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EFFECT OF POLYSORBATE-80 CONCENTRATION ON G-CSF FORMULATION USING LIQUID CHROMATOGRAPHY

AMAND BHASKAR, KUNAL MUKHOPADHYAY, MANISH KUMAR*

**Department of Bio-Engineering, Birla Institute of Technology, Mesra, Ranchi 835215, Jharkhand, India.
Email: manish@bitmesra.ac.in**

ABSTRACT

Objective: The aim of the study was to find the dual effects of polysorbate-80 concentration on oxidation and aggregation in the formulation of granulocyte colony stimulating factor.

Methods: The effect of polysorbate-80 at different concentration was monitored and analyzed using high performance liquid chromatography. Oxidative degradation and aggregate generation was studied using reverse phase and size exclusion chromatography method respectively.

Results: With increase in concentration of polysorbate-80 the amount of oxidation as well as aggregate formation increases in a concentration dependent manner. The aggregates present at higher concentration of polysorbate-80 formulation are not found with low concentration or without polysorbate-80. This result shows that higher concentration of Tween-80 force the formation of oligomers and leads to increased level of oxidation of granulocyte colony stimulating factor.

Conclusion: The dual effect of aggregation and oxidation of polysorbate-80 on the recombinant granulocyte colony stimulating factor indicate that during formulation development studies it is crucial to evaluate the amount of polysorbate-80 to be used in the formulation.

Keywords: Surfactants, Aggregates, Polysorbate-80, Oxidation, G-CSF.

INTRODUCTION

Granulocyte colony stimulating factor (G-CSF) or colony stimulating factor, a cytokine that is produced by macrophages that induce proliferation of neutrophil colonies and differentiation of precursor cells to neutrophils (1). They are responsible for detecting and destroying harmful bacteria and some fungi. The G-CSF is being used for more than two decades to treat congenital and acquired neutropenia and to reduce febrile neutropenia before or during courses of intensive cytoreductive therapy (2). G-CSF is used as medications to stimulate production of infection fighting white blood cells (WBCs), febrile neutropenia (3).

Polysorbates are the most extensively used non-ionic surfactants in biopharmaceuticals to stabilize proteins to prevent aggregation (4), minimize surface absorption of proteins (5,6) and withstand various stresses like shaking (7), freeze thawing (8) and lyophilization (9,10). However some of their effects on protein formulation need to be examined in more details. The most commonly used non-ionic surfactants are Tween 20 (Polysorbate 20) and Tween 80 (Polysorbate-80). Beside these Triton X-100, Pluronic F-68, Pluronic F-88, Pluronic F-127 (poloxamers) (11-12) and Brij 35 (polyoxyethylene alkyl ether) are also used. It is estimated that 70 % of monoclonal antibody biotherapeutics uses polysorbate-80 (13).

Biotherapeutic protein like monoclonal antibody also has a tendency to form aggregates. There are more than 30 monoclonal antibodies (MABs) marketed in United States and Europe. In 2010, the US only has sales of 18.5 billion dollar which is expected to continue at a combined annual growth rate (CAGR) > 10% (37).

The general process involved during manufacturing of biopharmaceuticals like filtrations (14), pumping (15), shaking or stirring (8), freeze thawing (16) may lead to generation of aggregates (17). Aggregation has a number of deleterious effects on biotherapeutics including the loss of efficacy (18), induction of unwanted immunogenicity (19), altered pharmacokinetics (20) and reduced shelf life. Aggregation is ameliorated by the inclusion of surfactants in biotherapeutic formulations, typically non-ionic polymeric ether surfactants. The generation of aggregates may lead to loss of yield and increase in manufacturing cost. Even the small percentage of generation of aggregates may alter the pharmacokinetic or pharmacodynamic profile of the drug by reducing bioavailability and finally the efficacy. The most serious problem is the induction of unwanted immunogenicity response thereby decreasing the efficacy as in "pure red-cell aplasia" during treatment with erythropoietin (20), neutralizing antibodies against interferon beta led to higher relapse rate and more disease activity (18,21).

Polysorbates contain low levels of residual peroxides, which may accumulate during storage and cause instant or long-term damage to the active biopharmaceutical ingredients (22, 23). Polysorbates are known to be degraded by auto-oxidation and hydrolysis yielding reactive peroxides (22, 24). The presence of residual levels of peroxide in bulk polysorbate is also important. The European Pharmacopeia has specified a limit of peroxide number (PN)≤ 10 (25). The oxidative damaging effect of peroxides in surfactants on drug molecules has been extensively reported (26-28). The main sites of oxidation within proteins are the methionine and tryptophan residues (29-30). The problem of immunogenicity is aggravated when both oxidation and aggregation takes place simultaneously (31). The dual effect of aggregation and oxidation on protein stability of polysorbate-80 has been reported, eg. IL-2 (32).

The main objective of our present study was to check the dual effect of Tween-80 concentration upon oxidation and aggregation on G-CSF drug formulation. Size Exclusion High Performance Liquid Chromatography (SE-HPLC) was utilized to study the generation of aggregates while Reverse Phase High Performance Liquid Chromatography (RP-HPLC) was utilized for detection of oxidized impurity.

MATERIALS AND METHODS

Sample preparation

The stock solution of polysorbate-80 (60 mg/ml) was prepared in G-CSF formulation buffer. From this stock, different concentration of G-CSF containing 0.04%, 0.1% and 0.2% polysorbate-80 was prepared. Final concentration of G-CSF was≈ 0.79 mg/ml in formulation buffer. All samples were incubated for 36 h at room temperature.

High-performance liquid chromatography

The analytes were separated using a Waters 1525 HPLC system (Waters Corporation Milford, MA, USA). The columns used for SEC was TSK-GEL 3000 SW$_{xl}$ (7.8 mm and 30 cm; 10 μm particle size). Different mobile phase buffer and conditions were tested to eliminate the chances of any contribution of buffer in the formation of oligomer. Initially mobile phase used were phosphate buffer (pH-6.9) having KH_2PO_4 (2 mM), Na_2HPO_4 (3 mM), NaCl (110 mM) containing 10 % methanol. The flow rate was maintained at 0.5 ml/min with a run time of 30 min. The detector was set at a wavelength 215 nm. The second mobile phase used was ammonium bicarbonate (0.395%-pH-7.0).

For RP-HPLC Grace Vydac C18 reverse phase column were used (4.6 X 25 cm, 300Å, 5 μm particle size). A linear gradient program was applied as follows: 0-5 min: 25% B; 5-8 min: 40% B; 8-12 min: 46% B; 12-37 min: 66% B; 37-50: 80% B; 50-52 min/;100 %B; 52-54 min: 25% B; 54-60 min: 25 % B. The flow rate was maintained at 0.8 ml/min and the temperature of the column was set at 60º C. The detector was set at 214 nm with a run time of 60 min. The binary mobile phase consisted of (A) water and (B) acetonitrile containing 0.1% trifluoroacetic acid. All the chemicals were of analytical grade purchased from Merck and Sigma (USA).

RESULTS AND DISCUSSION

RP-HPLC

The reverse phase HPLC was performed using Grace Vydac C18 column with 0.1% TFA in Acetonitrile and Water.
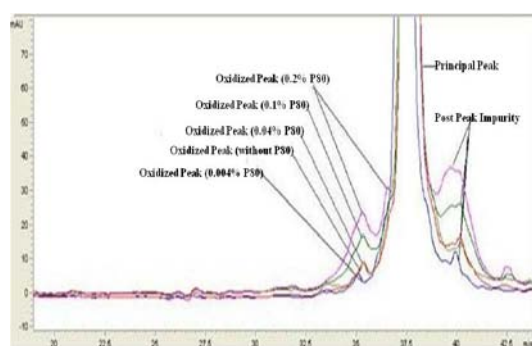
Fig. 1: Overlay chromatogram of RP-HPLC of drug product with different concentration of Polysorbate-80. The amount of oxidation as well as post peak impurity of G-CSF increases with respect to polysorbate-80 concentration.

A decrease in retention time of the protein was observed upon oxidation. It was also observed that with increase in the

concentration of polysorbate-80, the amount of oxidation also increases in a concentration dependent manner as shown through overlay of chromatograms (Fig. 1). Not only the oxidation but the amount of post peak impurities also increases with respect to increase in polysorbates concentration (Fig. 1).

Size exclusion Chromatography

Size Exclusion chromatography was performed to analyze the effect of polysorbate-80 concentration using a TSK- GEL 3000 SW$_{xl}$ column. The overlay of SEC chromatograms showed that as the concentration of polysorbate-80 was increased the amount of aggregates also increased. Significant variation has been observed in the SE-HPLC chromatogram of sample with low concentration or without polysorbate-80 as compared to higher concentration. The aggregates or oligomer present at high concentration of polysorbate-80 is not found to be generated in sample with 0.004% polysorbate-80 and without polysorbate-80; rather only dimer formation is occurred (Table 1, Fig. 2).

The dimer had a retention time of 20 min whereas oligomer had 16 min. This result showed that higher concentration of polysorbate-80 force the formation of oligomer. The HP-SEC buffers were modified in order to check that if there is any effect of the buffer in formation of oligomer. Ammonium bicarbonate at pH 7.0 was used as (mobile phase). The overlay of chromatogram (Fig. 3) showed that as the concentration of polysorbate-80 increased the amount of aggregate also increased suggesting buffer, component has no role in generation of aggregate.
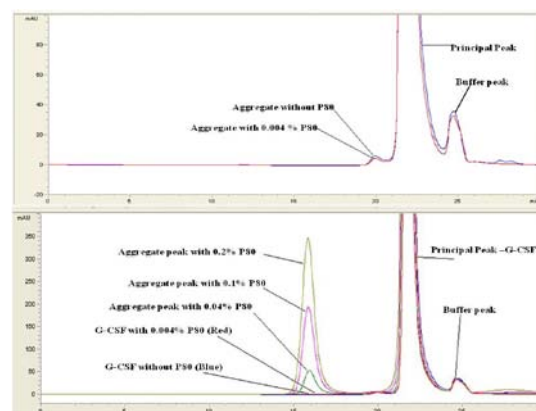
Fig. 2: Overlay SE-HPLC Chromatogram showing that as the concentration of Tween-80 increases the amount of aggregate increases. The aggregates or oligomer present at high concentration of polysorbate-80 is not found to be generated in sample with 0.004% polysorbate-80 and without polysorbate-80 rather only dimer formation is favored.

**Table 1: Shows the retention time of peak of RP-HPLC and SE-HPLC**

S. No.	Polysorbate concentration	RP-HPLC Rt (min.)		SEC Rt (min.)		
		Principal pk	Oxidized pk	Principal pk	Aggregate pk	Oligomer pk
1	0.0 %	37.5	35.0	22.0	19.8	Not detected
2	0.004 %	37.5	35.25	22.0	19.8	Not detected
3	0.04 %	37.5	35.25	22.0	19.8	16.0
4	0.1 %	37.5	35.25	22.0	19.8	16.0
5	0.2 %	37.5	35.25	22.0	19.8	16.0

Surfactants are essential components of many biotherapeutic formulations. They may induce unwanted immunogenicity by generation of aggregated and oxidized forms of proteins. Polysorbates are effective in preventing protein aggregation, but they contain ether linkages and polysorbate-80 has unsaturated alkyl chains that spontaneously auto oxidize in aqueous solution to produce damaging peroxides, epoxy acids and reactive aldehydes (25). Degradation of polysorbates decreases its ability to protect the

formulation and the degraded molecules damage the protein stability.

The peroxide generated cause oxidation of proteins and aldehyde induces unwanted immunogenicity in proteins by reaggregating proteins (22, 34). The types of aggregate vary in size and differ according to the nature of the protein, like it may be soluble, insoluble, covalent or non-covalent, reversible or irreversible.

In our study the dual effects of polysorbate-80 concentration on aggregation and oxidation on G-CSF has been observed which is the most serious problem associated with the biosimilar drugs. The result obtained shows that aggregate formation is favored by the partially unfolded structure of protein, since polysorbate-80 is a detergent and its high concentration causes structural change which favor the formation of aggregate. The high concentration of polysorbates generates more amount of peroxide which led to not only oxidation of G-CSF but also to post peak impurity. There are damaging effects of polysorbate on proteins even if the concentration is less, which is not observed immediately, immunogenicity may arise only after repeated administration over an extended period or use of higher doses. So the damaging effect of all potential source of unwanted immunogenicity of biotherapeutics must be dealt with strong risk management. Therefore, proper planning is essential for development of new drug to make sure it is safe, reliable and stable.
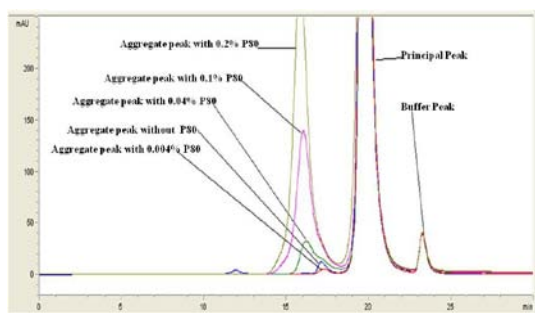
Fig. 3: Overlay SE-HPLC Chromatogram showing the increase in amount of aggregate on increasing concentration of Polysorbate-80 when using ammonium carbonate as mobile phase.

Polysorbate produces reactive species which cause chemical modification of aminoacyl side chains of proteins producing a non-similar molecule which can lead to immune response and subsequently generate antibodies against the drug in use. This creates a technical challenge for manufacturer of biosimilars where it is required to demonstrate not only the similarity of the drug but also the similarity of impurities of excipients.

Due to the severe nature and problems coupled with the development of unwanted immunogenicity of the biotherapeutics, the Food Drug and Administration with European Medicinal Agencies has highlighted their concern regarding aggregation in its new guidelines (35). The Committee for Medicinal Products for Human Use (CHMP) in 2009 at the European Medicines Agency issued marketing authorization guidelines for general recommendations on how to assess an unwanted immune response following the administration of a biotherapeutic drug (36). The evaluation of the equivalence of different excipients for the stabilization of biotherapeutic has not been well defined which may be included in future.

Most studies carried out on the use of polysorbate have looked on the avoidance of aggregation for reducing immunogenicity. While oxidative damage to protein on storage has to be included in future studies by modifiing aminoacyl groups in a biotherapeutic during stability analysis rather than to measure the reactive contaminants (like peroxide) in final product. Although "High purity" polysorbates are commercially available having head space filled with inert gas like nitrogen or argon, but as soon as it comes in contact with oxygen oxidation starts.

CONCLUSION

In this present work we have demonstrated the dual effect of aggregation and oxidation of polysorbate or Tween-80 on the recombinant G-CSF. The formation of aggregates and oxidized species are a concern when using polysorbates in formulation of biotherapeutics. Alternative surfactant must be tried in future to prevent aggregation and oxidation of biotherapeutics.

CONFLICT OF INTERESTS

Declared None

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REFERENCES

1. Metcalf D. Granulocyte-macrophage colony-stimulating factors. Sci 1985;229:16-22.
2. Beekman R, Ivo PT. G-CSF and its receptor in myeloid malignancy. Blood 2010;115:5131-6.
3. Aapro MS, Cameron DA, Pettengell R, Bohlius J, Crawford J, Ellis M, *et al.* European Organisation for Research and Treatment of Cancer (EORTC) Granulocyte Colony-Stimulating Factor. Guidelines Working Party EORTC guidelines for the use of granulocyte-colony stimulating factor to reduce the incidence of chemotherapy-induced febrile neutropenia in adult patients with lymphomas and solid tumours. Eur J Cancer 2006;42:2433–53.
4. Bondos SE, Bicknell A. Detection and prevention of protein aggregation before, during and after purification. Anal Biochem 2003;316:223-31.
5. Duncan MR, Lee JM, Warchol MP. Influence of surfactants upon protein/peptide adsorption to glass and polypropylene. Int J Pharm 1995;120(2):179–88.
6. Mahler HC, Huber F, Ravuri SKK, Reindl J, Ruckert P, Muller R. Adsorption behaviour of a surfactant and a monoclonal antibody to sterilizing-grade filters. J Pharm Sci 2010;99(6):2620-7
7. Hillgren A, Lindgren J, Alden M. Protection mechanism of Tween 80 during freeze-thawing of a model protein, LDH. Int J Pharm 2002;237:57–69.
8. Kiese S, Pappenberger A, Friess W, Mahler HC. Shaken, not stirred: mechanical stress testing of an IgG1 antibody. J Pharm Sci 2008;97:4347–66.
9. Carpenter JF, Chang BS, Garzon RW, Randolph TW. Rational design of stable lyophilized protein formulations: theory and practice. Pharm Biotechnol 2002;13:109–33.
10. Carpenter JF, Pikal MJ, Chang BS, Randolph TW. Rational design of stable lyophilized protein formulations: some practical advice. Pharm Res 1997;14:969–75.
11. Katakam M, Bell LN, Banga AK. Effect of surfactants on the physical stability of recombinant human growth hormone. J Pharm Sci 1995;84(6):713-6.
12. Wei G, Lu LF, Lu WY. Stabilization of recombinant human growth hormone against emulsification-induced aggregation by Pluronic surfactants during microencapsulation. Int J Pharmaceutics 2007;338(1-2):125-32.
13. Hawe A, Filipe V, Jiskoot W. Fluorescent molecular rotors as dyes to characterize polysorbate-containing IgG formulations. Pharm Res 2010;27(2):314-26.
14. Maa Y-F, Hsu CC. Investigation on fouling mechanisms for recombinant human growth hormone sterile filtration. J Pharm Sci 1998;87(7):808–12.
15. Cromwell MEM, Hilario E, Jacobson F. Protein aggregation and bioprocessing. AAPS J 2006;8(3):E572–E9.
16. Eckhardt BM, Oeswein JQ, Bewley TA. Effect of freezing on aggregation of human growth hormone. Pharm Res 1991;8(11):1360–4.
17. Mahler HC, Friess W, Grauschopf U, Kiese S. Protein aggregation: pathways, induction factors and analysis. J Pharm Sci 2009;98(9):2909–34.
18. Kappos L, Clanet M, Sandberg WM, Radue EW, Hartung HP, Hohlfeld R, *et al.* Neutralizing antibodies and efficacy of interferon β-1a: A 4-year controlled study. Neurology 2005;65:40–7.

19. Jefferis R. Aggregation, immune complexes and immunogenicity. MAbs 2011;3(6):503–4.
20. Casadevall N, Nataf J, Viron B, Kolta A, Kiladjian JJ, Martin DP, *et al*. Pure red-cell aplasia and antierythropoietin antibodies in patients treated with recombinant erythropoietin. New Engl J Med 2002;346:469–75.
21. Vartanian T, Sorensen PS, Rice G. Impact of neutralizing antibodies on the clinical efficacy of interferon beta in multiple sclerosis. J Neurol 2004;251:1125–30.
22. Donbrow M, Azaz E, Pillersdorf A. Autoxidation of polysorbates. J Pharm Sci 1978;67:1676–81.
23. Jaeger J, Sorensen K, Wolff SP. Peroxide accumulation in detergents. J Biochem Biophys Methods 1994;29:77–81.
24. Ha E, Wang W, Wang YJ. Peroxide formation in polysorbate-80 and protein stability. J Pharm Sci 2002;91(10):2252–64.
25. Kishore RS, Kiese S, Fischer S, Pappenberger A, Grauschopf U, Mahler HC. The degradation of polysorbates 20 and 80 and its potential impact on the stability of biotherapeutics. Pharm Res 2011;28(5):1194-210.
26. Van Beers MMC, Sauerborn M, Gilli F, Brinks V, Schellekens H, Jiskoot W. Oxidized and aggregated recombinant human interferon beta is immunogenic in human interferon beta transgenic mice. Pharm Res 2011;28(10):2393-402.
27. Knepp VM, Whatley JL, Muchnik A, Calderwood TS. 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 1996;50:163–71.
28. Azaz E, Donbrow M, Hamburger R. Incompatibility of non-ionic surfactants with oxidisable drugs. Pharm J 1975;211:5.
29. Simat TJ, Steinhart H. Oxidation of free tryptophan and tryptophan residues in peptides and proteins. J Agric Food Chem 1998;46(2):490–8.
30. Milzani A, Rossi R, Di Simplicio P, Giustarini D, Colombo R, Dalledonne I. The oxidation produced by hydrogen peroxide on Ca-ATP-G-actin. Protein Sci 2001;9:1774–82.
31. Van Beers MMC, Sauerborn M, Gilli F, Brinks V, Schellekens H, Jiskoot W. Oxidized and aggregated recombinant human interferon beta is immunogenic in human interferon beta transgenic mice. Pharm Res 2011;28(10):2393-02.
32. Wang W, Wang YJ, Wang DQ. Duel effects of Tween 80 on protein stability. Intl J Pharmaceut 2008;347:31-8.
33. Katakam M, Bell LN, Banga AK. Effect of surfactants on the physical stability of recombinant human growth hormone. J Pharm Sci 1995;84(6):713-6.
34. Maggio ET. Polysorbates, peroxides, protein aggregation and immunogenicity: a growing concern. J Excip Food Chem 2012;3:45–53.
35. http: //www. fda. gov/ Drugs/ Guidance Compliance Regulatory Information/Guidances/default. html.
36. Jahn EM, Schneider CK. How to systematically evaluate immunogenicity of therapeutic proteins regulatory considerations. N Biotechnol 2009;25(5):280-6.
37. Buss NA, Henderson SJ, McFarlane M, Shenton JM, de Haan L. Monoclonal antibody therapeutics: history and future. Curr Opin Pharmacol 2012;12(5):615–22.