Quantification of polysorbate 80 in biopharmaceutical formulations implementing an optimized colorimetric approach

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Objectives. We hereby describe an improvement of a previously developed quantification technique for polysorbate 80 in biopharmaceutical formulations (darbepoetin alfa and eculizumab) and report the validation of the new approach.

Methods. Polysorbate was isolated from analyte samples by protein precipitation using an organic solvent, followed by supernatant evaporation in vacuum. Polysorbate was derivatized using a ferric thiocyanate reagent and extracted into an organic phase; the relevant optical density measurements were performed.

Results. We established the optimal conditions for each step of the analysis procedure. The accuracy was 97–102% in the tested analytical range, the relative standard deviation did not exceed 5%, and the limit of quantification was 0.01 mg/mL.

Conclusions. The reported approach is highly sensitive; polysorbate isolation and quantification do not depend on the matrix or, most importantly, the protein.

Keywords: validation, darbepoetin alfa, eculizumab, polysorbate, precipitation, ferric thiocyanate.

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Определение полисорбата 80 в биофармацевтических препаратах с помощью оптимизированной колориметрической методики

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Цели. В данной работе была усовершенствована ранее разработанная методика определения полисорбата 80 в биотехнологических препаратах (дарбэпоэтин альфа, экулизумаб), а также проведена ее валидация.

Методы. Полисорбат извлекали из пробы осаждением белка органическим растворителем, затем выпаривали суспензат в вакууме. Полисорбат дериватизировали оптимизированным железо–тиоцианатным реагентом; дериват экстрагировали в слой органического растворителя и измеряли оптическую плотность.

Результаты. Были установлены оптимальные условия для каждой стадии методики. Правильность находится в диапазоне степени извлечения 97–102%, относительное стандартное отклонение составляет не более 5%, предел количественного определения методики 0.01 мг/мл.

Выводы. Представленная методика имеет высокую чувствительность. Извлечение и определение полисорбата не зависят от матрикса пробы – прежде всего, от присутствующего белка.

Ключевые слова: валидация, дарбэпоэтин альфа, экулизумаб, полисорбат, осаждение, тиоцианат железа.

INTRODUCTION

Polysorbates (PSs), especially polysorbate 20 (PS20) and polysorbate 80 (PS80), are very common surfactants in biopharmaceutical formulations, due to their low toxicity, reasonably low cost, and high efficacy at low concentrations. The addition of PSs to biopharmaceuticals affords a mitigation of protein adsorption, denaturation, degradation [2], and aggregation [3, 4] that may occur in stress conditions, such as agitation [5], freeze–thaw cycling [6], and contact with an air–water interface in the course of downstream purification and storage [7]. In the course of biopharmaceutical downstream production and storage, PS concentration can significantly change because they tend to be adsorbed onto surfaces and filter membranes, so the target PS concentration, which ensures protein stability, in intermediate downstream products, drug substances, and drug dosage forms must always be maintained. Therefore, in this context, access to a simple and relatively fast method for PS quantification is a necessity.

PSs are characterized by heterogeneous structures, a lack of chromophoric groups, and a low tendency to bind proteins [8, 9], so PS analysis is not a very straightforward proposition. Numerous analytical methods have been developed, including high-performance liquid chromatography relying on spectrophotometry [10, 11], mass-spectrometry [12], evaporative light scattering [9], fluorometry [13], or gas chromatography [14] for analyte detection. In these approaches, laborious and time-consuming sample pre-treatment procedures, such as alkaline or acidic hydrolysis, solid-phase extraction, and protein removal, are usually required to obtain reliable analytical results. Notably, complex sample pre-treatment procedures represent a challenge also from the standpoint of possible mistakes in experiment execution.

Since PSs are, in fact, a group of closely related molecules lacking a well-defined structure, their properties and chemical composition may differ substantially from batch to batch. Therefore, quantification methods that detect only some PS components—e.g., oleic [10–12] or lauric acid, total fatty acid content [9, 13], or ethylene glycol released through hydrolysis [14]—often provide imprecise data. Conversely, the results of detection methods that rely on the ability of PSs to form micelles are not affected by PS batch-to-batch variability or even by the use of PS samples supplied by different manufacturers. A commonly used analytical approach is colorimetry, which is based on the formation of complexes between PSs and iron, cobalt, or molybdenum thiocyanates [15, 16], followed by extraction of the said complexes into an organic solvent. Exploitation of the formation of a polysorbate–iodine–starch complex has also been suggested for PS quantification [17]. Another possible analytical technique is based on the inclusion of a fluorescent dye into PS micelles, followed by fluorescence detection and quantitation [18, 19].

The aim of this work was to develop an optimized spectrophotometric method for PS quantification that relies on PS reactivity with ferric thiocyanate, so as to improve the previously described technique [1]. We also report the validation of PS80 analysis in the presence of darbepoetin alfa and eculizumab, the drugs that appeared to interfere with the results of the analysis performed with the previously developed approach [1].
MATERIALS AND METHODS

Chemicals and reagents
All reagents (analytical grade) were purchased from Sigma-Aldrich, USA. Biopharmaceuticals were drug substance solutions (or active pharmaceutical ingredients) of darbepoetin alfa and ecuizumab, both manufactured by PHARMAPARK, Russia.

Assay procedure
The ecuizumab drug substance, normally containing a PS80 concentration of 0.02%, was diluted five-fold before analysis. Conversely, the darbepoetin alfa drug substance, containing 0.005% PS80, was analyzed without dilution.

In detail, 1000 µL of acetone was added to 400 µL of standard (0.002–0.008% PS80 solutions in ddH2O) and sample solutions. The contents were briefly mixed and centrifuged at 10000 rpm for 10 min in a 5417C centrifuge (Eppendorf, Germany). Subsequently, 1000 µL of each supernatant was placed in a fresh tube and the solvent removed by evaporation on a vacuum rotary evaporator RVC2-18HCL (Martin-Christ, Germany) for 2 h at 25°C. The dried samples were dissolved in 100 µL of 2 M sodium chloride. To the obtained solutions were then added 400 µL of freshly prepared derivatization reagent, which consists of equal volumes of 1 M iron(III) chloride and 6.4 M ammonium thiocyanate. Finally, 500 µL of dichloromethane (kept at −20°C) were added, and the capped tubes were vigorously shaken for 5 min using a Bullet Blender BBX24 (Biostep, Germany). The tubes were then subjected to centrifugation at 10000 rpm for 10 min at room temperature. The optical density (OD) of the lower phase (the organic solvent layer) was measured at 510 nm on an Ultrospec 7000 instrument (General Electric, USA) against a blank consisting of deionized water. The concentrations of PS80 were determined by linear regression using a calibration curve obtained with the standard samples.

Validation
All validation procedures were carried out in accordance with the State Pharmacopeia of the Russian Federation (14th edition) and the ICH Q2(R1) guidelines. Specificity, linearity, analytical range, precision, accuracy, limit of detection (LOD), and limit of quantification (LOQ) were all evaluated.

RESULTS AND DISCUSSION

Specificity
To evaluate the analytical technique’s specificity, we used pre-formulated PS-free protein substances of darbepoetin alfa and ecuizumab that were characterized by concentrations of 2.1 and 15.1 mg/mL, respectively. The values of the optical density of these solutions, OD_matrix, were compared with the OD of the PS80 standard solution at the LOQ (OD_LOQ), 0.01 mg/mL. The matrix interference (MI) index can be used to quantitatively appreciate interference from sample matrix on method specificity. MI was calculated by the following equation:

\[
MI = \frac{OD_{\text{matrix}}}{OD_{\text{LOQ}}} \times 100\%
\]

For darbepoetin alfa, the MI was 12.9%, and for ecuizumab it was 15.1%. Both values did not exceed the MI limit of 20% [15].

Linearity
The relationship between the OD and PS80 concentration was evaluated for five PS80 concentrations in the 0.02–0.08 mg/mL range—each in triplicate. The calibration curve thus obtained is reported in Fig. 1a, and the residuals’ plot (the plot of the deviation of the actual data points from the regression line) is reported in Fig. 1b.

![Fig. 1. Regression of the optical density vs. the concentration of polysorbate 80 (a).](image)

Residual values of the optical density plotted against polysorbate 80 concentration (b).
As can be evinced from Fig. 1a, the value for the correlation coefficient $R^2$ of the regression of the OD vs. PS80 concentration is 0.9966. Based on this high value and the fact that the residual values (reported in Fig. 1b) do not depend on PS80 concentration, we conclude that the method meets the linearity criterion.

**Accuracy**

For the estimation of the method’s accuracy, darbepoetin alfa and eculizumab solutions, initially PS80-free, were spiked with PS80 to reach the final concentration of 0.02, 0.05, and 0.08 mg/mL, in the case of darbepoetin alfa, and 0.08, 0.20, and 0.36 mg/mL, in the case of eculizumab. After PS80 quantification (as described), the recovery rate at every concentration was calculated. In the case of darbepoetin alfa, the recovery rate remained in the 98–102% range for all concentrations; in the case of eculizumab, the corresponding range was 97–100% (Table 1). The recovery results for both drug substances were thus narrowly scattered around the 100% value, and no sample exceeded the recovery range limit of 85–115%; within this range, the values are considered to be free of systematic error and provide true PS80 concentration for the tested samples.

**Repeatability and precision**

The analytical technique’s repeatability was assessed using the data of the linear regression ($n = 3$). The relative standard deviation (RSD) was calculated for each concentration. Since protein precipitation could affect PS80 extraction and alter analysis results, commercial samples of darbepoetin alfa and eculizumab (already containing 0.05 and 0.2 mg/mL PS80, respectively) were also analyzed ($n = 6$). For the evaluation of the intermediate precision, the same analyses were conducted in two additional replicates, and the RSD values of overall PS80 concentrations for each sample were compared. The relevant data are listed in Table 2. In the case of no sample, the repeatability limit of 5% or intermediate precision limit of 8% was exceeded.

**LOD and limit of quantification**

The LOD and LOQ values were determined using the standard deviation of the response, and the slope of the calibration plot. The LOQ was determined to be 0.010 mg/mL and the LOD 0.003 mg/mL.

**Robustness**

In order to evaluate the analytical method’s robustness, we analyzed the effect of varying the following parameters (the usual value is shown in **bold**):

- evaporation temperature (25, 30, and 35°C);
- derivatization time (2, 5, 10, and 20 min).

Standard solutions of PS80, as well as darbepoetin alfa and eculizumab drug substances spiked with PS80, were analyzed. The recovery of eculizumab and darbepoetin alfa was used as robustness criterion. One should expect PS80 concentration differences of no greater than 8% compared with the results obtained using standard procedure.

### Table 1. Accuracy estimation

| Product       | Theoretical polysorbate 80 concentration, mg/mL | Obtained polysorbate 80 concentration, mg/mL | Recovery rate, % |
|---------------|-----------------------------------------------|---------------------------------------------|------------------|
| Darbepoetin alfa | 0.02                                         | 0.0202                                      | 101              |
|               |                                               | 0.0198                                      | 99               |
|               |                                               | 0.0196                                      | 98               |
|               | 0.05                                         | 0.0512                                      | 102              |
|               |                                               | 0.0505                                      | 101              |
|               |                                               | 0.0511                                      | 102              |
|               | 0.08                                         | 0.0810                                      | 101              |
|               |                                               | 0.0817                                      | 102              |
|               |                                               | 0.0814                                      | 102              |
|               | 0.08                                         | 0.0802                                      | 100              |
|               |                                               | 0.0794                                      | 99               |
|               |                                               | 0.0788                                      | 99               |
| Ecuizumab     | 0.2                                          | 0.1936                                      | 97               |
|               |                                               | 0.1952                                      | 98               |
|               |                                               | 0.1930                                      | 97               |
|               | 0.36                                         | 0.3531                                      | 98               |
|               |                                               | 0.3597                                      | 100              |
|               |                                               | 0.3553                                      | 99               |

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Table 2. Repeatability and intermediate precision

| Sample                  | RSD of repeatability, % | RSD of intermediate precision, % |
|-------------------------|-------------------------|----------------------------------|
| PS80, mg/mL             |                         |                                  |
| 0.020                   | 2.6 (n = 3)             | 2.7                              |
| 0.035                   | 3.4 (n = 3)             | 3.1                              |
| 0.050                   | 1.6 (n = 3)             | 1.8                              |
| 0.065                   | 1.2 (n = 3)             | 2.0                              |
| 0.080                   | 1.4 (n = 3)             | 2.1                              |
| Darbepoetin alfa, 2.1 mg/mL | 2.5 (n = 6)             | 3.4                              |
| Eculizumab, 16.2 mg/mL  |                         | 4.6 (n = 6)                      | 5.2                              |

Note: PS80: polysorbate 80; RSD: relative standard deviation.

When the evaporation temperature was elevated to 35°C, no effect was observed on the recovery of darbepoetin alfa and eculizumab. However, the recovery RSD value increased two-fold at this temperature with respect to the 25°C case, which could be explained by an increased rate of evaporation, splashing, and/or partial loss of the sample. Changes in the derivatization time did not have much impact on the recovery of the drug formulations either. Notably, implementation of a 2-min derivatization resulted in a decrease of the samples’ OD.

Subsequently, the stability of the polysorbate–iron thiocyanate complex was monitored for 1 h. The PS80 standard and darbepoetin alfa drug substance samples spiked with PS80 (at 0.02, 0.05, and 0.08 mg/mL final concentrations) were analyzed at five time points: 10, 20, 30, 40, and 60 min. Between measurements, the solutions were stored in polypropylene tubes at room temperature. In Fig. 2 is reported the graph whereby the OD is plotted against the incubation time. The OD values did not differ from the initial value by more than 8% over the 1-h period, for any sample.

![Graph showing the effect of incubation time on the optical density of polysorbate 80 (PS80) standards at different concentrations and darbepoetin alfa drug substance samples spiked with PS80 to the specified final concentrations (DEPO).](image)

Analytical range

We have established the linearity, accuracy, and precision of our technique to determine the concentration of PS80 in the 0.02–0.08 mg/mL range. Therefore, evidence indicates that the PS80 analytical range is 0.02–0.08 mg/mL when darbepoetin alfa is present. However, we need to note that eculizumab was diluted five-fold before analysis in all experiments; therefore, in this case the PS80 analytical range is 0.1–0.4 mg/mL.

Selection of the derivatization reagent

PS80 is able to form colored complexes with thiocyanates of transition metals, especially iron(III), molybdenum(V), and cobalt(II). Notably, PS80–metal thiocyanate complexes are soluble in organic solvents [16]. In non-polar organic solvents, furthermore, all these complexes display absorbance peaks in the UV-Vis spectrum. The PS80–cobalt(II) thiocyanate complex displays two UV-Vis peaks, at 320 nm (higher intensity) and 620 nm; PS80–molybdenum(V) thiocyanate displays two peaks, at 323 and 475 nm; and PS80–iron(III) thiocyanate displays one peak at 510 nm. The extinction coefficients of the peaks just listed decrease in the following order: Fe (510 nm) > Co (320 nm) > Mo (323 nm) ≈ Mo (475 nm) > Co (620 nm). As we previously discussed [1], the most useful complex from the standpoint of our analysis is iron(III) thiocyanate because its detection relies on a relatively long wavelength; it interferes minimally with the background, sample matrix, and the presence of UV-absorbing impurities in reagents, and it is characterized by the highest sensitivity among the tested metal thiocyanates.

A considerable issue associated with PS80 derivatization is the high volatility of dichloromethane, which could result in quantification errors and increased RSD values. Attempts to replace dichloromethane with polar organic solvents (e.g., ethyl acetate, butyl acetate, or their mixtures with acetonitrile) have failed due to the intense color and almost opaque appearance of the organic layer, even in the case of blank samples, which is caused by the high solubility of the complexes in these solvents. However, the use of non-polar organic solvents, such as chloroform, carbon tetrachloride, toluene, and benzene, yielded similar results to those obtained using dichloromethane, although analyte sensitivity was 2–4 times lower in all these solvents than in dichloromethane. Moreover, collecting the toluene...
and benzene layers proved difficult, since these solvents’ densities were lower than that of the aqueous layer. In order to minimize dichloromethane volatility, we cooled the metal thiocyanate complex solutions to −20°C before adding them to the PS80-containing solutions.

**Cuvette fouling**

The measurements of the OD were carried out in quartz cuvettes. The cuvettes’ surfaces were noticeably stained after 3–4 measurements. In order to lessen cuvette fouling and maintain the stability of the colored complexes, we added 10% sulfuric acid to the derivatization reagent [16]. In our previous report [1], we recommended rinsing the cuvettes with 96% ethanol and dichloromethane after each measurement, although complete stain removal was not achieved by this approach. In the present study, we revised the composition of the derivatization reagent.

According to some sources [20, 21], sodium and potassium ions stabilize polyoxyethylene complexes with iron(III) thiocyanate. To investigate the effect of such ions on PS-based complexes, we added either sodium or potassium chloride into the derivatization reagent to the final concentrations of 0.1, 0.4, 1.0, and 2.0 M. To estimate the stability of the complexes in quartz cuvettes, the absorbance of derivatized 0.4 mg/mL PS80 samples was determined in six replicates for all sodium and potassium chloride ion concentrations. When a derivatization reagent containing at least a 0.4 M concentration of either sodium or potassium was utilized, the cuvette was observed to be much cleaner than in the case whereby the derivatization reagent contained no sodium/potassium ions or a 0.1 M concentration of them. We conclude that the addition of 0.4 M sodium chloride (or potassium chloride) is useful for preventing cuvette fouling.

**Removal of proteins from PS solutions**

Our previous attempt to quantify PS80 in darbepoetin alfa and eculizumab formulations using solid-phase extraction [1] produced ambiguous results, due to PS80 adsorption onto the proteins. Performing a preliminary protein denaturation with chaotropic agents, such as guanidine hydrochloride or urea, partially addressed the problem. In this study, we observed no interaction between the precipitated protein and PS80 in organic solvent–water mixtures. To investigate this issue further, we used different organic solvents to precipitate the protein, while causing no PS80 loss. Darbepoetin alfa, being a highly sialylated protein, was difficult to precipitate, and only the use of a mixed water–organic solvent with at least 50% acetone content afforded the complete removal of the protein from solution. Eculizumab precipitation was carried out in the same manner, and the recovery rate was quite similar, close to 100% (see **Accuracy** subsection in RESULTS AND DISCUSSION section).

**CONCLUSIONS**

The reported spectrophotometric method based on ferric thiocyanate complexation was suitable for PS quantification in biopharmaceutical formulations in the PS concentration range of 0.02–0.08 mg/mL. PS extraction was achieved by protein precipitation using an organic solvent, followed by supernatant evaporation in vacuum. PS was derivatized using an optimized, stable ferric thiocyanate reagent, and the derivative was extracted into dichloromethane to conduct OD measurements.

The optimal conditions for each step of the analysis were identified. The accuracy of the technique in the mentioned analytical range was 97–102%; the RSD of the repeatability did not exceed 5%; the LOQ value was 0.01 mg/mL. The approach was validated for PS80 quantification in darbepoetin alpha and eculizumab drug substances. The proposed method was more sensitive and precise than the previously reported technique [1], and we did not detect any MI.

*The authors declare no conflicts of interest.*

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