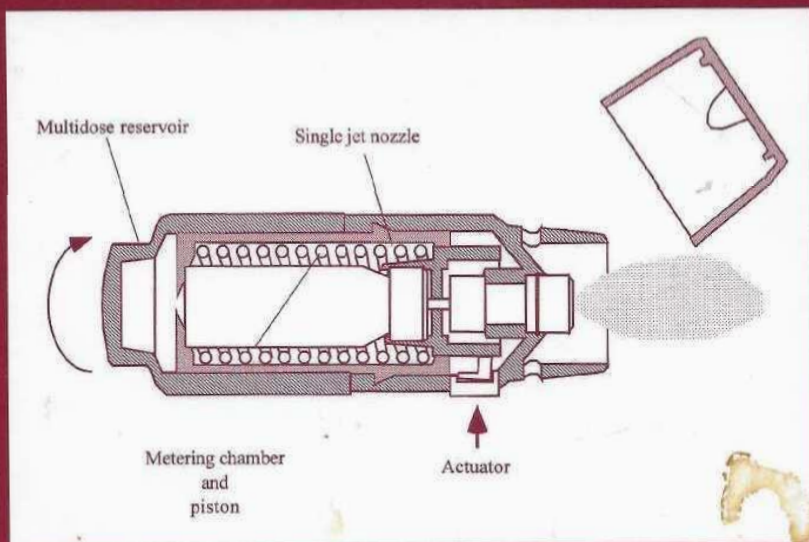


DRUGS AND THE PHARMACEUTICAL SCIENCES

VOLUME 99

Protein Formulation and Delivery



edited by
Eugene J. McNally

CELLTRION EXHIBIT - 1013

Celltrion, Inc. v. Regeneron Pharmaceuticals, Inc.

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Solution Formulation of Proteins/Peptides

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I. INTRODUCTION

The simplest and most economical way to formulate a protein is to develop a solution formulation. Lack of adequate protein stability in solution may ultimately require development of an alternative, more complex formulation such as a lyophilisate. A well-designed formulation study will allow the formulation scientist to determine whether a solution formulation will be acceptable for a given protein.

The first requirement for a formulation study is the availability of significant quantities of purified protein and/or peptide. Along with this protein or peptide should come a vast amount of practical knowledge obtained during various stages of process development. This knowledge will not only be anecdotal in nature but will also provide some specific physicochemical characteristics of the molecule. Purification in-process analytical tests should be available to assess basic parameters such as purity, concentration, and some measure of activity. Experiences during early stages of process development can contribute to this initial knowledge base. For example, problems such as poor solubility or aggregation that may occur during fermentation, harvesting, and purification of the molecule can result in a reduced recovery yield. Knowledge of the conditions and circumstances under which these problems occurred can aid in focusing and prioritizing the initial formulation studies. For

example, a particular pH or mix of buffer components used during processing may reduce solubility or induce aggregation. Even physical processing steps like diafiltration can expose the protein to shear and denaturing liquid–solid or liquid–air interfaces, which can ultimately cause protein aggregation as well. Alternatively, investigating the effect of freezing might be advisable, since freezing is often used as a convenient process-hold step. Feedback from these experiences can be used to design formulation experiments to gain insight on how to avoid, minimize, or explain process problems. Conversely, the results from these formulation experiments can aid in refining the purification process as well as in guiding future formulation development to address specific potential stability problems.

The second prerequisite for starting protein solution formulation studies is availability of analytical test procedures for characterizing the physicochemical properties of the protein. Methodologies for the characterization, beyond the basic ones used to assess purity during stages of purification, are necessary. As part of preformulation studies, specific stability indicating analytical methods will need to be developed. These methods must be capable of detecting changes in protein samples that have been altered by physical or chemical stress. Typical properties monitored include changes in charge [studied by means of isoelectric focusing (IEF), native–polyacrylamide gel electrophoresis (native-PAGE), and ion exchange chromatography], conformation [size exclusion chromatography (SEC), circular dichroism, fluorescence spectroscopy, native-PAGE, and capillary zone electrophoresis], size [SEC, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), laser light scattering, matrix-assisted laser desorption ionization time-of-flight (MALDI TOF) mass spectrometry, native-PAGE, and capillary electrophoresis], hydrophobicity (hydrophobic interaction chromatography, reversed phase chromatography, and micellar electrokinetic chromatography), and biological activity (cell-based assay, enzymatic assay, and enzyme-linked immunosorbent assays).

Additionally, knowledge of a protein's unique structural and functional characteristics can often indicate which analytical tests will be most useful in assessing stability. For example, when one is probing for changes that effect hydrophobicity, such as denaturation or methionine oxidation, hydrophobic interaction chromatography is generally more useful for large proteins (immunoglobins), whereas reversed phase techniques are more appropriate for small proteins ($MW < 40$ kD) and peptides. Many glycosylated proteins contain negatively charged sialic acid residues, which create surface charge heterogeneity and the potential for complicated IEF gel patterns and poorly resolved native-PAGE gels. Such complexity might mask charge changes that one

might expect due to deamidation, for example. Extensive glycosylation can also result in size heterogeneity that may contribute to zone broadening in SDS-PAGE or SEC. Calculations based on amino acid composition or sequence can be used to estimate charge as a function of pH (including isoelectric point) for a particular protein (1). This is useful in choosing electrophoretic or ion exchange conditions and may predict solubility behavior, since solubility can be strongly influenced by charge. Coupling specific structural knowledge of a protein with general knowledge of protein degradation will help in choosing the most appropriate analytical methods for a particular protein. In addition, it will give one an idea of what results to expect when a particular analytical method is applied.

Consideration of all the aforementioned prerequisites will allow for some prioritization and flexibility in designing solution formulation studies. In the end, formulation studies should address solubility as a function of pH and salt and the influence of increased temperature, solution pH, buffer ion, salt, protein concentration, and other excipients and preservatives (when necessary) on stability. Other studies to include are photostability, cavitation/shaking, and freeze-thaw cycling. Finally, material compatibility studies should be performed with any storage containers or medical device the molecule/formulation may contact.

II. SOLUTION FORMULATION

A. Solubility Studies

Typically, the relevant pH range over which solubility needs to be determined, for protein formulation studies, is 4 to 9. Although processing may expose the protein or peptide to pH values varying from 2.5 to 11, extremes in pH are avoided in formulations, since many degradative processes become more prevalent below pH 5 (acid hydrolysis) and above pH 7 (deamidation). To assess the minimum solubility required to formulate and deliver a protein drug, it is useful to consider the maximum dosing limit. A survey of the PDR (*Physician's Desk Reference*) reveals that for direct injection intravenous (IV) formulations, the upper limits on the volume and dose for currently marketed protein therapeutics, are 10–20 mL and 10 µg–100 mg of protein, respectively. Therefore, this means that one may need to achieve a minimum solubility in the range of 0.1–5 mg/mL. If the solubility is less than 0.1 mg/mL in this pH 4–9 range, additional excipients or sodium chloride may need to be examined as a means of increasing solubility.

If lyophilized, excipient-free protein is not readily available or obtainable owing to specific protein chemistry considerations, an alternative (and more laborious) way to perform solubility studies is to diafilter a protein stock solution into the desired buffer and then concentrate it as high as is practically possible. A variety of small-volume, centrifuge-driven, pressure-driven, and vacuum-driven devices are commercially available for this purpose. The concentrated stock solution can then be diluted with the appropriate series of buffers to be used in the solubility studies. An example of a solubility study protocol is as follows:

A ready supply of either lyophilized, excipient-free protein or diafiltration equipment is needed.

Start with protein in dry form and incrementally add weighed amounts to a small volume (typically ≤ 1 mL) of test buffer.

When the solubility limit is exceeded (i.e., when precipitation occurs), the undissolved protein pellet is centrifuged and the supernatant is analyzed for concentration.

Limitations on the amount of protein to add are discretionary as long as acceptable solubility is achieved.

Typically, if protein solubility exceeds 50 mg/mL at the pH of interest, no further solubility studies are necessary. The solubility desired should be minimally two fold and maximally 10-fold higher than the maximum conceivable dose.

If protein solubility is limited at relevant formulation pH values, additional measures will need to be taken to increase solubility. Some of the ways in which solubility can be increased include increasing/decreasing sodium chloride, adding other salts, varying the buffer species at a given pH, or including glycerol, lipids, polymers, cyclodextrins, or surfactants. Generally, one or the other of these approaches has successfully been applied to increasing solubility in protein solution formulations (2,3).

B. Isotonicity Considerations

Since isotonicity alone may have a tremendous influence on solubility, the basic approach should be to adjust the sodium chloride concentration before other excipients are added to the formulation. From a physicochemical standpoint, it is best to have as simple a formulation as possible, with fewer opportunities for potential complications due to undesired excipient-protein interactions. Furthermore, when problems do arise, sorting out what is happening is much easier. When considering salt concentration, our recommendation is to

formulate at or near isotonic conditions. While hypotonicity is not typically a problem for injectables, nasals, and topical products, hypertonic solutions may cause undesirable local tissue irritation or a burning sensation upon administration.

C. Solution Stability Studies

Generally, a formulation pH stability study should be limited to the physiologically relevant pH range of 4–9. Preformulation studies will typically address conditions and degradation pathways outside this pH range. The choice of buffer species should be physiologically compatible and listed as GRAS (generally regarded as safe) by the U.S. Food and Drug Administration. A useful source of excipients used in formulations is the FDA guide to inactive ingredients (4). Often at a given target pH, where multiple buffer choices are available, there will be differences between specific buffer ions with respect to effect on certain degradative pathways or rates of degradation.

D. Specific Buffer Ion Effects

To illustrate the importance of considering the specific ion effects of the buffer chosen, we discuss an example for a nine amino acid peptide (RMP-7). One clinical formulation for RMP-7 drug product consisted of a pH 4.0 unbuffered solution in normal saline (0.9% sodium chloride, pH adjusted with acid/base). This product was reformulated into a buffered system at the same pH to minimize pH drift due to leaching of hydroxyl ions from the surface of the glass vials of the drug product. The two choices for buffer species were citrate (pK 2.5, 4.5, 6.0) and acetate (pK 4.5), both of which possess good buffering capacity near the target pH of 4.0. The study consisted of formulating RMP-7 drug substance in a series of citrate buffers ranging in pH from 2.5 to 6.0 and in acetate buffers at pH 4.0, 4.5, and 5.0. Solutions were stored for up to 1 month at 60°C in a well-controlled temperature stability cabinet. Potency and purity of RMP-7 solution formulations were assessed as a function of time using a validated reversed-phase high performance liquid chromatography (HPLC) assay. Figure 1 graphically illustrates the effect of the two buffer species on the stability of RMP-7, as measured by drug purity as a function of solution pH versus the unbuffered formulation. In this case, the use of citrate ion caused more rapid peptide degradation than the acetate formulations over the pH range of 4.0–5.0. The acetate formulations were equivalent to the unbuffered formulation at pH 4.0.

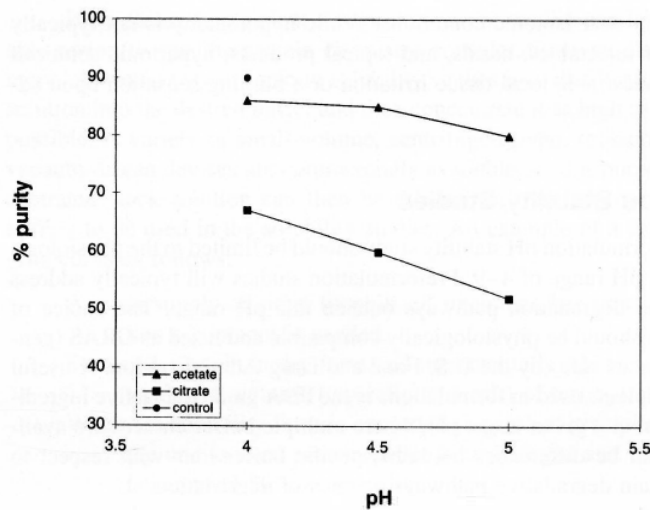


Fig. 1 Degradation of the peptide RMP-7 as a function of pH in two types of equivalent molarity buffer. The control solution was unbuffered with pH adjusted to 4 by addition of NaOH and HCl. All solutions were adjusted with NaCl to isotonicity and stored for one month at 60°C in molded glass vials. The difference in degradation rate demonstrates the buffer ion effect on RMP-7 stability.

This was an important finding in the formulation development of RMP-7 and illustrates the importance of choosing the appropriate buffer species for a formulation. This choice will always be dependent on the characteristics of the individual drug substance and cannot easily be predicted in advance nor generalized to other drugs. Once the likely pH of the final product formulation has been determined (from earlier preformulation studies), testing more than one buffer species at multiple concentrations at the chosen pH is advisable.

To measure the buffer ion effect on a degradative pathway, the buffer ion concentration in a formulation is varied and the tonicity is kept constant by addition of sodium chloride. A plot of the observed degradative rate constant versus buffer concentration will have a slope proportional to the buffer ion activity. Extremes in temperature and pH will accelerate degradation. Study design will require frequent sampling (i.e., hourly). Once the pH range has been narrowed to where major degradative processes are slowed, if degradation is still unacceptable (projected shelf life at 4°C <2 years), the effects of adding other potentially stabilizing excipients should be studied. However, if projected degradative rate is acceptable within this narrowed range, formula-

tion choice becomes more discretionary and can be based upon experience and other dosing or delivery considerations. With proteins, one should be cautious about projecting 4°C stability from accelerated temperature studies. At elevated temperatures other pathways/mechanisms can become more prevalent, often confusing analytical results and resulting in erroneous shelf life predictions. Some degradative processes have nonlinear plots of rate as a function temperature or may have plateaus, complicating projection [5–9].

E. Excipients

The inclusion of stabilizing excipients leads to the most interesting phase of formulation development studies. Here exists a vast sea of folklore and anecdotal information alongside excellent case history literature (3). Personal preferences often dictate excipient choices based on past experiences with different proteins (not always with positive results). Every protein has unique physicochemical characteristics and the potential to behave differently stability-wise in the presence of a particular excipient. For example, the common usage of surfactants to stabilize small recombinant proteins does not automatically warrant this inclusion in monoclonal antibodies formulations. Excipients, if necessary, should be chosen for their known functionality in slowing or arresting specific degradative pathways. An example of specific excipient choice based on known functionality is the use of hydroxypropyl- β -cyclodextrin in stabilizing interleukin (IL-2) aggregation (2,10).

F. Temperature Stability

Normally, proteins in solution are formulated for storage at 4°C with a minimum target shelf life of 2 years. As stated in the International Conference Harmonization (ICH) stability guideline (11):

Since most biotechnological/biological products need precisely defined storage temperatures, the storage conditions for the real-time/real temperature stability studies may be confined to the proposed storage temperature. However, it is strongly suggested that studies are conducted on the drug substance and drug product under accelerated and stressed conditions. Studies under stress conditions may be useful in determining whether accidental exposure to conditions other than those proposed (e.g. during transportation) are deleterious to the product. Conditions should be carefully selected on a case-by-case basis.

Most solutions of proteins are not thermally stable above room temperature for prolonged periods of time; therefore accelerated studies are typically con-

ducted at 25, 30, and/or 40°C, and generally no higher. However, in the case of peptides, accelerated studies at higher temperatures are often permissible because their inherent temperature stability is greater. Conducting a temperature rate study of degradation may allow not only a projection of stability at 4°C, but also an estimation of the activation energy of a particular process (12).

G. Protein Concentration

Another important formulation parameter, that needs to be studied is the effect of protein concentration on stability. Of course early on in development, availability of sufficient amounts of protein active may limit the extent to which high concentration studies can be conducted. However, such studies should be performed because rates of degradation typically vary as a function of protein concentration. Based on overall clinical experience with protein drugs and their maximum inherent solubilities, we recommend starting in the 1–10 mg/mL protein concentration range. This assumes that the final drug product will be administered by IV injection in a volume of 1–10 mL.

H. Photostability

Another ICH guideline states: “The intrinsic photostability of new drug substances and drug products should be evaluated to demonstrate that, as appropriate, light exposure does not result in unacceptable change. Normally, photostability testing is carried out on a single batch of material” (13). This particular test will be most relevant for products formulated as solutions where there is potential for storage under lighted conditions. Since most biologicals are stored refrigerated (the light goes out when you close the door), the assessment of photodegradation is a minor consideration, but nonetheless a regulatory requirement. After controlled light exposure of RMP-7 drug product, the nine amino acid peptide discussed earlier, an increase in one impurity was observed, but the product still met acceptance criteria of $\leq 1\%$ impurity. This is in contrast to RMP-7 drug substance, which exhibited no photodegradation when exposed to the same conditions.

I. Preservatives

1. General Considerations

If the protein formulation is intended for multidose use, a preservative will need to be included to prevent microbial growth. However, preservatives are

toxic, and there are maximum limits described for injectables (14). Table 1, assembled from a search of the PDR, lists preservatives used in some approved protein and peptide pharmaceuticals. Selection of an appropriate preservative is not trivial and requires the consideration of many factors, which complicates formulation. Focus initially will be to choose several potential preservative candidates based on current use and route of drug delivery, ideal solution pH of formulation, and compatibility with other formulation components. Once a selection has been made, accelerated and real-time stability testing of the protein in the presence of these selected preservative candidates should be performed. Chapter 4 on preformulation contains a discussion on the use of differential scanning calorimetry to screen preservatives with respect to protein interactions. In addition, an article on the effect of benzyl alcohol on the stability of γ -interferon is available (15). These authors do not identify the mechanism of interaction but do show that by picking the correct buffer salt and minimizing the amount of preservative, a stable formulation can be developed.

2. Timing for Inclusion in a Formulation

Performing extensive stability testing of the protein without a preservative will provide one with an idea of the buffer system and pH on which to focus stability testing. If the objective of the initial phase I clinical trial is proof of concept, the simplest approach is formulation without a preservative. However, this approach will mean repeating the phase I trial with a second preserved formulation, and additional stability studies will be required. If it is known that the final drug product will be used in a multidose form, a preserved formulation must be developed at some point. The timing of the decision to develop and test the preserved formulation will depend heavily on a cost-risk benefit assessment.

A decision to develop a preserved formulation will require developing methods to assay both protein and preservative. Processing equipment and container compatibility with the preservative, as with the protein, will need to be addressed. Finally, preservative efficacy in the protein formulation will need to be performed on the preservative candidates.

Quick elimination of a particular candidate formulation due to stability problems will reduce the amount of samples that need to be tested by preservative efficacy.

3. Choosing a Preservative

There are a number of prerequisites for selecting a potential preservative candidate. Is it generally regarded as safe and pharmaceutically acceptable

Table 1 Currently Used Preservatives for Proteins and Peptides from the PDR

Product, manufacturer, and drug	Route of administration ^a	Container	pH ^b	Buffer system ^b	Preservative concentration
Proteins					
Alferon N, Interferon Sciences, Inc.; interferon alfa-n3	IL	1 mL vial	7.4	Sodium and potassium phosphate	Phenol: 3.3 mg/mL
Epogen, Amgen, recombinant erythropoietin (epoetin alfa)	IV or SC	2 mL vial	6.1	Sodium citrate	Benzyl alcohol: 1%
Intron A, Schering; recombinant interferon alfa-2b	IM, SC, or IL	0.5, 1.0, and 3.2 mL vials		Sodium phosphate	1.5 mg/mL <i>m</i> -cresol, 0.1 mg/mL EDTA
Nutropin AQ, Genentech; somatropin	SC	2 mL vial	6.0	Sodium citrate	2.5 mg/mL phenol
Procrit, Ortho Biotech; recombinant erythropoietin (epoetin alfa)	IV or SC	2 mL vial	6.1	Sodium citrate	Benzyl alcohol: 1%
Roferon-A, Roche; recombinant interferon alfa-2a	SC or IM	1, 0.9, and 3 mL vials	NA	NA	Phenol: 3.3 and 3.0 mg/mL

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Peptides	SC or IM	2 mL vial	NA	Sodium acetate	Phenol: 5 mg/mL
Calcimar, Rhone Poulenc; calcitonin	SC or IM	2 mL vial	NA	Sodium acetate	Phenol: 5 mg/mL
Desmopressin, Ferring; desmopressin acetate	IN and IV	2.5 and 10 mL vial	4	No additional	Chlorbutanol: 5 mg/mL
DDAVP injection, Rhone Poulenc; desmopressin acetate	IV	10 mL vial	4	No additional	Chlorbutanol: 5 mg/mL
DDAVP nasal spray, Rhone Poulenc; desmopressin acetate	IN	2.5 mL vial, 5 mL bottle	NA	Citric acid, sodium phosphate	Benzalkonium chloride; 0.1 mg/mL
Iletin II pork, Lilly; pork insulin	SC	20 mL vial	NA	No additional	<i>m</i> -cresol: 0.25% (w/v)
Miacalcin, Novartis Pharmaceuticals; calcitonin	SC or IM	2 mL vial	NA	Sodium acetate	Phenol: 2.25 mg/mL
Miacalcin, Novartis Pharmaceuticals; calcitonin	IN	2 mL vial	NA	No additional	Benzalkonium chloride: 0.1 mg/mL
Pitocin, Parke Davis; oxytocin	IV	10 mL vial	NA	Acetic acid	Chlorbutanol: 0.5%
Sandostatin, Novartis Pharmaceuticals; octreotide acetate	Deep SC or IV	5 mL vial	4.2	Lactic acid	Phenol: 5 mg/mL
Synarel, Searle; nafarelin acetate	IN	10 mL bottle	NA	Acetic acid	Benzalkonium chloride

^a IN, intranasal; IV, intravenous; IL, intralesional; SC, subcutaneous.

^b NA, not available.

(GRAS-listed)? The intended route of administration is also an important consideration. A preservative appropriate for IV administration may not be appropriate for nasal administration. Some preservatives that are acceptable for IV administration may have a strong odor or taste (phenolics); others may dry nasal mucosa or be cilia static or toxic (16). Sensitivity of target patient population also may be an important consideration. For example, asthmatics have been shown to be more sensitive to certain types of preservative. Benzalkonium chloride and EDTA from nebulized solutions have both been reported to induce dose-related bronchoconstriction in asthmatics (17).

Formulation pH is another important consideration in choosing a preservative. Preservatives are most effective against microbes within specific pH ranges. When formulated outside these ranges, many preservatives change ionization state and lose efficacy. The candidate should have a wide spectrum of activity, preventing the growth of fungi, as well as exhibiting cidal activity against various types of Gram-positive and -negative bacteria. Preservatives can often be used in combination to achieve synergistic effects (18,19).

Compatibility with other formulation components is another important consideration. Certain preservatives (e.g., mercurial preservatives are incompatible with metals; others (e.g.) phenolics, quaternary ammonium, paraben preservatives), are incompatible with surfactants. Choice of preservative concentration to use can initially be based upon current use in the industry (PDR) and specific literature references for preservative safety and efficacy against various types of microbe (19). Eventually, preservative efficacy studies performed with the protein will determine the optimum preservative concentration.

4. Analytical Methods

Many preservatives have characteristics that interfere with analytical methods that work extremely well for the protein in their absence. For example, many preservatives exhibit high ultraviolet adsorption, making it necessary to have analytical techniques capable of separating the protein active from the preservative. Often the preservative needs to be removed before cell-based activity assays are performed, since the preservatives are toxic to the cells. Ideally, it would be nice to have quantitative separation methods capable of resolving the preservative(s) from the protein with no interfering interactions from any of the components. We have seen that paraben preservatives interact with hydrophilic (SEC) media, complicating results. In contrast, a reversed-phase method that worked well to separate and quantitate parabens irreversibly adsorbed the protein and thus modified column performance. Developing analytical techniques to get around these problems can be a challenge.

5. Compatibility

In addition, container considerations may change when a preservative is added to a protein formulation. Some preservatives are sensitive to light and/or air and may require storage in brown glass containers and/or airtight or purged containers. Phenolics, benzyl alcohol, chlorobutanol, parabens, and mercurial preservatives are examples.

Container-preservative compatibility can be a problem. Parabens and benzalkonium chloride adsorb to and chlorobutanol diffuses through plastic containers and delivery devices, reducing preservative strength (20,21). Furthermore, rubber stoppers adsorb many preservatives. Additionally adsorption/interaction with processing equipment that contacts preservative, such as vessels, tubing, and filters, can be potentially problematic. Adsorption studies should be performed to address these potential pitfalls.

6. Preservative Efficacy

Preservative efficacy studies are performed on preserved formulations as described under the following general test chapter of the U.S. Pharmacopeia: *Antimicrobial Preservatives—Effectiveness* •51* (USP 23, p. 1681) and are useful in identifying the most appropriate preservative for a particular protein at the optimum preservative concentration to maintain a growth-free solution. The assay involves challenging the formulation with bacteria of three types (*Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*) and fungi of two types (*Candida albicans* and *Aspergillus niger*). The effective concentration range of the preservative is determined by adding various levels to the formulation and performing USP <51>. For example, if the intended formulated preservative concentration of phenol is 0.3 % w/v, the effectiveness of this concentration is tested at this level and several lower levels (e.g., 0.2, 0.15, 0.1 % w/v). The lower concentration levels are tested to ensure a margin of safety, since preservatives can become less effective owing to either degradation or adsorption over time.

J. Cavitation/Shaking

Proteins in solution denature at air liquid interfaces. Under significant stress—for example, during filtration or physical shaking—a portion of the soluble protein will often denature and irreversibly aggregate. A typical result of this denaturation process is a turbid, cloudy solution or a solution containing insoluble protein particles. Therefore, it is a good idea to test the ruggedness of a formulation to withstand shaking or agitation that that might occur during

handling. To test a formulation's ruggedness, ultimately the final dosage form (in its final container) should be shaken or cavitated. A mechanical shaker is used to cavitate the protein solution for days and up to a week at room temperature. If the solution becomes cloudy or SEC reveals soluble aggregates, adding volume to fill container often reduces the problem. Another approach would be to supplement the formulation by adding glycerol, polymer, or a surfactant, but this ultimately depends on the sensitivity of the protein. If the solution does become cloudy, consider adding the instruction "DO NOT SHAKE" to the container label.

K. Freezing Studies

Freezing is another handling consideration. At various stages in processing, it may be necessary to freeze bulk protein solutions. Although the storage condition of final drug product is generally 2–8°C, there is always the possibility of inadvertent exposure to freezing temperatures during shipping and storage. Freezing might occur during air shipments of protein solutions, since packages may end up in uncontrolled-temperature cargo bays or in refrigerated storage units having colder areas that are below freezing. Thus it is important to do freeze–thaw cycling studies to determine the ruggedness of the protein solution. In the processing of proteins, the availability of containers of many types and sizes makes it a challenge to design a relevant scalable freeze–thaw simulation at the small scale. Variations in the process of freezing can radically affect the rate of freezing and ultimately the integrity of the protein. The temperature of the freezer (–20°C or –80°C), the type of freezer, the method of freezing (contact of shelf, liquid, or air), the location within the freezer, the volume of solution, and the container geometry all affect the way in which the solution freezes. Details of how these parameters affect protein integrity should be carefully considered in freeze–thaw cycling experimental design. One approach is the use of minitanks to assess the potential damage caused by repeated freeze–thaw cycles in a scaled-down version of process tanks. Studies of these types have been used to support the freezing and subsequent thawing of bulk drug substances stored in large-scale tanks. Protein solutions are analyzed both visually and by (SEC) for aggregation.

For final drug product, such studies are somewhat easier, because the container is a limiting constraint. Freeze–thaw studies using the final drug product are considered relevant because product may encounter hostile environmental conditions en route to its destination or accidentally in the hands of health care workers (doctors, nurses, pharmacists).

L. Materials Adsorption

Materials adsorption is mainly a consideration for low concentration protein/peptide formulations, generally at concentrations less than 1 mg/mL. Increasing surface area, contact time, and temperature all can enhance adsorption. Adsorption has the potential to occur during processing and during storage in final drug product container. Materials that contact the protein solution during processing stages and in the final container will determine what compatibility testing needs to be done. Table 2 shows what types of material may need to be tested and at the stage during processing at which contact occurs.

If the active drug is formulated at a low concentration and adsorbs to the container of choice, there are several options. One option is to fill the container to maximum volume. Filling a container to near capacity can reduce surface area contact between protein/peptide solution and the container, thus minimizing potential adsorption. The second and more difficult option is to change container material type. As a last resort, one may consider the addition of another excipient (e.g., human serum albumin, polymers, or surfactants) to the formulation, to inhibit absorption.

M. Placebo Considerations

In developing placebos for protein solution formulations, one must consider that the properties of the placebo solution will differ slightly from those of the active substance. There will be a difference in the solution viscosity, and a shaken protein solution will exhibit a certain degree of foaming, which may allow a clever clinician the opportunity to unblind a trial. As far as stability is concerned there should be no difference between the placebo and active solutions. To demonstrate this, it is prudent to manufacture and monitor the stability of the placebo product prior to initiating clinical trials. This will en-

Table 2 Material Contact at Various Processing Stages

Surface material	Drug substances	Formulated bulk	Final drug product
Plastics/glass	✓	✓	✓
Membranes/filters	✓	✓	✓
Tubing	✓	✓	
IV bags/catheters			✓
Syringes			✓
Stoppers			✓

sure that placebo of acceptable quality and adequate blinding properties is available.

III. MANUFACTURING OF DRUG PRODUCT: ASEPTIC PROCESS CONSIDERATIONS

A. Terminal Sterilization

The FDA requires manufacturers of drugs administered parenterally to provide data to support the necessity of aseptic processing. If a product can be autoclaved, it must be. If the manufacturer claims a product cannot be terminally sterilized, there must be data to support this claim. In the case of RMP-7 drug product, several vials of product were subjected to normal autoclave cycles to determine whether aseptic processing could be supported. The results of this study indicated that an unacceptable level of degradation occurred during even reduced autoclave cycle times (1, 3, 10, 15, 20, and 30 min). This study provided enough evidence to support aseptic processing as the only acceptable means of sterilizing RMP-7 final drug product.

B. Process Equipment Leachates

In filling low concentration (2 µg/mL) products, highly sensitive HPLC methods are capable of detecting and quantitating very low level process/formulation contaminants. Guidelines for impurities in peptide drug products allow for 1% or less of single impurities (ICH guideline). There is the potential during processing and formulation stages to leach into final drug product formulations low level contaminants that can be detected in final filled drug product as impurities. One should take care in testing all potential product contact equipment/containers for potential leachates. It is not always obvious which individual pieces of process/formulation equipment come in contact with drug product during filling operations. Caution should be exercised in determining which pieces of process equipment will contact drug formulations. One should thoroughly investigate this potential problem area before embarking on the formulation of low concentration drug products. All process and formulation equipment, should be thoroughly cleaned and rinsed, and the rinsate solutions analyzed for leachates, before active drug product is processed. This precaution could save a great deal of time, money, and lost product, in addition to avoiding a lengthy investigation.

C. Incompatibilities Between Process and Product

In addition to monitoring the product during the autoclaving process, it is important to bear in mind that the container closure system can be adversely affected by the terminal sterilization process. Several publications have addressed the issue of extractables from container closure systems and the associated problems of these leaching into a product. For example, leaching of zinc salts from various rubber closures has been reported to result in precipitation, discoloration, and contamination of products (22). Such leaching processes can be accelerated at elevated temperature, as would be encountered during the sterilization process.

An example of this was encountered during the aseptic filling and terminal sterilization of a phosphate buffered saline solution that was being manufactured for use as a placebo. During visual inspection of the autoclaved product vials, insoluble particulates were observed in the solution and on the gray butyl rubber vial closure. Upon isolation and examination of the particulates by X-ray microanalysis, the insoluble particulates were identified as zinc phosphate. A series of experimental compatibility studies was performed and the source of the particulates was identified as the gray butyl rubber stoppers encountered during autoclaving. These stoppers contained zinc in the form of zinc oxide, and the hypothesis was that zinc was leaching out of the stopper and in the presence of the phosphate buffer yielded water-insoluble zinc phosphate crystals. When Teflon-coated gray butyl rubber stoppers were used in the manufacture and autoclaving of the phosphate buffer, no particulates were generated.

IV. LINK OF FORMULATION BACK TO MANUFACTURING

Well-designed formulation studies yield much knowledge of the inherent degradative pathways to which a particular protein (or peptide) molecule is susceptible. They also reveal under which conditions these degradative processes proceed most rapidly. This in-depth understanding of the molecule's physico-chemical stability can aid in explaining problems that might have occurred during purification or how subtle changes in pH or storage and handling conditions might effect the final recovery and integrity of the protein. For example, knowledge of how freeze-thaw cycling affects the protein might aid in making decisions on storage of bulk drug substance at various in-process stages. A protein molecule known (from formulation cavitation studies) to be suscepti-

ble to air–water interface denaturation may need to be treated more gently, or a protective excipient may need to be added to the formulation during filtration. A change in the process that reduces air–water interface exposure might be another option in handling the sensitive molecule.

In our experience, changes in the large-scale cell culture process have resulted in changes in the IEF pattern of the purified and formulated protein. Knowledge of deamidation rates under cell culture conditions and exposure times to the media components in the cell culture process allowed prediction of the IEF banding pattern when the process was changed. Such projections are analogous to shelf life projections performed for final products. Modeling of this deamidation process based on solution formulation studies allowed extension of this model to the actual cell culture process conditions. Ultimately the model was used to explain that the changes seen in the purified molecule were only minor charge changes resulting from the extended exposure time of the protein in the modified cell culture process.

V. CONCLUSION

The following step-by-step, how-to- protocol should enable the formulation scientist to proceed through a protein solution formulation development study. While it is important to address all aspects of protein solution formulation, there is a natural sequence to the order in which individual parameters are investigated. Ideally, the protein concentration and solubility are determined first to establish a working range for the initial pH stability study. It is also advisable to assess freeze–thaw stability early in the plan. Once these parameters have been investigated, the initiation of a simple range-finding stability study, as outlined below, is recommended.

A. Step 1 Protocol

1. Protein concentration: 1 and 10 mg/mL
2. pH range in 1-degree increments from 4.0 to 9.0
3. Temperature: 2–8°C only
4. Additional excipients: NaCl to near isotonic
5. Time frame: maximum 3 months
6. Sampling interval: weekly at pH extremes; monthly at remainder

B. Step 2 Protocol

The results of the initial range-finding studies will enable one to refine and narrow the scope of future experiments. The next phase of experiments should focus on a narrower pH and concentration range and should study higher temperatures, possibly adding stabilizing excipients and extending the time frame of the study to at least 1 year, sampling at monthly intervals. Specific buffer ion effects could be investigated during this phase.

The final phase step 3, would involve a series of individual studies to address the remainder of the solution formulation parameters.

C. Step 3 Protocol

1. Autoclave study
2. Photostability study
3. Preservative study (if multidose product)
4. Cavitation/shaking
5. Materials adsorption
6. More in-depth freeze-thaw studies

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