Rational Design of Stable Protein Formulations

Theory and Practice

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High Throughput Formulation: Strategies for Rapid Development of Stable Protein Products

Rajiv Nayar and Mark C. Manning

INTRODUCTION

With the growing demand for new and innovative medicines, drug companies are spending record amounts of money on research and development. In the U.S. alone, research and development (R&D) investments are expected to exceed $24 billion in 1999, with investments in biotechnology totaling about $7 billion (Pharmaceutical Research and Manufacturers of America, 1999). As a result, new technologies are increasing the efficiency of the drug discovery process, and the drug pipelines have more products than ever in development. Nearly 350 biotechnology-related products are currently in clinical trials and over 50 are on the market (Pharmaceutical Research and Manufacturers of America, 1999). Pharmaceutical companies can now typically assess potential activities of up to 100,000 compounds a day using high throughput screening systems. In the biotechnology arena, recent advances in genomics, functional genomics, proteomics, bioinformatics and pharmacogenomics are facilitating the development of protein drug candidates at a much faster rate than was possible during the early years of the biotechnology industry. Couple these changes with the impending

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publication of the complete sequence of the human genome by 2001 and there is a potential for an additional 15,000 protein drugs from the predicted 150,000 potential genes in the human genome.

This new wave of protein drugs will include compounds that can function as growth factors, act as specific stimulators or suppressors of certain functions, or exhibit activities that have not been observed before. Many experts anticipate a golden age of protein-based therapeutics in the twenty-first century. As various research enterprises gear up to search for these new candidates using high throughput cellular and molecular screening assays, there undoubtedly will be a need for a corresponding ability to formulate these drugs much more quickly than in the past. In addition, there will be increased use of novel delivery systems to administer these protein therapeutics efficiently, making stabilization even more challenging. In short, the pharmaceutical biotechnology industry must find new ways to speed up the drug development process, in order to keep pace with the drug discovery process. We think that the limiting factor for introduction of new biotechnology-derived products will not be finding new drug candidates, but efficient and rapid formulation of these compounds into acceptable dosage forms for delivery and testing in humans.

The potential of many new drug candidates flowing through the pipeline will require implementation of an entirely new approach towards product development. The particular challenge addressed here is the demand that will be placed upon formulation groups, where there will be increased expectations to develop stable formulations of:

more proteins,
in a shorter period of time,
with less material, and
with little, if any, increase in personnel.

Therefore, one must consider new strategies for meeting such challenges.

This chapter is intended to serve as a forum for discussing and evaluating what factors must be considered in trying to implement new approaches to formulating protein pharmaceuticals in a rapid, rational fashion. As a comprehensive strategy for developing stable formulations of protein pharmaceuticals is not currently available, we have assembled what we consider to be the key elements necessary to accomplish this critical task. This chapter should not be viewed as a finished product, but as a work-in-progress, encouraging pharmaceutical scientists to comment on the concept and to modify the approach to their particular situations.

Again, the overarching goal is to possess a unified strategy for accommodating many more proteins at one time, while meeting shortening timelines. It should be expected that there would be a limited supply of well-characterized material and a fixed personnel head count. We envision a scheme that we term "high throughput formulation" or HTF. It is based upon our current

understanding regarding stabilization of proteins, but it also identifies areas where technical improvements must be made, especially in the area of analytical methodology and software. If the HTF strategy is successful, it should be possible to match the output of stable formulations by development groups with potential leads by discovery groups.

OVERALL STRUCTURE OF THE HTF APPROACH

The HTF concept includes four key elements, which are briefly outlined in Table 1. The heart of the HTF strategy is a decision plan, where the formulation scientist must decide on (a) the specific excipients to be used as well as (b) the final dosage form. Central to the HTF scheme is the use of a limited set of additives as well as dosage forms. Especially for lyophilized or freeze-dried formulations, our understanding of the behavior is such that some standard formulations can be envisioned (see Chapter 5). Also, the choice of basic solution conditions will be based upon preformulation studies that examine the behavior of the protein as a function of pH, ionic strength, and buffer type (see Chapters 1 and 3).

The second key aspect is an increased role for software and information databases. The preformulation studies could require a significant number of independent experiments to be conducted. Therefore, experimental design software should be used to reduce the number of experimental trials and optimize the results through statistical evaluation. In addition, there are a number of databases and associated programs for assessing physical and chemical properties based upon primary sequence of the protein through a number of websites (e.g. SWISS-PROT). These can provide insight into protein structure and other critical characteristics (e.g., pI, hydrophobicity and hydroflexibility plots and overall charge), and even identify residues that might be sensitive to chemical decomposition. We believe such capabilities will expand over time, and will play an increasingly important role in setting a rational plan for preformulation studies. Finally, we anticipate that specific protein stability databases will emerge, allowing scientists to gather data on specific development issues, without searching all of the open literature. Because information on protein stability comes from a multitude of disciplines (e.g., biochemistry, biophysics, food science, engineering, pharmaceutics, surface science and polymer science), open searches often miss crucial publications and documents. The challenge is not unlike Internet search engines trying to find a very specific subset of information on an ever-increasing world wide web. As these resources become more available and refined, they will emerge as invaluable tools for development of pharmaceutically acceptable formulations.

Table 1.
Key Elements of the HTF Approach

1. Decision Plan to Finalize Choice of Excipients and Dosage Form
   - A. Choice of Dosage Form
     1. Frozen Solution
     2. Liquid Formulation
     3. Lyophilized Formulation
     4. Protein Suspension
     5. Specialized Drug Delivery System
   - B. Choice of Excipients
     1. Proper pH/Buffer Species
     2. Salt
     3. Stabilizer
     4. Bulking Agent (for lyophilized formulations)
     5. Surfactant
     6. Chelators
2. Use of Software and Databases to Assist in Formulation Development
   - A. Experimental Design Software
   - B. Software and Databases for Analysis of Structure/Function/Properties/Stability
   - C. Protein Stability Databases
3. Availability of Essential Analytical Methods
4. Design of Appropriate Stability Testing Protocols

The third aspect of HTF is assembling the correct analytical methods to assay the stability, both chemical and physical, of a wide range of proteins. The mechanisms by which proteins degrade have been widely reviewed (Manning et al., 1989; Pearlman and Nguyen, 1992; Cleland et al., 1993), so they will not be discussed here. However, given this backdrop, we will describe certain key methods that must be available in order to conduct proper HTF studies. All of the required instruments do not necessarily need to be available in-house, as many can be accessed through contract organizations and through academic collaborations. In addition, it should be noted that there exists a tremendous opportunity to modify existing analytical techniques for use with smaller sample sizes and for rapid, parallel sampling. One of the expected limitations will be lack of well-characterized material. Whereas access to a minimum of 200–500 mg of protein for formulation studies is desirable, the genomic approach will tax the ability of process scientists to provide adequate amounts of purified proteins for all compounds under development. Therefore, in some cases, the total amount of protein available may be an order of magnitude less than the optimum. This situation should serve as an incentive to identify new approaches for gathering stability data. We will discuss below possible new methodologies that would be useful in the HTF scheme, but have not yet been applied to formulation development.

Finally, one must have a clear plan for stability testing, along with minimum criteria for a "stable" formulation. Certainly, that definition may change as a product matures. Minimum stability for the initial clinical studies may be quite different than the expectations for the final formulation tested in Phase III. Presently, there are debates within companies on how robust a preliminary formulation development or purification process must be in order to demonstrate efficacy in humans. Some companies will accept any minimal formulation in order to initiate clinical trials. However, even these formulations would likely have to have at least six to twelve months of shelf life. Our goal in the HTF approach is to develop a final formulation as early as possible in the drug development and testing process. If this goal can be accomplished, then fewer resources will need to be diverted to redesign the formulation later.

ROLE OF AN ESTABLISHED DECISION TREE FOR FORMULATION DESIGN

The central feature in the HTF scheme is a rational decision plan for choosing both the formulation components and the final dosage form. This decision tree must be flexible enough to accommodate a wide variety of proteins, it must allow for both liquid and solid dosage forms, and it must provide a rationale at each decision point. This framework allows the pharmaceutical scientist to decide what types of formulations are worth considering, both in terms of stability and the demands and desires of the marketing and clinical groups.

Often the design of a formulation of a protein pharmaceutical is not well developed. Companies attempt to expedite formulation selection by neglecting critical issues in formulation and stability. It is not uncommon to find sub-optimal formulations such as phosphate-buffered saline, possibly with the addition of human serum albumin as a ubiquitous protein stabilizer. For a few proteins, this approach has been sufficient, even resulting in commercially successful products, such as erythropoietin and granulocyte colony stimulating factor. However, there is a distinct danger in assuming that such successes with minimal formulations are generally applicable. Moreover, the current regulatory environment, and concerns in the general public surrounding the use of human-derived products in pharmaceuticals, makes the use of human serum albumin increasingly more difficult or even not acceptable. Therefore, there is pressure for any company selling a product that contains albumin to develop an albumin-free formulation. The same pressures are forcing companies to move from animal-derived surfactants to those obtained from vegetable sources. In addition, there is a realization that albumin, being a protein, is not indefinitely stable, and should have an expiration date above and beyond that of the active ingredient. All of these issues affect the

list of possible excipients that can be employed in a protein pharmaceutical product.

Constraints on a Pharmaceutically Acceptable Protein Formulation

Before we discuss the choice of dosage forms and excipients in the context of maximizing protein stability, we need to recognize the myriad of constraints placed upon the formulation by the rest of the company and by regulatory bodies. The first constraint, given the global nature of the pharmaceutical industry, is to have excipients that are acceptable in the major markets. This includes avoiding animal-derived additives, as discussed above. It also means that the list of possible additives is effectively limited to those already found in approved products in North America, Europe, and Japan. While this is not an immutable rule, few companies are willing to bear the added cost of getting a new excipient on the market while seeking approval for a new drug product. A major impact of this constraint is to move away from formulations containing albumin, which is still found in a number of protein products. One can anticipate that these older formulations will disappear in the coming years and be reformulated into second-generation products. A recent example has been the development of an albumin-free formulations for recombinant Factor VIII.

The second consideration is the tonicity of the final formulation. If a protein drug is to be administered by intravenous bolus injection or subcutaneously, rather than by continuous infusion, there are strict isotonicity and pH considerations that have to be met for a pain-free injection. Similarly, there are constraints on the choice of excipients, as some (e.g., citrate) have been shown to be irritating upon injection. This is one example of restrictions being set by the clinical groups, which should be clearly defined prior to the start of the formulation process.

Likewise, the eventual route of administration must be taken into account. A convenient administration system is highly desirable for ease of use in the clinical setting, so it is both a marketing and clinical issue. For example, in the case of a subcutaneous injection, there is a maximal volume (~1 ml.) that can be given to a patient without discomfort. Given the expected dose range, this will define the protein concentration that must be used. For other routes of administration, the ability to manipulate the protein concentration as a variable may be possible. Otherwise, it will be fixed, and the decision point regarding protein concentration is removed from the process described below.

The fourth issue is to work with a well-characterized bulk drug substance. This criterion will require cooperation and collaboration with the process science groups involved in fermentation and purification. Variation in the quality of the

starting material will compromise the validity of any preformulation or stability work done to date, as well as potentially invalidate data from toxicological tests.

The fifth constraint to be determined is the minimal stability required to complete proof-of-principle investigations, meaning animal studies and initial clinical trials. These investigations can extend for up to one year. Hence, at least a one-year shelf life for even the initial formulation may be required.

Finally, the manufacturing of the protein product must be possible at reasonable costs and allow adaptation to standard manufacturing processes. It is imperative that manufacturing economic analysis be conducted as soon as possible in the drug development process, including an assessment of whether sufficient manufacturing capability exists if the product is successful. Novel formulations that require special manufacturing setups and processes may not offer the advantages of speed which is necessary when one is confronted with development of numerous protein drug candidates. Most likely, specialized dosage forms and/or drug delivery systems will only be investigated with partners who possess the technology and capacity to solve these critical manufacturing issues. In addition, as the in-house pilot plants are challenged with manufacturing multiple products, with limited facilities and resources, there undoubtedly will be pressures to outsource the first clinical manufacturing because of both cost-effectiveness and timescale issues.

Proper Choice of Dosage Form

Considering all the therapeutic protein products on the market, it appears that three dosage forms are viable. The most preferred would be a solution formulation that is typically stored in the refrigerator and preferably in a pre-filled syringe. Second, a frozen solution loaded in a syringe or vial and thawed at the site of administration. Third, a lyophilized formulation, which can be stored at room temperature and reconstituted when needed. Each one of these dosage forms offers various advantages and disadvantages, in the speed of development, manufacturing, packaging and shipment logistics, and in administration of the product to the patient.

Generally, a frozen protein formulation offers the advantages of rapid development provided the protein is stable after multiple freeze/thaw cycles. It is also important to note that freezing can lead to selective precipitation of certain buffer species, producing possibly large pH shifts (van den Berg, 1959; van den Berg and Rose, 1959). This variation in pH, and the potential for damage at the water-ice interface (Chang et al., 1996; Hsu et al., 1995; Krielgaard et al., 1998), might limit the use of such a dosage form. Furthermore, there are disadvantages associated

with a frozen formulation concerning the packaging complexities involved in labeling, storage and maintaining the cold chain during transportation of a frozen product. These factors can add significantly to the development costs.

The second option for dosage form is a stable liquid formulation that can be stored in the refrigerator or at room temperature. This dosage form offers the advantages of potentially rapid development and ease of administration. However, for most proteins maintaining physical and chemical stabilities in aqueous solution for an extended period of time is extremely difficult. In addition, because the protein is free in solution there is potential for adsorption to the vial, whether it is made of glass or some type of plastic. A liquid product will have to be shown to be impervious to damage at the air-water interface during transportation, because agitation-induced denaturation and aggregation is a common problem with therapeutic proteins. There is also the possibility of leaching of metals and organics from the container and stopper, which is why some of the vials will need to be inverted during stability testing. These problems have been reported for drug products of all types (Airaudo et al., 1990; Nedich, 1983). Leached materials are particularly problematic with plastic vials and non-Teflon-coated stoppers. With glass vials the leachables could include ionic species that alter the pH and ionic strength of the solution during storage. Finally, the integrity of the container closure must be demonstrated, in order to ensure sterility of the product. For multi-use vials, preservatives can be added, but some are known to foster degradation of proteins (Lam et al., 1997; Rodrigues-Silva et al., 1999). Therefore, development of a stable liquid protein formulation has to be considered very carefully. Determination of the primary pathways responsible for decomposition of the active therapeutic protein is critical for developing this particular dosage form. All of these issues can add significant costs in terms of resources and analytical testing.

Most protein pharmaceuticals currently on the market are sold as lyophilized formulations. Placing proteins in the dried state significantly increases the shelf life, if the proper formulation and lyophilization cycle are used. The combined stresses of drying and freezing are inherently damaging to proteins, and only proper formulation will ensure that a stable product will be obtained. Fortunately, enough is understood now about freezing and drying of proteins that rational design of lyophilized formulations can be undertaken. Moreover, there are some formulations that seem to work well for a variety of proteins. We suggest that part of the strategy for rapidly identifying viable formulations is to make liberal use of some standard protein formulations. The rationale for these compositions has been described in detail in a number of review articles and book chapters, and is discussed in Chapter 5 (MacKenzie, 1976; Franks, 1990; Pikal, 1985, 1994; Carpenter and Chang, 1996; Carpenter et al., 1997). An approach towards developing a lyophilized formulation is described below.

As other formulation possibilities become more developed, one can modify the decision tree to accommodate them. For example, one can imagine alternatives to lyophilization for obtaining solid dosage forms. Both spray-drying and spray-freeze drying are being studied. However, it is expected that the formulation strategy for these processes will be quite similar to that used for lyophilization.

The most likely future additions to the dosage form list will be suspensions and specialized drug delivery systems. Protein suspensions can be produced by a number of different methods. The protein could be dried and suspended in a non-aqueous medium. Alternatively, the protein could be salted-out, using preferentially excluded solutes, such as ammonium sulfate or sucrose, leading to an aqueous vehicle containing most of the protein in its native state but in a solid form, because the solubility limit has been exceeded. The same effect might be accomplished by adjusting the pH to be near the isoelectric point of the protein. For the current version of HTF, we have chosen not to include these dosage forms until their use becomes more widespread and manufacturing facilities are developed to accommodate production of clinical batches.

The same can be said of specialized drug delivery systems, such as polymeric microspheres. Poly lactide/poly glycolide microspheres for sustained delivery of peptides have been in use for years. Recently, reports of their use for proteins, such as human growth hormone, have appeared. These systems bring their own specific stability issues (Cleland, 1998) in addition to making analysis of the stability of the active ingredient more challenging (Yang et al., 1999).

As discussed above, input from various departments, such as discovery research, pharmacokinetics, toxicology, purification, manufacturing, clinical and marketing, must be considered when deciding on the most appropriate dosage form for a given product. In the past, the drug development process has been more or less sequential, going from research to development to clinical, to manufacturing, and then to marketing. A change in strategy is required where there is more interactive, simultaneous communication between the various drug development departments. With the proper information from the other units, one should rapidly be able to decide on dosage form and develop candidate formulations.

Preformulation Studies

The exquisite sensitivity of protein structure, function, and stability to the primary sequence does not readily lend itself to a generic approach for protein formulation. Therefore, determination of the relative importance of various degradation pathways and elucidation of instability mechanisms for a given protein are essential. Even for closely related proteins, the relative stability and major

pathways for degradation might be quite different. Thus, the preformulation studies of new protein entities become very important and crucial tasks in the overall HTF strategy.

Preformulation refers to experimental studies designed at understanding the chemical and physical behavior of the protein or drug candidate. The major focus is on the solubility and stability as a function of a number of extrinsic factors, such as pH, protein concentration, ionic strength, buffer composition and temperature. This information will guide the appropriate choice of excipients, buffers and final protein concentration. Although a number of studies could be envisioned, HTF requires that these studies be very specific and focused. Any information that may narrow the choices would be essential for speedy formulation development. Relevant data can be gathered from the process-engineering group responsible for purification or literature studies on similar proteins, even if it is anecdotal. The final dosage form must also include constraints from marketing, clinical and regulatory groups addressing issues related to eventual approval of a pharmaceutically acceptable formulation for market. Ultimately, all of these data may be required to ascertain the most beneficial composition of excipients and the most appropriate dosage form for the protein product in hand.

Typical time scales for preformulation can range from one to three months depending on what assay systems have been established by the research groups and whether they can be utilized as stability-indicating. Therefore, early involvement of all of the formulation experts is critical for rapid development of lead formulations during the developmental phase. An additional driving force is also the fixed time required to generate the stability data to validate the formulation choice.

Proper Choice of Excipients

Liquid Formulations. If a solution dosage form is indicated, then there will be a finite set of possible excipients, restricting choices to those that are found in approved products and have been shown to be effective in protein formulations. For solution formulations, a list of possible excipients is given in Table 2.

Given that most protein formulations will exist at pH values between 4 and 9, there is a limited set of buffers that will exhibit sufficient buffering capacity. A number have been used in protein formulations and they are listed in Table 2. The main criteria for selection are good buffering capacity at the desired pH and lack of ability to accelerate specific chemical and physical reactions. Some proteins can preferentially bind certain buffer species, altering the conformational stability of the protein. In addition, some buffers (e.g., phosphate, Tris) have been

Table 2.
Possible Excipients for Use in Liquid Formulations

Excipient Class	Choices
Buffers	Histidine, Succinate, Acetate, Citrate Phosphate, Tris, Carbonate
Salts	Sodium Chloride, Calcium Chloride Magnesium Chloride
Non-Specific Stabilizers	Sucrose, Trehalose, other sugars, Amino acids (e.g., lysine, glycine)
Specific Stabilizers	depends upon the protein
Surfactants	Tween 20, Tween 80, Pluronic F-68 Sodium Dodecyl Sulfate
Chelators	EDTA

reported to catalyze certain hydrolytic reactions, such as deamidation (Patel et al., 1990).

Because proteins are polyelectrolytes, the ionic strength of the medium can often have an effect on structural stability. The most common salt used in protein formulations is sodium chloride. For an isotonic product, often it is advantageous to keep the concentration of salt as low as possible. Otherwise, the ability to employ stabilizing excipients will be limited.

Structural stabilization of proteins by specific ligands or cofactors in solution formulations can be important for formulation optimization. The mechanism by which these compounds increase thermodynamic stability is described by Timasheff (1992, 1995, 1998) and in other Chapters in this book. Several pharmaceutically important proteins have been stabilized by addition of specific ligands (including metals ions such as calcium), including fibroblast growth factor (Volkin et al., 1993) and DNAse (Chen et al., 1999).

To stabilize proteins (both in aqueous solution and during freezing) with non-specific compounds (e.g., sugars), relatively high concentrations (ca. > 0.2 M) of ligand (solute) are needed to affect protein stability. The mechanism of such stabilization is described in more detail in Chapters 2 and 3. The main point for the current discussion is that in the presence of nonspecific stabilizing excipients fewer protein molecules are unfolded at any point in time, either partially or completely, which reduces the amount of aggregation-competent species and the rate of aggregation (Kendrick et al., 1997, 1998). Addition of small amounts of a specific ligand should theoretically accomplish the same outcome. The most effective non-specific stabilizers tend to be disaccharides, such as sucrose and trehalose. However, certain salts, amino acids, and polymers are preferentially excluded as well. Still, unless there is evidence for advantage in use of a

particular compound from this group, sucrose and trehalose should remain the first-line choices.

Finally, one must decide on whether to include a surfactant in the formulation. Surfactants have been shown to inhibit protein aggregation during agitation (e.g., Bam et al., 1995; Krielgaard et al., 1998). The mechanisms by which surfactants stabilize proteins are discussed in a separate Chapter. The surfactant often will be most effective at or above the critical micelle concentration. In Table 2, only the surfactants that have been approved for use in parenteral products in the U.S. have been listed. Note that one potential drawback of including surfactants in the formulation are contaminants (e.g., peroxides) that could cause chemical degradation of proteins (e.g., oxidation).

Lyophilized Formulations. It can be assumed that most proteins will not exhibit sufficient stability in aqueous solution to allow a liquid formulation to be developed. Our understanding of the basic requirements for obtaining a stable lyophilized protein formulation is relatively well developed. The question then is what combination of excipients will allow such a formulation to be prepared. The minimal composition includes a buffer species, an additive capable of forming an amorphous glassy state and inhibiting lyophilization-induced unfolding in which the protein remains entrapped, a bulking agent to provide cake stability, and possibly a surfactant to retard surface-induced damage and/or promote refolding. The rationales for the minimal criteria for a stable lyophilized formation and the choice of appropriate excipients are described in detail in another Chapter.

While a comprehensive formulation development algorithm is yet to be published, following the HTF process outlined above, one can imagine obtaining a rational approach towards selecting final candidate formulation that can be used routinely, even with new classes of therapeutic proteins. Clearly, the details of each case will be guided by the outcomes of the preformulation studies. However, even without a complete data set from the preformulation experiments, implementation of a few standard formulations should be possible. Ultimately, we believe there will be a relatively small number (six of less) of generic, standard lyophilized formulations that will work for nearly all proteins. Each of these will have a corresponding lyophilization cycle that can be used to achieve optimal results. When the HTF process has evolved to this optimal state, the personnel demands be greatly reduced (in terms of full-time equivalents, or FTEs) and the time lines should shrink significantly from the current average of nearly one year.

Estimates of Resources Needed for Formulation Development

The current estimates of the resources required and the possible timelines for formulation development are given in Table 3. Specialized formulations

Table 3.
Estimated Resources Needed for Development of Final Stable Formulations of Protein Pharmaceuticals

Resource	Current Status	Initial HTF Targets	Optimal HTF Targets
• Personnel			
Standard Formulations	2 FTE	2 FTE	2 FTE
Complex Formulations	3–5 FTE 3–4 FTE	3 FTE	
• Time Scale			
Standard Formulations	12 months	6–9 months	3–6 months
Complex Formulations	12–24 months	12 months	6–9 months
• Minimal Amount of Material			
Standard Formulations	200–500 mg	100–200 mg	50–100 mg
Complex Formulations	400–2000 mg	200–500 mg	100–200 mg

(suspensions and controlled release dosage forms) will require more resources and less aggressive time lines (see Table 3). However, even this situation should improve as more of these types of products enter the marketplace. We anticipate that a significant number of these types of products will be approved over the next ten years. By then, most large pharmaceutical companies will have acquired the necessary expertise to develop them at rates comparable to the standard formulations of today. Once the HTF scheme has evolved to this stage, a company should be able to develop final formulations quickly and easily, even if personnel levels are relatively constant.

Interestingly, while a streamlined approach towards final formulation selection is the heart of the HTF strategy, it is the one aspect that can be introduced into any current product development scheme almost immediately, as it can be accomplished solely based on the current state of understanding of protein stabilization. Other aspects of the HTF package will demand more time to implement, as they will require significant advances to be made, particularly in the areas of analytical methodology and information technology. These two features of the HTF scheme are described below.

USE OF SOFTWARE AND DATABASES TO ASSIST IN THE HTF PROCESS

Unless formulation scientists have access to all pertinent information, critical choices for formulation components could be delayed or, even worse, incorrect. Therefore, we propose that a key aspect of the HTF process is to develop an integrated information system that can assist the scientist in making critical

decisions. Each and every step in the formulation development process is predicated upon having the most recent and comprehensive information on the physicochemical properties of the protein under development, as well as on the effects of additives on protein structure and stability. Clearly, the latter part of this goal is ever evolving as our knowledge of protein chemistry increases. Therefore, the final HTF scheme will involve a central database of essential information. While a large amount of this type of information is currently available, it appears in a multitude of sources, generated by researchers in disparate disciplines, ranging from materials science to biochemistry to food science to pharmaceutics. This makes access to the data difficult. Unless efforts are made to assemble this data in a cohesive package soon, the challenge will become even greater. Once the task is accomplished, sufficient information should be available to predict the performance of certain types of formulations.

In the absence of such resources, one can still make use of existing software and databases. These are summarized below. Briefly, they cover three areas: predictive protein algorithms based upon primary sequence, sequence homology analysis software, and programs aimed at predicting relative stability of proteins. Summaries of the locations and availability of these programs and databases has recently been published (Cook, 1999; Apweiler, 1999).

Predictive algorithms allow one to analyze a primary sequence and predict a variety of chemical and physical properties. For example, there has been enormous effort to develop schemes whereby one can predict the three-dimensional folding of a protein based solely on its sequence. Although there have been some successes, it has been demonstrated in double-blind studies that analysis of new sequences, when they have no clearly related partner in the existing database, fail to provide an accurate prediction of the folded conformation (Orengo et al., 1999; Vencloyas et al., 1999). When the analysis can be guided by sequence homology, the accuracy rises dramatically. Likewise, one can imagine developing structure-stability relationships, based solely upon primary sequence. Some studies of this nature have been reported. For example, Pandit and co-workers relate the frequency of certain dipeptide sequences to instability in proteins (Reddy, 1996; Guruprasad et al., 1990). The result is an algorithm that predicts the relative stability of a protein. Although the degradation criteria are based upon intracellular stability, this approach does provide researchers with an expectation of the overall fragility of the protein in question.

In another example of this type of sequence analysis, Roger et al. (1986) identified regions in proteins that were high in proline, acidic side chains (Asp and Glu), serine, and threonine. The presence of these regions was associated with more rapid degradation *in vivo*. Similar correlations should exist for *in vitro* stability. Even programs to predict likely deamidation sites (where Asn is converted into Asp-like residues) would be helpful, especially as our ability to remove those sites by recombinant DNA technology improves. There is now a

solid basis on which to predict both primary and secondary structure effects on deamidation rates (Wright, 1991; Xie and Schowen, 1999), which is considered in more detail in Chapter 4.

Given that numerous protein sequences, whether determined directly or extrapolated from nucleotide data, are available in databases that can be accessed via the world wide web, focused studies generating data on specific instabilities (e.g., deamidation, oxidation, aggregation) could lead to similar structure-stability relationships. The number of available sequences has been doubling every 18–24 months over the last ten years (Cook, 1999). Similarly, there has been an explosion in algorithms and software to analyze these data. Many focus on sequence homology and identification of functional and genetic relationships. Given these advances, it is not unreasonable to expect that stability-indicating profiles could be developed as well.

In addition to stability predictions, many programs are available to calculate important chemical and physical properties such as the isoelectric point, the hydrophobicity profile, the positioning of secondary structure elements, and the conformational flexibility. Knowledge of these properties would help the formulation scientist anticipate the behavior of a new molecule provided for development into a potential drug product. Furthermore, algorithms to predict correlations of these physical properties with physical and chemical stabilities of therapeutic proteins should be straightforward to design and implement with current programs.

ESSENTIAL ANALYTICAL METHODS

One must have access to certain equipment in order to characterize properly protein degradation pathways and effects of formulation variables on protein stability. In addition, we will discuss some areas where technological advancements must be made in order to allow formulation scientists to work with smaller amounts of protein. While a number of reliable and accurate stability-indicating assays exist, there needs to be efforts to miniaturize some of them. This will allow development of proteins where the amount of material is limited, e.g., less than 100 milligrams total.

The first analytical methods that are needed are those that can quantify the protein concentration. As determination of accurate extinction coefficients is straightforward, ultraviolet absorption spectroscopy is inexpensive and preferred over chemical methods, such as the Lowry method. One also needs to be able to monitor the global structure of the protein. Circular dichroism (CD) spectroscopy allows one to follow changes in both secondary and tertiary structure of proteins in solution (Manning, 1994). Infrared spectroscopy can also be used to detect

secondary structural changes (see below), and fluorescence and derivative UV spectroscopies can be used to study tertiary structure.

The second category of required analytical methods are those that monitor retention of native primary structure, namely, chromatographic methods, such as reversed phase HPLC and ion exchange HPLC, or alternative methods like capillary electrophoresis. Among the most useful applications of these HPLC methods is the generation of a "tryptic map", which is accomplished by digesting the protein with a sequence-specific enzyme (e.g., trypsin) and separating the resulting peptide fragments by reversed phase HPLC. The pattern then becomes a sensitive fingerprint of the protein. Any chemical modification of the protein will cause one or more of the peaks to shift, indicating damage to the protein. Mass spectrometry can be used to determine the specific chemical modification leading to the peak shift.

Among the most important degradation pathways for proteins is aggregation. A number of methods are available to characterize and quantify aggregation levels in proteins, including polyacrylamide gel electrophoresis (PAGE), size exclusion chromatography (SEC), light scattering techniques and analytical ultracentrifugation. Although each of these methods has its advantages and disadvantages, all four should be considered essential, as they can be complementary. They allow both qualitative and quantitative characterization of the soluble and insoluble aggregates in a bulk substance or final formulation.

For lyophilized formulations; a certain set of equipment is considered to be the minimum required for conducting formulation development properly. These include an infrared (IR) spectrometer, a Karl Fischer titrator, a differential scanning calorimeter (DSC), and x-ray diffractometer. The IR instrument will allow one to determine the extent of structural damage (at a secondary structure level) in the solid state. In addition, it can be used to monitor the secondary structure composition in the original solution (prior to lyophilization), in the frozen solid and in the reconstituted product (Carpenter et al., 1998). X-ray diffraction of solids allows one to determine the crystallinity of a solid sample. In formulations where the bulking agent is crystallizing during the lyophilization process, one needs to know whether the crystallization is complete. Incomplete crystallization can lead to degradation during storage (cf. Carpenter et al., 1997). The Karl Fischer apparatus allows one to measure the amount of water in the final cake. Typically, moisture contents need to be at 1–2 % in order for the product to have the optimal glass transition temperature (T_g) and maximal stability. Finally, DSC is required to measure the T_g of the final formulation. The storage temperature must below the T_g. As the minimal acceptable storage temperature for lyophilized formulations is room temperature, a minimum T_g would be about 40° C. In addition to these instruments, access to a microscope equipped with a lyophilization stage is valuable.

The issue of how to develop formulations with limited amounts of material is a critical concern. One can imagine adapting a number of existing

technologies to assist in assessing the viability of a given formulation while using very small amounts of material. These would include:

1. microtiter plate assays,
2. robotics to assist in sample preparation and analysis,
3. ELISA or other antibody-based recognition assays for intact drug,
4. multi-sample adaptations of existing analytical techniques (e.g., DSC or analytical ultracentrifuge)
5. nanoseparation methodology (the so-called "lab-on-a-chip" approach),
6. surface-immobilized analytical methods, such as the BIAcore.

Each of these methods could provide significant increases in the capability to monitor the stability of a protein in a microformulation (less than 10μg of active drug). For example, new chip technologies (either DNA- or protein-based) are becoming powerful tools that are increasingly used in all areas of drug discovery. They offer significant advantage over older technologies in terms of speed, sensitivity and cost. It is important to consider the use or implementation of these tools in formulation studies. One can envision protein chips doing for the study of proteins what DNA chips have done to the study of genes. That is, they can be used to identify critical parameters required for rapidly developing a stable formulation. Similarly, any of the other methods could be modified to assist the formulation scientist.

Finally, there has been a steady improvement in protein analysis tools such as mass spectrometry and more powerful and versatile biochemical techniques, which are not only more sensitive but offer the advantages of speed and low cost. So with the emerging technological advances in analytics and implementation of these enabling technologies in preformulation and formulation studies, one can envision a more time and resource efficient process for developing stable protein pharmaceuticals. Most of these technologies are being developed to meet the needs of efficient drug discovery research. The challenge here will be the speed at which the formulation scientists adapt these technologies into their programs. Unfortunately, these tools have not yet reached their maturity, so the implementation of micro-analytical methods for preformulation and formulation is crucial for developing HTF strategies for protein pharmaceuticals.

STABILITY PROTOCOLS

In addition to formal storage stability protocols that would be acceptable to regulatory agencies for real-time data, one must have standard approaches for assessing the possible long-term fate of the candidate formulations. Given the aggressive time line of the HTF strategy, one must rely, to some degree, upon accelerated storage studies to guide decision about suitable excipients. These

could be done as isothermal studies, storing the protein at room temperature and 40° C, for example. In certain cases, exposure to higher temperatures, such as 50° C or even 60° C, may be warranted. However, it is important to note that at higher temperatures, there is the distinct possibility that the rate-determining pathway may change compared to that at the projected storage conditions.

An alternative to isothermal testing that needs to be considered is the use of non-iosothermal stress testing. In this procedure, the sample is exposed to a linear increase in temperature over time (or approximately linear with respect to time). Samples are taken at set intervals and assayed for protein damage. Using an algorithm developed by Lee and Stavchansky (1998), it is possible to determine the Arrhenius parameters in a single experiment, which would otherwise require at least three separate isothermal studies. This approach has been demonstrated for a pentapeptide (Lee and Stavchansky, 1998), and should be extended to larger proteins in the future.

UNIFIED STRATEGY FOR HTF

The requirements for HTF can be summarized in four points. First, integration of speed, sensitivity and cost can be achieved by having in place good analytical methodologies for monitoring both chemical and physical instability mechanisms. These methods can serve as tools for elucidating the mechanism of instability of protein drugs and also as stability indicating assays during analysis of the final formulation. Second, identification of the instability mechanism of proteins can lend itself to developing a fast-track stable liquid protein formulation, although the disadvantages of addressing biocompatibility and transportation issues have to be addressed with such dosage forms. A third requirement for HTF is to have obviously a safe and a convenient dosage form for administration. Coupled with this criterion should be the requirement for manufacturing the formulation using standard processes, so that if needed the production can be out-sourced to a contract facility.

Finally, technological advances will be a major driver for HTF and in general biotechnology development. The ability to work at low microgram to milligram scale of protein is essential for the ability to start the process before the drug candidate achieves development status. In many cases, companies may initially neglect formulation and stability issues that will haunt them in later stages of development. The development of successful formulations is dependent upon the ability to study both the *in vitro* and *in vivo* characteristics of the protein drug, as well as its intended application in the clinic. Thus, it is essential for the formulation group to work closely with the discovery research, the

pharmacokinetics and the toxicology departments prior to the decision to move the drug into full-scale development. Resolving the difficulties in the early research and development stages will not only help in targeting the appropriate drug candidate for development, but also speed up the selection of a pharmaceutically acceptable formulation for clinical trials. What we propose is the involvement of the formulation group in a multi-disciplinary environment where interactions start early in the research environment. The appropriate lead candidates would be selected not only based on efficacy studies, but also on pre-formulation studies. We envision these studies to take one to three months to establish appropriate profiles for formulation development. As a result, the lead formulation can have the advantages of demonstrating efficacy and potential stability of the protein drug.

During the development process of a biotechnology drug we envision formulation to take typically three to six months. This period would encompass preformulation studies and evaluation of the formulation options determined by the planned clinical studies. Hence, protein formulation and purification can be developed concurrently using limited quantities of the product, and identification of the lead formulation could occur at the time of establishment of the purification process. What we propose is not a sequential development pathway for protein drugs, but a concurrent scenario where formulation design is initiated very early in the drug development pipeline. We are quite confident that the HTF can be coupled successfully with high throughput screening strategies of protein drugs and facilitate drug development within the inherent time and resource constraints of the pharmaceutical industry.

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