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Evaluation of Freeze-Drying Process of Aqueous Bovine Serum Albumin Dispersions

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ABSTRACT

Freeze drying (FD) has gained more importance as a method for heat sensitive drugs, such as proteins, hormones, and vaccines, as it could be used for improving long-term stability on distribution and storage. The aim of this work was to prepare and characterize freeze dried powders of bovine serum albumin (BSA). BSA was lyophilized in aqueous dispersions alone, and with excipients such as lactose, sucrose, and mannitol. To evaluate FD process, tests such as particle size, residual moisture, yield, and BSA assay were performed at both, directly after preparation, as well as during different storage conditions. The best results were obtained by using sucrose of 2% concentration, and the best result for stability during storage was at temperature of 4˚C. Our results confirmed that freeze-drying method is an efficient and suitable to obtained stable dried powders for heat sensitive pharmaceuticals such as albumin, and it could offer improved storage stability. In conclusion, successful lyophilization process must take into account two important factors, including the optimal formulation components and the understanding of critical parameters of the process.

Keywords: Freeze Drying; Nanoparticles; Bovine Serum Albumin; Particles Size; Residual Moisture

Introduction

Freeze drying [FD], also known as lyophilization, is a technical process widely used for pharmaceutical preparations for many reasons, such as for improving stability and long-term storage stability, especially for temperature sensitive drugs, such as proteins, hormones, and vaccines. It is used now as a novel method for loading drugs and targeting sick cells [1-3]. Suitable parameters of process application allow us to obtain best quality products compared to products dried with traditional methods. Very good physical and chemical properties make this method the best for drying exclusive products [2,4]. Freeze drying is the process that could take days to finish, and that one of its principal disadvantages. The time depends mainly on the design and optimization of the FD process. Freeze drying works on the principle of sublimation, where free water present in the material under process is frozen and directly converted into vapor state without entering the liquid phase [4,5]. A typical freeze-drying process consists of three stages which is: Freezing, primary drying, and secondary drying [2,6,7]. The freezing stage typically takes several hours to finish. Annealing is after freezing is simply holding the product at a temperature above the final freezing temperature for a defined period to crystallize the potentially crystalline components in the formulation during the freezing stage [1,2,8]. An annealing step is frequently necessary to allow efficient crystallization of the crystalline bulking agent, such as mannitol or glycine [1,2]. Primary drying, or ice sublimation, begins whenever the chamber pressure is reduced, and the temperature is raised to supply the heat removed by ice sublimation. The primary drying stage is the longest stage of freeze drying and optimization of this stage has a large impact on process economics [4,5]. Secondary drying is the stage where water is desorbed from the freeze concentrate at elevated temperature and low pressure. Secondary drying normally takes only hours, and the opportunity for time reduction by process optimization is limited [6-8].

The stability of the drug during freeze drying and storage, and the duration of the cycle are the two major considerations for freeze drying process optimization, although other parameters such as excipients, pressure, and primary and secondary drying temperatures have a great role on the FD process design [9-11]. To develop a successful protein formulation using freeze-drying procedure, physical properties, such as glass transition temperature (Tg) and residual moisture content, and operational parameters, should be considered, as well as the selection of suitable excipients [9,10]. Sugars, for example, as sucrose, lactose, mannitol and trehalose proved to stabilize various proteins during FD. They are the cryoprotectants of choice, and their stabilization mechanisms are well described by the water replacement and vitrification/ particle isolation theories [7,9,11]. Another example, PEG conjugation masks the protein's surface during FD and increases the molecular size of the polypeptide, thus preventing the approach of antibodies or antigen processing cells and reducing the protein's degradation [12]. The benefits of freeze-drying mainly for conserving proteins derived from its reduction in the deleterious chemical reactions as the diffusion of protein molecules is greatly inhibited in the vitrified solid-state [11,12]. Freeze-drying technology is not only used to prepare stable proteins, but also it is suitable for a wide range of applications, such as manufacturing biocarriers (e. g. Artificial oxygen carriers to reduce the need of patients for erythrocyte concentrates) [13], or for preserving the characteristics of the initial pharmaceuticals (e. g. Attenuated virus vaccines) [14], or for conserving natural biomaterials and cells in a dry form [15,16], or conserving food with best quality [17], or for increasing drug solubility [6,8,18] or for drug targeting and controlling release [19-21]. Bovine Serum Albumin (BSA), was chosen in our study as a model of protein. It is a non-glycosylated protein of 66 kDa, produced by the liver, and it is the most abundant protein in plasma. When it is heated to 50°C or above, albumin quite rapidly forms hydrophobic aggregates which do not revert to monomers upon cooling [4,5]. The aim of this work was to prepare stable freeze-dried powder of albumin, and to evaluate the optimal freeze-drying formulation.

Materials and Methods

Bovine Serum Albumin (BSA, fraction V) was purchased from Biowest- USA. Glutaraldehyde 8% aqueous solution, mannitol, sucrose, and lactose were purchased from Sigma- Germany. Bradford reagent, ethanol, phosphate buffered saline, sodium chloride, were purchased from Merck- Germany. All other chemicals and reagents were of analytical grade and used as received. Water was used as ultra-pure.

Preparation of BSA Suspension

200 mg BSA in 10 ml of 10 mM NaCl solution, adjusted to pH= 7.4 with phosphate buffer, then were transformed into nanoparticles by continuous addition of 8 ml ethanol 96% under constant stirring at room temperature. After obtaining a clear solution, 235 ml of glutaraldehyde solution was added with stirring for 24 hours at room temperature, then the resulting particles were centrifuged (using Sigma 3-16 KL centrifuge- Germany), then separated and redispersed to 10 ml of water to obtain a concentration of 2% of BSA only before lyophilization process [4].

Preparation of Sugar Solutions

Specific quantities of mannitol, sucrose and lactose were separately dissolved in water to obtain concentrations of 2,4, and 6%.

Preparation of Albumin-Sugar Suspensions

10 ml of freshly prepared sugar solution was added, before FD, to 10 ml of freshly prepared BSA suspension with continuous stirring for 2 h.

Freeze- Drying Process

1% and 2% of BSA suspension, sugar solutions of 1%, 2%, 3%, and mixtures of them were freeze dried as the following conditions:

a) Freezing: at -60˚C: 3 hours.

b) Annealing: at -40˚C: 6 hours.

c) Primary drying: -60˚C →-30˚C. Pressure: 0.1 mbar.

d) Secondary drying: -30˚C →+25˚C. Pressure: 0.1 mbar.

e) Using FreeZone Plus 2.5 Liter Cascade Freeze Dry Systems of Labconco apparatus. (Labconco Corp. Kansas City. MI. USA).

Determination of the Residual Moisture

The lyophilized samples (about 100 mg) were analyzed by a Karl Fischer titrator (Mettler DL 18- Mettler Toledo- USA).

Determination of Size and Size Distribution

The samples before lyophilization, were reconstituted by 10 ml of water, and analyzed by Coulter LS230-France, and this also applied to the lyophilized samples after preparation, and during storage.

Determination of yield%

Yield was determined by microgravimetry. (50 µl) of particles was put in a pan and dried for 2 h at 80˚C. The pans were weighed and the difference between the empty and filled pans was calculated [4,8].

BSA- Assay (Bradford Assay Method)

The Bradford protein assay, named after its developer Marion M. Bradford, is specifically used to calculate the concentration of total protein in a sample or solution.

Bradford Reagent Preparation: 100 mg of the reagent was dissolved in 50 ml of 95% ethanol and 100 ml of 85% phosphoric acid, mixed until completely dissolved, then water was added to 850 ml in volume.

BSA Standards Preparation: A set of 7 concentrations of BSA: 2, 1.5, 1, 0.75, 0.5, 0.25, 0.125 mg/ml) each was prepared by dissolving the accurate weight of BSA in the phosphate buffer (pH= 7.4), then 1.5 ml of Bradford reagent was added and incubated 10 min. The absorbance was measured at 595 nm (using T80 spectrophotometer from PG Instruments- UK), and the standard curve was performed. The blank, which is used to set the instrument to 0 absorbance was prepared in the same buffer as in the tested samples without BSA [22].

Sample Preparation: 20 mg of lyophilized BSA was dissolved in 3 ml of phosphate buffer (pH= 7.4) with stirring for 10 minutes at room temperature, then 1.5 ml of Bradford reagent was added, and the absorbance was measured [22].

Long- Term Stability

The lyophilized powders were stored at 2-8˚C, and 25˚C /60 RH for three months. Samples were characterized every month with regard of particle size, size distribution, and BSA assay. Table 1 below, shows the twelve studied formulations.

Statistical Analysis

All the data were presented as mean ± standard deviation (SD) of three separate experiments, and analyzed with t-test, $p < 0.05$ was indicative of significant difference between the test groups. The null hypothesis used in the statistical analyses propose there were no differences between certain characteristics of the generated data. When BSA was lyophilized without any excipient (in F1 and F2), the resulted powders were wet, sticky, and the residual moisture was nearly 5%. After reconstitution of the freeze-dried powder, large aggregates were produced (as shown in Table 2), and the cake appearance was visually evaluated especially in 2% BSA concentration. These observations ascertained the need of stabilizing the protein [4-6].

Results and Discussion

Table 2 shows the measurements of particles size: Z1 and Z2, before and after FD respectively, yield, and residual moisture. The obtained results indicated to the increasing in size of particles and decreasing in the residual moisture after freeze drying to almost the formulations. Aggregates were reduced as the particles size measurements showed, and that due to the stabilizing effect of the lactose sucrose, and mannitol. The formulations F7, F10, and F12 were selected after a statistical study for further investigations (long-term stability). These three formulations were stored either at a temperature = 4˚C or 25˚C / RH 60% for three months. The results of particles size and residual moisture are shown in Tables 3 & 4. As shown in Table 3, there is no considerable changes in size or residual moisture, which indicates the good storage conditions. The results showed, no considerable changes in size or residual moisture for F7 (Sucrose 2%), which indicates the good role of sucrose as a stabilizing agent in lyophilization process. The increase in particles size in F10 and F12 could be either because of mannitol crystallization or because of albumin aggregation. For further investigations, BSA assay in the three formulations F7, F10, and F12 was performed. BSA assay results for F7 (Figure 1) shows nearly the same results after three months of storage at 4˚C (97.5%), whereas a little bit less values at 25˚C (96.7%). This indicates no considerable change in albumin structure in both conditions, however the best values were when the temperature was at 4˚C. BSA assay results for F10 (Figure 2) shows nearly the same results after three months of storage at 4˚C (96.2%), but a decrease in values after three months of storage at 25˚C (from 96.5% to 92.1%). This indicates some changes in albumin structure or aggregations. BSA assay results for F12 (Figure 3) shows nearly the same results after three months of storage at 4˚C (96.7%), but a decrease in values after three months of storage at 25˚C (from 97.4% to 93.2%). This could be because of albumin aggregation or degradation.

Table 1: Freeze- dried BSA formulations.

Formulation	BSA %	Lactose %	Sucrose %	Mannitol%
F1	1	-	-	-
F2	2	-	-	-
F3	1	1	-	-
F4	1	2	-	-
F5	1	3	-	-
F6	1	-	1	-
F7	1	-	2	-
F8	1	-	3	-
F9	1	-	-	1
F10	1	-	-	2
F11	1	-	-	3
F12	1	-	1	1

Table 2: Particle size, yield, and residual moisture measurements.

Formulation	Z1 nm	Z2 nm	Yield %	RM%
F1	192.8 ± 2.4	1890 ± 20.9	73.3 ± 5.3	5.1 ± 0.2
F2	185.3 ± 4.3	1854 ± 28.5	78.2 ± 6.8	5.3 ± 0.1
F3	179.2 ± 5.1	285.8 ± 6.2	84.1 ± 2.9	2.1 ± 0.1
F4	174.3 ± 3.0	290.2 ± 5.3	87.0 ± 3.2	1.85 ± 0.1
F5	175.7 ± 5.1	261.3 ± 8.7	85.7 ± 4.2	1.9 ± 0.2
F6	201.0 ± 2.4	321.1 ± 2.3	90.8 ± 1.8	1.6 ± 0.1
F7	195.6 ± 3.6	316.2 ± 2.1	95.1 ± 0.9	1.2 ± 0.1
F8	191.5 ± 2.9	312.9 ± 3.2	92.2 ± 1.9	1.9 ± 0.2
F9	197.9 ± 5.7	426.1 ± 2.5	90.3 ± 2.4	1.8 ± 0.1
F10	188.4 ± 3.9	421.6 ± 3.6	93.1 ± 0.9	1.4 ± 0.1
F11	198.3 ± 2.8	409.2 ± 2.4	89.0 ± 1.1	1.9 ± 0.1
F12	199.4 ± 6.8	353.0 ± 4.5	92.8 ± 0.8	1.3 ± 0.1

Table 3: Particle size, and residual moisture measurements (Storage at 4˚C).

Storage at 4˚C	F7	F10	F12
After preparation	Z= 316.2	Z= 421.6	Z= 352.0
	RH= 1.2	RH= 1.4	RH= 1.3
After 3 months storage	Z= 320	Z= 424.8	Z= 360.1
	RH= 1.2	RH= 1.5	RH= 1.3

Table 4: Particle size and residual moisture measurements (Storage at 25˚C).

Storage at 25˚C	F7	F10	F12
After preparation	Z= 316.2	Z= 421.6	Z= 352.0
	RH= 1.2	RH= 1.4	RH= 1.3
After 3 months storage	Z= 320	Z= 520.2	Z= 470.6
	RH= 1.3	RH= 1.9	RH= 1.5

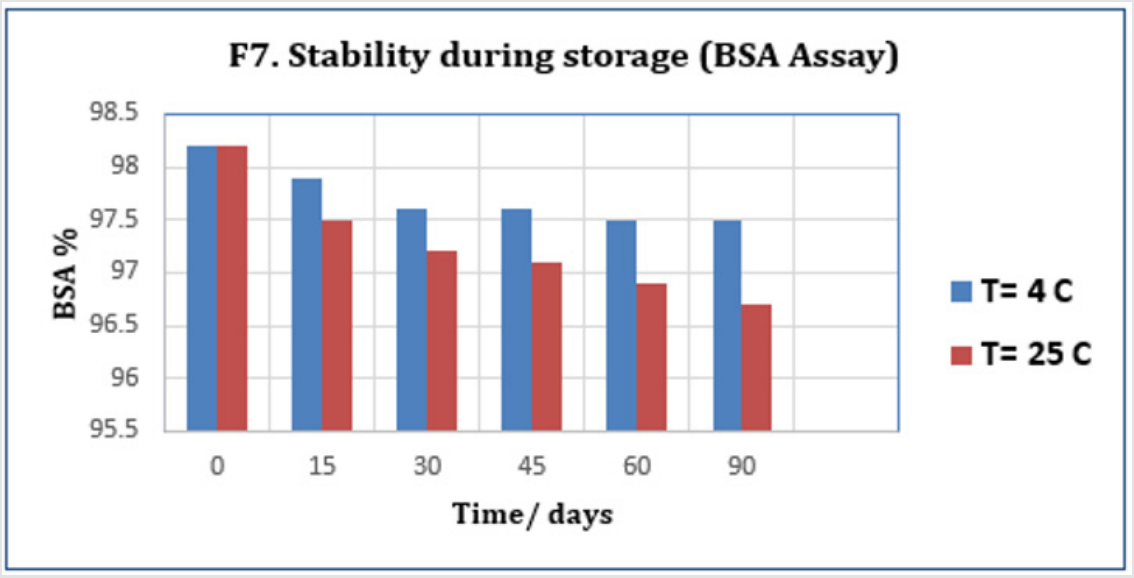

Figure 1: BSA assay for F7 during storage (T= 4 ºC or 25 ºC).

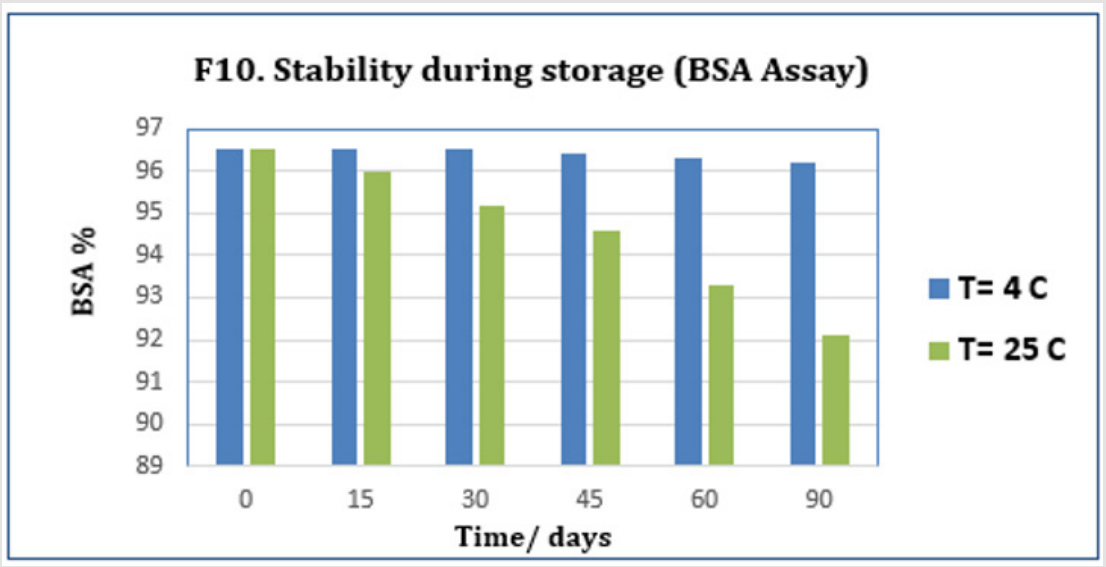

Figure 2: BSA assay for F10 during storage (T= 4 ^{O}C or 25 ^{O}C).

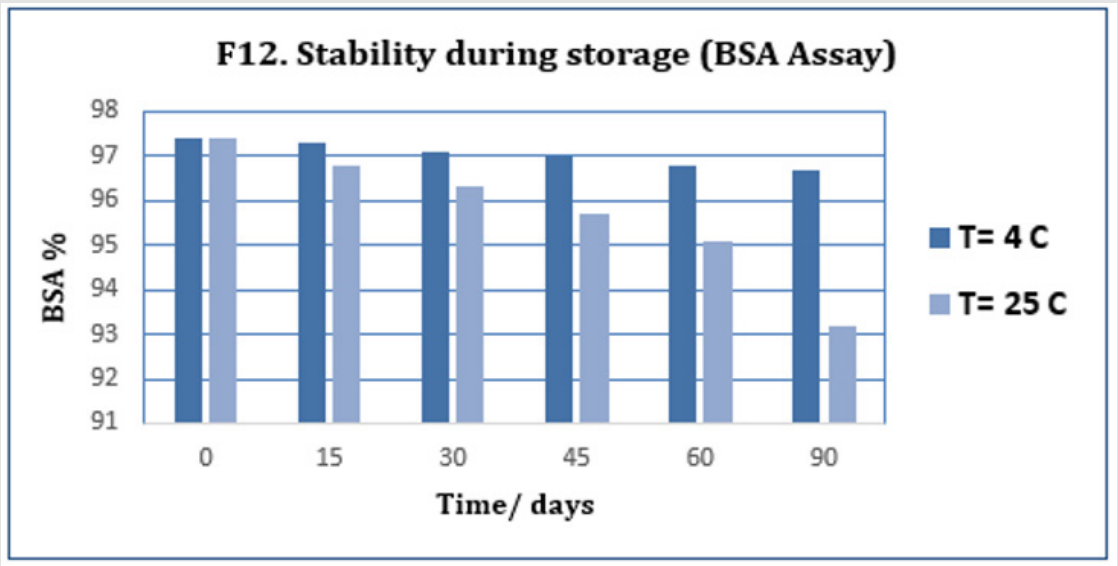

Figure 3: BSA assay for F12 during storage (T= 4 ^{o}C or 25 ^{o}C).

Conclusion

Freeze drying used to obtain stability of heat sensitive active pharmaceuticals during distribution and storage conditions. The present work shows that when BSA was lyophilized alone, without any excipient, large aggregates in the micrometer size range were observed. Stabilizing excipients in formulation, such as lactose, sucrose, and mannitol were necessary to obtain a stable freeze dried BSA both during the freeze-drying process, as well as for long term storage. Stability was assessed by aggregation state (particle diameter), residual moisture, and protein assay. The best results were with sucrose 2%, and storage temperature at 4 ˚C. In conclusion, successful lyophilization process must take into account two important factors, including the optimal formulation components and the understanding of critical parameters of the process.

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