the apparatus, taking care to exclude air bubbles from the surface of the dosage-form unit, and immediately operate the apparatus at the rate specified in the individual monograph. Within the time interval specified, or at each of the times stated, withdraw a specimen from a zone midway between the surface of the *Dissolution Medium* and the top of the rotating basket or blade, not less than 1 cm from the vessel wall. Perform the analysis as directed in the individual monograph. Repeat the test with additional dosage form units.

Where capsule shells interfere with the analysis, remove the contents of not less than 6 capsules as completely as possible, and dissolve the empty capsule shells in the specified volume of *Dissolution Medium*. Perform the analysis as directed in the individual monograph. Make any necessary correction. Correction factors greater than 25% of the labeled content are unacceptable.

Interpretation—Unless otherwise specified in the individual monograph, the requirements are met if the quantities of active ingredient dissolved from the units tested conform to the accompanying acceptance table. Continue testing through the three stages unless the results conform at either S_1 or S_2. The quantity, Q, is the amount of dissolved active ingredient specified in the individual monograph, expressed as a percentage of the labeled content; both the 5% and 15% values in the acceptance table are percentages of the labeled content so that these values and Q are in the same terms.

Acceptance Table

Stage	Number Tested	Acceptance Criteria
S_1	6	Each unit is not less than $Q + 5\%$.
S_2	6	Average of 12 units ($S_1 + S_2$) is equal to or greater than Q, and no unit is less than $Q - 15\%$.
S_3	12	Average of 24 units ($S_1 + S_2 + S_3$) is equal to or greater than Q, not more than 2 units are less than $Q - 15\%$, and no unit is less than $Q - 25\%$.

⟨721⟩ DISTILLING RANGE

To determine the range of temperatures within which an official liquid distils, or the percentage of the material that distils between two specified temperatures, use Method I or Method II

[3] Available from USP-NF Reference Standards, 12601 Twinbrook Parkway, Rockville, MD 20852.

as directed in the individual monograph. The *lower limit* of the range is the temperature indicated by the thermometer when the first drop of condensate leaves the tip of the condenser, and the *upper limit* is the Dry Point, i.e., the temperature at which the last drop of liquid evaporates from the lowest point in the distillation flask, without regard to any liquid remaining on the side of the flask, or the temperature observed when the proportion specified in the individual monograph has been collected.

[NOTE—Cool all liquids that distil below 80° to between 10° and 15° before measuring the sample to be distilled.]

Method I

Apparatus—Use apparatus similar to that specified for *Method II*, except that the distilling flask is of 50- to 60-mL capacity, and the neck of the flask is 10 to 12 cm long and 14 to 16 mm in internal diameter. The perforation in the upper asbestos board, if one is used, should be such that when the flask is set into it, the portion of the flask below the upper surface of the asbestos has a capacity of 3 to 4 mL.

Procedure—Proceed as directed for *Method II*, but place in the flask only 25 mL of the liquid to be tested.

Method II

Apparatus—Use an apparatus consisting of the following parts:

Distilling Flask—A round-bottom distilling flask, of heat-resistant glass, of 200-mL capacity, and having a total length of 17 to 19 cm and an inside neck diameter of 20 to 22 mm. Attached about midway on the neck, approximately 12 cm from the bottom of the flask, is a side-arm 10 to 12 cm long and 5 mm in internal diameter, which forms an angle of 70° to 75° with the lower portion of the neck.

Condenser—A straight glass condenser 55 to 60 cm in length with a water jacket about 40 cm in length, or a condenser of other design having equivalent condensing capacity. The lower end of the condenser may be bent to provide a delivery tube, or it may be connected to a bent adapter that serves as a delivery tube.

Asbestos Boards—Two pieces of asbestos board, 5 to 7 mm thick and 14 to 16 cm square, suitable for confining the heat to the lower part of the flask. Each board has a hole in its center, and the two boards differ only with respect to the diameter of the hole, i.e., the diameters are 4 and 10 cm, respectively. In use, the boards are placed one upon the other, and resting on a tripod or other suitable support, with the board having the larger hole on top.

Receiver—A 100-mL cylinder graduated in 1-mL subdivisions.

Thermometer—In order to avoid the necessity for an emergent stem correction, an accurately standardized, partial-immersion thermometer having the smallest practical subdivisions (not greater than 0.2°) is recommended. Suitable thermometers are available as the ASTM E-1 series 37C through 41C, and 102C through 107C (see *Thermometers* ⟨21⟩). When placed in position, the stem is located in the center of the neck and the top of the contraction chamber (or bulb, if 37C or 38C is used) is level with the bottom of the outlet to the side-arm.

Heat Source—A small Bunsen burner or an electric heater or mantle capable of adjustment comparable to that possible with a Bunsen burner.

Procedure—Assemble the apparatus, and place in the flask 100 mL of the liquid to be tested, taking care not to allow any of the liquid to enter the side-arm. Insert the thermometer, shield the entire burner and flask assembly from external air currents, and apply heat, regulating it so that between 5 and 10 minutes elapse before the first drop of distillate falls from the condenser. Continue the distillation at a rate of 4 to 5 mL of distillate per minute, collecting the distillate in the receiver. Note the temperature when the first drop of distillate falls from the condenser, and again when the last drop of liquid evaporates from the bottom of the flask or when the specified percentage has distilled over. Correct the observed temperature readings for any variation in the barometric pressure from the normal (760 mm), adding if the pressure is lower or subtracting if the pressure is higher than 760 mm, and apply the emergent stem correction where necessary. Unless otherwise specified in the individual monograph, allow 0.1° for each 2.7 mm (0.037° per mm) of variation.

⟨724⟩ DRUG RELEASE

This test is provided to determine compliance with drug-release requirements where specified in individual monographs. Use the apparatus specified in the individual monograph.

Apparatus 1, Apparatus 2, Apparatus Suitability Test, Dissolution Medium, and **Procedure**—Proceed as directed under *Dissolution* ⟨711⟩. [NOTE—Replace the aliquots withdrawn for analysis with equal volumes of fresh *Dissolution Medium* at 37° or, where it can be shown that replacement of the medium is not necessary, correct for the volume change in the calculation. Keep the vessel covered for the duration of the test, and verify the temperature of the mixture under test at suitable times.]

Extended-release Articles—General Drug Release Standard

Time—The test-time points, generally three, are expressed in terms of the labeled dosing interval, D, expressed in hours. Specimens are to be withdrawn within a tolerance of ±2% of the stated time.

Interpretation—Unless otherwise specified in the individual monograph, the requirements are met if the quantities of active ingredient dissolved from the units tested conform to *Acceptance Table 1*. Continue testing through the three levels unless the results conform at either L_1 or L_2. Limits on the amounts of active ingredient dissolved are expressed in terms of the percentage of labeled content. The limits embrace each value of Q_i, the amount dissolved at each specified fractional dosing interval.

Acceptance Table 1

Level	Number Tested	Criteria
L_1	6	No individual value lies outside each of the stated ranges and no individual value is less than the stated amount at the final test time.
L_2	6	The average value of the 12 units (L_1 + L_2) lies within each of the stated ranges and is not less than the stated amount at the final test time; none is more than 10% of labeled content outside each of the stated ranges; and none is more than 10% of labeled content below the stated amount at the final test time.
L_3	12	The average value of the 24 units (L_1 + L_2 + L_3) lies within each of the stated ranges, and is not less than the stated amount at the final test time; not more than 2 of the 24 units are more than 10% of labeled content outside each of the stated ranges; not more than 2 of the 24 units are more than 10% of labeled content below the stated amount at the final test time; and none of the units is more than 20% of labeled content outside each of the stated ranges or more than 20% of labeled content below the stated amount at the final test time.

Delayed-release (Enteric-coated) Articles—General Drug Release Standard

Use *Method A* or *Method B* and the apparatus specified in the individual monograph. Conduct the *Apparatus Suitability Test* as directed under *Dissolution* ⟨711⟩. All test times stated are to be observed within a tolerance of ±2%, unless otherwise specified.

Method A:

Procedure (unless otherwise directed in the individual monograph)—

Acid stage—Place 750 mL of 0.1 *N* hydrochloric acid in the vessel, and assemble the apparatus. Allow the medium to equil-

ibrate to a temperature of 37 ± 0.5°. Place 1 tablet or 1 capsule in the apparatus, cover the vessel, and operate the apparatus for 2 hours at the rate specified in the monograph.

After 2 hours of operation in 0.1 *N* hydrochloric acid, withdraw an aliquot of the fluid, and proceed immediately as directed under *Buffer stage*.

Perform an analysis of the aliquot using the *Procedure* specified in the test for *Drug release* in the individual monograph.

Unless otherwise specified in the individual monograph, the requirements of this portion of the test are met if the quantities, based on the percentage of the labeled content, of active ingredient dissolved from the units tested conform to *Acceptance Table 2*. Continue testing through all levels unless the results of both acid and buffer stages conform at an earlier level.

Acceptance Table 2

Level	Number Tested	Criteria
A_1	6	No individual value exceeds 10% dissolved.
A_2	6	Average of the 12 units ($A_1 + A_2$) is not more than 10% dissolved, and no individual unit is greater than 25% dissolved.
A_3	12	Average of the 24 units ($A_1 + A_2 + A_3$) is not more than 10% dissolved, and no individual unit is greater than 25% dissolved.

Buffer stage—[NOTE—Complete the operations of adding the buffer, and adjusting the pH within 5 minutes.] With the apparatus operating at the rate specified in the monograph, add to the fluid in the vessel 250 mL of 0.20 *M* tribasic sodium phosphate that has been equilibrated to 37 ± 0.5°. Adjust, if necessary, with 2 *N* hydrochloric acid or 2 *N* sodium hydroxide to a pH of 6.8 ± 0.05. Continue to operate the apparatus for 45 minutes, or for the time specified in the individual monograph. At the end of the time period, withdraw an aliquot of the fluid, and perform the analysis using the *Procedure* specified in the test for *Drug release* in the individual monograph. The test may be concluded in a shorter time period than that specified for the *Buffer stage* if the requirement for minimum amount dissolved is met at an earlier time.

Interpretation—Unless otherwise specified in the individual monograph, the requirements are met if the quantities of active ingredient dissolved from the units tested conform to *Acceptance Table 3*. Continue testing through the three levels unless the results of both stages conform at an earlier level. The value of *Q* in *Acceptance Table 3* is 75% dissolved unless otherwise specified in the individual monograph. The quantity, *Q*, specified in the individual monograph, is the total amount of active ingredient dissolved in both the acid and buffer stages, expressed as a percentage of the labeled content. The 5% and 15% values in *Acceptance Table 3* are percentages of the labeled content so that these values and *Q* are in the same terms.

Acceptance Table 3

Level	Number Tested	Criteria
B_1	6	Each unit is not less than $Q + 5\%$.
B_2	6	Average of 12 units ($B_1 + B_2$) is equal to or greater than *Q*, and no unit is less than $Q - 15\%$.
B_3	12	Average of 24 units ($B_1 + B_2 + B_3$) is equal to or greater than *Q*, not more than 2 units are less than $Q - 15\%$, and no unit is less than $Q - 25\%$.

Method B:

Procedure (unless otherwise directed in the individual monograph)—

Acid stage—Place 1000 mL of 0.1 *N* hydrochloric acid in the vessel, and assemble the apparatus. Allow the medium to equilibrate to a temperature of 37 ± 0.5°. Place 1 tablet or 1 capsule in the apparatus, cover the vessel, and operate the apparatus for 2 hours at the rate specified in the monograph. After 2 hours of operation in 0.1 *N* hydrochloric acid, withdraw an aliquot of the fluid, and proceed immediately as directed under *Buffer stage*.

Perform an analysis of the aliquot using the *Procedure* specified in the test for *Drug release* in the individual monograph.

Unless otherwise specified in the individual monograph, the requirements of this portion of the test are met if the quantities, based on the percentage of the labeled content, of active ingredient dissolved from the units tested conform to *Acceptance Table 2* under *Method A*. Continue testing through all levels unless the results of both acid and buffer stages conform at an earlier level.

Buffer stage—[NOTE—For this stage of the procedure, use buffer that previously has been equilibrated to a temperature of 37 ± 0.5°.] Drain the acid from the vessel, and add to the vessel 1000 mL of pH 6.8 phosphate buffer, prepared by mixing 0.1 *N* hydrochloric acid with 0.20 *M* tribasic sodium phosphate (3:1) and adjusting, if necessary, with 2 *N* hydrochloric acid or 2 *N* sodium hydroxide to a pH of 6.8 ± 0.05. [NOTE—This may be accomplished also by removing from the apparatus the vessel containing the acid and replacing it with another vessel containing the buffer and transferring the dosage unit to the vessel containing the buffer.] Continue to operate the apparatus for 45 minutes, or for the time specified in the individual monograph. At the end of the time period, withdraw an aliquot of the fluid, and perform the analysis using the *Procedure* specified in the test for *Drug release* in the individual monograph. The test may be concluded in a shorter time period than that specified for the *Buffer stage* if the requirement for minimum amount dissolved is met at an earlier time.

Interpretation—Proceed as directed for *Interpretation* under *Method A*.

Transdermal Delivery Systems—General Drug Release Standards

Time—The test-time points, generally three, are expressed in terms of the labeled dosing interval, *D*, expressed in hours. Specimens are to be withdrawn within a tolerance of ± 15 minutes or ± 2% of the stated time, the tolerance that results in the narrowest time interval being selected.

Apparatus 3—

PADDLE OVER DISK—

APPARATUS—Use the paddle and vessel assembly from *Apparatus 2* as described under *Dissolution* ⟨711⟩, with the addition of a stainless steel disk assembly[1] designed for holding the transdermal system at the bottom of the vessel. The temperature is maintained at 32 ± 0.5°. A distance of 25 ± 2 mm between the paddle blade and the surface of the disk assembly is maintained during the test. The vessel may be covered during the test to minimize evaporation. The disk assembly for holding the transdermal system is designed to minimize any "dead" volume between the disk assembly and the bottom of the vessel. The disk assembly holds the system flat and is positioned such that the release surface is parallel with the bottom of the paddle blade (see Fig. 1).

Apparatus Suitability Test and Dissolution Medium—Proceed as directed for *Apparatus 2* under *Dissolution* ⟨711⟩.

Procedure—Place the stated volume of the *Dissolution Medium* in the vessel, assemble the apparatus without the disk assembly, and equilibrate the medium to 32 ± 0.5°. Apply the transdermal system to the disk assembly, assuring that the release surface of the system is as flat as possible. The system may be attached to the disk by applying a suitable adhesive[2] to the disk assembly. Dry for 1 minute. Press the system, release surface side up, onto the adhesive-coated side of the disk assembly. If

[1] Disk assembly (stainless support disk) may be obtained from Millipore Corp., Ashley Rd., Bedford, MA 01730.

Other appropriate devices may be used, provided they do not sorb, react with, or interfere with the specimen being tested.

[2] Use Dow Corning, 355 Medical Adhesive 18.5% in Freon 113, or the equivalent.

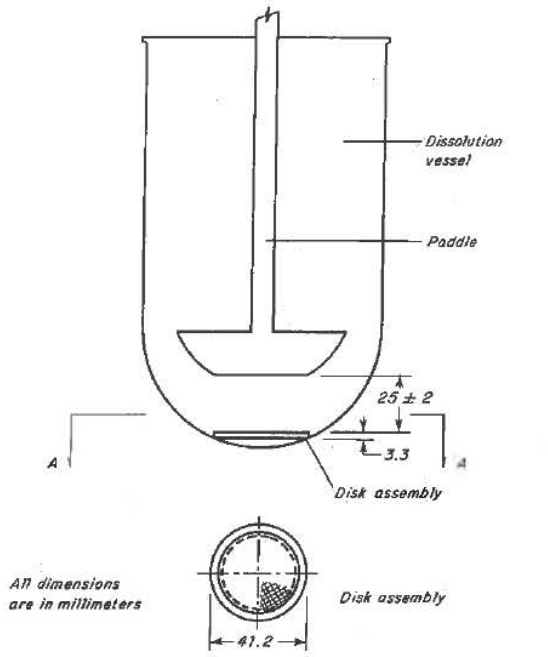


Fig. 1. Paddle Over Disk.

a membrane[3] is used to support the system, it is applied so that no air bubbles occur between the membrane and the release surface. Place the disk assembly flat at the bottom of the vessel with the release surface facing up and parallel to the edge of the paddle blade and surface of the *Dissolution Medium*. The bottom edge of the paddle is 25 ± 2 mm from the surface of the disk assembly. Immediately operate the apparatus at the rate specified in the monograph. At each sampling time interval, withdraw a specimen from a zone midway between the surface of the *Dissolution Medium* and the top of the blade, not less than 1 cm from the vessel wall. Perform the analysis on each sampled aliquot as directed in the individual monograph, correcting for any volume losses, as necessary. Repeat the test with additional transdermal systems.

Interpretation—Unless otherwise specified in the individual monograph, the requirements are met if the quantities of active ingredient released from the system conform to *Acceptance Table 4* for transdermal drug delivery systems. Continue testing through the three levels unless the results conform at either L_1 or L_2.

Acceptance Table 4

Level	Number Tested	Criteria
L_1	6	No individual values lies outside the stated range.
L_2	6	The average value of the 12 units ($L_1 + L_2$) lies within the stated range. No individual value is outside the stated range by more than 10% of the average of the stated range.
L_3	12	The average value of the 24 units ($L_1 + L_2 + L_3$) lies within the stated range. Not more than 2 of the 24 units are outside the stated range by more than 10% of the average of the stated range; and none of the units is outside the stated range by more than 20% of the average of the stated range.

[3] Use Cuprophan, Type 150 pm, 11 ± 0.5-μm thick, an inert, porous cellulosic material, which is available from ENKA AG, 1601 Castle Cove Circle, Corona DelMar, CA 92625, or LifeMed Corp., 2107 Delano Blvd., Compton, CA 90220.

Apparatus 4—Cylinder

APPARATUS—Use the vessel assembly from *Apparatus 1* as described under *Dissolution* ⟨711⟩, except to replace the basket and shaft with a stainless steel cylinder stirring element and to maintain the temperature at 32 ± 0.5° during the test. The shaft and cylinder components of the stirring element are fabricated of stainless steel to the specifications shown in Fig. 2. The dosage unit is placed on the cylinder at the beginning of each test. The distance between the inside bottom of the vessel and the cylinder is maintained at 25 ± 2 mm during the test.

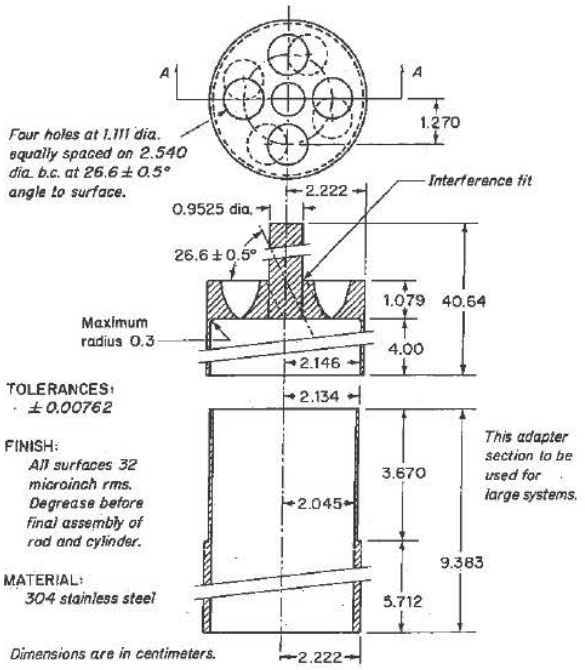


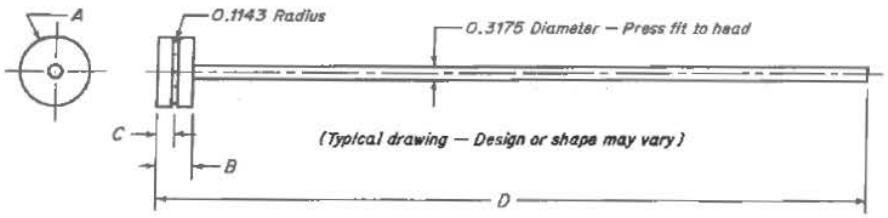
Fig. 2. Cylinder Stirring Element.[4]

Dissolution Medium—Use the medium specified in the individual monograph (see *Dissolution* ⟨711⟩).

Procedure—Place the stated volume of the *Dissolution Medium* in the vessel of the apparatus specified in the individual monograph, assemble the apparatus, and equilibrate the dissolution medium to 32 ± 0.5°. Unless otherwise directed in the individual monograph, prepare the test system prior to test as follows: Remove the protective liner from the system, and place the adhesive side on a piece of Cuprophan[3] that is not less than 1 cm larger on all sides than the system. Place the system, Cuprophan covered side down, on a clean surface, and apply a suitable adhesive[2] to the exposed Cuprophan borders. If necessary, apply additional adhesive to the back of the system. Dry for 1 minute. Carefully apply the adhesive-coated side of the system to the exterior of the cylinder such that the long axis of the system fits around the circumference of the cylinder. Press the Cuprophan covering to remove trapped air bubbles. Place the cylinder in the apparatus, and immediately rotate at the rate specified in the individual monograph. Within the time interval specified, or at each of the times stated, withdraw a quantity of *Dissolution Medium* for analysis from a zone midway between the surface of the *Dissolution Medium* and the top of the rotating cylinder, not less than 1 cm from the vessel wall. Perform the analysis as directed in the individual monograph, correcting for any volume losses as necessary. Repeat the test with additional transdermal drug delivery systems.

Interpretation—Unless otherwise specified in the individual monograph, the requirements are met if the quantities of active ingredient released from the system conform to *Acceptance Table*

[4] The cylinder stirring element is available from Accurate Tool, Inc., 25 Diaz St., Stamford, CT 06907, or from Van-Kel Industries, Inc., 36 Meridian Rd., Edison, NJ 08820.



Dimensions are in centimeters.

	HEAD				ROD		O-RING
System[a]	A (Diameter)	B	C	Material[b]	D	Material[c]	(not shown)
1.6 cm²	1.428	0.9525	0.4750	SS/VT	30.48	SS/P	Parker 2-113-V884-75
2.5 cm²	1.778	0.9525	0.4750	SS/VT	30.48	SS/P	Parker 2-016-V884-75
5 cm²	2.6924	0.7620	0.3810	SS/VT	8.890	SS/P	Parker 2-022-V884-75
7 cm²	3.1750	0.7620	0.3810	SS/VT	30.48	SS/P	Parker 2-124-V884-75
10 cm²	5.0292	0.6350	0.3505	SS/VT	31.01	SS/P	Parker 2-225-V884-75

[a] Typical system sizes.
[b] SS/VT = Either stainless steel or virgin Teflon.
[c] SS/P = Either stainless steel or Plexiglas.

Fig. 3. Reciprocating Disk Sample Holder.[6]

4 for transdermal drug delivery systems. Continue testing through the three levels unless the results conform at either L_1 or L_2.

Apparatus 5—Reciprocating Disk—

APPARATUS—The assembly consists of a set of volumetrically calibrated or tared solution containers made of glass or other suitable inert material,[5] a motor and drive assembly to reciprocate the system vertically and to index the system horizontally to a different row of vessels automatically if desired, and a set of disk-shaped sample holders (see Fig. 3). The solution containers are partially immersed in a suitable water bath of any convenient size that permits maintaining the temperature inside the containers at 32 ± 0.5° during the test. No part of the assembly, including the environment in which the assembly is placed, contributes significant motion, agitation, or vibration beyond that due to the smooth, vertically reciprocating sample holder. Apparatus that permits observation of the system and holder during the test is preferable. Use the size container and sample holder as specified in the individual monograph.

Dissolution Medium—Use the dissolution medium specified in the individual monograph (see *Dissolution* ⟨711⟩).

Procedure—Remove the transdermal system from its backing. Press the system onto a dry, unused piece of Cuprophan[3] or equivalent with the adhesive side against the Cuprophan, taking care to eliminate air bubbles between the Cuprophan and the release surface. Attach the system to a suitable size sample holder with a suitable O-ring such that the back of the system is adjacent to and centered on the bottom of the sample holder. Trim the excess Cuprophan with a sharp blade. Suspend each sample holder from a vertically reciprocating shaker such that each system is continuously immersed in an accurately measured volume of *Dissolution Medium* within a calibrated container pre-equilibrated to 32 ± 0.5°. Reciprocate at a frequency of about 30 cycles per minute with an amplitude of about 1.9 cm for the specified time in the medium specified for each time point. Perform the analysis as directed in the individual monograph. Repeat the test with additional transdermal drug delivery systems.

Interpretation—Unless otherwise specified in the individual monograph, the requirements are met if the quantities of active ingredient released from the system conform to *Acceptance Table 4* for transdermal drug delivery systems. Continue testing through the three levels unless the results conform at either L_1 or L_2.

[5] The materials should not sorb, react with, or interfere with the specimen being tested.

[6] The reciprocating disk sample holder may be purchased from ALZA Corp., 950 Page Mill Rd., Palo Alto, CA 94304 or Van-Kel Industries, Inc.

⟨726⟩ ELECTROPHORESIS

Electrophoresis refers to the migration of electrically charged proteins, colloids, molecules, or other particles when dissolved or suspended in an electrolyte through which an electric current is passed.

Based upon the type of apparatus used, electrophoretic methods may be divided into two categories, one called *free solution* or moving boundary electrophoresis and the other called *zone electrophoresis*.

In the *free solution* method, a buffered solution of proteins in a U-shaped cell is subjected to an electric current which causes the proteins to form a series of layers in order of decreasing mobility, which are separated by boundaries. Only a part of the fastest moving protein is physically separated from the other proteins, but examination of the moving boundaries using a schlieren optical system provides data for calculation of mobilities and information on the qualitative and quantitative composition of the protein mixture.

In *zone electrophoresis*, the sample is introduced as a narrow zone or spot in a column, slab, or film of buffer. Migration of the components as narrow zones permits their complete separation. Remixing of the separated zones by thermal convection is prevented by stabilizing the electrolyte in a porous matrix such as a powdered solid, or a fibrous material such as paper, or a gel such as starch, agar, or polyacrylamide.

Various methods of zone electrophoresis are widely employed. *Gel electrophoresis*, particularly the variant called *disk electrophoresis*, is especially useful for protein separation because of its high resolving power.

Gel electrophoresis, which is employed by the compendium, is discussed in more detail following the presentation of some theoretical principles and methodological practices, which are shared in varying degrees by all electrophoretic methods.

The electrophoretic migration observed for particles of a particular substance depends on characteristics of the particle, primarily its electrical charge, its size or molecular weight, and its shape, as well as characteristics and operating parameters of the system. These latter include the pH, ionic strength, viscosity and

temperature of the electrolyte, density or cross-linking of any stabilizing matrix such as gel, and the voltage gradient employed.

Effect of charge, particle size, electrolyte viscosity, and voltage gradient—Electrically charged particles migrate toward the electrode of opposite charge, and molecules with both positive and negative charges move in a direction dependent on the net charge. The rate of migration is directly related to the magnitude of the net charge on the particle and is inversely related to the size of the particle, which in turn is directly related to its molecular weight.

Very large spherical particles, for which Stokes' law is valid, exhibit an electrophoretic mobility, u_0, which is inversely related to the first power of the radius as depicted in the equation

$$u_0 = \frac{\nu}{E} = \frac{Q}{6\pi r\eta},$$

where ν is the velocity of the particle, E is the voltage gradient imposed on the electrolyte, Q is the charge on the particle, r is the particle radius, and η is the viscosity of the electrolyte. This idealized expression is strictly valid only at infinite dilution and in the absence of a stabilizing matrix such as paper or a gel.

Ions, and peptides up to molecular weights of at least 5000, particularly in the presence of stabilizing media, do not obey Stokes' law, and their electrophoretic behavior is best described by an equation of the type

$$u_0 = \frac{Q}{A\pi r^2\eta}$$

where A is a shape factor generally in the range of 4 to 6, which shows an inverse dependence of the mobility on the square of the radius. In terms of molecular weight, this implies an inverse dependence of mobility on the $\frac{2}{3}$ power of the molecular weight.

Effect of pH—The direction and rate of migration of molecules containing a variety of ionizable functional groups, such as amino acids and proteins, depends upon the pH of the electrolyte. For instance, the mobility of a simple amino acid such as glycine varies with pH approximately as shown in Figure 1. The pK_a values of 2.2 and 9.9 coincide with the inflection points of the sigmoid portions of the plot. Since the respective functional groups are 50% ionized at the pH values where $pH = pK_a$, the electrophoretic mobilities at these points are half of the value observed for the fully ionized cation and anion obtained at very low and very high pH, respectively. The zwitterion that exists at the intermediate pH range is electrically neutral and has zero mobility.

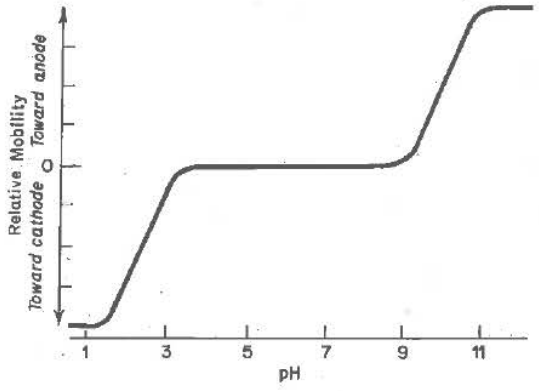


Fig. 1.

Effect of ionic strength and temperature—Electrophoretic mobility decreases with increasing ionic strength of the supporting electrolyte. Ionic strength, μ, is defined as

$$\mu = 0.5\Sigma C_i Z_i^2,$$

where C_i is the concentration of an ion in moles per liter and Z_i is its valence, and the sum is calculated for all ions in the solution. For buffers in which both the anion and cation are univalent, ionic strength is identical with molarity.

Ionic strengths of electrolytes employed in electrophoresis commonly range from about 0.01 to 0.10. A suitable strength is somewhat dependent on the sample composition, since the buffer capacity must be great enough to maintain a constant pH over the area of the component zones. Zones become sharper or more compact as ionic strength is increased.

Temperature affects mobility indirectly, since the viscosity, η, of the supporting electrolyte is temperature-dependent. The viscosity of water decreases at a rate of about 3% per °C in the range of 0° to 5° and at a slightly lower rate in the vicinity of room temperature. Mobility, therefore, increases with increasing electrolyte temperature.

Considerable heat is evolved as a result of current passing through the supporting electrolyte. This heat increases with the applied voltage and with increasing ionic strength. Particularly in larger apparatus, despite the circulation of a coolant, this heat produces a temperature gradient across the bed which may lead to distortion of the separated zones. Therefore, practical considerations and the design of the particular apparatus dictate the choice of ionic strength and operating voltage.

Effect of a stabilizing medium, electroosmosis—When an electrical current is passed through an electrolyte contained in a glass tube or contained between plates of glass or plastic, a bulk flow of the electrolyte toward one of the electrodes is observed. This flow is called electroosmosis. It results from the surface charge on the walls of the apparatus, which arises either from ionizable functional groups inherent in the structural material or from ions adsorbed on the cell walls from the electrolyte contacting them. The effect is usually increased when the cell is filled with a bed of porous substance, such as a gel, used to stabilize the supporting electrolyte and prevent remixing of separated zones by thermal convection or diffusion. The solution immediately adjacent to the surface builds up an electrical charge, equal but opposite to the surface charge, and the electrical field traversing the cell produces a movement of solution toward the electrode of opposite charge.

The substances commonly used as stabilizing media in zone electrophoresis develop a negative surface charge, and therefore electroosmotic flow of the electrolyte is toward the cathode. As a result, all zones, including neutral substances, are carried toward the cathode during the electrophoretic run.

The degree of electroosmosis observed varies with the stabilizing substance. It is appreciable with agar gel, while it is negligibly small with polyacrylamide gel.

Molecular sieving—In the absence of a stabilizing medium or in cases where the medium is very porous, electrophoretic separation of molecules results from differences in the ratio of their electrical charge to their size. In the presence of a stabilizing medium, differences in adsorptive or other affinity of molecules for the medium introduces a chromatographic effect that may enhance the separation.

If the stabilizing medium is a highly cross-linked gel such that the size of the resultant pores is of the order of the dimensions of the molecules being separated, a molecular sieving effect is obtained. This effect is analogous to that obtained in separations based on gel permeation or molecular exclusion chromatography, but in gel electrophoresis the effect is superimposed on the electrophoretic separation. Molecular sieving may be visualized to result from a steric barrier to the passage of larger molecules. Small molecules pass through pores of a wide size range, and therefore their electrophoretic passage through the gel will not be impeded. As size increases, fewer pores will permit passage of the molecules, causing a retardation of the migration of substances of large molecular weight.

Gel Electrophoresis

Processes employing a gel such as agar, starch, or polyacrylamide as a stabilizing medium are broadly termed gel electrophoresis. The method is particularly advantageous for protein separations. The separation obtained depends upon the electrical charge to size ratio coupled with a molecular sieving effect dependent primarily on the molecular weight.

Polyacrylamide gel has several advantages that account for its extensive use. It has minimal adsorptive properties and produces a negligible electroosmotic effect. Gels of a wide range of pore size can be reproducibly prepared by varying the total gel concentration (based on monomer plus cross-linking agent) and the

percentage of cross-linking agent used to form the gel. These quantities are conveniently expressed as

$$T(\%) = \frac{a + b}{V} 100,$$

$$C(\%) = \frac{b}{a + b} 100,$$

where T is the total gel concentration in %, C is the percentage of cross-linking agent used to prepare the gel, V is the volume, in mL, of buffer used in preparing the gel, and a and b are the weights, in g, of monomer (acrylamide) and cross-linking agent (usually *N,N'*-methylenebisacrylamide) used to prepare the gel. Satisfactory gels ranging in concentration (T) from about 3% to 30% have been prepared. The amount of cross-linking agent is usually about one-tenth to one-twentieth of the quantity of monomer (C = 10% to 5%), a smaller percentage being used for higher values of T.

In the preparation of the gel, the bed of the electrophoresis apparatus is filled with an aqueous solution of monomer and cross-linking agent, usually buffered to the pH desired in the later run, and polymerized in place by a free radical process. Polymerization may be initiated by a chemical process, frequently using ammonium persulfate plus *N,N,N',N'*-tetramethylenediamine or photochemically using a mixture of riboflavin and *N,N,N',N'*-tetramethylenediamine. Polymerization is inhibited by molecular oxygen and by acidic conditions. The gel composition and polymerization conditions chosen must be adhered to rigorously to ensure reproducible qualities of the gel.

Apparatus for Gel Electrophoresis—In general, the bed or medium in which electrophoresis is carried out may be supported horizontally or vertically, depending upon the design of the apparatus. A series of separations to be compared may also be carried out in several individual tubes or by placing different samples in adjacent wells, cast or cut into a single slab of gel. A vertical slab assembly such as that depicted schematically in Figure 2 is convenient for direct comparison of several samples. A particular advantage derives from the comparison of the samples in a single bed of gel which is likely to be more uniform in composition than gels cast in a series of chambers.

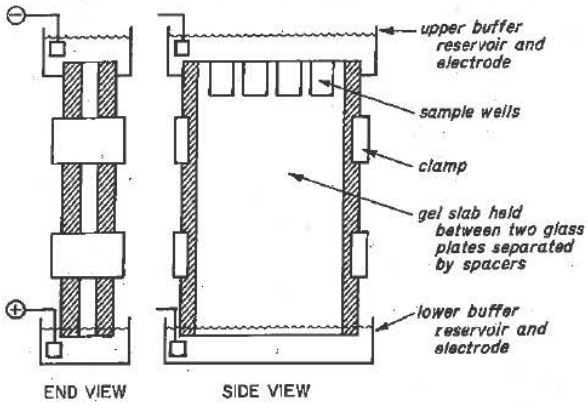


Fig. 2. Vertical Slab Gel Electrophoresis Apparatus.

A feature of many types of apparatus, not illustrated in the schematic view, seals the lower buffer chamber to the base of the bed and allows the level of the buffer in the lower chamber to be made equal to that in the upper chamber, thereby eliminating hydrostatic pressure on the gel. In addition, some units provide for the circulation of coolant on one or both sides of the gel bed.

In the preparation of the gel, the base of the gel chamber is closed with a suitable device and the unit is filled with the solution of monomer, cross-linking agent, and catalyst. A comb, having teeth of an appropriate size, is inserted in the top, and polymerization is allowed to proceed to completion. Removal of the comb leaves a series of sample wells in the polymerized gel.

In simple gel electrophoresis, an identical buffer is used to fill the upper and lower buffer chambers as well as in the solution used to prepare the gel. After filling the chambers, the samples, dissolved in sucrose or other dense and somewhat viscous solution to prevent diffusion, are introduced with a syringe or micropipet into the bottoms of the sample wells, and the electrophoresis is begun immediately thereafter.

DISK ELECTROPHORESIS

An important variant of polyacrylamide gel electrophoresis, which employs a discontinuous series of buffers and often also a discontinuous series of gel layers, is called disk electrophoresis. The name is derived from the discoid shape of the very narrow zones that result from the technique. As a result of the narrow zones produced, this technique exhibits an extremely high resolving power and is to be recommended for the characterization of protein mixtures and for the detection of contaminants that may have mobilities close to that of the major component.

The basis of disk electrophoresis is outlined in the following paragraphs with reference to an anionic system suitable for separating proteins bearing a net negative charge. To understand disk electrophoresis, it is essential to have a knowledge of the general aspects of electrophoresis and the apparatus already described.

Basis of Disk Electrophoresis—The high resolution obtained in disk electrophoresis depends on the use of a buffer system that is discontinuous with respect to both pH and composition. This is usually combined with a discontinuous series of two or three gels that differ in density.

A typical system is illustrated schematically in Figure 3.

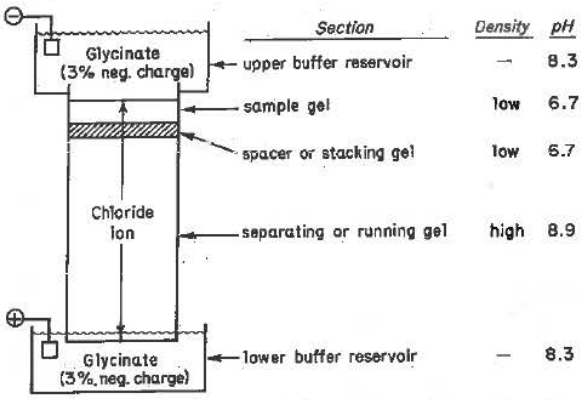


Fig. 3. Terminology, Buffer pH, and Buffer Composition for Acrylamide Gel Disk Electrophoresis.

A high density (T = 10% to 30%) separating gel several centimeters high is polymerized in a tris-chloride buffer in the bed of the apparatus. During polymerization the buffer is overlayered with a thin layer of water to prevent fixation of a meniscus in the top of the gel. The overlayer of water is then removed and a thin layer, 3 mm to 10 mm thick, of low density (T = 3%) gel, called the spacer or stacking gel, is polymerized in a tris-chloride buffer on top of the separating gel. An overlayer of water is again used to ensure a flat surface. The sample is mixed with a small amount of the spacer gel monomer solution which is applied on top of the spacer gel and allowed to polymerize. The pH of the separating gel is typically 8.9, while that of the spacer and sample gels is 6.7. All three gels are prepared using chloride as the anion.

The upper and lower buffer reservoirs are filled with a pH 8.3 buffer prepared from tris and glycine. At this pH about 3% of the glycine molecules bear a net negative charge.

When a voltage is applied across the system, the glycinate-chloride interface moves downward toward the anode. It was initially positioned at the junction of the buffer in the upper reservoir and the top of the sample gel layer. The chloride anion, by virtue of its small size, migrates faster than any of the proteins present in the sample. The pH of the sample and spacer layers was chosen to be about 3 units below the higher pK_a of glycine. Therefore, in traversing these layers, only about 0.1% of the glycine molecules bear a net negative charge. Consequently, glycine migrates more slowly than chloride. The tendency for the faster-moving chloride to move away from glycinate lowers the con-