Review Article

Propulsive PAT paradigm: Optimization of Freeze Drying Process.

Piyush P. Mehta[1*], Vividha V. Dhapte[2]

[1*]Department of Quality Assurance, Bharati Vidyapeeth University, Poona College of Pharmacy, Erandwane, Pune, Maharashtra, India.
[2]Department of Pharmaceutics, Bharati Vidyapeeth University, Poona College of Pharmacy, Erandwane, Pune, Maharashtra, India.
***Corresponding author's E-mail:** piyu053@gmail.com

Accepted on: 16-08-2014; **Finalized on:** 30-09-2014.

ABSTRACT

Process analytical technology (PAT) is a propulsive paradigm moving across the pharmaceutical and biotechnological arenas to strengthen the process with better understanding of formulation parameters and process variables. It is increasingly being acquired and utilized for improved operational control and compliance via process analysing. PAT can be defined as a unique approach for planning and implementing principles of validation. It is mainly the part of 'Control strategy'. It combines techniques, procedures and technologies that enable online verification of key process parameters. In our article, application studies of PAT would be described in context of various modern instruments and tools used in analysis, control and design of freeze drying process. This would involve the in-depth assessment of primary drying, secondary drying like mechanisms and their correlation for successful scale up.

Keywords: Freeze drying, PAT, Primary drying, CQA, and Risk assessment.

INTRODUCTION

Many pharmaceutical entities lose their viabilities in the liquid state and readily deteriorate if dried in air at normal atmospheric pressures. These entities are either thermo labile or may react readily with oxygen. So, they must be dehydrated to a solid state for stabilization. To prevent deterioration, specialized drying technique are used such as freeze drying (FD), drying by sublimations or gelsication. FD of pharmaceuticals in actual practice is processed at temperature of -10 °C to -40 °C, pressure 2000 to 100 micron[1]. Over the years, FD has proven to be an effective conventional method for the large scale production of dried pharmaceuticals[2]. FD is a method to preserve bioactive molecules (DNA, enzymes, and proteins), pharmaceutical products (antibiotics) and other delicate solvent-impregnated materials[3,4]. FD is a multidimensional, time dependent process mainly used in pharmaceutical industries to retain initial attributes and efficacy of material (biological and biological drug products) after dehydration. FD involves three steps such as:

a. Frozen the material

b. Subjected it to high vacuum

c. Sublimation/ thawing of frozen liquid.

In primary drying, frozen ice is sublimated while secondary drying removes residual water from the porous matrix. These steps and environmental conditions influence the final product quality and sensitivity significantly along with various processing and formulation variables. Assessment and monitoring effects of all such factors individually and collectively is cumbersome and practically unfeasible. Thus, there is a need for systematic and simultaneous analysis of all variables and PAT is one of the few approaches that suffice this requirement[5].

Process analytical technology (PAT)

PAT is defined as 'a system for designing, analyzing and controlling manufacturing through timely measurements (in and after processing) of critical quality and performance attributes of raw and in-process materials and processes with the goal of ensuring final product quality'[6]. PAT is a unique approach for process validation. It combines techniques, procedures and tools like quality by design (QbD) and real time release (RTR) that enable online and offline verification of key process parameters[7]. PAT creates a robust control strategy for the ongoing process monitoring[8]. PAT is thus, increasingly explored and adopted by pharmaceutical and biotechnological set ups for enhanced process understanding and risk management[9]. PAT from an implementation perspective is visualized as a three-step process:

a. Design

b. Analysis and

c. Control [10,11].

'Design' phase starts early in process development. In this phase, the critical quality attributes (CQA) that are being affected by the process steps are identified along with the critical process parameters (CPP). During 'Analysis' phase, a suitable analyzer is identified for monitoring of the CQA and CPP. 'Control' phase aid in understanding the process, monitoring CPP so as to achieve consistent CQA. Four types of process analysis measurements are performed as per **Table 1**[12].

Table 1: Process analysis measurements.

Type of PAT measurements	Method	Characteristics
in-line	No removal of the sample.	Quick and quality result obtained.
on-line	Sample is diverted from the main process, analyzed and may be returned.	
at-line	Sample is removed and analyzed closed to the process.	More time consuming.
off-line	Sample is removed and analyzed away from the process.	Difference between at-line and off-line measurements in lab scale processing is tough to define.

FD process

The principal function of FD process is to separate the solvent from solute. Solvent like water form ice crystals and solutes occupy the interstitial region between the ice crystals to form frozen matrix structure. In primary drying stage, pressure is reduced and heat is applied to initiate the sublimation of the ice crystals. It leads to plunging of the ice-gas interface through the cake. As soon as ice crystals are removed and volume occupied by the resulting cake equates with the frozen matrix, primary drying process completion occurs. This is followed by secondary drying wherein residual moisture content of the product is reduced to acceptable level (desorption of water vapour) that will no longer support any biological or chemical alterations. It is a step which controls the kinetic clock of the active constituents[13].

Primary drying

It is usually the rate determining step of the FD process and significant efforts are required to design and optimize this stage[14-16]. To prevent gross collapse of the system and to obtain the pharmaceutically acceptable cake-structure solids, product temperature in primary drying should be below the collapse temperature (T_c) or the glass transition temperature of the frozen solution ($T_{g'}$) (**Fig. 1**). In primary drying, product temperature is controlled by change of shelf temperature and chamber pressure. The primary drying is usually conducted at above -40 °C to accomplish ice sublimation on a practical timescale[17-19]. Manometric temperature measurement (MTM) is a PAT tool that greatly improves the FD process design and control. It is an alternative method of in-process product temperature measurement during primary drying. MTM measure the product temperature at the sublimation interface without placing any device in the vial. The MTM method records the pressure versus time data[20].

Secondary drying

In case of secondary drying, temperature is elevated up to maximum tolerable temperature of the dried product. When there is no more ice, final temperature is applied so as to gain dry product per vial. A relatively high or low pressure does not influence the secondary drying from the point of heat transfer as pressure in secondary drying is below 0.1 mbar. Hence, heat transfer coefficient is practically independent of pressure. Pressure has a major influence to play when partial vapour pressure inside the product is equal to the value of desorption isotherm. To avoid pressure interference, pressure must be maintained below this equilibrium point (**Fig. 1**). Desorption doesn't change physical structure or chemical constitution of product but desorption rate is critical in determining the residual moisture. Residual moisture is essential in assessing the product stability[21].

Drying rate of the product is affected by the depth of product in container. Hence the product depth in the container should be limited. For large volume product, slanted position of container is useful to increase the surface area. Amount of solid, their precipitated particle size (smaller particle size illustrate faster rate of drying) and their thermal conductance affect the rate of drying[22].

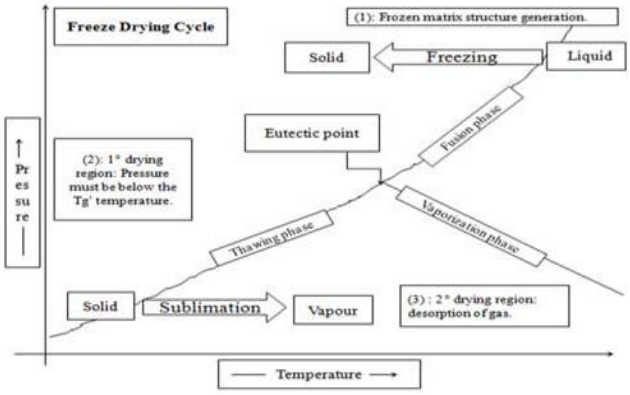

Figure 1: Phases of freeze drying (FD) cycle.

Process variables

Moisture and temperature control are two critical parameters in FD process. As a PAT strategy, these parameters are monitored by following techniques in **Table 2.**

Formulation variables

Active constituents

The formulation development or design in FD is dependent on the active pharmaceutical ingredient (API) involved and the intended route of administration for that substance. Various synthetic [fluconazole[42], flurbiprofen[43]] or natural [human spermatozoa[44], black raspberries, blackberries and strawberries[45,46]] products can be subjected to FD. FD also overcomes protein instability in aqueous systems by removing water [47]. It can be applied to different micro-organism [*Lactobacillus salivarius*[48], *P. pentosaceus*[49]] to enhance the stability. In presence of API, selection of excipients so to develop a simple, stable and consistent FD formulation with an economical process is judicious activity. Excipients include but are not limited to buffers, bulking agents, stabilizers, solvent, cryoprotectants and lyoprotectants.

Table 2: Techniques used to monitor critical parameters like moisture and temperature.

Technique	Characteristics
A. Determine % residual moisture during late primary and early secondary drying	
Karl Fischer technique.	Not suitable for small samples[23].
Gravimetric method.	Accuracy depends upon hygroscopic nature of sample[24].
Gas chromatography.	Based on the adsorption of water from the sample using organic solvents[23].
Multipoint NIR spectroscopy.	In-line quantification of moisture content and hence used for drying end point determination of a residual moisture level [25].
Multipoint NIR spectroscopy.	Detection of unequal sublimation rates within a freeze-dryer shelf [26].
B. Detect the end of primary drying	
Tunable Diode Laser Absorption Spectroscopy.	Spectroscopic and non-invasive sensor for monitoring secondary drying and targeting intermediate moisture contents in product [27].
	Also, provides continuous measurements of water vapour concentration, velocity of vapour flow and mass flow rates in the real time FD [28].
Thermal conductivity gauge.	Measuring the thermal conductivity of the gas in the drying chamber. It is the best method for evaluation of end point of primary drying, a batch technique, which is economical, steam sterilizable and easy to install without requiring any modification to the existing dryer [24].
Gas plasma spectroscopy. (Optical emission spectroscopy)	Measures water vapor concentration during the drying process [24].
Pressure rise test.	Manometric temperature measurements [24].
Condenser pressure, dew point, product thermocouples.	Commonly use methods to assess the drying process [24].
C. Temperature measurement during the FD process.	
Temperature Remote Interrogation System. (TEMPRIS)	Wireless temperature sensors to observe temperature profiles [29].
Manometric temperature measurement. (MTM)	A valid measurement of product temperature during primary drying even at temperatures as low as -45°C. It measure dry-layer resistance and vial heat transfer coefficients [30,31,32].
Freeze-drying microscope (FDM) based on time-domain Optical Coherence Tomography (OCT).	Measure T_c of product formulations in standard pharmaceutical vials without loss of product quality, Provides quantitative justification for FD above T_c and provides an upper limit to the temperature at which a FD cycle may run without macroscopic product collapse. It helps to reduce the time for primary drying and increase process efficiency for FD products with more accuracy than light transmission or differential scanning calorimetry; other benefits are product microstructure visualization.[33].
D. Miscellaneous Monitors	
Raman spectroscopy (in-line) and NIR spectroscopy and X-ray powder diffractometry (XRPD) (at-line)	Real time monitoring of FD processes in combination with experimental designs. Both techniques not only complement each other, but they also provide mutual confirmation of specific conclusions [34,35].
Raman spectroscopy	Useful technique to monitor physical changes during FD [36].
Cold plasma ionization device	Plasma tool, a relevant method for monitoring FD processes [37].
XRD technique	Characterize the phase transitions during FD and useful in developing a mechanistic understanding of the solid state alterations during FD of complex, multi-component, pharmaceutical systems [38].
Frequency Modulation Spectroscopy (FMS)	Helps to demonstrate the uniformity and create a map of headspace moisture (HSM) for vials which allow inspection and play an important part in process validation and quality assurance [39].
The Optical Fiber Sensors (OFS)	Allow easy handling and positioning along with detection of excipient crystallization events. It helps to obtain three-dimensional temperature profiles with an OFS helix configuration which enables non-invasive, automatic loading compatible monitoring of FD process [40].
Vial impedance spectroscopy (off-line)	Useful in the concurrent product formulation development and FD cycle without any uncertainty introduced so as to define the critical process parameters [41].

Buffers

In FD, selection of buffer for pH control was essential to prevent physicochemical changes in the product. Low concentrations of tris, citrate and histidine buffers [50] lead to minimal pH change during freezing. Buffering agent should have high collapse temperature (for rapid primary drying), non-volatile (to prevent pH drift which ultimately show impact on product stability) and high T_g (ensure stability during storage)[51,52]. Amorphous (citrate buffer) buffer was the most preferred as it retained amorphous nature, with negligible shift in pH as compared to crystalline buffer components (sodium and potassium phosphate salts, succinate and tartrate) which lead to a drastic pH shift resulting in degradation of the active component [53,54].

Bulking agents

They help to provide adequate structure to cake. These are generally used for low dose, high potency drugs and are more important when the total solid content is less than 2%. Bulking agents emerge as crystalline or amorphous solids at end of FD process. Amorphous solids are preferred as it least affects the physical stability.

Nature of lyophilized cake also depends on the ratio of drug and bulking agent; increased proportion of bulking agent increased crystallization. Mannitol and glycine are the most commonly used bulking agents as compared to other sugars and amino acids, respectively[55].

Stabilizing agents

During FD, stabilizing agents like disaccharides stabilize liposomes and protein products more effectively by forming an amorphous sugar glass[56]. Disaccharides such as sucrose and trehalose stabilize proteins both, thermodynamically as well as kinetically in aqueous solutions and freeze-dried solids[57].

Hypothesis postulating stabilizing effects of the disaccharides are the water replacement hypothesis and the vitrification hypothesis[58]. Controlling hydrogen-bonding and electrostatic interactions is a key element to optimize the physical properties of multi-component amorphous FD pharmaceutical formulations[19].

Solvents

Solvents may play minor role in stabilization of the active constituent but improve the aesthetic property of the final product. In case of FD, water is the most preferred solvent. High vapour pressure of co-solvents facilitates their faster removal from the product during drying process thus, speeding up the FD process. As a co-solvent, terbutyl alcohol speeds up the FD process and does not require additional cooling to attain frozen state as compared to ethanol[13].

Nevertheless, non-aqueous solvents control degradation rate of active constituents in the presence of water[55].

Cryoprotectants and lyoprotectants

Primary function of cryoprotectants is to protect the active constituents during FD process. Example includes glycerol and dimethyl sulfoxide (DMSO). Lyoprotectants are the excipients which prevent degradation of active constituents during freezing-thawing process. These molecules are typically polyhydroxy compounds such as sugars (mono-, di-, and polysaccharides), polyalcohols and their derivatives. Trehalose (anhydrobiosis) and sucrose are natural lyoprotectants[59]. Cryopreservation allows the use of very low temperatures to preserve structurally intact living cells and tissues. Cryoprotectants, simply by increasing the total concentration of all solutes in the system, reduce the amount of ice formed at any given temperature; but to be biologically acceptable they must be able to penetrate into the cells and have low toxicity. These include ethanediol and propanediol[60].

FD generates various stresses, during both, freezing and drying; to protect against such stresses, cryoprotectants/ lyoprotectants are used. Optimization of both, type and concentration of cryoprotectants is essential. Freeze-thaw study is short and quick pre-test for screening of type and concentration of cryoprotectants used for FD[61].

CQA for FD process and product

FD is a typical pharmaceutical process that incorporates various unit operations and variables as discussed above. Each unit operation or variable can be potentially regulated by implementation of one or more PAT tools. For effective implementation of PAT, CQA and CPP of each unit operation in FD process are selected (**Fig. 2**).

Understanding interaction between CQA and CPP assist in continuous analysis and improvement of FD product as well as process. Diversity of the available automated PAT tools (**Table 2**) and their capabilities to visualize the process helps to design FD process in a way such that at the end of each unit operation, assurance can be provided that the step performed its function in a satisfactory manner.

Risk assessment

Risk assessment is an imperative module of PAT strategy. ICH Q9 summaries the three basic components of risk assessment: risk identification, risk analysis and risk evaluation. These basic components deal with the systematic use of information derived from PAT tools to identify the potential harm, qualitative or quantitative linking of the likelihood of occurrence and severity of harm and compares the identified, analyzed risk against given risk criteria.

Purpose of risk assessment is to highlight the risk factors and accordingly frame a robust FD process and quality FD product. Quality risk management supports a scientific and practical approach to decision-making and problem-elucidation[62].

Risk assessment involves different tools in it such as Fault Tree Analysis. This tool assumes failure of the functionality of a product or process. The results are represented pictorially in the form of a tree of fault modes which can be used to investigate the errors or deviations.

This helps to fully understand the root cause of problem and ensure that intended improvement will resolve the issues without creation of any other problem. Application of this tool for addressing one of the risk issues regarding product appearance is as depicted in **Fig. 3.**

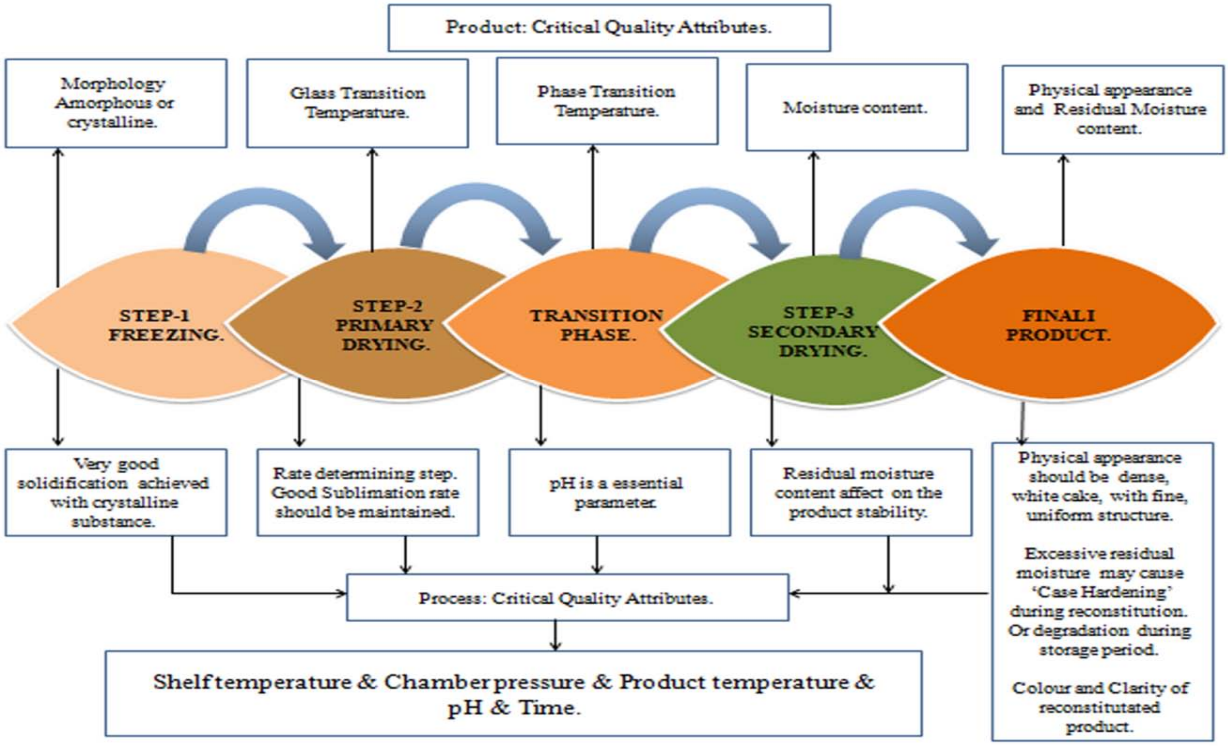

Figure 2: CQA for FD product and process.

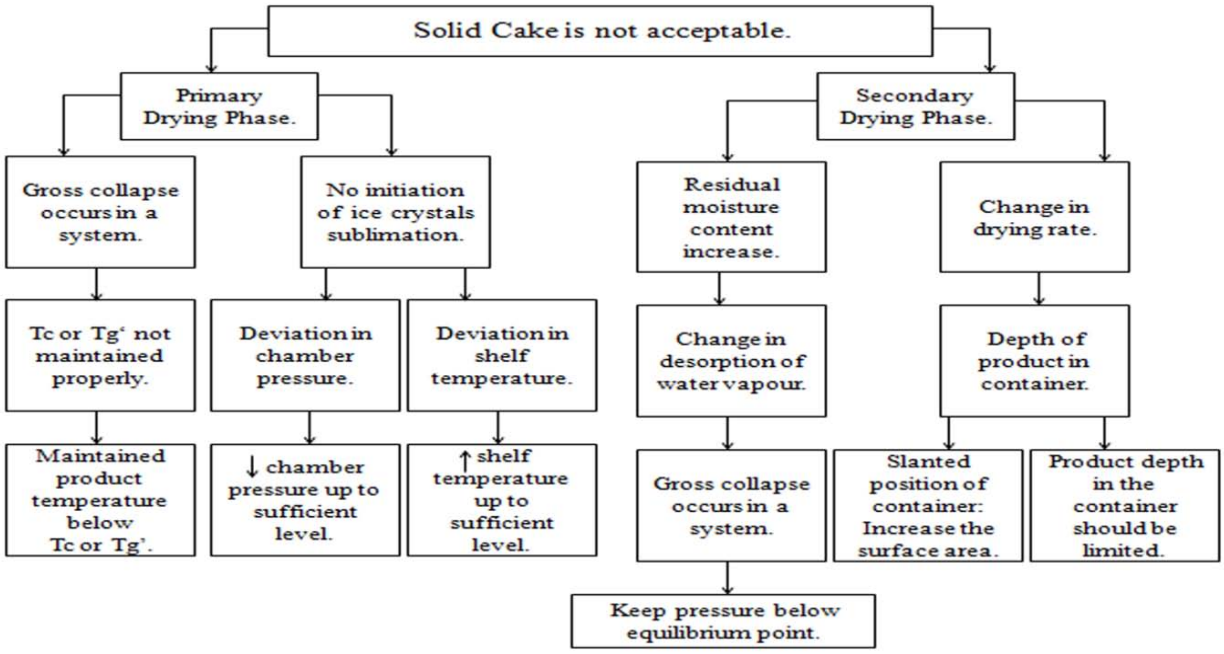

Figure 3: Risk assessment via Fault Tree Analysis in FD process.

8. Conclusion:

PAT provides wide range of tools for continuous optimization of the freeze drying process. It provides valid knowledge about freeze drying cycles. In order to design a robust process and quality product, all complementary and alternative devices as process analyzers (PAT tools) aid in the non-invasive or invasive, in, on, at, off-line and real-time monitoring of a freeze drying process. Simultaneous application of different PAT tools allows complete monitoring of a freeze drying process at negligible risk. Thus, PAT will be useful for better understanding of the real time interactions among all process and product variables thereby, attaining the most coherent design space for freeze drying of foods and pharmaceuticals.

REFERENCES

1. Khar RK, Vyas SP, Ahmad FJ, Jain GK (Eds) Lachman, L., Lieberman, H. A.: The theory and practice of industrial pharmacy. CBS publishers and distributors pvt. ltd., India, 2013, 98-99.
2. Allison SD, Molina M, Anchordoquy TJ, Stabilization of lipid/DNA complexes during the freezing step of the lyophilization process: the particle isolation hypothesis, Biochimica. et. Biophysica. Acta. 1468, 2000, 127-138.
3. Keskintepe L, Pacholczyk G, Machnicka A, Norris K, Curuk MA, Khan I, Brackett BG, Bovine blastocyst development from oocytes injected with freeze-dried spermatozoa, Biol. Reprod., 67, 2002, 409-415.
4. Kusakabe H, Kamiguchi Y, Chromosomal integrity of freeze-dried mouse spermatozoa after 137Cs gamma-ray irradiation, Mutat. Res., 556, 2004, 163-168.
5. Rathore AS, Bhambure R, Ghare V, Process analytical technology (PAT) for biopharmaceutical products, Anal. Bioanal. Chem., 398(1), 2010, 137-154.
6. U.S. Department of Health and Human Services, Food and Drug Administration Guidance for industry: PAT- a framework for innovative pharmaceutical development, manufacturing and quality assurance http://www.fda.gov/downloads/Drugs/Guidance Compliance Regulatory Information/Guidances/ucm070305.pdf Accessed 25 may 2013.
7. Kamble R, Sharma S, Varghese V, Mahadik KR, Process analytical technology (PAT) in pharmaceutical development and its application, Int. J. Pharm. Sci. Rev. Res., 23(2), 2013, 212-223.
8. Rathore AS, Roadmap for implementation of quality by design (QbD) for biotechnology products, Trends Biotechnol., 27(9), 2009, 546-553.
9. Chen Z, Lovett D, Morris J, Process analytical technologies and real time process control a review of some spectroscopic issues and challenges, J. Process. Control., 21, 2011, 1467-1482.
10. Read EK, Park JT, Shah RB, Riley BS, Brorson KA, Rathore AS, Process analytical technology (PAT) for biopharmaceutical products: part I. concepts and applications, Biotechnol. Bioeng., 2009, 105(2), 276-284.
11. Read EK, Park JT, Shah RB, Riley BS, Park JT, Brorson KA, Rathore AS, Process analytical technology (PAT) for biopharmaceutical products: part II Concepts and applications, Biotechnol. Bioeng., 105(2), 2009, 285-295.
12. Rathore AS, Follow-on protein products: scientific issues, developments and challenges, Trends Biotechnol, 27(12), 2009, 698-705.
13. Jennings TA, Lyophilization, introduction and basic principles. Informa health care, New York, London. 2008.
14. Pikal MJ, Freeze-drying of proteins part I: process design, BioPharm., 3, 1990, 14-26.
15. Tang XC, Pikal MJ, Design of freeze-drying processes for pharmaceuticals: practical advice. Pharm. Res., 21, 2004, 191-200.
16. Nail SL, Gatin LA, Freeze-drying: principles and practice. Pharmaceutical Dosage Forms: Parenteral Medications. Marcel Dekker, Inc. New York. 1993.
17. Pikal MJ, Roy ML, Shah S, Mass and heat transfer in vial freeze-drying of pharmaceuticals: role of the vial, J. Pharm. Sci., 73, 1984, 1224-1237.
18. Pikal MJ, Use of laboratory data in freeze-drying process design: heat and mass transfer coefficients and the computer simulation of freeze-drying, J. Parenter. Sci. Technol., 39, 1985, 115-139.
19. Kadoya S, Izutsu K, Yonemochi E, Terada K, Yomota C, Kawanishi T, Glass-state amorphous salt solids formed by freeze-drying of amines and hydroxy carboxylic acids: effect of hydrogen-bonding and electrostatic interactions, Chem. Pharm. Bull., 56(6), 2008, 821-826.
20. Milton N, Pikal MJ, Roy ML, Nail SL, Evaluation of manometric temperature measurement as a method of monitoring product temperature during lyophilization, PDA J. Pharm. Sci. Technol., 51, 1997, 7-16.
21. Willemer H, Experimental freeze drying: producer and equipment. (Eds) Ray L, May JC, Freeze drying /Lyophilization of pharmaceutical and biological product, New York, 1999, 105-108.
22. Alers MJ, Parenteral preparation, in: Remington-The science and practice of pharmacy. Lippincott Williams and Wilkins, USA, 2006, 828-831.
23. Robinson LC, A gas chromatographic method of measuring residual water in freeze-dried smallpox vaccine, Bull. Org. mond. Sante and Bull. Wld Hlth Org., 47, 1972, 7-11.
24. Patel SM, Doen T, Pikal MJ, Determination of end point of primary drying in freeze-drying process control, AAPS. Pharm. SciTech., 11(1), 2010, 73-84.
25. Kauppinen A, Toiviainen M, Korhonen O, Aaltonen J, Jarvinen K, Paaso J, Juuti M, Ketolainen J, In-line multipoint near-infrared spectroscopy for moisture content quantification during freeze-drying, Anal. Chem., 85(4), 2013, 2377-2384.
26. Genin N, Rene F, Corrieu G, A method for on-line determination of residual water content and sublimation end-point during freeze-drying, Chem. Eng. Process. Process Intensif., 35(4), 1996, 255-263.
27. Schneid S, Gieseler H, Kessler WJ, Optimization of the secondary drying step in freeze drying using TDLAS Technology, AAPS. Pharm. SciTech. 12(1), 2011, 379-387.
28. Schneid S, Gieseler H, Process analytical technology (PAT) in freeze drying: tunable diode laser absorption spectroscopy as an evolving tool for cycle monitoring, Eur. Pharm. Rev., 13(6), 2009, 18-25.
29. Schneid S, Gieseler H, Evaluation of a new wireless temperature remote interrogation system (TEMPRIS) to measure product temperature during freeze drying, AAPS. Pharm. SciTech., 9(3), 2008, 729-739.
30. Tang XC, Nail SL, Pikal MJ, Evaluation of Manometric Temperature Measurement, a Process Analytical Technology Tool for Freeze-Drying: Part I, Product Temperature Measurement, AAPS. Pharm. SciTech., 7(1), 2006, E1-E9.
31. Tang XC, Nail SL, Pikal MJ, Evaluation of manometric temperature measurement, a process analytical technology tool for freeze-drying: part II measurement of dry-layer resistance, AAPS. Pharm. SciTech., 7(4), 2006, E1-E8.
32. Tang XC, Nail SL, Pikal MJ, Evaluation of manometric temperature measurement (MTM), a process analytical technology tool in freeze drying, part III: heat and mass transfer measurement., AAPS. Pharm. SciTech., 7(4), 2006, E1-E7.
33. Mujat M, Greco K, Galbally-Kinney KL, Optical coherence tomography-based freeze-drying microscopy, Biomed. Opt. Express., 3(1), 2012, 55-63.
34. De Beer TRM, Alleso M, Goethals F, Coppens A, Vander Heyden Y, Lopez De Diego H, Rantanen J, Verpoort F, Vervaet C, Remon JP, Baeyens WRG, Implementation of a process analytical technology system in a freeze-drying process using Raman spectroscopy for in-line process monitoring, Anal. Chem., 79(21), 2007, 7992-8003.
35. De Beer TRM, Vercruysse P, Burggraeve A, Quinten T, Ouyang J, Zhang X, Vervaet C, Remon JP, Baeyens WR, In-line and Real-time process monitoring of a freeze drying process using raman and NIR spectroscopy as complementary process analytical technology (PAT) tools, American Pharmacists Association J. Pharm. Sci., 98, 2009, 3430-3446.

36. Romero-Torres S, Wikstrom H, Grant ER, Taylor LS, Monitoring of mannitol phase behavior during freeze drying using non-invasive raman spectroscopy, PDA J. Pharm. Sci. Technol., 61(2), 2007, 131-145.

37. Mayeresse Y, Veillon R, Sibille PH, Freeze drying process monitoring, PDA J. Pharm. Sci. Technol., 61(3), 2007, 160-174.

38. Cavatur RK, Suryanarayanan R, Characterization of phase transitions during freeze drying by in situ X-ray powder diffractometry, Pharm. Dev. Technol. 3(4), 1998, 579-586.

39. Cook IA, Ward KR, Headspace moisture mapping and the information that can be gained about freeze-dried materials and processes, PDA J. Pharm. Sci. Technol., 65(5), 2011, 457-467.

40. Kasper JC, Wiggenhorn M, Resch M, Friess W, Implementation and evaluation of an optical fiber system as novel process monitoring tool during lyophilization, Eur. J. Pharm. Biopharm., 83(3), 2013, 449-459.

41. Arshad MS, Smith G, Polygalov E, Ermolina I, Through-vial impedance spectroscopy of critical events during the freezing stage of the lyophilization cycle: The example of the impact of sucrose on the crystallization of mannitol, Eur. J. Pharm. Biopharm., 87(3), 2014, 598-605.

42. El-Nesr OH, Yahiya SA, El-Gazayerly ON, Effect of formulation design and freeze-drying on properties of fluconazole multilamellar liposomes, Saudi Pharmaceutical Journal, 18, 2010, 217-224.

43. Vega E, Egea M, Calpena AC, Espina M, Garcia ML, Role of hydroxypropyl-β-cyclodextrin on freeze-dried and gamma-irradiated PLGA and PLGA-PEG diblock copolymer nanospheres for ophthalmic flurbiprofen delivery, Int. J. Nanomedicine., 7, 2012, 1357-1371.

44. Kusakabe H, Yanagimachi R, Kamiguchi Y, Mouse and human spermatozoa can be freeze-dried without damaging their chromosomes, Hum. Reprod., 23(2), 2008, 233-239.

45. Stoner GD, Chen T, Kresty LA, Aziz RM, Reinemann T, Nines R, Protection against esophageal cancer in rodents with lyophilized berries: potential mechanisms, Nutr. Cancer., 54(1), 2006, 33-46.

46. Stoner GD, Wang L, Zikri N, Chen T, Hecht S, Huang C, Sardo C, Lechner J, Cancer prevention with freeze-dried berries and berry components. Semin. Cancer. Biol. 17(5), 2007, 403-410.

47. Wang W, Lyophilization and development of solid protein pharmaceuticals, Int. J. Pharm., 203(1-2), 2000, 1-60.

48. Pingitore EV, Bru E, Nader-Macias ME, Effect of lyophilization and storage temperature on the activity of salivaricin CRL 1328, a potential bioactive ingredient of a urogenital probiotic product, J. Gen. Appl. Microbiol., 58, 2012, 71-81.

49. Borges S, Costa P, Silva J, Teixeira P, Effects of processing and storage on pediococcus pentosaceus sb83 in vaginal formulations: lyophilized powder and tablets, BioMed. Research International, 2013, 2013, 1-8.

50. Carpenter JF, Pikal MJ, Chang BS, Randolph TW, Rational design of stable lyophilized protein formulations: some practical advice, Pharm. Res., 14, 1997, 969-975.

51. Shalaev EY, The impact of buffer on processing and stability of freeze-dried dosage forms, part 1. Solution freezing behaviour, Am. Pharm. Rev. 8, 2005, 80-87.

52. Shalaev EY, Wang W, Gatin LA, Rational choice of excipients for use in lyophilized formulations, in: McNally EJ, Hastedt JE, (Eds), Protein formulation and delivery, Informa Healthcare, New York, 2008, 197-217.

53. Gomez G, Pikal MJ, Rodriguez-Hornedo N, Effect of initial buffer composition on pH changes during far-from equilibrium freezing of sodium phosphate buffer solutions, Pharm. Res., 18, 2001, 90-97.

54. Shalaev EY, Johnson-Elton TD, Chang L, Pikal MJ, Thermo physical properties of pharmaceutically compatible buffers at sub-zero temperatures: implications for freeze- drying. Pharm, Res., 19, 2002, 195-201.

55. Baheti A, Kumar L, Bansal A, Excipients used in lyophilization of small molecules, J. Excipients and Food Chem., 1(1), 2010, 41-54.

56. Nireesha GR, Divya L, Sowmya C, Venkateshan N, Niranjan Babu M, Lavakumar V, Lyophilization/Freeze Drying-An Review, International Journal of Novel Trends in Pharmaceutical Sciences, 3(4), 2013, 87-98.

57. Izutsu K, Kadoya S, Yomota C, Kawanishi T, Yonemochi E, Terada K, Freeze-drying of proteins in glass solids formed by basic amino acids and dicarboxylic acids, Chem. Pharm. Bull., 57(1), 2009, 43-48.

58. Bedu-Addo KF, Understanding lyophilization formulation development. Pharmaceutical technology, Lyophilization, 2004, 10-18.

59. Ramesh K, Dwivedi A, Tushar B, Sharma YP, Amol G, Lyophilization Of Pharmaceuticals: An Overview, Journal of Scientific Research in Pharmacy, 1(3), 2012, 12-17.

60. Day JG, Stacey GN, Cryopreservation and freeze-drying protocols, Second Ed. Humana Press Inc, Totowa, New Jersey. 2007.

61. Date PV, Samad A, Devarajan PV, Freeze Thaw: A simple approach for prediction of optimal cryoprotectant for freeze drying, AAPS. Pharm. Sci. Tech., 11(1), 2010, 304-313.

62. Quality risk management (Q9), ICH Harmonised Tripartite Guideline, 2005.

Source of Support: Nil, **Conflict of Interest:** None.