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Article

Effect of Lyophilization on Stability of PEG-Protein Conjugate: A Case Study with Peginterferon alfa-2b

Patel Chintan[1], Patel Gayatri[1*]

[1]Department of Pharmaceutics & Pharmaceutical Technology, Ramanbhai Patel College of Pharmacy, Charotar University of Science and Technology (CHARUSAT), CHARUSAT campus, Changa 388421, India

The purpose of the present study was to develop stable lyophilized formulation of peginterferon alfa-2b which is acquiescent to the short lyophilization process. The present study evaluates the effect of buffering components and cryoprotectant(s) on depegylation of the peginterferon alfa-2b in combination with lyophilization process. Finally, a short lyophilization process was identified which can produce a stable pharmaceutical form of peginterferon alfa-2b without any depegylation during long-term storage. Formulations were analyzed mainly for depegylation by HP-size exclusion chromatography and *in-vitro* antiviral activity. Residual moisture content in the lyophilized product was also used as a key indicating parameter to check its role with respect to depegylation upon storage under various temperature conditions. It was observed that the peginterferon alfa-2b when formulated in presence of cryoprotectant like sucrose requires longer lyophilization process of about 5 days, irrespective of the buffering components used, to reduce the level of residual moisture content and thereby to produce the stable formulation without depegylation. A stable formulation in presence of high concentration of lactose as a cryoprotectant was developed which can withstand stresses exerted to protein-polymer conjugate during lyophilization phases without any significant depegylation. A short lyophilization process of about 48 hours can be utilized for peginterferon alfa-2b when formulated in presence of lactose as a cryoprotectant through which a stable lyophilized formulation can be produced as against longer process required when sucrose is used a cryoprotectant, which is essential from commercial point of view as lyophilization is a costly process.

Keywords: Depegylation. Lyophilization. Stability. Residual moisture content. Peginterferon alfa-2b.

INTRODUCTION

Interferons exhibits both antiviral and antineoplastic effect. Interferon alfa-2b (Intron® A) and interferon alfa-2a (Roferon®-A), either as monotherapy or in combination with other agents are approved for various indications. Due to its short half-life, interferon alfa-2b needs to be injected at least 3 times per week for the treatment duration as low as 3 weeks to 24 months (Murtaugh *et al*., 1996). The short half-life of interferon alfa leads to development of its long acting pegylated versions (McHutchison, Fried, 2003). Currently, both the interferons are available as their pegylated versions i.e. peginterferon alfa-2b (PEGINTRON®/ViraferonPeg®) and peginterferon alfa-2a (Pegasys®) (Foster, 2010). PEGINTRON®/ViraferonPeg® is approved as a part of a combination regimen for treatment of chronic hepatitis C (CHC) in patients with compensated liver disease. Pegasys® is indicated for chronic hepatitis C and chronic hepatitis B.

Peginterferon alfa-2a is produced by covalent conjugation of recombinant interferon alfa-2a with a single branched 40 kDa monomethoxy polyethylene glycol. Peginterferon alfa-2b is produced by conjugating 12 kDa succinimidyl carbonate polyethylene glycol (PEG) reagent with interferon alfa-2b (Matthews, McCoy, 2004). In case of peginterferon alfa-2b, the conjugation happens through covalent carbamate (urethane) linkage.

***Correspondence:** G. Patel. Email: gayatripatel26@gmail.com. ORCID: https://orcid.org/0000-0002-2523-1281

Peginterferon alfa-2b contains 14 positional isomers unlike 4 major isomers in peginterferon alfa-2a with majority of pegylation happening at Histidine 34 (His^{34}) residue (Wang *et al*., 2002). Although conjugation at His^{34} is covalent in nature it is highly susceptible to have hydrolysis as shown in Figure 1. While, protein-polymer conjugate in peginterferon alfa-2a is stable in the liquid form, it is not stable in case of peginterferon alfa-2b. It is known that pegylation on the side-chain of Histidine of interferon alfa-2b protein does not form a stable covalent bond between PEG and protein molecule due to high electron mobility in the imidazole ring of Histidine residue and, therefore, is susceptible to have spontaneous hydrolysis in solution as a function of time (Turecek *et al*., 2016). With such protein-polymer conjugates, one has to use techniques such as lyophilization/freeze-drying whereby water is sublimed from a composition after it is frozen which can provide stable formulation with a desired shelf-life (Ishihara, 2013; Kozlowski, Harris, 2001). To make a stable formulation of peginterferon alfa-2b one needs to carefully lyophilize the formulation with suitable buffering components along with cryoprotectant(s) or stabilizer(s) that stabilizes the conjugate to prevent depegylation during and after lyophilization. As the peginterferon alfa-2b is susceptible to have depegylation due to hydrolysis of the carbamate (urethane) linkage, the level of residual moisture in the final lyophilized product is very critical parameter for the long term storage stability of the peginterferon alfa-2b (Mosharraf, Malmberg, Fransson, 2007). It is important to utilize a lyophilization process which is cost effective and can produce the product without any significant degradation. Impact of various lyophilization process steps have been studied published elsewhere. In one of such studies, impact of reduced primary drying time on protein formulated in sucrose and sucrose/glycine formulations and compared it with conventional processes where longer primary drying was utilized (Bjelošević *et al.,* 2018*).* Aggressive primary drying process did not show any micro collapse and impact on product quality at different storage conditions. Reconstitution time and residual moisture remained within the acceptable limits. Similarly, effect of increased rate of ramp to attain the shelf temperature for primary drying step on model protein bovine serum albumin has been studies (Ohori, Akita, Yamashita, 2018). Slow ramp cycle is observed to collapse which was attributed to higher amount of residual moisture in the dried matrix. Protein appeared to show less degradation due to the presence of moisture when lyophilized using fast ramp process compared to slow ramp process. These observations indicate the importance of ramp rate during lyophilization process. Effects of annealing steps during the freeze drying process of etanercept using various analytical methods was studies earlier (Lim *et al.,* 2018). Annealing appeared to show lower residual moisture and higher monomer content with reduced aggregation. Drying process was observed to impact the secondary structure of protein as evaluated by circular dichroism. It was demonstrated that annealing is beneficial for protein stability with reduction of overall process time.

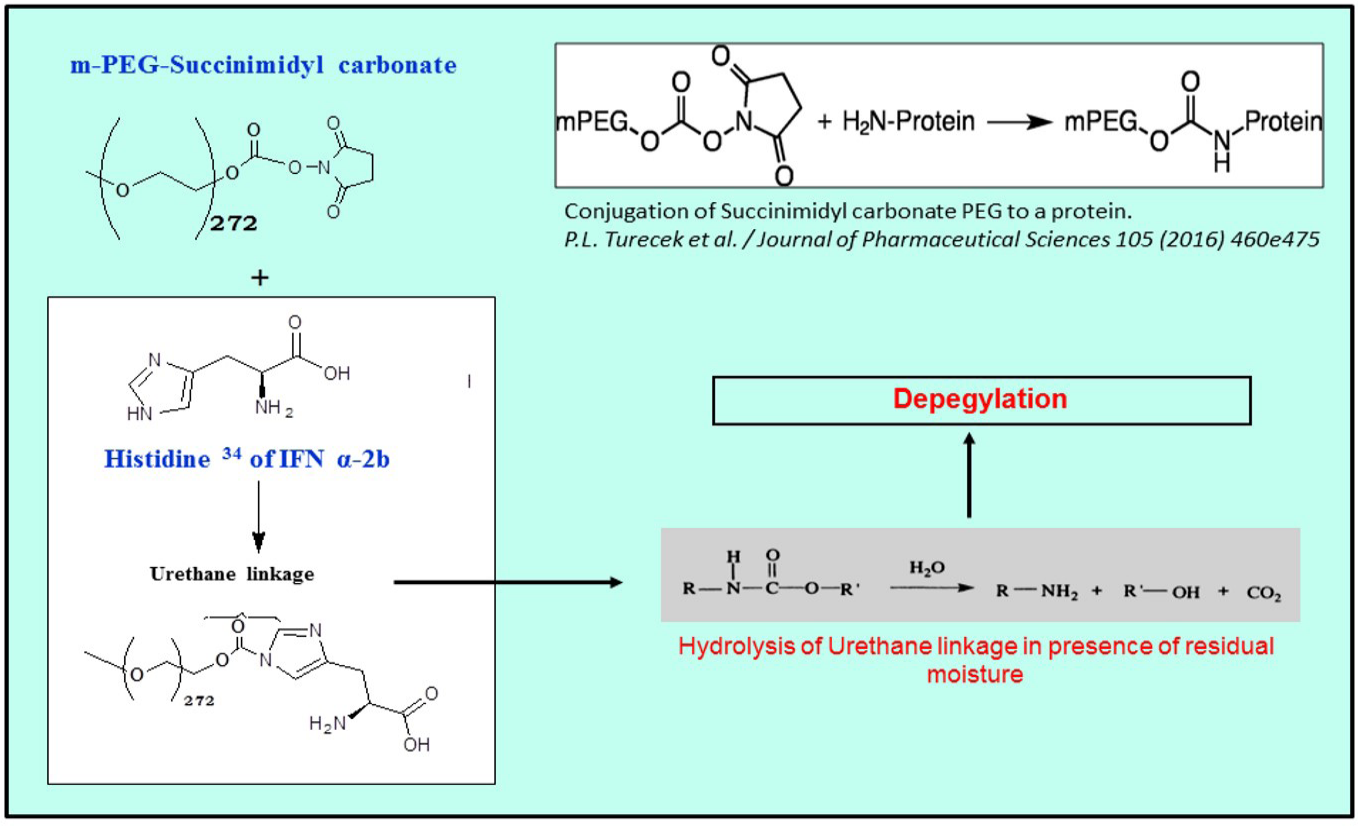

FIGURE 1 - Histidine depegylation in Peginterferon alfa-2b.

While the commercial formulation of PEGINTRON®/ViraferonPeg® which utilizes sucrose as a cryoprotectant is successful it is nevertheless associated with several problems like longer lyophilization cycles (about 5 days) leading to increase in cost of manufacturing and higher moisture content associated with the commercial formulation as elaborated in patent application US Patent 7,632,491 (WO/2006/020720). Therefore, it is important to have a lyophilization process with residual moisture content as low as possible without any depegylation of the peginterferon alfa-2b protein. These reasons makes it legitimate to develop a formulation of peginterferon alfa-2b in presence of suitable buffer(s) or cryoprotectant(s) or bulking agent(s) alone or in combination and an appropriate lyophilization process which can provide a stable lyophilized peginterferon alfa-2b drug product.

MATERIAL AND METHODS

Preparation of peginterferon alfa-2b

Interferon alfa-2b was produced in-house using recombinant DNA technology. Peginterferon alfa-2b was produced by pegylation of 12 kDa monomethoxy polyethylene glycol N-hydroxysuccinimide with interferon alfa-2b. PEG conjugation is carried out through integration of a carbamate (urethane) linkage either between N-atoms of the imidazole side-chain of His34 or the μ-NH2 group of N-terminal Cysteine residue or the e-NH2 group of Lysine side-chains of interferon alfa-2b and a 12 kDa monomethoxypolyethylene glycol-succinimidyl carbonate molecule (PEG-SC), at pH 6.5, in the presence of molar-excess amount of the said PEG molecule over the protein concentration. Peginterferon alfa-2b was isolated mainly in monopegylated form by using conventional column chromatography methods.

Formulation and lyophilization

Peginterferon alfa-2b was formulated in Water for Injection in presence of different excipients followed by 0.2 µm filtration and filling in the glass vials before lyophilization. Peginterferon alfa-2b was formulated in two different buffer systems i.e. sodium succinate and sodium phosphate in presence of sucrose as cryoprotectant and polysorbate 80 as a bulking agent as described in Table I. Peginterferon alfa-2b was also formulated in sodium succinate buffer with lactose alone or in combination with other cryoprotectants and polysorbate 80 as summarized in Table II. After filling in the glass vials formulations were subjected to lyophilization process utilizing VirTis bench top or pilot-scale lyophilizer. Lyophilization was performed by freezing peginterferon alfa-2b formulated bulk in glass vials between – 40 °C to – 55 °C. Primary drying was performed at various temperatures between – 32 °C to 0 °C while maintaining the vacuum pressure ranging from 200 mTorr to 20 mTorr followed by secondary drying at about 25 °C. Dibasic sodium phosphate USP, Monobasic sodium phosphate USP, Succinic acid NF, lactose monohydrate NF, Trehalose NF, Glycine USP and Polysorbate 80 NF were procured from MERCK KGA (Germany). Reagents used for analysis of the samples were of GR or analysis grade and procured from either Merck or Sigma. Formulations were lyophilized and stored in USP Type I borosilicate glass vial procured from Schott and stoppered with slotted rubber stopper purchased from West Pharmaceuticals, USA. Flip-off seals were used to seal the vials.

TABLE I - Peginterferon alfa-2b formulated in two different buffer systems

Ingredients	Quantity (mg per mL)	
	Formulation A	Formulation B
Peginterferon alfa-2b	0.160 mg	0.160 mg
Dibasic sodium phosphate	1.58 mg	-
Monobasic sodium phosphate	1.58 mg	-
Sodium Succinate	-	2.57 mg
Sucrose	115 mg	115 mg
Polysorbate 80	0.005 mg	0.005 mg
Water for Injection	1 mL	1 mL
pH	6.8	6.8

Formulation A: Prepared utilizing inactive ingredients same as innovators' product PEGINTRON®/ViraferonPeg®; Formulation B: Change in buffering component from Sodium phosphate to sodium succinate

TABLE II - Peginterferon alfa-2b formulated in succinate buffer with different cryoprotectant(s)

Ingredients	Quantity (mg per mL)			
	Formulation C	Formulation D	Formulation E	Formulation F
Peginterferon alfa-2b	0.178	0.178	0.178	0.178
Sodium Succinate	1.89	1.89	1.89	1.89
Lactose	18	100	57.1	57.1
Trehalose	-	-	31.4	22.8
Glycine	-	-	1.05	1.05
Polysorbate 80	0.1	0.1	0.1	0.1
Water for Injection	1 mL	1 mL	1 mL	1 mL
pH	6.8	6.8	6.8	6.8

Storage and stability

Peginterferon alfa-2b samples of different formulations were stored in the stability chambers (Thermo) set at different storage conditions i.e. between +2 °C and +8 °C, 25 °C ± 2 °C; 60 % RH ± 5 % RH and 40 °C ± 2 °C; 75 % RH ± 5 % RH. Samples were withdrawn after various time-intervals and reconstituted with Water for Injection before analysis wherever applicable. Samples were analyzed mainly for visual clarity and color, residual moisture content, purity, level of free interferon alfa-2b and potency.

Analytical evaluations

Formulations were evaluated for various physical, physico-chemical and biological properties. Appearance of the lyophilized cake was checked by visual inspection before reconstitution. Lyophilized formulations were reconstituted with Water for Injection and checked for clarity of the solution after reconstitution by visual inspection of the solution in the glass vial. Drug product samples of peginterferon alfa-2b were reconstituted with 0.7 mL of Water for Injection (WFI) as instructed for the reference product, ViraferonPeg® and evaluated for clarity and color of the reconstituted solution against the standards prepared as described in the pharmacopeial (Ph. Eur.) general chapters 2.2.1 and 2.2.2. Fist primary opalescent suspension was prepared using the said reagent solutions followed by dilution of the primary opalescent suspension to obtain standard of opalescence. Reference suspension I was prepared by diluting the standard of opalescence in water as described in Ph. Eur. Chapter 2.2.1. The reference solution B9 was prepared by diluting standard solution B (Brown) in hydrochloric acid (1:99). The standard solution B was prepared by mixing the three color solutions i.e. Yellow solution, Red solution and blue solution prepared by diluting ferric chloride, chopper chloride and copper sulphate pentahydrate in hydrochloric acid as described in Ph. Eur. Chapter 2.2.2. Presence of any visible particles in the reconstituted solution was also checked manually.

High performance size exclusion chromatography (HP-SEC) with UV detection was used to check the level of depegylation by measuring free interferon alfa-2b content in the product preparation (yu *et al*., 2007). Analysis was performed on LC2010-CHT series Shimadzu HPLC systems to check purity and level of free interferon alfa-2b content in the peginterferon alfa-2b drug product preparations. Samples were analyzed using TSK GEL 3000 SWXL column (Tosoh, Tokyo, Japan) in isocratic manner using 0.2 M phosphate buffer containing 10 % ethanol at pH 6.8.

Level of residual moisture was measured using Karl-Fischer technique after lyophilization and upon storage under different temperature conditions (MacLeod, 1991).

In-vitro anti-proliferation activity was measured on WISH cells challenged with EMCV in comparison with NIBSC standard. Cells were propagated and incubated with varying concentration of reference standard and test samples. Relative potency was determined with respect to reference standard (Yang *et al.,* 2013). In-vitro anti-viral activity against the cytopathic effect of the specified virus in WISH cells was measured. Cells were propagated and incubated with varying concentration of reference standard and test samples and EC50 values were derived for both the samples. Single cell suspension of the WISH cell was prepared after propagation in culture media. Test samples and NIBSC standard were further serially mixed / diluted with culture media in round bottom tissue culture plate. Test and control samples (virus and cell control) were incubated in the 96 well plate containing monolayer of the WISH cells. EMCV was spiked in different wells containing test and control samples. After incubation virus infected cells were washed with phosphate buffered saline and checked for cell viability by measuring absorbance at 570 nm. EC50 dose of the test and standard samples was determined. Relative potency was determined with respect to reference standard as shown below in equation below considering the dilutions of the test samples done during the assay procedure.

$$Relative\ potency = \frac{EC50\ of\ the\ reference\ standard}{EC50\ of\ the\ test\ sample}$$

Protein concentration of peginterferon alfa-2b samples was performed on Shimadzu UV1200 spectrophotometer by measuring maximum absorbance (optical density i.e. OD) at 280nm±2nm(5).

RESULTS AND DISCUSSION

Correlation between moisture content and histidine depegylation

In order to check the correlation between residual moisture content in the lyophilized drug product and histidine depegylation, peginterferon alfa-2b was formulated in two different compositions i.e. in presence of sodium phosphate buffer pH 6.8 containing sucrose as a cryoprotectant and polysorbate 80 as utilized for innovators' product PEGINTRON®/ViraferonPeg® (Formulation A) and in presence of an alternate buffering system of sodium succinate, pH 6.8 keeping the cryoprotectant and bulking agent same as innovators' product PEGINTRON®/ViraferonPeg® (Formulation B). Formulations were lyophilized following the process described above with primary drying duration of about 35 hours and secondary drying duration of about 8 hours.

Samples were exposed to higher temperature (40 °C ± 2 °C; 75 % RH ± 5 % RH) up to one month to check the rate of depegylation over the period of time. Samples of both the Formulation A and Formulation B observed to show about 2.6 to 3.5 fold depegylation when analyzed for content of free interferon alfa-2b by using HP-Size exclusion chromatography. Depegylation may occur due to hydrolysis of ester bond in the presence of residual moisture content and therefore lyophilization process was extended at both primary and secondary drying steps to reduce the level of residual moisture content.

Both the formulations were then lyophilized using extended lyophilization process with increase in primary drying duration ranging from 40 to 50 hours and secondary drying duration of about 34 hours. Formulations prepared using extended lyophilization process were further exposed to higher temperature to check depegylation of interferon alfa-2b upon storage under stressed conditions. When exposed to stress conditions (40 °C ± 2 °C; 75 % RH ± 5 % RH), both the formulations lyophilized using extended primary and secondary process observed to show fold depegylation ≤ 1.5 as compared to that of about 2.6 to 3.5 obtained with samples produced using shorter lyophilization process. This indicates that the formulation containing sucrose as a cryoprotectant requires longer lyophilization process (extended primary and secondary drying) to produce product with lower rate of depegylation.

Based on these observations, Formulation A and Formulation B were further prepared following a lyophilization process with extended primary and secondary drying to establish the correlation between residual moisture content and histidine depegylation. Formulation A and Formulation B were stored under stressed storage (40 °C ± 5 °C; 70 % RH ± 5 % RH) conditions and under accelerated storage conditions (25 °C ± 5 °C; 60 % RH ± 5 % RH) up to 30 days and up to 10 months, respectively. Samples were withdrawn on different time-points and analyzed for visual clarity after reconstitution, presence of free interferon alfa-2b, residual moisture content and potency. Results obtained with both the formulations stored under stressed and accelerated storage conditions are shown in Table III.

TABLE III - Peginterferon alfa-2b in two different buffer systems: exposed to different temperature conditions

Time intervals	Formulation A			Formulation B		
	Potency† (IU / mg)	% RMC	% Free IFN	Potency† (IU / mg)	% RMC	% Free IFN
	Stressed storage: 40 °C ± 2 °C; 75 % RH ± 5 % RH					
Initial	0.38×10^8	0.05 %	3.29 %	0.47×10^8	0.17 %	3.06 %
15 d	0.35×10^8	1.33 %	4.24 %	0.47×10^8	0.97 %	4.49 %
30 d	0.47×10^8	1.60 %	5.68 %	0.72×10^8	1.49 %	6.01 %

TABLE III - Peginterferon alfa-2b in two different buffer systems: exposed to different temperature conditions

Time intervals	Formulation A			Formulation B		
	Potency† (IU / mg)	% RMC	% Free IFN	Potency† (IU / mg)	% RMC	% Free IFN
Accelerated storage: 25 °C ± 2 °C; 60 % RH ± 5 % RH						
Initial	0.38×10^8	0.05 %	3.29 %	0.47×10^8	0.17 %	3.06 %
1 m	0.57×10^8	1.16 %	4.16 %	0.44×10^8	0.94 %	3.69 %
4 m	0.34×10^8	0.95 %	4.47 %	0.49×10^8	0.92 %	4.90 %
10 m	0.45×10^8	1.63 %	6.29 %	0.41×10^8	1.50 %	6.22 %
Real-time storage: between +2 °C and +8 °C						
Initial	0.38×10^8	0.05 %	3.29 %	0.47×10^8	0.17 %	3.06 %
1 m	0.36×10^8	0.10 %	3.33 %	0.44×10^8	0.11 %	2.87 %
4 m	0.44×10^8	0.14 %	3.77 %	0.48×10^8	0.09 %	2.40 %
10 m	0.40×10^8	0.91 %	3.18 %	0.37×10^8	0.61 %	3.26 %

Formulation A: Buffering components – sodium phosphate, Formulation B: Buffering components – sodium succinate; All the samples were found clear colorless upon reconstitution; IFN – Interferon alfa-2; †measured by in-vitro antiviral assay (Range: 0.1 to 0.9×10^8 IU / mg); RMC – Residual Moisture Content; m – month(s), d – days

Peginterferon alfa-2b when formulated in sodium phosphate or sodium succinate buffer and exposed to higher temperature conditions (Stressed: 40 °C ± 2 °C; 75 % RH ± 5 % RH or Accelerated: 25 °C ± 2 °C; 60 % RH ± 5 % RH) observed to show increase in free interferon alfa-2b over the period of time with increase in moisture content as shown in Figure 2. When samples of both the formulations (Formulation A and Formulation B) were stored under real-time storage conditions (between +2 °C and +8 °C) up to at least 10 months no considerable increase level of moisture content and depegylation were observed as summarized in Table III and shown in Figure 2. Based on these observations, the rate of increase in free interferon alfa-2b (depegylation) can be correlated to the increase in residual moisture content of the product upon storage. Additionally, degradation pattern in terms of depegylation of Peginterferon alfa-2b protein found to be comparable when formulated either in sodium phosphate or sodium succinate buffer with sucrose as a stabilizer and Polysorbate 80 as bulking agent. Peginterferon alfa-2b when formulated in sodium succinate buffer observed to remain as stable as that of sodium phosphate formulation.

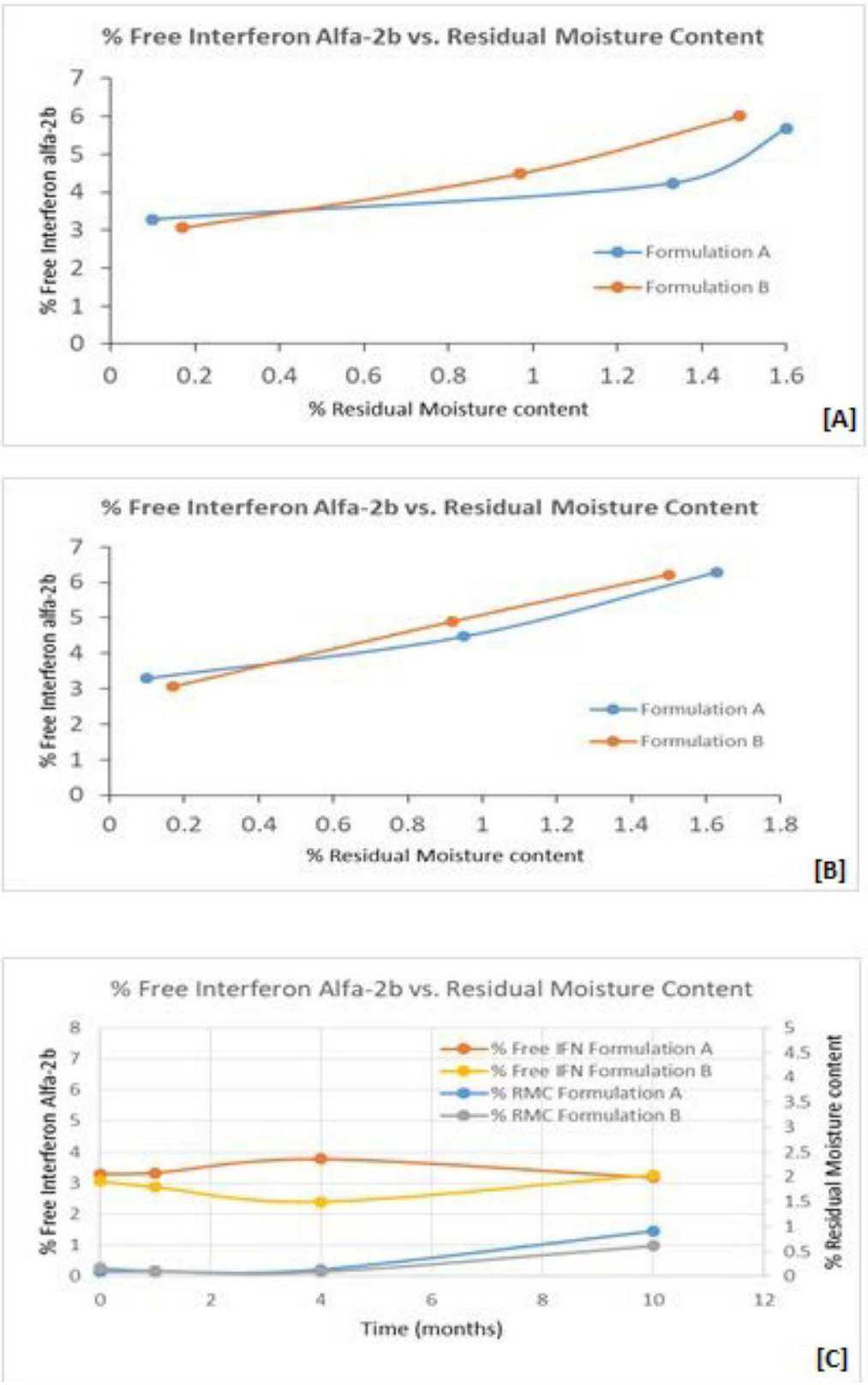

FIGURE 2 - Increase in free interferon alfa-2b (% Free IFN) with respect to increase in residual moisture content over the period of time: A) Formulation A and B exposed to stressed storage conditions (40 °C ± 2 °C; 75 % RH ± 5 % RH) B) Formulation A and B exposed to accelerated storage conditions (25 °C ± 2 °C; 60 % RH ± 5 % RH) C) Formulation A and B stored under real-time condition (between +2 °C and +8 °C).

Peginterferon alfa-2b is susceptible to have depegylation due to hydrolysis of the carbamate (urethane) linkage and therefore the level of residual moisture in the final lyophilized product is very critical parameter for the long term storage stability of the peginterferon alfa-2b. It is important to employ a lyophilization process which can yield a product with residual moisture content as low as possible without impacting PEG-protein conjugate and does not show any significant depegylation during and after lyophilization. Lyophilization process contributes in the cost of the product in terms of plant occupancy and operational cost. Therefore, shorter lyophilization process which does not destabilize the protein molecule is most desirable.

Development of stable formulation compatible with short lyophilization process

Selection of buffering system

For further work in the current investigation sodium succinate was used as a buffering agent as against sodium phosphate in the commercially available innovator's formulation as Peginterferon alfa-2b when formulated in sodium succinate buffer, pH 6.8 (Formulation B) observed to show similar degradation profile as that of the formulation prepared in sodium phosphate buffer (Formulation A) keeping sucrose as a cryoprotectant same as that of innovators' product PEGINTRON®/ ViraferonPeg® (Formulation A). When evaluated for long term stability under real-time storage conditions no considerable differences observed in terms of depegylation of peginterferon alfa-2b in comparison to innovators' compositions as summarized in Table III.

Utilizing the alternate cryoprotectant to evaluate stability of peginterferon alfa-2b in terms of depegylation

Lactose monohydrate either alone or in combination with trehalose or glycine was evaluated as a cryoprotectant with polysorbate 80 as a surfactant as against sucrose as a cryoprotectant used in case of innovators' composition PEGINTRON®/ViraferonPeg®. Two different concentration of lactose were also used to check the effect of concentration of lactose on depegylation when used alone as a cryoprotectant. Compositions for different formulations are described in Table II. Lyophilization was performed following the process described in sections above with primary drying duration of about 35 hours and secondary drying duration of about 8 hours (short lyophilization process). Samples were withdrawn after three months of storage between +2 °C and +8 °C and analyzed for visual clarity after reconstitution, residual moisture content, level of free interferon alfa-2b and potency. Results obtained with different formulations are summarized in Table IV.

TABLE IV - Depegylation of peginterferon alfa-2b conjugate – Formulation C, D, E and F stored under Real-Time storage conditions

Formulation	Time intervals	Potency† (IU / mg)	% RMC	% Free IFN	Fold depegylation
C	Initial	0.48×10^8	1.4 %	0.63 %	3.14
	After 3 m	0.43×10^8	2.5 %	1.98 %	
D	Initial	0.58×10^8	0.25 %	0.84 %	No change
	After 3 m	0.72×10^8	ND	0.79 %	
E	Initial	0.72×10^8	0.23 %	0.91 %	No change
	After 3 m	0.51×10^8	ND	0.92 %	

TABLE IV - Depegylation of peginterferon alfa-2b conjugate – Formulation C, D, E and F stored under Real-Time storage conditions

Formulation	Time intervals	Potency† (IU / mg)	% RMC	% Free IFN	Fold depegylation
F	Initial	0.48×10^8	1.96 %	0.84 %	1.91
	After 3 m	0.26×10^8	2.43 %	1.61 %	

All the samples were found clear colorless upon reconstitution; IFN – Interferon alfa-2; †measured by in-vitro antiviral assay (Range: 0.1 to 0.9×10^8 IU / mg); RMC – Residual Moisture Content; ND – not detectable; m – months

Formulation C and F observed to show 3.14 and 1.91 fold depegylation over the period of 3 months with residual moisture content between 1.4 to 2.5 % whereas Formulation D and E observed to show essentially no increase in free interferon alfa-2b content in the preparations after 3 months of storage as compared to that of initial amount with residual moisture content < 1 %. Based on this data it can be concluded that lactose as a cryoprotectant when used alone at 100 mg / mL concentration (Formulation D) observed to show comparable stability as observed with the composition where in it is used in combination with other cryoprotectant like trehalose and glycine (Formulation E). Based on this observation and with the aim of keeping formulation simple during manufacturing process only lactose with two different concentrations i.e. 18 mg / mL (Formulation C) and 100 mg / mL (Formulation D) was further evaluated for its stabilization effect on peginterferon alfa-2b against depegylation. To keep the residual moisture content lower than 2 % with the formulation containing 18 mg / mL lactose (Formulation C) extended primary drying duration of about 53 hours and secondary drying duration of about 34 hours were applied with total of about 89 hours lyophilization process. Formulation C was then compared with formulation containing 100 mg / mL lactose (Formulation D) lyophilized following same extended lyophilization process. Samples were stored under real-time temperature condition (between +2 °C and +8 °C) and accelerated temperature conditions (25 °C ± 2 °C; 60 % RH ± 5 % RH) up to 3 months. Samples were also exposed to stress temperature conditions (40 °C ± 2 °C; 75 % RH ± 5 % RH) up to 21 days to check the effect of high temperature on depegylation of peginterferon alfa-2b. After exposure samples were analysed for level of free interferon alfa-2b and thereby fold depegylation by HP-size exclusion chromatography.

Formulation D (with 100 mg / mL lactose) observed to show no increase in free interferon alfa-2b under real-time storage conditions. Under accelerated and stressed storage conditions formulation D observed to show 1.86 fold and 2.18 fold increase in level of free interferon alfa-2b, respectively. Formulation C (containing 18 mg / mL lactose) observed to have higher level of depegylation with 1.31 fold increase in free interferon alfa-2b upon storage under real-time conditions whereas 4.85 fold and 5.84 fold increase in free interferon levels under accelerated and stressed storage conditions, respectively. These results indicate that the amount of lactose makes significant impact on prevention of depegylation upon storage. Further, four different strength of Peginterferon alfa-2b was prepared with extended lyophilization process of about 89 hours using lactose (100 mg / mL) as cryoprotectant with Polysorbate 80 as a bulking agent and analysed for different physical, physico-chemical and biological properties as summarized in Table V. The results obtained with all the strengths observed to be comparable with the innovators' product ViraferonPeg® mainly in-terms of purity by HP-SEC and specific activity as measured by antiviral assay.

TABLE V - Physico-chemical and biological test results: Formulation D with different strengths

Parameters	50 µg / 0.5 mL	80 µg / 0.5 mL	120 µg / 0.5 mL	180 µg / 0.5 mL
Appearance	Intact cake	Intact cake	Intact cake	Intact cake
pH*	6.87	6.8	6.87	6.85
% RMC	< 1 %	< 1 %	< 1 %	< 1 %
% Purity	94.96	95.0	94.42	94.11
% HMW	3.25	3.0	3.36	3.27
% Free interferon	1.79	2.0	2.22	2.62
Specific activity†	0.44×10^8 IU / mg	$0.50 \times 10^{8.}$ IU / mg	0.42×10^8 IU / mg	0.50×10^8 IU / mg

*Target pH – pH 6.8; HMW – High molecular weight species; †measured by in-vitro antiviral assay (Range: 0.1 to 0.9×10^8 IU / mg); RMC – Residual Moisture Content; Results obtained for ViraferonPeg® (Average of two separate analysis): Specific Activity – 0.57×10^8 IU / mg, % Purity – 94.26 %, % HMW – 2.93 %, % Free interferon – 2.79 %

Peginterferon alfa-2b in Formulation D with one of the strength, 80 µg / 0.5 mL, was stored between +2 °C and +8 °C to check stability upon long-term storage up to at least 24 months. Samples were also exposed to higher temperature under accelerated storage conditions (25 °C ± 2 °C; 60 % RH ± 5 % RH) and stressed storage conditions (40 °C ± 2 °C; 75 % RH ± 5 % RH). Samples were withdrawn at different time intervals and analysed for various stability indicating parameters i.e. visual clarity after reconstitution, level of free interferon alfa-2b, residual moisture content and potency. Results obtained with samples exposed to different temperature conditions are summarized in Table VI.

TABLE VI - Formulation D with 80 µg / 0.5 mL strength – exposed to different temperature conditions

Time intervals	Potency† (IU / mg)	% RMC	% Free IFN
Real-time storage: between +2 °C and +8 °C			
Initial	0.51×10^8	0.16 %	2.38 %
3 m	0.53×10^8	0.40 %	2.27 %
6 m	0.37×10^8	0.78 %	2.46 %
9 m	0.33×10^8	1.21 %	2.59 %
12 m	0.51×10^8	2.21 %	3.27 %
18 m	0.47×10^8	0.91 %	3.30 %
24 m	0.54×10^8	2.01 %	3.79 %

TABLE VI - Formulation D with 80 µg / 0.5 mL strength – exposed to different temperature conditions

Time intervals	Potency† (IU / mg)	% RMC	% Free IFN
Accelerated storage: 25 °C ± 2 °C; 60 % RH ± 5 % RH			
Initial	0.51×10^8	0.16 %	2.38 %
3 m	0.67×10^8	1.50 %	4.45 %
6 m	0.29×10^8	2.05 %	6.85 %
9 m	0.29×10^8	2.44 %	6.98 %
Stressed storage: 40 °C ± 2 °C; 75 % RH ± 5 % RH			
Initial	0.51×10^8	0.16 %	2.38 %
7 d	0.85×10^8	0.93 %	3.88 %
14 d	0.53×10^8	1.32 %	4.69 %
21 d	0.28×10^8	1.73 %	5.19 %

All the samples were found clear colorless upon reconstitution; IFN – Interferon alfa-2; †measured by in-vitro antiviral assay (Range: 0.1 to 0.9×10^8 IU / mg); RMC – Residual Moisture Content; m – months, d – days

As expected, when stored under accelerated (25 °C ± 2 °C; 60 % RH ± 5 % RH) and stressed storage conditions (40 °C ± 2 °C; 75 % RH ± 5 % RH) level of free interferon alfa-2b observed to increase with increase in residual moisture content over a period of about 9 months and 21 days, respectively. Peginterferon alfa-2b in Formulation D observed to remain stable up to at least 24 months with no considerable increase in the content of free interferon alfa-2b when stored under Real-Time storage condition (between +2 °C and +8 °C). Residual moisture content observed to remain about 2 % upon storage up to 24 months under real-time storage conditions. Peginterferon alfa-2b protein observed to remain biologically active up to 24 months when lyophilized and stored in presence of lactose and polysorbate 80 as assessed by in-vitro antiviral assay.

Lactose when evaluated as cryoprotectant shows better stability at higher concentration compared to that when used at lower concentration. It is important to keep residual moisture content as low as possible in the vials and therefore longer lyophilization process can be utilized to lyophilize Peginterferon alfa-2b. It is known that sucrose (used as a cryoprotectant in commercial formulation) requires longer lyophilization process to manufacture stable formulation of Peginterferon alfa-2b. However, in order to keep manufacturing process cost effective sodium succinate as a buffering system along with lactose as a cryoprotectant was further evaluated to formulate and lyophilize Peginterferon alfa-2b with shorter lyophilization process.

Short lyophilization process for peginterferon alfa-2b

Peginterferon alfa-2b is susceptible to have depegylation due to hydrolysis of the carbamate (urethane) linkage and therefore the level of residual moisture in the final lyophilized product is very critical parameter for the long term storage stability of the peginterferon alfa-2b. It is important to employ a lyophilization process which can yield a product with residual moisture content as low as possible without impacting PEG-protein conjugate and does not show any significant depegylation during and

after lyophilization. Lyophilization process contributes in the cost of the product in terms of plant occupancy and operational cost. Therefore, shorter lyophilization process which does not destabilize the protein molecule is most desirable.

Peginterferon alfa-2b formulation with lactose as a cryoprotectant was lyophilized with lyophilization process of about 48 hours with primary drying of about 12 hours and secondary drying of about 31 hours as against earlier process of 89 hours as described above. Two different strengths of 50 µg / mL and 150 µg / mL were prepared from three different batch materials using shorter lyophilization process and samples were analyzed for level of free interferon alfa-2b using HP-Size exclusion chromatography. Results were compared with the formulation prepared with different strengths using longer lyophilization process of about 89 hours. Results obtained are shown in Figure 3. Formulations prepared with two different strengths of 50 µg and 150 µg using short lyophilization process of 48 hours observed to show low level of free interferon alfa-2b within the range obtained with five different strengths prepared using lyophilization process of 89 hours. Samples were also stored between +2 °C and +8 °C for evaluating long term storage stability of the peginterferon alfa-2b drug products prepared using shorter lyophilization process of 48 hours. Results obtained with two different strengths are summarized in Table VII.

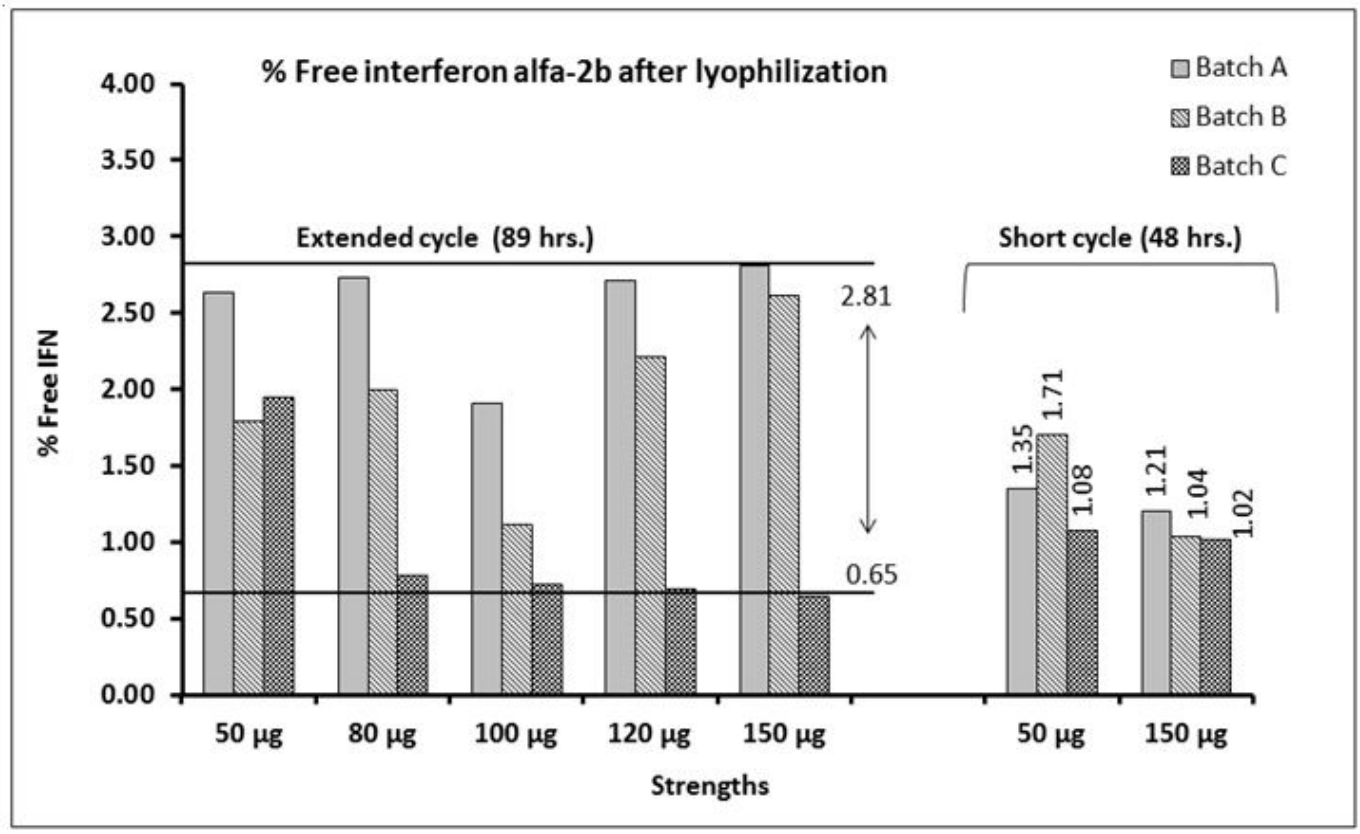

FIGURE 3 - % free interferon alfa-2b obtained with short lyophilization process.

TABLE VII - Formulation D with two different strengths lyophilized using short lyophilization process

Strength	Real-Time storage stability (+2 °C to +8 °C):			
	Time intervals	Potency† (IU / mg)	% RMC	% Free IFN
50 µg / 0.5 mL	Initial	0.62×10^8	0.63 %	1.21 %
	3 m	0.81×10^8	0.84 %	1.65 %
	6 m	0.73×10^8	0.84 %	1.67 %
	9 m	0.43×10^8	0.92 %	1.42 %
	18 m	0.59×10^8	0.73 %	2.25 %
150 µg / 0.5 mL	Initial	0.71×10^8	0.31 %	0.89 %
	3 m	0.60×10^8	0.44 %	1.73 %
	6 m	0.59×10^8	0.52 %	1.95 %
	9 m	0.39×10^8	0.67 %	2.01 %
	18 m	0.63×10^8	0.48 %	2.41 %

All the samples were found clear colorless upon reconstitution; IFN – Interferon alfa-2; †measured by in-vitro antiviral assay (Range: 0.1 to 0.9×10^8 IU / mg); RMC – Residual Moisture Content; m – months

Peginterferon alfa-2b formulation with lactose as a cryoprotectant prepared using short lyophilization process was observed to remain stable without any radical increase in level of free interferon alfa-2b (no considerable depegylation) when stored under real-time storage conditions (between +2 °C and +8 °C) for at least up to 18 months. Residual moisture content observed to remain below 1 % over a period of 18 months under real-time storage conditions. Peginterferon alfa-2b protein observed to remain biologically active as assessed by in-vitro antiviral assay.

Present work investigates the correlation between moisture content of the lyophilized peginterferon alfa-2b and removal of PEG moiety from protein backbone (depegylation mainly at His^{34}). Also lactose was evaluated as an alternative cryoprotectant to develop a stable pharmaceutical formulation of peginterferon alfa-2b. Lactose when evaluated as cryoprotectant shows better stability at higher concentration of 100 mg / mL compared to that when used at lower concentration of 18 mg / mL. It is important to keep residual moisture content as low as possible in the vials and therefore longer lyophilization process can be utilized to lyophilize Peginterferon alfa-2b. It is known that sucrose (used as a cryoprotectant in the innovators' formulation) requires longer lyophilization process to manufacture stable formulation of Peginterferon alfa-2b. However, in order to keep manufacturing process cost effective sodium succinate as a buffering system with lactose was evaluated as against sucrose as a cryoprotectant to formulate and lyophilize Peginterferon alfa-2b with shorter lyophilization process. It is observed that the composition derived through the present work can produce stable biologically active lyophilized formulation of Peginterferon alfa-2b utilizing shorter lyophilization process.

CONCLUSION

Depegylation of Peginterferon alfa-2b can be correlated with the presence of residual moisture content

present in the glass vials. It is observed that the level of free interferon alfa-2b increases as a function of residual moisture content under accelerated and stressed storage conditions. In summary, peginterferon alfa-2b can be stabilized in the lyophilized form without any significant degradation using lactose as a cryoprotectant in sodium succinate buffer with polysorbate 80 as a bulking agent utilizing a short cost effective lyophilization process.

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