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In situ precipitation and vacuum drying of interferon alpha-2a: Development of a single-step process for obtaining dry, stable protein formulation

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ABSTRACT

Feasibility studies were performed to develop a process for obtaining stable dry protein formulations based on *in situ* polyethylene glycol (PEG)-induced precipitation and vacuum drying of interferon alpha-2a (IFNα2a) solution in a vial. Using a laboratory scale freeze dryer, the process was carried out in two phases: first, protein solution containing PEG was concentrated to achieve protein precipitation, and second, remaining water was removed by further reducing the chamber pressure. Drying conditions, i.e. temperature and pressure, and solution composition were selected to ensure maximal precipitation (solubility of IFNα2a), to achieve precipitation without boiling, and to ensure stability. Dried formulations were subjected to stability studies (40 °C). Concentration and precipitation could be achieved at a fast rate by utilizing pressures slightly above the vapor pressure of water. Fluorescence and circular dichroism (CD) studies showed that precipitated IFNα2a maintained its native structure. Fourier transform infrared spectroscopy (FTIR) studies showed that IFNα2a when dried in the presence of trehalose, maintained its secondary structure. Trehalose also prevented formation of aggregates during drying. Moisture contents of 1% (w/w) were achieved within 48 h of drying. Dry formulation containing 1:20:100 (w/w) IFNα2a:trehalose:mannitol was stable against aggregation and oxidation (6% oxidized at 40 °C, 6 months). Stability profile was comparable to a similar lyophilized formulation.

1. Introduction

Stability problems often arise in protein formulations due to the complex native structure of protein molecules. These problems are usually associated with manufacturing and processing as well as with the long-term storage stability and manifest in the form of physical instability (aggregation, precipitation or unfolding) and/or chemical instability (oxidation, deamidation or β-elimination) (Arakawa et al., 1993; Wang, 1999; Cleland and Langer, 1994; Manning et al., 1989). In order to minimize these instabilities, proteins are frequently formulated in the dried state. Freeze drying or lyophilization has been the process of choice for the preparation of dry protein powders (Pikal, 1994; Wang, 2000). However, in recent years there has been an increased level of interest towards the development of alternative drying technologies. This is because freeze drying, though successful for several proteins, has certain disadvantages. These include the instabilities incurred on proteins due to the inherent steps involved in the process of lyophilization, long processing times, and expensive setup and maintenance of the lyophilization plant (Gomez et al., 2001; Strambini and Gabellieri, 1996; Chang et al., 1996; Izutsu et al., 1998).

Other methods that are being evaluated and reported in literature include spray drying (Munmenthaler et al., 1994; Maa et al., 1998a), spray-freeze drying (Munmenthaler and Leuenberger, 1991), bulk crystallization (Shenoy et al., 2001), supercritical fluid drying (Moshashaee et al., 2000; Winters et al., 1996), simple evaporative or vacuum drying (Roser, 1991; Mattern et al., 1997) and foam drying (Brohnstein, 2003). Although all of the above mentioned techniques have shown promise, limitations still exist in each of these processes. For example, use of organic solvents in supercritical fluid technology can lead to the perturbation of the native structure of protein molecules, and presence of large air–water interface has been associated with protein aggregation in spray drying (Winters et al., 1996; Maa et al., 1998b). Similarly, simple evaporative drying may require long drying times (Mattern et al., 1997). Hence, despite the continued emergence of the new techniques, there is a need for a drying process which is economical,

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does not create protein stability problems and can be utilized for a given protein, resulting in a desired shelf-life of the product.

Recently, Sharma and Kalonia have reported that precipitation of proteins by polyethylene glycols (PEGs) under favorable conditions followed by vacuum drying of the precipitate can be used for the formulation of proteins as dry stable powders (Sharma and Kalonia, 2004). The process is simple, economical, less time consuming and preserves protein structure and conformation during drying and upon storage at accelerated temperatures. This method offers several advantages including prevention of the protein from cold denaturation, freezing induced denaturation and short duration of drying process (1–2 days). However, this process involved multiple steps (precipitation, filtration, mixing of the precipitate with the stabilizer, vacuum drying of the mixture and powder filling in vials) in order to achieve the final dry protein formulation in a vial. Nevertheless, the advantages offered leads to opportunity for further development of this process as a single-step technology that can be utilized in industry in a setting similar to freeze drying.

The above authors have also demonstrated the utility of PEGs as the precipitating agents of choice during the development of their drying process (Sharma and Kalonia, 2004). PEGs have also been used earlier to carry out the crystallization of proteins for therapeutic use (Shenoy et al., 2001). Utility of PEGs as the precipitating agents of choice is because of their following properties: (1) negligible effect on the native structure of the protein (higher molecular weight PEGs may affect the tertiary structure); (2) required in relatively small amounts for precipitation; and (3) approved for parenteral use (Sharma and Kalonia, 2004; Polson et al., 1965; Zeepezauer and Brishammar, 1974; Haire et al., 1984; Ingham, 1978). Though, phase separation of proteins can also be carried out by utilizing some of the other precipitating agents such as salts (ammonium sulfate) or organic solvents (ethanol), their utility in a protein drying process is limited because of the disadvantages associated with the use of these precipitating agents (Melander and Harvarth, 1977; Askonas, 1951). For example, salts are required in high concentrations while use of organic solvents may result in the denaturation of proteins. Since the principle objective for any drying process is the transformation of the protein to the solid state by removal of water while maintaining the native structure of the protein, it is worthwhile to mention that phase separation of proteins by PEGs with least denaturation is by far the most important advantage that PEGs offer, and hence makes them of immense use in the development of precipitation related drying process.

Polyethylene glycols are non-ionic polymers with a general formula $HO–(CH_2CH_2O)_n–H$. The mechanism of the precipitation of proteins by PEGs has been well documented. Phase separation of proteins by different molecular weight PEGs has been explained on the basis of steric exclusion mechanism, according to which proteins are excluded from the immediate vicinity of the PEG molecules due to steric effects (Atha and Ingham, 1981; Arakawa and Timasheff, 1985a). Steric exclusion of PEGs leads to the preferential hydration of proteins resulting in the maintenance of the protein native structure.

The present paper describes the development of a process based on *in situ* protein precipitation by PEGs followed by evaporative drying. The process was designed to achieve dry protein formulation from its aqueous solution directly within the vial (*in situ* stresses on the fact that protein precipitation and drying are performed within the vial once the vials filled with a protein solution have been loaded within the dryer). The process is thus a modification of the process that was developed by Sharma and Kalonia. The modified technique eliminates the need for the steps of centrifugation/filtration and bulk powder filling thus making the process more practical for industrial use with minimal concerns about sterility issues. In this method, the precipitation of protein is carried out from its aqueous solution containing the suitable precipitating agent by removal of water under moderate vacuum, thus increasing the concentration of the precipitating agent until desired precipitation of the protein is achieved. Further drying is achieved by utilizing high vacuum thus facilitating rapid removal of remaining water resulting in dried protein ready for pharmaceutical and therapeutic use. The overall objective of this work was to develop a single-step drying process that is available to protein formulators as an alternative to lyophilization.

Based on the previous study, Recombinant human interferon alpha-2a (IFNα2a) was used as the model protein for these studies (Sharma and Kalonia, 2004). IFNα2a has 165 amino acids and has a molecular weight of 19.2 kDa (Hochuli, 1997). IFNα2a is an all helical protein (65% alpha helical) with a p*I* in the range of 6.0–7.0 (Klaus et al., 1997). The protein is found to maintain its native structure in the pH range of 4.0 and 8.0 (Sharma and Kalonia, 2003a). IFNα2a is chosen as the model protein for the drying studies since it is well characterized, is susceptible to oxidation (Gross et al., 1998) (since PEGs have peroxides, which accumulate on storage (Johnson and Taylor, 1984; Kumar and Kalonia, 2006), this provides an opportunity to evaluate effect of peroxides, if present, on oxidation of IFNα2a) and has propensity to unfold during the normal vacuum-drying process (Sharma and Kalonia, 2003b). IFNα2a in solution is also known to undergo aggregation on storage (Gross et al., 1998).

This study shows that *in situ* protein precipitation by PEGs followed by vacuum drying can be used as a suitable process for the formulation of proteins in the dried state. Results are presented in detail on the selection and optimization of the formulation, development of the process for drying and on the stability studies of various formulations that were prepared by PEG precipitation and vacuum drying. Finally results from the accelerated storage stability studies on PEG precipitated and vacuum dried IFNα2a are compared with that of similar freeze dried formulation in order to compare the long-term stability of dry protein produced by the two methods.

2. Materials and methods

2.1. Materials

All buffer reagents were of the highest purity grade available from commercial sources and were used without further purification. PEG 1450 was obtained from Acros Organics (Geel, Belgium) and PEG 3350 was obtained from Fischer Scientific (Fair Lawn, NJ). D-Mannitol was obtained as fine crystalline powder United States Pharmacopeias grade from Cerestar USA Inc. (Hammond, IN). Trehalose dihydrate was obtained from Sigma–Aldrich (St. Louis, MO). IFNα2a was donated generously by Hoffmann-La Roche and was supplied as 1.6 mg/ml solution in 25 mM acetate buffer, containing 120 mM NaCl (total ionic strength = 142 mM). Protein was stored at −80 °C in small vials and each vial was thawed before use. 5-cm^3 tubing clear vials with 20 mm finish (diameter × height: 22 mm × 40 mm, i.d. × o.d.: 13 mm × 20 mm, catalogue no. 223685) were purchased from Fischer Scientific (Millville, NJ). Fluoretec stoppers (V10 451, Single vent, 20 mm lyophilization) were purchased from West Pharmaceuticals (Lititiz, PA).

2.2. Solubility studies

Solubility of IFNα2a in the presence of PEG 3350 was obtained as a function of PEG concentration, ionic strength and excipients (trehalose and mannitol). All studies were conducted at 25 °C and pH 6.5. pH 6.5 is the midpoint of the isoelectric pH range of IFNα2a (protein has lowest apparent solubility at the isoelectric point

(Sharma and Kalonia, 2004) and hence pH 6.5 was chosen in order to minimize the concentration of PEG required to precipitate the protein out of the solution). Stock solutions of the protein, PEG and excipients were prepared at pH 6.5 utilizing phosphate buffer. Adjustments of ionic strengths if required were made using NaCl. IFNα2a was precipitated by adding aliquots of buffered PEG stock solution to the solution of IFNα2a (in order to study the effect of excipients, PEG stock solution was added to the solution of IFNα2a containing the required excipients) to vary the PEG concentration. The final volume of the solution was adjusted to 1.0 ml.

The final solutions obtained were mixed well and equilibrated for 2 h. The protein suspensions thus obtained were centrifuged at $3600 \times g$ for 30 min to separate the protein precipitate. Protein solubility was obtained by measuring absorbance of the supernatant at 280 nm ($E^{1\%}$ 9.3 for IFNα2a). Since precipitation by PEG results in the formation of amorphous protein phase, the solubility is referred to as apparent solubility rather than true solubility (McPherson, 1985; Smatanova, 2002).

2.3. Structural characterization of the precipitated IFNα2a

Addition of PEG to the solution of IFNα2a results in the precipitation of the protein. Fluorescence spectra of the protein suspension obtained after the protein has been precipitated out of the solution by PEG and that of the native protein in solution were compared. The protein precipitate was collected after centrifugation and reconstituted in acetate buffer pH 5.0, and the secondary and tertiary structure of the reconstituted IFNα2a was compared with the structure of the native IFNα2a using Far and near UV circular dichroism (CD) spectroscopy.

2.3.1. Fluorescence spectroscopy

Fluorescence measurements were carried out using a PerkinElmer LS-50 Luminescence spectrometer. The excitation wavelength was fixed at 295 nm and the emission scans were collected between 310 and 400 nm. An excitation slit width of 10 nm and emission slit width of 5 nm was used to collect all the emission scans. A scan speed of 20 nm/min was selected to improve the signal to noise ratio. For every solution, 5 spectra were accumulated and averaged to get the final spectrum. The solutions or the suspensions were equilibrated at 25 °C and were continuously stirred with a small magnetic bar. All spectra were corrected for the Raman peak by subtraction of the emission scan of the buffer from the fluorescence emission scan of the solution or the suspension. The fluorescence emission spectra were normalized to an intensity of 1 at the λ_{max} using FL-winlab software (PerkinElmer Instrument Corporation (Wellesley, MA).

2.3.2. CD spectroscopy

Measurements were performed using a Jasco-710 spectropolarimeter. The far UV-CD studies were performed in a 0.1 cm path length cell using a protein concentration of 0.1 mg/ml and a scan speed of 20 nm/min from 200 to 260 nm. The near UV-CD studies were performed in a 0.2 cm path length cell using a protein concentration of 2 mg/ml and a scan speed of 50 nm/min from 250 to 310 nm. A total of 5 spectra were accumulated and averaged.

2.4. Vacuum-drying studies

The first step in the development of the drying process was to determine conditions under which water can be evaporated without boiling from solutions containing the desired formulation in order to concentrate the components to an extent such that the protein could be completely precipitated (≥90% precipitation) by utilizing minimal amounts of the precipitant. These studies were conducted by placing the vials with water or with the appropriate formulation in a laboratory scale Durastop lyophilizer (FTS/Kinetics Thermal Systems, Stoneridge, NY). Vials were stoppered with the vents open and placed on the loading tray in a hexagonal arrangement (each vial is surrounded by six other vials except the ones that are on the edges which were always empty). The load was then transferred from the tray to the shelf of the dryer. Conditions for the initial slow evaporation were hence determined.

Formulations for drying were prepared by mixing appropriate aliquots of protein, PEG, mannitol and trehalose in the desired ratios from their stock solutions. Vacuum drying of the formulations was performed in two steps. In the first step the formulations were concentrated to the desired volume of the solution by utilizing appropriate conditions (see Section 3). Once the solution had been reduced to desired volumes and complete precipitation achieved, the second step was initiated by reducing the pressure to 100 mTorr while maintaining the shelf temperature. The drying was performed at this pressure for a maximum of 48 h. The volume of the solution kept for drying was 1.0 ml. All formulations that were dried contained 0.4 mg of the protein. Formulations that were dried contained protein:trehalose:mannitol in different weight ratios (the formulation will thus be referred to as *x*:*y*:*z* formulation). In addition formulations also contained PEG 3350 as the precipitating agent. The amount of the precipitating agent was different for different formulations (see Section 3).

Karl Fischer titrimetry was used to determine the moisture content of the dried formulations. Secondary structure of IFNα2a in the dried formulations was done by using second derivative transmission Fourier transform infrared spectroscopy (FTIR). Presence of soluble and insoluble aggregates of IFNα2a in the reconstituted dried formulations was done by size exclusion chromatography (SEC-HPLC) and UV spectroscopy respectively.

2.4.1. Moisture analysis

Moisture content was determined using an Orion AF7LC Coulometric Karl Fischer Titrimeter (Orion Research Inc., Boston, MA). Samples were analyzed dissolving or dispersing in methanol/formamide (75%/25%) and using dry methanol/formamide (75%/25%) as blank.

2.4.2. Analysis of secondary structure

The secondary structure of IFNα2a was evaluated by obtaining area normalized second derivative FTIR spectra on a Nicolet Magna 560 FTIR spectrometer (Nicolet Inc., Madison, WI). FTIR spectra of original IFNα2a sample were recorded using a demountable pathlength cell with CaF_2 windows. The pathlength was fixed by placing a 6 μm mylar spacer. A solvent blank spectrum was collected with water. Transmission spectra of dry samples were obtained by KBr pelletization. Approximately 0.5 mg of IFNα2a was mixed with 200 mg of KBr (dried overnight at 40 °C) and transferred to a stainless steel die. The die was placed on a 12 ton hydraulic press (Carvar, Wabash, IN) and evacuated with an attached vacuum pump. Pellets were prepared by pressing at 12,000 psi for approximately 2 min. A total of 128 scans were accumulated in 4000–400 cm^{-1} region at 4 cm^{-1} instrument resolution. The second derivatives of these scans were obtained in 1700–1600 cm^{-1} region (amide I). The second derivatives were baseline corrected and finally area normalized to unit area for relative comparisons (Dong et al., 1995; Heller et al., 1997). Quantitative analysis of the second derivative spectra was carried out using the area overlap method. The area of overall values quantitates the similarity between two spectra. The values are on a scale 0–100 (the greater the similarity, the higher the value). The values were calculated using the method described by Gribenow et al. (1999). It should be realized that the authors had normalized

the spectra to an area of 100 (unit area in the present case). Hence, the values obtained were multiplied by 100.

2.4.3. Estimation of insoluble and soluble aggregates

The amount of insoluble aggregates that may have formed during drying was determined by UV spectroscopy. The dried samples were reconstituted and filtered through a 0.45 μm filter. The concentration of the protein in the filtrate was determined by measuring the absorbance at 280 nm.

The amount of soluble aggregates was determined by analyzing reconstituted and filtered samples using size exclusion chromatography (SEC-HPLC) attached online to a UV detector set at 280 nm and a 90° light scattering detector by Precision Detectors (Bellingham, MA). A waters YMC-Pack Diol-60 column, DL06S05-3008WT (300 mm × 8.0 mm inner diameter, 5 μm particle size), which is a diol end-capped silica based column (Waters Corp., Milford, MA), was used. The mobile phase was 25 mM acetate buffer, pH 4.5, with 125 mM NaCl and the flow rate was 1 ml/min.

2.5. Stability studies

Stability studies were performed on samples that were dried by using precipitation and vacuum-drying technique. Accelerated stability studies were carried out at 40 °C for 6 months. Stoppered vials were sealed with aluminum seals and stored in desiccators containing anhydrous calcium sulfate (Fischer Scientific, Pittsburg, PA) to maintain 0% relative humidity. All control samples were placed at −20 °C in order to stay consistent with the previously published results (Sharma and Kalonia, 2004). Different formulations that were dried and kept on stability included: (1) IFNα2a:trehalose:mannitol (1:20:100, w/w); (2) IFNα2a:trehalose:mannitol (1:20:0, w/w), (3) IFNα2a:trehalose:mannitol (1:0:0, w/w); and (4) IFNα2a:trehalose:mannitol:methionine (1:20:100:2.5, w/w). Methionine was included in the last formulation to study its utility as an antioxidant. Please note that all these formulations included PEG 3350 as the precipitating agent. For comparison, a formulation (without PEG) containing 1:20:100 (w/w) IFNα2a:trehalose:mannitol was also prepared by lyophilization and subjected to stability studies. The lyophilized formulation was prepared in FTS Durastop lyophilizer. The lyophilization cycle was as follows: samples were cooled to 5 °C for 30 min followed by cooling at −5 °C for 30 min before freezing at −40 °C for 2 h. The shelf temperature was increased to −25 °C at 0.3 °C/min and the chamber pressure was decreased to 100 mTorr for 20 h. The shelf temperature was then increased to 25 °C at 0.2 °C/min and the vacuum was retained at 100 mTorr. The secondary drying was performed for 10 h. Stability samples at various time points were assessed for insoluble aggregates, soluble aggregates and for oxidized species. Methodology for the determination of soluble and insoluble aggregates has already been described earlier.

Oxidation of IFNα2a at different time points was assessed utilizing reverse phase HPLC. The studies were performed by using a 30 nm, 250 mm × 2.1 mm inner diameter Vydac C_{18} column, 218TP54 (Grace Vydac, Hesperia, CA) attached to a UV detector with wavelength set at 220 nm. The mobile phase contained solvent A (0.1% trifluoroacetic acid [TFA] in water), B (0.1% TFA and 43.5% acetonitrile in water) and C (0.1% TFA and 50.0% acetonitrile in water). An isocratic elution with flow rate 1 ml/min was used for separation according to the following program; mobile phase B for 25 min followed by mobile phase C for 30 min. The column was equilibrated for 30 min with mobile phase A between injections. The oxidized species were identified by comparing the chromatograms of the samples to that obtained by the oxidation of the IFNα2a at pH 5.0 by 100 mmol/l H_2O_2 in 5 min. Oxidized species eluted at 41 min while the native species eluted at 42 min (Fig. 1).

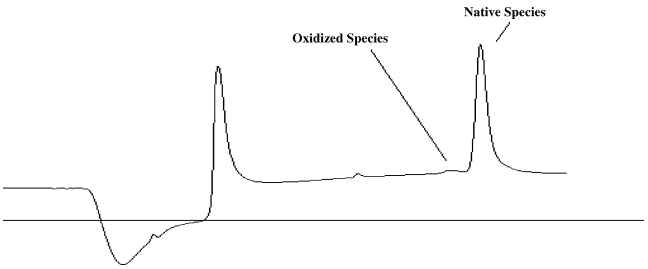

Fig. 1. Separation and quantification of IFNα2a and its oxidized species by RP-HPLC.

3. Results and discussion

3.1. Drying process

Conceptually, the process of drying was designed to be divided into two phases. During the first phase of drying, concentration of components of the protein solution (containing protein along with the desired precipitating agent) would be increased by evaporation to achieve precipitation of the protein. Once the desired precipitation has been achieved, the second phase would be initiated, wherein high vacuum would be used to facilitate rapid removal of water.

The first aim of the studies was to determine conditions under which water can be evaporated without boiling from solutions containing the desired formulation. This was necessary to concentrate the components, through removal of water, to an extent such that the protein could be completely precipitated (>90% precipitation) out of the solution by utilizing low amounts of the precipitant, i.e. by achieving precipitation at low volumes of the solution. Development of this first phase of drying was essential because of the following two reasons: (1) since the process was aimed to be a single-step *in situ* process, wherein the used precipitant would form an integral part of the final dried formulation, it was important that the amount of the precipitant used is minimized, and (2) incorporation of the slow evaporation step eliminates any issues such as viscosity that may be associated with the handling of the concentrated protein suspensions (it should be realized that the precipitation could well be performed at low volumes and that these low volume precipitated suspensions could well be placed in the dryer for drying).

The primary objective of the first phase of drying was to achieve evaporation without boiling, at a fairly fast pace. The importance of time is obvious. Boiling on the other hand would result in the generation of air–water interface which is undesirable as proteins are prone to denaturation at interfaces (Lechevalier et al., 2003; Postel et al., 2003). Additionally, complete precipitation of the protein during this first phase of the drying process would minimize the exposure of the protein to the air–water interface that would be generated during the second phase of drying. Moreover, boiling of dilute solution that is present during this initial phase of drying may result in significantly compromised product appearance.

Different factors that were anticipated to affect the removal of water such as chamber pressure, dryer load and temperature were investigated. Fig. 2 shows that rate of removal of water at 25 °C and 25 Torr is relatively fast. The rate of removal of water (the rate was determined from the slope of the curve) was 4.4 mg/min when 10 vials (1.0 ml fill) were loaded onto the shelf. The rate of removal of water decreased with increase in the vial load. The rate of evaporation was 4.37 mg/min when 20 vials were loaded onto the shelf

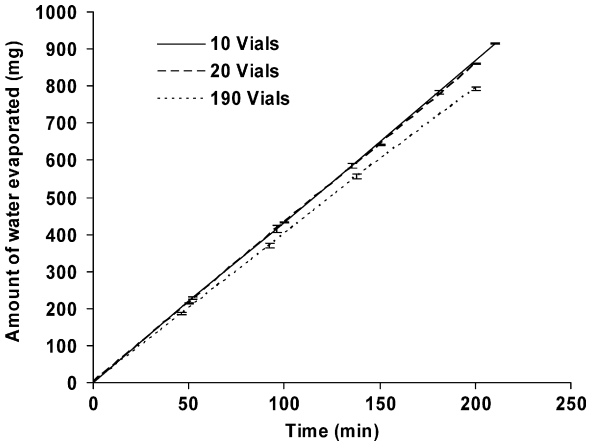

Fig. 2. Effect of vial load on the evaporation of water from 5 ml vials (22 mm × 40 mm) with 1 ml fills at 25 °C and 25 ± 0.5 Torr.

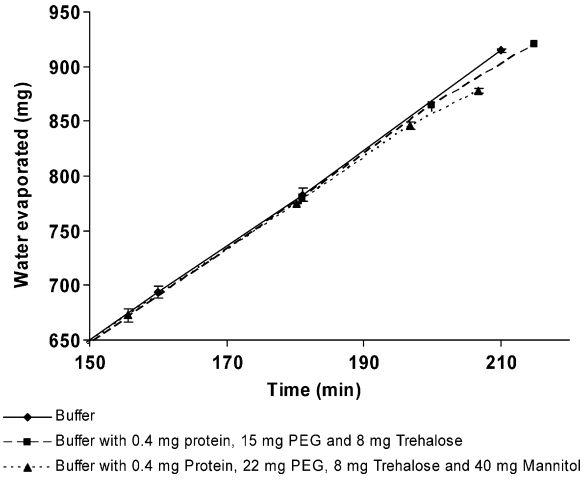

Fig. 3. Effect of formulation (pH 6.5, ionic strength 15 mM) constituents on the evaporation of water from 5 ml vials (22 mm × 40 mm) with 1 ml vial fills and a vial load of 10 at 25 °C and 25 ± 0.5 Torr.

and was 3.95 mg/min when 190 vials were loaded onto the shelf. Visual inspection of the evaporation process did not show any boiling. At 25 °C, pressures lower than 20 Torr resulted in boiling of the solution (vapor pressure of water at 25 °C is ≈24 Torr). Since a decrease in temperature results in a decrease in the vapor pressure of water, lower shelf temperatures at lower pressures did not significantly affect the rate of removal of water (data not shown). Sharma et al. conducted a temperature dependent CD study and showed that subtle change in the tertiary (and secondary) structure of IFNα2a started occurring as low as 30 °C and hence temperatures higher than 25 °C were not investigated in this regards (Sharma and Kalonia, 2003a). 25 °C and 25 Torr were hence chosen as the parameters of choice for the first phase of vacuum drying.

The next step was to select the time point to initiate second phase of evaporation to achieve maximal removal of residual water. In general, process of evaporation under moderate vacuum was continued until complete precipitation of the protein was achieved at low volumes of the solution (confirmation of complete precipitation was done by doing solubility studies that are discussed in one of the following sections). The shelf pressure was then reduced to 100 mTorr and rapid evaporation was initiated and the second phase of drying was continued for 48 h.

Selection of the time point and/or volume of the solution at which lower pressure is switched (time point when the second phase of drying is initiated) is important because of following three reasons: (1) moisture content of the final dried product is anticipated to be dependent on the time point at which rapid evaporation is initiated (rapid evaporation from solutions of differing viscosities will result in differential consistencies/porosities of the product) (Sankat and Castaigne, 2004; Abdul-Fattah et al., 2007); (2) differential consistency/porosity of the product will result in differential appearance of the dried formulation (because of differential foam thickness) (Sankat and Castaigne, 2004), and (3) the rate of evaporation of water under moderate vacuum was anticipated to decrease (and hence the drying time) with increase in the concentration of components (due to the elevation in boiling point). It was also anticipated that the effect of the factors mentioned above would be different for different formulations.

Though, in the present studies it was found that switching on the lower pressures at different times did not significantly affect the moisture content of the dried product, going below 100 μl for 1:0:0, 1:1:0, 1:5:0 and 1:20:0 formulations and below 170 μl for 1:20:100 formulation did compromise the product appearance and resulted in the formation of compact products at the bottom of the vial. Additionally, Fig. 3 shows that the rate of evaporation of water from formulations began to deviate from linearity when low solution volumes were reached (visual inspection, linear regression also started to give low *r*-square values). The desired volume for complete precipitation was hence set at 150 μl for 1:0:0, 1:1:0, 1:5:0 and 1:20:0 formulations and at 220 μl for 1:20:100 formulation.

3.2. Characterization of the precipitated IFNα2a/selection of precipitating agent

Although, a range of different molecular weight PEGs are available, the choice of PEG for this study was limited to PEG 1450 and PEG 3350. PEGs with molecular weight 8000 or higher were not considered for use as these have been observed to affect the native structure of the precipitated IFNα2a (perturbation of the tertiary structure of IFNα2a was observed by CD spectroscopy) (Sharma and Kalonia, 2004). PEGs with molecular weight less than 1000 were discarded because of the following two reasons: (1) these PEGs are liquids at room temperature and hence would have affected the handling and appearance of the final dried product, and (2) these would have been required in high concentrations as it is well documented in literature that precipitating power of PEGs decreases with a decrease in their molecular weight (Sharma and Kalonia, 2004). PEG 1450 and PEG 3350 were hence evaluated for their effect on the structure of the precipitated and reconstituted IFNα2a in order to select the PEG of choice for the drying studies. PEG 1450 and PEG 3350 were chosen because of the following two reasons. First, authors had previously utilized these two PEGs for the precipitation of IFNα2a (Sharma and Kalonia, 2004) and the data showed promise in regards to the use of the two PEGs in the present scenario. Secondly, the two PEGs were easily available to us.

Fig. 4 shows the near and far UV-CD spectra (A and B, respectively) for native IFNα2a solution and the reconstituted IFNα2a solution when precipitated from 10% (w/v) PEG 1450 and PEG 3350 at pH 6.5. Near UV-CD spectra indicated that the tertiary structure of IFNα2a on reconstitution was not significantly altered when precipitated using any of the two PEGs. Similarly, far UV-CD spectra indicated that the secondary structure of reconstituted IFNα2a was retained when precipitated by PEG 1450 or PEG 3350. Though these results clearly show that the structure of the native IFNα2a and that of the reconstituted IFNα2a are similar, they do not essentially indicate that the structure of IFNα2a in the precipitated state and that of IFNα2a in solution are similar. This is because any alter-

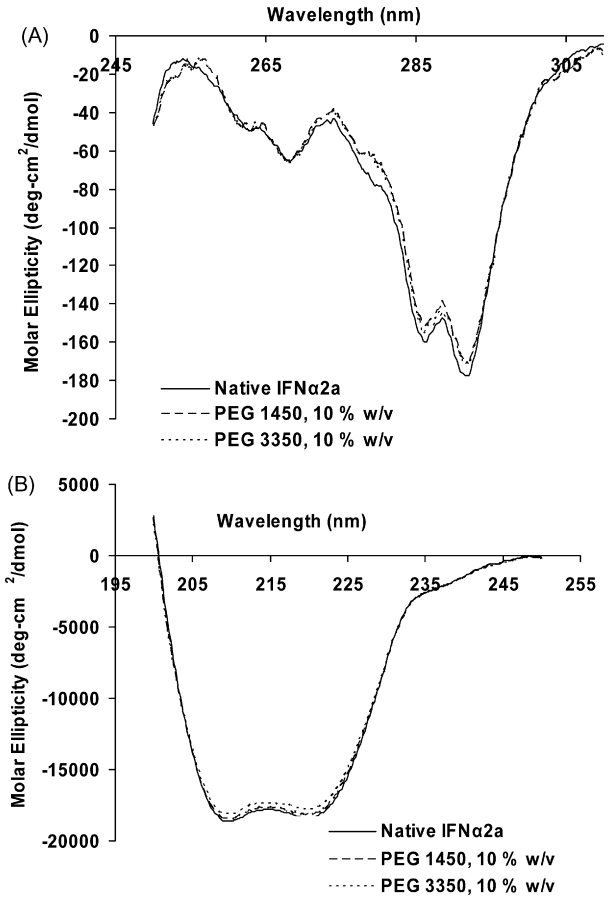

Fig. 4. Near UV-CD spectra (A) and far UV-CD spectra (B) of reconstituted IFNα2a. Reconstitution of the protein was done in acetate buffer at pH 5.0 following precipitation from PEGs.

ation of the structure of the protein in the precipitated state could well have been reversed upon reconstitution.

In order to ascertain the structure of the precipitated IFNα2a, a fluorescence study was done. Fig. 5 shows the normalized fluorescence emission scans of IFNα2a solution and IFNα2a suspension when precipitated using 10% (w/v) PEG 3350. Removal of the precipitated protein by centrifugation showed that >95% of the fluorescence emission intensity from the protein suspension was due

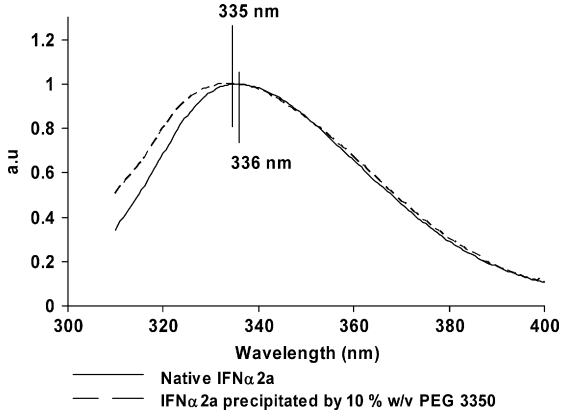

Fig. 5. Normalized fluorescence (normalized at λ_{max}) spectra of native IFNα2a in solution and of IFNα2a suspension obtained following precipitation by 10% (w/v) PEG 3350 at pH 6.5 and ionic strength of 15 mM. Wavelength of excitation was 295 nm.

to the precipitated protein. λ_{max} of emission for IFNα2a in solution was observed at 336 nm, while the λ_{max} of emission for the protein suspension was observed at 335 nm. Slight blue shift of the λ_{max} of emission in the case of protein suspension is attributed to the scattering of light by the suspended protein particles (note that the fluorescence intensity is higher for protein suspension in 310–335 nm region). On the other hand there is near complete overlap of the two emission scans in 335–400 nm region. This indicates that the contribution due to scattering by the suspended protein particles is minimal in this region. In an earlier report, it was shown that complete loss of the tertiary structure of IFNα2a at pH 2.0 results in a red shift of the λ_{max} of emission by ≈3.5 nm (Kumar et al., 2005). Additionally, it was shown in this report that the emission scans of the native protein at pH 6.5 and unfolded protein at pH 2.0 showed a distinct difference in 335–400 nm region. Hence, the near overlap of the emission scans in 335–400 nm region observed in the present study indicates that structure of the protein is not significantly altered on precipitation. Since it has been shown previously that higher molecular weight PEGs are required in lower amounts to precipitate the protein out of the solution (Sharma and Kalonia, 2004), PEG 3350 was selected as the precipitating agent of choice (and hence fluorescence study was not conducted with PEG 1450).

3.3. Solubility studies

Solubility of proteins is affected by factors such as PEG concentration and ionic strength of the solution (Sharma and Kalonia, 2004; Arakawa and Timasheff, 1985b). Additionally, the solubility of IFNα2a may also be affected by the presence of excipients such as trehalose (Antipova and Semenova, 1996; Conti et al., 1997; Paleg et al., 1984). Solubility studies were hence conducted in order to study the effect of different excipients, and hence determine the experimental solution conditions at which complete *in situ* precipitation of IFNα2a could be achieved, when a desired volume of the solution is reached during the process of slow evaporation. It has been observed earlier that the apparent solubility of IFNα2a (since PEGs result in the formation of amorphous precipitate, solubility in the presence of PEGs will be referred to as apparent solubility) is lowest around the p*I*, i.e. in the pH range of 6.0–7.0 (Sharma and Kalonia, 2004). The amount of precipitating agent required to precipitate IFNα2a out of the solution would thus be minimum at pH 6.5, and hence all solubility studies (and subsequently the drying studies) were conducted at this pH.

During the development of the drying process, it was found out that stabilizer such as trehalose was required to protect the secondary structure of the protein during drying and keep the protein in soluble form on reconstitution. Hence, in addition to the protein (0.4 mg/ml), formulations that were dried typically contained PEG as the precipitating agent, trehalose as the stabilizer and mannitol as the bulking agent. In order to obtain information about the amount of trehalose required for optimum stability, formulations that were dried contained 1:1:0, 1:5:0, 1:20:0 and 1:20:100 (w/w) protein:trehalose:mannitol. Formulations that were finally selected to undergo accelerated storage stability had 1:0:0, 1:20:0, and 1:20:100 (w/w) protein:trehalose:mannitol. Since such a wide variety of formulations were to be dried, solubility studies were conducted on solutions that would mimic the actual formulations.

Fig. 6A shows the effect of PEG concentration on the solubility of IFNα2a in the absence and presence of 0.8% (w/v) trehalose at pH 6.5 and ionic strength of 15 mM. Fig. 6B shows the effect of PEG concentration on the solubility of IFNα2a in the absence and presence of 5.33% (w/v) trehalose at pH 6.5 and ionic strength of 100 mM. The conditions used to generate data in Fig. 6A mimic the conditions of a typical formulation that contains 0.4 mg of the protein, and

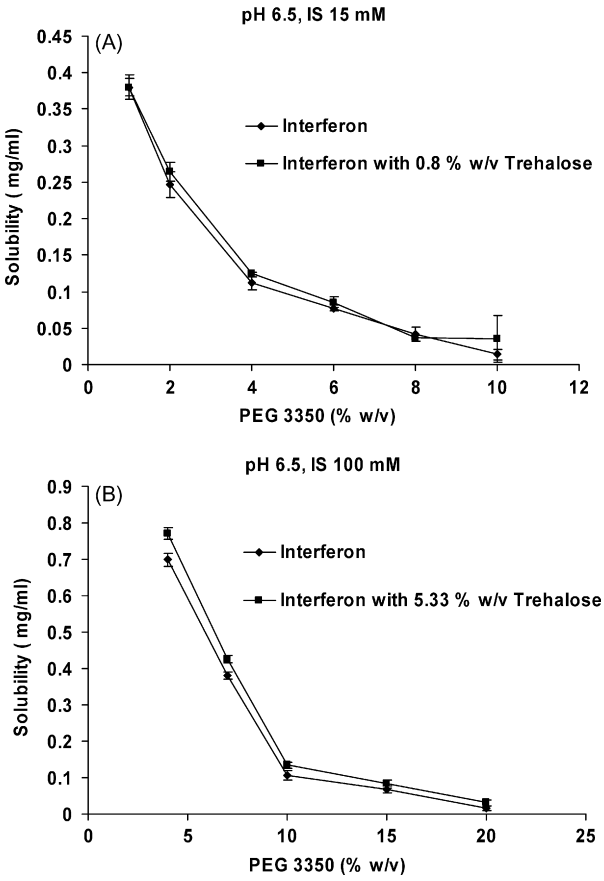

Fig. 6. Effect of PEG concentration on the solubility of IFNα2a in the absence and presence of trehalose at pH 6.5 (A) Solubility of IFNα2a in the absence and presence of 0.8% (w/v) trehalose at an ionic strength of 15 mM and (B) Solubility of IFNα2a in the absence and presence of 5.33% (w/v) trehalose at an ionic strength of 100 mM. Lines are guide to the eyes.

1:20:0 (w/w) protein:trehalose:mannitol (the formulation condition mimics those conditions that would be present at the onset of drying). Similarly, the conditions used to generate data in Fig. 6B mimic the conditions of the same formulation at the time when the volume of the solution has been reduced to 150 μl during the process of slow evaporation.

Several significant observations can be made from Fig. 6A and B. Solubility of IFNα2a decreased non-linearly with an increase in the concentration of PEG under both solution conditions. Solubility of IFNα2a increased with an increase in the ionic strength of the medium. Trehalose increased the apparent solubility of IFNα2a. The effect of PEG concentration and ionic strength on the solubility of proteins is well documented in literature (Haire et al., 1984; Ingham, 1978; Arakawa and Timasheff, 1985b). However, the increase in the solubility of IFNα2a by trehalose in the presence of PEGs is a new finding.

In order to ascertain the effect of sugars, the apparent solubility of IFNα2a was also studied in the presence of several other sugars such as sucrose, glucose and mannitol (data not shown). These studies indicated that sugars in general increased the apparent solubility of IFNα2a. It is intriguing that the observed effect of sugars on the apparent solubility of proteins is in contradiction of the widely accepted preferential exclusion theory of protein stabilization by sugars (Lee and Timasheff, 1981). Since preferential exclusion of sugars from proteins immediate vicinity results in an increase in the chemical potential of the protein molecules, preferentially excluded co-solvents tend to favor the solid state over the dissolved state and hence should result in a decrease in the solubility of proteins (Arakawa and Timasheff, 1985b; McClements, 2002). Detailed investigation of the mechanism of the observed effects of sugars on the solubility of proteins is currently being pursued in our lab.

Once the solubility of IFNα2a was determined, the plots (Fig. 6A and B) were used to determine the optimum amount of PEG that should be used in the formulation such that complete precipitation of IFNα2a could be obtained at the desired solution volume of 150 μl. For example, it can be observed from Fig. 6 that if a 1:20:0 formulation that contains 0.4 mg of IFNα2a and 15 mg of PEG 3350 (1.5%, w/v) in 1 ml of solution is kept for drying, complete precipitation of the protein would be obtained when the solution volume reaches 150 μl. This is because the solubility of IFNα2a under the conditions that would be present when the volume reaches 150 μl is only 0.1346 mg/ml (see Fig. 6B). Thus, the amount of the protein remaining soluble when the volume of the solution reaches 150 μl would only be 0.020 mg. Similarly, if the initial solution has 25 mg of PEG, the amount of the protein remaining soluble when the volume reaches 150 μl would only be 0.008 mg. However, since it was desirable to minimize the amount of PEG required to precipitate the protein out of the solution completely, 15 mg was chosen as the amount of PEG that would be added to this 1:20:0 formulation.

Solubility studies were also conducted to determine the optimum amount of PEG required for the drying of formulations that contained 1:20:100 (w/w) protein:trehalose:mannitol. The results are presented in Fig. 7. It can be observed from the figure that addition of 22 mg of PEG in the initial formulation would result in complete precipitation of the protein at the desired solution volume of 220 μl. This is because the solubility under the conditions that would be present when the solution volume reaches 220 μl (see Fig. 7B) is only 0.1376 mg/ml. Hence, the amount of protein remaining soluble at this point of time would only be 0.030 mg.

In situ precipitation studies were then conducted in order to ascertain if indeed protein precipitation proceeds as anticipated from the solubility studies. The total volume of the solution kept for slow evaporation was 1 ml. The solution contained 0.4 mg of the protein and the requisite amount of the precipitating agent as determined from the solubility studies. Fig. 8 shows that for 1:20:0 formulation, amount of the protein remaining soluble was only ≈0.05 mg at the time when the volume of the solution had reduced to ≈200 μl. From these results it was clear that complete *in situ* precipitation of IFNα2a can be achieved at any desired volume of the solution by utilizing appropriate amount of PEG in the formulation. The amount of PEG required is formulation dependent and can be easily determined from the solubility or *in situ* precipitation studies.

3.4. Drying studies and characterization of the dried protein

Once the formulation and drying conditions had been established, various formulations were subjected to *in situ* precipitation and drying. Formulations that contained 1:0:0, 1:1:0, 1:5:0 and 1:20:0 (w/w) protein:trehalose:mannitol were subjected to slow evaporation until the volume reached 150 μl and formulations that had 1:20:100 (w/w) protein:trehalose:mannitol was subjected to slow evaporation until the volume reached 220 μl. All formulations that were subjected to drying by this technique contained 0.4 mg of IFNα2a. 1:0:0, 1:1:0, 1:5:0 and 1:20:0 formulations had 15 mg of PEG and 1:20:100 formulation had 22 mg of PEG. The initial volume of solution that was kept for slow evaporation was 1.0 ml. Once complete precipitation was achieved the shelf pressure was reduced to 100 mTorr and the drying was continued for 48 h. Dried products were analyzed for moisture content, and % soluble protein remaining after reconstitution of the dried samples.

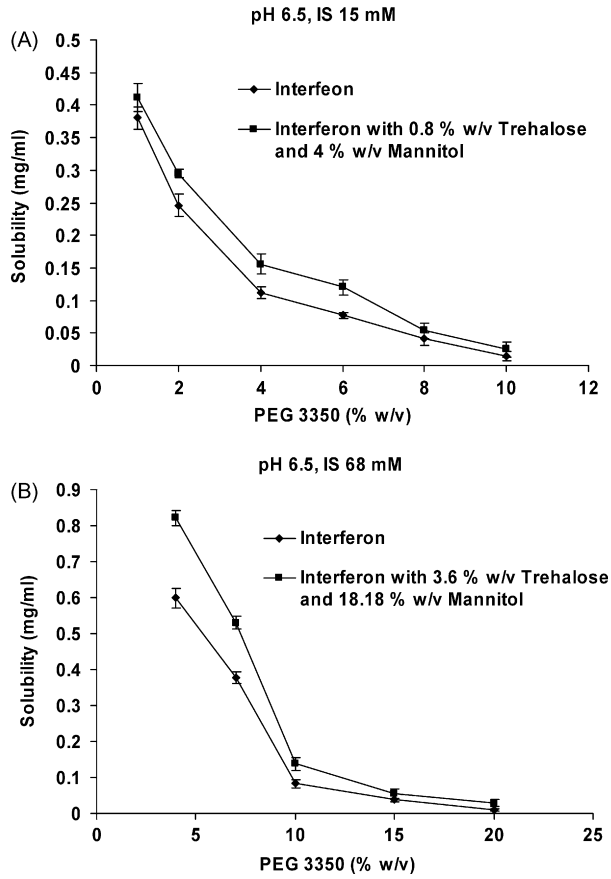

Fig. 7. Effect of PEG concentration on the solubility of IFNα2a in the absence and presence of trehalose and mannitol at pH 6.5. (A) Solubility of IFNα2a in the absence and presence of 0.8% (w/v) trehalose and 4% (w/v) mannitol at an ionic strength of 15 mM and (B) solubility of IFNα2a in the absence and presence of 3.63% (w/v) trehalose and 18.18% (w/v) mannitol at an ionic strength of 68 mM. Lines are guide to the eyes.

Table 1 shows the effect of protein:trehalose:mannitol (w/w) ratio on the percentage soluble protein remaining upon reconstitution at the end of drying. It was observed that absence of any stabilizer (trehalose) resulted in formation of insoluble aggregates

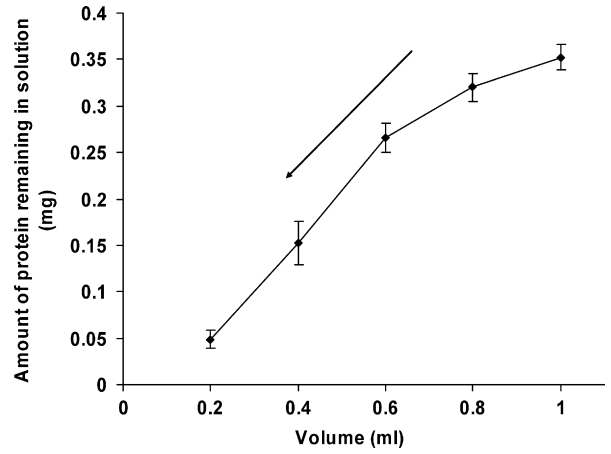

Fig. 8. Effect of evaporation of water from formulation containing 1:20:0 (w/w) protein:trehalose:mannitol and 15 mg of PEG 3350 at pH 6.5 and ionic strength of 15 mM on the precipitation of IFNα2a. Total volume of the solution kept for evaporation was 1 ml with a protein concentration of 0.4 mg/ml. Lines are guide to the eyes.

Table 1
Effect of IFNα2a:trehalose:mannitol (w/w) ratio on the % of protein remaining soluble in the solutions reconstituted from samples dried with precipitation/vacuum-drying technique

IFNα2a:trehalose:mannitol (w/w)	Soluble protein (%)	Moisture (%w/w)
	Mean ± S.D., $n = 3$[a]	Avg. ± dev., $n = 2$[b]
Before drying	100.00 ± 0.52	–
1:0:0	94.98 ± 0.43	0.72 ± 0.03
1:1:0	98.25 ± 1.00	1.02 ± 0.30
1:5:0	98.70 ± 1.20	0.78 ± 0.11
1:20:0	99.60 ± 0.40	0.66 ± 0.20
1:20:100	99.61 ± 0.45	0.58 ± 0.04

High vacuum drying was done at 100 mTorr for 48 h at 25 °C. Table also shows the residual moisture content of the formulations at the end of drying.
[a] Percentage recovery as compared with initial prior to drying.
[b] (–) indicates not determined.

in the reconstituted samples. Addition of trehalose to the drying formulation prevented the formation of insoluble aggregates during drying. Addition of trehalose in 1:20 weight ratio provided complete protection (within the experimental error) to IFNα2a during drying. Table 1 also shows the data for the amount of moisture remaining in the dried products. It can be seen from Table 1 that all formulations that were dried had moisture contents of less than 1.5% by weight. Achievement of low moisture content is important for the long-term stability of the formulation. Moisture contents achieved here compare well with those usually obtained in freeze drying.

Fig. 9A and B shows the secondary structure of IFNα2a in the native state in solution and after drying by precipitation and vacuum-drying technique in the presence of 1:0:0, 1:1:0 and 1:20:0 (w/w) protein:trehalose:mannitol as determined by area normalized second derivative FTIR spectra of the amide I region. As seen from this figure, native IFNα2a shows an intense peak at ≈1655 cm^{-1}, a characteristic of the presence of alpha helices in this protein (Dong et al., 1990). Additionally, the native protein also shows a small peak at 1633 cm^{-1}. Peaks in this region are usually indicative of β sheets (Gribenow et al., 1999). Upon drying after precipitation with PEG 3350 in the absence of any stabilizer, a loss in the intensity of peak at 1655 cm^{-1} is observed (Fig. 9A), indicating a loss in the content of the alpha helices of this protein. Interestingly, the peak at 1633 cm^{-1} seem to have disappeared (although from Fig. 9B it appears that following drying, the peak may have become very small). In addition increase in the intensities of peaks in 1680–1695 and 1624 cm^{-1} regions could indicate the presence of β turns and/or antiparallel β sheets and aggregates and/or side chains, respectively (Bandekar, 1992; Gribenow et al., 1999). Upon drying in the presence of trehalose, the loss in the intensity of the peak at 1655 cm^{-1} was regained. Additionally, the changes that were observed in 1680–1695 and 1624 cm^{-1} regions became less pronounced. The extent of retention of the secondary structure varied and increased with the increase in the amount of added trehalose. Best structural retention was observed at 1:20 weight ratio of protein:trehalose. It should be noted that trehalose is known to preserve protein structure against dehydration stress by acting as water substituent by hydrogen bonding to the protein, and due to its high T_g (glass transition temperature) (Prestrelski et al., 1993; Carpenter and Crowe, 1989).

Table 2 shows the reconstitution times of various dried formulations. It was expected that the density of the vacuum dried material obtained by utilizing the current methodology would be greater than comparable freeze dried formulations. As anticipated, the reconstitution time of the vacuum dried formulations were found to be higher than similar freeze dried products. However, it is encouraging to note that the times do not differ significantly and that the

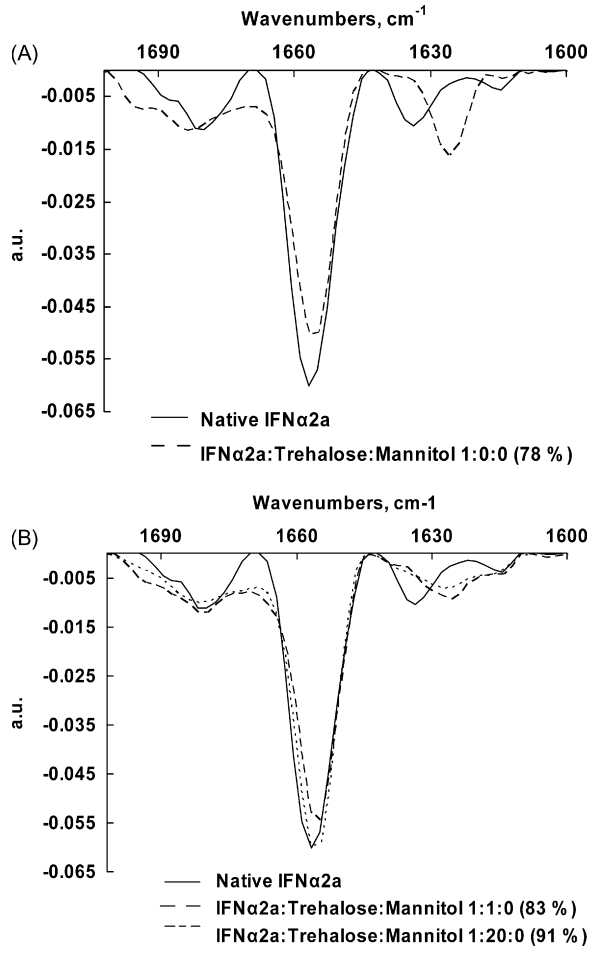

Fig. 9. Area normalized second derivative FTIR spectra of IFNα2a in solution and of the solid IFNα2a following drying in the absence and presence of trehalose (A) IFNα2a in solution and IFNα2a dried in the absence of any stabilizer and (B) IFNα2a in solution and IFNα2a dried from formulations containing 1:1:0 and 1:20:0 weight ratio of protein:trehalose:mannitol. The numbers in brackets indicate the area of overlap (%) between the given spectrum and the native IFNα2a in solution. The IR spectra of the solid IFNα2a were taken after KBr pelletization.

reconstitution times of a 1:20:100 vacuum dried formulation were only about twice that of similar freeze dried formulations.

3.5. Accelerated storage stability studies

Storage stability studies were conducted on a 1:20:100 formulation that was prepared by utilizing precipitation and vacuum-drying method. Formulations were also prepared containing 1:0:0 and 1:20:0 (w/w) protein:trehalose:mannitol. A lyophilized formulation containing 1:20:100 (w/w) protein:trehalose:mannitol but

Table 2
Reconstitution times of various dried formulations

	IFNα2a:trehalose:mannitol (w/w)	Reconstitution time (s) Avg. ± dev.[a], $n = 2$
Vacuum dried	1:0:0	261 ± 2
	1:20:0	216 ± 4
	1:20:100	362 ± 11
Freeze dried	1:20:100	189 ± 13

The formulations were reconstituted in pH 5.0 acetate buffer.
[a] Deviations from average.

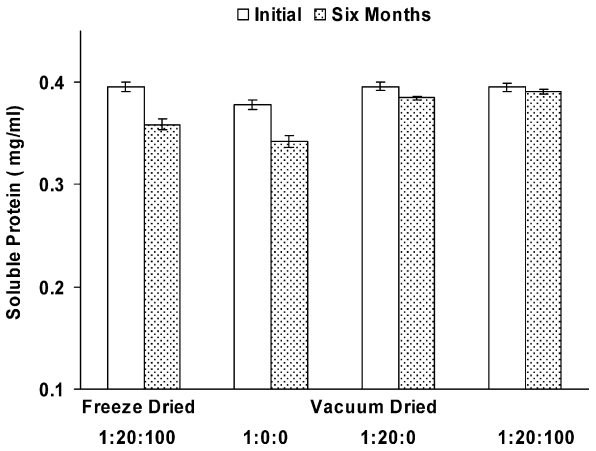

Fig. 10. Storage stability of various formulations of IFNα2a at 40 °C dried by precipitation/vacuum drying and lyophilization showing soluble protein remaining in the initial samples and in samples after 6 months of storage.

no PEG was also kept on stability in order to compare the storage stability of the product prepared by precipitation and vacuum-drying method and that prepared by freeze drying. All samples were stored at 40 °C for a period of 6 months. Stability of IFNα2a was monitored by following the formation of insoluble and soluble aggregates and the formation of oxidized species.

Fig. 10 represents the physical stability of various formulations stored for 6 months at 40 °C as indicated by the concentration of soluble protein remaining in the reconstituted samples. The figure clearly shows that the formulation containing protein:trehalose:mannitol in 1:20:100 weight ratio that was prepared by precipitation and vacuum drying showed the best stability as compared with the other vacuum dried and freeze dried formulations. This is because minimum loss of protein due to the formation of insoluble aggregates occurred in this sample as compared to the initial samples. The stability of formulation containing 1:20:0 (w/w) protein:trehalose:mannitol was also better than the lyophilized formulation. Trehalose is known to provide structural stabilization to the protein in the dried state. The present results are in clear agreement with this statement.

Fig. 11 shows the SEC result for the 1:20:100 formulation prepared by vacuum drying and stored at 40 °C for 6 months as compared with that of native IFNα2a. It is clear that no soluble

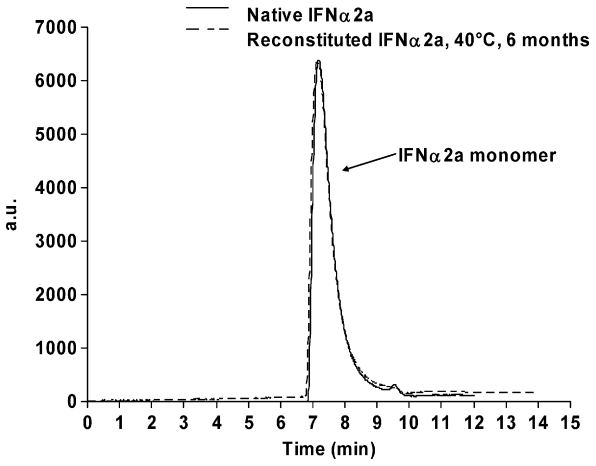

Fig. 11. SEC-HPLC chromatograms of native IFNα2a in solution and that of IFNα2a reconstituted from a formulation dried with 1:20:0 (w/w) protein:trehalose:mannitol by precipitation/vacuum-drying technique and stored for 6 months at 40 °C.

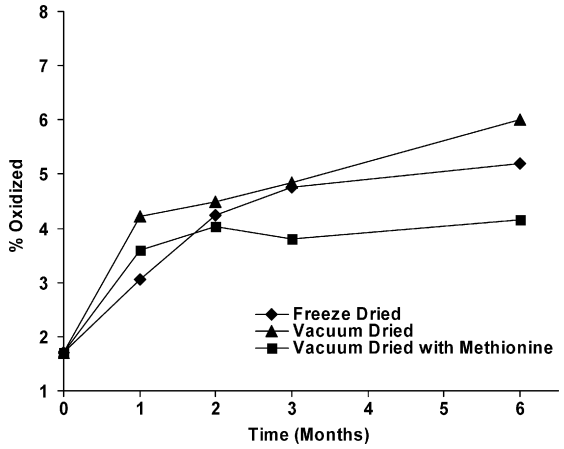

Fig. 12. Percentage oxidized IFNα2a in solutions reconstituted from 1:20:100 (protein:trehalose:mannitol) and 1:20:100:2.5 (protein:trehalose:mannitol:methionine) formulations dried by precipitation/vacuum drying and 1:20:100 (protein:trehalose:mannitol) formulation dried by lyophilization and stored for 40 °C for 6 months. The points represent mean of duplicate studies. The difference was less than 5% in the duplicate studies.

aggregates were formed in this sample. In general, none of the formulations that were dried by either vacuum drying or freeze drying were found to have any soluble aggregates even at the end of 6 months.

It is well established in literature that PEGs have low levels of residual peroxides which accumulate on storage and could potentially affect the oxidation stability of sensitive pharmaceuticals (Johnson and Taylor, 1984; Kumar and Kalonia, 2006). Since, IFNα2a is known to oxidize under neutral and acidic conditions, it was essential to study the long-term oxidative stability of the formulations dried by precipitation and vacuum-drying technique. In fact, one of the reasons of selecting IFNα2a for the present studies was its oxidative susceptibility.

Fig. 12 compares the percentage of IFNα2a oxidized at various time points for 1:20:100 formulations that were prepared by freeze drying and vacuum drying. As anticipated, the percent of IFNα2a oxidized was higher in the case of the protein that was dried by precipitation and vacuum-drying technique (contained PEG). However, the percent of the protein oxidized was not significantly different among these two formulations. In a recent report, it was shown that the level of residual peroxides in PEGs is significantly reduced when PEGs are vacuum dried at low pressures (Kumar and Kalonia, 2006). Hence, the observation that only a small difference is seen for the amount of the protein oxidized in the formulations that were dried by freeze drying (without PEG) and vacuum drying (with PEG) may not be unusual. Though, not the case with IFNα2a, addition of PEGs may significantly affect the oxidation susceptibility of proteins. Hence, oxidative storage stability studies were also conducted on a formulation that contained methionine as an antioxidant (Yin et al., 2004). Formulation containing 1:20:100:2.5 (w/w) protein:trehalose:mannitol:antioxidant was dried by precipitation and vacuum-drying technique and kept at 40 °C for 6 months. The results are included in Fig. 12. Clearly, addition of methionine resulted in a significant decrease in the amount of the oxidized protein. The percent of protein oxidized was even less than the similar freeze dried formulation. An antioxidant could thus be utilized in formulations that are dried by precipitation and vacuum-drying technique in order to minimize the effect that peroxides present in PEGs might have on the oxidation of the protein.

Feasibility of developing a process to formulate proteins in the dried form has been systematically investigated and reported in this paper. The process involves *in situ* polyethylene glycol-induced protein precipitation and vacuum drying of the solution and the entire process of precipitation and vacuum drying is conducted within the vial (and the freeze dryer). It is shown that the technique offers several advantages and hence presents itself as a suitable alternative to lyophilization for the preparation of proteins in the dried state.

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