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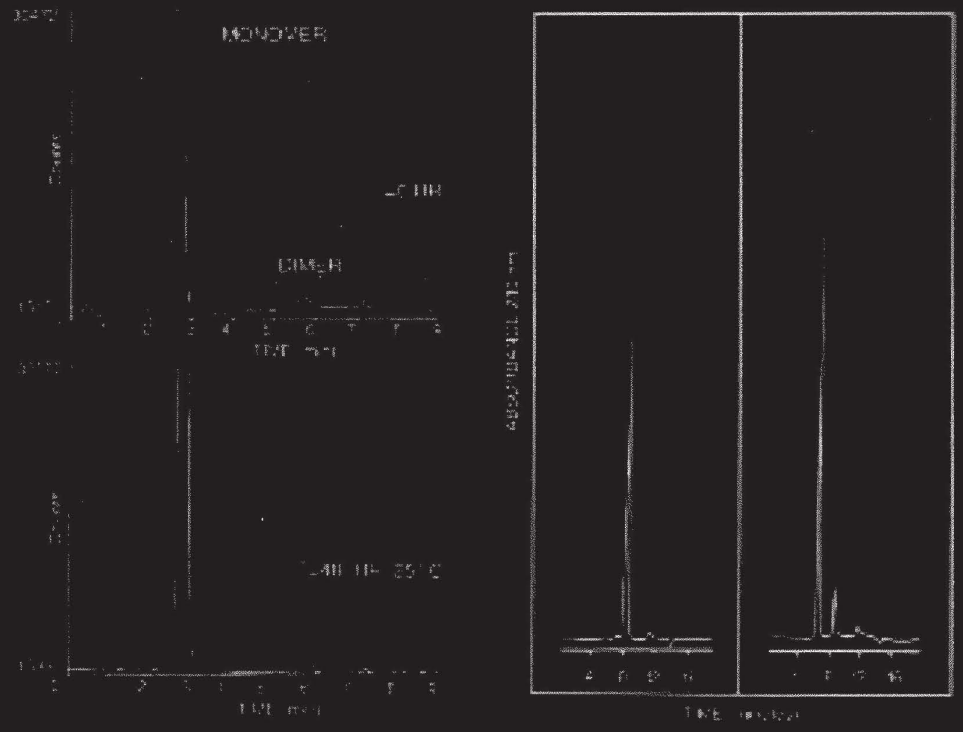
Development of Biopharmaceutical Parenteral Dosage Forms



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Preformulation Development of Parenteral Biopharmaceuticals

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I. INTRODUCTION

Preformulation research studies of protein therapeutics encompass biopharmaceutical, physicochemical, and analytical investigations in support of subsequent stable formulations for preclinical, clinical, and market usage.

In this highly competitive protein therapeutics field, it is very important to obtain significant, measurable progress with preformulations studies in a timely manner. How extensive these studies are will depend on the availability of the crude, active drug substance and the intended route of administration. Most often, these studies begin with extremely small amounts of crude bulk active substance and, as more material becomes available with greater purity, more studies are initiated.

From an industrial point of view, the preformulation studies are designed to cover a wide range of properties in a short time to learn as much as possible, but not in great depth. The pharmaceutical formulation scientist is very much interested in identifying potential problems early enough to evaluate potential alternatives to stabilize future formulation(s).

As previously stated, there must be a strong interdisciplinary collaboration team to review, identify, and maximize the most productive leads toward formulation development. Preformulation studies are short in duration, two to three months, and some of these are performed under varying stress conditions which will be described later in this chapter. It is important to remember that no two proteins are alike and studies designs will vary case by case.

Prior to the onset of preformulations, the pharmaceutical team must review some very important factors which will have an impact on the preformulation and formulation development.

A. Considerations of Domestic and International Distribution of the Product

Many global joint ventures and partnerships today in the biopharmaceutical industry dictate various pharmaceutical, clinical, and marketing strategies. The regulatory requirements and acceptance of formulation excipients, packaging components, unit dose versus multidose product, and stability conditions vary from continent to continent. Constituted and/or lyophilized dosage forms must also be considered. The development of formulation considerations should be on a worldwide acceptance basis.

B. Points to Consider for Constituted Versus Lyophilized Formulations

Some of the key points to be considered for a constituted formulation are:

- A constituted formulation may be less stable than a lyophilized one
- Effect of agitation during manufacturing and shipping
- Interaction of the liquid with the inner wall of the glass vial and with the elastomeric closure
- Aggregation problems
- Head space within the vial
- Preservative effectiveness

Some of the key points to be considered for a lyophilized formulation are:

- Better stability than a constituted product
- Determination of an optimal lyophilization cycle
- Effects of residual moisture on the activity and stability of the product
- Ease of reconstitutability. Clinicians, nurses and trained home users, prefer reconstitutability of the product within two minutes.

- Stability of the reconstituted product
- Preservative effectiveness (if this is a multidose product)
- Cost effectiveness. Lyophilization technology is expensive along with cost of utilities

At the onset of preformulations studies, it is difficult to predict with certainty which of the two types of formulations will have a marketable advantage for an extended shelf life. At this early stage of development, there are usually very small amounts of the bulk active drug substance available. The formulator must make very efficient use of the active drug substance. Nevertheless, both formulations should be considered and started at the same time. Stability results should be the deciding factor as to which form will be selected for further development.

C. Unit Dose or Multidose

The decision to select unit dose versus multidose should be based upon input from clinical investigators, focus groups, marketing surveys, and competitors' products. A multidose formulation will require significantly more time for development.

The multidose will require the screening and incorporation of compatible preservative(s) with the protein formulation. This formulation will be tested to determine if it is efficacious enough to meet the United States Pharmacopeia (USP) requirements. Meeting these requirements, it can qualify as a "multidose" for the U.S. market. However, if the formulation is also designated for international market, there are three additional factors that must be taken into account. The *first* is that for the "antimicrobial effectiveness test," a particular country may or may not accept the preservative selected. *Secondly*, the concentrations of the preservative present in the formulation may be different from the USP requirements. *Thirdly*, the time periods required for the inhibition of the bacteria and fungi strains tested may also differ. Consequently, I strongly suggest that the international regulatory requirements for compliance should be well researched and understood by the scientific and management staff. Other excipients should also be thoroughly reviewed for international acceptance.

D. Physicochemical Factors to Be Considered for Protein Drug Formulations

Some of the most important physicochemical properties of protein drugs required for the development of parenteral preformulations and formulations are found in Table 1.

TABLE 1 Physicochemical Factors to Be Considered for Protein Drug Formulations

Structure of the protein drug	Agents affecting stability
Isoelectric point	pH
Molecular weight	Temperature
	Light
Amino acid composition	Oxygen
	Metal ions
Disulfide bonds	Freeze–thaw
	Mechanical stress
Spectral properties	
Agents affecting solubility:	Polymorphism
Detergent	Stereoisomers
Salts	Filtration media compatibility
Metal ions	Shear
pH	Surface denaturation

Since this may be an early stage of process development, some of the properties listed in Table 1 may not be available initially, simply because there was not enough time or personnel to perform the work.

II. INITIAL PREFORMULATION STUDIES: PARAMETERS AND VARIABLES TO BE TESTED

The pharmaceutical formulation scientist will consider several factors in the preformulation designs. The data received from the Process/Purification section are reviewed for structure, pH and purity of the substance, preliminary bioassay, and an immune assay used in terms of semiquantitative measurements.

Other important information that may or may not be available are product solubility, preliminary stability, potential degradation routes. From personal experience, there is only minimal crude bulk active substance at this early stage.

A. Initial Variables to Be Tested

Perhaps 10 or more initial preformulation combinations should be considered. The initial variables to be tested with various protein concentrations are the effects of buffer species, ionic strength, pH range, temperature,

initial shear, surface denaturation, agitation, and aggregation. Since it has been well documented that protein solutions are unstable, some selective excipients from various classes of stabilizers should also be included in order to evaluate stability requirements. Stabilizers will be discussed later in this volume.

B. Preliminary Analytical Development

In order to determine the initial stability results, it is necessary to have developed, or to have under development, analytical methods to measure the potency of the specific formulation under various experimental conditions. Ultimately some of these analytical methods will be needed to monitor stability to detect physical and chemical degradation. Regulatory compliance for the beginning of Phase I Clinical Studies may require at least two different methods that are "stability indicators," most often fully validated. Dr. Sharma, in Chapter 6, will cover the bioanalytical development.

TABLE 2 Bioanalytical Methods to Evaluate Initial Preformulation Development

Method	Function
Bioassay	Measure of activity throughout shelf life of a formulation
Immunoassay	Purity assessment and measures concentration of a particular molecular species
pH	Chemical stability
SDS-PAGE (Reduced & nonreduced)	Separation by molecular weight, characterization of proteins and purity
RP-HPLC	Estimation of purity, identity, and stability of proteins. Separation and analysis of protein digests.
IEF	Determines the isoelectric point of the protein and detects modifications of the protein
SE-HPLC	Method of separating molecules according to their molecular size and purity determination
N-terminal sequencing	Elucidation of the C-terminus, identity
UV	Detection of individual component, concentration, and aggregation
CD (circular dichroism) in the UV region	Detects secondary and tertiary conformation and quantitates various structures

However, some of the following bioanalytical methods listed in Table 2 can be applied to begin initial evaluation of the preformulations degradation (if any) under test.

C. Experimental Conditions for the Initial Preformulation Studies

Protein Concentration

Protein drugs are extremely potent; therefore, very low concentrations are required for their respective therapeutic levels. Dosage forms development need to be tested at varying ranges of activity. The respective concentrations may range from nanograms to micrograms to milligrams and the concentration will vary from protein to protein.

pH Range

Initially, a range of pHs should be selected, for example, 3, 5, 7 and 9. Specific pH units will be determined during the formulation studies. The pH changes may have varying impacts on the solubility and stability of the formulation. pH control in pharmaceutical dosage forms is very critical (1). The proper pH selection is one of the key factors in developing a stable product.

Buffers

The buffer(s) selection should be made from the USP physiological buffers list and should be selected based upon their optimal pH range. Some of these buffers are acetate pH 3.8–5.8, succinate pH 3.2–6.6, citrate pH 2.1–6.2, phosphate pH 6.2–8.2, and triethanolamine pH 7.0–9.0. These pH ranges will differ from protein to protein.

Buffer concentrations should be in the range of 0.01 to 0.1 molar concentration. As buffer concentration goes up, so does the pain upon injection. In selecting the proper buffer, phosphate should be the last in one's choice. Phosphate buffer reacts with calcium from the glass vial and zinc from the rubber stopper to cause glass laminates and eventually haziness of the solution during stability periods.

Other Excipients to Be Considered

As it was stated previously, the objective of a preformulation study is to select potentially compatible excipients in order to hasten the development of stable formulations. Based upon protein chemical and physical instabil-

ity, it is highly probable that some excipients may be included in the preformulation. In so doing, the designs of formulations to follow can be more specific in selecting the proper excipient(s) to control specific degradation pathways.

Chelating Agents

The crude bulk protein drug during fermentation and purification steps has passed through and contacted surfaces such as metal, plastic, and glass. If metal ions are present in the liquid bulk active, it is highly recommended to use a chelating agent such as ethylenediamine tetraacidic acid (EDTA) to effectively bind trace metals such as copper, iron, calcium, manganese and others. A recommended dose of (EDTA) would be about 0.01 to 0.05%.

Antioxidants

Since oxidation is one of the major factors in protein degradation, it is highly recommended, should the use of a specific antioxidant be required, to include into the preformulation an antioxidant such as ascorbic acid, sodium disulfide, monothio-glycerol, or alpha tocopherol. The role of an antioxidant is to deplete or block a specific chain reaction. Antioxidants will be the preferential target and eventually be depleted, or may block a specific chain reaction. Argon and/or nitrogen gas can also be used to flood the head space of a vial or ampule during sterile filling to prevent or retard oxidation. A recommended antioxidant dose would be about 0.05 to 0.1%.

Preservatives

If a multidose formulation is required, an antimicrobial agent, called preservative, is required to be incorporated into the formulation. The preservative effectiveness must comply with the USP requirements to be qualified as multidose. The most often used preservatives and respective concentrations are phenol (0.3 to 0.5%), chlorobutanol (0.3 to 0.5%) and benzyl alcohol (1.0 to 3.0%). Additional details are provided in Chapter 5.

Surfactants

Judicious selection of surfactants can result in the prevention of aggregation and stabilization of proteins (2). Polysorbate 80, poloxamer 188, and pluronic 68 have been used in injectable formulation. The purity of the surfactant may have an impact on the chemical stability of the preformulation. Peroxide residues in the surfactant have been implicated in oxidations of protein.

Glass Vial Selection

Type I glass, as classified in the USP, should be used. The selection of a glass vial must also be taken into consideration when dealing with adsorptive properties of the respective protein. Adsorption of proteins will be treated later in this volume.

Rubber Stopper Selection

In studying both the liquid and reconstituted protein drugs, the selection of a rubber stopper is also of major concern considering the potential reactivity of a protein solution with a rubber stopper, as well as the reactivity of the reconstituted lyophilized solution during storage conditions prior to use. For parenteral formulations, the biopharmaceutical industry has been using rubber stoppers with a very thin film of various inert polymers in order to achieve greater compatibility, flexibility, low levels of particulates, and machinability. In addition, adsorption, absorption, and permeation through the stopper are essentially eliminated. Extensive details may be found in Chapter 8.

Membrane Filter Selection

Membrane filtration is the most often used technique to sterilize protein solutions. The chemical nature of the filter and the pH of the protein solution are the two most important factors affecting the protein adsorption (3). However, there are other issues that require consideration. The formulation scientist must be aware of particles or fibers released during the filtration, the potential extractables that may occur, the potential toxicity of the filter media and the product compatibility with the membrane. Of all the filters tested (unpublished data) polyvinylidene difluoride, polycarbonate, polysulfone, and regenerated cellulose were found to be the most compatible with various proteins and with minimal amounts of protein binding and deactivation.

III. MECHANICAL AND PHYSICAL STRESSES

A. Shaking Effect on Protein Solution at the Preformulation Level

Some of the various physical modes of vialed protein solution can undergo begin with the bulk active formulation, filling of formulated solution into vials or ampules, visual inspection, labeling, packaging, shipping, and receiving. Simulation of some of the functions described above need to be

performed by doing some shaking experiments to determine their affect on aggregation induction.

Some of these preformulation experiments should also contain varying concentrations of surfactant(s) with appropriate controls. These short and inexpensive experiments can be set up on reciprocal shakers for periods of time from 1 to 6 to 24 hours, shaking from 10, 30, and 60 reciprocal strokes per minute. Reciprocal strokes disrupt and break up the flow of the liquid, while rotary strokes move the liquid circularly without breakup. These studies are intended to determine precipitation and aggregation effects. Detailed aggregation experiments and results will be described later in these chapters.

B. Freeze–Thaw Experiments

These experiments will also be described in later chapters and will be part of Chapter 5. These experiments require a fair amount of active drug substance as well as a fair amount of work. At this point of development there may not be enough active drug substance available.

C. Filling Systems

Of all the filling types employed to dispense liquid, such as time-pressure, piston, and rotary pump, the rolling diaphragm metering pump is the one of choice for filling biopharmaceutical solutions. The internal parts of the pump do not come in contact with one another where the liquid solution flows. This is the "TL Systems Rolling Diaphragm Liquid Metering Pump" (4). One of the most important features of this pump is that it eliminates the principal cause of particulate generation which is most often induced by parts coming together creating shedding of microscopic particles.

There are three other important parameters to control while dispensing protein solutions. (1) The speed at which liquid is filled into the vials. With protein solutions the maximum speed is between 25 to 30 vials per minute, delivering 0.5 to 2.0 mL volume per 5- or 10-mL vial per single filling head. If a large number of vials need to be filled, this filling system can accommodate variable numbers of filling heads, thus allowing it to fill a large number of vials. Filling at a faster rate will result in protein precipitation and aggregation. (2) The inner diameter of the filling cannula should not be so very small as to induce shearing and aggregation of the protein solution. (3) The tip of the cannula for the filling head should be bent at such an angle as to deliver the fluid against the inner wall of the vial and not perpendicular to the bottom of the vial. This will result in a gentle flow

touching the inner wall of the vial when the cannula enters the vial and delivers the required amount of fluid. The proper bend on the tip of the cannula may also eliminate aggregation and/or shearing of the protein solution.

D. Stability Evaluation

The development of acceptable analytical methods while isolation, characterization, and purification of a bulk active drug substance are going on is very important. It can be an aid in generating semiquantitative and quantitative measurements of the active bulk drug at various stages of the process.

Significant marketing advantages in this competitive pharmaceutical market would be to achieve a longer shelf life of the product and storage temperature at room temperature. Today the lyophilized protein drug offers refrigerated temperature storage between 2 and 8°C.

The present storage conditions set up by the USP on storage requirements are as follows:

- Cold storage. Any temperature between 2 and 8°C
- Cool. Any temperature between 8 and 15°C
- Room temperature. Temperature prevailing in a working area
- Controlled room temperature. Temperature controlled thermostatically between 15 and 30°C
- Excessive heat. Temperature exceeding 40°C

Table 3 summarizes the initial guideline time points and temperatures that preformulation solutions should be exposed to. The results from the preformulations will allow the review team to determine directions to manipulate the excipients to obtain better stability.

TABLE 3 Guideline for Preformulation Stability Studies

Temperatures	Timepoint
Frozen controls (–80 and –20°C)	Reference control sample as needed
Refrigerated (2–8°C)	T = 0, 6, 12, 24 & 48 weeks Continue if stable
Intermediate (20, 30, 37°C)	T = 0, 4, 8, 12, 18, 24 weeks Continue if stable
High temperature (40, 45, 50°C)	T = 0, 1, 2, 4, 8, 12 weeks Continue if stable

IV. DEGRADATION MECHANISMS

To predict degradation pathways of new biopharmaceuticals is very difficult. Depending on the stress conditions, each protein may react differently than another protein. As stated previously, the objectives of preformulation are to evaluate stress conditions such as pH, temperatures, and buffers and begin evaluation of some initial breakdown products. At this particular stage of development, it is necessary to have some analytical method(s) with some reliability to detect initial degradation. It is difficult to begin evaluation of degradation products without the reliability of these preliminary assay methods.

The purpose of initial preformulation studies is to begin understanding of protein instability via chemical and physical stress conditions (5). In order to stabilize potential useful pharmaceutical products, it is important to understand how proteins degrade, how they are affected by the composition of the formulation, and the effects of stability conditions. The major pathways of protein degradation are chemical and physical. Under chemical degradation, changes and modifications occur due to bond formation or cleavage, yielding new chemical entities. One or more of the following can occur: oxidation, deamidation, hydrolysis, racemization, isomerization, beta elimination, and disulfide exchange. Physical instability can occur in the form of denaturation, aggregation, precipitation, and adsorption without covalent changes.

A. Oxidation

Oxidation of protein is perhaps one of the most common degradation mechanisms that can take place during various stages of the processing, such as fermentation, purification, filling, packaging, and storage of the biopharmaceuticals. Under oxidative stress and in the presence of trace metals, amino acids such as methionine (Met) can be oxidized to methionine sulfoxide, cysteine (Cys) to cysteine disulfide, as well as tryptophane (Try) and histidine (His) via other modifications.

Oxidation can be controlled or minimized by (1) the addition of antioxidants, (2) having strict controls on the processing operations, (3) using nitrogen gas to flood head space of the container.

Oxidized human growth hormone (hGH) retains only 25 percent the activity of the native molecule, recombinant interferon-beta loses considerable antiviral activity due to oxidation (5). Oxidation can be detected by reversed phase HPLC (RP-HPLC), high-performance isoelectric chromatography (HP-IEC), peptide mapping, amino acids analysis, and mass spec-

trometry (MS) (6). In terms of total protein concentration, ultraviolet spectrophotometry is the method most often used (7).

B. Deamidation

Deamidation is another more frequent degradation mechanism affecting pharmaceutical protein stability. Deamidation is the hydrolysis of the side chains amide on asparagine (Asp) and glutamine (Gln) to form Asp and/or Gln residues. Extensive reports have elucidated mechanisms of deamidation reactions (8).

Deamidation can be detected by isoelectric focusing, ion exchange chromatography, tryptic mapping and HPLC (9).

C. Hydrolysis

Hydrolysis is another most likely cause of degradation of proteins. It involves a peptide (amide) bond in the protein backbone (5). The most influential factor affecting the hydrolytic rate is the solution pH.

D. Racemization

Proteins may also degrade via other modifications (10) such as racemization. This mechanism involves the removal of the alpha proton from an amino acid in a peptide to yield a negatively charged planar carbanion. The proton can then be replaced into this optically inactive intermediate, thus producing a mixture of D and L enantiomers (2). Racemization can yield enantiomers in both acidic and alkaline conditions.

E. Isomerization

Protein degradation is also induced by isomerization. Hydrolysis of cyclic amides of asparagine, glutamine, and aspartic acid will result in isomerization. Low pH accelerates hydrolysis of asparagine and glutamine. However, high pH accelerates hydrolysis of aspartic acid and glutamic acid (2,11,12).

F. Disulfide Exchange

Disulfide exchange may result from a degradation other than covalent modification. These reactions may include the disulfide exchange of cysteine. This reaction is base, catalyzed and promoted by thiol antioxidants (13).

Disulfide exchange can occur in misfolded conformers due to incorrect intramolecular disulfide bonds (14).

G. Beta-Elimination

Another degradation residue can be the beta-elimination of ser, thr, cys, lys and phe residues. These reactions are accelerated by basic pH, temperature, and the presence of metal ions (16).

V. PHYSICAL DEGRADATIONS

Aggregation

Protein aggregation can be of a covalent or noncovalent nature (17,18).

A. Covalent Aggregation

This pathway involves modification of the chemical structures resulting in new chemical structures and may include reactions, such as oxidation, deamidation, proteolysis, disulfide interchanges, racemization, and others.

B. Noncovalent

This instability may be induced by agitation, shear, precipitation, and adsorption to surfaces.

C. Aggregation

Protein aggregation derived from either physical or chemical inactivation, is presently a major biopharmaceutical problem (17–21). Aggregation can be either covalent or noncovalent, occurring during any phase of product development from purification to formulation. An early detection of aggregation via biochemical or spectrophotometric methods, or both, can be of significant guidance to formulation scientists in selecting compatible excipients to minimize and/or prevent its formation in the experimental formulation.

Formation of aggregation can begin by the formation of initial particles from protein molecules via the Brownian movement. This is followed by collision of these molecules and aggregates of varying sizes can be formed. These aggregates can be generated by shear or collisional forces (22).

Detection and measurements of aggregations can be performed by a number of techniques. Visual observations, light scattering, polyacrylamide gel electrophoresis, UV, spectrophotometry, laser light diffraction particu-

late analysis, fluorescence spectra and differential scanning colorimetry (DSC), RP-HPLC, and SE-HPLC (7,14). Conformational changes can also lead to aggregations and can be measured by DSC (23).

A formulation scientist should focus on some important observations that need to be made to answer some potential problems on aggregation.

- Determination of initial approximate number of aggregates
- Determination of approximate size and distribution of aggregates
- Do the aggregates increase in size and number over time?
- Do the aggregates affect the efficacy of the proteins?
- What is the effect of aggregation on the long-term storage of the potential marketable product?

D. Denaturation

Denaturation of proteins can be the result of several processes and reported by several investigators (27).

Factors which induce denaturation are heat or cold, extreme pHs, organic solvents, hydrophilic surfaces, shear, agitation, mixing, filtering, shaking, freeze–thaw cycles, ionic strength, and others. Thermal inactivation processes will induce conformational side reactions and destruction of amino acids (28). The loss of biological function may well be attributed to the effect of the temperature on the higher-ordered structure of the protein.

Thermal denaturation of proteins is of great interest to the formulation scientist. Thermal probes offer tools to study protein structure and stability that ultimately can be of significant use to stabilize protein drug formulations. Modifications of protein thermal effects have been reviewed (8).

The ability of the protein to refold from a denatured state, a reversible heat denaturation, is also of considerable interest for the stability of a protein formulation. These processes of renaturation are very complex (29), and each protein does have its own unique renaturation mechanisms.

Since filtrations and volume reductions occur from the fermentation to process purification, there is very likely inactivation of the protein attributable to shearing effect.

E. Precipitation

Precipitation in formulations can occur by a variety of mechanisms such as shaking, heating, filtration, pH, and chemical interactions. Aggregation is the initial onset of precipitation. The protein molecules form aggregations of varying sizes first, and later when the aggregates reach a critical mass, precipitate out of solution and are clearly visible.

From a biopharmaceutical formulation point of view, precipitation can occur in membrane filters, filtration equipment, pumps, and tubing and loss of activity is very often recorded.

Eventually, as the aggregation mechanisms are controlled and prevented, precipitation is essentially reduced or avoided. Details on the functions of stabilizers are discussed later in this volume.

F. Adsorption

Some of the most prevalent, ubiquitous factors of deactivation (30,31, 32) that the protein biochemists and formulation scientists face, are the surface areas interactions from the purification, formulations, and stability stages.

Essentially, at each point that the protein solution has encountered air during mixing (process), filtration (process), and air in the process steps, a significant surface area has been encountered to yield interphases.

During the actual final manufacturing of vials, ampules, syringes, catheters, pumps, and their respective storage conditions, the proteins could be adsorbed at the interphase and removed from the solution.

Several researchers (22,33,34,35,36) have investigated these biochemical mechanism problems. Since proteins have surfactant characteristics, they have a high affinity to adsorption at the air–liquid and solid–liquid interphase. Hydrophobic and hydrophilic interactions which are concentration dependent, determine the extent and the rate of adsorption. The adsorption effect on the protein is the unfolding of the protein. When this occurs at an interphase, it can lead to (1) inactivation of the protein solution, (2) insoluble protein aggregates being formed at the adsorbed site, (3) additional conformational changes occurring, and (4) chemical degradation of the protein continuing during stability periods.

VI. SUMMARY

The initial critical parameters of preformulations have been addressed in this chapter. The formulation team, at this point of development, will review and evaluate all the results obtained from the preformulation studies. The pharmaceutical formulator will design several approaches for the next stage of formulation development taking into account all the parameters that may achieve one or more stable marketable formulations. In the formulation studies ahead, a number of stabilizing ingredients should be considered to achieve acceptable industrial stability.

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5

Formulation Development

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I. FORMULATION REQUIREMENTS

Preformulation Evaluation From the preformulation studies, there should be some key parameters that can be of significant aid in the designs of experimental formulations. These key parameters are (1) Initial compatibility testing of the active drug substance with some excipients, (2) Effect of stability factors such as temperature, light, packaging components, (3) Initial degradation products in the preformulation, and (4) the performance of stability assays for the preformulation.

The following are some of the major considerations to be taken into the experimental formulation designs:

A. Characterization, Homogeneity, and Reproducibility of the Bulk Active Drug

Characterization, homogeneity, and lot-to-lot reproducibility of the bulk active drug substance is of paramount importance. At this stage of dosage form development, a great deal of characterization of the bulk has been obtained. Regulatory compliance demands that the process in place yields reproducibility of the active drug substance, as well as whatever impurities

may be present. What is important is that they are quantitatively reproducible from lot-to-lot and that whatever impurities found have no toxicological and biological effects on the host.

B. pH Effect on a Formulation

The pH has a critical impact on formulations of proteins and peptides. It has a solubility and a stability impact on the formulations. With a monoclonal antibody at pH 4.2 in two different buffers, there was significant degradation as opposed to pHs 5.2 and 6.7—an optimal pH range for further development (1). At these higher pHs, further formulation development was pursued.

Some peptides can be formulated at acidic pH 2.5–4.5; however, at low pH, deamidation of asparagine and glutamine occurs. At higher pH, however, oxidation of methionine, cysteine, and tryptophan can occur, as well as other degradative mechanisms (2). The optimal pH is essential for better stability.

A change of one pH unit will change the reaction one way or another. The solution pH may be one of the most effective ways to stabilize a liquid formulation (3).

C. Stabilizers Used in Protein Formulations

Degradation of proteins can be a major biopharmaceutical problem during purification, characterization, preformulation, formulation development, and possibly during storage. Selective excipients are incorporated into the formulation in order to improve the physical and chemical stability of the protein drug substance.

A variety of molecules have been used as stabilizers, such as surfactants, amino acids, polyhydric alcohols, fatty acids, proteins, antioxidants, reducing agents, and metal ions. Some of the most often used excipients are stabilizers, and an explanation for their mode of action has been reported in the literature and listed in Table 1 (4–19).

D. Surfactants

Protein surfactant interactions have also been investigated by other researchers (20–22). Most recently, the interaction of Tween 20, Tween 40, Tween 80, Brij 52, and Brij 92 were studied with recombinant human growth hormone and recombinant human interferon gamma for surfactant:protein binding stoichiometry.

TABLE 1 Stabilizers Used in Protein Formulations

Stabilizer	Action/uses
Proteins	
Human serum Albumin (HSA)	Prevents surface adsorption Conformational stabilizer Complexing agent Cryoprotectant
Amino acids	
Glycine	Stabilizer
Alanine	Solubilizer
Arginine	Buffer
Leucine	Inhibit aggregation
Glutamic acid	Thermostabilizer
Aspartic acid	Isomerism inhibitor
Surfactants	
Polysorbate 20 & 80	Retard aggregation
Poloxamer 407	Prevent denaturation Stabilize cloudiness
Fatty Acids	
Phosphotidyl choline	Stabilizer
Ethanolamine	
Acethyltryptophanate	
Polymers	
Polyethylene glycol (PEG)	Stabilizer
Polyvinylpyrrolidone (PVP) 10, 24, 40	Prevent aggregation
Polyhydric alcohol	
Sorbitol	Prevent denaturation Aggregation
Mannitol	Cryoprotectant
Glycerin	May act as antioxidant
Sucrose	
Glucose	Strengthen conformational
Propylene glycol	Prevent aggregation
Ethylene glycol	
Lactose	
Trehalose	
Antioxidants	
Ascorbic acid	Retard oxidation
Cysteine HCl	
Thioglycerol	
Thioglycolic acid	
Thiosorbitol	
Glutathione	

TABLE 1 Continued

Stabilizer	Action/uses
Reducing agents	
Several thiols	Inhibit disulfide bond formation Prevent aggregation
Chelating agents	
EDTA salts Gluthamic acid Aspartic acid	Inhibit oxidation by removing metal ions
Metal ions	
Ca^{++}, Ni^{++}, Mg^{++}, Mn^{++}	Stabilize protein conformation

This stoichiometric relationship can be applied to protein formulations to determine stability. Poloxamer 407 (Pluronic F-127) was also tested with interleukin-2 and urease resulting in increased stability when the formulation was subjected to strong agitation (23). Recombinant urokinase losses were reduced by the addition of human serum albumin (HSA), Tween 80, and Pluronic F-68 (24).

Interleukin-2 and ribonuclease A, when reconstituted with a variety of surfactants, amino acids, sugars and other substances, reduced aggregation significantly (25).

The formation of particulates with a monoclonal antibody was inhibited by Tween 80 and recorded by visual and laser light diffraction particulate analysis methods (26).

Proteins will adsorb at interphases such as liquid/air or liquid/solid. When protein molecules are adsorbed they undergo physicochemical changes. Insoluble particles begin to form, eventually resulting in aggregation and precipitation and this, in turn, may lead to partial or full loss of bioactivity.

The addition of surfactants poloxamer 188 (Pluronic 68), or polysorbate to a liquid formulation can prevent or reduce denaturation of the protein at a liquid/air or liquid/solid interface of the protein in solution (27).

The most recent literature concerning the use of nonionic surfactants, indicate that during bulk storage and usage, hydroperoxides may be formed and can degrade many proteins (28).

It is for this reason that, when these surfactants are purchased, a client must ask the vendor for a certificate of analysis specifying all the tests performed, including hydroperoxides.

E. Buffer Selection

The primary objective in selecting suitable buffers is that the buffer should have considerable buffering capacity to maintain the pH of the product at a stable value during storage condition in its marketed final container. These should be physiological buffers, USP approved. The ionic strength should also be taken into consideration since it can affect stability and isotonicity and when administered intramuscularly, the higher the ionic strength, the higher the pain in situ. In Table 2, U.S.P. physiological acceptable buffers for parenteral administration are listed.

F. Polyols

Polyols are substances with multiple hydroxyl groups, including polyhydric alcohols and carbohydrates. These include mannitol, sorbitol, and glycerol. These have been found to stabilize proteins in solution in varying concentration from 1.0 to 10%. Although the mode of action of protein stabilization is not yet clear, it is suggested that the sugar exerts pressure to reduce the surface contact between the protein and the solvent (29,30).

G. Antioxidants

Oxidation is one of the major factors in protein degradation. A protein solution, from purificatioin to final product for an end user, goes through various equipment made of metal, glass, or plastic. At some points during the process, the protein solution comes in contact with catalyzing metals such as copper, iron, calcium, and manganese, thus inducing the potential loss of protein activity. A probable solution to this problem will be the incorporation of a compatible antioxidant in the formulation. Some of the most often used antioxidants for parenteral preparations are ascorbic acid, sodium bisulfite, sodium metabiosulfite, monothio-glycerol, alpha tocopherol, and others. The most frequently used concentrations are in the 0.1% range and higher. The optimal concentrations are determined by the data the formulator obtains from experimental results on a case by case evaluation. Nitrogen and argon gas is also used to retard or prevent oxidative reactions and the gas is used by flooding the head space of a vial or ampule during sterile filling.

Antioxidants fall into one or more of the following categories (31):

1. *Chelating agents.* Oxidative reactions catalyzed by metal ions. Chelating agents such as EDTA and citric acid decrease their effectiveness.

TABLE 2 USP Compatible Buffers for Parenteral Use

Buffering agent	KA Values (PKA)	Approximate buffering range
Monobasic acids		
Acetic	1.8×10^{-5} (4.8)	3.8–5.8
Benzoic	6.5×10^{-5} (4.2)	3.2–5.2
Gluconic	2.5×10^{-4} (3.6)	2.6–4.6
Glyceric	2.8×10^{-4} (3.55)	2.6–4.6
Lactic	8.4×10^{-4} (3.1)	2.1–4.1
Dibasic acids		
Aconitic	(1) 1.58×10^{-3} (2.8) (2) 3.5×10^{-5} (4.46)	2–5.5
Adipic	(1) 3.9×10^{-5} (4.41) (2) 5.29×10^{-6} (5.28)	3.4–6.3
Ascorbic	(1) 6.76×10^{-5} (4.17) (2) 2.51×10^{-12} (11.6)	3.2–5.2
Carbonic	(1) 4.3×10^{-7} (6.4) (2) 5.6×10^{-11} (10.3)	5.4–7.4
Glutamic	(1) 7.4×10^{-3} (2.1) (2) 4.9×10^{-6} (4.3)	2–5.3
Malic	(1) 3.0×10^{-4} (3.4) (2) 7.8×10^{-6} (5.1)	2.4–6.1
Succinic	(1) 6.9×10^{-5} (4.2) (2) 2.5×10^{-6} (5.6)	3.2–6.6
Tartaric	(1) 1×10^{-3} (3.0) (2) 4.55×10^{-5} (4.3)	2.0–5.3
Polybasic acids		
Citric	(1) 8.4×10^{-4} (3.14) (2) 1.7×10^{-5} (4.8) (3) 6.4×10^{-6} (5.2)	2.1–6.2
Phosphoric	(1) 7.5×10^{-3} (2.1) (2) 6.3×10^{-8} (7.2) (3) 2.2×10^{-13} (12.7)	2–3.1 6.2–8.2
Bases		
Ammonia (ammonium chloride)	5.6×10^{-10} (9.25)	8.25–10.25
Diethanolamine	1.0×10^{-9} (9.0)	8.0–10.0
Glycine	1.7×10^{-10} (9.8)	8.8–10.8
Triethanolamine	1×10^{-8} (8.0)	7.0–9.0
Tromethamine (Tris, Tham)	8.3×10^{-9} (8.1)	7.1–9.1

2. *Reducing agents.* These are reducing substances and inactivate oxidizing agents. Some of the reducing agents used in pharmaceuticals are sodium bisulfite, thioglycerol and ascorbic acid.
3. *Oxygen scavengers.* These compounds are more readily oxidized than the substance they are supposed to protect, thereby preferentially reducing the amount of oxidant in solution. These are ascorbic acid and sodium bisulfite.
4. *Chain terminator.* Oxidation reactions occur via free radical procedure. Chain terminators such as thiols (cysteine and thioglycerol) react with radicals in solutions to produce a new species which does not reenter the radical propagation cycle.

H. Antimicrobials (Preservatives)

For the development of multidose formulations, it is mandatory that an antimicrobial agent is selected and incorporated into the formulation. These antimicrobial agents are called preservatives and their function is to kill or inhibit growth of bacteria and fungi that could be accidentally introduced into a vial in the process of withdrawing dosages from the vial, thus rendering the solution adulterated. The most common preservatives used in pharmaceutical and biopharmaceutical injectable products are phenol, benzyl alcohol, chlorobutanol, metacresol, and parabens. The formulator must address the following critical issues in selecting the proper preservative:

- Antimicrobial activity
- Use concentrations
- Solubility
- Optimum pH
- Stability
- Compatibility
- Inactivation

Each of these preservatives has its own characteristic reactivity with the drug substance, the excipients and the pH. Some of these preservatives have binding properties with several proteins (unpublished data). Several papers have been published (32–36) documenting binding of pharmaceutical substances by various preservatives. The most often used concentrations of preservatives are: phenol at 0.3%–0.5%, parabens-methylparaben at 0.18%, propyl-paraben at 0.02%, metacresol at 0.3–0.5%, chlorobutanol up to 0.5%, and benzyl alcohols at 1.0–3.0%.

An injectable pharmaceutical substance meets the qualification of a "multidose" if it complies with the Antimicrobial Effectiveness Test, as

described in the USP No. 23. If any multidose product is designed to be marketed in Europe or the Far East, it is imperative to know the exact test procedure requirements since the preservative test requirements vary in the United States, European and Far Eastern countries. Complete preservative characteristics are found in Table 3.

I. Tonicity

The pharmaceutical scientist, as we have read thus far, must fulfill several key requirements for a successful formulation. Another key requirement to consider is tonicity. Parenteral injectables are most desirable as isotonic solutions. In controlling isotonicity, we can control tissue damage irritation, pain, hemolysis, and crenation of the red blood cells. Hypertonic solution causes shrinkage (crenation) of the red blood cells and is reversible. Hypotonic solution will cause swelling and bursting of the red blood cells (hemolysis).

To control tonicity at all times may not be possible because of the high drug concentrations and low volumes required by some injections. When necessary, tonicity modifiers such as dextrose, sodium, and potassium chloride can be used, but it is more advisable to use sugars in place of salts. Tonicity can be calculated by several methods (37).

II. CONTAINER-CLOSURE INTERACTIONS

A. Glass Vials

Parenteral vial containers must be designed and packaged in such a way as to maintain package integrity. It must maintain product sterility, it must be convenient for shipping and storage, and prevent leakage.

The type of glass recommended for protein formulation is the USP Type I glass because it is the most unreactive of the glasses available. In order to achieve an excellent seal with the rubber stopper, proper dimensions of the vial and the stopper are required, thus assuring a good contact. Chapter 8 of this book covers various aspects of the elastomeric closures with focus on protein interaction.

B. Leakage Tests

Parenteral solutions in a finished vial must prevent liquid leakage either in or out of the vial. In some cases, vacuum or gas headspace need to be controlled. There are three main tests to be performed (38). These are leakage for gas, liquid, and microorganisms (39).

TABLE 3 Characteristics of Preservatives

	Benzyl alcohol	Chlorobutanol
Antimicrobial activity	Bacteria, weak against fungi	Bacteria, fungi
Use concentrations	1.0–3.0%	Up to 0.5%
Solubility	1:25 in water	Soluble in water (1:125), more soluble in hot water Soluble in ethanol
Optimum pH	4–7	Up to 4.0
Stability	Slowly oxidizes to benzaldehyde	Decomposed by alkalies
Compatibility/inactivation	Inactivated by nonionic surfactants (Tween 80)	Incompatible with some nonionic surfactants (10% Tween 80) Decomposes at 65°C
Comments	Bacteriostatic Used for parenteral and ophthalmic products Local anesthetic action	Wide range of compatibility Local anesthetic action Widely used

There are also three mechanical tests that need to be performed (40), namely, needle penetration, coring, and vapor transmission.

C. Plastic Vials

The pharmaceutical industry introduced plastic containers because of some of the advantages plastic appeared to have, including durability, easier manufacturing, more flexibility, and perhaps more biocompatibility. How-

Metacresol	Parabens (Hydroxybenzoates: methyl, propyl)	Phenol
Bacteria, fungi	Primarily fungi and gram positive bacteria Poor vs. pseudomonads	Bacteria, fungi
0.3–0.5%	Methylparaben 0.18% Propylparaben 0.02%	0.3–0.5%
1:50 in water	Methylparaben (in water) 1:400 Propylparaben (in water) 1:2000 Alcohol 1:2:5	1:15 in water
2–8	(3–8)	Wide range (2–8)
Activity decrease at high pH	Essentially good	Activity decreases at high pH
May be inactivated by iron and certain nonionic surfactants	Serum reduces activity, also nonionic surfactants	May be inactivated by iron, albumin and oxidizing agents May be incompatible with with some nonionic surfactants
The meta isomer is most effective and least toxic, ortho is the weakest	Binds to PEG	Mode of action is physical damage of the cell wall and enzyme inactivation by free hydroxyl group
Mode of action is apparently related to solubility in fatty portions of organisms	Slightly soluble Stable and nonirritating Proposed to block essential enzyme system of microorganism	
Combine with and denature proteins		

ever, plastic containers were found to be prone to sorption, gas permeation, and leachables (41).

D. Sorption of Preservatives by Plastic

Interactions of preservatives with plastics have been reviewed (42). Several preservatives were studied, including benzyl alcohol paraben, benzalkonium, and benzethionium chloride with plastic materials such as polycarbon-

ate, polystyrene, polypropylene, polyvinyl chloride, and others. There occurred 20 to 40% loss of concentration after three months stability. This could be a significant problem that warrants study case by case.

E. Siliconization of Elastomeric Closures

Siliconization of elastomeric closures, with a 2.0% solution of Dow Corning 360, was usually necessary to give an elastomeric closure better insertion into the neck of a glass vial. High speed filling certainly required this treatment. Without it, all kinds of problems arose during manufacturing. However, with proteins and peptides, significant problems were encountered in dealing with potential adsorptive problems between the protein-silicone-elastomeric interactions (unpublished data). Silicone traces also interfered with the development of analytical methodology, for it complexed readily with the proteins.

The latest advance in closure development is the application of a very thin flexible coating of nonreactive polymer on the elastomeric closure, such as teflon. This technology improves the insertion of the elastomeric closure into the vial, gives good seal integrity, reduces particulates associated with elastomer manufacture and washing, and eliminates silicone treatment. Pharmaceutical elastomeric closure manufacturing companies are solving these problems by researching adequate and nonreactive polymers to coat their elastomeric surfaces.

F. Siliconization of Vials

Siliconization of glass vials has been an industry practice for some time in order to achieve complete drainage of the formulation from the walls of the container. However, with biopharmaceutical products such as proteins and peptides, siliconization has generated some difficult problems. Even though the vials, after siliconization, are baked in an oven at about 250°C for about five to six hours, during stability storage, at varying temperatures, the silicone layer begins to flake off over a period of several months. When this occurs, there could be initially visible a light haze formed by the interactions of silicone residues with the formulations. In addition, the light haze interferes with quantitative analytical development (unpublished data). The use of siliconization should judiciously be determined case by case, while perhaps new research on more inert coatings are discovered to reduce adsorptive surface properties.

III. OTHER FORMULATION CONSIDERATIONS

A. Shake Test

Determine the amount of physical stress the formulation in the final container can withstand by using various modes and different temperatures to simulate some shipping conditions. Some of the results will be applicable to the design of an applicable and suitable shipping container.

B. Freeze–Thaw Cycles

Again, the physical stress of freeze–thaw cycles can have significant detrimental effect(s) on the formulation compounds; therefore, as part of the shipping validation studies, the dosage form storage is simulated from –40°C or –20°C to 2–8°C. These temperature ranges are product-to-product specific.

The cycle will begin from a frozen state at –20°C or –40°C, to a 2–8°C temperature, and subsequently to room temperature, over specific time periods. A typical freeze–thaw cycle is a 24 hour period.

After each thawing, samples are taken and assayed. The samples are frozen again, and so on. The most frequent freeze–thaw cycle is 5 days. Freezing and thawing cycles can be performed with rapid or slow cooling and with slow or fast warming. Fast warming should not exceed 25°C temperature.

The amounts of dimer formation increase with the number of freeze–thaw cycles. These dimers may or may not be reversible; this is protein and formulation dependent (43).

C. Mechanical Stressing

Physical factors that must be controlled during the formulation development and varying stability conditions are: shaking, shearing, freeze–thaw freezing rates, liquid filtration, and filling under pressure can have significant detrimental effects, such as denaturation, adsorption, and aggregation (44–46).

D. Stability Evaluation

The objectives of stability studies are to determine, and comply with cGMPs and regulatory requirements to establish, an expiration date and the appropriate storage conditions. The stress conditions used in preformulation stability evaluation, both physical and chemical, will be of significant guidance

in formulation approaches and indicate specific excipients to be used to improve stabilization and integrity of the formulations.

E. Setting Up Potential Formulation Candidates

To ensure that at least two or three different formulations will survive the rigorous screening, leading to a desirable marketable dosage form, the formulator should design six or more final candidate formulations. These should represent varying concentrations of the active drug substance, buffers, and selected excipients in order to achieve the most stable formulation with acceptable shelf life (47–50). Presently the majority of protein drugs on the market are stored at 2–8°C for 15–18 months.

The various excipients selected for each formulation should be acceptable by regulatory agencies. This is very important because each excipient selected by a formulating scientist must be justified for its use and at the concentration selected. *More is not better in formulations.* On the contrary, it is wise to select only those ingredients that are necessary to impart desirable stability for product superiority.

F. Points to Consider in Setting Up Stability Studies

Analytical Assay Methodologies. When the formulations reach this stage of development, it is highly necessary that at least two methods of assay have been developed that are "stability-indicating" assays. One assay alone cannot be considered sufficient, and not accepted by CBER, to monitor the potential degradation products induced by chemical or environmental routes. These assays will eventually be rigorously validated to assure measurable quantities of degradants over time. Dr. Sharma, in Chapter 6, will focus on the development of these assays that will have accuracy, precision, linearity, sensitivity, show spiked recovery, potency, strength, and stability indicator.

Table 4 summarizes the characterization and control of biopharmaceuticals.

Calculate the number of vials required for each test for each specific time point, taking into consideration the following:

- Number of batches
  At least three, if possible and available
- Active drug substance.
  At least three different lots from the final process
- Batch size
  Enough for stability requirements, plus large overage (for unexpected testing and FDA requirements)

TABLE 4 Characterization and Control of Biopharmaceuticals. Methods of Biopharmaceutical Characterization and Control, Their Uses, and References

Amino acid analysis for identity, structural analysis, and quantity
Amino acid sequencing (N- and C-terminal) for identity and structural analysis
Biochemical and colorimetric assays for activity, identity, and quantity
Biosensor assays for identity, activity, and quantity
Capillary electrophoresis for quantity, purity, heterogeneity, and stability
Carbohydrate mapping, compositional, sequence and linkage analysis for heterogeneity and structural analysis
Cell-based bioassays for activity
Differential scanning calorimetry for stability
HPLC for quantity, purity, heterogeneity, and stability
Immunoassays for quantity, impurity, and identity
Isoelectric focusing for identity and heterogeneity
Mass spectrometry for identity, heterogeneity, and stability
Microbiological testing for impurity
Nuclear magnetic resonance for structural analysis
Peptide mapping for identity
Residual DNA analysis for impurity
Residual moisture analysis for lyophilization efficiency
SDS-PAGE for purity, heterogeneity, and identity
Spectroscopy (UV, CD/ORD, infrared, fluorescence) for quantity and structural analysis
Ultracentrifugation (analytical) for heterogeneity, stability, and structural analysis
Western blots for impurity
Whole animal assays for activity
Hyphenated techniques (LC-MS, CE-MS) for identity, heterogeneity, stability, and quantity

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- Specifications
  Samples for both in-house and regulatory requirements

In setting up temperature stability studies, the highest temperature points require the least amount of sample. At 40–45°C, protein products are not anticipated to be stable for more than several days. However, at 2–8°C, the temperature most likely to have the longest stability for proteins, the most samples will be required.

- Control or reference samples
  Enough samples should be stored for *retest* purpose and *retained* samples. These samples are normally stored at –40°C.
- Shipping conditions
  Final Market Container. Summer and winter conditions should be considered in the design.
- Storage position
  Upright and inverted and, if enough samples are available, place the vials in a horizontal position.
- Testing frequency
  The frequency of testing will be determined by the number of samples needed for each time point. The frequency will vary from product to product.

IV. SUGGESTED GUIDELINES FOR MAJOR STABILITY STUDIES OF FINISHED PRODUCT AND BULK ACTIVE DRUG SUBSTANCE

- A Formulation Development Stability Program is summarized in Table 5.
- A proposed ICH storage condition is summarized in Table 6.

Bulk active drug substance stability must also be performed in order to determine stability profiles at various time points and temperature. This information has direct impact on the flexibility of how long a bulk active drug substance can be stored, and at what temperature, for manufacturing purposes.

A. Breakdown Products

During the various time points of stability at each condition selected, the specific formulation is evaluated for characteristics such as color changes, clarity, pH, moisture transfer, extractables, tonicity, binding, adsorption, potency, stopper appearance, aggregation, particulates, and container closure integrity, and for a multidose formulation test for residual preservative(s). In addition, select the most appropriate analytical methods to monitor degradation, such as SDS-PAGE, HPSEC, IEF, CZE, RPHPLC, and others, if necessary. Understanding of degradation products is very important in both the initial preIND and postIND evaluation, in terms of toxicology and other pharmacological effects.

TABLE 5 Formulation Development Stability Program

- Preformulation
  - Time: 0, 1W, 2W, 1M, 2M, 3M
  - Temp: C°: 2–8°, 25°, 37°, 45°
- Experimental formulation
  - Time: 0, 1M, 3M, 6M, 9M, 12M, 18M, 24M
  - Temp: C°: 2–8°, 25°, 37°, 45°
- Primary formulation
  - Time: 0, 1M, 3M, 6M, 12M, 18M, 24M, 36M, 48M, 60M
  - Temp: C°: 2–8°, 25°, 37°, 45°
- Market formulation
  - Time: 0, 1M, 3M, 6M, 12M, 24M, 36M, 48M, 60M
  - Temp: C°: 2–8°, 25°, 37°, 45°
  - Lots from this formulation can be qualified as conformity lots
- Relative humidity in percent (RH)
  - At 25°C and 30°C, use 60% RH; at 40°C, use 75% RH

W = Week, M = Month.

B. Specifications

The development of specifications for protein and peptide drugs is a control mechanism that is capable of assuring that the purification process is in place, yielding consistency from lot to lot to lot. Specifications apply to both bulk active protein drug and the finished dosage forms to insure the integrity and safety of the product throughout its shelf life and compliance with regulatory requirements governing the product.

In designing specifications of a specific protein, and a peptide drug product, the following are the key characteristics to consider: potency, purity, identity, microbiological, sterility, and physical tests. Depending on the physicochemical makeup of the active bulk drug substance, appropriate

TABLE 6 Proposed ICH[a] Storage Conditions

Temperature	Time	
A. 25°C/60% RH	0, 3, 6, 9, 12, 18, 24, 36	Months
B. 30°C/60% RH	0, 3, 6, 9, 12, 24, 36	Months
C. 40°C/75% RH	0, 1, 3, 6	Months

[a]International Conferences on Harmonization.
Federal Register, September 22, 1994.

TABLE 7 Specifications for a Protein/Peptide Drug Finished Dosage Form

Test methods
1. Physical evaluation
Appearance
pH
Volume/container
Moisture (lyophilized product)
Total protein
Particulates (for both liquid and lyophilized formulations)
2. Potency tests
In vitro assays
Radioimmunoassays
Enzyme immunoassays
Chromatographic methods
Bioassays (animal model or cell-line derived)
Protein content
3. Identity
Peptide mapping
NH_2 Terminal analysis
Western blot
Isoelectric focusing
SDS-PAGE
Coomassie stain (reduced and unreduced)
Biological activity
4. Purity
SDS-PAGE
Coomassie stain
HPLC-RP
HPLC-SEC
HPLC-Gel filtration
DNA contamination
Other specifications can be included depending on the specific requirement of the protein.
5. Microbiological tests
Sterility
Pyrogens
Mycoplasma
6. Safety
7. Degradation assays
SDS-PAGE
ELISA
HPLC
Electrophoresis

TABLE 8 Specifications for Purified Bulk Drug Concentrate

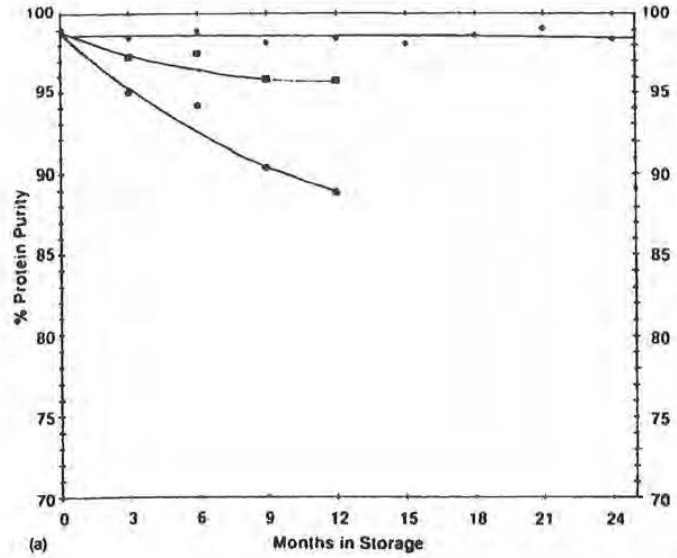
Test methods
1. Physical evaluation
Appearance
pH
2. Identity
Bioassay
Peptide mapping
Amino acid analysis
3. Protein potency
Nitrogen content
HPLC
4. Biological potency
Specific activity
5. Purity
HPLC
SDS-PAGE
CZE
IEF
6. DNA
7. Endotoxins
8. Sterility
Other specifications can be included depending on the specific requirements of the individual protein.

specifications may be required. Specifications are product to product requirements.

In Table 7, some of the most applicable specifications are identified for a finished drug form and in Table 8, for a bulk active drug.

C. Stability—Case Studies Graphs

As previously cited in this chapter, biopharmaceutical substances have their own specific physicochemical characteristics; consequently, it is very difficult to demonstrate degradation by a single bioanalytical method of assay. In order to support true stability characteristics, the formulator must judiciously select different methods in order to demonstrate the final marketable purity and identity of the product.



Recombinant Interleukin-2

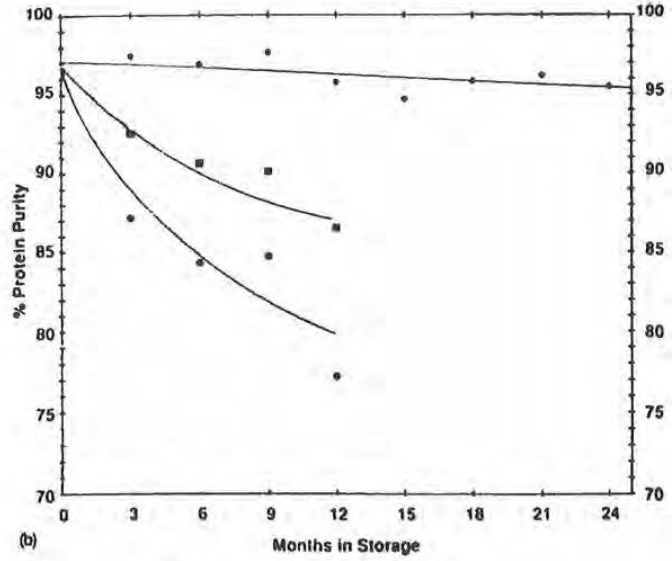


FIGURE 1 A Proleukin formulation showed decrease in purity when tested by SDS-PAGE (a) and corroborated by RP-HPLC when samples were stored at various temperatures over periods of time (b): ♦, 40°C; ■, 25°C; ●, 37°C.

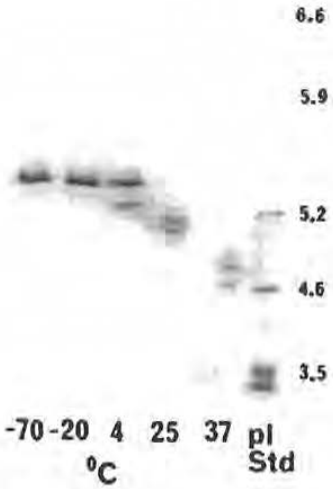


FIGURE 2 A TNF product was undergoing deamidation during storage, as shown by the appearance of bands at pI value below 5.3; however, decomposition in SDS-PAGE method could not be detected.

Figures 1 to 8, reproduced with permission from various investigators, demonstrate the results obtained using different analytical methodologies to monitor stability.

D. Investigational New Drug (IND) Requirements

The preparation of an IND for filing an application for a new drug to be tested in humans can be a complex, difficult, and a frustrating enterprise if the people who are responsible for preparing a specific portion of the IND have had no instruction or experience.

There are several scientific groups involved, each responsible for their specific scientific task. Table 9 represents a compilation of scientific tasks required by an IND and how long each of these tasks may take. This generic template is a summary of several INDs prepared from my experience. As it

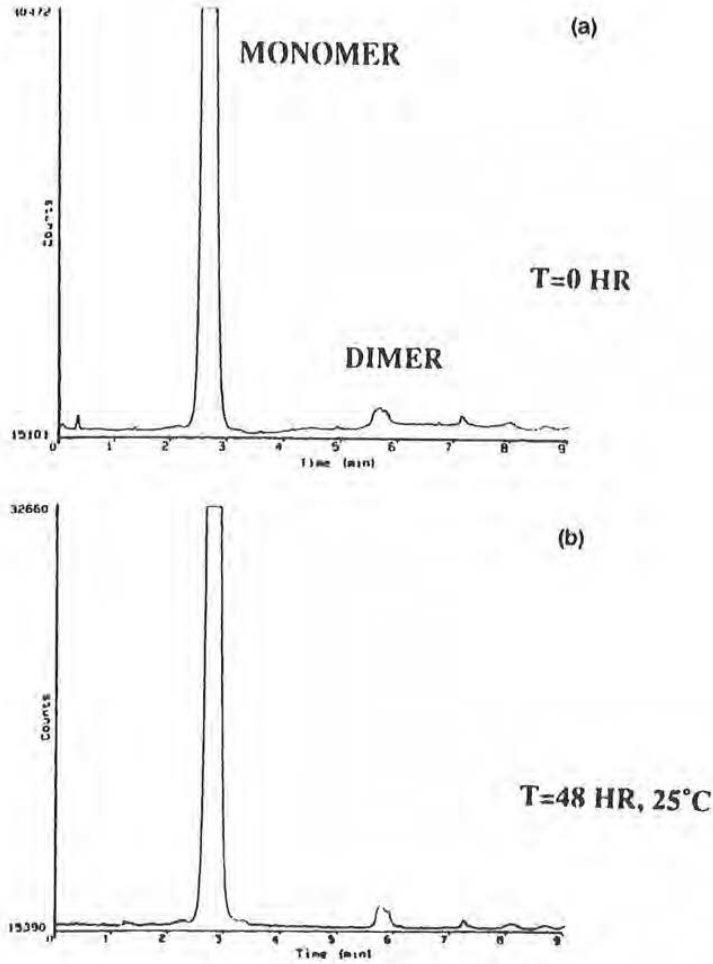
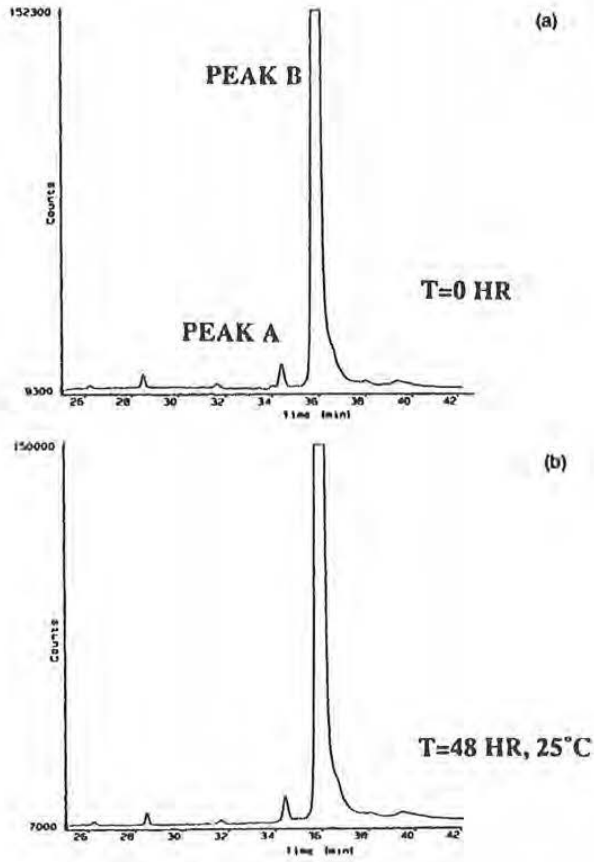


FIGURE 3 Stability samples of Proleukin (IL-2), determined by SDS-PAGE method (nonreducing) (left panel) corroborated by RP-HPLC method (right panel) immediately after reconstitution (a), and after 48 hours (b), at room temperature.



can be seen, there are several tasks that go on simultaneously to minimize the overall time requirements. When all these tasks are completed, it shows that an IND preparation may take from 9 to 12 months.

The three most critical segments are the analytical assay development, the preformulation, and formulation of the product, followed by the preclinical pharmacology.

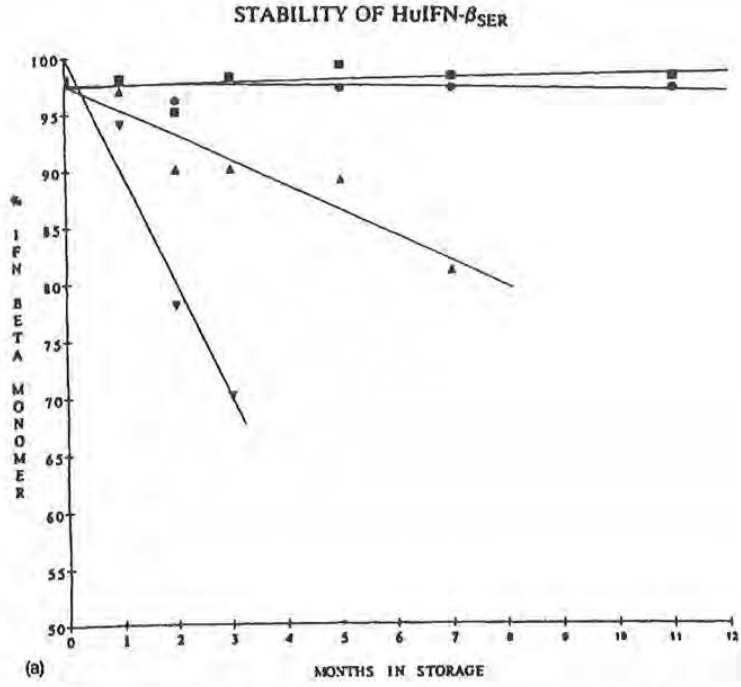


FIGURE 4 Stability purity of HuIFN-beta$_{SER}$ by (a) SDS-PAGE and by (b) RP-HPLC tested at monthly time intervals at various temperature levels (■, –70°C; ●, 4°C; ▲, 25°C; and ▼, 37°C). The two methods yielded similar results. [Reproduced with permission from J. Geigert (15).]

From the analytical development, two stability indicating assay methods should be identified. From the preformulation and formulation development, three or four formulation candidates should be identified for long range stability studies. In the present competitive market, the management of companies exert a great deal of pressure on the scientific and regulatory staff to shorten these timelines. We can be realistically aggressive and make a risky decision on a limited amount of data; however, it is when we become unrealistically aggressive that we may very well be forced to return to square one and to start all over again, very painfully.

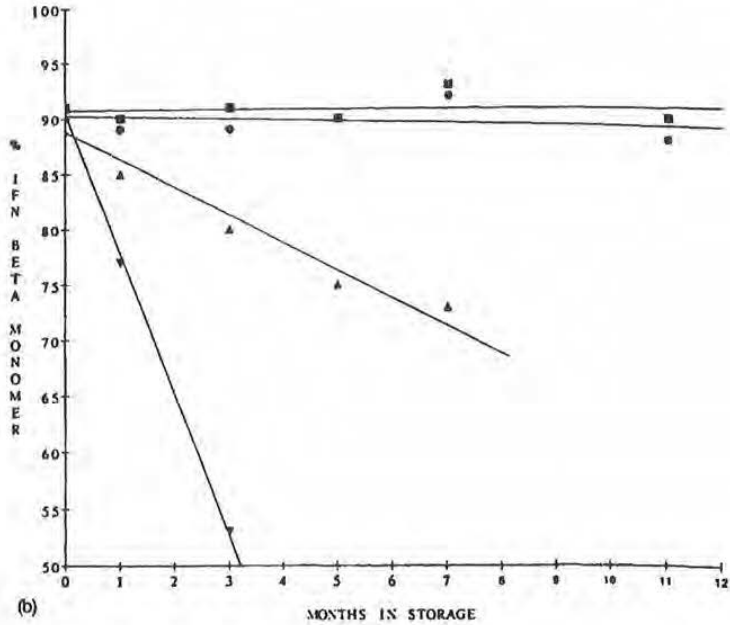


TABLE 9 IND Task/Time—Overall Requirements

- Dvlp. 100-l ferm. proc. 2M
- Dvlp. ferm. mfg. doc. 1M
- Dvlp. mfg. doc. 1M
- Dvlp. specs & assays 3M
- Dvlp. lab recov. process 2M
- Dvlp. 100-l recov. process 2M
- Prepare for tox. studies 2M
- Tox. data/report. 2M
- Analyt. assay development and qual. lots 9M
- (Qual. lots stability) 3M
- Preformulation screening (liquid/lyoph.)—select formul. 8M
- Preclinical pharmacology for clinical protocols 7M
- Write clinic. protoc. 2M

Write ind 1.5 M

Ind approv. 1 M

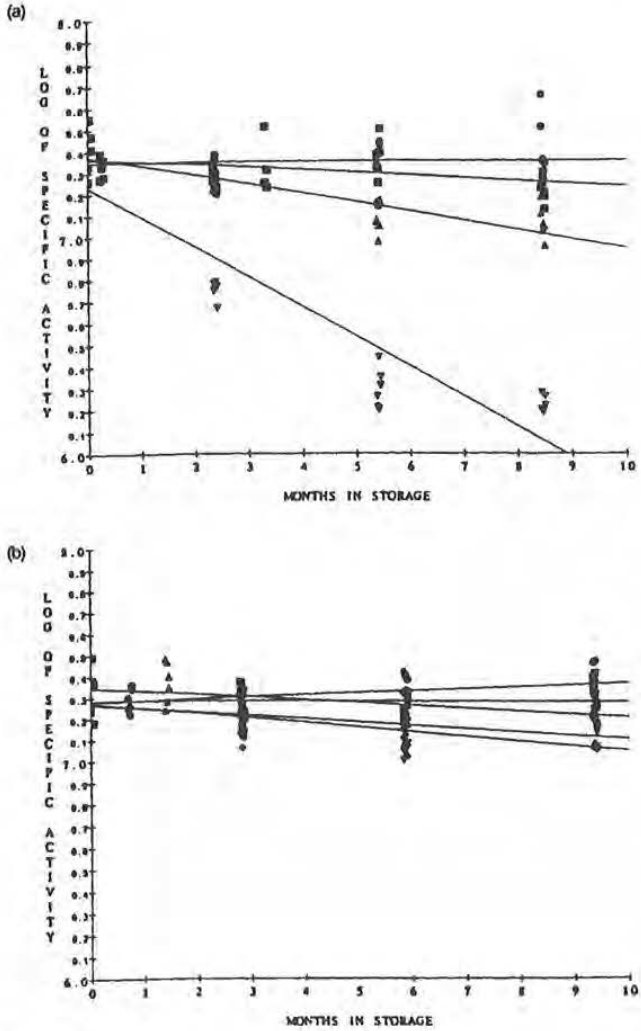
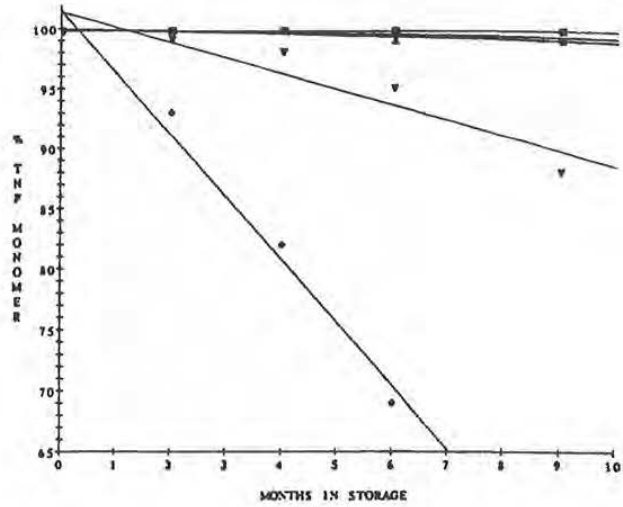
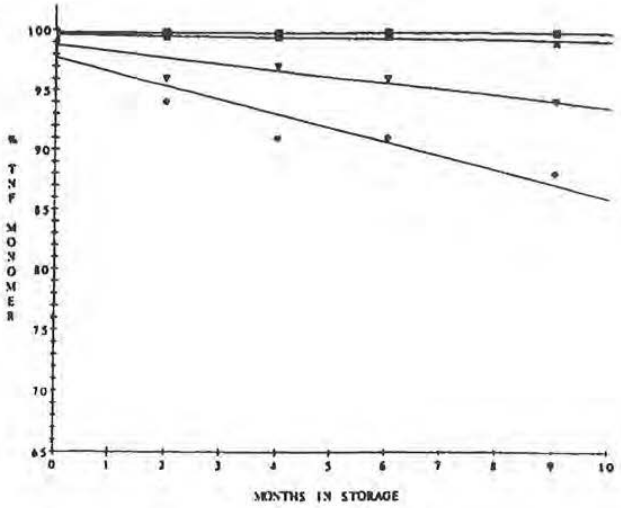


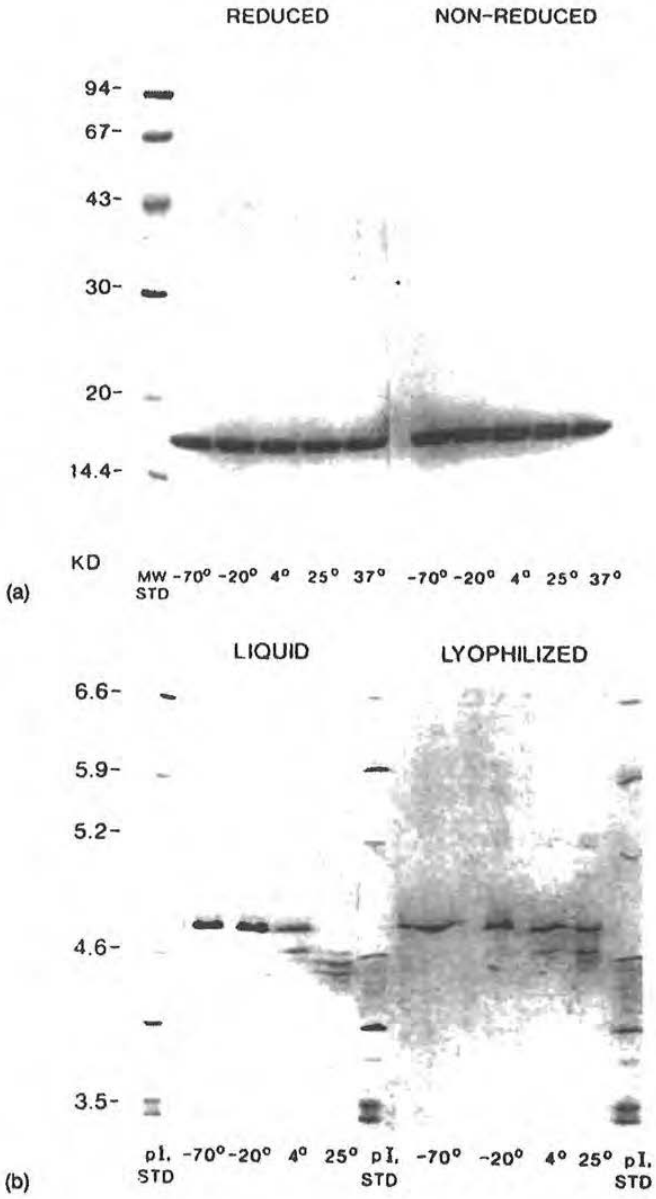
FIGURE 5 Demonstration of (a, b) stability and (c, d) purity of a liquid (a, c) versus a lyophilized preparation (b, d) of TNF. Specific activity of TNF and SDS-PAGE non-reducing gel showed similar results. ■, –70°C; ●, –20°C; ▲, 4°C; ▼, 25°C; and ♦, 37°C. [Reproduced with permission from J. Geigert (15).]

(c)



(d)




REDUCED
NON-REDUCED
94-
67-
43-
30-
20-
14.4-
KD
MW STD
-70°
-20°
4°
25°
37°
-70°
-20°
4°
25°
37°
(a)
LIQUID
LYOPHILIZED
6.6-
5.9-
5.2-
4.6-
3.5-
pI. STD
-70°
-20°
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pI. STD
-70°
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4°
25°
pI. STD
(b)

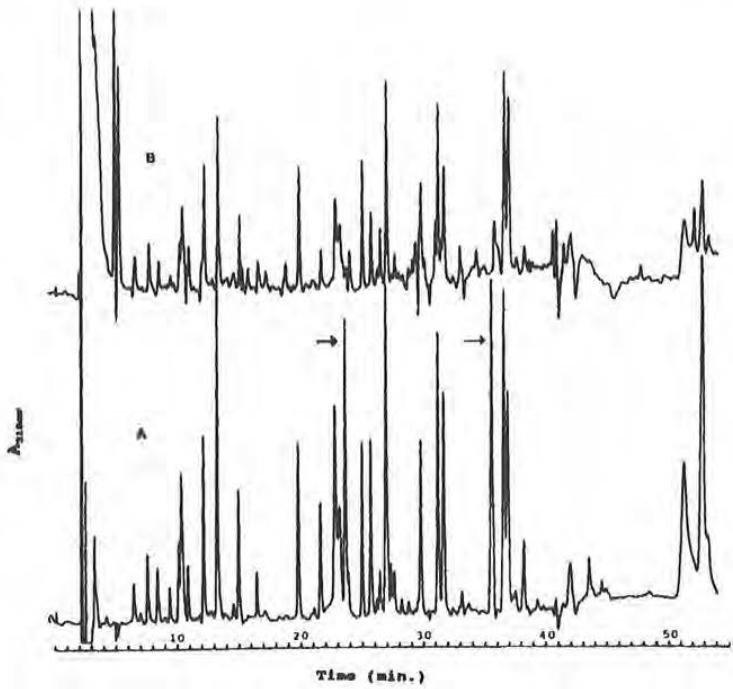


FIGURE 7 This monoclonal antibody, OKT3 peptide mapping shows chromatogram A is the standard and B is the map of the material from the degraded sample. The results suggest that an oxidative step is involved in the mechanism of its formulation. (Reproduced with permission from D. Kroon.)

FIGURE 6 (a) A lyophilized sample of TNF showed no detectable deterioration by SDS-PAGE analysis, reduced and nonreduced, or by IEF after 6 months under various storage conditions. (b) However, after 9 months by IEF, both liquid and lyophilized formulations showed additional bands at lower pI value, indicating the onset of deamidation, Figure 11. [Reproduced with permission from J. Geigert (15).]

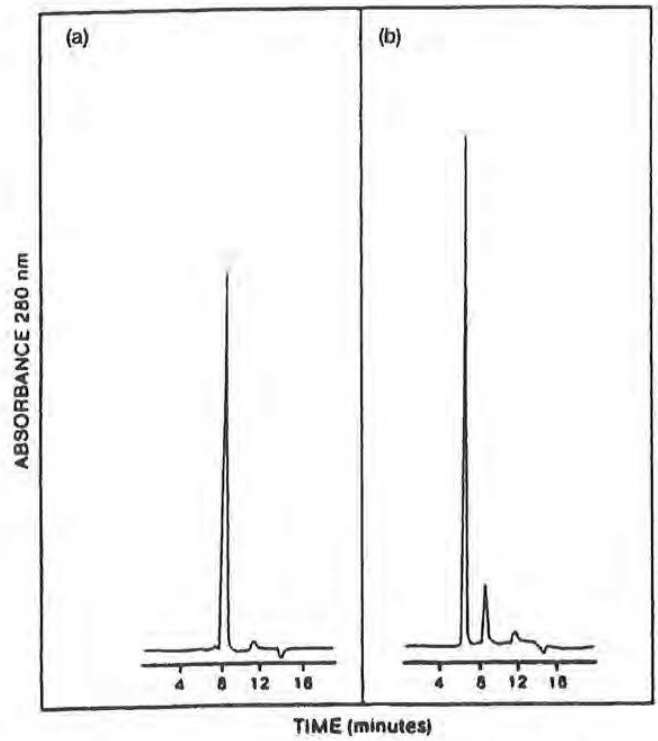
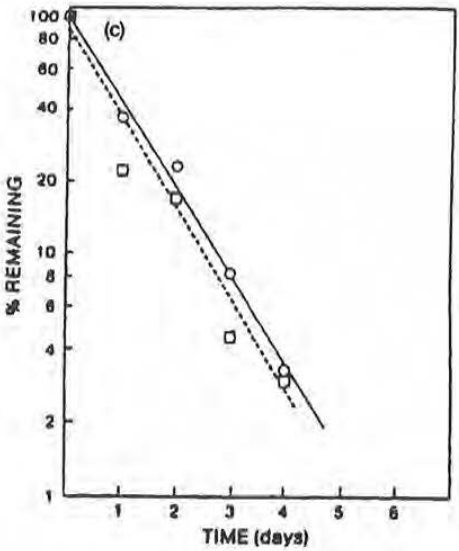


FIGURE 8 A demonstration of aggregation (a, b) and loss of activity (c) of an IL-2 preparation has occurred when exposed at 80°C for 5–8 minutes. These data demonstrate excellent correlation of aggregation and the amount remaining analyzed by HPSEC (○) and bioassay (□). From Watson, E. and Henney, W.C. (1988).

While all the IND activities are going on, additional product development issues to address are (1) research directions for scale-up, (2) preparation of preclinical supplies, and (3) preparation of initial clinical supplies.

The research directions for scale-up will require:

- Batch sheets preparation
- SOP's preparations



- Equipment selection and testing
- Master file preparation

The preparation of pre-clinical supplies will require dosage forms for:

- Pharmacology
- Toxicology
- Sensitization
- Irritation
- Bioburden
- Preservative efficacy
- Product stability
- GLP/GMPs compliance

Preparation of initial clinical supplies:

- Stability evaluation of the dosage forms
- Training of personnel

- Evaluate preclinical testing
- Technology transfer from research to scale-up that eventually will be transferred to pilot scale and production for clinical and market supplies.

E. Formulation Development Scale-Up Considerations

This section of scale-up is addressed only as general highlights in this chapter. The massive documentation, such as scale-up procedures, technical documentation, validation, and final acceptance required to comply with cGMPs and Regulatory Compliance is subject matter that I am sure will be addressed by Process Development Scientists in future books or other publications, in great detail.

At this stage of formulation development, if the data demonstrate acceptable stability to warrant scale-up development, it is necessary to put the scale-up process in place. Several equipment variables will be screened and ultimately the most appropriate for the product will be selected in order to bring the product from formulated liquid bulk active drug to filled product in its final packaging configuration.

There are, however, some key considerations that do have major impact on the process. First of all, the design of the scale-up process. The process will be required to give reproducibility from lot to lot. The design of the process will reflect the choice of the final container selected, such as vials, syringes, ampules. In scaling up, another consideration of major importance will be the selection of excipients. The composition of the excipients should be the same if more than one supplier is utilized. There is, in addition, the international acceptance of the excipients and ultimately, but not last, is the cost consideration.

As covered previously, formulation and manufacturing, at this stage of development, need to focus on the physical and chemical problems associated with biopharmaceuticals. The physical adsorption of the product on surfaces such as glass, metal, and plastic and on any prefilters can have significant loss of the product, inconsistent concentration per unit container, poor yield, and ultimately rejection of the lot.

The chemical denaturation can be induced by several factors such as temperature and pressure, metal particles shedding from equipment surfaces, shear and oxidation at the air–liquid interfaces. The technology of scale-up must yield reproducible, quality attributes in the final product. When these objectives are met, we achieve a successful product development.

F. Summary

Preformulations with biopharmaceuticals have a significant role in identifying and solving potential formulation problems. Preformulation results allow the formulation scientists to make rational designs for the experimental formulations to be tested.

Key phases of successful product development place emphasis on close, collaborative, and productive interactions of the interdisciplinary sciences within a pharmaceutical group.

Physicochemical properties of a protein–peptide drug must be identified in order to approach preformulations and formulations studies with rational designs. Selective protein stabilizers play a major role in imparting stability of the product under specific experimental conditions.

Analytical methods able to determine the potential stability or degradation products of a formulation must be developed. These methods must be validated and qualified as *stability indicators*.

The ultimate goals of the formulation and product development scientists, from fermentation to production, are to deliver to the health field a protein drug which is safe, effective, pure, stable, elegant, suitable for production, cost effective, and marketable.

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