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Protein Pegylation: Impact of PEG Quality on Thermal Stability of Protein

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Abstract: Successful protein PEGylation, require pure functionalized Polyethylene glycol (PEG) reagents, which exhibit high stability against hydrolysis and maintain a level of reactivity with protein functional groups under mild reaction conditions. This paper describes the effect of slight change in quality parameters such as functional activity, polydispersity and diol content of PEG on pegylation yield, storage stability, thermal denaturation, structural conformation and pharmacokinetic parameters of pegfilgrastim i.e. Pegylated Granulocyte colony stimulating factor. For pegylation, two different brands of 20kDa methoxy-PEG (mPEG) were used. It was evaluated for multiple factors including pegylation yield, storage stability at different temperatures, structural elucidation by Dynamic light scattering (DLS) and fluorescence analysis, thermal denaturation or unfolding by Differential scanning calorimetry (DSC) and Pharmacokinetics (PK) study. The pegylation yield was found to increase with increased functional activity. The PEG with higher polydispersity and diol content was found to have less storage stability of protein as evident from storage stability and thermal denaturation studies. It also leads to formation of cross-linked conjugates and degradation of proteins as evident from structural conformation and pharmacokinetic (PK) studies. An increase in the renal clearance was seen although the half life remained unaffected.

Key words: Pegylation · Stability · Polydispersity · Pharmacokinetic (PK) Studies · Aggregation and DSC

INTRODUCTION

Small changes in protein's amino acid composition, structural conformation and stress factors can cause protein unfolding and degradation, leading to loss of biological activity and immunogenicity. There are other factors such as oxidation and depegylation which have deleterious impact on pharmacokinetic parameters, effector functions and also immunogenicity reactions, all of which complicate effective delivery of biotherapeutics in humans. There are ways such as sequence changes, peg attachment to proteins, glycosylation advancement and mutation of cysteine residues by which desired stability and solubility of proteins can be achieved. Currently, one of the most successful method is to attach a polymer molecule i.e. polyethylene glycol (PEG) to an active molecule or protein to increase stability and half life of the molecule. This process of PEG attachment is termed as PEGylation.

Uniquely, PEG occupies three to four times larger hydrodynamic volume compared to globular proteins of equal molecular weight [1], thus covalent attachment of PEG to protein leads to considerable increase in hydrodynamic volume above the filtration size of kidney. Therefore PEG-protein conjugate caused to have a PK extension [2]. As renal clearance is reduced due to hydrodynamic volume, the compounds could accumulate in the liver, leading to macromolecular syndrome. PEG is also a non-biodegradable polymer.

So, considering the problem of accumulation as well as non-biodegradability of PEG, molecular weights of PEG below renal clearance threshold are commonly used. Till now, the highest molecular weight of PEG approved for human use is used in Pegasys i.e. 40 kDa. The threshold for easy kidney filtration [3] is 40-60 kDa (hydrodynamic volume approximately 45A°) and over this limit, polymer remains in circulation for longer time and accumulates in liver [4].

PEGylation also has several advantages other than increasing circulating life [5] such as reduced dosage frequency, with potentially reduced toxicity, improved protection from proteolytic degradation and augmented drug stability [6].

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Besides, these advantages, one shortcoming of pegylation is decrease in biological activity of protein e.g. in pegasys, which retains only 7 % of antiviral activity of the protein. However, this is often compensated by longer bioavailability of protein.

Although pegylation technology or type of pegylation affect the plasma life, immunogenicity and stability of protein, quality of PEG used for pegylation also influences all these parameters, to a greater extent.

An ideal PEG reagent should have the following characteristics:

- Monodispersity or at least a dispersity index close to 1.01 for low molecular weight PEGs to 1.1 for higher molecular weights in order to assure a reproducible quality [11].
- Availability of one single terminal reactive group for the coupling reaction, in order to avoid cross-linking between drug molecules.
- Water solubility and high mobility in solution [12].
- Ready clearance from the body, altered distribution in the body.
- Non-toxic and non-immunogenic, biochemically stable linker
- Options for site-specific PEGylation.

In this research, linear propionaldehyde (mPEG 20kDa) was used for site-specific pegylation of Granulocyte colony stimulating factor (GCSF) under transient denaturation conditions. As this PEG (mPEG) is having methoxy group at one end, making it unreactive at this end and cannot be used for any further chemical modification, the other end carrying a reactive group can only form a conjugate with protein. The properties of methoxy PEG which can affect the stability, solubility; immunogenicity and plasma life of protein are listed below:

Diol Content: Commercially available mPEG contains a considerable amount of diol PEG due to the presence of water during polymerization. Activation of diol leads to difunctional contaminant that can produce cross-linking and aggregation [13]. High diol concentrations will also yield unwanted cross-linked conjugates. [14] So, the use of pure monomethoxy-PEG is critical in order to avoid the formation of cross-linked conjugates [15].

Polydispersity: Traditional PEG systems are polydispersed in nature. In order to quantify the distribution of the molecular weight, the Polydispersity is defined as the ratio between the weight average molecular weight and the number average molecular weight. The Polydispersity should be used close to 1.05 for PEGs less than 30kDa and 1.1 is acceptable for higher molecular weight PEGs [8]. High polydispersity leads to a population of drug conjugates, which might have different biological properties, mainly in body-residence time and immunogenicity. However, whenever a PEGylated new drug compound needs to be approved by the FDA and other authorities; it is easier and faster, if this compound shows only one signal in the mass spectrum and not a distribution pattern.

Purity or Functional Activity: The PEG should be highly pure and functional so that there are more chances of getting pegylation yield and also avoidance of toxicity or immunogenicity issues related with PEG and PEG metabolites.

This study was carried out to evaluate the effect of PEG quality on GCSF pegylation yield, stability during storage, through the evaluation of structural conformation, thermodynamic stability and pharmacokinetic parameters.

MATERIALS AND METHODS

The liquid formulation of PEG-GCSF (formulated with Sorbitol, Polysorbate 20, Sodium Hydroxide and Glacial Acetic acid, pH 4.0) was processed and filled at Intas Biopharmaceuticals Ltd., Ahmedabad. Chemicals and Reagents used for High-performance liquid chromatography (HPLC) and other analysis were of analytical grade.

Sample A- PEG-GCSF (10mg/ml) PEG Brand A

Sample B- PEG-GCSF (10mg/ml) PEG Brand B

Quality Attributes of PEG Used in Study: Both the PEGs used in this study are of equal molecular weights while there is differences in Purity (functional group activity), polydispersity and diol content. PEG brand A is having less functional group activity and higher polydispersity and diol content compared to PEG brand B.

Evaluation of Impact of PEG Quality: The studies viz. process comparability, comparability stability study, structural elucidation, thermodynamic stability or unfolding, PK studies were done to evaluate the effect of PEGs (Brands A and B) on process parameters, product stability, structural conformation, clearance rate and half life.

Process Comparability: The pegylated products manufactured with the same process (flow chart) by having two different PEGs as raw material were evaluated for pegylation yield and recovery of protein.

Process Flow Chart:
↓
Pegylation of GCSF
↓
Chromatographic Purification
↓
Concentration and Diafiltration
↓
Drug Substance

Comparability Stability Study: Both the pegylated products (with two different Polyethylene glycol brands, PEG A and PEG B) were kept at real time real temperature (5°C±3°C), Accelerated (25°C±2°C) and Stress (40°C±2°C) conditions for 12 months, 6 months and 7 days, respectively to evaluate comparability and effect of PEG quality on stability of pegylated product.

The samples were withdrawn at intermediate time points and analyzed by stability indicating methods such as Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Size Exclusion chromatography (SE-HPLC), Reverse Phase liquid chromatography (RP HPLC), Cation exchange chromatography (CEx-HPLC) and In-vitro bioassay. The description of all the tests employed are mentioned below–:

SDS-PAGE (Silver Staining): Gel electrophoresis in the presence of SDS was carried out on 4-20% (Non-reducing) polyacrylamide gels (160 x 160 mm) using the procedure of Laemmli. The gels were silver stained to detect aggregates and depegylated species.

Reverse Phase Liquid Chromatography: RP-HPLC was performed using a Jupiter C18 column on an Agilent 1200 system with U.V detector. Column temperature was maintained at 60°C. The mobile phases consisted of water and Acetonitrile (ACN) containing trifluoroacetic acid (TFA) and principal peak elutes at 66 % of ACN. The injection amount was 30 μg and the flow rate was 0.8 ml/min.

Size Exclusion Chromatography: SEC-HPLC was performed using a TOSHO TSK 3000 SWXL column on an Agilent 1200 system. The mobile phase consisted of 50 mM sodium phosphate, 100 mM sodium chloride, 10% ethanol (pH 6.9). The analysis was done in isocratic condition.

Cation Exchange Chromatography: CEx-HPLC was performed using a TOSOH BIOSCIENCE SP-5PW column on an Agilent 1200 system. The mobile phase consisted of 20 mM Sodium acetate trihydrate (pH 5.4) with gradient of increasing salt NaCl.

In-Vitro Bioassay: The specific activity of both pegylated products was checked at intermediate time points. The method is a cell proliferation assay. It utilizes the ability of murine myeloblastic cell line M-NFS-60 to proliferate in the presence of pegfilgrastim in a dose dependent manner.

Structural Elucidation:

Fluorescence Analysis: The fluorescence analysis of both pegylated products was done using Fluorescence spectrometer (Perkin Elmer LS55). The sample concentration was used as 0.025 mg/ml for intrinsic fluorescence and 0.1 mg/ml for extrinsic fluorescence. Both the products were analyzed in 0.1 M Tris-Cl, pH 6.8. For extrinsic fluorescence analysis, 1-Anilino-8-naphthalene sulfonate (ANS) was added to a final concentration of 0.5 mM. The excitation wavelength for intrinsic fluorescence was 280 nm, so both the products were scanned from 300 nm to 500 nm. The excitation wavelength for extrinsic fluorescence was 380 nm, so both the products were scanned from 400 nm to 600 nm.

DLS Analysis: Both pegylated products were used in the concentration of 2.0 mg/ml. The set parameters were refractive index of material and dispersant at 1.34 and 1.33 respectively, viscosity of dispersant at 1.0 cP, dielectric constant of dispersant at 75 and absorption of coefficient material at 0.86. The samples were then analyzed by zetasizer nanoseries (Zetasizer nano S).

Thermodynamic Denaturation or Unfolding of Pegylated Products: DSC measurement of both pegylated products was performed using VP-DSC instrument (Microcal, GE) at heating scan rate of 1°C/min. in scanning range of 10°C to 100°C. The baseline was established by running multiple buffer-buffer scans. Both pegylated products were diluted to 2 mg/ml for sample analysis. Data evaluation was done with the origin software, version 7.0 VP-DSC 2000. The buffer-buffer baseline was subtracted from sample data. The data were corrected and fitted by using non two-state transition model. A comparison was established for melting transition temperature (Tm) and change in enthalpy (ΔH) for both the products.

PK Studies: Pharmacokinetic study of both the products was carried out using rodent model (Rats) dosed 50 µg/kg subcutaneously for all the drugs. Samples were collected at different time intervals upto 120 hrs by Retro-orbital mode of sampling. Serum samples were analyzed using Human GCSF Instant ELISA, commercial ELISA kit from Bender MedSystems (Product code BMS2001INST) in duplicates. Calibration standard range was used in the range of 78 pg to 5000 pg/ml.

Pharmacokinetic parameters were calculated after analyzing Time Versus Concentration data by Non Compartmental analysis using WinNonlin Software.

RESULTS

In this study, the effect of quality of PEG on process performance parameters was examined on stability, thermodynamic denaturation and pharmacokinetics of pegfilgrastim.

Process Comparability: Pegylated products tend to aggregate during processing of protein. The pegylation yield is solely dependent on reactivity of PEG. As shown in Table 1, the protein pegylated with brand B has higher amount of monopegylated species in comparison to unpegylated species (Free GCSF) than product A corresponding to higher functional activity of PEG (Brand B). On the contrary, dipeg is observed higher with pegylated product B as compared to brand A. However, it was removed in subsequent purification steps.

Comparability Stability Study: The effect of quality of PEG was also examined on the storage stability of pegfilgrastim at 5°C, 25°C and 40°C.

Product related Impurities by RP HPLC: The related impurities viz. oxidized impurities, depegylated species etc. were observed of same percentage in both the pegylated products at zero time point as shown in Fig. 1a, b and c. These impurities were observed to be increased with time at 5°C, 25°C and 40°C in pegylated product A compared to pegylated product B which is clearly evident from trend analysis (Fig. 1a, 1b and 1c) respectively. The level of these impurities in pegfilgrastim increased linearly with time for brand A but brand B showed a slightly upward trend with time at 25°C while no significant increase in impurities were observed at 5°C (Fig. 1a) and 40°C (Fig. 1c). Pre and post-peak impurities were also found to be higher in product A compared to product B after 6 months of exposure at 25°C (Fig. 1e) and 7 days of exposure at 40°C (Fig. 1f). Two additional post-peak impurities were observed in pegylated product A at 5°C (Fig. 1d).

Aggregation and Depegylation by SE-HPLC: Fig. 2a, 2b and 2c shows the aggregation and depegylation (impurities) of pegfilgrastim during storage at 5°C, 25°C and 40°C respectively. The SE-HPLC results showed no increase in impurities with time in both the products at 5°C as shown in Fig. 2a. However, the representative overlapped chromatogram (Fig. 2d) of last time point (12 months) at 5°C shows higher percentage of impurities in product (A) compared to product (B) which is clearly evident from the zero time point data (Fig. 2a). The level of these impurities is showing a considerable linear increase with time in product A compared to product B, where product B is showing less increase at 25°C (0.1 degree compared to 0.2 degree of degradation rate) (Fig. 2b) and 40°C (0.03 degree compared to 0.1 degree of degradation rate) (Fig. 2c). The degradation rate is calculated from slope of increase in impurities. The principal peak in product A is observed broader than product B at 5°C (Fig. 2d) which could be due to higher polydispersity in PEG brand A.

Impurities by SDS-PAGE: After incubation at real temperature (5°C) for 12 months (Fig. 3b), accelerated (25°C) for 6 months (Fig. 3c) and stress (40°C) for 7 days (Fig. 3d), intensity of high molecular weight impurities band in both the products increased. However the amount of aggregates was considerably low in pegylated product B compared to product A at all the temperatures (Fig. 3b, 3c and 3d). Depegylated species were also increased and intensity of these species for pegylated

Table 1: Pegylation yield:

Pegylation Yield				
PEG Used	Aggregate (%)	DiPEG GCSF (%)	Mono PEG GCSF (%)	GCSF (%)
Brand A	0.42	6.46	79.87	13.21
Brand B	0.69	8.19	81.99	9.15

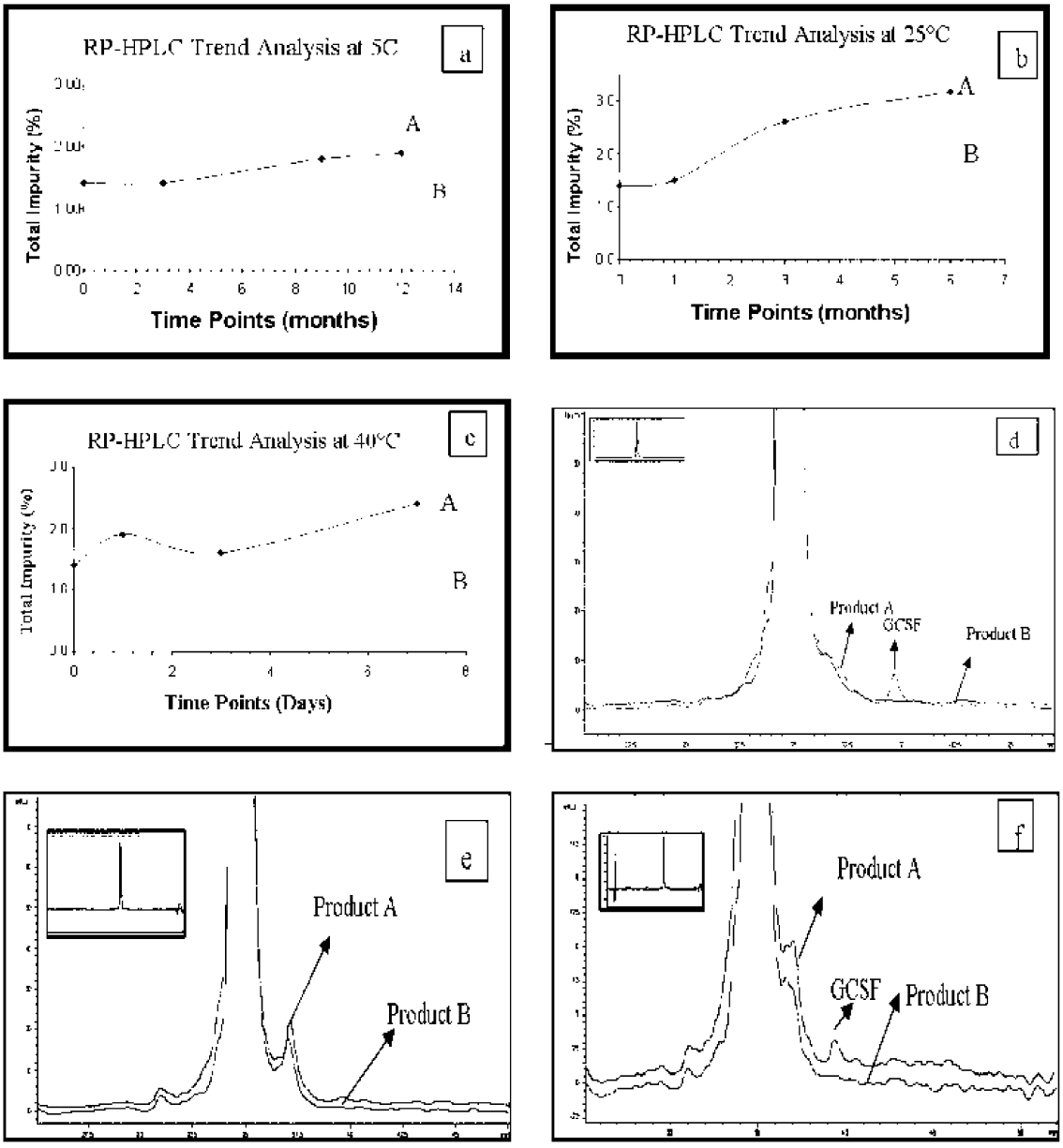


Fig. 1: Trend of product related impurities by RP-HPLC at 5°C (a), 25°C (b), 40°C (c) and Overlapped chromatograms after 12 months at 5°C (d), 6 months at 25°C (e), 7 days at 40°C (f)

product A again seems to be stronger than product B as in case of aggregates at all the temperatures (Fig. 3b, 3c and 3d). The intensity of smear is also observed higher with product A comparative to product B after 12 months incubation at 5°C (Fig. 3b). An aggregate ladder i.e. multiple aggregate bands are observed in pegylated product A considerably higher in intensity than product B at 25°C (Fig. 3c). One high molecular weight impurity band strike (Fig. 3b, 3c and 3d) was observed in product A at all temperatures.

Specific Activity by *In-Vitro* Bioassay: The specific activities of both the products were observed to be varying with time but within the assay variability (20 %) at 5°C (Fig. 4a) and 25°C (Fig. 4b). However at 40°C, the specific activity of product B was found to be consistent with time while with product A, it was found to be decreasing.

Cation Exchange - HPLC : The pegylated product A was found to have significant higher (1.7 % in A compared to

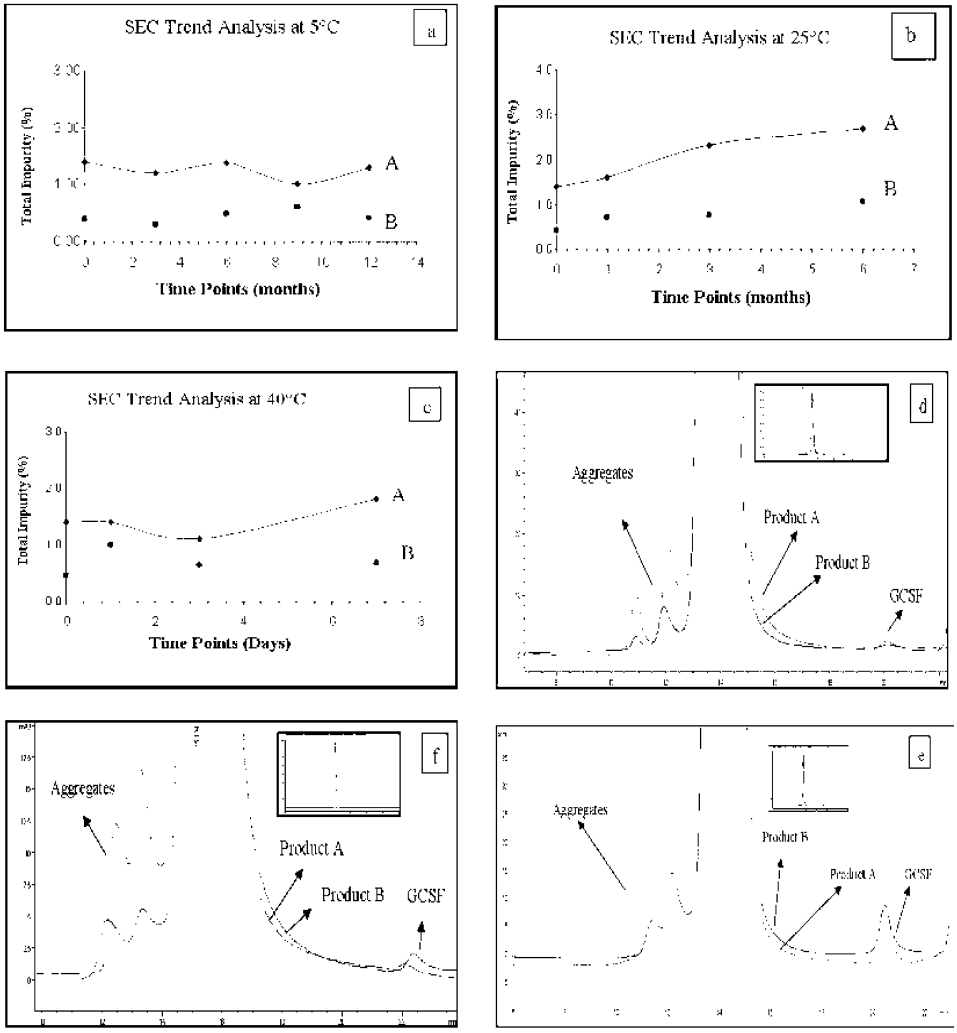
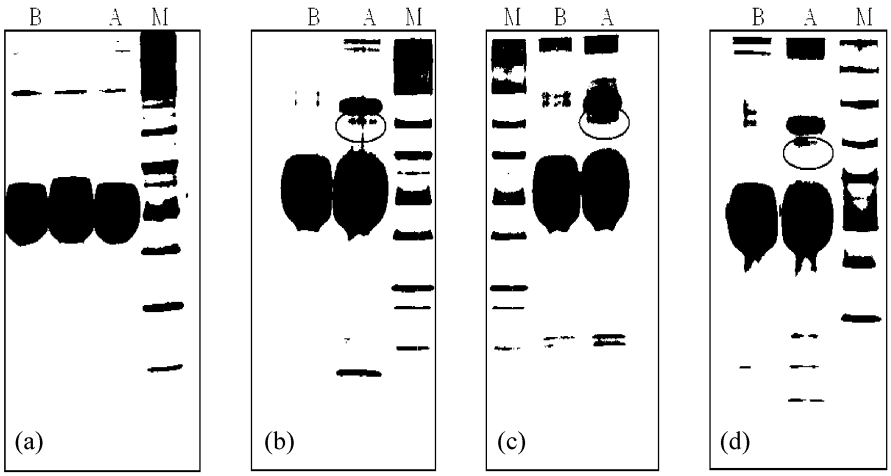


Fig. 2: Trend of Aggregation and depegylation impurities by SE-HPLC at 5°C (a), 25°C (b), 40°C (c) and Overlapped chromatograms after 12 months at 5°C (d), 6 months at 25°C (e), 7 days at 40°C (f)



B = brand B, A = brand A and M = Marker

Fig. 3: SDS-PAGE Gel at Zero time point (a) after 12 months at 5°C (b), 6 months at 25°C (c), 7 days at 40°C (d)

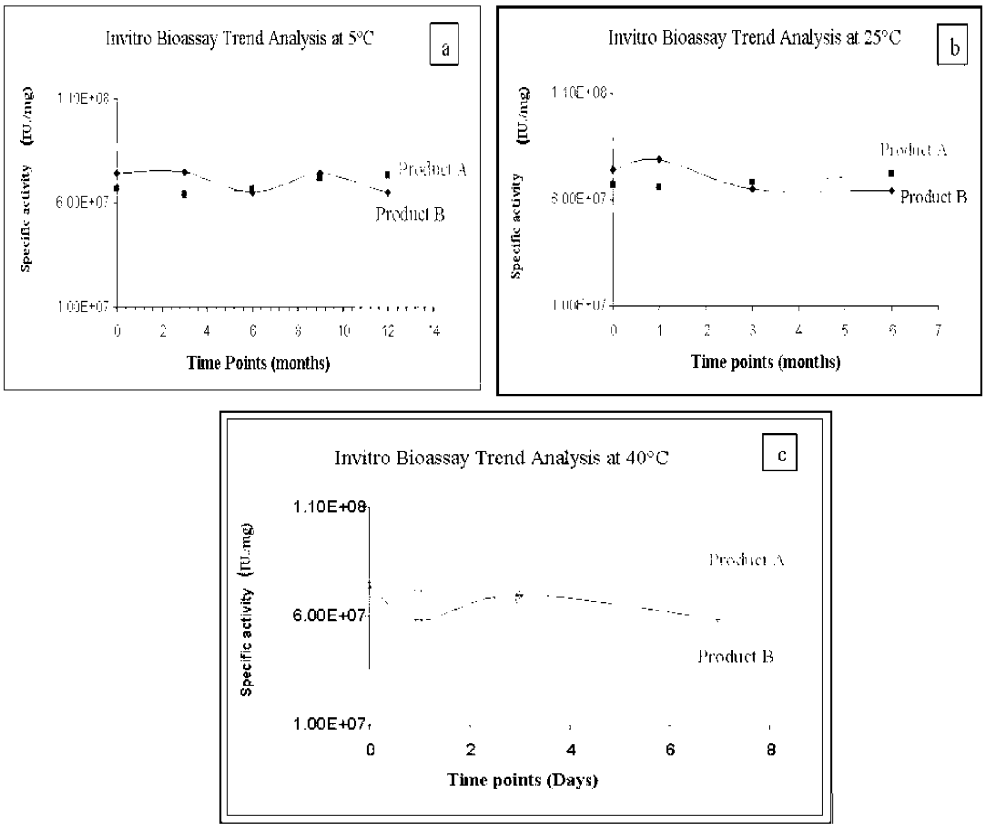


Fig. 4: Trend analysis of Specific activity by In-vitro bioassay at 5°C (a), 25°C (b), 40°C (c) CEx-HPLC:

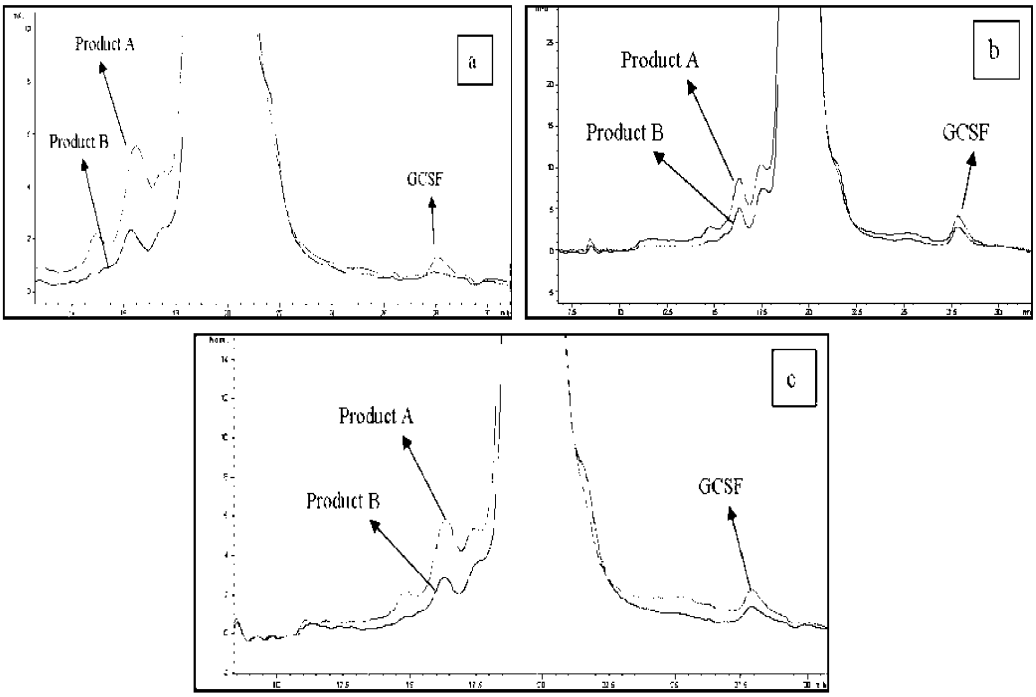


Fig. 5: Overlapped CEx chromatograms, Real time 12M (a), Accelerated 6M (b) and Stress 7D (c)

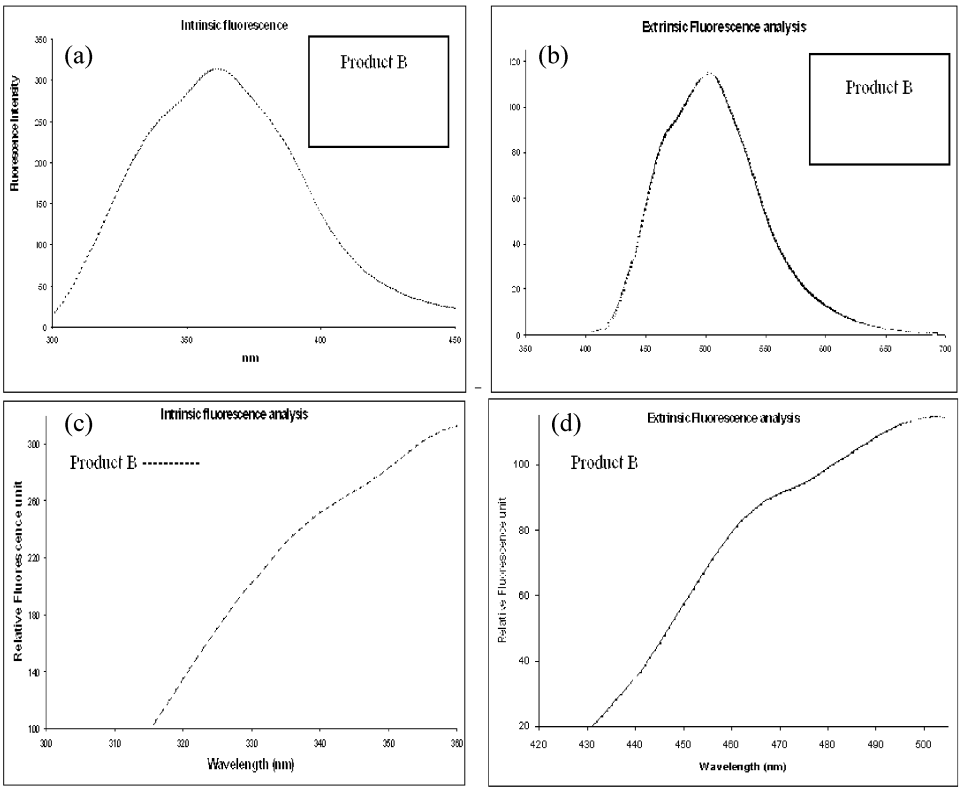


Fig. 5: Emission spectra of Intrinsic Fluorescence (a), Extrinsic Fluorescence (b), Zoomed version Intrinsic (c), Extrinsic fluorescence (d)

0.8 % in B) amount of aggregates and depegylated species compared to pegylated product B after 12 months of exposure at 5°C (Fig. 5a). The aggregates were observed to be increased in both the products at 25°C (Fig. 5b). However, the amount of aggregates was higher (3.8 % in Product A compared to 2.3 % in product B) in case of pegylated product A. The depegylated species (GCSF) was also observed to behave similar as the case of aggregates. Similarly, at stress temperature of 40°C there was an increase in the proportion of aggregated and depegylated species and the difference is also similar that amount of % impurities is almost double in product A (2.1 %) compared to product B (1.1 %).

Structural Elucidation

Fluorescence Analysis: In intrinsic fluorescence (Fig. 5a), a minor blue shift was observed in the product A when compared with product B.

In extrinsic fluorescence (Fig. 5b), a distinct blue shift was observed in the product A when compared with product B.

Thermodynamic Denaturation or Unfolding

Thermograms: The thermal unfolding of both pegylated products was studied using differential scanning calorimetry. The thermograms depicting the thermal unfolding of pegfilgrastim with brand A and brand B from VP-DSC are presented in Figures 6a and 6b respectively. The difference between transition midpoint of pegylated product B and product A is 1°C. This increase in transition midpoint of pegylated product B is indicative of higher stability of this product which is also correlated with the data of stability study at all three temperatures (5°C, 25°C and 40°C).

DLS Analysis: By DLS analysis (Fig. 7), pegfilgrastim brand B showed fewer amounts of aggregates (56.4 % intensity) compared to pegylated product A (82.7 % intensity). The amount of monomer having diameter (approx. 8 nm) was observed more in pegylated product B (39.4 % intensity) compared to pegylated product A (17.6 % intensity).

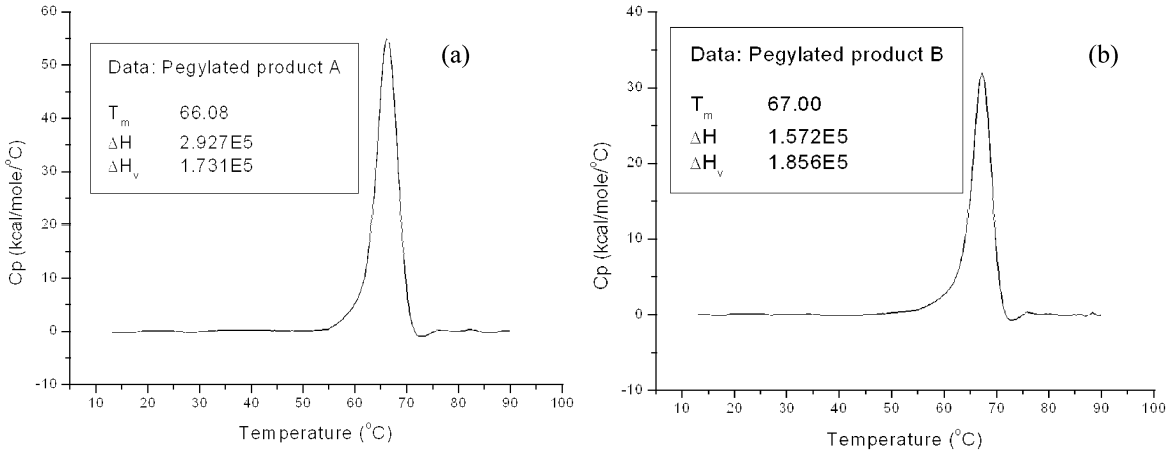


Fig. 6: Thermodynamic profile of pegylated product A (a) and product B (b)

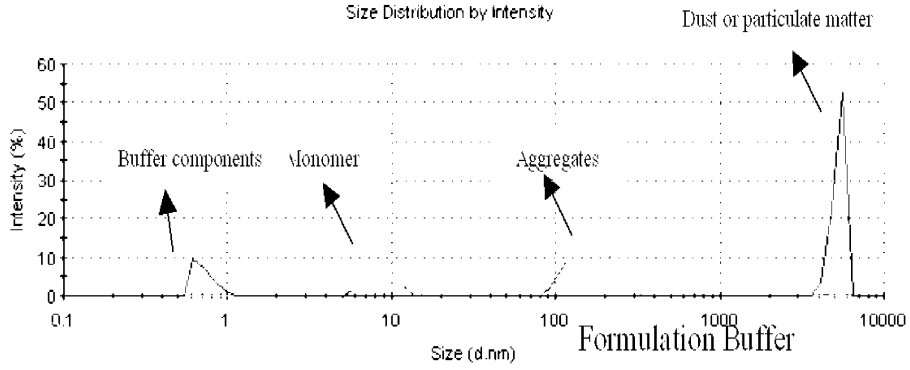


Fig. 7: Size distribution by DLS analysis

Table 2: Comparison of thermodynamic properties of both pegylated products

Product	Transition midpoint (T_m)°C	ΔH_{cal} (Kcal/mol)	ΔH_v (Kcal/mol)	ΔH_{cal} / ΔH_v
Pegylated product (Brand A)	66.1	292 ± 2	173 ± 1	1.69
Pegylated Product (Brand B)	67.0	157 ± 1	186 ± 2	0.84

Table 3: Comparison of pharmacokinetic parameters of both pegylated products

	PK Parameters	Product (Brand A Peg)	Product (Brand B Peg)
Time of peak serum concentration	Tmax (hr)	12.00	12.00
Peak Serum concentration	Cmax (ng/ml)	131.78	138.85
Half Life	$T_{1/2}$ (hr)	11.55	12.05
Rate of elimination	K_{el} (1/hr)	0.06	0.06
Bioavailability	AUC last (hr*ng/ml)	3531.19	4576.17
Volume of Distribution	Vz-obs (ml)	43.54	31.96
Clearance	Cl-obs (ml/hr)	2.57	1.84

PK Studies: To estimate the concentration during time course of the PEGprotein conjugate in the blood sera with satisfactory sensitivity, specificity, reproducibility and accuracy, a universal analytical method for detecting the conjugate in complex biological samples is necessary. So, pharmacokinetics study of both the products was carried out using rodent model (rats). Pharmacokinetic parameters were calculated after analyzing Time Vs Concentration data by Non Compartmental analysis using WinNonlin Software.

Bioavailability of pegylated product B was observed higher than product A which could be an indication of the effect of quality of PEG on pharmacokinetics as product A was observed to be less stable than product

B, evaluated in comparability stability study and unfolding studies. The clearance rate and volume of distribution was also observed less in pegylated product B compared to product A which could be due to less stability of product A (Table 3).

DISCUSSION

In this study, the effect of quality of PEG (raw material) on pegylation yield was demonstrated. As functional activity of PEG brand A is less than product B, the pegylation yield was found to be reduced by 2 % in product A. The unpegylated species (GCSF) is also found higher by 4% in product A compared to product B due to less functional activity of PEG brand A. Higher functional activity increases the chances of pegylation by having functional groups more reactive and ready to pegylate with protein. The higher functional activity may also contribute to formation of Dipeg which had been observed in pegylation reaction (2 % more in pegylated product B). A small amount of dipeg is necessary to have optimum specific activity. As amount of dipeg is observed higher in pegylated product B, the dipeg had been reduced in further purification steps.

In the Comparability Stability study, the storage stability of both pegylated products and impact of PEG quality on stability of product was demonstrated. The comparability study at temperatures viz. 5°C, 25°C and 40°C was found to have pegylated product B more stable than pegylated product A. Since hydrophobic interactions increases with increasing temperature, a stronger effect would be expected at high temperatures. This seemed to be the case in the study, where significant aggregates and depegylated species were observed in increasing order (5°C < 25°C < 40°C) with temperature. Since high polydispersity has an impact on degradation rate of protein, the pegylated product A (with high polydispersity) was found to have higher rate of degradation than pegylated product B (with low polydispersity). High polydispersity and diol content also directed to population of drug conjugates [13] which was seen in the case of pegylated product A by SDS-PAGE at all the storage conditions studied. The same case had been observed in RP-HPLC and CEx results of pegylated product A. These properties were found to be the case of higher degradation and conjugate formation in pegylated product A compared to product B in our study. The smear in SDS PAGE was also observed to be higher in pegylated product A, which is due to PEG polydispersity as high polydispersity caused to disperse the protein and appeared to have smear.

In Structural Elucidation study, the effect of PEG quality on structure of the protein was demonstrated. The intrinsic fluorescence spectra of pegylated product A was found to have minor blue shift (Fig. 5a) which is due to more high molecular weight species [19] as observed in the case of comparability stability study and DLS analysis. The extrinsic fluorescence of pegylated product A was found to have distinct blue shift (Fig. 5b) in emission maxima. In ANS fluorescence, blue shift indicated higher dye binding which happens in the case of exposed hydrophobic patches. So, it is well determined that pegylated product A had more exposed hydrophobic patches in comparison to pegylated product B. Higher amount of aggregates observed in DLS were found as true indicator of high molecular weight species observed in case of fluorescence analysis and comparability stability study. These results clearly defined that high polydispersity caused to open up the protein by exposing hydrophobic pockets in pegylated product A. The differences in structural elucidation are the same as in the case of comparability stability study.

Thermodynamically, the free energy of unfolding (ΔG_{unf}) is the true indicator of stability. However the equilibrium between N and D is often studied in terms of the transition melting temperature or Tm.

$$N \underset{}{\overset{Tm}{\Longleftrightarrow}} D \overset{Ka}{\Longrightarrow} A$$

The Tm is the temperature at which half of the protein is in native conformation while the other half is unfolded or denatured. Although there is no defined relationship between Tm and free energy of unfolding, it is generally accepted that any increase in Tm should lead to an increase in protein stability [20]. Importantly, an increase in Tm has been correlated to improve real time stability of proteins.

The thermal unfolding of both pegylated products is characterized by a single, cooperative, endothermic peak, which is indicative of two-state transition. (Fig. 6a and Fig. 6b). Thermodynamic denaturation of pegylated product A is showing a drop in transition melting point (T_m) of 1°C compared to pegylated product B. Higher the T_m, better the stability. The higher T_m in pegylated product B corresponds to higher stability of product which is directly correlated with the stability of protein under storage, given in comparability stability study.

In this case, the free PEG formed due to depegylation, can interact with the hydrophobic patches and stabilize the unfolded form. This stabilization causes a shift in the unfolding equilibrium towards the D form and is manifested as a Tm drop.

Unfolding of protein is showing difference in ΔH_{cal} / ΔH_v ratio in both the products. ΔH_{cal} / ΔH_v ratio should be close to 1 for a perfect two-state transition [20]. However, as seen from (Table 2), the ΔH_{cal} / ΔH_v ratio for pegylated product A is 1.69. As the ratio is more than 1, it can be inferred that the denaturation of protein molecule involves a multi-state process, or in other words, it consists of several stages. Analysis of calorimetric representing multistate transitions is not straightforward and usually requires the use of deconvolution procedures. From DSC studies with complex proteins, it appears that proteins generally consist of independent cooperative domains whereas small proteins usually consist of single domain. This higher ratio corresponds to higher aggregation and depegylation in pegylated product A as evidenced in comparability stability study.

In case of Pegylated product B, the ΔH_{cal} / ΔH_v ratio is slightly less than 1 (0.84). This may be indicative of intermolecular cooperatively that is introduced in the unfolding process as a result of pegylation. The PEG chains may chaperone GCSF through its unfolding process by progressively patching the hydrophobic interior as the protein unfolds and inhibit protein-protein interactions [21]. Although the value of ratio is close to 1, so it has no significant impact on stability of protein.

These results of T_m and ΔH_{cal} / ΔH_v ratio are supporting the comparability stability study by showing pegylated product B to be more stable than pegylated product A. This data also confirms the effect of quality of PEG on thermodynamic denaturation or unfolding of protein.

Pegylated product A is showing less body-residence time (Fast clearance) [3] compared to pegylated product B. These results could be due to formation of cross-linked conjugates, reported due to high polydispersity and diol content [14] as also evidenced in data of comparability stability study. The formation of these cross-linked conjugates is also evidenced from the fact that pegylated product A is showing unfolding by multi-state process, indicative of degradation in a number of steps. As PK study is done immediately after manufacturing of both the products, so no pronounced effect on pharmacokinetic parameters were evidenced. If study was done after storage of protein within the shelf life time period, it could have certain impact on pharmacokinetic parameters, which needs to be further investigated.

CONCLUSION

The quality of PEG used in pegylation process significantly impacts pegylation yield, storage stability, structural conformation, thermal unfolding and pharmacokinetic behavior of pegfilgrastim. An increase in polydispersity of PEG increase the chances of aggregation, depegylation, increase in product related impurities during storage at real storage conditions as well as elevated temperatures in pegfilgrastim. Higher polydispersity and increase in Diol content leads to formation of cross-linked conjugates and variants in pegfilgrastim. These conjugates further contribute to increase in thermal denaturation and unfolding of the product, increased renal clearance and decreased bioavailability.

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