UDC: 615.012:547.96
Professional paper

LYOPHILIZATION (FREEZE-DRYING) OF PROTEINS: CRITICAL ROLE OF PROCESS OPTIMIZATION

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Abstract

Freeze-drying (lyophilization) is a technique in pharmaceutical manufacturing, for stabilizing protein-based and - heat-sensitive small molecules. This method enables long-term preservation by removing water under low temperature and pressure, thereby minimizing degradation and maintaining product efficacy. This review outlines the main principles of lyophilization, emphasizing the importance of components making up the formulation such as stabilizers, bulking agents and buffer. It also highlights how the careful selection and balance of these components are crucial for ensuring product stability, efficacy, and successful freeze-drying performance. Specific attention is also given to the behavior and stability of the active pharmaceutical ingredient (API), which has a certain sensitivity to environmental and process conditions, and whose requires careful selection of excipients and processing parameters. It explores key process parameters, including freezing rate and drying conditions and highlights critical thermal properties such as glass transition and collapse temperature. Analytical techniques, such as DSC, FTIR, HPLC and bioassays are reviewed for their role in ensuring protein stability and product quality. This review addresses current challenges, including raw materials variability, limitation in equipment and evolving regulatory demands, while pointing toward future innovation such as PAT tools and sustainable technologies. Despite its advantages, freeze-drying faces challenges such as raw material variability, equipment limitations, and evolving regulatory expectations. Addressing these issues involves integrating Process Analytical Technology (PAT), Quality by Design (QbD), and real-time monitoring systems. Finally, the successful development of lyophilized pharmaceuticals depends on an integrated understanding of formulation science, process engineering and regulatory compliance.

Keywords: freeze-drying (lyophilization), protein stability, pharmaceutical formulation, analytical techniques, excipients

1. Introduction

Lyophilization is a key process in the pharmaceutical industry, especially for small molecules which are intended for ready-to-use formulations. Lyophilization is defined as a dehydration process frequently used to preserve thermolabile and sensitive active ingredients or to make them more convenient for transportation and storage. The process starts with freezing of the formulation, then reducing to the surrounding pressure to allow the frozen water in the material to sublimate directly from solid phase to the gas phase. This technique is widely used in the pharmaceutical industry for the stabilization of vaccines, biologics and other heat sensitive drugs (Tang & Pikal, 2004). The process helps to maintain the structural integrity and biological activity of the product, making it ideal for long term storage (Patel et al., 2010). Despite its advantages, this process is extensive energy and time consuming, which contributes to higher production costs (Franks, 1998).

2. Objective of the Review

This review aims to consolidate current methodologies, identify critical process parameters, and propose best practices for the successful implementation of lyophilization in small molecule ready-to use kit production. It provides a comprehensive overview of the formulation design, selection of excipients, and the optimization of freeze-drying cycles tailored specifically to the physicochemical properties of small molecule drugs. Special emphasis is placed on the challenges unique to kit-based formulations, such as ensuring component compatibility, maintaining reconstitution efficiency, and preserving drug potency during storage and transport.

Formulation Composition

The term formulation composition refers to the specific combination of active pharmaceutical ingredients (API) and excipients, that are used to create a pharmaceutical product, which will have the desired therapeutic effect, stability and patient acceptance. While the API is the core molecule responsible for the intended pharmacological activity, excipients have various roles such as bulking agents, buffers, disintegrants, stabilizers and preservatives (Aulton & Taylor, 2013). The selection and proportion of these components plays a crucial role in ensuring optimal bioavailability, manufacturability and shelf life of the product. Moreover, formulation design must comply with regulatory requirements and also take into account factors such as the route of administration, targeted patient population and potential interactions between ingredients (Allen, Popovich, & Ansel, 2011). The choice of excipients plays a significant role in the success of lyophilization:

Stabilizers: Such as sugars (eg. trehalose, sucrose). Stabilizers are excipients that are added to pharmaceutical formulations to maintain the physical, chemical and microbiological stability of the active pharmaceutical ingredient throughout the product's shelf life. Their primary role is to prevent degradation caused by environmental factors: light, heat, oxygen, moisture or interactions with other ingredients (Carstensen & Rhodes, 2000). Depending on the formulation, stabilizers can include antioxidants (ascorbic acid, butylated hydroxytoluene), chelating agents (EDTA), buffering agents to maintain pH and preservatives to inhibit microbial growth. The selection of an appropriate stabilizer is essential for ensuring therapeutic efficacy, safety and patient compliance (Kibbe, 2000).

Bulking Agents: Also known as diluents or fillers. These are excipients used to increase the volume or mass of a pharmaceutical formulation, particularly when API is present in very small quantities. These agents are essential in ensuring uniformity of dosage, improving the mechanical properties of tablets or capsules and facilitating manufacturing processes (Aulton & Taylor, 2013). Common bulking agents include lactose, microcrystalline cellulose, mannitol and dicalcium phosphate. The choice of bulking agent depends on factors such as compatibility with the API, solubility, compressibility and the intended route of administration. In lyophilized products, bulking agents like mannitol and glycine are especially important for maintaining cake structure and appearance during lyophilization (Pikal, 2007).

Buffers: Buffers are excipients used in pharmaceutical formulations to maintain a stable pH, which is crucial for the chemical stability, solubility and bioavailability of API. Many APIs are sensitive to pH changes, and even slight shifts can lead to degradation or reduced efficacy (Florence & Attwood, 2011). Buffer systems typically consist of a weak acid and its conjugate base (acetic acid/sodium acetate, citric acid/sodium citrate or phosphate buffers), and they work by neutralizing small amounts of added acid or base to keep the pH within a desired range. In parenteral, ophthalmic and other sensitive dosage forms, buffers play a critical role in ensuring safety and comfort for the patient, as well as in preventing precipitation or microbial growth (Allen, Popovich, & Ansel, 2011).

An optimal balance of the above components is necessary to achieve desired product characteristics.

3. Process Parameters

Process parameters refer to the specific conditions and variables that must be controlled during the manufacturing of pharmaceutical products to ensure consistent quality, safety and efficacy. These include factors such as temperature, pressure, pH, humidity, drying time and, depending on the type of dosage form being produced (FDA, 2011). Critical Process Parameters (CPPs) are those that have a direct impact on Critical Quality Attributes (CQAs) of the final product, and their identification and control are central to Quality by Design (QbD) approaches (ICH Q8, 2009). Optimizing and validating these parameters is essential for achieving reproducibility, minimizing batch to batch variability and complying with Good Manufacturing Practice (GMP) standards. Key process parameters include:

Freezing rate: Influences ice crystal formation; slower freezing can lead to larger crystals, affecting the sublimation rate.

Primary drying pressure and temperature: Must be carefully controlled to ensure efficient sublimation without exceeding critical temperatures.

Secondary drying conditions: Aim to remove residual moisture, enhancing product stability.

Employing Design of Experiments (DOE) methodologies can aid in optimizing these parameters to achieve a robust lyophilization cycle.

4. Critical points in lyophilization of protein molecule formulations

Lyophilization of protein-based pharmaceuticals is a complex process that must be carefully optimized to preserve the structural integrity, biological activity, and stability of the protein throughout manufacturing and storage. Several critical points must be addressed:

Freezing phase: The rate and method of freezing significantly affect ice crystal formation and the distribution of solutes. Rapid freezing creates smaller ice crystals, which can lead to a more porous cake but may increase stress on proteins due to higher concentration gradients (Carpenter et al., 1997).

Primary drying (sublimation): Maintaining product temperature below the formulation's collapse temperature (Tc) or eutectic temperature is crucial. Exceeding these temperatures can cause structural collapse or denaturation of proteins (Tang & Pikal, 2004).

Secondary drying (desorption): This phase removes bound water. Inadequate drying can result in residual moisture, promoting protein degradation or aggregation during storage. However, excessive drying can cause brittleness and potential loss of activity (Allison et al., 1999).

Stabilizers and excipients: The choice of excipients, such as cryoprotectants (eg. sucrose, trehalose) and bulking agents (eg. mannitol) is critical. These substances help protect the protein from freezing and drying stress by forming a glassy matrix and maintaining hydrogen bonding (Arakawa et al., 2001).

Reconstitution: The formulation must allow for easy and complete reconstitution. Improperly designed formulations can lead to insoluble aggregates or long reconstitution times, affecting usability and safety.

Each of these points requires precise control and thorough pre-formulation studies to ensure the successful development of a stable and effective protein-based lyophilized product.

4.1. Thermal properties in lyophilization of pharmaceutical formulations: Understanding the thermal behavior of pharmaceutical formulations is critical for designing an effective and safe lyophilization process. Two key thermal parameters must be carefully determined and controlled:

Glass Transition Temperature (Tg'): This is the temperature at which the maximally freeze-concentrated solution transitions from a glassy (rigid) state to a rubbery (viscous) state. Below Tg', molecular mobility is greatly reduced, which helps stabilize labile molecules such as proteins and peptides during freezing and primary drying. Operating below this temperature minimizes degradation reactions and preserves the product's physical structure (Allison et al., 1999; Franks, 1998).

Collapse Temperature (Tc): This refers to the temperature at which the structure of the frozen cake begins to collapse during primary drying due to insufficient mechanical strength of the amorphous matrix. Exceeding Tc leads to loss of the porous structure, resulting in poor reconstitution, longer drying times, and potentially reduced efficacy or stability of the product (Pikal & Shah, 1990; Tang & Pikal, 2004).

Maintaining product temperature below both Tg' and Tc throughout the freeze-drying cycle is essential for preserving the cake's integrity, preventing denaturation of sensitive biomolecules, and ensuring the final product has acceptable appearance, stability, and performance characteristics. Accurate thermal analysis using tools such as differential scanning calorimetry (DSC) and freeze-drying microscopy is often employed during development to identify these critical points and optimize cycle parameters accordingly.

5. Key contributions of analytical techniques for protein based lyophilized formulations

Analytical techniques play a central role in the development, optimization, and quality control of protein lyophilization processes. Given the sensitivity of proteins to environmental and processing stresses, the use of robust analytical tools enables researchers and manufacturers to monitor structural integrity, detect degradation, and ensure the consistency and efficacy of the final product. These methods provide insights into thermal behavior, physical stability, and biological activity before, during, and after the lyophilization cycle (Kasper & Friess, 2011).

Thermal analysis techniques, such as Differential Scanning Calorimetry (DSC) and Freeze-Drying Microscopy (FDM), are essential during formulation development. DSC helps identify key thermal transitions, including the glass transition temperature (Tg') and crystallization events, while FDM is used to determine the collapse temperature (Tc), which guides the setting of safe product temperatures during primary drying (Tang & Pikal, 2004). These parameters are critical for preserving the protein's native structure and avoiding physical collapse or denaturation during processing.

Spectroscopic methods, including Fourier Transform Infrared Spectroscopy (FTIR) and Circular Dichroism (CD), provide information on protein secondary and tertiary structures. These tools are vital for confirming that no significant conformational changes occur as a result of lyophilization. FTIR, in particular, has been widely used to assess protein unfolding and hydrogen bonding interactions in the dried state, often in combination with stabilizers like sugars (Allison et al., 1999; Prestrelski et al., 1993).

Chromatographic and electrophoretic techniques, such as High-Performance Liquid Chromatography (HPLC), Size-Exclusion Chromatography (SEC), and SDS-PAGE, are employed to quantify the purity and aggregation state of proteins. Aggregation is a common issue in lyophilized formulations and can reduce therapeutic efficacy or increase immunogenicity. These techniques help in detecting degradation products, monitoring post-reconstitution stability, and confirming the presence of intact protein species (Manning et al., 2010).

Circular dichroism can be used to study both secondary and tertiary protein structures by measuring the difference in absorbance. Solid-state nuclear magnetic resonance (ssNMR) is used to characterize both structural and dynamic changes. Differential scanning calorimetry (DSC) is useful for characterizing molecular mobility, crystallization kinetics, degree of crystallinity and denaturation, and for determining glass transition temperature (Tg). Dielectric relaxation spectroscopy (DRS) as a complementary method to DSC can provide insights into protein dynamics, while X-ray diffraction (XRD) analysis can be used to study the powder structure (amorphous/crystalline) of lyophilized proteins. Moreover, recently developed methods based on mass spectrometry, such as solid-state hydrogen-deuterium exchange mass spectrometry (ssHDX-MS), can be used to study the protein structure and conformation in the solid state with high resolution. In addition to structural characterization, monitoring protein aggregation is also very important for the stability, quality, safety, and efficiency of the final drug. Aggregation, along with protein denaturation and surface adsorption, can affect the amount of native protein and, thus, the activity of the drug (Bolje, A., & Gobec, S., 2021).
Lastly, biological activity assays remain indispensable, as they directly assess the functional integrity of proteins post-lyophilization. Even when structural analyses show no visible changes, biological assays can reveal subtle loss of activity due to microenvironmental changes or incomplete reconstitution. Therefore, combining physical, chemical, and biological analytical techniques offers a comprehensive assessment of protein stability and ensures product quality and regulatory compliance.

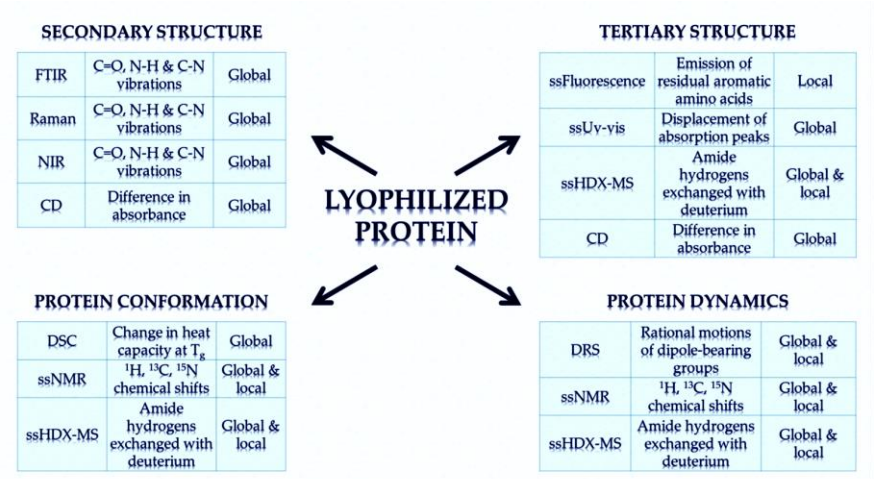

Figure 1. The most common analytical techniques for the structural characterization of proteins in solid pharmaceutical forms are presented with corresponding type of measurements. Changes in secondary/tertiary structure and conformation can be studied on a global and local scale. Protein dynamics can also be traced using some of the above methods. FTIR - fourier transform infrared; NIR - near-infrared; CD - circular dichroism; ss - solid-state, HDX-MS - hydrogen-deuterium exchange mass spectrometry; DSC - differential scanning calorimetry; NMR - nuclear magnetic resonance; DRS - dielectric relaxation spectroscopy.
(Figure: Bolje, A., & Gobec, S. (2021). Analytical Techniques for Structural Characterization of Proteins in Solid Pharmaceutical Forms: An Overview. Pharmaceutics, 13(4), 534.)

6. Challenges and future directions in protein lyophilization

Despite the widespread use and advantages of lyophilization in stabilizing protein-based pharmaceuticals, the process still faces several challenges that can affect product quality, efficiency, and scalability. Addressing these issues is essential for the continued development of safe, effective, and accessible biopharmaceutical products.

Variability in Raw Materials is one of the major challenges in protein lyophilization. Differences in the physical and chemical properties of the API - such as polymorphic form, hygroscopicity, and impurity levels - can significantly influence the lyophilization behavior and final product stability (Jameel & Hershenson, 2010). Likewise, variability in excipients, especially those of natural origin like sugars (e.g., sucrose or trehalose), can affect glass transition temperature (Tg'), collapse temperature (Tc), and protein stabilization capacity. Ensuring consistent raw material quality and employing advanced characterization techniques are critical for maintaining batch-to-batch reproducibility (Kasper & Friess, 2011).

Equipment limitations also pose a significant constraint in the lyophilization process. Differences in lyophilization models, condenser capacities, shelf temperature uniformity, and vacuum control can result in non-uniform drying, affecting critical quality attributes (CQAs) such as moisture content, cake appearance, and reconstitution time. Scale-up from laboratories to commercial lyophilizators introduces additional challenges due to non-linear heat and mass transfer dynamics (Tang & Pikal, 2004). Innovative technologies such as controlled nucleation, real-time process monitoring, and continuous lyophilizing systems are being developed to address these limitations and improve process efficiency and robustness (Pisano et al., 2011).

Regulatory considerations are another evolving area, as agencies like the FDA and EMA increasingly emphasize process understanding and control under Quality by Design (QbD) frameworks. Regulatory expectations now require detailed knowledge of critical process parameters (CPPs), critical material attributes (CMAs), and risk-based validation strategies for lyophilized products (ICH Q8–Q10). Additionally, compliance with Good Manufacturing Practice (GMP) standards and the need for comprehensive analytical documentation, including stability and microbiological testing, remain vital for market approval (FDA, 2011).

Future directions in protein lyophilization include the integration of process analytical technology (PAT) tools for real-time monitoring and control, the use of artificial intelligence and machine learning for predictive modeling, and the design of next generation lyoprotectants and stabilizers tailored to specific protein structures. These innovations aim to enhance product quality, reduce development timelines, and meet the increasing demand for biotherapeutics, especially in personalized medicine and global distribution.

7. Conclusion

Lyophilization remains a cornerstone technique in the stabilization of protein-based pharmaceutical products, offering significant benefits in terms of product shelf-life, structural integrity, and storage stability. A deep understanding of the formulation composition, especially the roles of stabilizers, bulking agents, and buffers, is essential for protecting sensitive proteins throughout the drying process. Equally critical is the careful control of thermal properties such as the glass transition temperature (Tg′) and collapse temperature (Tc), which guide safe operating conditions during freezing and primary drying to avoid physical or chemical degradation (Tang & Pikal, 2004; Franks, 1998).

Analytical techniques, including thermal analysis (DSC, FDM), spectroscopy (FTIR, CD), chromatography (HPLC, SEC), and biological assays, play a pivotal role in monitoring protein integrity and optimizing formulation and process parameters. These methods enable researchers to detect early signs of denaturation, aggregation, or loss of activity, ensuring the final product meets stringent quality and regulatory standards (Allison et al., 1999; Manning et al., 2010).

Nonetheless, the lyophilized of protein formulations faces persistent challenges such as variability in raw materials, equipment limitations, and increasing regulatory expectations. Addressing these issues requires a multidisciplinary approach combining material science, engineering, and regulatory science. The future of lyophilization lies in innovation, through

real-time monitoring tools, predictive modeling, controlled nucleation technologies, and the application of Quality by Design (QbD) principles (Jameel & Hershenson, 2010; FDA, 2011). Altogether, the successful development and commercialization of protein-based lyophilized pharmaceuticals depend on a holistic understanding of the interplay between formulation science, process engineering, and advanced analytical methods. As the demand for biotherapeutics continues to grow, so too must our capability to produce them safely, efficiently, and at scale.

In addition to scientific and technical considerations, environmental sustainability and waste management are gaining importance in the future of lyophilization. The process is energy-intensive, requiring prolonged freezing and vacuum operation, which contributes to high energy consumption and environmental impact (Franks, 1998). As environmental regulations tighten and the pharmaceutical industry moves toward greener practices, optimizing energy efficiency and reducing carbon footprints will become essential. Approaches such as cycle time reduction, energy-efficient equipment, and alternative drying technologies (e.g., spray lyophilization or microwave-assisted lyophilization) are being explored to make the process more sustainable without compromising product quality (Kasper & Friess, 2011).

Moreover, personalized medicine and biologics innovation are driving demand for more flexible and scalable lyophilized solutions. With the rise of personalized therapies, such as individualized vaccines, gene therapies, and monoclonal antibodies, there is a need for smaller batch sizes, rapid production cycles, and adaptable lyophilization platforms. This shift requires new technologies like miniaturized and continuous lyophilization systems, along with automation and digitalization through smart sensors and machine learning algorithms to monitor and control the process in real time (Pisano et al., 2011). As these innovations mature, they will play a vital role in ensuring that lyophilization remains a viable and essential technology in the evolving pharmaceutical landscape.

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