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Development and validation of a method for the determination of the specific activity of recombinant monoclonal antibody eculizumab

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Objectives. *Developing reliable and accurate analytical methods is necessary for comparative pharmaceutical analysis using physicochemical, biological (in vitro), preclinical, and clinical trials. The main objective of this study was to develop and validate an in vitro method for determining the specific activity of the recombinant monoclonal antibody eculizumab.*
Methods. *The method of indirect enzyme immunoassay was used in the study.*
Results. *A method for determining the specific activity of the humanized recombinant monoclonal antibody eculizumab was described and validated for the first time. A comparative evaluation of the specific activity of Soliris® (Alexion Pharmaceuticals Inc., USA), and its biosimilar PRK-001 (Pharmapark, Russia) was performed using the developed method.*
Conclusions. *The similarity of PRK-001 and the original Soliris® in relation to their specific activity, that is, binding to the human complement system C5 protein, was proved.*

Keywords: *validation; paroxysmal nocturnal hemoglobinuria; reproduced drug; specific in vitro activity; enzyme-linked immunosorbent assay; complement system.*

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Разработка и валидация метода определения специфической активности рекомбинантного моноклонального антитела экулизумаб

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Цели. *При подтверждении биоподобности препаратов необходимо создание надежных и точных аналитических методов сравнительных исследований для доказательства схожести препаратов по результатам физико-химических, биологических (in vitro), доклинических и клинических испытаний. Основной задачей настоящей работы является разработка и валидация метода определения специфической активности рекомбинантного моноклонального антитела экулизумаб.*
Методы. *В работе использован метод непрямого иммуноферментного анализа.*
Результаты. *Впервые разработан метод определения специфической активности гуманизированного рекомбинантного моноклонального антитела экулизумаб и проведена его валидация. С использованием разработанного метода проведена сравнительная оценка специфической активности оригинального препарата Солирис® (Alexion Pharmaceuticals Inc., USA) и его биоаналога PRK-001 (ООО «Фармапарк», Россия).*
Выводы. *Доказана биоаналогичность препаратов Солирис® и PRK-001 в отношении их специфической активности.*

Ключевые слова: *валидация, воспроизведенный лекарственный препарат, пароксизмальная ночная гемоглобинурия, специфическая активность in vitro, иммуноферментный анализ, система комплемента.*

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INTRODUCTION

Paroxysmal nocturnal hemoglobinuria (PNH) is an extremely rare disease (an average of three cases per million [1]) accompanied by serious epiphenomena, such as hemolytic anemia, atypical thrombosis, bone marrow failure, and renal failure. The etiology of PNH is associated with a somatic mutation in the PIG-A gene, which blocks the biosynthesis of glycosylphosphatidylinositol, a glycolipid called GPI anchor, necessary to retain a number of proteins on the cell membrane, in particular CD55 and CD59, which are inhibitors of the membrane attack complex (MAC) complement systems. The deficiency of CD55 and CD59 on the surface of the cell membrane leads to dysfunction in the suppression of MAC production, which is the main cause of erythrocyte hemolysis in PNH [2, 3]. This is determined by flow cytometry [4, 5].

Currently, the only commercially available drug, Soliris® (*Alexion Pharmaceuticals Inc.*, USA) is used for PNH therapy. The active substance of Soliris® is a recombinant kappa-monoclonal antibody of mixed type IgG2/4—eculizumab consisting of a human constant chain and determining the complementarity of murine regions grafted onto human framework regions in the variable and heavy chain with a total molecular mass of 148 kDa[1]. The mechanism of eculizumab action is the binding of the complement system to protein C5 followed by its lytic destruction in lysosomes, blocking the formation of a MAC, and subsequent cell lysis [6–8].

Due to the high cost of therapy (USD 600 000 per year for one patient) [9] and the need for its lifelong

[1] DRUGBANK. Eculizumab. Available from: https://www.drugbank.ca/drugs/DB01257 [Accessed October 22, 2019].

implementation, the number of pharmaceutical companies actively developing a bioanalog of Soliris® increases (*Generium Pharmaceutical,* Russia; *Samsung Bioepis,* South Korea; *Amgen*, USA). Therefore, the need for the development of reliable methods for controlling the quality of the resulting product becomes obvious. One of the most important quality indicators is its binding to protein C5 [10–12].

Currently, no method has been described to evaluate the specific activity of eculizumab with accuracy and reproducibility at all stages of production. Although the Soliris®/Eculizumab enzyme-linked immunosorbent assay (ELISA) Kit (*Arsh Biotech Pvt. Ltd.,* India) is already at the market and can help to evaluate the activity of the drug in biological fluids, because the main part of eculizumab molecules in the human body is bound to an antigen, it is impossible to evaluate its content and activity with the required level of accuracy without using additional sample preparation. In addition, it is unclear whether the use of the kit is effective at various stages of drug production, in particular, after the stages of isolation, purification, and filtration, which can significantly affect the property of the final product.

In this work, we present a new method for determining the specific activity of eculizumab. Our method is suitable for both monitoring the drug quality at various stages of development and implementing the output control of the finished product. The suggested method was used for the comparative evaluation of the specific activity of three series of Soliris® (*Alexion Pharmaceuticals Inc.,* USA) and PRK-001 (*Pharmapark,* Russia).

MATERIALS AND METHODS

We used ELISA to quantify the specific activity of eculizumab. The specific activity of a standard sample of eculizumab used in this study was confirmed by the manufacturer. Soliris® (*Alexion Pharmaceuticals Inc.,* USA) was used for comparison.

Preparation of standard solutions

Preparation of phosphate-buffered saline ("Buffer solution A")

One tablet of the dry prepared buffer (*Sigma Aldrich,* USA) was dissolved in 100 ml of deionized water. The pH of the resulting solution was adjusted to 7.4 using a pH meter *(Mettler Toledo,* USA). The resulting solution was stored in a tightly closed container at 4°C.

Preparation of phosphate-buffered saline with 0.01% Tween 20 ("Buffer solution B")

One tablet of a dry finished buffer containing 0.01% Tween 20 (*Sigma Aldrich,* USA) was dissolved in 500 ml of deionized water under constant stirring at 25°C. The solution pH was adjusted to 7.4. The resulting solution was stored in a tightly closed container at 4°C.

Blank solution preparation

1 g bovine serum albumin (*Sigma Aldrich,* USA) was dissolved in 100 ml buffer solution A. Then, the solution was stored in a tightly closed container at 4°C.

Immobilization of C5 protein on an ELISA plate

A solution of C5 protein (*Complement Technology,* USA) was diluted to a concentration of 1.5 μg/ml using buffer solution A. 100 μl of the resulting solution was added to the wells of the plate and incubated for 48 h at 4°C.

Preparation of calibration solutions

Soliris® with a specific activity of 9138000 IU per 300 mg was diluted with a blank solution according to Table 1.

Preparation of the solution of secondary antibodies

A working solution of secondary antibodies conjugated to horseradish peroxidase was diluted with a blank solution according to the manufacturer's recommendations (*Genway Biotech Inc.,* USA).

Determination of the specific activity of eculizumab

The test solution of eculizumab diluted in the blank solution, and S1–S6 calibration solutions were added into the wells of a tablet with pre-immobilized C5 protein in an amount of 100 μl per well in three repetitions each. The plate was incubated for 2 h at 4°C. Next, the wells of the plate were washed with 200-μl buffer solution B in three repetitions to remove the matrix of the sample. 100-μl solution of secondary antibodies was added into the wells of the plate and incubated for 2 h at 37°C. After incubation, the wells were washed with 200-μl buffer solution B in three repetitions to remove unbound antibodies. After removing buffer solution B, 100-μl 3,3', 5,5'-tetramethylbenzidine containing hydrogen peroxide (*Sigma Aldrich,* USA) was added into the wells of the plate. The plate was incubated for 10 min at 25°C, and the reaction was stopped using 0.5 M sulfuric acid (*CHIMMED,* Russia). The optical density was measured at 450 nm and 650 nm (reference) using a Tecan Infinite 200 Pro plate reader (*Tecan,* Switzerland). A sigmoidal curve was plotted using the calibration solutions of eculizumab in the optical density–activity coordinates. The equation describing the curve was used to calculate the activity of eculizumab in the test sample.

The results were processed using Origin 9.1 software (*OriginLab Corp.,* USA).

Method validation

The validation of the developed method was carried out in compliance with the requirements of the

Russian Federation State Pharmacopeia XIV in terms of specificity, linearity, repeatability, intermediate precision, accuracy, analytical area, and stability.

RESULTS AND DISCUSSION

First, the immobilized protein C5–eculizumab complex was obtained. Second, secondary antibodies specific for the Fc fragment of type 4 IgG and conjugated to horseradish peroxidase were bound. Finally, a chromogenic reagent was added, the reaction was stopped, and the optical density was determined. A schematic representation of the method is shown in Fig. 1.

Specificity evaluation

The method specificity was determined by the ability to evaluate the content of the studied component against the background of related substances in the protein solution [13]. To confirm the method specificity, we studied the effect of buffer solutions on PRK-001 and Soliris® (hereinafter referred to as SP1 and SP2, respectively) prepared in accordance with the manufacturer's instructions. At the same time, the effect of the components of the buffer solutions used at each stage of drug purification was evaluated. When using sample SP3, the buffer solution after purification using an affinity sorbent was used. When using sample SP4, the buffer solution after purification using an ion-exchange sorbent was used. The optical density of the investigated solutions should be in the range of ±10% of the optical density of the blank solution. The results of the study are presented in Table 2.

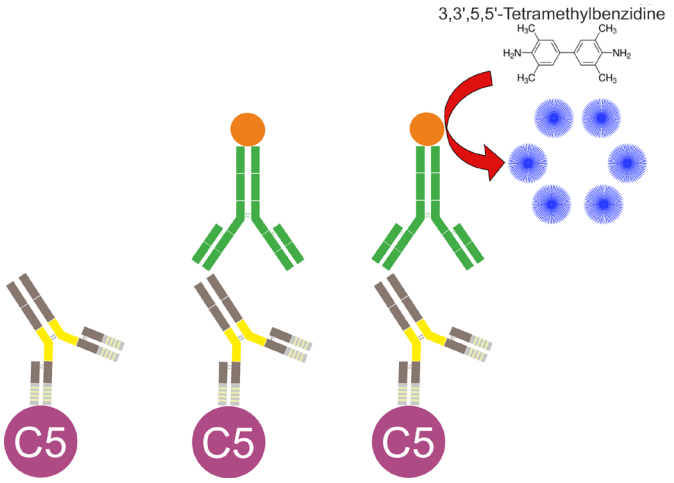

Fig 1. Schematic for determining the specific activity of eculizumab. The stages of the method are described in the text below.

Table 1. Dilution of a standard sample for the preparation of calibration solutions

Sample name	Sample volume, µl	Volume of blank solution, µl	Specific activity, IU/ml
Sample ITD 1*	10 µl of Soliris®	294.6	300 000
Sample ITD 2	100 ITD1	900	30 000
Sample ITD 3	100 ITD2	900	3000
Sample ITD 4	100 ITD3	900	300
Sample S1	500 ITD4	500	150
Sample S2	500 S1	250	100
Sample S3	400 S2	400	50
Sample S4	400 S3	400	25
Sample S5	400 S4	400	12.5
Sample S6	400 S5	140	9.3

*Samples labeled "ITD" are used as intermediate dilutions.

The obtained results indicate that the auxiliary components of the drug do not affect the analysis results. Thus, the specificity of the method was established.

Linearity evaluation

Our method does not demonstrate a linear dependence between specific activity and optical density for the selected range (from 9.3 IU/ml to 150 IU/ml). Therefore, we used a sigmoid curve described by the following equation [14–15]:

$$y = A_1 + \frac{A_2 - A_1}{1 + 10^{(LOGx_0 - x) \times p}},$$

where A_1 and A_2 are the asymptotes, x is the value of activity, IU/ml; p is the Hill coefficient; x_0 is the coordinate of the inflection point.

When plotting the calibration curve, six standard solutions with the following values of specific activity were used: 150, 100, 50, 25, 12.5, and 9.3 IU/ml. The resulting curve is presented in Fig. 2. The correlation coefficient was 0.9987, which is above the minimum allowable value of 0.99. Thus, the linearity of the method was established.

Repeatability evaluation

The repeatability of the method was evaluated by analyzing standard samples vis-à-vis specific activity values (150, 25, and 12.5 IU/ml) in six replicates each. The relative standard deviation (RSD) for each concentration should not exceed 3%. The results obtained for the method repeatability are presented in Table 3. The value of the RSD for all concentration levels does not exceed 3%, proving the repeatability of the method.

Intermediate precision and accuracy

Intermediate precision and accuracy were evaluated by conducting six analytical sessions over six days by two operators. In each analytical session, a calibration curve was plotted, and five test solutions were studied with the following values of specific activity: 150, 75, 37.5, 18.7, and 9.3 IU/ml. To establish the method accuracy, the value of the degree of extraction (R) should not go beyond 85–115%. The intermediate precision of the method is considered to be established if the RSD according to the results of six sessions for each concentration level does not exceed 15%. The results are presented in Table 4.

The obtained results meet the requirements. Thus, the intermediate precision and accuracy of the method were established.

Evaluation of the analytical field

The analytical region of the method was evaluated on the basis of the results obtained when determining the method linearity, repeatability, and accuracy

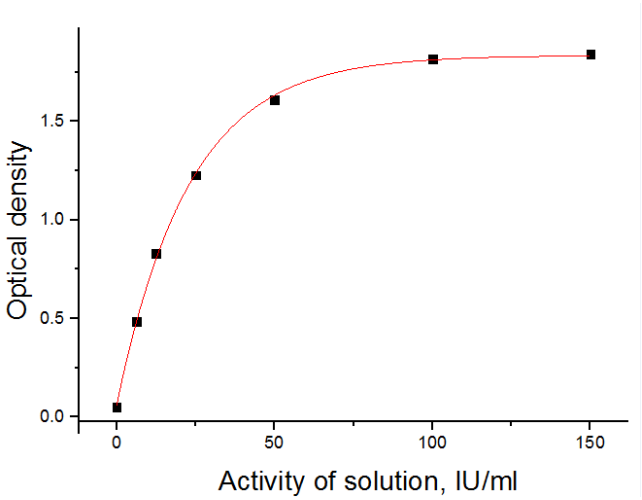

Fig. 2. The calibration curve obtained when evaluating the linearity of the method.

Table 2. Determination of method specificity

Sample name	Optical density*	Optical density* of blank solution
Sample SP1	0.0154 ± 0.0004	0.0151 ± 0.0004
Sample SP2	0.0153 ± 0.0004	
Sample SP3	0.0151 ± 0.0003	
Sample SP4	0.0155 ± 0.0004	

*The data in the table are given in the following format: average value ± standard deviation (n = 3).

for the activity range of 9.3–150 IU/ml. Since the technique meets all the validation requirements, we can assume that the analytical region of the method is in the range of 9.3–150 IU/ml.

Stability evaluation

The most critical parameter of the method, which may affect the result, is the storage time of the tablet with immobilized C5 protein. To evaluate the method stability, plates with immobilized C5 protein were incubated at 4°C for 7 and 14 days. Next, the test sample was analyzed with parallel analysis in the tablet (storage time 0 days). The results of determining the activity in the tablets subjected to storage were compared with the results obtained in the tablet without any long-term storage.

The deviation of the activity of the test sample from the true value obtained in the tablets subjected to storage, both for 7 and for 14 days, did not exceed the set limit (±5%). This proves the method stability with respect to the storage time of the tablet with immobilized protein C5.

Comparative study of the specific activity of PRK-001 and Soliris®

The drug manufactured by Pharmapark—PRK-001—is a reproduced biological drug. Thus, it is necessary to confirm its similarity to the original [16]. In this work, we performed a comparative evaluation of the specific activity of three series of Soliris® and PRK-001 to confirm their similar bioactivity. Soliris® 1000325 series with a known activity value was used as the standard.

Figure 3 presents the optical density dependence on the specific activity of all the drugs studied in the work.

For all the series of studied drugs, the specific activity was determined using the developed method. The study results are shown in Fig. 4.

The obtained results confirm the compliance of PRK-001 and Soliris® preparations with respect to their specific activity, that is, binding to the human complement system C5 protein. The measured specific activity of Soliris® and PRK-001 samples differed no more than 9%.

Table 3. Results of the method repeatability study

Theoretical specific activity of the sample, IU/ml	The value of the measured specific activity of the sample*, IU/ml	RSD, %
150	154.9 ± 4.4	2.8
25	25.5 ± 0.7	2.5
12.5	12.3 ± 0.1	0.8

*The data in the table are given in the format average value ± standard deviation (n = 6).

Table 4. Evaluation of the intermediate precision and accuracy of the method

Theoretical specific activity of the sample, IU/ml	The value of the measured specific activity of the sample*, IU/ml	RSD (n = 6), %	R, %
150.0	157.3 ± 15.3	9.7	104.9
75.0	75.9 ± 6.0	7.9	101.2
37.5	37.5 ± 2.2	5.9	100.0
18.7	19.9 ± 1.7	8.5	106.1
9.3	9.3 ± 0.9	8.7	108.1

*The data in the table are given in the format average value ± standard deviation (n = 6).

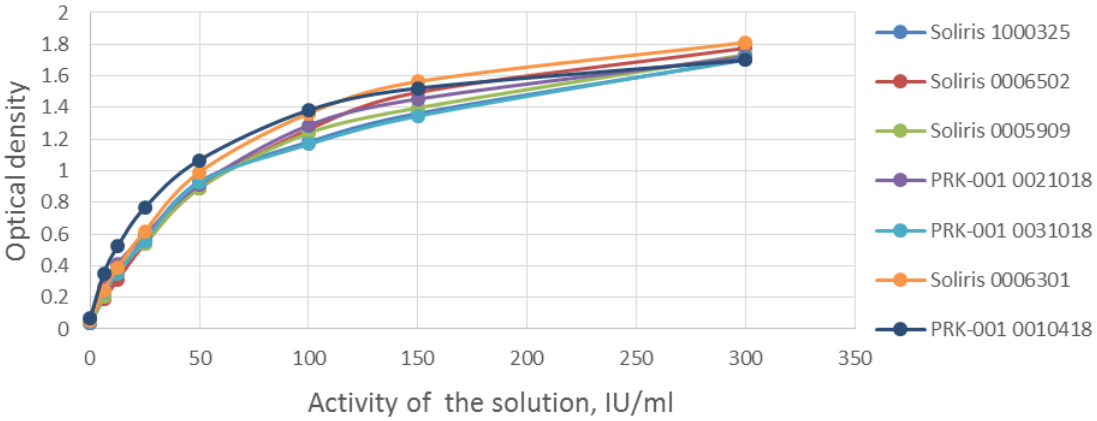

Fig. 3. "Optical density-specific activity (binding)" dependence plot obtained for Soliris® (Series 0006502, 0005909, and 0006301) and PRK-001 (Series 0010418, 0021018, and 0031018) in comparison with Soliris® Series 1000325.

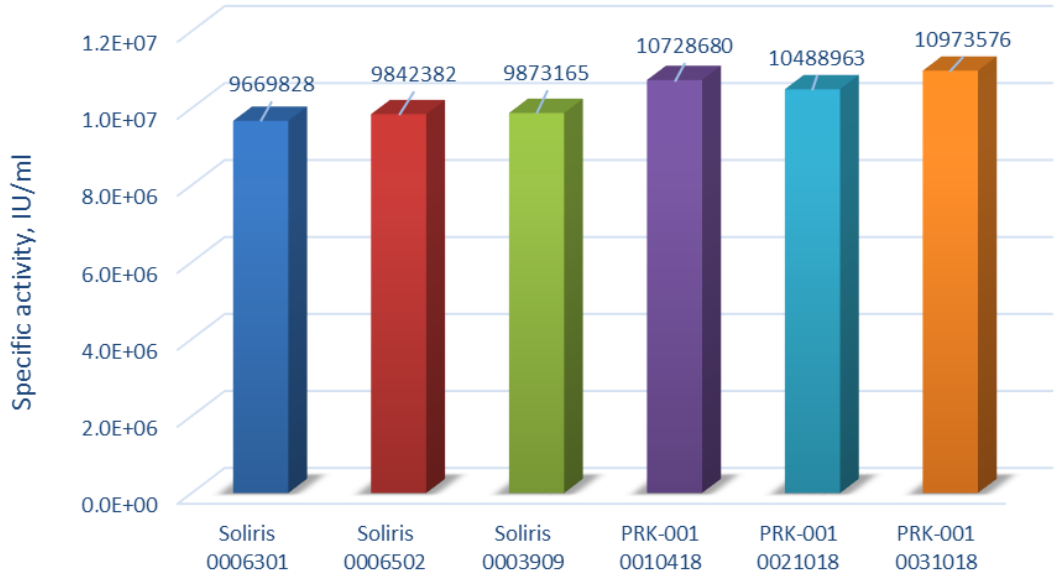

Fig. 4. Specific activity of three series Soliris® and PRK-001.

CONCLUSIONS

In this work, a method for determining the specific activity of the humanized recombinant monoclonal antibody eculizumab was described and validated for the first time. A comparative evaluation of the reproduced drug PRK-001 and the original—Soliris®—was performed. The similarity of both drugs in relation to their specific activity was proved.

The authors declare no conflicts of interest.

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