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RESEARCH ARTICLE

Development, characterization, and stability assessment of a lyophilized Eculizumab formulation for use as a reference material

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Abstract

Objectives. The study set out to develop a stable lyophilized formulation of the monoclonal antibody Eculizumab, comprehensively characterize the resulting material, and assess its stability for qualifying it as a reference material. This involved developing a matching placebo formulation, determining the optimal lyophilization conditions, and conducting a rigorous stability study.

Methods. In the development of the formulation and lyophilization conditions for Eculizumab, we tested various buffer systems and cryoprotectants. The residual moisture content in the resulting lyophilized samples was determined by Karl Fischer titration. Peptide mapping was performed using reversed-phase high-performance liquid chromatography (RP-HPLC) following enzymatic hydrolysis with trypsin. The structural, physicochemical, and biological properties were analyzed using various analytical methods, including RP-HPLC, high-performance liquid chromatography mass spectrometry, capillary sodium dodecyl sulfate electrophoresis, size-exclusion high-performance liquid chromatography, and enzyme-linked immunosorbent assay.

Results. A placebo solution for lyophilization of Eculizumab was selected with the following composition: 20 mM sodium phosphate, 4% trehalose, 0.2% polysorbate 80, pH 7.0. The results demonstrated a high degree of similarity between the candidate reference material and Eculizumab EU. Stability studies under storage conditions at 2–8°C demonstrated the material's stability for one year, with control points at 3, 6, 9, and 12 months.

Conclusions. The absence of any effect of the drying process on the primary and spatial structure, post-translational modifications, content of related impurities, composition of isoforms, and specific activity was confirmed. Furthermore, stability studies demonstrated no significant changes in protein quality during storage at 2–8°C for at least 12 months, which represents the entire available data period at the time of manuscript preparation. The results indicate that the developed lyophilized material is a viable candidate for an international reference material, although its official qualification would require additional collaborative trials and long-term stability data.

Keywords
reference material, lyophilization, monoclonal antibody, Eculizumab

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НАУЧНАЯ СТАТЬЯ

Разработка, характеризация и оценка стабильности лиофилизированной формы экулизумаба для использования в качестве стандартного образца

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Аннотация

Цели. Целью данного исследования была разработка стабильной лиофилизированной формы моноклонального антитела экулизумаб, характеризация полученного материала и оценка его стабильности для аттестации в качестве стандартного образца. Для достижения поставленных целей требовались разработка соответствующего состава плацебо, определение оптимальных условий лиофилизации и проведение исследования стабильности.

Методы. В ходе разработки состава плацебо и условий лиофилизации экулизумаба были протестированы различные буферные системы и криопротекторы. Содержание остаточной воды в лиофилизированных образцах определяли методом титрования по Карлу Фишеру. Пептидное картирование проводили методом обращенно-фазовой высокоэффективной жидкостной хроматографии (ВЭЖХ) после ферментативного гидролиза трипсином. Структурные, физико-химические и биологические свойства анализировали с использованием широкого ряда аналитических методов, включая обращенно-фазовую ВЭЖХ, жидкостную хромато-масс-спектрометрию, капиллярный гель-электрофорез, эксклюзионную ВЭЖХ и иммуноферментный анализ.

Результаты. Для лиофилизации экулизумаба был выбран буферный раствор следующего состава: 20 мМ фосфат натрия, 4% трегалозы, 0.2% полисорбата 80, pH 7.0. Полученные результаты продемонстрировали высокую степень сходства между кандидатом на стандартный образец и референсным образцом экулизумаба. Исследования стабильности при хранении при 2–8°C показали стабильность материала в течение одного года с контрольными точками через 3, 6, 9 и 12 месяцев.

Выводы. Подтверждено отсутствие влияния процесса лиофилизации на первичную и пространственную структуру, посттрансляционные модификации, содержание родственных примесей, состав изоформ и специфическую активность. Кроме того, исследования стабильности показали отсутствие значимых изменений качества белка при хранении при 2–8°C в течение по меньшей мере 12 месяцев, что соответствует всему доступному объему данных на момент подготовки рукописи. Полученные результаты свидетельствуют о том, что разработанный лиофилизированный материал является перспективным кандидатом на роль международного стандартного образца, однако его официальная аттестация потребует проведения дополнительных межлабораторных исследований и получения данных по долгосрочной стабильности.

Ключевые слова

стандартный образец, лиофилизация, моноклональное антитело, экулизумаб

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1. INTRODUCTION

A biosimilar medicinal product is defined as a biological product that has no clinically significant differences in purity, safety or efficacy as compared to the reference medicinal product [1]. The scale of application of biosimilars is increasing every year. The increasing number of biosimilar monoclonal antibodies (mAbs)—both registered and mAbs—currently under development dictates the necessity for primary reference materials. The National Institute for Biological Standards and Control (NIBSC) is the official laboratory in the United Kingdom approved by the World Health Organization (WHO) for performing the control of biological medicines. This institute is the world's largest manufacturer and distributor of international reference materials (supplying more than 95% of WHO standards worldwide). However, due to the current lack

of an international reference material of Eculizumab, we publish for the first time in this paper the conditions of lyophilization technology, the results of the stability study, as well as the methods for validation of the authenticity of the reference material of the Eculizumab monoclonal antibody. Both the aim and the number of the studies involving the use of reference samples (RSs) depend on the type of the sample and the purposes of its use. RSs can be categorized as primary or secondary depending on the certification procedure. In addition, there are RSs of the following categories: international, interstate (regional), state, pharmacopeial, industry RSs, as well as enterprise RSs[1]. Each RS is limited to a specific area of application and cannot be used for other purposes.

Eculizumab is a recombinant humanized IgG2/4k monoclonal antibody in which the CH1 and hinge regions are related to IgG2, while the CH2 and CH3 regions are related to IgG4, and the complementarity determining regions (CDRs) are murine [2]. Having an approximate mass (including glycosylation) of about 148 kDa, Eculizumab contains two light chains of 214 amino acid residues and two heavy chains of 448 amino acid residues with a conservative *N*-glycosylation site at the Asn297 position in both heavy chains. Eculizumab, which inhibits the terminal lytic pathway of complement by blocking of the enzymatic cleavage of complement factor 5 [3–5], is used to treat patients with paroxysmal nocturnal hemoglobinuria (PNH) [6–8], atypical hemolytic uremic syndrome (aHUS) [9–14], refractory generalized myasthenia gravis (gMG) in patients aged 6 years and above [15–17], and neuromyelitis optical spectrum disorder (NMOSD) [18–21]. The wide range of biosimilar drugs of Eculizumab approved for the use in Europe and Russia includes: EPYSQLI™ (*Samsung Bioepis*, South Korea) and Bekemv® (*Amgen*, USA), which was approved in Europe by the European Commission in 2023; Elizaria® (licensed by *GENERIUM* in the Russian Federation in 2019 and in Turkey in 2022); Eculizumab (licensed by *PHARMAPARK* in the Russian Federation in 2024).

Both the Soliris® (the original product developed by *Alexion Pharmaceuticals*, USA) and the various biosimilar products are supplied as the concentrates for solution for infusion. Although liquid forms of Eculizumab demonstrate high stability during the storage at 2–8°C for at least 2.5 years, they have a number of limitations that make them less suitable for the use as international reference materials. The use of liquid forms in international practice is significantly limited by their sensitivity to temperature fluctuations and the requirement of strict cold chain conditions at all stages of transportation and storage. Here, it can be noted that the instructions for medical use of all Eculizumab preparations explicitly prohibit state freezing, which may indicate potential instability of the molecule as a result of freezing and thawing (e.g., aggregation or denaturation). This is a particular problem in the course of developing reference materials due to the requirement of optimal resilience to external influences and consequent suitability for long-term storage. Typically, WHO international standards produced by NIBSC are supplied as lyophilized material in hermetically sealed glass ampoules under nitrogen. The absence of a lyophilized form of the Eculizumab protein to date is likely due to the sensitivity of the molecule to freezing and drying stress. However, our data demonstrate that optimal formulation and correct selection of the process parameters provide effective lyophilization of Eculizumab without loss of structural integrity and biological activity. Thus, the present study describes for the first time the production of a lyophilized form of the Eculizumab monoclonal antibody. In addition, an assessment of the stability of the obtained material for 12 months at a temperature of 2–8°C and 2 months at 25°C was carried out along with a study of its structural, physicochemical, and biological properties.

2. METHODS

2.1. Preparation of the formulation

Two of the most widely used buffer solutions [22] were tested, namely 20 mM phosphate buffer solution, pH 6.0 and 30 mM histidine buffer solution, pH 7.0 in combination with different cryoprotectants, such as sucrose, trehalose, and mannose. After concentrating the samples of Eculizumab (*PHARMAPARK,* Russia) (to a concentration of 12 mg/mL) using the concentrator Vivaspin 20 with polyethersulfone membrane (MWCO[2] 30000 Da; *Sartorius*, Germany), the buffer solution was exchanged using Slider-A-Lyzer dialysis cassettes (MWCO 10000 Da; *Thermo Scientific*, USA) for 24 h. The concentration after the dialysis was measured using UV5 Nano ultraviolet (UV) spectrophotometer (*Mettler Toledo*, Switzerland) at 280 nm. The content of Eculizumab in the samples was normalized to 10 mg/mL and 1 mL of the solution was transferred into 2R vials (*SCHOTT Pharma AG & Co. KGaA*, Germany) for lyophilization.

[1] World Health Organization. WHO good practices for pharmaceutical reference standards. Annex 3, WHO Technical Report Series, No. 943. Geneva: World Health Organization. 2007.

[2] Molecular weight cut-off.

2.2. Freeze-drying process

The samples were placed into a metal rack and pre-frozen for 4 h at the temperature of −30°C. Then they were transferred to a shelf of a laboratory-scale freeze-dryer Christ Beta 1-8K (*Martin Christ*, Germany) at a pre-equilibrated shelf temperature of −30°C. Primary drying was performed at the pressure of 0.10 mbar and a shelf temperature of −30°C for 20 h. Secondary drying was carried out at the pressure of 0.05 mbar with a gradual increase of the shelf temperature from −30 to 0°C for 3 h and from 0 to 25°C for 3 h. Desorption was carried out at the temperature of 25°C at the pressure of 0.05 mbar for 4 h.

2.3. Estimation of residual moisture content

The residual water content was estimated using V20 volumetric titrator (*Mettler Toledo*, Switzerland) using the Karl Fischer method according to the manufacturer's recommendations. The results were calculated as relative water content (w/w).

2.4. Peptide mapping by reversed-phase high-performance liquid chromatography (RP-HPLC)

The test samples were denatured with 6 M guanidine hydrochloride and reduced with dithiothreitol followed by alkylation with iodoacetic acid. Excesses of reagents were removed and samples were re-buffered into ammonium bicarbonate using Vivaspin 500 microcentrifuge concentrators retaining the particles with a MWCO 10000 Da (*Sartorius*, Germany). Trypsin proteolysis was carried out at 37°C for 16 h. Analysis of the hydrolysates was performed using Aeris WIDEPORE XB-C18 chromatography column (250 × 4.6 mm, 3.6 µm, purchased from *Phenomenex*, USA) with Vanquish Flex system (*Thermo Fisher Scientific*, USA). The flow rate was 0.5 mL/min, mobile phase A: HPLC-grade water containing 0.1% formic acid, mobile phase B: acetonitrile containing 0.1% formic acid. Separation was performed in a gradient mode (*t*, min / content of mobile phase A, %): 0/100, 4/100, 60/64, 62/50, 65/0, 66/100. Detection was performed at wavelength of 214 nm.

2.5. Mass spectrometric studies

A Bruker Q-TOF Maxis Impact mass spectrometric detector (*Bruker Corporation*, USA) with electrospray ionization and qTOF Control data management and processing software was used to study the protein structure. To remove *N*-linked glycans, 1 µL of PNGase F solution (*NEB*, USA) was added to 100 µL of 0.5 mg/mL sample and incubated at 37°C for 4 h. The end groups of cysteine were protected by addition of 4 µL of 500 mM iodoacetamide solution to 100 µL of 0.5 mg/mL sample followed by incubation in the dark for 30 min at 25°C. For heavy and light chain analysis, disulfide bonds were reduced by addition of 5 µL of 100 mM dithiothreitol to 100 µL of 0.5 mg/mL sample, followed by incubation for 30 min at 56°C. In order to analyze the full-length protein and the protein following removal of *N*-linked glycans, heavy and light chains, the samples were diluted with 0.1% formic acid to a concentration of 0.1 mg/mL. High-performance liquid chromatography mass spectrometry (HPLC-MS) was performed using an Elute liquid chromatograph (*Bruker Corporation*, USA) on a BIOshell 400A Protein C4 UHPLC[3] chromatographic column (100 × 2.1 mm) at a column temperature of 80°C. The following parameters were applied: mobile phase A: 0.1% formic acid in water, mobile phase B: acetonitrile, mobile phase flow rate: 0.3 mL/mL. Separation was performed in a gradient mode (*t*, min / content of mobile phase A, %): 0/80, 1/80, 2/50, 3/10, 4/80, 7/80. Mass spectrometric detector settings for full-length protein analysis were: desiccant gas flow rate, 8 L/min; gas pressure in the nebulizer, 26.1 psi; conductive capillary temperature, 220°C; capillary voltage, 4500 V. Detection in full ion current scanning mode with ion registration was carried out in the *m*/*z* range from 400 to 5000 21amu with positive ionization.

In order to determine the position of disulfide bonds, enzymatic hydrolysis in the presence of trypsin was performed using the SMART Digest Trypsin Kit (*Thermo Scientific*, USA). Separation of peptide fragments was performed on a Chromolith® Performance RP-18 end-capped chromatographic column (100 × 2.0 mm). The column temperature was 40°C. Mobile phase A: 0.1% formic acid in water, mobile phase B: acetonitrile, mobile phase flow rate 0.3 mL/mL. Separation was performed in a gradient mode (*t*, min / content of mobile phase A, %): 0/95, 1/95, 24/30, 25/10, 27/10, 28/95. Settings for peptide analysis were as follows: desiccant gas flow, 6 L/min; gas pressure in the nebulizer, 29.0 psi; temperature of the conducting capillary, 220°C; voltage on the capillary, 4500 V. Detection in AutoMS mode with registration of precursor ions and fragment ions was carried out in the *m*/*z* range from 150 to 2200 amu with positive ionization.

[3] Ultra-high-performance liquid chromatography.

2.6. Size-exclusion high-performance liquid chromatography (SEC-HPLC)

The analysis was performed using TSKgel G3000SWxl at 300 × 7.8 mm with a 5 µm chromatography column (*Tosoh*, Japan) with the Vanquish Flex system (*Thermo Fisher Scientific*, USA) in isocratic mode. The flow rate was 0.5 mL/min, mobile phase: 20 mM solution of sodium hydrogen phosphate, 150 mM solution of sodium chloride, pH 7.0. Elution time was 35 min. Detection was performed at wavelength of 214 nm.

2.7. Capillary electrophoresis sodium dodecyl sulfate (CE-SDS) in non-reduced form

CE-SDS in non-reduced form was performed using PA 800 Plus Pharmaceutical Analysis System (*SCIEX*, USA). Bare-fused silica capillaries, SDS-MW gel buffer, acidic and basic wash solutions, Tris/SDS sample buffer (pH 9.0) were purchased from *SCIEX* (USA). Separation was performed using a capillary with a total length of 30 cm (effective length 10 cm). Samples were injected from the short end of the capillary for 20 s at 5 kV (normal polarity). Separation was performed at 15 kV (normal polarity) for 25 min. Detection was performed using a UV detector at 214 nm. The capillary temperature was 25°C.

2.8. Capillary isoelectric focusing (cIEF)

cIEF was performed using PA 800 Plus Pharmaceutical Analysis System (*SCIEX*, USA). Pharmalyte 5–8 carrier ampholytes were purchased from *Cytiva* (USA); neutral capillary, pI marker set and ready-made gel for cIEF were obtained from *SCIEX* (USA). Phosphoric acid, sodium hydroxide, iminodiacetic acid, acetic acid, L-arginine, and urea for electrophoresis were purchased from *Sigma-Aldrich* (USA). Separation was performed using the capillary with a total length of 30 cm (effective length 20 cm). 200 mM phosphoric acid solution was used as anolyte, 300 mM sodium hydroxide solution was used as catholyte, 350 mM acetic acid solution was used as chemical mobilizer. The sample focusing stage was carried out at 25 kV for 13 min, while chemical mobilization was carried out at 30 kV for 25 min. Detection was performed using UV detector at 280 nm. The capillary temperature was 20°C.

2.9. Cation-exchange high-performance liquid chromatography (CEX-HPLC)

Analysis of Eculizumab charged forms distribution was performed using a YMC BioPro SP-F chromatography column (100 × 4.6 mm, 5 µm, purchased from *YMC*, Japan) with the Vanquish Flex system (*Thermo Fisher Scientific*, USA). At a flow rate of 0.5 mL/min, the following parameters were applied; mobile phase A: 20 mM MES, 0.02% isopropanol, pH 5.7; mobile phase B: 20 mM solution of sodium hydrogen phosphate, 100 mM solution of sodium tetraborate decahydrate, 0.05% isopropanol, pH 9.0. Separation was performed in a gradient mode (*t*, min / content of mobile phase A, %): 0/100, 5/85, 35/50, 40/50, 41/0, 43/0, 50/100. Detection was performed at wavelength of 280 nm.

2.10. C5-binding specific activity by ELISA (C5-ELISA)

In order to analyze the specific activity of Eculizumab, an enzyme-linked immunosorbent assay (ELISA) was used. Eculizumab specifically forms a complex of C5-Eculizumab, for the detection of which secondary antibodies to the Fc fragment of human IgG4 are subsequently introduced. Detection of the formed complex C5-Eculizumab–IgG4 HRP was performed by introducing a ready-made solution of 3,3',5,5'-tetramethylbenzidine containing hydrogen peroxide (*Sigma-Aldrich*, USA). The reaction was stopped with 0.5 M sulfuric acid. Data were read using an Infiniti M200 Pro plate spectrophotometer (*Tecan Austria GmbH*, Austria) at a main wavelength of 450 nm and a reference wavelength of 650 nm. The dependence of Eculizumab concentration on the obtained optical density was approximated using a 4-parameter logistic curve in GraphPad Prism 6.0 software (*GraphPad Software*, *Inc*., San Diego, CA, USA). A detailed description of the method was previously published along with the validation results in [23].

3. RESULTS AND DISCUSSION

Lyophilization is widely used to increase the stability and shelf life of biopharmaceutical products. In the freeze-drying process, a protein solution is successively subjected to freezing, primary drying (mainly to remove ice by sublimation) and secondary drying (to remove the retained water) [24, 25]. It is evident that protein molecules undergo significant environmental effects during both the freezing and the drying processes. Typically, even minor changes in the compositional characteristics of a drug solution, such as pH, buffer capacity, and drug or excipient concentration, lead to striking differences in the behavior of molecules during the freezing and freeze-drying.

The main object of the study was Eculizumab, a recombinant humanized monoclonal antibody of IgG2/4k subclass that specifically binds the

Table 1. Characteristics of the studied formulations

Abbreviations	PhT	PhS	HisT	HisS	PhSN	PhSM
Buffer solution	20 mM NaPh	20 mM NaPh	30 mM His	30 mM His	20 mM NaPh	20 mM NaPh
Trehalose	+	–	+	–	–	–
Sucrose	–	+	–	+	+	+
Mannose	–	–	–	–	–	+
NaCl, 0.9% w/v	–	–	–	–	+	–
Polysorbate 80, % w/v	0.2					
pH	7.0					
Eculizumab, mg	10.0					

Note: PhT—phosphate buffer with trehalose; PhS—phosphate buffer with sucrose; HisT—histidine buffer with trehalose; HisS—histidine buffer with sucrose; PhSN—phosphate buffer with sucrose and NaCl; NaPh—phosphate buffer; PhSM—phosphate buffer with a mixture of sucrose and mannose; His—histidine.

protein C5 of the complement to inhibit its cleavage. The initial preparation of Eculizumab produced by *PHARMAPARK* (Russia) as part of the development of a biosimilar drug (hereinafter referred to as Eculizumab Lyo) was subsequently lyophilized. The stock solution (bulk drug substance) at a concentration of 12 mg/mL and pH 7.0 contained 20 mM sodium phosphate and 0.2% (w/v) polysorbate 80. Prior to lyophilization, the sample was dialyzed to change the buffer solution and brought to a concentration of 10 mg/mL. The resulting solution was poured into 2R vials and lyophilized. All physicochemical and biological studies within the framework of the work were carried out using the samples obtained from the same batch. This sample is not certified as an RS, but is considered in this study as a preparation potentially suitable for the use as a national or international RS after passing of the appropriate qualification procedures.

The preparation was supplied as a concentrate for obtaining an infusion solution (300 mg/30 mL) and stored according to the manufacturer's instructions. It is should be noted that, unlike classical studies on the biosimilarity of drugs, for example [26], the main emphasis was placed in this study on the development of a lyophilized form of Eculizumab for possible use as a national or international standard sample.

To assess the structural and physicochemical comparability, the original drug Soliris® (Eculizumab, *Alexion Pharmaceuticals*, approved for use in the European Union (EU), hereinafter referred to as Eculizumab EU) was used. The substance was supplied as a concentrate for the preparation of an infusion solution (300 mg/30 mL) and stored in accordance with the manufacturer's instructions.

3.1. Determination of the optimal composition of the formulation buffer solution

In order to select the composition of the formulation buffer solution for lyophilization, we applied two widely used buffer systems based on phosphate and histidine. Selection of the appropriate composition was carried out at pH value of 7.0. This value was chosen taking into account our previous study of the stability of Eculizumab protein under various stress conditions [26], as well as the results of a study of the long-term stability of the protein in solution carried out as part of the development of the drug Eculizumab (licensed by *PHARMAPARK* in the Russian Federation in 2024). Trehalose and sucrose or mixture of sucrose and mannose were used as cryoprotectants. Mannose was included in one of the formulations during the screening phase due to its use for stabilizing of glycoproteins as it was described earlier [27–29]. Furthermore, an effect of sodium chloride adding to the formulation buffer solution was studied. As reported in [27], the addition of sodium chloride ensured a decrease in the residual water content and the formation of an optimal cake shape. The formulations shown in Table 1 were considered in the primary screening stage, whose purpose was to quickly identify the acceptable options in terms of moisture content and physical characteristics.

In the first experiment, combinations of phosphate (Ph) and histidine (His) buffer solutions with trehalose (T), sucrose (S), and a mixture of sucrose and mannose (SM) without the addition of sodium chloride were compared. Cryoprotectant/protein ratio was 5 : 1 in all cases. With the exception of the formulation with the addition of sucrose

Table 2. Compositions of the studied variants of the formulation buffer solution in the experiment with variation of the cryoprotectant content

Abbreviations	T2	T3	T4	S2	S3	S4	T4N	S4N
Buffer solution	20 mM NaPh							
Trehalose, % w/v	2	3	4	–	–	–	4	–
Sucrose, % w/v	–	–	–	2	3	4	–	4
NaCl, 0.9% w/v	–	–	–	–	–	–	+	+
Polysorbate 80, % w/v	0.2							
pH	7.0							
Eculizumab, mg	10.0							

Note: T2—phosphate buffer with 2% trehalose; T3—phosphate buffer with 3% trehalose; T4—phosphate buffer with 4% trehalose; S2—phosphate buffer with 2% sucrose; S3—phosphate buffer with 3% sucrose; S4—phosphate buffer with 4% sucrose; T4N—phosphate buffer with 4% trehalose and NaCl; S4N—phosphate buffer with 4% sucrose and NaCl.

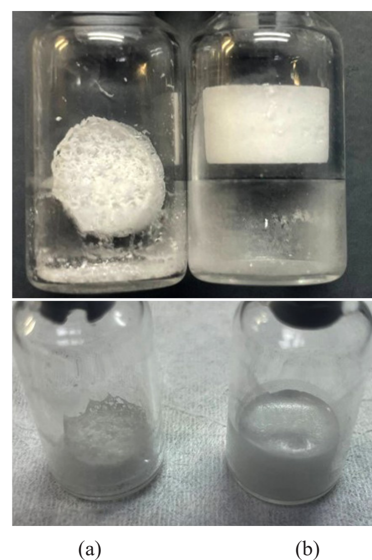
and mannose (PhSM), where the value was 3.7%, the residual water content in the samples after freeze-drying varied from 1 to 3%. Due to the high residual water content, this formulation was excluded from the further studies. Changes in the protein activity and increase in the number of aggregates were not detected in all of the studied formulations. According to WHO recommendations[4], satisfactory long-term stability is demonstrated by the preparations with a residual water content of less than 1% w/v, although higher values are acceptable in some cases. In a number of studies, the authors predictably pointed out the detrimental influence of low moisture content (less than 1% residual water) on the protein stability [28].

The next experiment was carried out to optimize the cryoprotectant/protein ratio and to evaluate the effect of addition of sodium chloride to the formulation. The content of cryoprotectant was varied only in the phosphate buffer solution. The use of this buffer solution was determined by the absence of significant differences between the used buffer systems in the first experiment, the relatively low cost, as well as the obtained data relating to the stability of Eculizumab during the development of the Eculizumab drug (licensed by *PHARMAPARK* in the Russian Federation in 2024). The compositions of the studied variants of the formulation buffer solution in the experiment with variation of the cryoprotectant content are given in Table 2.

The obtained (with varying cryoprotectant content) lyophilizates were evaluated for cake appearance, residual water content, pH shift, and changes in the protein purity and activity (using SEC-HPLC and C5-ELISA).

The samples containing sucrose demonstrated higher levels of residual water (2.0–2.7%) in comparison with the trehalose-containing formulation (1.0–1.5%). The addition of 0.9% w/v sodium chloride resulted in a number of cake defects as compared to other formulations. No pH shift or changes in the protein purity or activity were detected.

A scaled-up series of the samples of Eculizumab Lyo was prepared using the formulation buffer containing 20 mM sodium phosphate, 4% trehalose, 0.2% polysorbate 80, pH 7.0 (Fig. 1).



(a) (b)

Fig. 1. An appearance of the cakes after drying of the samples. (a) 4% trehalose with NaCl; (b) 4% trehalose without NaCl

[4] World Health Organization, Recommendations for the preparation, characterization and establishment of international and other biological reference standards (Revised 2004), WHO Tech. Rep. Ser., no. 932, Geneva: WHO, 2006.

The scaling up process presupposed use of the maximal number of available vials in the laboratory's equipment. Thus, to evaluate the stability and impact of the lyophilization process on the physicochemical and biological properties of Eculizumab, more than 400 vials filled with the same bulk solution were prepared. Analysis of the resulting samples confirmed the absence of pH shift, as well as the retention of the protein purity and activity. To analyze the residual water content, the samples were taken into vials from both the central part and the edges of the lyophilizer shelf. This approach ensured the data representativeness insofar as the distribution of temperature and heat transfer might differ between the center and the periphery of the shelf. The residual water content in the selected samples did not exceed 1.1 ± 0.2% (± standard deviation (SD), $n = 5$). Although the scalability of the process was limited in our case by the capabilities of the laboratory freeze drying plant, the used process can be directly transferred to a pilot plant providing the required drying parameters.

3.2. Study of physicochemical and biological properties

The study of the effect of lyophilization on the physicochemical and biological properties of Eculizumab Lyo was performed using various complementary analytical methods. The corresponding methods and the results of analysis of the structural, physicochemical, and biological properties are as presented in Table 3.

The intact protein mass before and after *N*-glycan removal, as well as the mass of the light and heavy chains of the antibody after disulfide bond reduction, were estimated using reversed-phase liquid chromatography with mass spectrometric detection. The average isotopic molecular mass of the main glycoform (G0F) of the studied protein was shown to be 147875 Da. The intact and subunit molecular mass, amino acid sequence, disulfide bonds positions, and glycosylation site corresponded to theoretical expectations.

Table 3. Methods used to assess the structure, physicochemical and biological properties of Eculizumab Lyo

Characteristic	Parameter and research method	Results
Primary structure	Intact molecular weight (HPLC-MS)*	G0F/G0F: 147875 ± 1 Da G0F/G1F: 148037 ± 1 Da G1F/G1F: 148199 ± 1 Da
	Intact molecular weight of deglycosylated molecule (HPLC-MS)*	144987 ± 1 Da
	Molecular weight of the heavy chain (HPLC-MS)*	G0F: 50817 ± 1 Da G1F: 50979 ± 1 Da
	Molecular weight of the light chain (HPLC-MS)*	23130 ± 1 Da
	Free thiols content (HPLC-MS)	0.2%
	Glycosylation site (HPLC-MS/MS)	Asn298
	Isoelectric point (cIEF)	Main form: 6.1
Product-related substances and impurities	Size variants (SEC-HPLC)	HMW: 0.3% Monomer: 99.6% LMW: 0.1%
	Non-reduced CE-SDS	Monomer: 98.5%
	Charge variants (CEX-HPLC)	Acidic form: 12.2% Main form: 82.7% Basic form: 5.1%
Biological activities	C5-component binding by ELISA	1053321 U/mg

*Data were expressed as mean ± SD, $n = 3$. HMW—high molecular weight, LMW—low molecular weight.

Peptide mapping of the protein after deglycosylation was performed by RP-HPLC with UV detection (Fig. 2). The query cover of amino acid sequence (obtained using mass spectrometric identification of peptides) was 100%. The chromatographic profile of the studied sample completely coincided with the RSs. The content of oxidized forms in Eculizumab Lyo, which was comparable to that of the reference product (Eculizumab EU), did not exceed 2% in either sample. Oxidation is presented as the most illustrative and representative post-translational modification (PTM), while the full range of PTMs was assessed during the studies.

The monomer of Eculizumab (recombinant IgG2/4k antibody) consists of two heavy and two light chains linked by disulfide bonds. As such, protein fragmentation and aggregation may occur during the production and storage. CE-SDS is typically used to assess the protein fragmentation, while size exclusion chromatography is used to determine the relative content of various aggregated forms. The profiles of high- and low-molecular impurities were obtained as a result of analysis of the sample after freeze-drying using such methods as SEC-HPLC and CE-SDS in non-reduced form in comparison with the RSs (these are shown in Figs. 3 and 4). The results demonstrated a high degree of similarity between the Eculizumab Lyo and Eculizumab EU, not only in the chromatographic profiles of the peptide map, but also in the relative content of aggregates and fragments. No statistically significant differences were observed between the two materials, thus confirming the compossibility of the candidate material with the reference product.

Charge heterogeneity is a consequence of post-translational modifications of a protein. As a rule, monoclonal antibodies have three groups of isoforms: the main isoform, as well as acidic and alkaline forms. Acidic isoforms have a different nature; the most common mechanism of their formation is deamidation of asparagine or glutamine residues, oxidation, and sialylation. Alkaline forms are mainly formed due to incomplete hydrolysis of *C*-terminal lysine residues by the action of carboxypeptidases. During the biosynthesis of heavy chains of antibodies, each chain has one lysine residue at the end, some of which are subsequently removed by the action of carboxypeptidases during the secretion and incubation in culture fluid. As a result, the purified antibody contains three forms having 0, 1, and 2 lysine residues [29]. In our studies, we used a cation exchange chromatography (CEX-HPLC) method to assess the charge heterogeneity in the sample. The obtained data confirmed the comparability of the profile of charged forms between Eculizumab Lyo and Eculizumab EU.

A comprehensive study of Eculizumab Lyo was carried out using complementary analytical methods (HPLC-MS, cIEF, SEC-HPLC, CE-SDS, CEX-HPLC, C5-ELISA, and RP-HPLC with UV detection). Figures 3 and 4 represent the quality attributes that are most relevant for assessing the lyophilization process and formulation, while the results from additional complementary methods are summarized in Table 3. The study demonstrated a high degree of comparability between Eculizumab Lyo and the reference product (Eculizumab EU). No statistically significant differences were observed in high molecular weight impurities, fragments, charge variant profiles, or specific activity. These results confirm that the lyophilization process does not affect the physicochemical or functional properties of Eculizumab Lyo.

3.3. Study of stability

The stability of medicinal products depends on many factors, such as storage temperature, illumination, composition of the surrounding atmosphere, technology of manufacturing of the dosage form, presence of

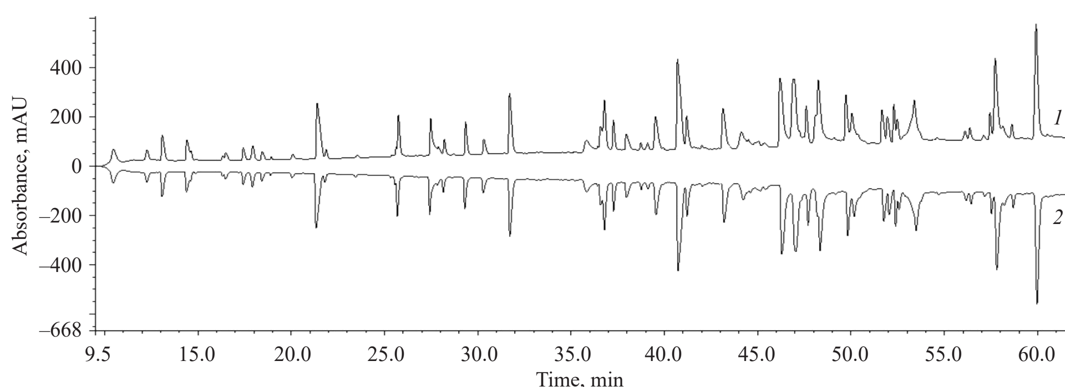


Fig. 2. Peptide mapping of Eculizumab samples. (*1*) Scaled up series of Eculizumab Lyo, (*2*) Eculizumab EU

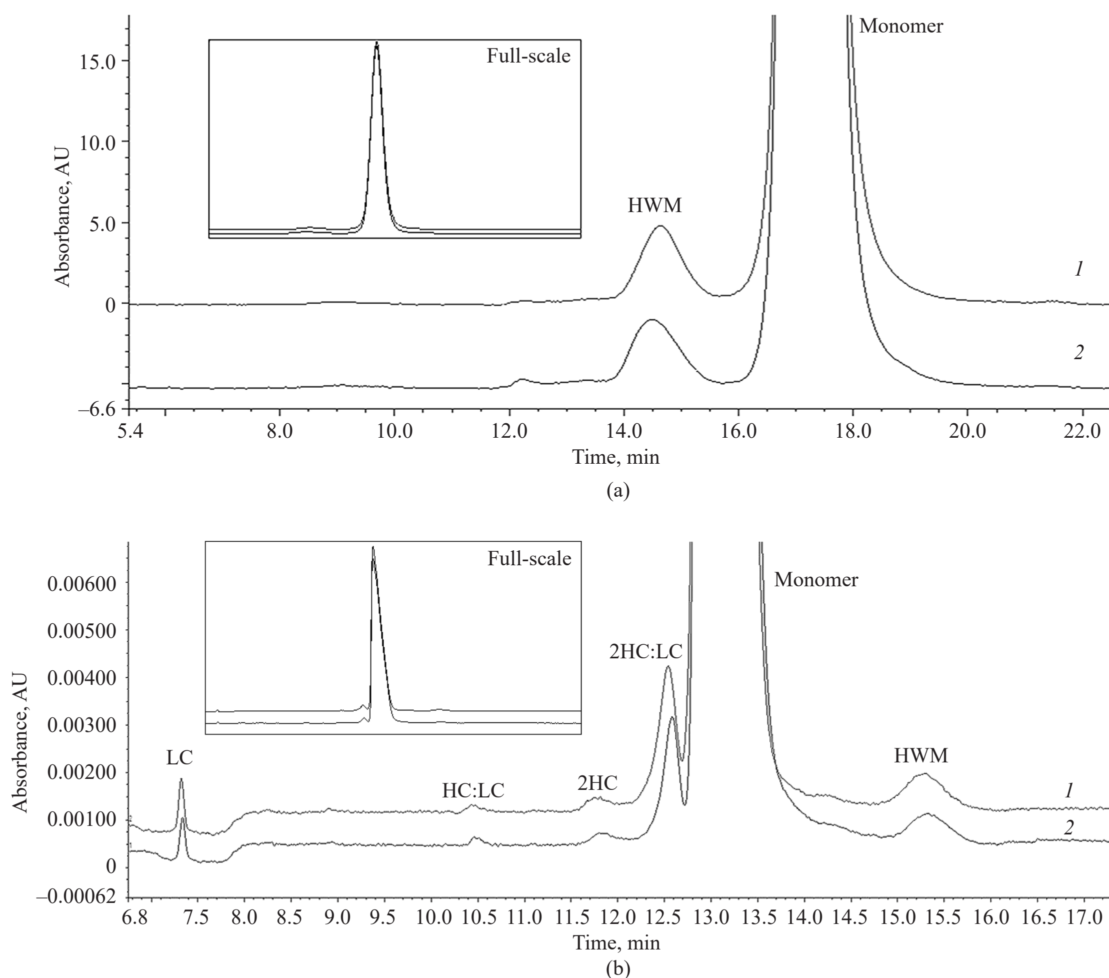


Fig. 3. Results of the study of Eculizumab using SEC-HPLC (a) and CE-SDS (b) in non-reduced form methods. (*1*) Scaled up series of Eculizumab Lyo, (*2*) Eculizumab EU. HC—heavy chain, LC—light chain, HMW—high molecular weight

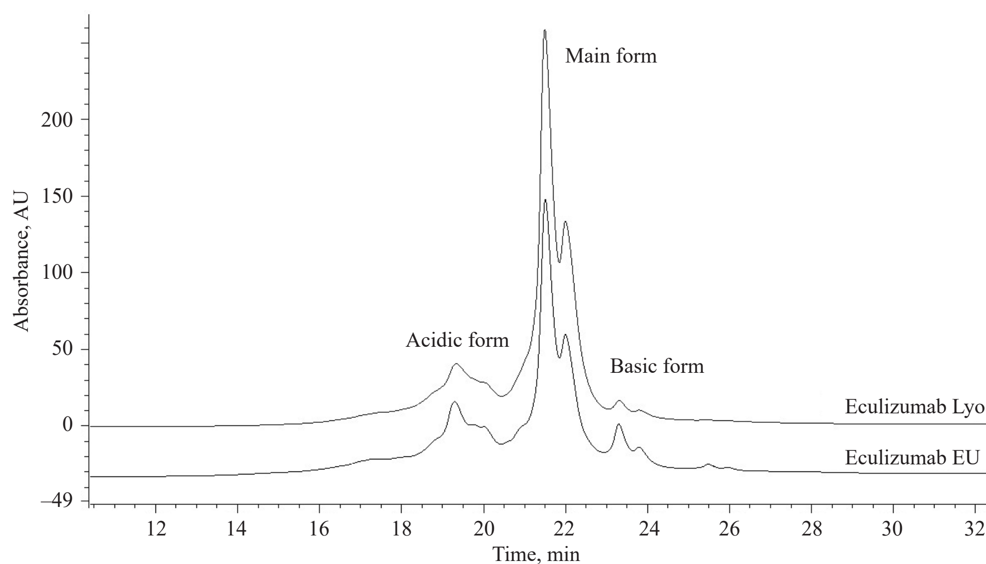


Fig. 4. Chromatograms of Eculizumab Lyo and Eculizumab EU (the last one is given for comparison) obtained by the CEX-HPLC method

excipients, type of dosage form, and especially their aggregate state, nature of material and method of packaging, etc. Classical programs for studying of the stability of biological medicinal products envisage long-term-, accelerated-, and stress tests, including assessment of photostability. Thus, the stability of a drug is a characteristic of a biologically active substance, which describes the degree of preserving of physicochemical properties and pharmacological activity during the established shelf life.

When determining the storage temperature, we wanted to avoid the necessity of the use of low and ultra-low temperatures (from −18 to −30 or −80°C), as this significantly complicates the transportation of the RS. Both the original Soliris® preparation and biosimilar drugs are supplied as a concentrate for solution for infusion at a storage temperature of 2–8°C. Based on the instructions for Eculizumab preparations, which were approved for the use in Europe and Russia, the storage temperature of 5 ± 3°C was identical for all manufacturers. The data regarding obtained by us regarding the stability of Eculizumab during the development of the Eculizumab drug (licensed by *PHARMAPARK* in the Russian Federation in 2024) confirm the absence of significant changes in protein quality over 3 years of storage. In view of this, additional stability studies at low and ultra-low temperatures were not performed.

Long-term stability of the scaled-up series was studied at the temperature of 2–8°C, protected from light, with checkpoints every 3 months during the first year and every 6 months thereafter. At the time of submission of the publication, the study had been carried out at four checkpoints: 3, 6, 9, and 12 months. Accelerated stability was studied at a temperature of 25 ± 2°C and humidity of 65 ± 5%, quality control of parameters was performed at checkpoints of 3 and 6 months. Stress stability tests were not performed due to the well-described degradation profile of Eculizumab in the scientific literature [26]. The results of stability assessment are presented in Table 4.

The data on stability at 25°C confirm that the sample retains its physicochemical properties and activity for at least 6 months. It suggests that short-term transportation and storage outside the cold chain is acceptable, for example, when moving between laboratories or under conditions of temporary lack of temperature control. The observed slight increase in the proportion of acidic isoforms (about 1%) is consistent with the expected degradation processes [26, 30] and is not accompanied by a decrease in biological activity. At the same time, when the preparations are stored at 2–8°C, all the monitored parameters remained stable for 12 months, confirming the suitability of this form for long-term storage under standard refrigeration conditions. Despite the advantages of lyophilization for long-term storage, the process carries inherent risks, such as protein denaturation during freezing and the potential possibility of oxidation or aggregation upon removal of the hydration shell. Although our data (e.g., SEC-HPLC, ELISA) confirm the structural and functional integrity of the protein after lyophilization and throughout the studied period, continuous monitoring of these quality attributes is essential to mitigate potential long-term risks associated with the mentioned degradation pathways.

CONCLUSIONS

The development of biosimilar medical preparations based on monoclonal antibodies represents a complex multi-stage process. The critical parameters of quality, such as primary and spatial structure, post-translational

Table 4. Results of the stability study

Quality parameter		Zero point	Long-term stability, months				Accelerated stability, months	
			3	6	9	12	3	6
Residual moisture, %		1.1	1.2	1.2	1.2	1.2	1.2	1.2
SEC-HPLC (Eculizumab monomer), %		99.5	99.5	99.5	99.5	99.5	99.2	98.9
Non-reduced CE-SDS (Eculizumab monomer), %		98.5	98.5	98.4	98.4	98.4	98.1	97.8
CEX-HPLC	Acidic form, %	12.3	12.3	12.4	12.5	12.5	12.6	13.5
	Main form, %	82.6	82.6	82.5	82.4	82.4	82.4	81.5
	Basic form, %	5.1	5.1	5.1	5.1	5.1	5.1	5.0
C5-ELISA, U/mg		1 052 213	1 046 524	1 053 314	1 052 781	1 053 256	1 042 249	1 041 994

modifications, product isoforms, and biological activity, must be studied in detail at each step of this process. When considering several products supplied by different manufacturers, there is always a risk of discrepancies in the results of determination of quality indicators between the biosimilar drugs and following their comparison with the reference preparation. The availability of an international reference material permits independent assessments of the quality of biosimilar preparations worldwide. The use of certified international reference materials thus facilitates the introduction of uniform quality standards for the production and registration of biosimilar preparations.

Currently, although a wide range of biosimilar Eculizumab drugs are approved for use worldwide, no international standard sample of Eculizumab has yet been released. In our study we obtained a lyophilized form of the monoclonal antibody Eculizumab, which was characterized by the following formulation buffer solution composition: 20 mM sodium phosphate, 4% trehalose, 0.2% polysorbate 80, pH 7.0. The absence of any effect of the drying process on the primary and spatial structure, post-translational modifications, content of related impurities, product isoforms and biological activity was rigorously demonstrated. Furthermore, stability studies demonstrated no significant changes in the critical quality attributes of the protein during storage at 2–8°C for the entire available data period of 12 months at the time of manuscript preparation. The confirmed stability provides a strong foundation for near-term application of the material. To substantiate the shelf-life required for an international reference material, continuous monitoring of long-term stability (up to 36 months) is currently in progress. Collectively, these results indicate that the developed lyophilized material is a viable candidate for an international reference material; however, its official qualification depends on the outcomes of the ongoing stability program and future collaborative trials.

Authors' contributions

D.I. Zybin—writing: original draft.

A.A. Klishin—visualization.

N.V. Orlova—conceptualization.

T.S. Sorokina—writing: review and editing.

D.V. Kapustin—writing: review and editing.

Conflict of interest

The authors work for *PHARMAPARK*. However, when writing this paper, the authors were guided by considerations of the scientific value of the material obtained; the authors declare their impartiality in its assessment.

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