

**CHEMISTRY AND TECHNOLOGY OF MEDICINAL COMPOUNDS
AND BIOLOGICALLY ACTIVE SUBSTANCES**

**ХИМИЯ И ТЕХНОЛОГИЯ ЛЕКАРСТВЕННЫХ ПРЕПАРАТОВ
И БИОЛОГИЧЕСКИ АКТИВНЫХ СОЕДИНЕНИЙ**

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RESEARCH ARTICLE

Quantitation of polysorbate 80 in recombinant protein formulation using high-performance liquid chromatography

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Abstract

Objectives. Polysorbate 80 (PS80) quantification in biopharmaceutical products has always been challenging owing to its minute content, absorption to the protein backbone, lack of specific chromophoric PS80 groups, and heterogenic nature. This work is aimed at developing an express method for PS80 analysis in biopharmaceutical products using hydrolysis and subsequent high-performance liquid chromatography analysis with ultraviolet detection that does not consume substantial amounts of sample ($\geq 35 \mu\text{L}$).

Methods. Five therapeutic protein formulations were chosen as model proteins. Alkaline hydrolysis formulation was applied, without protein precipitation and with a range of precipitation techniques to remove protein from the test solution and hydrolyze PS80, to free fatty acids. The obtained hydrolysate was analyzed using reverse-phase high-performance liquid chromatography.

Results. As a result of the high protein content of monoclonal antibody formulations, preliminary protein removal was required, which was achieved by precipitation with organic solvents. A specific

precipitant ethanol–isopropanol mixture (1:1 volumetric ratio) was developed to efficiently remove antibodies while keeping PS80 in the solution. The PS80 quantification method was developed for monoclonal antibody drugs. For three monoclonal antibody drug products (adalimumab, infliximab, and eculizumab), method validation was performed according to the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use, the United States Pharmacopeia, and the State Pharmacopeia of the Russian Federation guidelines. **Conclusions.** The optimal assay conditions for each group of recombinant monoclonal antibody substances were chosen. Protein precipitation with ethanol or ethanol–isopropanol mixtures before hydrolysis was introduced, allowing for a substantial reduction of sample to 35 μ L or even less if PS80 content is higher than 0.05 mg/mL. Accelerated hydrolysis (90 min) is preferable to slow hydrolysis (4–18 h). Method validation for protein products such as adalimumab, infliximab, and eculizumab was demonstrated for the first time. Both methods were validated for each drug product. The coefficients of variation for method specificity and high precision were $\leq 6.0\%$ for 3 analyses. The accuracy of the methods ranged from 96% to 109% for all of the tested drug products.

Keywords: polysorbate 80, hydrolysis, HPLC, oleic acid, therapeutic proteins, spectrophotometric detection

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НАУЧНАЯ СТАТЬЯ

Методика ВЭЖХ для определения полисорбата 80 в препаратах рекомбинантных терапевтических белков

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Аннотация

Цели. Определение полисорбата 80 в препаратах терапевтических рекомбинантных белков всегда являлось трудной задачей, ввиду низкого содержания, гетерогенной природы, присутствия белка в препарате, а также отсутствия хромофорных групп у данного аналита. Целью данной работы являлась разработка экспрессной и

экономичной методики определения полисорбата 80 в препаратах рекомбинантных моноклональных антител с использованием гидролиза с последующим определением высвобожденной олеиновой кислоты методом высокоэффективной жидкостной хроматографии (ВЭЖХ) с УФ детектированием.

Методы. В качестве модельных образцов выбраны пять терапевтических рекомбинантных белков. Использован щелочной гидролиз без удаления белка и с его удалением различными способами осаждения для выделения свободных жирных кислот. Полученный гидролизат проанализирован методом ВЭЖХ.

Результаты. Для субстанций моноклональных антител, ввиду высокого содержания белка, требовалось его удаление. В качестве наиболее простого способа удаления выбрана денатурация и последующая преципитация белка, что достигалось путем осаждения антитела органическим растворителем. Был выбран универсальный осадитель (смесь этанола и изопропанола в объемном соотношении 1:1), использование которого позволяло эффективно удалять моноклональное антитело, но в то же время не допускать потерь полисорбата 80. Была разработана экспрессная методика определения полисорбата 80 для субстанций моноклональных антител. Методика определения полисорбата 80 валидирована согласно требованиям International Council for Harmonisation, United States Pharmacopeia и Государственной фармакопеи Российской Федерации.

Выводы. Были проведены испытания условий пробоподготовки для моноклональных антител. Впервые было внедрено осаждение белка этанолом или смесью этанол-изопропанол перед проведением гидролиза и анализом полисорбата 80. Это позволило значительно уменьшить требуемое количество образца для анализа – до 35 мкл, при концентрации полисорбата 80 – 0.05 мг/мл или еще меньше при его большем содержании. Ускоренный гидролиз полисорбата 80 в течение 90 мин является более предпочтительным при проведении анализа в сравнении с продолжительным гидролизом в течение 4–18 ч, описанным в литературе. Впервые была проведена валидация методики пробоподготовки и анализа для моноклональных антител адалимумаб, экулизумаб и инфликсимаб. Высокая прецизионность методики (среднеквадратичное отклонение $\leq 6.0\%$), специфичность и удовлетворительные значения правильности (фактор отклика от 96 до 109%), говорят о пригодности методики для определения полисорбата 80 в лекарственных средствах на основе рекомбинантных моноклональных антител.

Ключевые слова: полисорбат 80, гидролиз, высокоэффективная жидкостная хроматография, олеиновая кислота, рекомбинантные терапевтические белки, спектрофотометрическое детектирование

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INTRODUCTION

PSs consist of sorbitol core bound to oxyethylene units, with one molecule of sorbitol containing one molecule of oleic (PS80) or lauric (PS20) acid. Its heterogenic nature [8], lack of chromophoric groups, and a tendency to bond with proteins present in formulation make it difficult to analyze [9]. Thus, several analytical approaches have been developed, including spectrophotometric detection, which is based on the extraction of PSs complexes with transition metals thiocyanates into organic solvent [10, 11] or inclusion of a fluorescent dye into PS micelles, followed by

fluorescence detection and quantification [12, 13] and gas chromatography [14, 15]. The given methods are relatively free of systematic error owing to differences of PSs lot and manufacturer, but their laboriousness and low precision make them unsuitable for routine quality control.

High-performance liquid chromatography (HPLC) is another approach. There are multiple methods, each with its level of sensitivity, sample pretreatment, and detection. Reversed-phase (RP) HPLC of native PSs is conducted using evaporative light scattering detection (ELSD), and PS chromatograms appear as a collection of peaks [9, 16]. The detection of one of

the PS components, mostly commonly liberated fatty acid, is based on HPLC with mass spectrometric [17, 18], fluorometric [19], or spectrophotometric [20–22] detection, or gas chromatography with flame ionization detector [23]. Although sample pretreatment is required, most of these approaches are less laborious than other quantitation methods. Currently, the presented methods require a substantial amount of sample for analysis or a prolonged hydrolysis time. Both of these conditions complicate routine PS80 analysis in TPs, which should be solved.

It should be noted that not every laboratory has the necessary equipment for analysis, thus HPLC with spectrophotometric detection is the most preferred and available, despite the following disadvantages:

Oleic acid (OA) extraction methods described in [21, 22, 24–26] require a substantial amount of sample (200–300 μL), which can be problematic in downstream technology development or drug product stability estimation.

Poor sensitivity, prolonged incubation time, or inability to quantify PS80 in high protein content formulations [20, 25].

Thus, the development of the low-demanding PS80 quantitation method is of interest.

In this work, a modified HPLC method for PS80 quantification was developed. The method begins with a protein precipitation stage using ethanol–isopropanol mixture, followed by fatty acid hydrolysis and ultraviolet spectroscopy with HPLC analysis. The developed procedure uses 5 to 6 times fewer samples than others presented in scientific literature and can quantify PS80 concentrations as low as 0.005 mg/mL. Monoclonal antibody (MAb) precipitation was performed in an ethanol–isopropanol mixture. The method was approbated for three recombinant MAb formulations with a protein content of about 10 mg/mL or more, validation was performed according to the International Council for Harmonization (ICH) of Technical Requirements for Pharmaceuticals for Human Use¹, the United States Pharmacopeia (USP), the United States Food and Drug Administration², and the State Pharmacopeia of the Russian Federation³.

¹ ICH Q 2 (R1) guidance. *Validation of Analytical Procedures: Text and Methodology*. EMEA. URL: https://www.ema.europa.eu/en/documents/scientific-guideline/ich-q-2-r1-validation-analytical-procedures-text-methodology-step-5_en.pdf. Accessed January 13, 2022.

² Analytical Procedures and Methods Validation for Drugs and Biologics. U.S. Department of Health and Human Services Food and Drug Administration. 2015. URL: <https://www.fda.gov/files/drugs/published/Analytical-Procedures-and-Methods-Validation-for-Drugs-and-Biologics.pdf>. Accessed January 13, 2022.

³ State Pharmacopeia of the Russian Federation, 14th edition. URL: <https://gmpreg.com/en/documentos/marco-normativo/state-pharmacopoeia-russian-federation-edition-xiv>. Accessed January 13, 2022.

MATERIALS AND METHODS

Materials

PS80 [the USP and the National Formulary (NF)], OA (65.0%–88.0%), formic acid (98%–100%), phosphoric acid (85%), and potassium hydroxide ($\geq 85\%$) were purchased from *Sigma-Aldrich* (USA). Acetonitrile and isopropanol (UHPLC grade) were purchased from *PanReac AppliChem* (Spain). HPLC-grade water (18.2 M Ω /cm) was obtained using the Millipore Milli-Q Advantage A 10 system (USA). The monoclonal antibodies, eculizumab (ECZB), adalimumab (ADMB), and infliximab (INFL), were manufactured by *Pharmapark* (Russia); 15.1 mg/mL for ECZB and INFL and 60.5 mg/mL for ADMB.

Method optimization

Method optimization was performed using the central composite design, which was done using the design of the Minitab19 experiment software (*Minitab*, USA).

Volume V_1 of precipitation reagent [X , % (v/v) isopropanol solution in ethanol] was mixed with 35 μL of ECZB substance with a 0.22 mg/mL PS80 content and Milli-Q water. After 10 min incubation at 80 °C, volume V_2 of supernatant was added to 35 μL of 0.5 M potassium hydroxide and incubated for 1.5 h at 80 °C. After the reaction was stopped with 35 μL of 4% formic acid in acetonitrile, 40 μL of the sample was injected. Input varied parameters (factors) are shown in Table 1.

All other parameters remained constant.

As criteria, recovery of PS80 in ECZB and signal to noise (S/N) ratio of the OA postpeak was chosen.

Sample preparation

PS80 standard solutions in Milli-Q water were prepared in seven concentrations ranging from 50% to 150% of the nominal PS80 concentration (NC) for each drug product.

ECZB (NC 0.22 mg/mL), ADMB (NC 1.00 mg/mL), and INFL (NC 0.05 mg/mL) formulations were prepared as follows. Followed by vortexing, sample incubation at 80 °C for 10 min, and centrifugation for 10 min at RT, 35 μL of standard or test sample were added to 20 μL of 1/1 (v/v) isopropanol/ethanol mixture. Twenty μL of obtained supernatant was mixed with 35 μL of 0.5 M potassium hydroxide solution. Subsequently, the mixture was incubated at 80 °C for 90 min, followed by addition of 35 μL of 4% formic acid in acetonitrile. All mixtures were transferred into chromatographic vials.

Chromatography

HPLC was performed on the Ultimate 3000 system (*Thermo Fisher Scientific*, USA), which included a quaternary gradient pump (LPG-3400SD), a split loop autosampler (WPS-3000), a column oven (TCC-3000), and an ultraviolet detector (DAD-3000). A C18 stationary

Table 1. Design of experiment variable parameters to optimize method

Input parameter	Value	
	Low level	High level
X (isopropanol concentration in ethanol), %	25	65
V_1 (volume of precipitation reagent), μL	20	40
V_2 (volume of supernatant), μL	20	40

HPLC column [YMC-Pack ODS-AQ, 150×4.6 mm, $3 \mu\text{m}$ particle diameter (YMC, Japan)] with end-capping was used. The mobile phase consisted of an 80:20 (v/v) mixture of acetonitrile and 0.85% phosphoric acid. The analysis was performed using isocratic elution for 13 min at a flow rate of 1.2 mL/min, a column temperature of 30°C , and a detection wavelength of 195 nm (the choice of that wavelength was based on low absorption at high wavelengths). The injection volume was set at $40 \mu\text{L}$.

Each injection in process of analysis or validation was derived from a separate vial containing a sample that had been prepared separately.

All validation procedures followed the ICH Q2 (R1), ICH, and USP guidelines. Selectivity, linearity, analytical range, precision, accuracy, limit of detection (LOD), and limit of quantification (LOQ) were evaluated.

Specificity

Specificity was evaluated by analyzing the pre-formulated PS80-free drug product, PS80 spiked drug product, Milli-Q water, standards of PS80, and OA.

Linearity

Linearity was analyzed in triplicate by preparing seven PS80 standards in Milli-Q water: 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, and 1.2 mg/mL. Linear regression was used to plot the OA peak area against PS80 concentration, and the correlation coefficient (R^2) was determined.

Accuracy

For the estimation of accuracy, each PS80-free recombinant protein solution was spiked with PS80 to obtain a final concentration of 100% and, at least 80% and 120% of the nominal PS80 concentration while containing protein at the level present in the substance. Sample pretreatment and analysis were performed in triplicate for each concentration level. Following PS80 quantification, the recovery (R , %) at every concentration level was calculated for each replica using the formula:

$$R = \frac{C_0}{C_t} \times 100,$$

where C_0 is the obtained PS80 concentration and C_t is the calculated PS80 concentration.

Repeatability and precision

The repeatability of the analytical technique was assessed using the accuracy assessment results ($n=3$). The coefficient of variation (CV, %) was calculated for each concentration level. For evaluation of the intermediate precision, the same analyses were conducted in two additional days, and the CV values of the overall PS80 concentration for each sample were compared.

LOD and LOQ

LOD and LOQ values were determined using consecutive injections of standard sample dilutions and estimation of the S/N ratio.

RESULTS AND DISCUSSION

Method development

This study aimed at developing a simple and reproducible PS80 quantification method for biopharmaceutical formulations of recombinant monoclonal antibodies protein products that require a relatively small amount of tested sample. The approbation of the method [18] showed relatively accurate PS80 quantification results, although it possessed certain drawbacks such as a large sample amount ($200 \mu\text{L}$) and difficulty in phase separation after hydrolysis.

The use of RP chromatography in analyzing nonhydrolyzed PS80 with spectrophotometric detection at 234 nm revealed multiple PS80 peaks, as indicated in previous works that used HPLC ELSD detection [8, 13]. PS80 quantification using a sum of multiple peak areas demonstrated reasonable method accuracy, but PS80 concentration had gradually increased over long-term TP stability assessment, making the method unfit for quantifying PS80 in long-term storage [20].

To develop the method, OA, which was the main fatty acid component, was chosen as the analyte, and hydrolysis with potassium hydroxide at a final concentration of 0.1 M was carried out without protein precipitation, followed by neutralization of the solution with 4% formic acid in acetonitrile. MAb preparation showed a tailing peak over all chromatograms owing to high concentration (≥ 10 mg/mL) and high tendency to aggregate in the presence of organic solvents, without protein removal (Fig. 1), resulting in OA quantification errors and column degradation with the MAb precipitate.

To remove MAb from test solutions, multiple methods, such as precipitation with organic solvents and heat denaturation, or both, were used. Heat denaturation of the test solution at 80 °C for 10 min resulted in protein precipitation, although further assessment the accuracy of the method revealed a decrease in PS80 content from 20% for ECZB and ADMB to 40% for INFL. For MAb precipitation, ethanol, isopropanol, and a 20% 1-butanol solution in ethanol were used at an initial volumetric 1:1 ratio (Fig. 2).

Chromatograms revealed the presence of close-eluting to the OA postpeak for all samples (standard PS80 solution, protein formulation, and water) with 20% butanol precipitation and, to a lesser extent, isopropanol, resulting in a loss of specificity and misestimation of PS80 content. The OA postpeak

was due to the extraction of leachables from the Eppendorf tube. There was no close-eluting peak for the ethanol when compared to OA. The preliminary results of the accuracy assessment are shown in Table 2.

Using ethanol precipitation, the method demonstrated satisfactory accuracy for ADMB, but not for ECZB or INFL.

The central composite design method was used to optimize the method for ECZB, resulting in 15 experiments with 3 variables. Contour plots were used to assess the optimum recovery (95%–105%), and the absence of OA postpeak (S/N = 0) was determined. The results of each experiment are depicted in Table 3.

The most ideal conditions, according to the obtained data, were $X = 50\%$ and $V_1 = V_2 = 20 \mu\text{L}$.

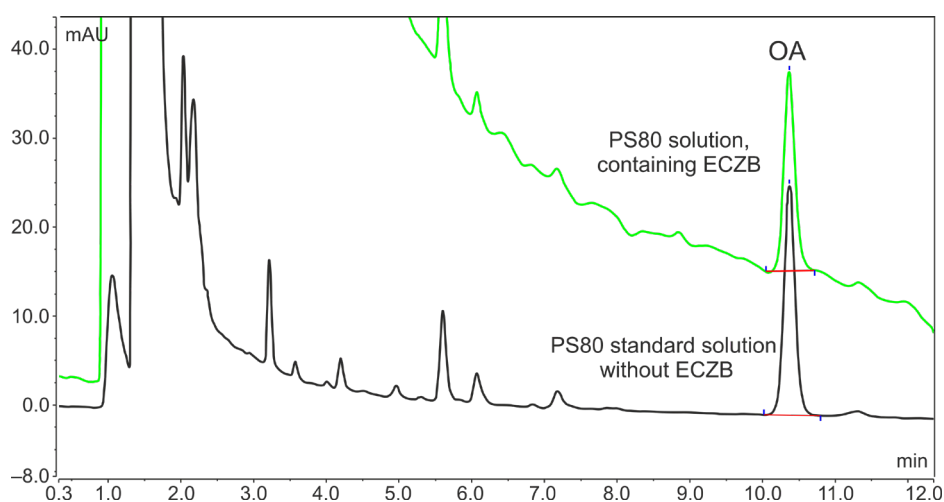


Fig. 1. Representative chromatogram of a 0.22 mg/mL PS80 solution. The OA retention time is 10.3 min.

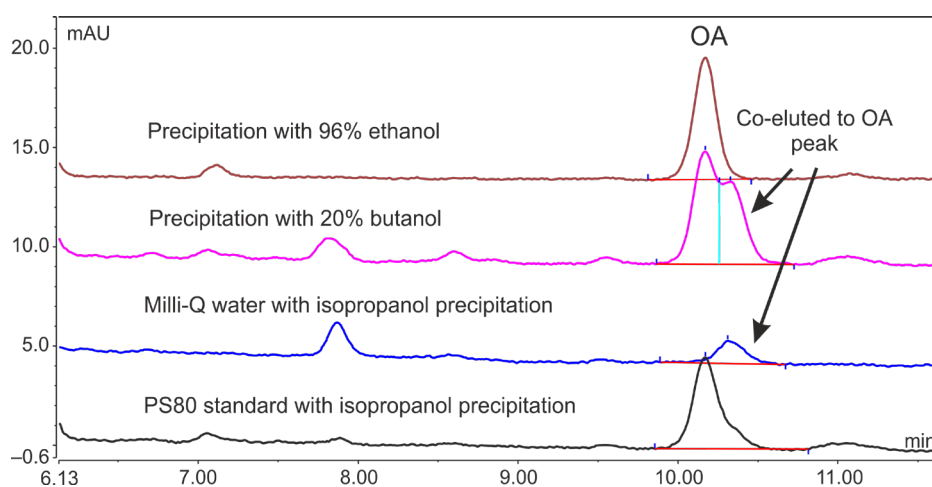


Fig. 2. Chromatograms obtained after PS80 solution hydrolysis and protein precipitation with organic solvents.

Table 2. Preliminary testing results of different precipitation reagents

Precipitation reagent	Recovery, %			OA postpeak presence
	ECZB	ADMB	INFL	
Ethanol 96%	84	102	82	–
Isopropanol	103	105	112	+
20% 1-butanol solution in ethanol	–*	–*	–*	+

*The results were incorrect owing to interference from a large postpeak presence.

Under the given conditions, recovery was about 99%, and there was no OA postpeak.

Under the given conditions, INFL substance testing demonstrated the same level of accuracy.

Method validation

The developed method demonstrated specificity. No peaks with OA retention time were observed in the Milli-Q water and PS80-free protein product. ECZB specificity assessments are shown in Fig. 3.

The method used for other drug products also demonstrated specificity. The correlation coefficient, R^2 , of the regression line was 0.996, obtained R^2 values were ≥ 0.99 , and the linearity of the methods was demonstrated. Accuracy estimation results for each biopharmaceutical are depicted in Table 4.

As a result, the recovery results for each biopharmaceutical were narrowly scattered around the

100% value, and no sample exceeded the 90%–110% recovery range limit. If the same PS80 lot was used as standard, the obtained values are considered free of systematic error and give actual PS80 concentration for the tested sample.

Repeatability and intermediate precision assessment results are presented in Table 5.

No sample exceeded the repeatability limit of 5% or the intermediate precision limit of 8%. In the one-day and day-to-day analyses, results obtained using both methods demonstrated narrow result distribution across the calibration range. Positive linearity, accuracy, and precision assessment results indicate that both methods are capable of persistently producing correct results in the range of 80% to 120% of nominal PS80 concentration. The LOQ was found to be 0.005 mg/mL ($S/N = 3.4$) while the LOD was found to be 0.002 mg/mL ($S/N = 11.2$).

Table 3. Method condition screening results

Experiment	Isopropanol content in ethanol (v/v), %	Volume of precipitation reagent (V_1), μL	Volume of supernatant for hydrolysis (V_2), μL	Criteria	
				Recovery, %	S/N of OA postpeak
1	25.0	20.0	20.0	84	0.0
2	25.0	20.0	40.0	85	0.0
3	25.0	40.0	20.0	92	0.0
4	25.0	40.0	40.0	93	0.0
5	65.0	20.0	20.0	99	0.0
6	65.0	20.0	40.0	98	3.2
7	65.0	40.0	20.0	102	5.2
8	65.0	40.0	40.0	104	6.1
9	11.4	30.0	30.0	85	0.0
10	78.6	30.0	30.0	103	15.2
11	45.0	13.2	30.0	98	0.0
12	45.0	46.8	30.0	99	5.2
13	45.0	30.0	13.2	97	0.0
14	45.0	30.0	46.8	96	0.0
15	45.0	30.0	30.0	96	0.0

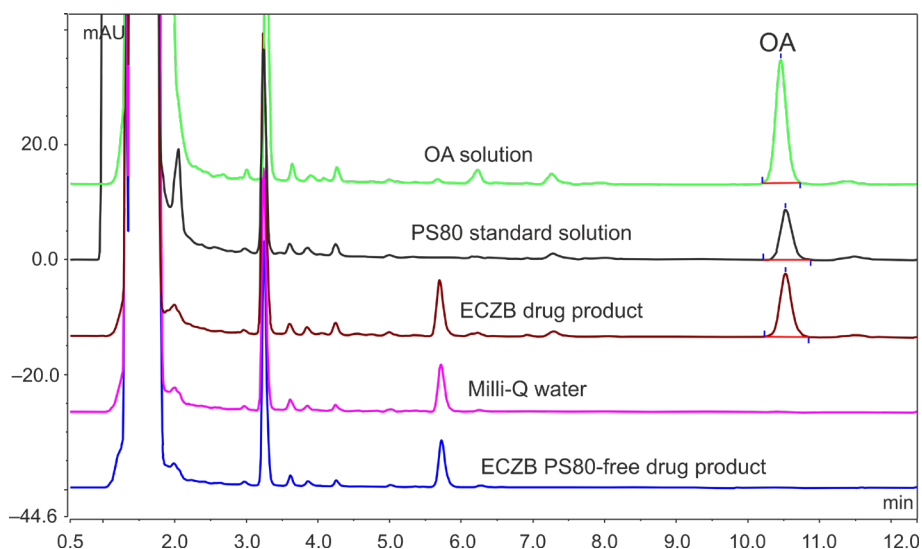


Fig. 3. Chromatograms of specificity assessment of PS80 determination in the ECZB substance.

Table 4. Accuracy estimation results

PS80 content from nominal concentration*, %	Recovery, %		
	ADMB	ECZB	INFL
NC, mg/mL	1.00	0.22	0.05
50	99.9	104.1	96.7
100	98.0	103.8	98.7
150	99.6	106.6	100.5

*Accuracy, precision, and repeatability for INFL and ECZB were assessed in the range of 40% to 160% and 80% to 120%, respectively.

Table 5. Repeatability assessment

Parameter	PS80 content from nominal concentration*, %	CV, %		
		ADMB	ECZB	INFL
Repeatability (n = 3)	50	2.6	1.0	3.0
	100	0.5	0.5	1.2
	150	2.5	3.9	2.9
Intermediate precision (n = 3)	50	5.4	4.3	3.5
	100	2.7	3.4	1.9
	150	4.8	5.6	2.3

*Accuracy, precision, and repeatability for INFL and ECZB were assessed in the range of 40% to 160% and 80% to 120%, respectively.

CONCLUSIONS

An HPLC method with spectrophotometric detection was developed for PS80 determination in TPs. The method is based on the hydrolysis of PS80 to free fatty acids, with OA as the main constituent being detected using RP HPLC. For monoclonal antibody removal, an additional step with organic solvent mixture precipitation was developed, while PS80 remained in solution, ensuring method accuracy. The method was

approved and validated for 3 therapeutic monoclonal antibody substances. The accuracy assessment demonstrated recovery ranging from 96% to 109%. The repeatability and intermediate precision values (n = 3) were less than 4.0% and 6.0%, respectively. The LOQ was 0.005 mg/mL. The current method requires minor quantities of protein products ($\leq 35 \mu\text{L}$). The method is also dependent on PS80 lot-to-lot variability, although the PS80 lots from a single manufacturer within two years of manufacture can be used as a standard to determine its

content in drug formulation. Since the method was only tested on 3 monoclonal antibody drug products, others may require sample pretreatment optimization, which can be achieved by varying the isopropanol/ethanol ratio or adding precipitants, such as sulfosalicylic acid, which was tested in another method for filgrastim analysis.

Authors' contributions

A.D. Askretkov – development of analytical methods, conducting experimental studies and methods validation, writing the text of the article;

D.O. Shatalov – writing the article, developing of the research methodology, editing the text of an article;

N.V. Orlova – development of the research methodology, experimental data processing, adjustment of experimental studies;

D.I. Zybin – data visualization, writing the text of the article;

V.V. Nikolaeva – conceptualization of the research methodology;

A.A. Klishin – conceptualization of the research methodology;

E.S. Tuzova – correction of the text of the article, conceptualization of the article methodology;

D.S. Minenkov – editing the text of the article, development of research methodology;

S.A. Kedik – writing an article, developing of the research methodology, editing the text of an article, conceptualizing of the article;

Yu.A. Seregin – analysis of literary sources, writing and editing the text of the article, conceptualization of the article.

The authors declare no conflicts of interest.

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