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Rational Design of Stable Protein Formulations

Theory and Practice

Edited by
John F. Carpenter
and
Mark C. Manning

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Edited by

John F. Carpenter

and

Mark C. Manning

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Denver, Colorado

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Rational Design of Stable Lyophilized Protein Formulations: Theory and Practice

John F. Carpenter[1,2], *Beyong S. Chang*[3], *William Garzon-Rodriguez*[1,2], *and Theodore W. Randolph*[1,4]

INTRODUCTION

For ease of preparation and cost containment by the manufacturer, and ease of handling by the end user, an aqueous therapeutic protein formulation usually is preferred. However, with many proteins it is not possible—especially considering the time constraints for product development—to develop sufficiently stable aqueous formulations. Unacceptable denaturation and aggregation can be induced readily by the numerous stresses to which a protein in aqueous solution is sensitive; e.g., heating, agitation, freezing, pH changes, and exposure to interfaces or denaturants (Arakawa et al., 1993; Cleland et al., 1993; Brange, 2000; Bummer

John F. Carpenter, William Garzon-Rodriguez, and Theodore W. Randdph • Center for Pharmaceutical Biotechnology. *John F. Carpenter, William Garzon-Rodriguez, and Theodore W. Randolph* • Department of Pharmaceutical Sciences, School of Pharmacy, University of Colorado Health Sciences Center, Denver, CO 80262. *Beyong S. Chang* • Amgen, Inc., Thousand Oaks, CA 91320. *Theodore W. Randolph* • Department of Chemical Engineering, University of Colorado, Boulder, CO 80309.

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and Koppenol, 2000). Furthermore, even under conditions that thermodynamically greatly favor the native state of proteins, aggregation can arise during months of storage in aqueous solution (e.g., Gu et al., 1991; Arakawa et al., 1993; Chen et al., 1994; Volkin and Middaugh, 1996; Chang et al., 1996a). In addition, several chemical degradation pathways (e.g., hydrolysis and deamidation) are mediated by water. In aqueous formulations, the rates of these and other (e.g., oxidation) chemical degradation reactions can be unacceptably rapid on the time scale of storage (e.g., 18–24 months) for pharmaceutical products (Manning et al., 1989; Cleland et al., 1993; Goolcharran et al., 2000; Bummer and Koppenol 2000).

In contrast, a properly lyophilized formulation can maintain adequate physical and chemical stability of the protein during shipping and long-term storage even at ambient temperatures. As will be outlined in this chapter, developing stable lyophilized protein formulations should be a rational, straightforward process, which for most proteins should be rapid. With liquid formulation development, it may only be possible to obtain adequate protein stability after lengthy studies. Furthermore, sometimes there are conflicting conditions (e.g., pH) needed to slow sufficiently multiple degradation pathways in aqueous solution. Considering these issues plus the fact that formulation scientists now have to deal with numerous proteins and/or variants of a given protein, lyophilization should be considered as a primary mode for product development. Only if a parallel effort to develop an aqueous formulation is successful, will a final lyophilized product not be needed.

Rapid formulation development has important financial ramifications. A drug product has a finite patent life, during which time the company has an exclusive market. Considering that even a moderately successful drug product has annual sales of hundreds of millions of dollars, potentially millions of dollars in sales are lost for each day of delay in bringing a product to market. Unfortunately there are often delays because the formulations designed during early stage development and clinical trials (e.g., frozen) were not adequate for the final product. With a rational approach to formulation development, pharmaceutical scientists and process engineers can minimize the risk of this problem and the time needed to obtain a successful, final formulation. The success of such efforts depends on frank, open communication between the groups involved. For example, it is critical that the formulation scientists learn from the process engineers the issues for large-scale lyophilization runs, which are usually conducted in units that do not have the capacity to match processing parameters obtained in a small-scale research lyophilizers.

Despite the best efforts of the scientists and engineers, all too often delays in formulation development arise because sufficient resources are not invested in product development. For example, sometimes, purchase of essential equipment

(e.g., a differential scanning calorimeter), which costs a minute fraction of a day's sale of product, is not allowed. To avoid unnecessary delays in product launch, which can have disastrous consequences for the company and for patients, it is essential that companies appreciate that product development is ultimately a key limiting factor in getting a therapeutic to market. Hence, development efforts need to be as well funded as the usually much more visible drug discovery research programs. If a product is not stable, it will not be marketed, no matter how dramatic an impact it can have on human health and the financial status of the company.

MINIMAL CRITERIA FOR A SUCCESSFUL LYOPHILIZED FORMULATION

Research over the past several years has demonstrated that five criteria define the minimal conditions necessary for a successful lyophilized protein formulation (Table 1).

The first four criteria can be met with use of the appropriate excipients and lyophilization cycle design. For information on the proper design of lyophilization cycles, the reader is directed to the numerous previous reviews in this area (Franks, 1990; Pikal, 1990; Nail and Gatlin, 1993; Gatlin and Nail, 1994; Carpenter and Chang, 1996; Rey and May, 1999; Cappola, 2000). For the current chapter we will only consider cycle design in terms of the interplay between formulation physical properties (e.g., collapse temperature) and process parameters (see below). The last criterion listed in Table 1 requires insight into the unique physicochemical properties of each therapeutic protein, which will be explained in more detail below. We will discuss in turn why each of these criteria is important. Then we will present an explanation of how to design rationally a formulation to meet these criteria.

Table 1.
Minimal Criteria for a Successful Lyophilized Protein Formulation

1. Protein unfolding during freezing and drying is inhibited.
2. The glass transition temperature of the product exceeds the planned storage temperature (e.g., $T_g > 30°C$).
3. The water content is relatively low (e.g., 1% by mass).
4. A strong, elegant cake structure is obtained (i.e., collapse and meltback are avoided).
5. Steps are taken to minimize specific routes of protein chemical degradation (e.g., product vials are sealed under nitrogen to reduce the rate of methionine oxidation).

Inhibition of Lyophilization-Induced Protein Unfolding

The stresses of freezing and drying cause protein unfolding, and the formulation must be designed to inhibit unfolding at each step (Prestrelski et al., 1993a,b; Carpenter et al., 1993; Prestrelski et al., 1995; Constantino et al., 1995, 1998; Griebenow and Klibanov, 1995; Allison et al., 1996, 1998, 1999, 2000; Chang et al., 1996b; Krielgaard et al., 1998a, 1999; Chen et al., 1999; Bell, 1999; Carrasquillo et al., 2000). Even if the formulation excipients and/or intrinsic thermodynamic stability of the protein prevent denaturation during freezing, unfolding can arise during subsequent drying (Carpenter et al., 1993; Prestrelski et al., 1993b; Allison et al., 1998; Carrasquillo et al., 2000). Conversely, once a protein unfolds during freezing, it will not regain native structure during dehydration.

For many proteins, unfolding during lyophilization leads to clinically unacceptable, non-native aggregates, even when samples are rehydrated immediately after lyophilization (Prestrelski et al., 1993a, 1995; Allison et al., 1996; Krielgaard et al., 1998a, 1999; Costantino et al., 1998). Aggregates are not necessarily formed during freezing and drying. Rather, during rehydration refolding of structurally perturbed protein molecules competes with formation of non-native protein aggregates (Prestrelski et al., 1993a). Aggregation can be minimized by including stabilizing excipients (e.g., sucrose or trehalose) in the formulation to inhibit lyophilization-induced unfolding (Prestrelski et al., 1993a, 1995; Allison et al., 1996; Krielgaard et al., 1998a, 1999; Costantino et al., 1998). Furthermore, fostering refolding during rehydration (e.g., with surfactants) can reduce aggregation (Chang et al., 1996c; Zhang et al., 1995, 1996).

In addition to minimizing protein aggregation during lyophilization/rehydration, maximizing retention of native protein structure in the dried solid is essential for optimizing long-term storage stability (Prestrelski et al., 1995; Chang et al., 1996b; Krielgaard et al., 1998a, 1999; Allison et al., 2000; Cleland et al., 2001). Both chemical and physical degradation in the dried solid can be accelerated if protein unfolding is not inhibited during lyophilization. With chemical degradation, a non-native structure may provide an environment conducive to covalent modification of one or more residues. For example, exposure of a methionine, which is normally buried deep in the interior of the native protein, on the surface of an unfolded dried protein may foster oxidation. Increased levels of aggregates noted after storage and rehydration of unfolded proteins could be due to formation of non-native intermolecular contacts within the dried solid, perturbation of refolding during rehydration because of chemical degradation, and/or other undefined processes.

Infrared spectroscopy has been used routinely to compare the secondary structures of a protein in lyophilized formulations to that of the native protein in aqueous solution (Prestrelski et al., 1993a,b; Prestrelski et al., 1995; Dong et al.,

1995; Constantino et al., 1995, 1998; Griebenow and Klibanov, 1995; Allison et al., 1996, 1998, 1999, 2000; Chang et al., 1996b; Krielgaard et al., 1998a, 1999; Carpenter et al., 1998; Chen et al., 1999; Carrasquillo et al., 2000). This method should be considered essential in the development of stable lyophilized formulations, because it allows one to assess rapidly the effectiveness of formulations at inhibiting protein unfolding. Technical details about how to employ infrared spectroscopy to design stable lyophilized protein formulations can be found in the papers cited above.

Storage at Temperatures Below Formulation Glass Transition Temperature

In the dried powder, the protein is a component of an amorphous phase that includes amorphous excipients and water. If this glassy matrix is held below its characteristic glass transition temperature (T_g), the rate of diffusion-controlled reactions, including protein unfolding/aggregation and chemical degradation, are greatly reduced, relative to rates noted at temperatures >T_g (Roy et al., 1991; Franks, 1990; Franks et al., 1991; Pikal, 1994, 1999). T_g can be determined with differential scanning calorimetry (DSC) or other thermal scanning methods (Nail and Gatlin, 1993; Chang and Randall, 1992; Craig and Royall, 1998; Verdonck et al., 1999).

Obtaining a formulation T_g in excess of the planned storage temperature (e.g., room temperature) is absolutely essential for optimal protein stability (e.g., Franks et al., 1991; Pikal, 1994, 1999; Carpenter and Chang, 1996; Duddu and Dal Monte, 1997). The T_g of a given amorphous phase is dependent on the T_g and mass percent of each component, including water (Angell, 1995; Franks et al., 1991; Levine and Slade, 1988, 1992; Pikal, 1994, 1999). Compared to excipients, dried proteins have relatively high T_g's (e.g., >150°C; Angell, 1995). Thus, with all other factors being held constant, the formulation T_g varies directly with the mass fraction of protein. However, care must be taken that the mass fraction of protein is not so high that there are not adequate levels of stabilizing excipients to prevent protein unfolding during lyophilization (Cleland et al., 2001; and see below).

Fortunately, sucrose and trehalose, which are the preferred excipients for inhibiting lyophilization-induced protein unfolding (see below), also provide a glassy matrix with acceptably high T_g values. For example, with water contents of 1% the T_g for pure sucrose and trehalose are about 100 and 65°C, respectively (Crowe et al., 1998).

It has now been documented with several proteins, that simply storing the formulation at temperature below T_g alone does not assure optimal stability. A

native protein structure is also required. For example, proteins lyophilized in dextran alone are usually unfolded, but in a glassy matrix with a relatively high T_g (e.g., > 75° C). Yet they still degrade at relatively rapid rates compared to those for native protein molecules lyophilized with either sucrose or trehalose (Kriel-gaard et al., 1998a, 1999; Lueckel et al., 1998; Allison et al., 2000; Yoshioka et al., 2000). On pharmaceutical time scales of several months of storage many degradative reactions are not coupled to the glass transition of a formulation. This is because on these times scales there is still significant molecular mobility, even at temperatures well below (e.g., more than 30° C) the T_g (Hancock et al., 1995; Duddu et al., 1997; Pikal, 1999; Yoshioka et al., 1999).

The Water Content is Relatively Low

Because of its very low T_g (−135°C), water is a potent plasticizer for glasses; increasing water content in the dried formulation will greatly reduce T_g. For example, increasing the water content of pure sucrose from 1 to about 3–4% (g H_2O/100 g dried powder) is sufficient to reduce the T_g to below room temperature (Crowe et al., 1998). It is critical to achieve a sufficiently low water level for a given formulation such that T_g exceeds the planned storage temperature. The lyophilization cycle dictates the initial water content (see reviews listed above). The most important parameter is the temperature for secondary drying, when the unfrozen water is desorbed (Pikal et al., 1990).

Water can also be transferred to the product from the vial stoppers during storage (Pikal and Shah, 1992; DeGrazio and Flynn, 1992; Hora and Wolfe, 1999). This effect can be dramatic. For example, let's consider a formulation containing 10 mg of dried protein, 40 mg of sucrose and initial water content of 1% by weight. The total amorphous fraction containing protein and sucrose has 0.50 mg of water. If 1.0 mg of water was transferred from the stopper to the product, the water content of this fraction would increase from 1% to 3.0%. This increase would be sufficient to lower the formulation T_g to below room temperature (Crowe et al., 1998). The risk of transfer of moisture from stoppers can be minimized by drying the stoppers before use, and, if acceptable for a given product, using stoppers coated with a material such as Teflon (see Hora and Wolfe, 1999).

A Strong, Elegant Cake Structure is Obtained

Often the most desired cake has strong, porous structure, without macroscopic collapse or meltback. This structure has a high surface area to volume

ratio, which aids in the rapid dissolution of product upon addition of water. A detailed account of how to obtain such a cake structure is beyond the scope of the current chapter, but is available in several previous reviews (see above). For the current purposes it is sufficient to focus on the impact of formulation composition on avoiding collapse or meltback. When a product is frozen, the protein and amorphous excipients (e.g., sucrose) are dispersed between ice crystals and any excipient used as a crystalline bulking agent (e.g., glycine). To obtain an appropriate cake structure during lyophilization, the product temperature during primary drying, when the water in ice is sublimed, must be below the characteristic collapse and eutectic melting temperatures of amorphous and crystalline solutes, respectively. Above the eutectic temperature, the melting of crystalline solutes leads to massive loss of porous structure and macroscopic dissolution of the frozen matrix into a "puddle". Above the collapse temperature, which closely coincides with the glass transition temperature (T_g') of the maximally freeze-concentrated amorphous phase, the amorphous phase cannot support its own weight. The result is also a loss of pore structure and a macroscopic shrinkage or collapse of the cake.

It should be noted that the T_g' thermal event, which can be measured with differential scanning calorimetry, is also referred to as a softening event (T_s), rather than the actual glass transition temperature of the freeze-concentrated amorphous phase (Shalaev and Franks, 1995). Whatever the exact nature of the thermal event, it can be detected with differential scanning calorimetry as a second order increase in the baseline of the thermogram, which usually occurs just prior to the onset of the endotherm for the melting of ice (e.g., Her and Nail, 1994). The transition can also be measured with electrothermal methods (Her et al., 1994).

Another powerful method, which is essential for rational development of lyophilized formulations, for determining collapse and eutectic melting temperatures is freeze-drying microscopy (e.g., Nail et al., 1994). With this approach, the formulation of interest is directly examined visually for its performance during a simulated freezing, annealing and drying cycle. All of the critical phase changes, including ice formation, solute crystallization, eutectic melting and collapse, can be detected easily, and the temperature of their occurrence can be measured accurately.

Formulation composition dictates collapse temperature. Each pure amorphous excipient has a characteristic T_g' and collapse temperature; the collapse temperature for the formulation is the mass averaged temperatures of all of the components in the amorphous phase. It is important to design a formulation with maximum collapse temperature, because the rate of drying is directly proportional to the sample temperature during lyophilization. To allow for a reasonable drying time the T_g' should not be lower than –40° C. The T_g' values for pure sucrose is –32° C, while that of pure trehalose is –30° C (Skrabanja et al., 1994; Chang and

Randall, 1992). In contrast, glucose, which should also be avoided because it is a reducing sugar, has a T_g' of –43° C. The collapse temperature of pure protein is about –10° C, which means increasing the protein:sugar mass ratio will increase collapse temperature. Finally, collapse temperature will be decreased if salts and excipients are not maximally crystallized. For example, glycine has a T_g' of –37° C, and its contribution to the amorphous phase can reduce collapse temperature to impractically low values (Carpenter and Chang, 1996). This problem can be avoided by using the appropriate annealing (see Carpenter and Chang, 1996) of the frozen product to maximize crystallization.

Meltback can be avoided by using crystalline solutes with relatively high eutectic melting temperatures. For example, eutectic melting temperatures for mannitol and glycine are –1 and –4° C, respectively. In contrast, additives such as calcium chloride have very low eutectic melting temperatures (e.g., –51° C for calcium chloride). If mannitol or glycine is used as a crystalline bulking agent, the T_g' of the amorphous excipient phase, which is lower that the eutectic melting temperatures of these excipients, will be the value that dictates the temperature of primary drying.

Collapse can also occur during secondary drying, when unfrozen water is desorbed, if the temperature is increased too rapidly. As water is removed from the amorphous phase the T_g of this phase increases. Thus, product temperature can be raised gradually and collapse can be avoided if product temperature at a given time point does not exceed the T_g. Directions for optimizing secondary drying can be found in previous reviews mentioned above.

Steps Taken to Minimize Specific Routes of Protein Chemical Degradation

It is essential that the major routes of chemical, as well as physical, degradation be characterized carefully for each protein, because all of the criteria listed above can be met and a protein might still be damaged during long term storage in the dried solid. For example, methionine oxidation is a common degradation pathway for therapeutic proteins (Manning et al., 1989; Ahern and Manning, 1992; Cleland et al., 1993; Goolcharran et al., 2000). Even in a formulation that prevents protein unfolding and has a T_g exceeding the storage temperature methionine oxidation can proceed at an unacceptably rapid rate. If a methionine residue is on the surface of the protein, maintaining native structure would not prevent this residue's exposure to reactive oxygen species in the dried solid. But why would the glassy matrix not sufficiently retard the mobility of the reactive species to prevent the reaction from occurring? On pharmaceutical time scales (i.e., of many months) relevant motion in a glass is not arrested unless the storage

temperature is about 50° C below the T_g (e.g., Hancock et al., 1995; Pikal, 1994; Pikal, 1999). Therefore, the mobility of a relatively small reactive oxygen species and the oxidation of methionine residues are probably not coupled to the glass transition of the formulation. An intriguing alternative, but not mutually exclusive, explanation has been suggested by Steve Prestrelski (personal communication). He proposes that reactive oxygen species accumulating during long-term storage might not be causing damage just in the dried solid. Rather upon rehydration oxygen radicals rapidly react with the protein. This is an important area for future research.

Fortunately, despite the rather complicated and poorly understood theoretical aspects of unacceptable methionine oxidation rates in dried protein formulations, from a practical viewpoint dealing with the problem is relatively straightforward. Methionine oxidation can be minimized by sealing vials under nitrogen and/or using formulation additives (e.g., free methionine) to compete with protein residues for reactive oxygen species. These approaches should be effective whether the oxidation of residues occurs in the dried solid, during rehydration or at both times.

RATIONAL DESIGN OF STABLE LYOPHIILIZED FORMULATIONS

Two critical practical issues need to be considered when choosing excipients for a therapeutic protein formulation. 1) From a financial standpoint, scientists should focus on using excipients and processing approaches that do not need to be licensed from a patent holder. Acceptable protein stability can be achieved readily using excipients and processing methods that are well known to someone, to use the legal term, "skilled in the art of protein formulation." Clearly, however, the specific applications of such compounds and processes to a given protein drug product and/or a class of proteins often can be patented. Such patents may be critical to the company developing that product.

2) From a regulatory standpoint (and financial perspective, also), the formulation scientist should choose from among excipients that are already used in approved parenteral products. For protein stabilizers, the best choices are the disaccharides, sucrose and trehalose. For bulking agents, the best choices are glycine, mannitol and hydroxyethyl starch. For surfactants, usually the Tweens (20, 40 or 80) are preferred.

Based on these practical concerns and the criteria for a successful lyophilized protein product (Table 1), prototypic rational formulations are presented in Table 2. If the formulations suggested are employed, and the criteria in Table 1 are met, then most likely the product will have acceptable stability.

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Table 2.
Example of Rational Lyophilized Protein Formulation

1. Buffer that does not acidify during freezing (e.g., Tris, histidine, citrate)
2. Specific ligands/pH that optimizes thermodynamic stability of protein
3. Trehalose or sucrose to inhibit protein unfolding and provide glassy matrix
4. Bulking agent (e.g., mannitol, glycine or hydroxyethyl starch)
5. Nonionic surfactant to reduce protein aggregation

It is important to emphasize that not all components listed in Table 2 may be needed for a given protein product. For example, it may not be necessary to include a nonionic surfactant to aid in reducing protein aggregation. If there is not clear evidence that a given component is beneficial for a formulation, then that component should not be included in the formulation. Also, some components that are used because of "tradition" and/or for purposes such as final formulation tonicity can cause great difficulties in lyophilization. The most common example is the use of NaCl as a tonicity modifier. During lyophilization, NaCl can greatly reduce the collapse temperature of a formulation, if a fraction of the salt does not crystallize (Her et al., 1994). Crystallization of NaCl during freezing and annealing can be inhibited by other excipients (e.g., bulking agents and stabilizing sugars). Because of the low collapse temperature, a low temperature cycle must be used, which increases production time and costs. Also, even if formulations with NaCl can be lyophilized successfully in small research lyophilizers, there is great risk that a large fraction of vials will collapse during large scale manufacturing runs. Thus, if at all possible, NaCl should not be used in lyophilized formulations. Alternative tonicity modifiers include mannitol and glycine, which can also serve as crystalline bulking agents.

Every protein and product has unique characteristics, some of which may cause difficulty in designing stable formulations. Thus, the suggestions in Table 1 should be considered only as a good starting point in formulation development. For the remainder of this chapter we will discuss the rationale for the choice of each of the formulation components, their mechanisms of action and other practical approaches that can be used to increase protein stability.

Choice of Buffer

In terms of protein stability, the main concern with choice of buffer is the potential of certain buffer salts to precipitate during freezing and cause large changes in pH. For example, crystallization of the dibasic form of sodium

phosphate results can result in a pH < 4 (van den Burg, 1959; van den Burg and Rose, 1959; Anchordoquy and Carpenter, 1996). Thus, whenever possible, sodium phosphate buffer should be avoided. Although somewhat obvious, it is important to realize that a sodium phosphate system will be present if one starts with potassium phosphate buffer salts and NaCl, as is the case with phosphate buffered saline. Alternative buffers that do not appear to have major pH changes during freezing include Tris and histidine (Anchordoquy and Carpenter, 1996).

A clear example of the detrimental effects of buffer acidification on the stability of a lyophilized protein formulation has been reported by Lam et al. (1996). These researchers documented that a succinate buffer with an initial pH of 5 exhibited a pH drop of 1–2 units during lyophilization. The result was a reduced stability of interferon-γ during storage of the lyophilized formulation, relative to that noted for formulations prepared with glycolate buffer, which does not undergo freezing-induced acidification.

Specific Ligands/pH that Optimizes Thermodynamic Stability of Protein

Often experience gained during purification, intermediate storage protocols and preformulation studies can provide great insight into specific solution conditions (e.g., pH, ionic strength, buffer type) that confer the greatest stability to a given protein. Before choosing the appropriate "general" stabilizers, which are effective at protecting most proteins, it is absolutely essential that the formulation be optimized for the specific factors that increase the physical and chemical stability of a given protein. For example, simply avoiding extremes in pH can drastically reduce the rate of deamidation (Manning et al., 1989; Goolcharran et al., 2000). Also, specific ligands that increase protein physical stability (e.g., by increasing the free energy of unfolding) should be investigated. The stabilizing effects of heparin and other polyanions on growth factors (e.g., Chen et al., 1994; Volkin and Middaugh, 1996) and calcium on DNase (Chen et al., 1999) provide good examples. As will be described below, increasing the free energy of unfolding directly correlates with increased resistance of proteins to denaturation during freezing. In addition, at least in the case of DNase, there was also dramatic increases in storage stability of the lyophilized protein in formulation containing calcium, which were not due to increased structural stabilization during lyophilization (Chen et al., 1999). The mechanism for this effect is unknown.

Moreover, sometimes protein unfolding during freeze-drying can be minimized by optimizing initial solution pH (e.g., Prestrelski et al., 1995). However, in other cases it has been found that initial solution pH does not impact the degree of unfolding arising during lyophilization (Costantino et al., 1995; Carrasquillo et al., 2000). For each protein it is necessary to investigate the effect of initial pH

(and potential pH changes during freezing) on structural retention during lyophilization. Infrared spectroscopy can be used to monitor secondary structure in the dried solid, and the resulting data can be used to choose the initial pH that results in the most native-like secondary structure in the dried solid.

Trehalose or Sucrose to Inhibit Protein Unfolding and Provide Glassy Matrix

In this section we will discuss the rationale for choice of stabilizing excipients. Then we will describe the mechanisms for protection of proteins by additives during freezing and drying. Mechanistic insight is important for a clear understanding of protein stabilization during lyophilization and for guiding critical practical choices, such as determining the level of disaccharide needed for optimal protein protection. Finally, we will address this and other practical issues in the use of stabilizing excipients to inhibit protein unfolding during freezing and drying.

Rationale for Choice of Stabilizing Sugar. Among the numerous compounds tested, it appears that the most effective stabilizers of proteins during lyophilization are disaccharides (reviewed in Carpenter et al., 1999). Trehalose and sucrose are the best choices for a stabilizing disaccharide for therapeutic proteins. Both sugars: 1) protect proteins during both freezing and dehydration; 2) are nonreducing; 3) tend to remain amorphous during lyophilization; and 4) have been used in approved parenteral therapeutic products. There are, however, some important differences in the physicochemical properties of these sugars. 1) Trehalose has a higher T_g at a given moisture content than sucrose and, thus, for formulations containing trehalose it may be easier to obtain an appropriate cake structure with an economical lyophilization cycle (Crowe et al., 1998). However, a skilled process engineer should be able to design economical, effective cycles for formulations containing either sugar. In addition, the condition of having a T_g greater than the product storage temperature will hold at higher residual water contents for trehalose. In products with a relatively high protein concentration, the protein could contribute to an increased T_g, which serves to minimize the advantages of trehalose. 2) Trehalose is more resistant than sucrose to acid hydrolysis. Hydrolysis of these disaccharides produces reducing sugars, which must be avoided. Usually this is not a problem, unless very low pH's (ca. < 4) are employed. It should be noted that acid catalyzed hydrolysis of sucrose can occur even in lyophilized solids (Shalaev et al., 2000), suggesting that buffer acidification during freezing (see below) could ultimately result in formation of reducing sugars and resulting damage to proteins in a lyophilized formulation. 3)

Sucrose appears to be somewhat more effective at inhibiting unfolding during lyophilization (Allison et al., 1999). This difference has been most obvious when there is a relatively high protein concentration and a need to employ a relatively high initial concentration of sugar. Evidence to date indicates that less effective stabilization by trehalose is due to the greater propensity of this sugar to phase separate from polymers (Isutzu et al., 1996) and proteins (S.D. Allison, T.W. Randolph, B.S. Chang and J.F. Carpenter, unpublished observation) during freezing and drying. Whether or not this is a problem with a given formulation cannot be predicted. Hence, the relative capacities of sucrose versus trehalose to inhibit lyophilization-induced unfolding of must be examined for each protein. The final choice of sugar for a given protein product should be based on a direct comparison of sucrose and trehalose as stabilizers during the lyophilization cycle and storage in the dried solid.

Reducing sugars, such as maltose or lactose, should not be used. These compounds may effectively inhibit protein unfolding during the lyophilization cycle, but during storage in the dried solid they can degrade proteins via the Maillard reaction between carbonyls of the sugar and free amino groups on the protein (Hageman, 1992; Li et al., 1996).

Mechanism for Freezing Protection. Many different compounds, such as sugars, polyols, certain amino acids, methylamines and salting-out salts, nonspecifically stabilize proteins during freezing or freeze-thawing ("cryoprotection"). The same compounds have also been shown in nonfrozen aqueous systems to increase protein thermodynamic stability (e.g., increase resistance to temperature- or chaotrope-induced unfolding). Protection of proteins by these compounds during freezing and freeze-thawing is due to the same universal thermodynamic mechanism that Timasheff and colleagues have defined for solute-induced protein stabilization in nonfrozen aqueous solution (reviewed in Carpenter and Crowe, 1988; Carpenter and Chang, 1996; Carpenter et al., 1999; Timasheff, 1998).

Usually relatively high concentrations (ca. > 0.3 M) of solute are needed to stabilize proteins because the interactions of the solute with the protein are relatively weak. Stabilizing solutes are excluded preferentially from the surface of the protein. This statement does not mean that no solute molecules bind to the surface of the protein, but rather that there is a lower concentration of solute in the immediate vicinity of the protein than in the bulk solution. The chemical potential of the protein is increased in the presence of a preferentially excluded solute, which is the basis for the effect of solute of protein thermodynamic stability. Considering a two-state model for protein folding ($N \leftarrow \rightarrow D$), the native state is favored thermodynamically (e.g., under physiological conditions) because it has a lower free energy than the denatured state. The key to increasing the thermodynamic stability of the native state is to increase the free energy barrier

between the native and denatured states. So how can increasing the chemical potential of the native state result in stabilization? The degree of preferential exclusion and concomitant increase in protein chemical potential correlates directly with the protein surface area. The denatured state has a much greater surface area than the native state. Thus, in the presence of a preferentially excluded solute, the magnitude for the increase in protein chemical potential will be much greater for the denatured than that for the native state; the native state will be stabilized.

This same mechanism applies to the inhibition of smaller scale structural expansions of the native state, which may be sufficient to promote irreversible protein aggregation, by compounds such as sucrose (e.g., Chang et al., 1996a; Kendrick et al., 1997, 1998; Kim et al., 2000). Many proteins are known to form non-native aggregates from species with conformations that are not greatly different from the most compact native state. One example is the molten globule that has perturbed tertiary structure, but native secondary structure. However, even expanded species within that native state ensemble can participate in intermolecular interactions leading to non-native protein aggregates (e.g., Chang et al., 1996a; Kendrick et al., 1997, 1998; Kim et al., 2000). Preferentially excluded solutes shift the equilibrium between protein species towards that with the lowest surface area, i.e., the most compact species in the native state ensemble. As a result aggregation is inhibited.

Timasheff's preferential interaction mechanism also explains the influence of solutes on the degree of assembly of multimeric proteins. Preferentially excluded solutes tend to induce polymerization and stabilize native oligomers since the formation of contact sites between constituent monomers serves to reduce the surface area of the protein exposed to the solvent. Polymerization reduces the thermodynamically unfavorable effect of preferential solute exclusion. In this case, the assembled protein is much more stable than the constituent monomer because of the direct solute effects, but also because of the increase in oligomer stability gained by intersubunit contacts (e.g., Neet and Timm, 1994). In one example of this effect during lyophilization, maintenance of the native tetramer during freezing has been shown to increase the resistance of lactate dehydrogenase to dissociation and inactivation during subsequent drying (Anchordoquy and Carpenter, 1996; Anchordoquy et al., 2001).

How do we know that the preferential exclusion mechanism is actually operative in the frozen state? It is not possible to measure directly the protein-solute interaction in the frozen state. However, the effects of solutes on protein chemical potential and the resulting protein stabilization can be inferred from a freezing study with hemoglobin, in which the protein was partitioned into separate polyethylene glycol-rich and dextran-rich solution phases (Heller et al., 1996). In a phase separated system, thermodynamic equilibrium requires that chemical potential of each component be equal in both phases. Hence, there should be

equivalent effects of the different solute conditions in the two phases on the increase in free energy of protein unfolding. In other words, the impact of preferentially excluded solutes on protein stability is equal in the two phases. As predicted from the preferential exclusion mechanism, the degree of structural protection of hemoglobin during freezing (as observed directly in the frozen state with infrared spectroscopy) was equivalent in both phases (Heller et al., 1996).

Mechanism for Inhibition of Dehydration-Induced Unfolding. The interaction of a protein's residues with water are intimately involved with the formation of the native, globular protein structure, and if the protein is dehydrated it will unfold. It has been documented by numerous studies that sucrose and trehalose prevent dehydration-induced unfolding by hydrogen bonding to the dried protein in place of the lost water (e.g., Carpenter and Crowe, 1989; Prestrelski et al., 1993a; Allison et al., 1999; Costantino et al., 1998; Wolkers et al., 1998; Tzannis and Prestrelski, 1999). This so called "water replacement mechanism" is supported by several different observations.

For example, with infrared spectroscopy, it has been found that the band at 1583 cm^{-1} in the spectrum for lysozyme, which is due to hydrogen bonding of water to carboxylate groups, is not present in the spectrum for the dried protein (Carpenter and Crowe, 1989; Remmele et al., 1997; Allison et al., 1999). When lysozyme is dried in the presence of trehalose or sucrose, the carboxylate band is retained in the dried sample, indicating that the sugar is hydrogen bonding in the place of water. Similar results have been obtained with α-lactalbumin and sucrose (Prestrelski et al., 1993a). The magnitude of the retention of the carboxylate band correlates directly with the level of trehalose or sucrose in the lyophilized formulation, as well as with the degree of inhibition of unfolding (Allison et al., 1999). These effects of sugars on proteins in the dried solid are not due to the presence of increased amounts of water in the formulations dried with sugars (Allison et al., 1999; Tzannis and Prestrelski, 1999). The level of water in formulations dried with the sugars is as low as that for the protein lyophilized from just buffer or water, and is so low that the hydration shell of the protein is essentially completely removed (Prestrelski et al., 1993a; Krielgaard et al., 1998a, 1999; Allison et al., 1999).

Furthermore, Farhat et al., (1998) used infrared spectroscopy to study dried gelatin-sugar mixtures and the hydration behaviors of the mixtures, and concluded that the sugars hydrogen bond to the dried protein. Costantino et al. (1997) studied water sorption of proteins lyophilized with sucrose or trehalose and found that the solid-state interactions between protein and sugar reduced the availability of water binding sites. Tzannis and Prestrelski (1999) found that water sorption behavior of spray-dried protein formulations containing stabilizing levels of sucrose indicated hydrogen bonding between dried protein and the sugar. However, if an excessively high sucrose:protein ratio was employed, due to

formation of protein- and sugar-rich phases, hydrogen bonding of sucrose to protein and resulting protein stabilization were reduced. Also, Suzuki et al. (1998; 1999) found that there was a high degree of stabilization of lactate dehydrogenase when sucrose remained amorphous and hydrogen bonded to the dried protein. Crystallization of sucrose abolished hydrogen bonding between the sugar and dried protein, as well as protein stabilization.

Further support for the water replacement mechanisms comes from two studies that compared the relative effectiveness of saccharides of increasing molecular weight for inhibiting lyophilization-induced unfolding. Tanaka and colleagues (1991) found that the capacity to protect catalase during freeze-drying was inversely related to saccharide size. They suggested that as the size of the saccharide increases, steric hindrance interferes with hydrogen bonding between the saccharide and the dried protein. In support of this contention, the carboxylate band is only minimally detectable in the infrared spectrum of lysozyme freeze-dried in the presence of glassy dextran and the protein is unfolded (Allison et al., 1999). With infrared spectroscopy, Prestrelski et al. (1995) found that as the molecular weight of a carbohydrate additive was increased the capacity to inhibit unfolding of interleukin-2 during lyophilization decreased, and the level of protein aggregation after rehydration increased. Also, it was clear that protection of the protein did not correlate directly with the formation of a glass (all samples were found to be amorphous) or with the glass transition temperature of the sample (the T_g increased as carbohydrate molecular weight increased). Rather, there was a negative correlation between stabilization and molecular weight, which is to be expected if protection during drying is due to the water replacement mechanism.

An alternative mechanism for stabilization of proteins during dehydration states that proteins are simply mechanically immobilized in a glassy, solid matrix during dehydration (e.g., Franks, 1991). The restriction of translational and relaxation processes is thought to inhibit protein unfolding, and spatial separation between protein molecules (i.e., "dilution" of protein molecules within the glassy matrix) is proposed to prevent aggregation. It is clear that protective additives must partition with the protein into the amorphous phase of the dried sample. If the compound crystallizes during lyophilization it does not inhibit protein unfolding (Carpenter et al., 1993; Izutsu et al., 1993; Krielgaard et al., 1999). Also, spatially separating protein molecules can help favor refolding over aggregation during rehydration (Allison et al., 1998, 2000). However, as evidenced by the failure of glassy dextran matrices to prevent unfolding (Prestrelski et al., 1995; Krielgaard et al., 1999; Allison et al., 1998, 1999, 2000), simply forming a glassy solid is not sufficient for inhibiting dehydration-induced unfolding.

Practical Considerations. The protein mass in a unit dose (or multidose) vial of therapeutic protein is dictated by the amount to be given to the patient.

With a lyophilized product, which can be reconstituted to a volume different from the prelyophilization volume, the initial protein concentration can be varied without changing the total protein mass. Changes in initial protein concentration can affect relative loss of protein due to interaction with vial surfaces (e.g., Page et al., 2000), protein stability during processing, the collapse temperature of the formulation, the T_g of the final dried formulation and the lyophilization process itself. Thus, protein concentration can be an important variable to investigate during formulation development.

Increasing initial protein concentration leads to apparent increased resistance to denaturation during freezing (Strambini and Gabellieri, 1996; Chang et al., 1996c; Krielgaard et al., 1998b). This phenomenon can be demonstrated by determining the percentage protein aggregated after freeze-thawing, which varies inversely with protein concentration. Increasing protein concentration can directly reduce freezing-induced protein unfolding because one component of protein damage during freezing appears to involve protein denaturation during formation of the ice-water interface (Strambini and Gabellieri, 1996; Chang et al., 1996c; Krielgaard et al., 1998b). Assuming that only a finite number of protein molecules can be unfolded per unit area at this interface (Krielgaard et al., 1998b), increasing the initial protein concentration will lead to a smaller percentage of damaged molecules. Of course, other factors such as direct cold denaturation and freeze concentration of salts may predominate over surface denaturation during freezing. For practical purposes, it is not necessary to sort out the relative contribution of the various stresses to freezing-induced damage. Rather, is important to include realative high protein concentrations during early formulation development in order to increase the "intrinsic" resistance of the protein to denaturation. The greater the intrinsic stability of the protein, the lower the concentration of excipient that will be needed.

Another advantage of employing a relatively high initial protein concentration is that increasing protein : disaccharide mass ratio increases both formulation collapse temperature and T_g' of the final dried product. In addition, the formulation volume will be reduced, which will reduce the duration and costs of lyophilization.

Of course, if the ratio of excipient to protein is not sufficiently high, there will not be adequate stabilization of the protein during freezing and dehydration. Freezing protection depends on the initial bulk concentration of the sugar, and if the given protein is freeze-labile sometimes concentrations exceeding 5% (wt/vol) are needed to maximize stabilization. However, often the stability of the protein is sufficient during freezing that the level of sugar needed for optimal protection during lyophilization is dictated by that required for inhibition of unfolding during dehydration. Protection during drying depends on the final mass ratio between the sugar and the protein (Pikal, 1994; Carpenter and Chang, 1996;

Cleland et al., 2001). Recently it has been demonstrated that the sugar:protein mass ratio predicts not only degree of native structural retention during lyophilization, but also the long-term storage stability of a lyophilized protein (Cleland et al., 2001). Generally, a weight ratio of sugar to protein of at least 1:1 is required for inhibiting lyophilization-induced unfolding, with optimal stability being reached at around 3–5:1. In practice, with the protein concentration held constant, a range of sugar concentrations can be tested during formulation screening to discern the optimal concentration needed for retention of native protein in the dried solid. In general, the optimal sugar concentration for stabilizing the protein during lyophilization will also provide storage stability, if the final dried powder has a T_g well above the storage temperature. Finally, it should be stressed that the minimal effective level of sugar should be employed, because of the effect of changing protein:excipient ratio on glass transition temperatures. Also, if excess levels of sugar are employed there is an increased risk of sugar crystallization if a product should happen to be held at temperatures near or above the formulation T_g (e.g., Krielgaard et al., 1999).

Bulking Agent (e.g., Mannitol, Glycine or Hydroxyethyl Starch)

If the product has a relatively low mass of protein per vial, often it will necessary to have a bulking agent in the formulation to prevent the protein from being lost from the vial during drying and to form the product cake. Mannitol and glycine are examples of bulking agents, which can also serve as tonicity modifiers, that usually crystallize to a substantial degree during lyophilization (Pikal, 1994; Carpenter and Chang, 1996). A disaccharide protein stabilizer will need to be used in combination with these bulking agents. One drawback of mannitol and glycine is that often an annealing step is required to assure maximum crystallization. If a fraction of the bulking agent remains amorphous during lyophilization, there may be problems with obtaining a suitable cake structure, as well as a risk of excipient crystallization during subsequent storage in the dried solid (e.g., Carpenter and Chang, 1996; Carpenter et al., 1997). An alternative, amophous bulking agent is the polymer hydroxyethyl starch, which is used clinically as a plasma volume expander. Its main advantages in lyophilization are that it has a high collapse temperature (ca. −10°C), it forms strong cakes and it has a relatively high T_g for a given moisture content (e.g., >200°C at 2% residual moisture). However, as is the case with dextran, the large polymer hydroxyethyl starch does not inhibit protein unfolding during dehydration, and may actually foster additional protein unfolding due to phase separation from the protein during freezing and drying. Again, stabilizing disaccharides must be used with hydroxyethyl starch to inhibit lyophilization-induced protein unfolding.

Nonionic Surfactant to Inhibit Aggregation

Surfactants are often included in protein formulations to minimize interfacial denaturation and aggregation, e.g., at vial surfaces, due to bubble entrainment during filling or because of agitation (Chang et al., 1996c; Krielgaard et al., 1998b; Page et al., 2000). For a lyophilized product, a surfactant can be beneficial to minimize protein aggregation during vial filling, freezing and rehydration. Generally, a surfactant will not inhibit protein unfolding during dehydration (Krielgaard et al., 1998a). The mechanisms by which surfactants inhibit protein aggregation will be described in a separate chapter in this volume. For the current discussion it is sufficient to stress that a surfactant should not be included in a lyophilized product, unless there is direct evidence that increases recovery of native protein in the rehydrated sample. Surfactants can decrease the free energy of unfolding of some proteins, which may cause the compound to actually foster aggregation. Also, they have been shown to inhibit the assembly of small soluble aggregates into higher order soluble oligomers and insoluble aggregates (e.g., Krielgaard et al., 1998b). This effect can manifest itself during lyophilization and rehydration. As a result, the presence of a surfactant can cause an undesirable increase in the level of soluble aggregates.

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8

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

Rajiv Nayar • HTD Biosystems, Hercules, CA. *Mark C. Manning* • Center for Pharmaceutical Biotechnology, University of Colorado Health, Sciences Center, Denver, CO 80262.

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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 U spectroscopies can be used to study tertiary structure.

The second category of required analytical methods are those that monitc retention of native primary structure, namely, chromatographic methods, suc as reversed phase HPLC and ion exchange HPLC, or alternative methods lik capillary electrophoresis. Among the most useful applications of these HPL methods is the generation of a "tryptic map", which is accomplished by digest ing the protein with a sequence-specific enzyme (e.g., trypsin) and separating th resulting peptide fragments by reversed phase HPLC. The pattern then become a sensitive fingerprint of the protein. Any chemical modification of the protei 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 modificatio leading to the peak shift.

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

For lyophilized formulations, a certain set of equipment is considered to b the minimum required for conducting formulation development properly. Thes include an infrared (IR) spectrometer, a Karl Fischer titrator, a differential scan ning calorimeter (DSC), and x-ray diffractometer. The IR instrument will allo 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 structur composition in the original solution (prior to lyophilization), in the frozen soli and in the reconstituted product (Carpenter et al., 1998). X-ray diffraction c solids allows one to determine the crystallinity of a solid sample. In formulation where the bulking agent is crystallizing during the lyophilization process, on needs to know whether the crystallization is complete. Incomplete crystallizatio can lead to degradation during storage (cf. Carpenter et al., 1997). The Ka Fischer apparatus allows one to measure the amount of water in the final cak Typically, moisture contents need to be at 1–2 % in order for the product to hav the optimal glass transition temperature (T_g) and maximal stability. Finally, DS is required to measure the T_g of the final formulation. The storage temperatur must below the T_g. As the minimal acceptable storage temperature for lyophilize formulations is room temperature, a minimum T_g would be about 40° C. In add tion to these instruments, access to a microscope equipped with a lyophilizatio stage is valuable.

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

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 10 μg 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 temperatu and 40° C, for example. In certain cases, exposure to higher temperature such as 50° C or even 60° C, may be warranted. However, it is important to no that at higher temperatures, there is the distinct possibility that the rat determining pathway may change compared to that at the projected stora conditions.

An alternative to isothermal testing that needs to be considered is the use non-iosothermal stress testing. In this procedure, the sample is exposed to a line 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 alg rithm developed by Lee and Stavchansky (1998), it is possible to determine t Arrhenius parameters in a single experiment, which would otherwise require least three separate isothermal studies. This approach has been demonstrated f a pentapeptide (Lee and Stavchansky, 1998), and should be extended to larg proteins in the future.

UNIFIED STRATEGY FOR HTF

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

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

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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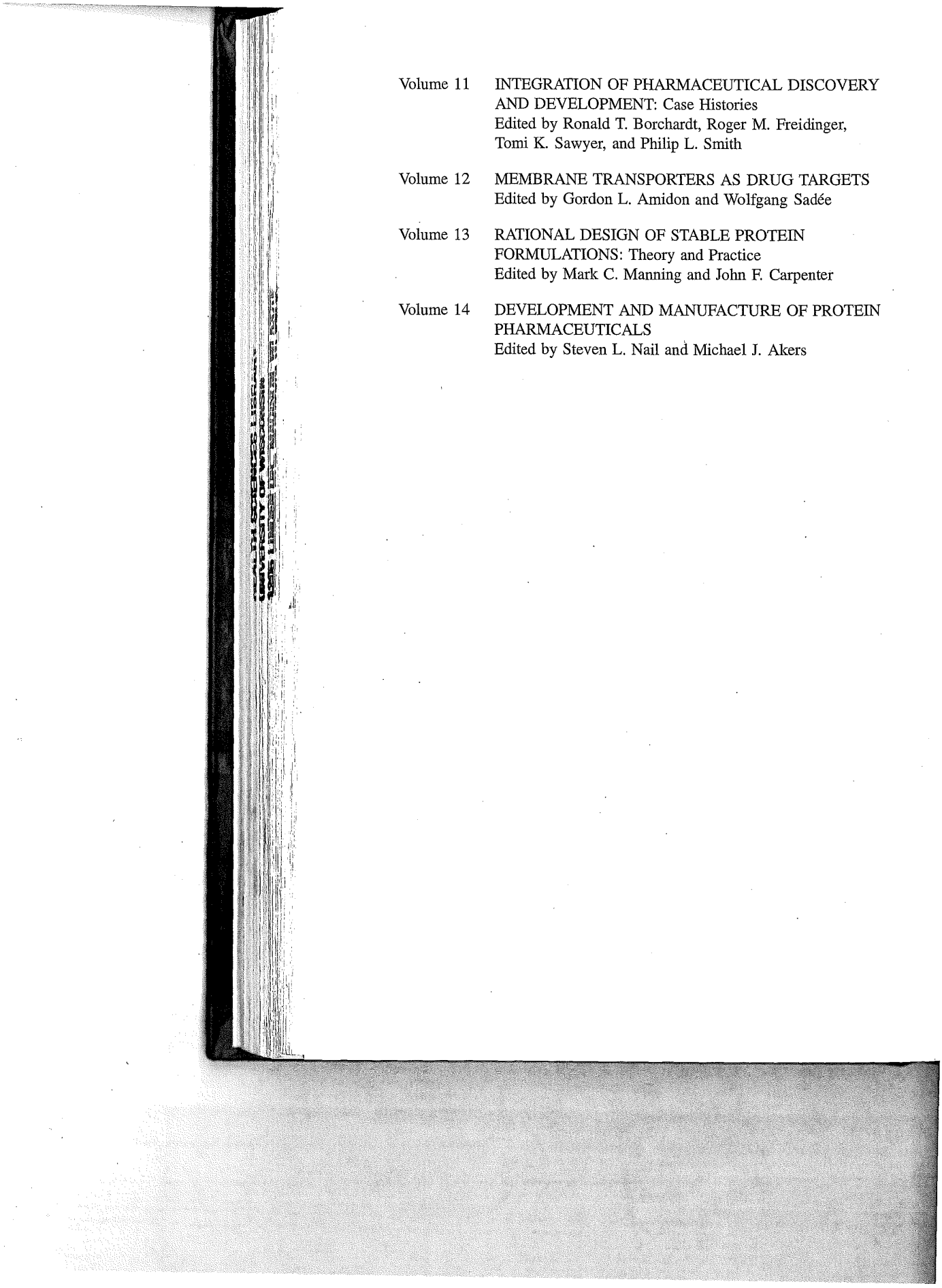

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