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Stable Tween 80 Free Formulation Development for Peginterferon Alpha 2b

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Abstract: The stability of peginterferon alpha 2b was studied in the presence and absence of tween 80. Similarly, the stability was also assessed in formulation containing poloxamer other than tween 80. Analysis of aggregated, depegylated and fragmented species were done by SDS PAGE, CEX-HPLC, RP-HPLC and SEC-HPLC. From the results obtained, it is evident that formulation C (10mM phosphate buffer containing 84.6 mg/ml sucrose and 0.01 mg/ml poloxamer) is the best among the three formulation buffers tried, followed by Formulation A (10 mM phosphate buffer, 84.6 mg/ml sucrose) which do not contain any surfactant and formulation B (10 mM phosphate buffer containing 84.6 mg/ml sucrose and 0.01 mg/ml tween 80). Hence, it is concluded that tween 80 can be removed safely from the formulation of interferon alpha 2b with out affecting the product quality with respect to its stability and efficacy.

Key words: Peginterferon · Protein stability · Tween · Poloxamer · SDS PAGE · CEX-HPLC · RP-HPLC and SEC-HPLC

INTRODUCTION

Surfactants decrease the surface tension of protein solutions as predicted by Gibbs adsorption isotherm and force the protein to change its order so that more hydrophilic groups are solvent exposed and there will be less chances of protein adsorption. There are two classes of surfactants ionic and non-ionic. Ionic surfactants such as SDS have been used in the formulation of many products viz., IL-2, BSA, RNase, aFGF. These surfactants are charged, they can bind to charged groups of proteins which will further assist in denaturation [1]. Nonionic surfactants are the preferred ones for use in pharmaceutical industry, as low concentrations of these surfactants offer sufficient protection against aggregation and adsorption.

A popular class of non-ionic surfactants widely used in the pharmaceutical industry are tweens. Their popularity is largely due to their effectiveness at low concentrations and relatively having low toxicities. These characteristics have made them the most usable surfactant in the formulation research of biotherapeutics in the past two decades. The surfactants inhibit surface adsorption of the proteins [2], aggregation during processing and formulation [3], mixing [4], freeze-thawing [5], freeze-drying [6] and reconstitution [7]. The surfactants can also inhibit chemical instability of protein e.g., prevention of deamidation and loss of mitogenic activity in hEGF [8]. As a result, several protein (pharmaceutical products) both in liquid and solid dosage forms, contain tweens as inactive pharmaceutical ingredients, including actimmune, activase and intron A [9].

However, one rising issue in using tweens for protein formulations is their potential adverse effect on protein stability, which has not been extensively reported. The contamination of peroxides in tween preparation and autooxidation of tween during processing or storage [10,11] pose a threat to proteins for oxidative damage. The oxidative degradation of proteins [12] can be a serious concern for immunogenicity and bioactivity of proteins. There are few examples of this phenomenon including oxidative degradation (dimerization by alkyl peroxides) of recombinant human ciliary neurotrophic factor (rhCNTF) in solution [13] and recombinant human granulocyte colony-stimulating factor (rhG-CSF) in solution during storage [14] by the residual peroxides in tween 80 (a single oleic acid ester of polyoxyethylene sorbitan).

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In addition to oxidative damage, there are certain concerns related to adverse reactions from the injection of product containing tween as formulation excipient. Patients who developed hypersensitivity reactions while receiving darbepoetin and erythropoietin were studied and through skin testing, the causative agent was identified [15] as polysorbate 80 (tween 80) which was used as an excipient in these formulations. Tween has also been reported to cause both respiratory and dermatologic side effects when used as an emulsifier in inhalation suspensions. The additives are causative agents of allergic reactions and the patient was found to be sensitized to polysorbate 80, contained in the penicillin preparations [16]. A case of severe recurrent asthma was observed in a patient due to polysorbate 80, contained in the budesonide preparation [17].

Pegylated interferon-ribavirin combination therapy is a treatment for chronic hepatitis C. This therapy has higher injection site reactions compared to interferon and ribavirin alone. The common side effects of ribavirin and peginterferon treatment are hemolytic anemia and cutaneous site manifestations. The patient in this case, developed erythematous edematous vesiculobollus patches after injection of peginterferon alfa 2b which ultimately come out to be generalized rash [18]. As peginterferon alfa 2b contains additional additives such as sodium phosphate monobasic and dibasic, sucrose, Polysorbate 80 etc. and some of these additives are reported to have hypersensitivity reactions. It could be the same case if further diagnostic testing such as patch, prick or intra-cutaneous testing would have been done.

Due to raising concerns over usage of tween 80 in protein formulations, the present study was carried out to evaluate its affect on the quality attributes of protein during storage, in terms of aggregation, isomers profile, oxidation and other types of degradation. Formulations without surfactant and with different non-ionic surfactants were also studied in order to develop stable formulations for peginterferon.

MATERIALS AND METHODS

Materials: USP grade sodium phosphate monobasic ($NaH_2PO_4.H2O$), sodium phosphate dibasic (Na_2HPO_4), sucrose, poloxamer was procured from J.T baker (USA) and used for the study. Low peroxide tween 80 was procured from Sigma (USA).

Peginterferon alpha 2b (31 KD) was prepared by using site specific pegylation of interferon alpha 2b. The protein was then processed by several chromatographic steps to achieve more than 95% purity by SE-HPLC. The peginterferon bulk was then diafiltered to achieve a protein concentration of approximately 0.5 mg/ml in a buffered solution (Formulation A, B and C) at pH 7.0 and stored at-70°C in a Polyethylene Terephthalate Copolyester (PETG) bottle from Nalgene.

Preparation of Samples: Frozen peginterferon alpha 2b was thawed and exchanged against three different formulations (A, B and C) (Table 1) buffered with phosphate at pH 7.0. The final compositions of these formulations are listed in Table 1.

These formulations were then sterile filtered using 0.2 µ filter and were aliquoted in glass vials for analysis at different time points as mentioned in Table 2. The filled vials were then incubated at 25 and 40 °C and withdrawn periodically for analysis as enlisted in Table 2.

The quality attributes of these formulations were evaluated by means of methods given below.

Methods: Sodium dodecyl sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE):

- SDS PAGE was performed using 14% acrylamide gel. The gel was loaded with 5µg of sample. The sample was run at constant voltage of 130 V and variable current of 200 mA. Visualization was done by silver staining.

Table 1: Different compositions of peginterferon alpha 2b

S. No.	Composition Type	Excipients in formulations
1.	Formulation A	Phosphate buffer (10 mM) and sucrose (84.6 mg/ml)
2.	Formulation B	Phosphate buffer (10 mM), sucrose (84.6 mg/ml) and tween 80 (0.01 mg/ml)
3.	Formulation C	Phosphate buffer (10 mM), sucrose (84.6 mg/ml) and poloxamer (0.01 mg/ml)

Table 2: Time points for withdrawal and Analysis

S. No.	Formulations Type	Storage temperature (°C)	Time points for analysis (days)
1.	A-C	25	0D, 4D, 7D, 14D
2.		40	0D, 4D, 7D, 14D

D→Days

Size Exclusion High-Performance Liquid Chromatography (SE-HPLC): SE-HPLC was performed on Agilent HPLC having temperature controlled autosampler (4°C). This method employs TOSHO TSK 3000 SWXL column, mobile phase of 100 mM sodium phosphate (NaH_2PO_4), 150 mM sodium chloride (NaCl), 10% ethanol (pH 5.0). The analysis was done in isocratic condition. The elution time for protein and its related impurities was between 12-25 min.

Cation Exchange High-Performance liquid Chromatography (CEX HPLC): IEC was performed using a TOSOH SP-5PW column on an Agilent HPLC system. The mobile phase consist of binding buffer 3.7 mM Sodium acetate (CH_3COONa, pH 4.3) and 10% ethanol and elution buffer containing 10 mM phosphate (pH 6.4) and 10% ethanol. The elution was done by linear gradient of 1% per minute with elution buffer.

Reversed Phase High-Performance Liquid Chromatography (RP HPLC): RP-HPLC was performed using a C18 column. Separation was achieved using a linear gradient from 30 to 68% of mobile phase organic modifier (ACN + 0.1% TFA) in 33 minutes.

RESULTS AND DISCUSSION

Effect of different surfactant on stability of peginterferon alpha 2b was studied under different environmental conditions. The impurities generated were studied by SDS PAGE (under reducing and non reducing conditions), RP-HPLC, SE-HPLC and CEX-HPLC.

Effect of Surfactant on Aggregation, Depegylation and Fragmentation During Storage: Non-reducing SDS PAGE results under non-reduced conditions are shown in Fig. 1a, 1b, 1c, 1d and 1e, where all formulations are compared at different time intervals at both temperature levels.

The aggregation and depegylation of protein increased in all the formulations at both the temperatures but comparatively higher at 40°C as evidenced in Fig. 1. At both the temperatures the smear between monopeg and IFN (representative of histidine isomer) was found to be declining. The declination at 40°C was comparatively higher which could have a strong influence on biological activity of the protein. After 14 days of temperature exposure, formulation B showed one band lower to native IFN at 40°C (Fig. 1C, lane 15) which was not found in formulation A (Fig. 1C, lane 12) or C (Fig. 1e, lane 22). One light fragment band was also observed in formulation A at 40°C after 7 days of exposure (Fig. 1b, lane 9) but not evidenced after 14 days (Fig. 1c, lane 12), so the band observed on 7th day is an outlier. Aggregation in all the formulations at 40°C were observed in the order of--- Formulation C > B > A, although it is observed same at 25°C. Depegylation (IFN) in all the formulations was observed approximately similar at both the temperatures (Fig. 1a-1e).

The results of SDS PAGE under reduced conditions are shown in fig. 2a, 2b, 2c, 2d and 2e, where all formulations are compared at different time intervals of both the temperatures.

The depegylation is observed similar in case of all the formulations at both temperatures. Fragmentation is

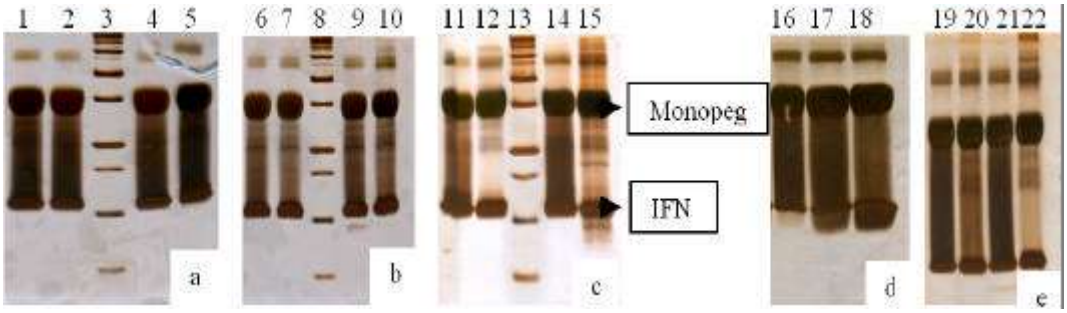

Fig. 1(a-e): Non-reducing SDS PAGE profile of peginterferon alpha 2b (different formulations) at different temperatures and time points. Lane 3, 8 and 13 contain molecular weight marker. Lane 1 and 2 contains Formulation A (25°C; 4D) and Formulation B (25°C; 4D) sample respectively. Lane 4 to 7 contain Formulation A (25°C; 7D), Formulation B (25°C; 7D), Formulation A (40°C; 4D) and Formulation B (40°C; 4D) samples, respectively. Lane 9 to 12 contains Formulation A (40°C; 7D), Formulation B (40°C; 7D), Formulation A (25°C; 14D) and Formulation A (40°C; 14D) samples respectively. Lane 14 to 21 contains Formulation B (25°C; 14D), Formulation B (40°C; 14D), Formulation C (0 day), Formulation C (25°C; 4D), Formulation C (40°C; 4D), Formulation C (25°C; 7D), Formulation C (40°C; 7D), Formulation C (25°C; 14D) and Formulation C (40°C; 14D) samples respectively.

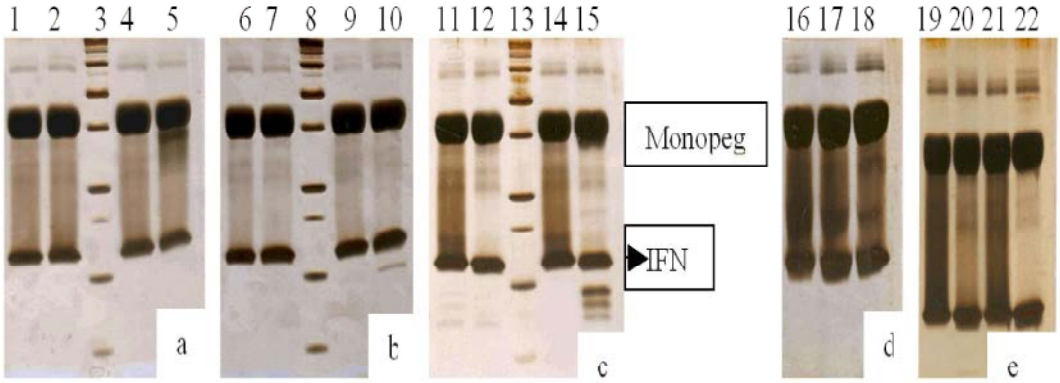

Fig. 2(a-e): Reducing SDS PAGE profile of peginterferon alpha 2b (different formulations) at different temperatures and time points. Lane 3, 8 and 13 contain molecular weight marker. Lane 1 and 2 contains Formulation A (25°C; 4D) and Formulation B (25°C; 4D) sample respectively. Lane 4 to 7 contain Formulation A (25°C; 7D), Formulation B (25°C; 7D), Formulation A (40°C; 4D) and Formulation B (40°C; 4D) sample respectively. Lane 9 to 12 contains Formulation A (40°C; 7D), Formulation B (40°C; 7D), Formulation A (25°C; 14D) and Formulation A (40°C; 14D) samples respectively. Lane 14 to 21 contains Formulation B (25°C; 14D), Formulation B (40°C; 14D), Formulation C (0 day), Formulation C (25°C; 4D), Formulation C (40°C; 4D), Formulation C (25°C; 7D), Formulation C (40°C; 7D), Formulation C (25°C; 14D) and Formulation C (40°C; 14D) samples respectively.

observed in case of formulation A (Fig.1c, lane 12) and B (Fig.1a, lane 15) after 14 days of exposure at both the temperatures. The sign of fragment bands is significantly less in case of formulation A while the fragment bands were observed significantly higher in case of formulation B at 40°C from 7th day of exposure (Fig. 1b, lane 10). No sign of fragment bands were observed in case of formulation C at both the temperatures. Fragmentation order for all the formulations is as follows---Formulation B > A > C. A significant amount of fragmentation observed in case of formulation B which could be the cause of acetic acid or formic acid formation due to auto-oxidation of tween 80 [19]. These fragment bands were also observed at higher temperatures as the case of oxidized impurities in RP HPLC (Fig. 13c). These fragment bands seen at 40°C on 7th day and 15th day of exposure are indicative of the IFN fragments co-eluting with formulation buffer components as shown in Fig. 3d.

Size Exclusion High Performance Chromatography: The aggregation, depegylation and fragmentation of peginterferon formulations A, B and C were checked by SE-HPLC at time intervals specified in Table 2. The representative chromatograms and trend analysis are mentioned below:

Overlapped Reference Chromatograms: Depegylation (LMW species and IFN) is observed to increase at 25°C for all the formulations while no significant increase in aggregation was observed as shown in Fig. 3a, 3c and 3e. One extra peak (strike in Fig. 3c) between HMW species and monopeg (Retention time of ~17.5 min) was observed in case of formulation B which disappeared at 14 days at both temperatures i.e. 25°C and 40°C. This peak is observed to decrease continuously with time as seen in Fig. 3d. HMW species at retention time of 12.5 (approx.) were observed to increase considerably with time at 40°C in formulations (A and B) as shown in Fig. 3b and 3d. HMW species adjacent to monopeg does not show any increase at any temperature which implies that it is a dipeg. As seen in Fig. 3d, one pre-peak is also observed in case of formulation B at 40°C which is not the case of formulation A (Fig. 3b). This peak could be due to formation of micelle or a new HMW species (yet to be characterized) and this peak is showing increase or emergence at 7th day and 14th day time point at 40°C. The peaks which are labeled as formulation buffer components are showing significant increase at 7th day and 14th day time point at 40°C (Fig.3d). This increase is a clear indication of co-elution of fragments pertaining to interferon along with formulation buffer as clear fragment band were observed at 7th day and 14th day time point of 40°C in reducing gel (Fig. 2b, lane 10 and Fig. 2c, lane 15, respectively).

To identify an extra peak between HMW species and monopeg, formulation buffer of all the formulations were run.

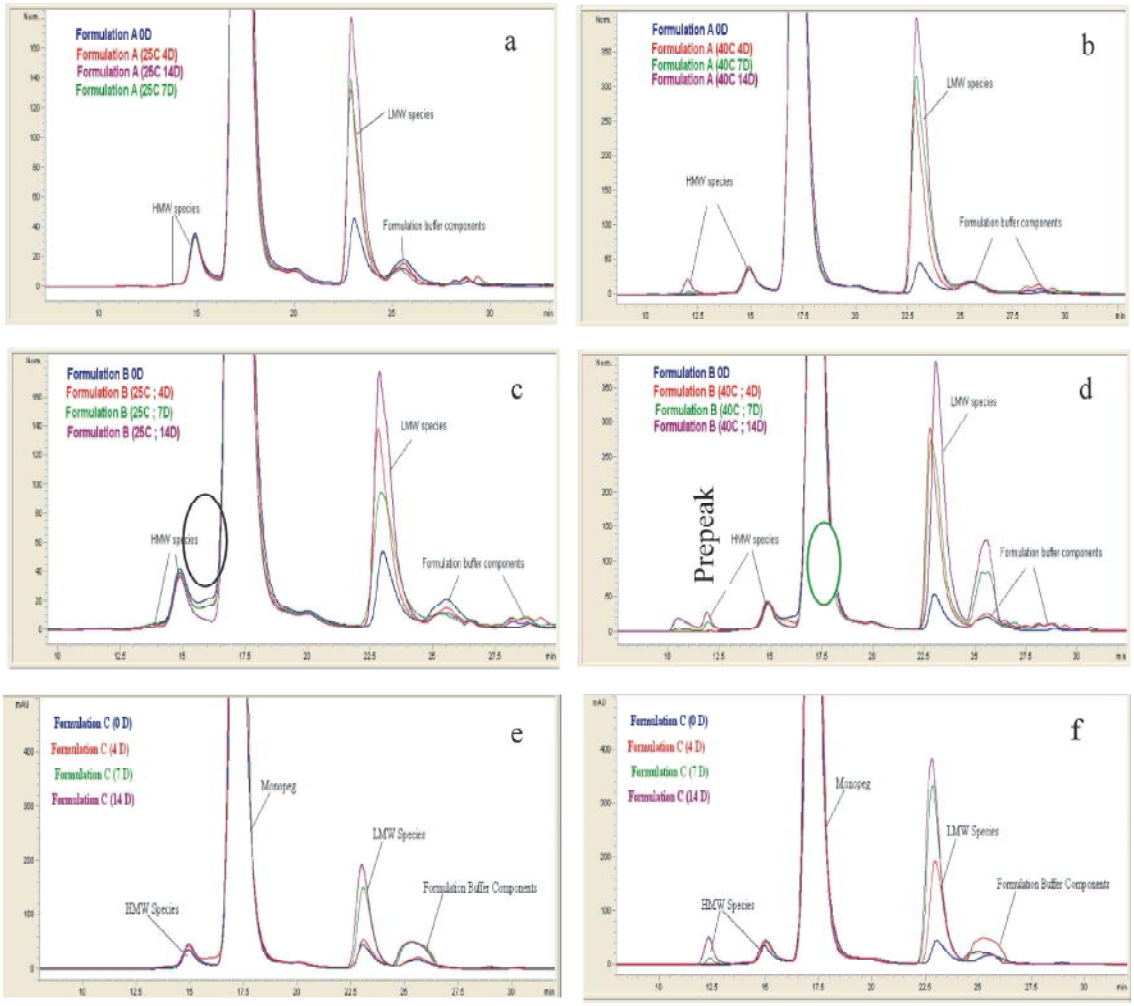

Fig. 3a: Formulation A at 25°C b-Formulations A at 40°C, c-Formulations B at 25°C d-Formulations B at 40°C

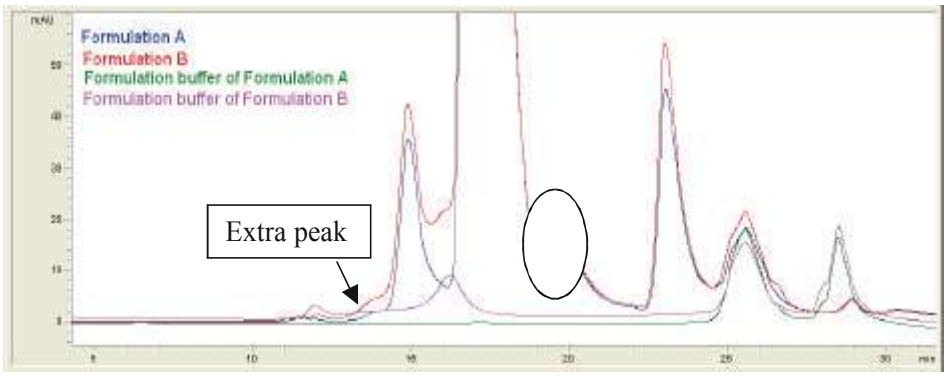

Fig. 4: Overlapped chromatogram of formulation A and B at Zero days against formulation buffers of both the formulations

The peak shown in circle is extra peak observed in formulation B which is also observed in formulation buffer of formulation B (Fig.4). This result indicates that the extra peak in formulation B compared to formulation A is due to tween 80 as only difference in formulation A and B is the presence of tween 80. One peak (labeled as extra peak) is also observed at exact position of HMW species except dipeg in case of formulation buffer of

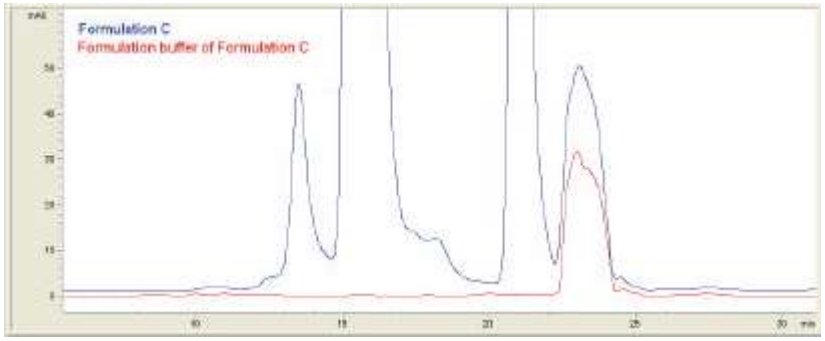

Fig. 5: Overlapped chromatogram of Formulation C against formulation buffer

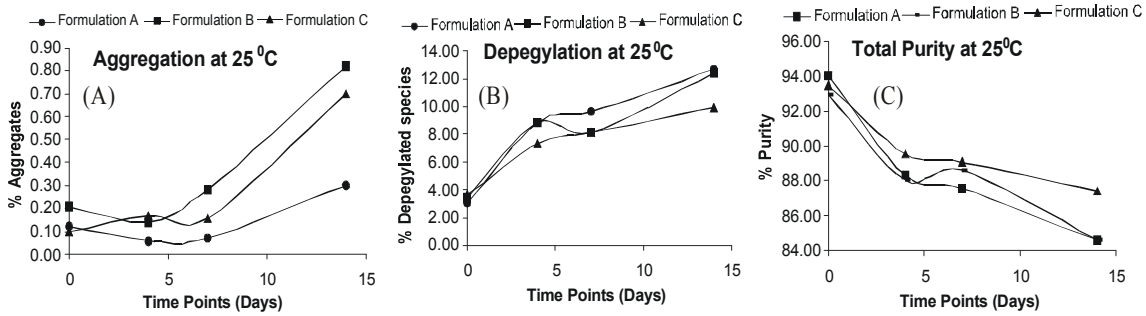

Fig. 6: Trend analysis of aggregation (a), depegylation (b) and total purity (c) at 25°C.

formulation B (Fig. 4). This result signifies that there could be chances of overestimation of HMW species in formulation containing tween 80. A paper [20] by Annabelle *et al.* on "Interaction of polysorbate 80 with erythropoietin" concluded that polysorbate 80 associates with erythropoietin in a defined stoichiometric ratio. It could be the same case in this study also.

Formulation buffer of formulation C is also not having any co-elution with the peginterferon related substances as shown in Fig. 5.

The depegylation (Fig. 6a) in all the formulations is observed to increase at 25°C which contributes to corresponding decrease in purity of Peg-IFN as shown in Fig. 6b. The trend of depegylated species in formulation B is showing considerable decrease in depegylated species at 7th day time point (Fig. 6a) which could be due to fragmentation of interferon in formulation B as fragmentation is observed from 7th day onwards (Fig. 2b, lane 10). This is the same case as trend of purity of Peg-IFN (Fig.6b).

As shown in Fig.7a, the aggregation was observed to increase considerably with time at this temperature, while the formulations containing surfactant were shown to have higher rate of aggregation. In Fig. 3d, the peak observed at retention time of 10 min (approx.), is the one which contribute to significant increase in aggregates of formulation B. The aggregation in formulations containing surfactant is higher compared to formulation without surfactant which could be due to direct interaction of surfactant with the protein, resulting in an exposure of buried and less reactive groups. These results are perfectly in correlation with the results obtained for IL-2 mutein during storage in presence of tween 80 [21]. The aggregation at 25°C was not observed to increase while a significant increase at 40°C was observed since hydrophobic interactions increases with increase in temperature. It is reported that poloxamer increases solubility of urease at 50°C but decreases its stability at 75°C [22], tween 20 at 0.03% reduces unfolding free energy of rhIFNã [23]. This explains the destabilization of protein in presence of nonionic surfactants in a temperature dependent manner. The depegylation in all the formulations are observed to increase considerably with time at 40°C (Fig.7b) while highest and lowest depegylation is observed in case of formulation A and formulation C, respectively. Same is the case at 25°C (Fig. 6a).

The degradation rate of Peg-IFN formulations was calculated by trend of purity obtained at both temperatures till 14 days.

Degradation rate of Peg-IFN formulations at both the temperatures follows the order of Formulation C < B < A as shown in Fig. 8a and 8b. This degradation rate in all the formulations at 25°C is exclusively due to depegylation

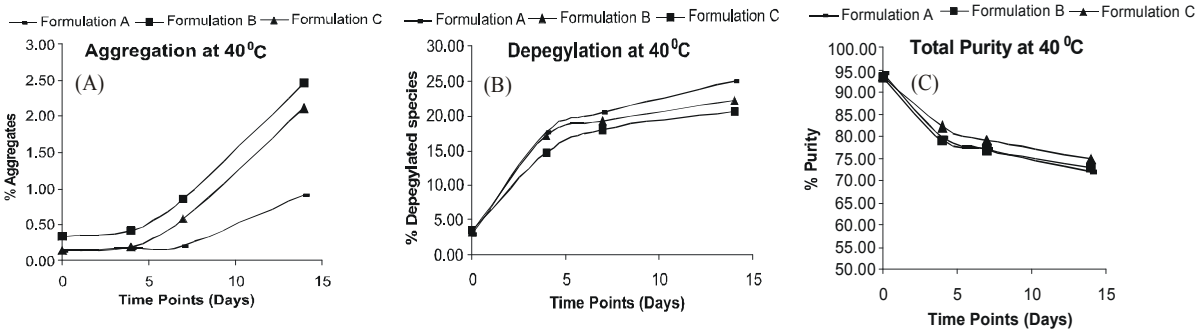

Fig. 7: Trend analysis of aggregation (a), depegylation (b) and total purity (c) at 40°C.

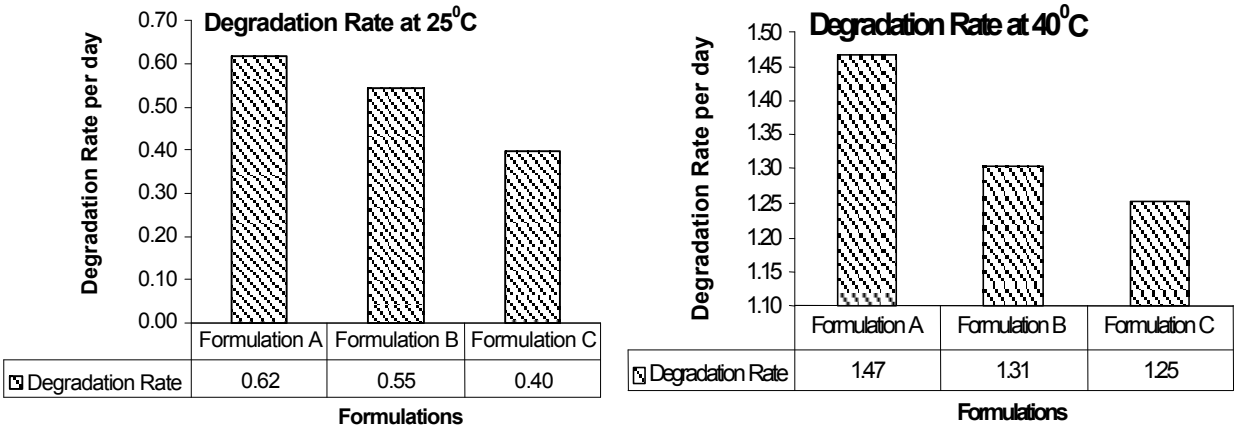

Fig. 8: Degradation rate of formulation A, B and C at 25°C (a) and 40°C (b).

(Fig.6a and 6b) while at 40°C, both aggregation and depegylation (Fig. 7a-7c) contributes to degradation rate. As degradation rate of Peg-IFN formulation without surfactant is showing highest degradation rate. So it is evident that surfactant helps in preventing depegylation to some extent. As in our study, surfactant increases hydrophobic interactions between the protein molecules and these interaction cause the unfolding of protein. So, this loosening of protein fold and hydrophobic interactions by burying pegylation bond would prevent depegylation in the protein. Formulation C shows least degradation rate.

Effect of Surfactant on Isoforms Pattern During Storage: The isoforms pattern and degradation rate of all the three formulations of peginterferon alpha 2b was studied at 25 and 40°C by CEX HPLC.

The histidine isomer in all the formulations was observed to decrease with time at both the temperatures. The decrease in histidine isomer in all the formulations is considerably higher at 40°C compared to 25°C which is the same case observed by SDS PAGE.

The degradation rate in all Peg-IFN formulations was calculated from the trend of decrease in histidine isomer (major isomer for biological activity) [24] which are as follows:

- According to degradation rate calculated from trend analysis of all the formulations, formulation (A) stands to be the best at both the temperatures (25 and 40°C), followed by formulation (C) and formulation (B) which was found to be least stable among all formulations. The degradation rate in formulation B and C is observed to be higher compared to formulation A due to formation of HMW species which were also observed by SDS PAGE and SE-HPLC. The generation of new impurities due to auto-oxidation of tween 80 could be the primary cause of major degradation associated with formulation (B) at both the temperatures as oxidized impurities, aggregates and fragments were observed higher in case of formulation (B). These results indicate that tween 80 can be safely omitted from the formulation of peginterferon alpha 2b.

Effect of Surfactant on Oxidation During Storage: The effect of surfactant on oxidation of peginterferon alfa 2b was studied by RP HPLC.

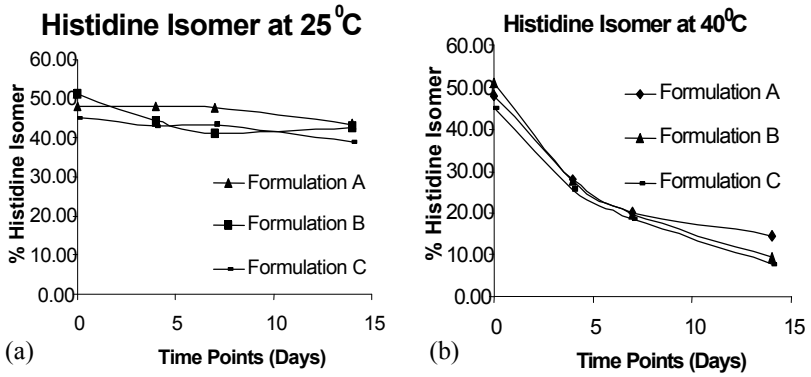

Fig. 9: Trend analysis of histidine isomer at 25°C (a) and 40°C (b).

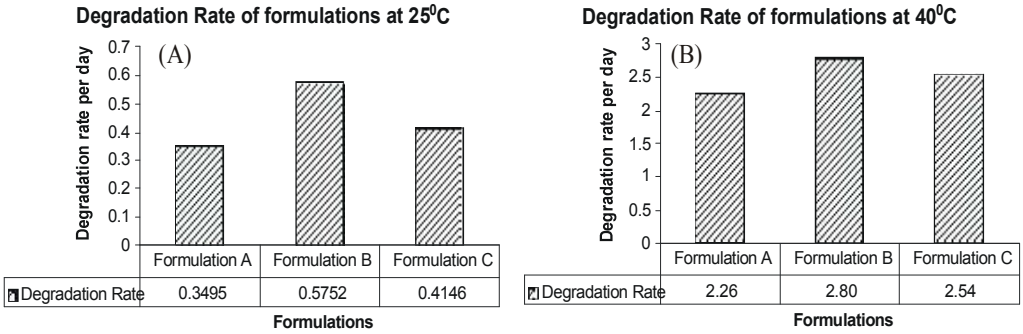

Fig. 10(a-b): Degradation rate of Peg-IFN in all formulations A, B and C at 25°C (a) and 40°C (b).

The samples were analyzed by RP HPLC on First day and 14th day for calculation of degradation rate.

The impurities are observed to increase in all the formulations with time at both the temperatures. The impurities in formulation (B) were observed to increase considerably higher compared to other formulations at both the temperatures. The trend is observed similar in case of formulation A and C as shown in Fig. 11b while a slightly upward trend is observed in formulation A compared to formulation C at 25°C as shown in Fig. 11a.

The degradation rate of Peg-IFN in all the formulations was calculated from the trend of increase in impurities. The chart of degradation rates are as follows:

- According to degradation rates calculated, formulation (A) was observed to be the best among all the formulations which is also evidenced from results of IEC and SDS PAGE. Although a slightly higher degradation, in case of formulation (A) was observed in comparison to formulation (C) at 40°C but the difference (Fig. 12b) is not much significant (0.03). The degradation rate for formulation (B) is found to be the highest at both the temperatures and rate of degradation is almost double at 40°C (Fig. 12b) compared to that at 25°C (Fig. 12a). The degradation rate in formulation (C) is observed equivalent (Fig. 12a and 12b) at both the temperatures. At 40°C, the rate of degradation (Fig. 12b) in formulation (B) is almost double to that of formulation (A) and (C).

As seen in Fig. 13b, no significant increase in related impurities except native molecule (IFN) was observed in all Peg-IFN formulations at 25°C. IFN was found to increase which contributes further to the degradation phenomenon (shown in Fig. 12a). All formulations were found to have an increase in related impurities at 40°C, while significant increase in oxidized impurities was observed in case of formulation B (Fig. 3c) which has tween 80 in the formulation buffer. The presence of these impurities in formulation containing tween could be due to auto-oxidation of tween which generates peroxide at 40°C and this peroxide can be the root cause of oxidation in formulation B. The polysorbates undergo autooxidation [10] and cleavage at the ethylene oxide subunits [25, 26], as well as hydrolysis of the fatty acid ester bond [27]. Autooxidation of ethylene oxide results in hydroperoxide formation, side-chain cleavage and eventually formation

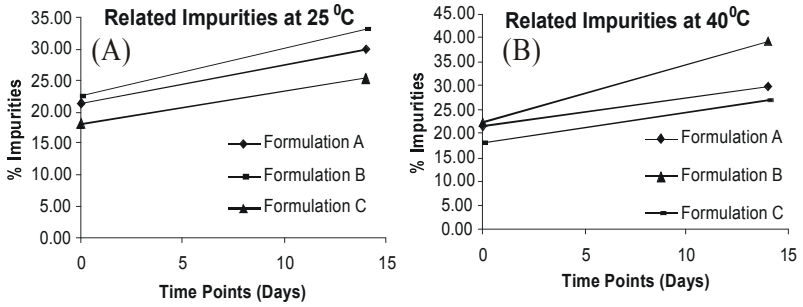

Fig. 11: Trend analysis of related impurities by RP HPLC in Peg-IFN formulations A, B and C at 25°C (a) and 40°C (b).

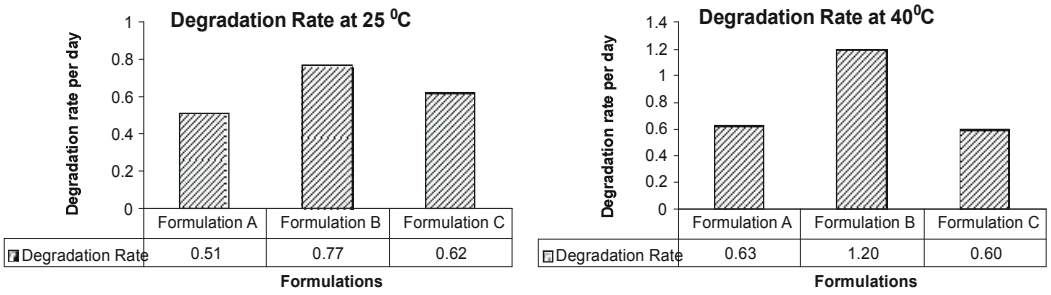

Fig. 12: Degradation rate of Peg-IFN in formulations A, B and C by RP HPLC at 25°C (a) and 40°C (b).

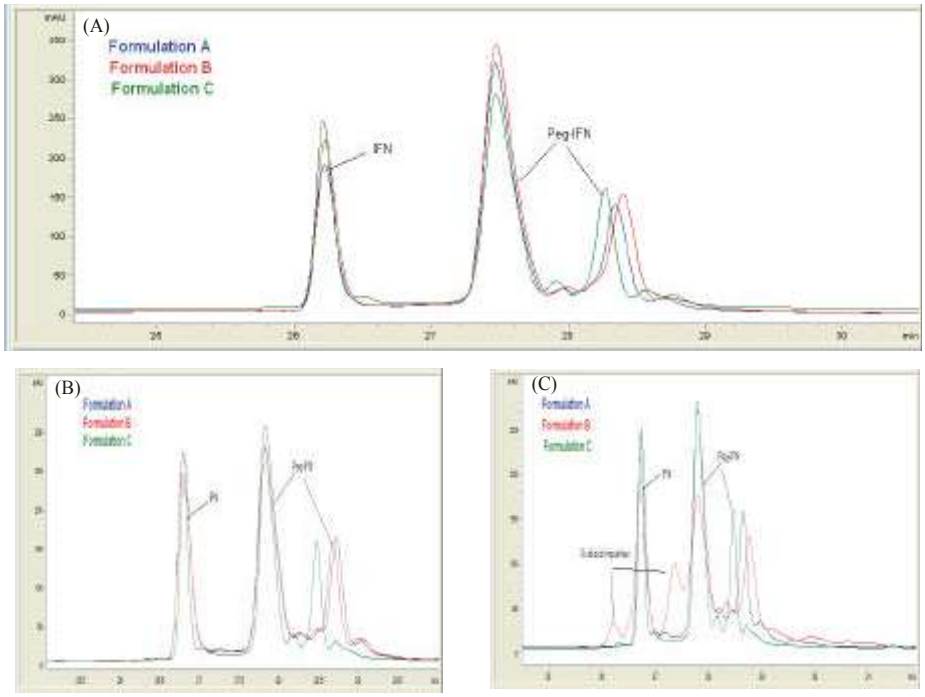

Fig. 13: Overlapped reference chromatograms of formulations A, B and C at zero day (a) and 25°C; 15D (b) and 40°C; 15D (c).

of formic acid [19]. This hydroperoxide formation is known to cause oxidation of proteins such as rhCNTF and rhNGF [13]. Storage of polysorbates at room temperature primarily results in hydrolysis of the fatty acid ester while storage at higher temperatures favors autooxidation of ethylene oxide subunits. This seems to be the case in this study where formulation B i.e., formulation containing tween 80 was found to have less amount of oxidized impurities at 25°C (Fig. 13b) while it was higher at temperature (40°C) which contributes to almost double

Table 3: Summary of quality attribute of three different formulations

	Formulations		
Quality Attributes	Formulation A	Formulation B	Formulation C
Aggregation	+++	+	++
Depegylation	+	++	+++
Purity	++	++	+++
Degradation	+	++	+++
Histidine isoforms	+++	+	++
Oxidatized impurity	++	+	+++
Total	12	9	16

+++ is the best desired quality followed by ++ and +

(Fig. 12a and 12b) rate of degradation compared to that at 25°C. This higher degradation rate at higher temperature is due to increase in autooxidation of ethylene oxide subunits. No significant difference in impurities was observed in case of formulation C at 25°C and 40°C. This is also evident from the degradation rate of both the temperatures specified in Fig. 12a and 12b. A slight increase in impurities such as oxidized impurities of IFN and post IFN peak in case of formulation A were observed at 40°C compared to 25°C in case of formulation A which contributes to increase in degradation rate at 40°C (0.63) compared to that at 25°C (0.51).

Significant amount of oxidized impurities which is observed in formulation B, increases the chance to generate immunogenicity or other adverse reactions. These results are also supported with the case report published by Toney and Agarwal [18] suggesting the influence of additive on adverse reactions associated with the patient.

CONCLUSION

Peginterferon easily form aggregates in temperature dependent manner as no significant increase in aggregates was observed at 25°C while at 40°C, considerable amount of aggregates are generated after 14 days of exposure. The formulation containing surfactants (poloxamer or tween 80) at 40°C are having significantly increased rate of aggregation compare to without surfactant formulation during storage. The increase in aggregates led to decreased histidine isomer which is the major isomer required for biological activity. Addition of surfactants helps to prevent depegylation in peginterferon while formulation containing tween 80 is observed to have fragmentation which could be a cause of less depegylation rate. Poloxamer helps to prevent depegylation and no sign of fragmentation is observed in this formulation. Tween 80 is found to create oxidative damage to the protein due to auto-oxidation of ethylene subunits and oxidation rate is strongly temperature dependent. The autooxidation of additive tween 80 is evidenced as the main cause for all types of degradation such as aggregation, oxidation, fragmentation and loss of isomers in peginterferon.

From the Table 3, it is also evident that Formulation C (10 mM phosphate buffer containing 84.6 mg/ml sucrose and 0.01 mg/ml poloxamer) is the best among the three formulation buffers tried, followed by Formulation A (10 mM phosphate buffer, 84.6 mg/ml sucrose) and Formulation B (10 mM phosphate buffer containing 84.6 mg/ml sucrose and 0.01 mg/ml tween 80).

Thus, it is suggested that tween 80 leads to generation of impurities in peginterferon preparations which ultimately can be a cause of adverse reactions associated with peginterferon therapy. Poloxamer was found to be a better surfactant than tween 80, with respect to promoting stability via generating less impurity. Formulation with out any surfactant can also be a choice when developing peginterferon formulations.

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REFERENCES

1. Giancola, C., C. De Sena, D. Fessas, G. Graziano and G. Barone, 1997. DSC studies on bovine serum albumin denaturation: Effects of ionic strength and SDS concentration. Int. J. Biol. Macromol., 20(3): 193-204.

2. Gombotz, W.R., S.C. Pankey, L.S. Bouchard, D.H. Phan and A.P. MacKenzie, 1996. Stability, Characterization, Formulation and Delivery System Development for Transforming Growth Factor-beta1. Pharm. Biotechnol., 9: 219-45.
3. Bam, N.B., J.L. Cleland and T.W. Randolph, 1996. Molten globule intermediate of recombinant human growth hormone: stabilization with surfactants. Biotechnol. Prog., 12(6): 801-809.
4. Katakam, M., L.N. Bell and A.K. Banga, 1995. Effect of surfactants on the physical stability of recombinant human growth hormone. J. Pharm. Sci., 84(6): 713-716.
5. Chang, B.S., B.S. Kendrick and J.F. Carpenter, 1996. Surface-induced denaturation of proteins during freezing and its inhibition by surfactants. J. Pharm. Sci., 85(12): 1325-1330.
6. Sarciaux, J.M., S. Mansour, M.J. Hageman and S.L. Nail, 1999. Effects of buffer composition and processing conditions on aggregation of bovine IGG during freeze-drying. J. Pharm. Sci., 88(12): 1354-1361.
7. Zhang, M.Z., K. Pikal, T. Nguyen, T. Arakawa and S.J. Prestrelski, 1996. The effect of the reconstitution medium on aggregation of lyophilized recombinant interleukinl-2 and ribonuclease A. Pharm. Res., 13(4): 643-646.
8. Son, K. And C. Kwon, 1995. Stabilization of Human Epidermal Growth Factor (hEGF) in Aqueous Formulation. Pharmaceutical Research, 12(3):451-454.
9. PDR, 1999. Physician's Desk Reference. Medical Economics Company, Montvale, NJ.
10. Donbrow, M., E. Azaz and A. Pillersdorf, 1978. Autoxidation of polysorbates. J. Pharm. Sci., 67(12): 1676-1681.
11. Jaeger, J., K. Sorensen and S.P. Wolff, 1994. Peroxide accumulation in detergents. J. Biochem. Biophys. Methods, 29(1): 77-81.
12. Ha, E., W. Wang and Y.J. Wang, 2002. Peroxide formation in polysorbate 80 and protein stability. J. Pharm. Sci., 91(10): 2252-2264.
13. Knepp, V.M., J.L. Whatley, A. Muchnik and T.S. Calderwood, 1996. Identification of antioxidants for prevention of peroxide-mediated oxidation of recombination human ciliary neurotrophic factor and recombinant human nerve growth factor. J. Pharm. Sci. Technol., 50(3): 163-171.
14. Herman, A.C., T.C. Boone and H.S. Lu, 1996. Characterization, Formulation and Stability of Neupogen (Filgrastim) a Recombinant Human Granulocyte-colony Stimulating Factor. Pharm. Biotechnol., 9: 303-28.
15. Steele, R.H., S. Limaye, B. Cleland, J. Chow and M.G. Suranyi, 2005. Hypersensitivity reactions to the polysorbate contained in recombinant erythropoietin and darbepoietin. Nephrology (Carlton), 10(3): 317-320.
16. Isaksson, M. and L. Jansson, 2002. Contact allergy to Tween 80 in an inhalation suspension. Contact Dermatitis, 47(5): 312-313.
17. Grims, R.H., B. Kränke and W. Aberer, 2006. Pitfalls in drug allergy skin testing: false-positive reactions due to (hidden) additives. Contact Dermatitis, 54(5): 290-294.
18. Toney, R.C. and R.M. Agrawal and M. Loeffert, 2009. Injection site bullous eruption and generalized rash induced by pegylated interferon treatment for hepatitis C. Gastroenterol. Hepatol., 5(10): 715-718.
19. Donbrow, M., R. Hamburger, E. Azaz and A. Pillersdorf, 1978. Development of acidity in non-ionic surfactants: Formic and acetic acid. Analyst, 103(1225): 400-402.
20. Villalobos, A.P., S.R. Gunturi and G.A. Heavner, 2005. Interaction of Polysorbate 80 with Erythropoietin: A Case Study in Protein-Surfactant Interactions. Pharmaceutical Research, 22(7): 1186-1194.
21. Wang, W., Y.J. Wang and D.Q. Wang, 2008. Dual effects of Tween 80 on protein stability. Int. J. Pharm., 347(1-2): 31-38.
22. Wang, P.L. and T.P. Johnston, 1993. Thermal-induced denaturation of two model proteins: effect of poloxamer 407 on solution stability. Int. J. Pharm., 96(1-3): 41-49.
23. Webb, S.D., J.L. Cleland, J.F. Carpenter and T.W Randolph, 2002. A new mechanism for decreasing aggregation of recombinant human interferon-gamma by a surfactant: slowed dissolution of lyophilized formulations in a solution containing 0.03% polysorbate 20. J. Pharm. Sci., 91(2): 543-558.
24. Wang, Y.S., S. Youngster, M. Grace, J. Bausch, R. Bordens and D.F. Wyss, 2002. Structural and Biological characterization of pegylated recombinant interferon alpha-2b and its therapeutic implications. Advanced Drug Delivery Reviews, 54(4): 547-570.
25. Hamburger, R., E. Azaz and M. Donbrow, 1975. Autoxidation of polyoxyethylenic non-ionic surfactants and of polyethylene glycols. Pharm. Acta. Helv., 50(1-2): 10-17.
26. Lever, M., 1977. Peroxides in detergents as interfering factors in biochemical analysis. Anal. Biochem., 83(1): 274-284.
27. Bates, T.R., C.H. Nightingale and E. Dixon, 1973. Kinetics of hydrolysis of polyoxyethylene (20) sorbitan fatty acid ester surfactants. J. Pharm. Pharmacol., 25(6): 470-477.