AAPS PharmSciTech 2006; 7 (4) Article 99 (http://www.aapspharmscitech.org).

Aggregation of Recombinant Human Interferon Alpha 2b in Solution: Technical Note

Submitted: June 16, 2005; Accepted: June 22, 2006; Published: December 22, 2006

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INTRODUCTION

Recombinant DNA technology has now made possible the large-scale production of proteins for pharmaceutical applications.[1] Consequently, proteins produced via biotechnology now compose a significant portion of the drugs currently under development. Isolation, purification, formulation, and delivery of proteins represent significant challenges to pharmaceutical scientists because of the unique chemical and physical properties of these biomolecules.[1] Current methodologies to stabilize proteins include the use of excipients, chemical modification, and site-directed mutagenesis to produce more stable protein species.

Interferons are cytokines with antiviral, antiproliferative, and immunomodulatory properties.[2] Specifically, the human alpha interferon family comprises 23 species of structurally similar proteins, with specific activity of approximately 1 to 2×10^8 international units per milligram and molecular masses from 17 to 28 kDa.[2] Additionally, the genes encoding human alpha interferons have been cloned and expressed in recombinant strains of microorganisms, and their products have been isolated in a very pure form.[3] In these conditions, these cytokines have been formulated in parenteral dosage forms[4] to treat a great number of disorders, including hairy cell leukemia, multiple myeloma, basal cell carcinoma, and hepatitis B and C.[5,6]

However, the development of stable dosage forms of alpha interferons remains a great challenge for pharmaceutical scientists because of the poor stability of these biomolecules.[7] This poor stability frequently increases the probability of protein degradation during some pharmaceutical processes such as purification, separation, freeze-drying, and storage; through chemical reactions (eg, proteolysis, oxidation, deamidation); and because of physical influences (aggregation, precipitation, and adsorption).[8]

The degradation pathways in therapeutic proteins have been problematic because they can increase the likelihood of adverse immunogenic effects and inconsistent dosing during therapy.[8] In addition, the solubility and biological function of the protein can be compromised.[8] Aggregation figures among such degradation pathways; it may reduce the activity or solubility and may alter the immunogenicity of the protein.[8]

The aim of this work was to study the influence of different factors (buffer species, stabilizers, and stressing agents) on the aggregation rate of recombinant human interferon alpha 2b (rhIFN-α2b).

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MATERIALS AND METHODS

Materials

The Center for Genetic Engineering and Biotechnology (CIGB, Havana, Cuba) supplied rhIFN-α2b with the characteristics previously described for this cytokine.[9] All chemicals used were of analytical grade. Type I borosilicate glass vials were acquired from Nuova OMPI (Piombino Dese, Italy), and rubber stoppers plus flip-off seals were from Helvoet Pharma (Alken, Belgium).

Influence of Buffer Species on Liquid Stability of rhIFN-α2b

Samples were diluted to 0.5 mg/mL in 50 mM sodium citrate buffer, pH 6.0; 50 mM sodium citrate-phosphate buffer, pH 6.0; or 50 mM sodium phosphate buffer, pH 6.0. Then, 0.5 mL was dispensed in borosilicate glass vials. Each vial was sealed with a chlorobutyl stopper and 13-mm flip-off aluminum seal and stored at 37ºC; 20 μg of rhIFN-α2b was analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS/PAGE) under reduced and nonreduced conditions[10] at time 0 and after 3, 6, 15, 21, and 30 days. The experiments were accomplished in triplicate.

Influence of Different Stabilizers on Aggregation of rhIFN-α2b in Solution

rhIFN-α2b was diluted to 0.5 mg/mL in 50 mM sodium phosphate buffer, pH 6.0, and then the following chemicals were added to the solution: 0.5 mg/mL polysorbate 20, 0.5 mg/mL polysorbate 80, 0.5 mg/mL SDS, and 0.5 mg/mL EDTA $Na_2 \times 2H_2O$. Samples were dispensed into borosilicate glass vials and stored at 37ºC; 20 μg of the cytokine was analyzed by SDS/PAGE under reduced and nonreduced conditions[10] at time 0 and after 3, 6, 15, 21, and 30 days.

rhIFN-α2b in 50 mM sodium phosphate buffer, pH 6, without additives was used as the control. The experiment was accomplished in triplicate.

Aggregation of rhIFN-α2b by Other Excipients

rhIFN-α2b was diluted to 0.5 mg/mL in 50 mM sodium citrate buffer, pH 6, and then $CuSO_4 \times 5H_20$, $ZnSO_4$, dithiothreitol (DTT), or reduced glutathione (GSH), at 0.01 mg/mL, was added to the solutions. Samples of 0.5 mL were dispensed into borosilicate glass vials and stored at 37ºC. rhIFN-α2b (20 µg) was then analyzed by SDS/PAGE under reduced and nonreduced conditions[10] at time 0 and after 3, 6, 15, 21, and 30 days. rhIFN-α2b in 50 mM sodium citrate buffer, pH 6, without additives was used as the control. The experiment was accomplished in triplicate.

Electrophoresis Analysis of the Cytokine

The samples were gently vortexed before the analysis by SDS/PAGE, as described by Laemmli.[10] The gels were scanned and densitometrically analyzed after the Coomassie blue staining in order to quantify the percentage of monomer and dimer. A reference standard of IFN alpha 2b provided by the Quality Control Division of CIGB was used as the positive control. Molecular weight standard markers were lysozyme (14 kDa), ribonuclease (18 kDa), tyrosine inhibitor (21 kDa), carbonic anhydrase (31 kDa), ovalbumin (45 kDa), and bovine serum albumin (66 kDa).

Statistical Analysis

Following a comparison of the homogeneity of variance (Bartlett test),[11] the statistical significance of the experimental data was determined by either the unpaired Student *t* or analysis of variance (ANOVA) tests. When $P \leq .05$, the ANOVA test was followed by a Duncan multiple range test to determine the specific groups showing significant differences.

Results and Discussion

Influence of Buffer Species on Stability of rhIFN-α2b in Solution

Herein the usefulness of different buffer species and excipients in preventing the aggregation of rhIFN-α2b was studied.

The impact of the evaluated buffer species on the diminishment of aggregation of rhIFN-α2b was significantly higher when both sodium citrate and sodium citrate-phosphate buffers were used (Figures 1 and 2). These 2 buffer species reduced the aggregation rate of rhIFN-α2b in the range of

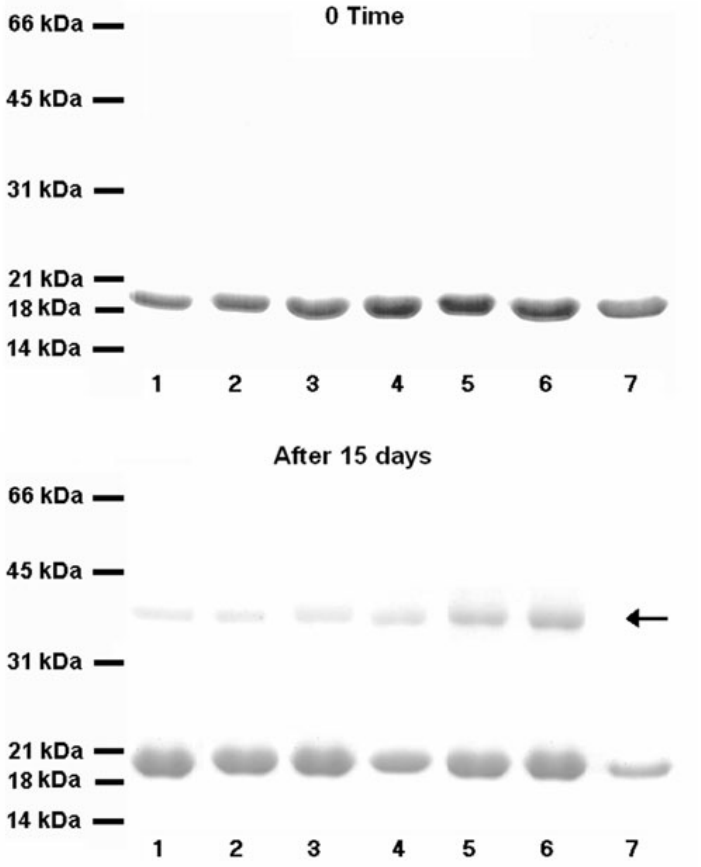

Figure 1. Effect of buffer species on the SDS/PAGE profile of recombinant human interferon alpha 2b under reduced conditions, at the beginning of the study (0 time) and after 15 days, at 37ºC. 1, 2: sodium citrate buffer; 3, 4: sodium citrate-phosphate buffer; 5, 6: sodium phosphate buffer; 7: control.

1.06- to 1.53-fold, as compared with samples in sodium phosphate buffer (Table 1).

The results showed that both reduced and nonreduced aggregates were induced during the aggregation of the cytokine (Table 1). In fact, the aggregation rate of rhIFN-α2b determined under reduced conditions was significantly lower than that determined under nonreduced conditions. These results suggest that this protein may aggregate through the formation of reducible linkages (disulfide bonds). However, reduced conditions were not enough to dissolve most aggregates (Figure 1). The failure of SDS and mercaptoethanol to dissolve aggregates suggests that these substances do not have access to the interfaces of protein molecules because of strong hydrophobic interaction.[8]

Human alpha interferons contain 2 disulfide bonds between Cys-1 and Cys-98 and between Cys-29 and Cys-138. Morehead et al determined that reduction of these bonds leads to an irreversible loss of antiviral activity.[12] Therefore, retaining the stability of disulfide bridges in alpha IFNs becomes

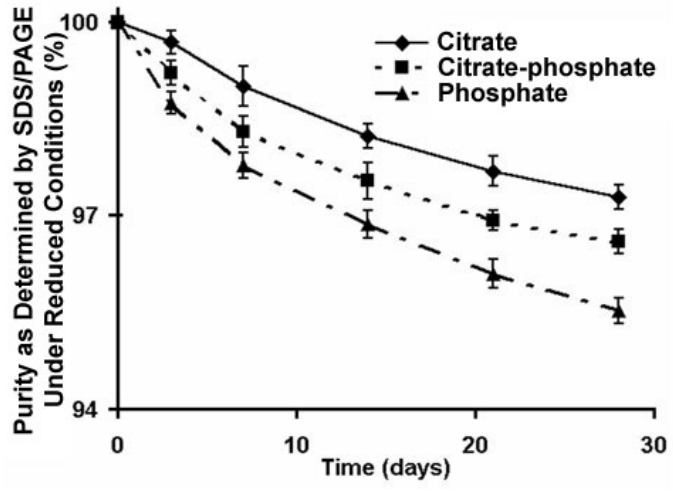

Figure 2. Effect of the buffer species on the recombinant human interferon alpha 2b purity decline, at 37ºC, as determined by SDS/PAGE under reduced conditions.

essential to maintaining the bioactivity. Buffers species seem to play a significant role in this process.

Differences in aggregation rates caused by the use of different buffer species have been previously discussed.[13] Chen et al found that citrates reduced the aggregation of keratinocyte growth factor.[13] The authors speculated that this effect could be due to the presence of negatives charges in the buffer ions, which may interact with the positive amino acid clusters on proteins. Therefore, the protein could be stabilized through this charge-charge interaction.

Following this logic, one might expect a significant stabilization of rhIFN-α2b by sodium phosphate buffer because of the negative charges of the phosphate ions. However, this did not occur (Table 1, Figure 2). Trace amounts of metal ions may contaminate the buffer salts and participate in the oxidation reactions of this protein.[14]

Therefore, the better behavior of sodium citrate and sodium citrate-phosphate buffer species can be explained by the combination of the above-mentioned mechanism (charge-charge interaction) and the ability of citrates to act as chelating agents of the trace amounts of metal ions that could contaminate the buffer solutions.

Table 1. Kinetic Parameters of rhIFN-α2b Aggregation in Different Experimental Conditions*

Experimental Conditions *Buffer Species*	SDS/PAGE (under reduced conditions) $k \times 10^3$ (day^{-1})	SDS/PAGE (under nonreduced conditions) $k \times 10^3$ (day^{-1})
Sodium citrate	1.01 ± 0.05	1.64 ± 0.08
Sodium citrate-phosphate	1.22 ± 0.04	1.71 ± 0.07
Sodium phosphate	1.55 ± 0.12	1.81 ± 0.08
Stabilizers		
Polysorbate 20	0.71 ± 0.06	0.84 ± 0.04
Polysorbate 80	0.85 ± 0.05	1.12 ± 0.05
SDS	1.48 ± 0.11	1.67 ± 0.07
EDTA $Na_2 \times 2H_2O$	0.52 ± 0.03	0.75 ± 0.03
Control	1.54 ± 0.11	1.82 ± 0.08
Stressing Agents		
Cu^{2+}	6.21 ± 0.26	6.77 ± 0.22
Zn^{2+}	4.54 ± 0.44	5.54 ± 0.51
DTT	2.96 ± 0.72	4.02 ± 0.72
GSH	1.92 ± 0.11	3.18 ± 0.25
Control	1.03 ± 0.05	1.63 ± 0.08

*Determination of the purity of the rhIFN-α2b main band. The results are expressed as mean (n = 3) ± SD. rhIFN-α2b indicates recombinant human interferon alpha 2b; SDS/PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis ; DTT, dithiothreitol; GSH, reduced glutathione.

Influence of Different Stabilizers on Aggregation of rhIFN-α2b in Solution

Currently, aggregation has mostly been attributed to the intermolecular association of partially denatured protein chains.[15] Some evidence suggests that aggregation may occur by specific interaction of certain conformations of protein intermediates rather than by nonspecific coaggregation.[16] Therefore, several excipients, including non-ionic detergents, have widely been used to prevent this physical instability.[17]

In this work, the influence of EDTA $Na_2 \times 2H_2O$, polysorbate 20, polysorbate 80, and SDS was evaluated.

EDTA $Na_2 \times 2H_2O$: Results from the use of this chelating agent showed a significant ($P < .05$) reduction in the aggregation rate (Table 1, Figure 3). Kinetic analysis indicated that EDTA $Na_2 \times 2H_2O$ reduced the aggregation rate of

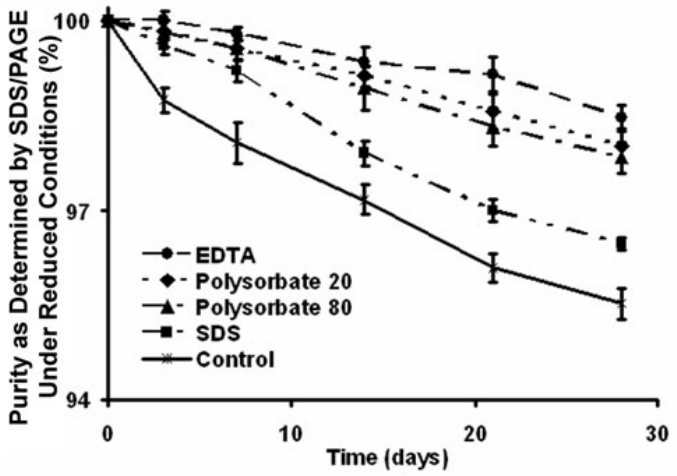

Figure 3. Effect of the different stabilizers on the recombinant human interferon alpha 2b purity decline, at 37ºC, as determined by SDS/PAGE under reduced conditions.

rhIFN-α2b by ~2.96- and 2.43-fold, as determined by SDS/PAGE under reduced and nonreduced conditions, respectively. rhIFN-α2b in sodium phosphate buffer without additives was used as the control of this experiment.

Under these experimental conditions, the role of EDTA $Na_2 \times 2H_2O$ might be to capture the trace amounts of metal ions in solution, which otherwise would accelerate several protein degradation reactions, including aggregation.[8]

EDTA $Na_2 \times 2H_2O$ has been used to stabilize proteins, including rhIFN-α2b, basically because of its ability to inhibit the oxidation reactions by chelating the metal ions in solutions.[8]

Polysorbates 20 and 80: These non-ionic detergents improved the stability of rhIFN-α2b and reduced the aggregation rate of the cytokine. However, kinetic analysis showed a lower effectiveness ($P < .05$) as compared with results from the use of the chelating agent (Table 1, Figure 3).

Taking into account the hydrophobic nature of the protein-surfactant interaction, these compounds could stabilize rhIFN-α2b by covering the hydrophobic sites at the protein surface and thus reducing the possibility of formation of noncovalent aggregates.[18] In general, surfactants drop the surface tension for protein solutions and decrease the force driving proteins to aggregation by hydrophobic interactions.[18]

However, polysorbates were less effective than EDTA $Na_2 \times 2H_2O$ in preventing the aggregation of this cytokine (Table 1, Figure 3). This result might be explained by the probable contamination of these compounds with alkyl peroxides.[19,20] These peroxides may accelerate the occurrence of some degradation reactions, which may in turn give rise to the aggregation of the protein.[19] Additionally, other factors such as the presence of trace amounts of metal ion contaminating the protein solution would increase the aggregation rate of rhIFN-α2b, even in the presence of polysorbates.

SDS: This anionic detergent had the lowest impact on the stabilization of rhIFN-α2b (Table 1, Figure 3). Kinetic analysis showed that SDS reduces the aggregation of the cytokine only ~1.04- and 1.09-fold, as determined by SDS/PAGE under reduced and nonreduced conditions, respectively (Table 1). rhIFN-α2b in sodium phosphate buffer without additives was the control for the experiment.

The low impact of this detergent on the stability of rhIFN-α2b might be explained by the capacity of SDS to bind proteins.[8] This union usually disrupts both hydrophobic interactions and hydrogen bonds. Therefore, the chemical potential of the protein and the free energy of denaturation are substantially diminished.[8]

Given the higher effectiveness of citrates and EDTA $Na_2 \times 2H_2O$ in reducing the aggregation rate of this cytokine, these data suggest that metal-catalyzed reactions could play an important role in the aggregation of rhIFN-α2b. In fact, the influence of metal ions could be higher than that of noncovalent forces (eg, hydrophobic forces) on the aggregation rate of rhIFN-α2b.

Aggregation of rhIFN-α2b by Other Excipients

Aiming to corroborate the evidence regarding the relevance of metal ions in the aggregation of rhIFN-α2b, we also evaluated the effect of 2 metal ions. Additionally, these results were compared with those obtained from the evaluation of DTT and GSH, which may accelerate the aggregation of rhIFN-α2b through covalent reactions like disulfide exchange.[8]

Cu^{2+} and Zn^{2+}: Kinetic analysis and primary data (Table 1, Figure 4) showed that these compounds increased the aggregation of rhIFN-α2b in the range of 3.4- to 6.03-fold, as compared with rhIFN-α2b in sodium citrate buffer without additives.

These metal ions have been used to stabilize proteins, especially metalloproteinases.[8] In contrast, other authors have studied the oxidation of proteins due to the presence of trace amounts of these metal ions.[8] Labile residues include Met, Cys, His, Trp, Tyr, Pro, Arg, Lys, and Thr.[8] The oxidation of such amino acids may increase the aggregation of proteins. Specifically, free Cys residues can be easily oxidized to form disulfide bond linkages or cause thio-disulfide exchanges between 2 or more molecules. Even though a protein does not have free Cys residues, disulfide bond scrambling may still occur, causing protein aggregation.[8]

DTT and GSH: The inclusion of DTT and GSH also destabilized rhIFN-α2b and increased the aggregation of the

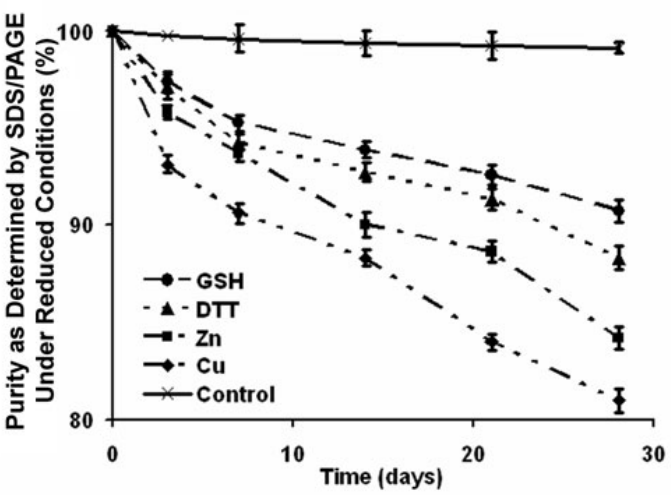

Figure 4. Effect of the stressing agents on the recombinant human interferon alpha 2b purity decline, at 37ºC, as determined by SDS/PAGE under reduced conditions.

protein in the range of 1.86- to 2.87-fold, as compared with rhIFN-α2b in sodium citrate buffer without additives (Table 1).

Reyes et al studied the capacity of GSH to inhibit the peroxide-mediated oxidation of recombinant human interleukin-2 (rhIL-2) in the freeze-dried state.[20] The general explanation has been focused on the competition with proteins for the oxidation of sulfhydryl groups. A similar mechanism explains the influence of DTT as an antioxidant in protein solutions.[7] Nevertheless, given their ability to be oxidized, it might be suggested that GSH and DTT could reduce disulfide bonds from rhIFN-α2b, causing disulfide scrambling and consequently the formation of aggregates of the protein. In this sense, Reyes et al demonstrated that GSH promoted the aggregation of IL-2 in solution.[20]

However, this increment was significantly lower ($P < .05$) than that induced by the metal ions (Table 1, Figure 4).

These results suggest that metal ions may induce some degradation reactions that significant increase the aggregation rate of the cytokine.

Summary and Conclusions

Sodium phosphate buffer increased the aggregation of rhIFN-α2b in the range of 1.55 to 1.810^3 day^{-1}, as determined by SDS/PAGE under reduced and nonreduced conditions. In contrast, sodium citrate buffer decreased the aggregation rate of this cytokine, as compared with those samples in sodium phosphate buffer. Results from sodium citrate-phosphate buffer were very similar to those obtained with sodium citrate solutions.

On the other hand, EDTA $Na_2 \times 2H_2O$ reduced the aggregation rate of rhIFN-α2b, showing an aggregation kinetic constant in the range of 0.52 to 0.75 $\times$ 10^3 day^{-1}. Polysorbates 20 and 80 were less effective than the chelating agent in preventing this degradation pathway.

Additionally, metal ions (Zn^{2+} and Cu^{2+}) increased the aggregation kinetic constant of rhIFN-α2b, probably through undetermined metal-catalyzing reactions.

Taken together, these data can be useful for the development of new formulations containing rhIFN-α2b as an active ingredient.

Acknowledgments

The authors are grateful for editorial assistance from Frank Batista and Julia Metzger.

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