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Review

Moisture content in proteins: its effects and measurement

John K. Towns

Biopharmaceutical Analytical Development, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN 46285, USA

Abstract

Residual moisture content has a significant impact on the solid-state stability of biopharmaceutical products. Protein degradation due to residual moisture is minimal at or below the monolayer level of hydration owing to low availability of water and limited dynamic activity of the protein. However, residual moisture content beyond a monolayer generally results in increased rates of decomposition due to the enhanced conformational flexibility of the protein and the ability of the less tightly bound water to mobilize reactants. In addition to moisture content, the temperature and the composition of the lyophilized plug are important variables dictating the stability of proteins in the amorphous solid state. Water can act as a plasticizer to reduce the glass transition temperature, T_g, of the amorphous polymer, thus an increase in temperature or plasticizer level can result in a phase transition from a dynamically constrained state to a dynamically relaxed state. The selection of excipients can have a large impact on water–protein interactions as small ions and/or crystallization of excipients can redistribute water available to the protein. Owing to the key role that water content plays in the conformational and/or chemical state of the protein, an accurate and precise moisture determination is essential in resolving stability issues. A wide variety of techniques for the determination of moisture content have been utilized, with special attention being placed on sample handling to minimize atmospheric moisture contamination.

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1. Introduction

Recombinant DNA techniques have resulted in great advances in biotechnology, making it possible to produce a variety of proteins for biopharmaceutical use. Many proteins, however, have poor stability in aqueous solution and the freezing of the solution or the addition of preservatives fails to provide the required product stability [1]. To prevent excessive loss of potency or excessive increase in the level of decomposition, pharmaceutical protein products are often freeze-dried (lyophilized). Freeze-drying, however, is still usually not sufficient, as many products must be refrigerated at 5°C to provide reasonable shelf-life (1–2 years).

The process of lyophilization removes water from a system based on the principle of ice sublimation at reduced pressure. When a protein solution is lyophilized, the bulk water that resides in ice matrices of the frozen solution sublimes first. The multilayer water surrounding the protein molecule is then removed, leaving a residual monolayer of water on the surface of the protein. This operation allows drying of heat-labile materials to low residual moisture content under moderate temperature conditions. If the product is intended for parenteral use, then the protein is usually lyophilized in a final container such as a flame-sealed glass ampoule or a glass container with rubber closure that is usually sealed under vacuum or nitrogen. The water content of the freeze-dried material in the final container may vary depending on the freeze-drying process and may increase during storage [2].

The amount of water present in the protein has a significant impact on stability and is a concern for both bulk solid and lyophilized formulations. Residual moisture refers to the low level of surface water, ranging from less than 1% to 5%, remaining in a freeze-dried biological product after the bulk of the aqueous solvent has been removed [3]. Residual moisture should not compromise the potency and integrity of the product. The appropriate level of residual moisture to optimize stability is largely dependent on the particular protein's decomposition pathway. The generally accepted view is the drier the better. However, levels of residual moisture for certain products should not be so low that overdrying adversely affects product stability. Water retention varies with the type of water present, bound, surface and/or trapped, and is different for each product. Because more than one type of water may exist in a freeze-dried biological product, different moisture results may be found when different methods are employed in determining the moisture content of the sample [3].

Regulations of the Center for Biologics Evaluation and Research (CBER) require that each lot of freeze-dried biological product be tested for residual moisture [4]. The regulations pertaining to residual moisture are published in Title 21 of the Code of Federal Regulations for Food and Drugs and require that moisture levels meet and not exceed established limits as specified by an approved method on file in the product license application [5]. Guidelines for the determination of residual moisture in dried biological products have been issued under 21 CFR 10.90 to describe residual moisture test methods and procedures used to set product residual moisture limits [6]. For most products, levels of residual moisture should range from less than 1.0% to 3.0% so that the chemical and/or conformational stability, and therefore potency of the product, are not compromised over time.

This paper addresses the importance of residual moisture on protein stability and the need to control and measure water content accurately. An overview of the binding of water and its effects on protein stability is included. Examples illustrate how the amount of water present in the lyophilized plug can have a significant impact on solid state stability. Many of the examples emphasize that a specific degradation pathway is contingent on the increased flexibility of the protein to expose amino acid residues to the surrounding environment. The methods employed in the measurement of residual moisture content and the importance of controlling atmospheric moisture contamination are discussed.

2. Water sorption and protein mobility

The importance of water sorption on the solid-state stability of proteins can be addressed through an understanding of the binding of the sorbed water. The binding of water to proteins is a result of numerous molecular and intermolecular interactions due to hydrophilic, hydrophobic and ionized groups on the protein. The water content is determined following equilibration at a given relative humidity. A sorption isotherm is generated by assessing equilibrium moisture content at varying relative humidities. The sorption isotherm for proteins can be roughly separated into three regions [7]. The first region is binding of water to highly active sites such as charged and highly polar groups. The second region is a transition region from monolayer to multilayer coverage. It occurs with the binding of water to weaker sorption sites such as the peptide backbone and polar surface groups. Additional water binding occurs via clustering at or near charged and highly polar groups and through filling of voids created by swelling of the polymer. The last region, or multilayer region, occurs with condensation of water at very weak binding sites and layering of loosely held water [8]. It is in this region, hydration of 30–40%, that the true monolayer coverage of a protein actually occurs [9].

The behavior of a system can be predicted using isotherms and existing mathematical models to identify several areas of the isotherms that represent distinctly different characteristics of the solid. The uptake of water by proteins occurs with penetration into the disordered structure of the solid and is not limited to surface adsorption [10]. At levels of water below the Brunauer–Emmet–Teller (BET) monolayer level, generally 5–9% water content, the mobility of the absorbed water and flexibility of the protein are limited [8]. At or near this level of hydration, there is some indication that secondary relaxation of the amorphous system may occur and many decomposition pathways become observable over a reasonable time frame. The dynamic mobility of the system then slowly increases with increasing hydration. The dynamic flexibility of a protein in the solid state is dependent on interrelated variables of temperature, hydration and composition of the amorphous protein.

The adsorbed water may act as a plasticizer, resulting in an increase in free volume and greater macroscopic mobility [11]. The outcome of this plasticization is a lowering of the glass transition temperature, T_g, below the temperature of the surroundings. The phase transition from the dynamically constrained "glassy" state to the dynamically relaxed viscoelastic state is possible when the temperature is raised above T_g [12]. This phase transition can also occur when sufficient water has been absorbed in the amorphous solid to lower T_g below the temperature of the surroundings.

Reactions dependent upon the mobility of the water and/or the protein are therefore greatly enhanced at temperatures above T_g [13]. Increases in both the mobility of the water and protein as a result of the plasticizing effect of water are generally related to the monolayer moisture content. An increase in plasticization of the protein due to water sorption has been observed to result in a rapidly decreasing T_g with increasing moisture up to the BET monolayer region [14]. The onset of internal protein flexibility correlates reasonably well with the attainment of the BET monolayer level of water [9]. Significant increases in internal motions of the protein begin at hydration levels slightly greater than the BET monolayer. For proteins, the monolayer approximates the amount of water vapor necessary to cover the highly active heterogeneous sorption sites. Reasonable estimates of the BET monolayer were found for methionyl human growth hormone (met-hGH) and rt-PA by considering one water molecule per polar group [1].

It is a concern that the BET equation may not be adequate for determining the monolayer water content in proteins, owing to the unequal affinity of water associated with the weak and strong binding sites [8]. The chemical composition of the protein may dictate the level of residual moisture adsorbed on to the surface of the protein. As an example, met-hGH BET

monolayer results are in good agreement with the theoretical calculation employing only strong polar groups [1]. It is likely that the weakly polar and non-polar groups do not contribute significantly to the formation of the monolayer adsorbed on the strong polar groups of met-hGH.

3. Water–protein interactions

Water in the monolayer is thought to have low thermodynamic activity owing to its strong interaction with the protein molecule. This is in contrast to water in the multilayer that is thought to retain more free water activity depending on its distance from the surface of the protein. It is not the absolute residual water content that is important for maintaining protein stability, but rather the way in which residual moisture is adsorbed and how it is available, i.e., its water activity. The hydration of proteins results from coulombic, hydrogen bonding, Van der Waals and hydrophobic interactions between water and specific functional groups of the protein [8]. The early stage of hydration involves water adsorption and its predominant interaction with charged groups. The strength of these interactions can vary significantly as the amino acid residues provides large differences in ionic, polar and non-polar sites. These interactions are further complicated by neighboring amino acids in the folded protein. Unless all of the decomposition pathways are similarly affected by moisture, the sensitivity of long-term stability will be dependent on the sensitivity of the relevant pathway to increased moisture content levels.

At high residual moisture content, the possibility of chemical reactions occurring (cleavage, oxidation, deamidation, denaturation, aggregation, etc.) is increased. This is due to conformational flexibility of the protein molecule, the availability of water for hydrolytic reactions and the increased mobility of reactants. Owing to high protein mobility and flexibility, the protein backbone segments and/or amino acids functional groups can have appropriate orientation and energy to participate in chemical reactions. When the moisture content is decreased, the probability of reactions occurring is consequently reduced. The appropriate level of moisture to optimize stability varies from product to product and is dependent on the protein and its particular degradation pathway(s) [1,15–23]. Protein reactions can be minimized by reducing the moisture content to the monolayer water level or less. However, an optimum moisture content is necessary to maintain protein activity during storage. Overdrying will remove water from the monolayer, leading to increased exposure of the protein surface and to various unwanted reactions. Too low residual moisture levels may cause aggregation, loss of activity and/or inadequate reconstitution.

The effect of water content and/or water activity on the solid-state stability of proteins results from either (1) changes in dynamic activity or conformational stability of the protein or (2) participation of water as a reactant or medium for mobilization of reactants. Studies on the hydration of proteins have identified several critical levels of hydration at which significant changes in properties of the protein and the bound water occur [9]. Most decomposition reactions are minimal at or below the monolayer level of hydration owing to the low availability of the water and limited dynamic activity of the protein. Residual water in excess of monolayer coverage increases molecular mobility in the solid protein, thereby increasing general reactivity, resulting in increased rates of decomposition.

Dynamic motions within proteins impose a significant degree of flexibility in the conformational structure. The impact of water on the conformational stability of proteins depends on its flexibility and the ease of conformation structure variations. The outcome of such unfolding can be irreversible physical aggregation induced by exposure of hydrophobic amino acid residues [2,16]. Increases in this flexibility are reasonably well correlated with decreases in conformational stability. Decomposition pathways related to covalent bond formation or cleavage are also expected to require flexibility, of at least a localized segmental motion, in order for orientational constraints to be overcome and chemical reactions to occur.

A high water content may decrease the protein stability in freeze-dried solids via several mechanisms. Chemical modification generally results in changes to the primary sequence and may or may not have a subsequent effect on conformational structure. Chemical modifications can result from both intra- and inter-molecular reactions or involve reactions with other components of a heterogeneous system. These increased rates are primarily due to the increased conformational flexibility of the protein and the ability of the less tightly bound water to mobilize reactants. This is observed in studies of the Maillard reaction, where reaction rates are increased due to increased reactant mobilization [24]. The reaction is initiated by Schiff base formation between amino nucleophiles of the protein, such as lysine, and the carbonyl groups of the reducing sugars. This leads to the "browning reaction" as the Schiff bases undergo further rearrangement with degradation to unsaturated carbonyls and eventually polymerization. At water levels below the BET monolayer level, the reaction is minimal owing to the low mobility of reactant and the limited mobility of the protein side-chains containing nucleophilic species. At water levels above the BET monolayer level, water is sufficiently mobile to solubilize reactants, that has been determined to correlate directly with the observation of the browning reaction [16].

The increased flexibility of the protein in the solid state due to the presence of water can lead to both reducible and non-reducible cross-linking reactions. Rates of reducible cross-linking of proteins containing free sulfhydryls are increased with increasing water content [25]. The pH of the solution to be freeze-dried is an important point to consider with this reaction, as an increase in the rate of cross-linking is observed with an increase in pH. Rates of non-reducible cross-linking reactions have been observed in the covalent dimerization of somatotropins at high water content [18]. Initial zero-order rates for covalent dimerization of bovine somatotropin in lyophilized formulations stored at 47°C were impacted by residual moisture. Levels of residual water of 1–5% resulted in significant increases in the amount of rBST lost due to non-reducible dimerization as compared with water levels below the BET monolayer level.

Enzymatic reactions are also impacted by the increased flexibility of the protein and the mobilization of reactants in the solid state. Self-proteolysis, however, is of minimal concern when formulating proteases, because the intermolecular reactions require a significant mobility to the segmental portions of the protein backbone [9]. Water is a reactant in the deamidation reaction, and high levels of water should increase the rate of deamidation [10]. Rates of acid-catalyzed [26] and base-catalyzed [17,27,28] deamidation reactions are impacted by residual moisture in the solid state. These reactions require either an increase in the mobility of the side-chain (acid-catalyzed) or a large change in the flexibility of the protein segment(base-catalyzed).

The effect of water on oxidation reactions is varied; it can have both anti- and pro-oxidant effects depending on the system. Generally, water acts as an antioxidant to facilitate the recombination of free radicals [29]. Free radicals are stable at water levels below the monolayer, but decay rapidly through recombination as the water content increases. This can result in an increase in oxidation reactions even though the moisture content is below the BET monolayer. At water levels exceeding the monolayer, the pro-oxidant effects of water become important as it mobilizes catalysts and increases protein flexibility, resulting in increased exposure of the reactions sites.

4. Examples of protein stability

Human growth hormone (hGH) is a good example of a protein that may be freeze-dried without significant degradation, but where the resulting lyophilized protein is potentially unstable [2,17]. Chemical decomposition via oxidation of methionine residues and deamidation of asparagine residues occurs in the solid state of hGH, in addition to aggregation to dimer and high order aggregates.

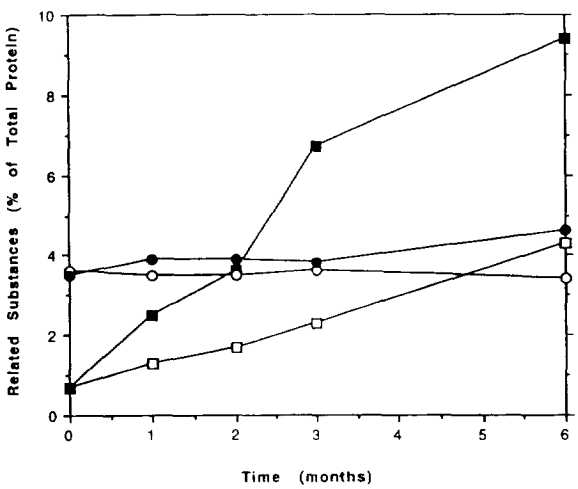

Fig. 1. Percentage of total protein for two major related products of hGH formulation stored for 6 months at 25°C. Squares represent the oxidation product (Met-14). Circles represent the deamidation product (Asn-149). Closed squares and circles represent the high-moisture content (3%) lot and open squares and circles represent the low-moisture content (1%) lot.

Fig. 1 shows the increase (as a percentage of total protein) of the two major degradation products of an hGH formulation. Two lots containing different amounts of moisture content were stored for 6 months at 25°C. The "high" and "low" lots contain approximately 3% and 1% moisture content, respectively, as determined by Karl Fischer titration measurements. The formulation contains a glycine–mannitol excipient system that offers optimum stability at low moisture levels when stored in a nitrogen headspace [2]. In the lyophilized plug, mannitol is thought to be crystalline, with glycine remaining amorphous [17]. The major oxidation product (at Met-14) and the major deamidation product (at Asn-149) have initial amounts of approximately 0.7% and 3.5%, respectively. Over the 6 months at 25°C, an increase is observed in the oxidation product for both lots, with a dramatic increase in the high-moisture lot. The deamidation product, on the other hand, showed little increase in degradation over the 6-month period for either lot. The large difference in degradation rates for the two amino acid residues points to the fact that the effect of moisture on protein stability is sensitive to the individual pathway. In this example, the oxidation of Met-14 is sensitive to moisture content, whereas deamidation at Asn-149 is unaffected.

A second oxidation product (Met-125) also shows an increase in oxidation for both the 1% and 3% moisture lots, with a much larger increase in the 3% moisture lot. The degradation, however, is much less than is observed for Met-14. The third methionine present in hGH(Met-171) shows no degradation over the 6-month storage period at 25°C. The difference in degradation among the three methionine groups is consistent with the observation that the extent of chemical degradation depends on the availability and sensitivity of individual amino acid residues to moisture content. Met-14 and Met-125 are located in relatively inflexible helices, leading to the conclusion that the mobility of the side-chain is only required for the oxidation reaction [8].

The dramatic increase observed in oxidation of Met-14 in the high-moisture lot is consistent with what would be expected at moisture levels above the BET monolayer moisture content. The monolayer water content for this hGH formulation has been previously determined to be 1.69% [17], and therefore the low- and high-moisture lots are on either side of the monolayer moisture content. Assuming that all the mannitol is crystalline, the water content of the amorphous phase at BET monolayer is 5.5%, which is consistent with the monolayer moisture content calculated for dry met-hGH(1). At 25°C, an increase in moisture content above the BET monolayer level showed a dramatic increase in the rate of chemical decomposition for Met-14, as opposed to moisture content below the monolayer level. This observation is consistent with the greater reactivity one would expect above the BET monolayer level due to increased protein flexibility and water mobility. In addition to chemical degradation, the extent of aggregation of freeze-dried human growth hormone after 10 days at 40°C has been found to be about five times greater at a moisture level of 3% than at less than 0.5% [1].

A separate oxidation pathway was observed in the increased level of hemoglobin to methemoglobin (oxidation of the heme group) in a freeze-dried sucrose-containing formulation as a result of an increase in residual moisture. The oxidation is twice as high for the formulation with 8% residual moisture as the same formulation containing 2% moisture content after 4 years of storage at room temperature [18]. In a separate study, the influence of hydration on the oxidation of lyophilized carbon monoxyhemoglobin to methemoglobin was determined [19]. The percentage of methemoglobin increased slightly over the moisture content range 0–12%. The rate of oxidation to methemoglobin then increased dramatically, reaching a maximum at approximately 18% water. The rate of oxidation at 18% moisture content is almost 35 times faster than in solution. Above 18% moisture content, the rate of oxidation decreases with increasing moisture content to the point where the oxidation rates are comparable to those obtained at very low water levels. In addition to oxidation, at water levels above 18% moisture content, the carbon monoxide ligand becomes replaced by the oxygen ligand. This oxygenation is possibly due to an increase in flexibility of the conformational structure, as the exchange is not observed below 12% moisture content. The oxygenation may prevent the oxidation of hemoglobin to methemoglobin, thus explaining the decreased rates of oxidation observed at high moisture content levels.

Maintaining low residual moisture is well illustrated for five rDNA cytokine preparations (three interleukins and two colony-stimulating factors) that were ampouled and lyophilized for use as internal standards [30]. The preparations were analyzed under accelerated storage conditions (56°C) in order to assess the procedure used to prepare cytokine standards and to assess the long-term stability of the preparations for use as standards. Each of the cytokines was ampouled in microgram amounts with milligram amounts of bulking agent such as albumin and carbohydrate. The moisture content after freeze-drying of a sample batch of any one cytokine preparation was between 1.1% and 0.64%. A low residual moisture content could be reproduced for the same or different cytokine preparations with a similar formulation. The cytokine preparations showed good retention of activity under the accelerated storage conditions as measured by flow cytometry. A sufficiently low moisture content was achieved such that the activity of the cytokines was not adversely affected by water-induced hydrolysis. The freeze-drying of cytokines under these conditions, for use as standards, enabled identical samples to be supplied without affecting the integrity or stability of the preparation.

Biological products are more likely to be adversely affected by over-drying than low-molecular mass drugs. Excessive drying may allow for the removal of structural water and disruption of the conformational integrity [31]. Studies to determine the optimum residual water level in formulations of tissue-type plasminogen activator have shown that the widely held view that the drier the better may not be appropriate for protein formulations [1]. The data suggest that the protein was mainly altered during the lyophilization process since subsequent degradation during storage was minimal. Drying the rt-PA protein to below the calculated monolayer water levels would appear to have an immediate and detrimental effect on the physical lots stability of the protein. Higher residual moisture lost more activity, suggesting that higher residual water levels permit more chemical degradation reactions to take place. The lower moisture content has an apparent higher level of aggregation that is largely due to the greater amount of aggregates initially generated during the lyophilization process. Results suggest that each protein may have a minimum moisture content that is necessary to shield the polar groups. This water may exist as clusters associated with the strong polar groups on the surface of the protein, rather than a continuous monolayer. Results of the stability on rt-PA show that it is desirable to achieve a balance between too little moisture (causing physical instability) and too much moisture (causing biological instability) [1].

5. Formulation and container considerations

Solid-state stability is especially complex in heterogeneous systems such as protein formulations, where the extent of product stability is sensitive to both formulation variables and the level of residual water in the dry solid. The residual water content varies with formulation largely because, while the percentage of water is based on the total sample, mass, the crystalline components do not retain significant amounts of water during freeze-drying, nor do crystalline components adsorb water from the stopper during storage. Formulations with excipients that adsorb very little moisture and contain a low ratio of protein to excipient are especially sensitive to small differences in water content. A small change in the moisture content of the lyophilized formulation can have a large effect on the amount of water associated with the protein and thus adversely affect stability. As an example, the greater stability of the hGH–mannitol–glycine formation described earlier is consistent with the concept that stability enhancement depends on the excipient system being at least partially amorphous to allow molecular interaction with the protein and/or to act as a "sink" for residual water [2]. The use of amorphous excipients provides more reproducible formulations from a stability standpoint, which is a necessity on the production scale owing to the difficulty in precisely controlling residual moisture levels.

In amorphous solids, the excipients can directly impact the stability by altering the effective protein concentration and pH. Adding excipients to the protein dilutes the effective concentration of the protein, increasing stability at high levels of hydration. The addition of buffer salts can also affect the stability of the protein. It is expected that low-molecular-mass species act as plasticizers to decrease the T_g of the amorphous systems and increase protein mobility. The addition of small ions can result in a lower moisture content needed to mobilize reactants. The ions preferentially bind to the proteins, thus excluding the water from the protein surface. This allows the water to be available to increase the mobility of the system again. The increasing availability of water as a medium for mobilization is also observed with the crystallization of excipients. This redistribution of water increases the moisture content in the remaining amorphous phase, leading to a decrease in stability through the lowering of the T_g.

In heterogeneous systems such as protein formulations, the rates of decomposition are influenced by additives, such as glycerol, propylene glycol or other polyhydric alcohols, which are commonly used in protein formulations as cryoprotectants during freezing and lyophilization. The presence of additives can increase water content at constant relative humidity or water activity. These additives act as plasticizers and the presence of such a liquid in the amorphous solid will decrease the water level necessary for reactant mobilization and protein flexibility. However, if the water content of the formulation is constant, the presence of these additives will decrease the water activity in the formulation. The presence of soluble salts will also increase water uptake at high relative humidities by dissolving in the loosely bound water and decreasing the vapor pressure, causing increased condensation. At low relative humidities, such electrolytes may actually decrease the water uptake by occupying proteins binding sites for water. Sugars such as trehalose and lactose can serve to satisfy partially the hydrogen-bonding requirements of the polar groups in dried proteins, and thus serve as water substitutes for dried proteins [31–33].

A low residual moisture content in the dried product upon freeze-drying is essential to maintain the stability of compounds. Low residual moisture after manufacture, however, does not ensure low moisture throughout the shelf-life of the product. The rubber stoppers used in the container hold a measurable amount of water that can transfer to the freeze-dried product, eventually coming to equilibrium with the water in the stopper [34,35]. The use of amorphous polymers protects the protein by serving as a moisture "sink" for the small amounts of mois-

ture that may transfer from the stopper. The use of amorphous excipients provides more reproducible formulations from a stability standpoint. During validation studies of each product and changes such as vial size, manufacturers should test several samples from several positions on each shelf in the freezer-dryer to determine that the lot meets the residual moisture specification of the product.

6. Moisture content determination

Owing to the key role water content plays in the solid-state stability of protein formulations, an accurate and precise moisture determination is essential in resolving stability issues. Accurate moisture determination will facilitate a more complete understanding of the role of moisture content on product stability and the setting of meaningful specifications and criteria for acceptable moisture content. Guidelines for the determination of residual moisture in dried biological products have been issued to describe residual moisture test methods and procedures used to set product residual moisture limits. These guidelines have been issued under 21 CFR 10.90 stating the principles and practices of general applicability. These are not legal requirements but can be relied upon by the user with the assurance of its acceptability to the FDA [6].

A number of chromatographic, spectroscopic, electrochemical, thermal and wet chemical methods have been used to determine moisture [6,36,37]. The most common of these are loss on drying (LOD), thermogravimetric analysis (TGA), gas chromatography using a thermal conductivity detector and the Karl Fischer titration. In addition to these methods where the sample is not recovered after analysis, the non-destructive technique of near-infrared spectroscopy has also been utilized [37]. In this technique a fiber-optic diffuse-reflectance probe measures reflectance through intact glass vials. These water content measurements, however, require that an accurate reference moisture value be obtained as a guide, with the Karl Fischer titration being the method of choice.

6.1. Gravimetric method (loss on drying)

The procedure for loss on drying involves dispensing the substance to be tested into a tared, glass-stoppered, shallow weighing bottle [38]. The optimum sample size is approximately 200 mg and may require that the contents from several final product containers be pooled. The particle size of the test substance may need to be reduced (down to 2 mm) to allow for proper drying if large crystals are present. The test specimen is prepared for analysis in a low-humidity glove-box and is weighed and evenly distributed along the bottom of the bottle for even drying. The test is performed in a temperature- and humidity-controlled environment to prevent ambient humidity from interfering in the test procedure. The unstoppered bottle and contents are then loaded in the drying chamber and the test specimen dried at a given temperature and time. The approved test method for residual moisture (Code of Federal Regulations, 21 CFR 610.13) in freeze-dried biological products measures the maximum loss in mass of a weighed sample equilibrated to constant mass over anhydrous phosphorus pentoxide at a pressure of not more than 1 mmHg and a temperature of 20–30°C for as long as it has been established is sufficient to results in a constant mass. After drying, the bottle is stoppered promptly and allowed to come to room temperature in a desiccator before weighing.

This method relies on the removal of water from the cake when exposed to heat in a vacuum oven. Measurements of the opened vial and cake are made before and after the addition of heat. The difference in mass is used to calculate the amount of water present. The gravimetric method measures surface moisture and loosely bound water of hydration [39]. Surface moisture is the classical definition of residual moisture. The major disadvantage of this method is that is very difficult to remove all the residual water, which may remain tightly bound to either the protein or the excipients used in the formulation buffer. Therefore, this method may underestimate the actual residual moisture level [40]. The general recommendation for most products is that the

residual moisture should not exceed 1.0% by the gravimetric method [3].

6.2. Thermogravimetric

In thermogravimetric analysis, the mass of a sample is recorded continuously as its temperature is increased linearly from ambient to as high as 1200°C [41]. This method can provide more useful information than LOD at a fixed temperature for a fixed time. The essential features of the equipment are a recording balance and programmable heat source. Variations on the equipment employed include the sensing of the specimen temperature, size of the sample holder and range of atmosphere control. In the determination of water content, the TGA will show a decrease in mass from ambient to 100°C and then plateau. The mass loss from the initial sample value to this plateau is due to the loss of water.

In determining the loss of mass upon drying, it is necessary that the method differentiates between measuring the "moisture" and not "total volatiles" content of the sample. Depending on the sample and test conditions, the amount of mass lost from the sample may include substances in addition to water (methanol, etc.). Care must be taken to choose the proper method to insure that the mass loss is due solely to moisture and does not include other species. The thermogravimetric method measures both surface and bound moisture in freeze-dried biological products. The TGA method can determine the moisture content in freeze-dried samples as small as two milligrams. CBER used TGA methodology as a second method to confirm Karl Fischer test results, especially when the Karl Fischer test results indicate that the sample has a falling moisture content [3].

In complex thermograms, the transitions attributed to residual moisture are verified for samples by thermogravimetry–mass spectrometry (TG–MS). This technique provides precise TG heating conditions and mass loss information along with mass spectral identification of volatiles evolved during the mass loss process. Mass spectra are taken of the TG off-gases continuously with ion intensities of mass peaks 18 and 44 being monitored to show the changes in the amounts of water and carbon dioxide. The mass spectral ion intensities verify the transition caused by moisture in the freeze-dried sample. This is done by differentiating between the water content of the sample and the water evolved from thermal decomposition of the sample, which coincides with the evolution of carbon dioxide.

6.3. Gas chromatography

In addition to the thermal methods of analysis, gas chromatography (GC) with a thermal conductivity detector has been used for the determination of water in proteins. In this method the sample is dissolved in an appropriate solvent and injected on-column along with an internal standard (i.e., methanol). In a method used in the European Pharmacopoeia monograph for hGH, 1.0 mg of the preparation is suspended in 0.1 ml of 2-propanol using anhydrous methanol as the internal standard [42]. The chromatographic procedure is carried out using a stainless-steel column packed with styrene–divinylbenzene copolymer using helium as the carrier gas. The water content of the sample is calculated taking into account its density (0.997 g/ml at 20°C) and any water detectable in the internal standard solution. Care must be taken to assure that the entire contents of the sample are well dissolved and that any contamination of the solvent(s) due to water is corrected for.

Gas chromatography may be utilized if the need exists to perform residual moisture determinations on a micro scale. GC analysis, however, is complicated to perform and is prone to overestimating the amount of moisture content in the sample. Precautions are needed, such as sample preparation in a glove-box purged with a dry inert gas, to prevent rehydration of the sample with moisture from the atmosphere [40].

6.4. Near-infrared spectroscopy

In addition to methods where the sample is not recovered after analysis, the non-invasive,

non-destructive technique of near-infrared spectroscopy has also been utilized [37]. In this technique, a fiber-optic diffuse-reflectance probe was used to measure reflectance from 1100 to 2500 nm through the bottom of intact glass vials. The correlation of the method with results obtained by Karl Fischer analysis was very good. The method is rapid, as analysis times are short (typically 20-s analysis times) and little sample preparation is required. In addition, the sample container remains closed during the analysis, removing the threat of erroneous results from atmospheric moisture. The water content measurements, however, require that an accurate reference moisture value be obtained as a guide, with the Karl Fischer titration being the method of choice.

6.5. Karl Fischer methodology

Of all these methods, the Karl Fischer titration, originally described in 1935 [43], is the approach most widely used in the determination of water content. The Karl Fischer method gives a better estimate of the total residual moisture, is very reproducible and can be automated. The titration can be run in either protic or aprotic media with the protic medium seeing wider use owing to the higher sensitivity of the titer to sample and solvent composition [44]. The reaction in protic media (i.e., alcohol) involves sulfur dioxide reacting with the alcohol to produce an alkyl sulfite in a buffered medium using an appropriate base to maintain the solution at the optimum pH. The method of adding iodine to the reaction differs according to the type of experiment, volumetric or coulometric. In the volumetric experiment, the iodine is contained in a buret and metered out as required. The amount of iodine per unit volume is determined empirically during a standardization step and the amount of water in the sample is calculated from this titer. In a coulometric experiment, the iodine is generated electrochemically from iodide present in the cell. Iodine together with pyridine, sulfur dioxide and methanol form the Karl Fischer reagent, which reacts quantitatively with water. The cake is dissolved or suspended in methanol and a coulometric oxidation of iodine is made titrimetrically. Alternative "pyridine-free" Karl Fischer reagents substitute another amine for pyridine [45]. The electrochemical efficiency of this method is generally 100%, and the amount of water in the sample is calculated from the number of moles of electrons used in the iodine generation.

The ability of the coulometric Karl Fischer method to measure residual moisture in about 10 mg of a freeze-dried biological sample makes it the most practical Karl Fischer method for the determination of residual moisture in freeze-dried biological products in single dose final containers. Freeze-dried biological products cannot be analyzed by the Karl Fischer methodology when (1) materials are present in the matrix of the biological product that interfere with the Karl Fischer reagents, (2) the sample does not dissolve adequately in the Karl Fischer reagent and (3) the sample moisture does not adequately extract into these solvents.

An important point to consider with the Karl Fischer titration is the possibility of erroneous results due to water contamination during sample handling. Water content of a protein at a given relative humidity can be influenced by its prior process and storage conditions. In studies involving the freeze-dried formulation of human growth hormone, an increase in water content arose from absorption of water from the stopper, not transmission of water through the stopper [33]. Titration of small amounts of water in coulometric systems requires correction for atmospheric moisture entering the system. Sample handling can have a significant impact on the results of a titration as a result of gain or loss of moisture between sampling and analysis. This becomes increasingly frustrating when one considers the increased variability of the assay due to wide fluctuations in relative humidity throughout the year [46]. Atmospheric moisture contamination can be partly circumvented by using a dry-box to minimize interference from ambient humidity, but care must be taken when setting the relative humidity in the glove-box so as not to remove or add moisture to the sample. An alternative to the glove-box is to add a known

amount of anhydrous methanol with a syringe to the freeze-dried biological product in a final container [3]. The methanol should dissolve the sample. Known amounts of sample and methanol are withdrawn and added to the Karl Fischer titration vessel for moisture determination. The moisture content of the anhydrous methanol is determined as the blank.

7. Conclusions

The relationship between water and protein is important in the understanding of the shelf-life stability of lyophilized formulations of biopharmaceuticals. The flexibility of a protein in the solid state is increased by temperature and moisture content. Water has the potential to act as a plasticizer in the amorphous polymer to reduce the glass transition temperature, T_g. If the T_g is decreased below the temperature of the surroundings, a phase transition from a glassy state to a dynamically relaxed state may occur. The level of moisture content resulting in a more mobile system can be extrapolated using isotherms to help predict the BET monolayer. At or near the BET monolayer, there is increased mobility of the absorbed water and the flexibility of the protein. The increased protein flexibility and water mobility may adversely affect the chemical and/or conformational stability of the protein. The observed decomposition pathways are sensitive to the flexibility of particular segments of the protein backbone and the orientation of exposed amino acid residues. In addition to temperature and moisture content considerations, care must be taken in the selection of the proper excipients. Excipients that exclude water from the protein surface (such as small ions) or that increase the water content in the amorphous phase (those that crystallize) can adversely affect protein stability. Strategies employed to maintain the solid-state stability of proteins need to minimize protein flexibility and water mobility by maximizing the T_g through low moisture content and careful selection of excipients. Owing to the close interplay between water and protein stability in the amorphous system, the accurate determination of moisture content in the lyophilized plug is imperative. A number of techniques have been utilized to determine moisture content, with each having its own set of advantages and limitations. The one common limitation, however, is the ability to prevent atmospheric moisture contamination during sample handling.

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