ANALYTICAL METHODS IN CHEMISTRY AND CHEMICAL TECHNOLOGY

АНАЛИТИЧЕСКИЕ МЕТОДЫ В ХИМИИ И ХИМИЧЕСКОЙ ТЕХНОЛОГИИ

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RESEARCH ARTICLE

Comparing the original and biosimilar biotherapeutics of the monoclonal antibody eculizumab by intact mass measurement and middle-up mass spectrometry analysis

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Abstract

Objectives. *In this biosimilar research, we compare the monoclonal antibody eculizumab obtained from different drugs [original Soliris® (Alexion Pharmaceuticals) and candidate Elizaria® (Generium)] by intact mass measurement and middle-up mass spectrometry analysis to enhance the role of mass spectrometry methods in biopharmaceutical development processes.*

Methods. *The intact mass measurement is performed using a high-resolution ESI-MS. The middle-up analysis is performed by reversed-phase high-performance liquid chromatography with ESI-MS detection, subsequent IdeS treatment of antibodies, and disulfide bond reduction.*

Results. *We have shown some small differences between the original and candidate drugs in the minor glycans level. Man5 glycan is only found in the original Soliris, and G0 is only found in the Elizaria. Glycation sites are also found in the light chain and Fd subunits of the original Soliris. The glycation level does not exceed 4.4%. The non-clipped C-end lysine level and G0F glycan levels are slightly lower in the original Soliris. All registered differences are not crucial for eculizumab's quality and do not affect its effectiveness and preclinical safety. Generally, the results show a high level of similarity between the original and candidate drugs.*

Conclusions. *The comparative mass spectrometry analysis of eculizumab in the original Soliris and Elizaria allows us to estimate their high degree of similarity by molecular masses and major modification profiles.*

Keywords: *Eculizumab, mass spectrometry, posttranslational modifications, biosimilar drugs, glycosylation, high-performance liquid chromatography-mass spectrometry, intact mass measurement, middle-up analysis, monoclonal antibody*

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НАУЧНАЯ СТАТЬЯ

Сравнение оригинального и биоаналогичного препаратов моноклонального антитела экулизумаб методами масс-спектрометрии интактного белка и «с середины вверх»

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Аннотация

Цели. *В целях исследования биоаналогичности, а также внедрения масс-спектрометрии в процессы биофармацевтической разработки было необходимо провести сравнительное масс-спектрометрическое исследование моноклонального антитела экулизумаб из оригинального лекарственного препарата «Солирис»® и кандидатного «Элизария»® производства АО «ГЕНЕРИУМ» методами измерения молекулярных масс интактных молекул (intact mass measurement) и их субъединиц (middle-up).*
Методы. *Измерение масс интактных белков и оценку долей их модификаций проводили при помощи инфузионной масс-спектрометрии высокого разрешения с электрораспылительной ионизацией. Измерение молекулярных масс субъединиц и оценку долей их модификаций проводили методом обращенно-фазовой ВЭЖХ, совмещенной с масс-спектрометрией высокого разрешения с электрораспылительной ионизацией после предварительного расщепления антител ферментом IdeS и разрыва дисульфидных связей.*
Результаты. *Нами был зарегистрирован ряд небольших отличий в содержании некоторых минорных гликанов: олигосахарид Man5 был обнаружен только в белке оригинального препарата, а G0 – только в кандидатном белке; в субъединицах LC и Fd оригинального белка были зарегистрированы сайты гликирования с содержанием данной модификации не выше 4.4%. Также доли неотщепленного С-концевого лизина и гликана G0F в оригинальном белке были несколько ниже, чем в кандидатном. Однако зарегистрированные отличия не являлись критическими параметрами качества экулизумаба и не влияли на активность молекул и их безопасность в доклинических испытаниях, и, в целом, сравниваемые молекулы продемонстрировали высокое сходство.*
Выводы. *Сравнительный хромато-масс-спектрометрический анализ экулизумаба из оригинального и кандидатного препаратов позволил установить высокую степень сопоставимости сравниваемых молекул по молекулярным массам и по профилям мажорных модификаций.*

Ключевые слова: *экулизумаб, масс-спектрометрия, посттрансляционные модификации, биоаналогичность, гликозилирование, ВЭЖХ-МС, intact mass measurement, middle-up*

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INTRODUCTION

One of the most promising areas in the pharmaceutical industry is biopharmaceuticals (e.g., therapeutic proteins). Therapeutic proteins exhibit much higher specificity and activity than chemically synthesized low-molecular-weight drugs. Since their development, they are found effective in treating many acute diseases previously inaccessible to therapy, increasing the patients' duration and quality of life. For the last five years, the US Food and Drug Administration[1] (FDA) has approved 213 drugs, of which 44 are monoclonal antibodies.[2]

One of the main driving forces for the development of biopharmaceuticals is the development of biosimilar drugs due to several reasons. The biosimilar drug's cost is typically lower than the original drug's cost; hence its release is attractive for pharmaceutical companies. Stringent requirements for the comprehensive characterization of a promising biosimilar molecule developed by leading regulatory bodies such as the FDA and the European Medicines Evaluation Agency (EMEA) minimize the risk of an ineffective or hazardous product entering the market. They also stimulate research by developing new analysis methods and deepening understanding of the structures and functions of therapeutic proteins, their targets, and the pathogenesis of various diseases.

Monoclonal antibodies account for most developed biosimilar drugs. Monoclonal antibodies are successfully used in treating serious diseases, including hereditary diseases. The potential of some monoclonal antibodies to expand indications for therapeutic use is also a stimulus for the development of biosimilar drugs. A striking example is eculizumab. It is a recombinant humanized monoclonal antibody (IgG2/4k) used for treating paroxysmal nocturnal hemoglobinuria, atypical hemolytic uremic syndrome, myasthenia gravis, and optic neuromyelitis. The development of biosimilar eculizumab requires a comprehensive, in-depth study of its physicochemical properties. Currently, an important method for studying proteins has become the method of mass spectrometry (MS). Hence, it is possible to determine the molecular mass of an intact molecule (by intact mass measurement) and its subunits (by middle-up analysis).

Intact mass measurement of a protein molecule for the comparison of biological products makes it possible to establish its authenticity by the correspondence of the measured value to the expected one, measure the content of some modified forms, and evaluate the purity of the drug by the number of proteoforms found with a polypeptide chain cleavage [1–3]. Suppose the measurement is carried out in the mode of native MS. In that case, the list of characteristics to be determined can also be supplemented with information on the molecule's spatial organization, on the content of proteins with different conformations and their aggregates in the sample [4, 5]. Intact mass measurement involves preliminary purification and fractionation of the protein before it is enter to the ion source of the mass spectrometer using offline (ultrafiltration, dialysis, and size exclusion purification) or online (high-performance liquid chromatography (HPLC) and capillary electrophoresis) methods followed by collecting mass spectra for a sample or its fractions, their mathematical processing (deconvolution), and interpreting obtained data [6, 7]. The method's main advantages are achieving high-speed research and obtaining a picture of the structure and shape of whole protein molecules [3, 4].

However, the high complexity and heterogeneity of therapeutic proteins limit intact molecule applicability for in-depth comparability studies. The method does not yet allow identifying minor modifications and establishing their positions in the molecule, interpreting and annotating the spectra of proteins with ultrahigh heterogeneity [8]. A significant part of these problems is solved by middle-up analysis, slightly reducing the analysis performance and minimizing the risk of losing data about the molecule under study. Its essence lies in the limited chemical or enzymatic cleavage of the protein molecule under the study of obtaining large fragments. Then, these fragments are separated using HPLC or capillary electrophoresis and entered to the mass spectrometer. The obtained mass spectra are interpreted, taking into account theoretical data on the molecule's structure, allowing a deeper characterization of the profile of modifications, degradant forms, and interpreting the results of an analysis of proteins characterized by high heterogeneity of forms [9].

[1] Guidance, Compliance & Regulatory Information (Biologics). https://www.fda.gov/vaccines-blood-biologics/guidance-compliance-regulatory-information-biologics (accessed 21.10.2020).

[2] Global Therapeutic Proteins Market Report 2020: Market was Valued at $93.14 Billion in 2018 and is Expected to Grow to $172.87 Billion through 2022. https://www.businesswire.com/news/home/20191223005228/en/Global-Therapeutic-Proteins-Market-Report-2020-Market-was-Valued-at-93.14-Billion-in-2018-and-is-Expected-to-Grow-to-172.87-Billion-through-2022---ResearchAndMarkets.com (accessed 21.10.2020).

[3] Biological guidelines. https://www.ema.europa.eu/en/human-regulatory/research-development/scientific-guidelines/biological-guidelines (accessed 21.10.2020).

[4] ASSESSMENT REPORT FOR Soliris. International Nonproprietary Name: ECULIZUMAB. Procedure No. EMEA/H/C/000791/II/0050. European Medicines Agency, Committee for Medicinal Products for Human Use (CHMP). March 21, 2013. EMA/CHMP/126714/2013

In this study, we evaluate the comparability of eculizumab from different sources by infusion MS after purification of the protein by size-exclusion filtration. To perform middle-up analyses, we have ensured that the antibody is cleaved in the hinge region with the IdeS protease before analysis. Then, the fragments' disulfide bridges are disrupted with TCEP-HCl. Before entering the mass spectrometer, the resulting antibody subunits (light chain (LC), Fc/2, and Fd: light chain and monoclonal antibody fragments located in the region from the hinge region to the C-terminus of the heavy chain and from the N-terminus of the heavy chain to the hinge region, respectively) are separated and purified from impurities using reverse-phase HPLC.

The results of mass spectrometric comparison of eculizumab from the original and candidate drugs show their similarity in the main quality parameters: the molecular masses of the main protein proteoforms coincide with each other and with the expected values; the glycosylation profiles of the compared samples turned out to be close with each other, as well as the content of some modified variants: oxidized forms of the Fc/2 subunit and forms with uncleaved C-terminal lysine. Significant differences are recorded in the content of high-mannose and fucosylated glycans and glycated variants of LC and Fd subunits: Man5 glycan and glycated variants are found in the eculizumab from the original drug, and G0 (fucosylated) glycan is found in Elizaria® drug. The content of these proteoforms does not exceed 4.4%. All reported differences are expected because of using different host cell lines. The differences are not significant in the effectiveness and safety of eculizumab. The data obtained have become the basis for further, deeper comparison of molecules by other methods.

MATERIALS AND METHODS

The candidate Elizaria drug was produced from *Generium JSC* (Moscow, Russia); the original drug was purchased as part of the Soliris® drug (*Alexion Pharmaceuticals*, Zürich, Switzerland). Deionized water was obtained using a MilliQ IQ 7000 system (*Merck*, Darmstadt, Germany). Acetonitrile (LC-MS grade) was also purchased from *Merck*. We use the IdeS protease manufactured by *Promega* (Madison, WI, USA). HPLC column BioResolve RP mAb Polyphenyl and difluoroacetic acid (DFA, LC-MS grade) were purchased from *Waters* (Finglas, Dublin, Ireland). Zeba Spin Desalting Columns (7K MWCO, 0.5 mL) and tris-(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl) were purchased from *Thermo Scientific* (Dreieich, Germany). The rest of the reagents and materials (Na_2HPO_4, NaH_2PO_4, NaCl, guanidine hydrochloride, ethylenediaminetetraacetic acid (EDTA), and trisaminomethane hydrochloride (Tris-HCl)) were purchased from *Sigma-Aldrich* (St. Louis, MO, USA). All studies were performed using a Nexera X2 HPLC system (*Shimadzu Co.*, Tokyo, Japan) connected to a 6550 QTOF mass spectrometer (*Agilent Technologies*, Santa Clara, CA, USA).

EXPERIMENTAL

Sample preparation for intact mass measurement

In this experiment, we use three series of original and candidate drugs. The antibody concentration in the solution is adjusted to 1.0 mg/mL. The buffer is replaced with a 0.15% solution of difluoroacetic acid by size exclusion filtration through Zeba Spin columns according to the instructions.

Sample preparation for middle-up analysis

The antibody concentration in the solution is adjusted to 2.0 mg/mL by dilution with 50-mM phosphate buffer with 150-mM NaCl (pH 6.6). Then, to 25-μL sample solutions, 2-μL IdeS solution (1 U/μL) is added and incubated at 37°C for 1.5 h. The samples are diluted twice with denaturing buffer containing 6-M guanidine-HCl, 1-mM EDTA, and 0.1-M Tris-HCl (pH 7.8); 2.5 μL of 1-M TCEP-HCl solution is added. The incubation periods last for 18 h at 4°C.

The mechanism of action of the IdeS enzyme comprises cutting the antibody molecule in the hinge region, resulting in the formation of two subunits [$F(ab')_2$ and Fc/2] (Fig. 1). Subsequent chemical cleavage of the disulfide bridges divided the $F(ab')_2$ subunit into two fragments: LC and the Fd subunit.

Intact mass measurement

We have performed the test in the infusion MS mode. The injection rate of the sample solution is 10 μL/min. Before ionization, it is mixed with a 0.15% DFA solution flow supplied at a rate of 100 μL/min. The device operated under the following conditions: positive ionization mode and high mass range with boundaries of 700–8000 Th with signal detection in the frontal scanning mode. The linear data collection rate is 1 Hz, and the evaporator pressure is 22 psi. We analyze each sample in three technical replicas. We process the experimental results using the MassHunter Qualitative Analysis B.09.00 (*Agilent Technologies*) and UniDec v.4.1.2 [10].

Middle-up analysis

We have performed the linear gradient reversed-phase HPLC-MS mode test using a BioResolve RP mAb Polyphenyl column (1.0 × 150 mm). Mobile phase A: 0.15% DFA in water; mobile phase B: 0.15% DFA in acetonitrile. Gradient scheme: 0 min (26% B), 22 min (41.5% B), 22.1 min (98% B), 24 min (98% B), 24.1 min (26% B),

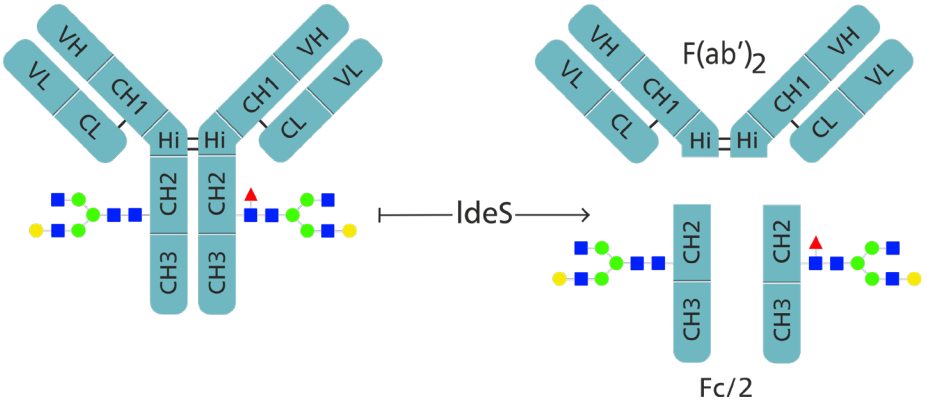


Fig. 1. Mechanism of action of the IdeS protease.

30 min (26% B) at 80°C and flow rate of 0.1 mL/min. We process the obtained mass spectra using MassHunter Qualitative Analysis B.09.00 and UniDec v.4.1.2 [10].

Statistical comparison of results

The analysis methods used for comparison are qualitative to establish the equivalence of the results, according to the currently accepted standards [11, 12]; the concept of a quality range is introduced. Its boundaries are determined by a threefold increase in the standard deviation of the measured parameters of the original samples from their mean values.

RESULTS AND DISCUSSION

Measuring the molecular mass of intact antibodies

The average molecular mass of the major proteoform of eculizumab, taking into account its amino acid sequence and main modifications (a pair of G0F glycans, a pair of cleaved C-terminal lysines, a pair of pyroglutamates at the N-termini of heavy chains, and 17 disulfide bonds) is 147874 Da. Comparative analysis of the raw mass spectra of eculizumab from samples of various manufacturers shows that the distribution of the protein's charge states in all cases ranges from 35+ to 65+, with the most intense states in the range from approximately 40+ to 50+ (Fig. 2).

We perform the deconvolution of mass spectra using UniDec software with the following settings: *m/z* range of 2250–4300 Th, automatic noise subtraction, range of charge states at 35–65, automatic smoothing of mass spectra, peak width at half-height of 0.8 Th, and normalized peak detection limit at 1%. It shows the presence in all studied samples of glycoforms' molecular mass distribution, monoclonal antibodies' characteristic, in the region expected for eculizumab. The molecular mass of the major glycoform G0F/G0F in all samples corresponds to the expected value of 148874 Da, and the deviation of its mass from the expected did not exceed 1 Da (7 ppm). Eight proteoforms are found in the original Soliris, and seven proteoforms in Elizaria, of which six and five, respectively, differed in the glycan profile; the remaining two differ in the content of uncleaved C-terminal lysine (Fig. 3 and Table 1).

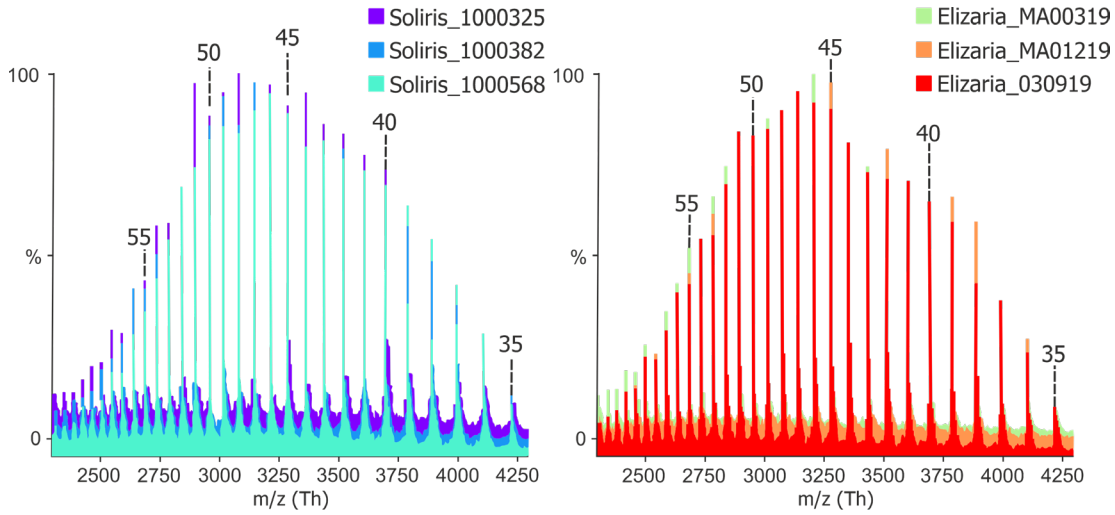


Fig. 2. Mass spectra of the original Soliris and Elizaria drugs before deconvolution. Individual charge states are annotated.

The content of the major glycoforms of eculizumab in the compared samples is similar. G0F/G0F, G1F/G0F, and G1F/G1F occupy the first, second, and third places, respectively. Glycoforms with smaller proportions are distributed more variably. Therefore, in the samples of the original Soliris, the variant G0F/G0F-GN is in fourth place, and in the drug Elizaria, the variant G0F/G0 is in fourth place. The fifth places in all cases are represented by the glycoform G2F/G1F, and the sixth place is registered only in the samples of the original Soliris, G2F/G2F. The content of galactosylated glycans of the main functional group of oligosaccharides in the samples of the original Soliris and Elizaria are 25.7–26.4% and 11.9–14.7%, respectively. The proportions of forms of antibodies with uncleaved C-terminal

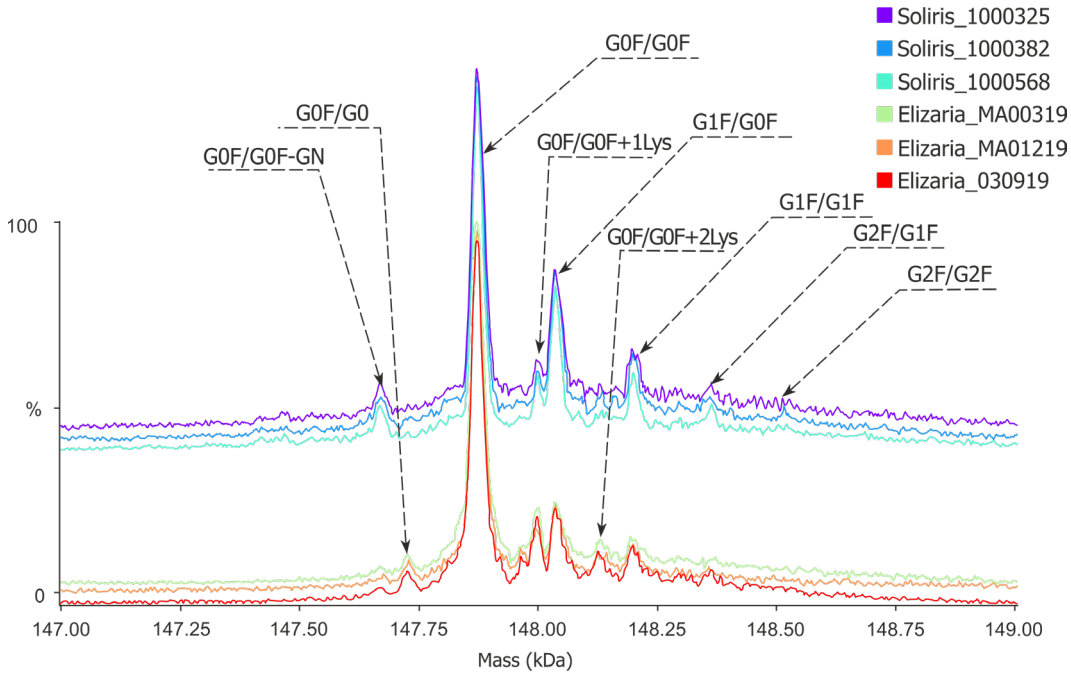


Fig. 3. Mass spectra of the original Soliris and Elizaria drugs after deconvolution. The identified proteoforms are annotated.

Table 1. Results of measuring the molecular masses of eculizumab proteoforms from the compared samples

Eculizumab proteoforms	Theoretical mass, Da	Measured mass			
		Original Soliris		Elizaria	
		Average ± SD, Da	Error, ppm	Average ± SD, Da	Error, ppm
G0F/G0	147728	–	–	147727 ± 2	−5
G0F/G0F-GN	147671	147669 ± 1	−11	–	–
G0F/G0F	147874	147873 ± 1	−5	147874 ± 0	0
G0F/G0F+1Lys	148002	147999 ± 3	−23	148000 ± 1	−16
G1F/G0F	148036	148036 ± 3	0	148036 ± 2	2
G0F/G0F+2Lys	148130	148130 ± 3	2	148128 ± 2	−14
G1F/G1F	148198	148197 ± 2	−7	148198 ± 2	−2
G2F/G1F	148360	148360 ± 2	0	148363 ± 1	20
G2F/G2F	148522	148513 ± 13	−61	–	–

Note: SD, standard deviation.

lysine, both one and two, are slightly higher in the samples of Elizaria (Fig. 4 and Table 2). All observed differences could be explained using different cell lines to produce the original Soliris and Elizaria. The original Soliris is produced using NS0 rodent myeloma cells; Elizaria is produced using a line of Chinese hamster ovary cells that produces more galactosylated forms [13, 14]. However, the proportion of galactosylated glycans is important only for antibodies that implement a therapeutic function through the mechanism of complement-dependent cytotoxicity. In eculizumab, this mechanism is deliberately inhibited [13–15]. Similar to the previous ones, differences in uncleaved C-terminal lysine content are due to different producers and usually do not significantly affect the antibody's therapeutic efficacy and safety.

Therefore, the samples of the original Soliris and Elizaria can be considered comparable in molecular masses and spectrum of the presented proteoforms, despite some differences in their content.

Middle-up analysis

Treating the samples with IdeS protease followed by destructing disulfide bonds using TCEP makes it possible to obtain from the eculizumab molecule three well chromatographically separated subunits, Fc/2, LC, and Fd. Figure 5 shows the recorded chromatographic peak of the coeluting oxidized form and the form with uncleaved C-terminal lysine of the Fc/2 subunit and a peak of the Fd subunit with one unbroken internal disulfide bond.

The molecular masses of all subunits coincide with the calculated ones; the maximum discrepancy from the expected value does not exceed 0.6 Da (40 ppm, Table 3).

The proportion of glycosylated forms in the compared samples slightly changes compared to the intact molecule analysis (Fig. 6 and Table 4). Thus, the difference between the content of the major glycan G0F decreases: its average levels in the original Soliris and Elizaria are 58.2 and 60.8%, respectively. The contents of G1F and G2F are approximately 14.0 and 2.5%, respectively, in all samples; no reliable difference is established for this parameter. In the middle-up analysis, we have confirmed the data on the uniqueness of oligosaccharides G0F-GN in the composition of the original Soliris and G0 in the composition of Elizaria, and another glycan, unique for the original Soliris, is discovered—Man5. Studying LC and Fd subunits makes it possible to understand why the difference in the content of G1F and G2F glycans in intact molecules. In the middle-up analysis, we have found minor proteoforms in LC and Fd subunits in the original Soliris by identifying by a characteristic mass shift of 162 Da as glycated variants. Their content ranges from 2.8 to 4.6%. The contribution of these forms overestimates the levels of G1F/G1F, G2F/G1F, and G2F/G2F in the analysis of intact molecule from the original Soliris. The content of galactosylated glycans in the samples of Elizaria fully corresponds to the original Soliris quality range. In the C-terminal lysine content of the compared samples, a rather close similarity is observed: compliance with the quality range is demonstrated by two out of three series of Elizaria medicinal products. The oxidation of the Fc/2 subunit becomes a new modification, which is established using the middle-up analysis. Its content in all candidate drug batches also corresponds to the quality range of the original Soliris.

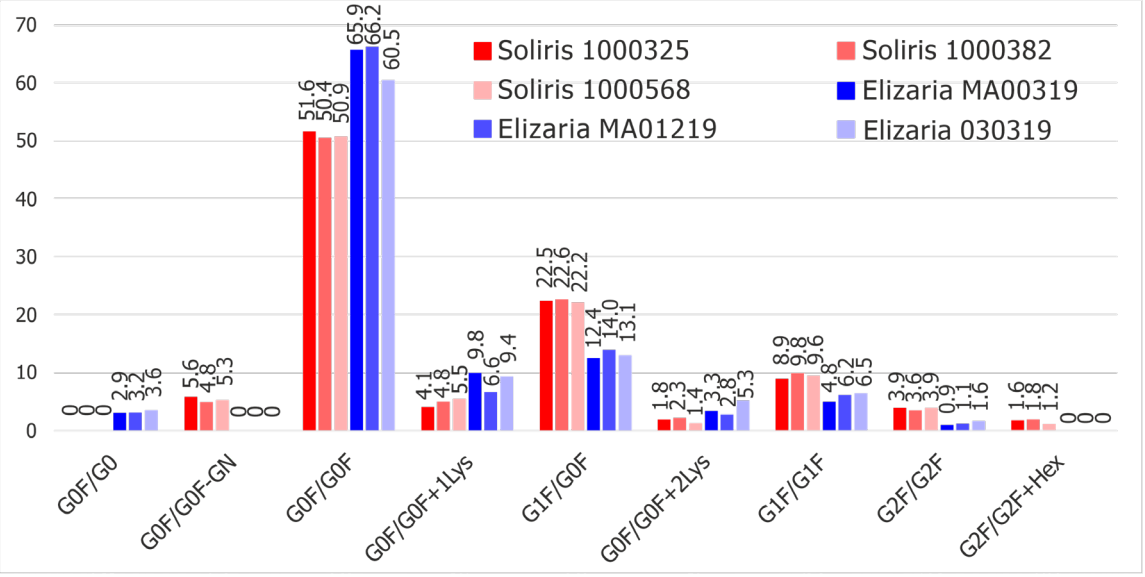
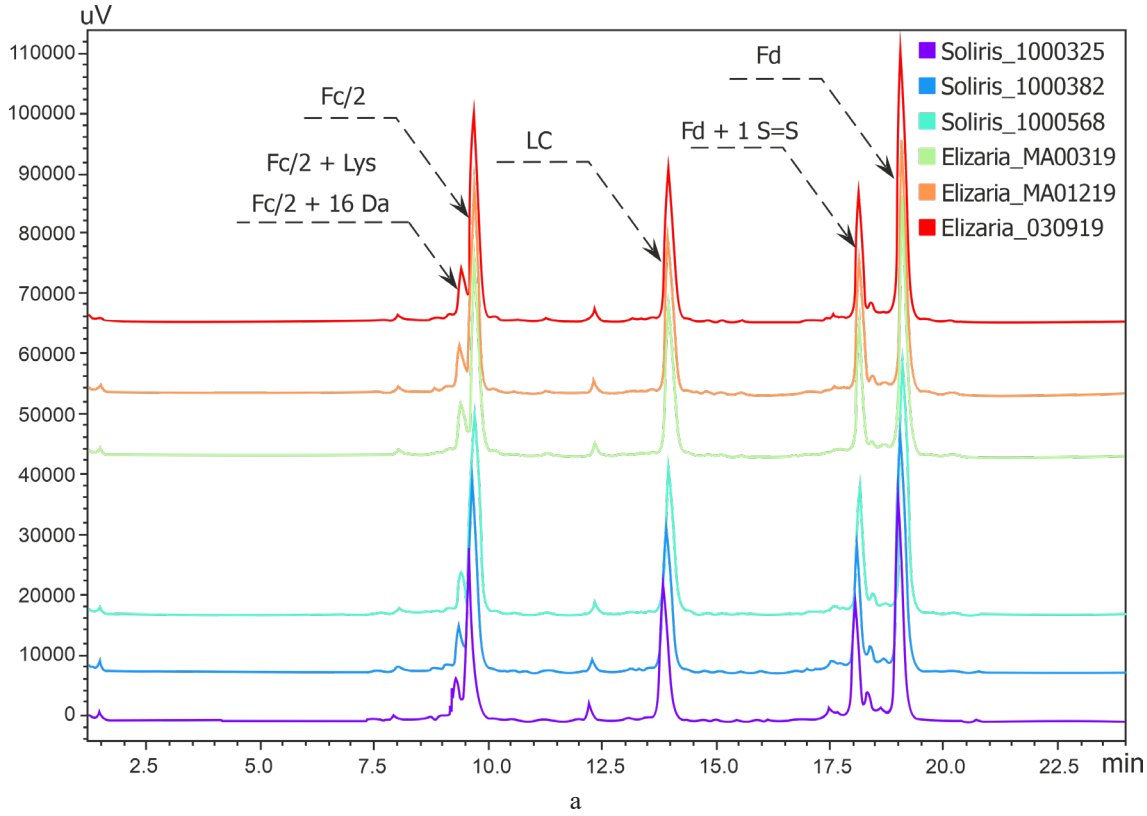


Fig. 4. The content of proteoforms in individual series of the original Soliris and Elizaria drugs.

Table 2. Comparison of the proportions of eculizumab proteoforms in the analyzed intact molecules

Eculizumab proteoforms	Original Soliris		Elizaria		Quality range, % (Average ± 3SD)	Compliance of Elizaria with the quality range, %
	Average	SD , %	Average	SD , %		
G0F/G0	–	–	3.2	0.3	–	None
G0F/G0F-GN	5.2	0.4	–	–	4.0–6.4	None
G0F/G0F	51.0	0.6	64.2	3.2	49.2–52.8	None
G0F/G0F+1Lys	4.8	0.7	8.6	1.7	2.7–6.9	33
G1F/G0F	22.4	0.2	13.2	0.8	21.8–23.0	None
G0F/G0F+2Lys	1.8	0.4	3.8	1.3	0.6–3.0	33
G1F/G1F	9.4	0.5	5.8	0.9	7.9–10.9	None
G2F/G1F	3.8	0.2	1.2	0.4	3.2–4.4	None
G2F/G2F	1.5	0.3	–	–	0.6–2.4	None
Galactosylated glycans, sum (0.5 × G0F/G1F + G1F/G1F + G2F/G1F + G2F/G2F)	26.0	0.4	13.6	1.5	24.8–27.2	None

Note: SD, standard deviation.



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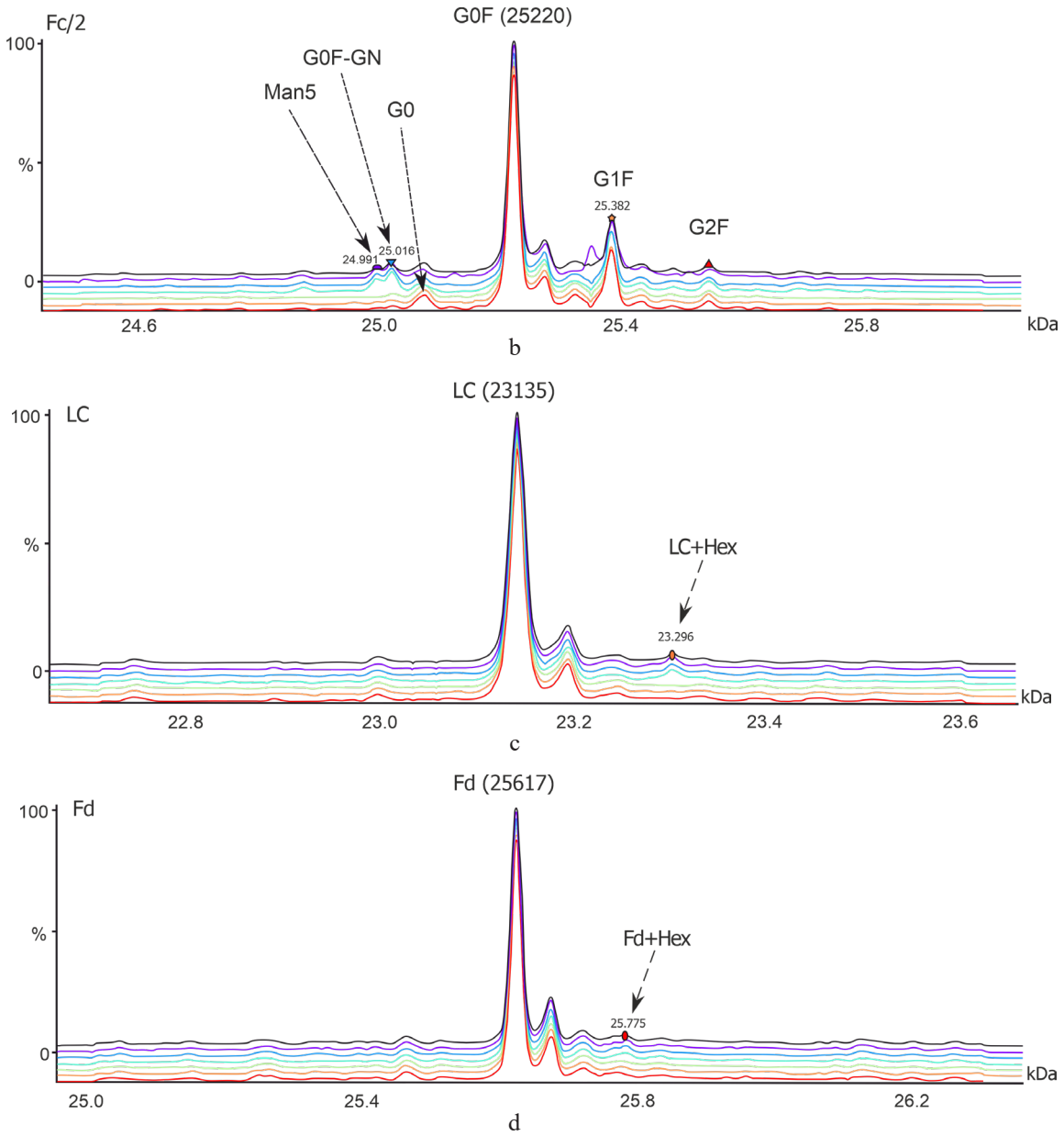

Fig. 5. Annotated chromatograms (a) and mass spectra (b–d) of eculizumab subunits of the original Soliris and Elizaria drugs.

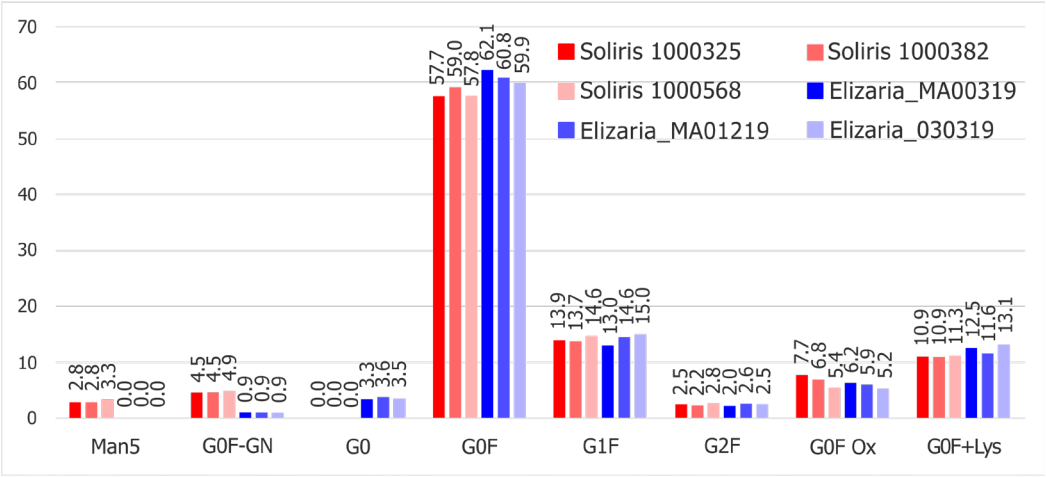

Fig. 6. The content of proteoforms in the Fc/2 subunit of separate series of the original Soliris and Elizaria drugs.

Table 3. Results of measuring the molecular masses of eculizumab subunits from the compared samples

Eculizumab proteoforms	Theoretical mass, Da	Measured mass			
		Original Soliris		Elizaria	
		Average ± SD, Da	Error, ppm	Average ± SD, Da	Error, ppm
Fc/2+Man5	24992	24992 ± 0.3	7	–	–
Fc/2+G0F-GN	25017	25017 ± 0.6	−13	25016	−40
Fc/2+G0	25074	–	–	25073	−40
Fc/2+G0F	25220	25220	0	25220	0
Fc/2+G1F	25382	25382 ± 0.3	−7	25382	0
Fc/2+G2F	25544	25544 ± 0.6	13	25544 ± 0.6	13
Fc/2 + 16 Da (oxidation)	148198	148197 ± 2	−7	148198 ± 2	−2
Fc/2+Lys	25348	25349	39	25349	39
LC	23135	23135	0	23135	0
Fd	25617	25617 ± 0.6	−13	25617 ± 0.6	−13

Note: SD, standard deviation.

Table 4. Comparison of the proportions of eculizumab proteoforms in the subunits of the analyzed samples

Eculizumab proteoforms	Original Soliris		Elizaria		Quality range, % (Average ± 3SD)	Compliance of Elizaria with the quality range, %
	Average	SD , %	Average	SD , %		
Man5	3.0	0.3	–	–	2.1–3.9	None
G0F-GN	4.6	0.2	0.9	0	4.0–5.2	None
G0	–	–	3.5	0.2	–	None
G0F	58.2	0.8	60.9	1.1	55.8–60.6	33
G1F	14.1	0.5	14.2	1.0	12.6–15.6	100
G2F	2.5	0.3	2.4	0.3	1.6–3.4	100
G0F Ox	6.6	1.2	5.8	0.5	2.0–10.2	100
G0F+Lys	11.1	0.2	12.4	0.8	10.5–11.7	67
LC+Hex	4.4	0.2	–	–	3.8–5.0	None
Fd+Hex	2.9	0.1	–	–	2.6–3.2	None
Galactosylated glycans, sum (G1F + G2F)	16.6	0.7	16.5	1.3	14.5–18.7	100

CONCLUSIONS

The development and registration of biosimilar therapeutic proteins require their comprehensive characterization, in the physicochemical part of which HPLC-MS occupies one of the leading positions. The options for this analysis are quite diverse because of the goals of the researchers. However, both in biopharmaceutical comparability studies and in developing a molecule, the entire protein molecule must be described. The results of this analysis become the basis for a deeper characterization of objects of comparison or development.

The study of the monoclonal antibody eculizumab in the original Soliris and Elizaria by mass spectrometric intact mass measurement shows the identity of the molecular masses of most of the proteoforms found in the samples. However, it demonstrates differences in their content, which consisted in a lower content of the major glycoform G0F/G0F and larger—glycoforms G1F/G0F, G1F/G1F, G2F/G1F, and G2F/G2F as part of the original Soliris. However, a detailed study of the antibody subunits by an HPLC-MS middle-up analysis allowed explaining the increased content of heavier glycoforms by glycation sites in the LC and Fd subunits of eculizumab from the original Soliris. As expected, the comparison of oligosaccharides attached to the canonical glycosylation site on the Fc/2 subunit establishes high comparability of the compared samples. Significant differences are observed in the content of the major glycoform G0F; however, the difference in the content of this glycan averaged 2.7%. In the intact molecule analysis, the analogous parameter for the G0F/G0F variant between the original and candidate drugs is at the level of 13.2%. The content of minor oligosaccharides Man5, G0F-GN, and G0 also differ, but their share does not exceed 4.6%.

This work becomes part of the report on the comprehensive comparative characterization of eculizumab of the original and candidate drugs and allowed obtaining Russian marketing authorization for the world's first biosimilar drug based on eculizumab. Mass spectrometric study of intact molecules and subunits of eculizumab from various sources shows the samples' fundamental comparability, which has become the basis for further and deeper HPLC-MS characterization, acting as an orthogonal analysis method for "classical" chromatographic and electrophoretic studies of the comparability of eculizumab.

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Authors' contribution

All authors equally contributed to the research work.

Potential Conflict of Interest Statement

The authors are employees of the Generium International Biotechnological Center, an organization that develops a biosimilar drug to eculizumab.

REFERENCES

1. Dillon T.M., Bondarenko P.V., Rehder D.S., Pipes G.D., Kleemann G.R., Ricci M.S. Optimization of a reversed-phase high-performance liquid chromatography/ mass spectrometry method for characterizing recombinant antibody heterogeneity and stability. *J. Chromatogr. A.* 2006;1120(1–2):112-120. https://doi.org/10.1016/j.chroma.2006.01.016

2. Ren D., Pipes G., Xiao G., Kleemann G.R., Bondarenko P.V., Treuheit M.J., Gadgil H.S. Reversed-phase liquid chromatography–mass spectrometry of site-specific chemical modifications in intact immunoglobulin molecules and their fragments. *J. Chromatogr. A.* 2008;1179(2):198–204. https://doi.org/10.1016/j.chroma.2007.11.088

3. Sawyer W.S., Srikumar N., Carver J., Chu P.Y., Shen A., Xu A., Williams A.J., Spiess C., Wu C., Liu Y., Tran J.C. High-throughput antibody screening from complex matrices using intact protein electrospray mass spectrometry. *Proc. Natl. Acad. Sci. USA.* 2020;117(18):9851–9856. https://doi.org/10.1073/pnas.1917383117

4. Haberger M., Leiss M., Heidenreich A-K., Pester O., Hafenmair G., Hook M., Bonnington L., Wegele H., Haindl M., Reusch D., Bulau P. Rapid characterization of biotherapeutic proteins by size-exclusion chromatography coupled to native mass spectrometry. *MAbs.* 2015;8(2):331–33. https://doi.org/10.1080/19420862.2015.1122150

5. Leney A.C., Heck A.J. Native Mass Spectrometry: What is in the Name? *J. Am. Soc. Mass Spectrom.* 2017;28(1):5–13. https://doi.org/10.1007/s13361-016-1545-3

6. Wehofsky M., Hoffmann R. Automated deconvolution and deisotoping of electrospray mass spectra. *J. Mass Spectrom.* 2002;37(2):223–229. https://doi.org/10.1002/jms.278

7. Lu J., Trnka M.J., Roh S.H., *et al*. Improved Peak Detection and Deconvolution of Native Electrospray Mass Spectra from Large Protein Complexes. *J. Am. Soc. Mass Spectrom.* 2015;26(12):2141–2151. https://doi.org/10.1007/s13361-015-1235-6

8. Wohlschlager T., Scheffler K., Forstenlehner I.C. *et al*. Native mass spectrometry combined with enzymatic dissection unravels glycoform heterogeneity of biopharmaceuticals. *Nat. Commun.* 2018;9:1713. https://doi.org/10.1038/s41467-018-04061-7

9. Lermyte F., Tsybin Y.O., O'Connor P.B., Loo J.A. Top or Middle? Up or Down? Toward a Standard Lexicon for Protein Top-Down and Allied Mass Spectrometry Approaches. *J. Am. Soc. Mass Spectrom.* 2019;30(7):1149-1157. https://doi.org/10.1007/s13361-019-02201-x

10. Marty M.T., Baldwin A.J., Marklund E.G., Hochberg G.R.A., Benesch J.L.P., Robinson C.V. Bayesian deconvolution of mass and ion mobility spectra: from binary interactions to polydisperse ensembles. *Anal. Chem.* 2015;87(8):4370–4376. https://doi.org/10.1021/acs.analchem.5b00140

11. Tsong Y., Dong X., Shen M. Development of statistical methods for analytical similarity assessment. *J. Biopharm. Stat.* 2017;27(2):197–205. https://doi.org/10.1080/10543406.2016.1272606

12. Chow S.-C. Challenging issues in assessing analytical similarity in biosimilar studies. *Biosimilars.* 2015;5:33–39. https://doi.org/10.2147/BS.S84141

13. Raju T.S., Jordan R.E. Galactosylation variations in marketed therapeutic antibodies. *MAbs.* 2012;4(3):385–391. https://doi.org/10.4161/mabs.19868

14. Bruce A., Hunter J., Malanson H.F. Recombinant glycosylated eculizumab and eculizumab variants: US Patent US20170073399A1. Priority 11.09.2015.

15. Reusch D., Tejada M.L. Fc glycans of therapeutic antibodies as critical quality attributes. *Glycobiology.* 2015;25(12):1325–1334. https://doi.org/10.1093/glycob/cwv065

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