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REVIEWS

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Past, current and future of legal regulation of drugs compounding in the Russian Federation

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Today the activities of compounding pharmacies in terms of the drug compounding and in-pharmacy packaging of approval drugs is considered as a priority social task of the Russian Federation, due to the need to solve problems that are aimed at ensuring the strategic independence of the state from external and internal challenges, as well as threats, widespread introduction of personalized pharmacotherapy methods, opportunities in the field of optimizing the costs of the healthcare system.

The aim of the study was to conduct a historical analysis and comprehensive review of the current state of legal and regulatory framework of the Russian pharmaceutical market in the field of compounding drugs, as well as to develop proposals for improving the regulatory field.

Materials and methods. The following methodological tools – empirical, theoretical, quantitative ones – have been used in the work. In particular, an analysis of a wide range of relevant sources of information was carried out and information was obtained from legal and regulatory framework for the activities of compounding pharmacies in the Russian Federation, which was implemented using a bibliometric method of analysis.

Results. The main elements of the Russian legislation in the field of drug circulation with the aim of a comprehensive understanding of the classification and determination of the role of extemporaneous drugs in the Russian healthcare system, are presented in the article. A historical and technical analysis of the regulatory practice development is consistently presented. The key issues of organizing the pharmaceutical business in the field of compounding and dispensing of drugs on the territory of the Russian Federation have been considered, and current problems that need to be solved when improving the regulatory field, are presented.

Conclusion. The review conducted makes it possible to clarify further actions to improve federal legislation and delegated legislation in the field of circulation of compounded drugs by pharmacy organizations. The work presents recommendations that will contribute to the development of compounding pharmacies in the constituent entities of the Russian Federation.

Keywords: drug circulation in the Russian Federation; drugs compounding; extemporaneous drugs; diluting (reconstitution) of drugs; compounding pharmacies; history of pharmaceutical compounding of drugs; Russian pharmaceutical market; sales volume of extemporaneous drugs; rules for compounding and dispensing of drugs

Abbreviations: MP – medicinal product / preparation; EU – European Union; ED – extemporaneous drug; PO – pharmacy organization; DF – dosage form; API – active pharmaceutical ingredient; MPTF – medical and preventive treatment facility; EMs – List of Essential Medicines; FD – approval drug; RPD – radiopharmaceutical drugs; MA – marketing authorization; SRMRs – State Register of Medicinal Remedies; FDA – Food and Drug Administration; GxP – Good Practice.

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ОБЗОРЫ
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Прошлое, текущее и будущее нормативного правового регулирования аптечного изготовления лекарственных препаратов в Российской Федерации

Д.Д. Мамедов[1], Д.С. Юрочкин[1], З.М. Голант[1], В.С. Фисенко[2], А.В. Алехин[3,4], И.А. Наркевич[1]

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На сегодняшний день деятельность производственных аптек в части изготовления лекарственных препаратов и внутриаптечной фасовки зарегистрированных лекарственных препаратов рассматривается в качестве приоритетной социальной задачи Российской Федерации, в связи с необходимостью решения задач, направленных на обеспечение стратегической независимости государства от внешних и внутренних вызовов, а также угроз, широкого внедрения методов персонализированной фармакотерапии, возможностей в сфере оптимизации затрат системы здравоохранения.

Цель. Провести исторический анализ и всесторонний обзор текущего состояния нормативного правового регулирования российского фармацевтического рынка в области изготовления лекарственных препаратов, а также разработать предложения по совершенствованию регуляторного поля.

Материалы и методы. В работе использованы методологические инструменты: эмпирические, теоретические, количественные. В частности, проведён анализ широкого перечня релевантных источников информации и получены сведения из нормативных правовых документов, регулирующих деятельность производственных аптек на территории Российской Федерации. В данном исследовании авторами применён метод библиометрического анализа.

Результаты. В статье представлены основные элементы российского законодательства в сфере обращения лекарственных средств с целью всестороннего понимания классификации и определения роли экстемпоральных лекарственных препаратов в российской системе здравоохранения. Последовательно изложен историко-технический анализ развития регуляторной практики. Рассматриваются ключевые вопросы организации фармацевтического дела в области изготовления и отпуска лекарственных препаратов на территории Российской Федерации, а также представлены актуальные задачи, которые предстоит решить при совершенствовании регуляторного поля.

Заключение. Проведённый обзор позволяет уточнить дальнейшие действия по совершенствованию федерального законодательства и подзаконных нормативных правовых актов в сфере обращения лекарственных препаратов, изготавливаемых аптечными организациями. В работе представлены рекомендации, которые могут поспособствовать развитию деятельности производственных аптек в субъектах Российской Федерации.

Ключевые слова: обращение лекарственных средств в Российской Федерации; изготовление лекарственных препаратов; экстемпоральные лекарственные препараты; разведение (восстановление) лекарственных препаратов; производственные аптеки; история аптечного изготовления лекарственных препаратов; фармацевтический рынок России; объём продаж экстемпоральных лекарственных препаратов; правила изготовления и отпуска лекарственных препаратов; надлежащая практика изготовления и отпуска лекарственных препаратов

Список сокращений: ЛП – лекарственный препарат; ЕС – Европейский союз; ЭЛП – экстемпоральный лекарственный препарат; АО – аптечная организация; ЛФ – лекарственная форма; ЛС – лекарственное средство; АФС – активная фармацевтическая субстанция; ЛПУ – лечебно-профилактическое учреждение; ЖНВЛП – жизненно необходимые и важнейшие лекарственные препараты; ГЛФ – зарегистрированный лекарственный препарат; РФЛП – радиофармацевтические лекарственные препараты; РУ – регистрационное удостоверение; ГРЛС – Государственный реестр лекарственных средств; FDA – Управление по санитарному надзору за качеством пищевых продуктов и медикаментов; GxP – система надлежащих практик.

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INTRODUCTION

With the development of industrial manufactories, in the USSR, the segment of pharmaceutical compounding of drugs experienced processes of stagnation in the framework of reference to compounding pharmacies, similar to the European Union (EU) [1] and the USA [2].

According to the data for 1939, in the Soviet Union, the drugs compounded by pharmacy organizations (POs) (i.e., extemporaneous drugs products, EDs) occupied a share equal to 60–70% of all the drugs sold in Pos [3], in 1961 – 55%, in 1980 –18% with a predominance of parenteral dosage forms (DFs) in the amount of up to 40–50% in medical and preventive treatment facilities (MPTFs). Despite this, based on the content analysis performed [1, 4–6], a systemic conclusion follows that in the 80^{s} of the 20th century, the USSR had the most developed network of compounding pharmacies in the world.

The collapse of the Soviet Union led to the formation of a market for the circulation of drugs in Russia with the parameters that resulted from the specifics of the previously functioning healthcare system, i.e., a delivery of health care as a public service and supplying the population with medicinal products [7]. The breakdown of production chains and the simultaneous economic crisis led to a rapid decline in local production of drugs and active pharmaceutical ingredients (API). For example, for the period from 1992 to 2008, the volume of production of the latter in the Russian Federation decreased by more than 20 times [8]. The lack of access to raw materials, coupled with the insufficient regulatory influence, led to the closure of the majority of compounding pharmacies, the decline of which is observed annually in the Russian Federation. In particular, according to the study [9], 36% of joint-stock companies ceased their activities in the production of drugs in the period from 2015 to 2019. It was at that time that pharmaceutical specialists reoriented their activities to industrial facilities producing drugs, as well as POs engaged in retail trade of approval drugs. Nowadays, the number of POs that have a license for pharmaceutical activities with the right to compounding and dispense drugs is estimated at less than 0.5% of the total number of POs [10].

Federal Law No. 61-FZ "On drug circulation" (FL No. 61) [1], adopted in 2010, was aimed at harmonizing domestic legislation with European legislation and, consequently, implementing a Good clinical practice (GCP / GxP) in all areas of the pharmaceutical market, which was implemented at all levels of drug circulation, with the exception of the pharmaceutical compounding segment of drugs.

Compounding pharmacies are an element of both the healthcare system and the social protection system in terms of providing certain categories of citizens entitled to receive government assistance, with medications.

Today, the Russian pharmacological support has encountered difficulties in relation to its basic tasks in terms of the transition from supplying patients with bulk drug substances regarding course prescriptions, including the ones associated with the absence in the legislation of one of the GxP elements – drugs preparation by the compounding pharmacies and intrapharmacy packing of approval drugs. In the next decade, according to the authors of this study, the latter will likely become one of the key areas for improving the legislation of the Russian Federation regarding drug circulation. Herewith, the need for changes in legal and regulatory framework will come from the already established and basic functions of compounding pharmacies and the newly created modern pharmacy infrastructure, i.e.:

1. Providing direct physical access to the drug.

Currently, there are cases when the pharmaceutical industry does not offer an alternative to packaging and volume, especially for expensive and high-cost drugs that are not registered in the Russian Federation. This fact affects the rationality of spending money, regardless of the source of financing – the state or citizens' own funds. Moreover, without a direct control of the drug provision costs for a particular patient in the required course volume of therapy, it is impossible to achieve a budgetary efficiency, since there are no comparable criteria for the comparison [1, 11].

2. Direct pharmacoeconomic advantage of compounding pharmacies:

– by packaging approval drugs in the "bulk" form (thereby achieving the aim of solving the problem in the field of systematization of healthcare in accounting for spent course prescriptions);

– due to direct savings in the drugs compounding in those nosologies where this is justified (orphan drugs, antitumor drug therapy, high-tech drugs, etc.).

This study significantly reveals the current legal and regulatory framework for the drug circulation in the Russian Federation, thereby demonstrating in it the place of pharmaceutical compounding of drugs, and also specifies the past and a current state of regulation of the compounding pharmacies, includig setting possible vectors of development of the latter.

THE AIM of the study was to conduct a historical analysis and comprehensive review of the current state of legal and regulatory framework of the Russian pharmaceutical market in the field of drug compounding, as well as to develop proposals for improving the regulatory field.

This article is a continuation of the series of works by the authors, which are devoted to the formation of a unified harmonized system of legal and regulatory framework in the field of circulation of the drugs compounded by joint-stock companies in the Russian Federation [1, 2, 9–12].

[1] Federal Law of April 12, 2010 No. 61-FZ "On the Circulation of Drugs". Available from: https://docs.cntd.ru/document/902209774

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MATERIALS AND METHODS

The following methodological tools – empirical, theoretical, quantitative ones – have been used in the work. In particular, an analysis of a wide range of relevant information sources was carried out and data was obtained from legal and regulatory framework for the activities of compounding pharmacies in the Russian Federation, which was implemented using the bibliometric method of analysis.

The data from various sources of information were used. In terms of the analysis of legal and regulatory framework, the following materials were used: the electronic fund of regulatory, technological and regulatory intelligence of the "Code" Consortium, the reference legal system "ConsultantPlus".

To analyze the results of research by other authors, relevant sources of information and data from search engines were used: PubMed, for biomedical research, scientific electronic library – elibrary.ru, Russian National Library, National Electronic Library, Google Academy. The search depth was 1917 to 2023. The choice of period was determined by historical events and the beginning of the active development of compounding pharmacies activities in the USSR. The literature search was carried out in Russian and English using the following keywords or combinations: drug circulation in the Russian Federation; drug compounding; extemporaneous drugs; diluting (reconstitution) of drugs; compounding pharmacies; history of pharmaceutical compounding of drugs; Russian pharmaceutical market; sales volume of extemporaneous drugs; rules for good compounding and dispensing of drugs; good practice in compounding and dispensing of drugs.

RESULTS AND DISCUSSION

Classification of extemporaneous drugs

The main legal and regulatory framework on the drug market in the Russian Federation is FL No. 61, according to which a separate definition of ED has not been established. Based on the systemic interpretation of Art. 4, 13 and 56 of FL No. 61, as well as other legal and regulatory framework, it follows that EDs belong to drugs. In addition, FL No. 61 does not contain a definition of the approval drugs approved by the Ministry of Health of Russian Federation [13] which is an authorized federal government body, a ministry of the Russian Federation, similar in functionality to the FDA; hereinafter ADs). According to Art. 14 and Art. 17 of FL No. 61, the fact of the ADs registration is a marketing authorization (MA) received by the applicant for the drug, issued as a result of the examination of the registration dossier. The distribution diagram of the main definitions used in FL No. 61 is presented in Figure 1.

Within the meaning of clause 5 of Article 13 of the FL No. 61, state registration [14] in the Russian Federation is not subject to:

– drugs compounded by POs that have a license for pharmaceutical activities, according to prescriptions for drugs and the requirements of medical organizations;

– drugs purchased by individuals outside the Russian Federation and intended for personal use;

– drugs imported into the Russian Federation to provide medical care according to the vital indications of a specific patient on the basis of a permission issued by the authorized federal executive body;

– drugs imported into the Russian Federation on the basis of a permission issued by the authorized federal executive body and intended for conducting clinical trials of drugs and (or) to be evaluated for the state registration of drugs;

– active pharmaceutical ingredient (API);

– radiopharmaceutical drugs (RPDs), compounded directly in medical organizations, in the manner established by the authorized federal executive body;

– drugs produced for export.

According to Art. 33 of FL No. 61, the list of ADs and the list of APIs included in the ADs are contained in the State Register of Medicinal Remedies (SRMRs)[2] [15]. According to paragraph 2 of Art. 33, an API produced for sale may be included in the SRMRs on the basis of an application from the developer or manufacturer of the drug, or a legal entity authorized by them, subject to an examination of the API quality in relation in the manner established by Art. 34 of FL No. 61.

Thus, on the Russian Federation drug circulation market, APIs can exist in two different states:

– as active APIs included in the MA can only be used by the holder of the MA (sale of such APIs to third party companies is prohibited);

– as APIs included in the SRMRs can be sold to all drug manufacturers, drug wholesalers and compounding pharmacies.

Licensing in the field of drug circulation

In accordance with FL No. 61, the production of drugs and pharmaceutical activities are separated [16]. Moreover, the latter consists of retail trade and wholesale trade of drugs. At the same time, a pharmaceutical activity for compounding is a type of retail trade of drugs (Fig. 2).

To produce drugs, it is necessary to obtain an advisory license (Art. 45 of FL No.61). The procedure for licensing activities for the drugs production was approved by the Decree of the Government of the Russian Federation No. 686 dated July 6, 2012 (hereinafter referred to as Resolution No. 686)[3]. Based on that, licensing of the drugs production is carried out

[2] State Register of Drugs of the Russian Federation. Available from: https://grls.rosminzdrav.ru/Default.aspx

[3] Decree of the Government of the Russian Federation of July 6, 2012 No. 686 "On approval of the Regulations on licensing the production of drugs." Available from: https://docs.cntd.ru/document/902356716

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by the authorized federal executive body – the Ministry of Industry and Trade of Russia [17]. To obtain a license, drug manufacturers must meet the criteria of the Good Manufacturing Practice Rules of the Eurasian Economic Union (hereinafter referred to as the EAEU GMP Rules[4]) – an adapted translation of the EU Good Manufacturing Practice Rules[5]. Confirmation of the production site for compliance with the EAEU GMP Rules is carried out through a preliminary inspection procedure. The authority to conduct inspections has currently been transferred to the Institution for Medicinal Products and Good Manufacturing Practice [18], subordinate to the Ministry of Industry and Trade of Russia.

According to Art. 54 of FL No.61, wholesale trade of drugs is permitted for drug manufacturers and drug wholesale trade organizations that meet the requirements of the Rules of Good Distribution Practice within the Eurasian Economic Union (hereinafter referred to as the EAEU GDP Rules)[6] [19]. It should be noted that a direct standard for compliance by drug manufacturers with these rules is not provided for by Resolution No. 686. The procedure for licensing pharmaceutical activities (wholesale and retail trade) was approved by the Decree of the Government of the Russian Federation No. 547 dated March 31, 2022[7].

The basic requirements for the retail drug trade procedure are described in Art. 55 of FL No.61 and are implemented by establishing the POs compliance with the Rules of Good Pharmacy Practice (hereinafter referred to as Order No. 647n)[8]. However, these rules are devoted exclusively to the retail drug trade, which differs significantly from the approaches to good pharmacy practices implemented, for example, in the EU, as well as in the countries of the Eurasian Economic Union (Republic of Kazakhstan, Republic of Belarus) [1].

In the Russian Federation, distance sale of drugs is permitted, with the exception of prescription drugs, narcotic and psychotropic ones, as well as alcohol-containing drugs with a volume fraction of ethyl alcohol over 25%, subject to a license for the retail drug trade and the corresponding permit[9] from the federal executive body, which carries out the functions of control and supervision in the field of healthcare – Federal Service for Surveillance in Healthcare (hereinafter referred to as Roszdravnadzor) [20]. At the time of submitting the manuscript for publication, distance trading of prescription drug is carried out in a pilot (test) mode in the territories of Moscow, Moscow and Belgorod regions according to the limited list of international nonproprietary names (INN)[10] [21]. The procedure for remote retail trade of prescription drugs was introduced by Federal Law No. 405-FZ dated October 20, 2022[11]. At the same time, by the Decree of the Government of the Russian Federation No. 2465[12] dated December 28, 2022, the online sale of EDPs is prohibited.

The production of drugs is regulated by Art. 56 FL No. 61 and is carried out by POs that have a license for pharmaceutical activities on the basis of prescriptions for drugs and the requirements of medical organizations (MO), in accordance with the Rules for Good Manufacturing and Dispensing Practices of Drugs, approved by the Order of the Ministry of Health of Russia dated May 22, 2023 (hereinafter – Order No. 249n)[13]. Types of joint stock companies are established in Order of the Ministry of Health of Russia No. 780n[14] dated July 31, 2020 [22] and include, but are not limited to (Figure 3):

[4] Decision of the EEC Council of November 3, 2016 No. 77 "On approval of the Rules of Good Manufacturing Practice of the Eurasian Economic Union." Available from: https://docs.cntd.ru/document/456026099

[5] Comparison and analysis of GMP requirements of the Russian Federation and the EAEU. Available from: https://gxpnews.net/2020/08/sravnenie-i-analiz-trebovanij-gmp-rossijskoj-federacii-i-eaes/.

[6] Decision of the EEC Council of November 3, 2016 No. 80 "On approval of the Rules of Good Distribution Practice within the framework of the Eurasian Economic Union." Available from: https://docs.cntd.ru/document/456026098

[7] Decree of the Government of the Russian Federation of March 31, 2022 No. 547 "On approval of the Regulations on licensing of pharmaceutical activities." Available from: https://docs.cntd.ru/document/350167126

[8] Order of the Ministry of Health of Russia dated August 31, 2016 No. 647n "On approval of the Rules of Good Pharmacy Practice of Drugs for Medical Use." Available from: https://docs.cntd.ru/document/564406688

[9] Order of Roszdravnadzor dated May 28, 2020 No. 4394 "On approval of the List of documents confirming the compliance of a pharmacy organization with the requirements giving the right to carry out retail trade in drugs for medical use remotely, the Procedure for maintaining a register of issued permits for retail trade in drugs for medical use remotely and forms of documents used by the Federal Service for Surveillance in Healthcare when issuing permission to retail trade in drugs for medical use remotely." Available from: https://www.consultant.ru/document/cons_doc_LAW_354200/.

[10] Decree of the Government of the Russian Federation of February 22, 2023 No. 292 "On approval of the Regulations on the procedure for conducting an experiment on the retail trade of drugs for medical use, dispensed with a prescription for a drug, remotely." Available from: https://docs.cntd.ru/document/1300876915

[11] Federal Law of October 20, 2022 No. 405-FZ "On Amendments to the Federal Law "On the Circulation of Drugs". Available from: http://publication.pravo.gov.ru/Document/View/0001202210200012

[12] Decree of the Government of the Russian Federation of December 28, 2022 No. 2465 "On approval of criteria for the inclusion of drugs and pharmacotherapeutic groups of medicinal products in the list of drugs and pharmacotherapeutic groups of drugs approved for sale within the framework of an experiment in the retail trade of medicinal products for medical use, dispensed with a prescription for a drug, remotely." Available from: http://publication.pravo.gov.ru/Document/View/0001202212290005

[13] Order of the Ministry of Health of Russia dated May 22, 2023 No. 249n "On approval of the rules for the compounding and dispensing of drugs for medical use by pharmacies licensed for pharmaceutical activities." Available from: https://docs.cntd.ru/document/1301699481

[14] Order of the Ministry of Health of Russia dated July 31, 2020 No. 780n "On approval of types of pharmacy organizations." Available from: https://docs.cntd.ru/document/565649073

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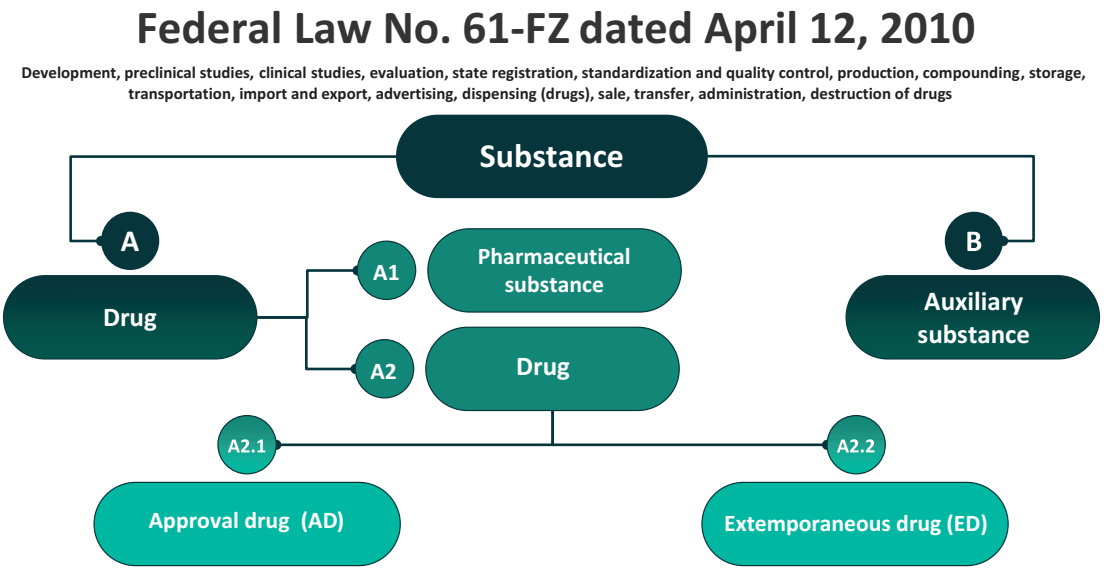

Figure 1 – Definition of drug according to Federal Law No. 61

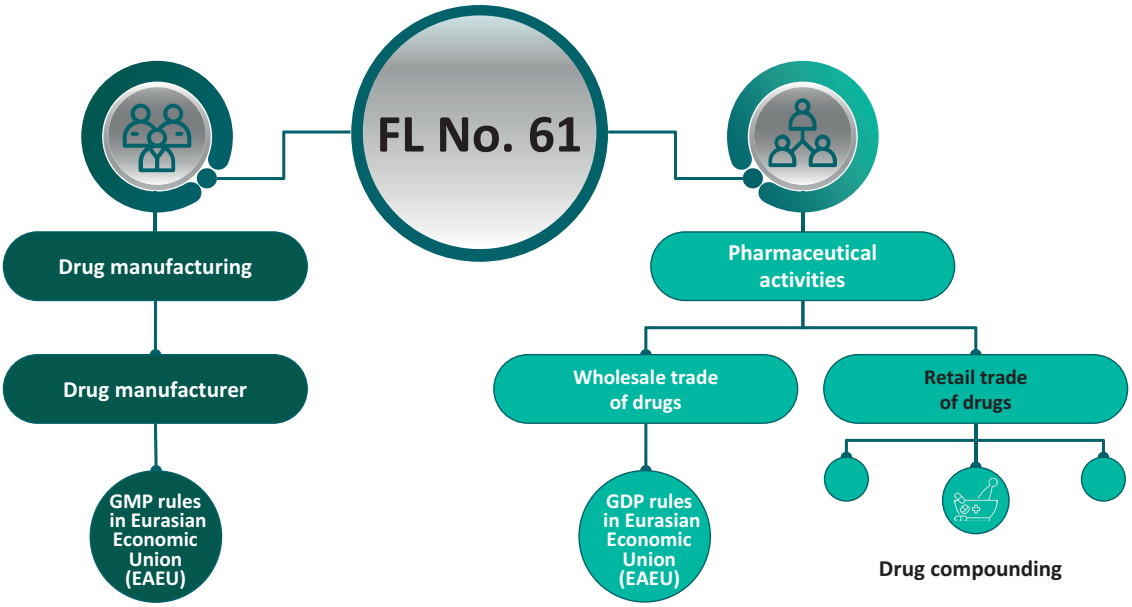

Figure 2 – Relationship between drug manufacturing and pharmaceutical activities

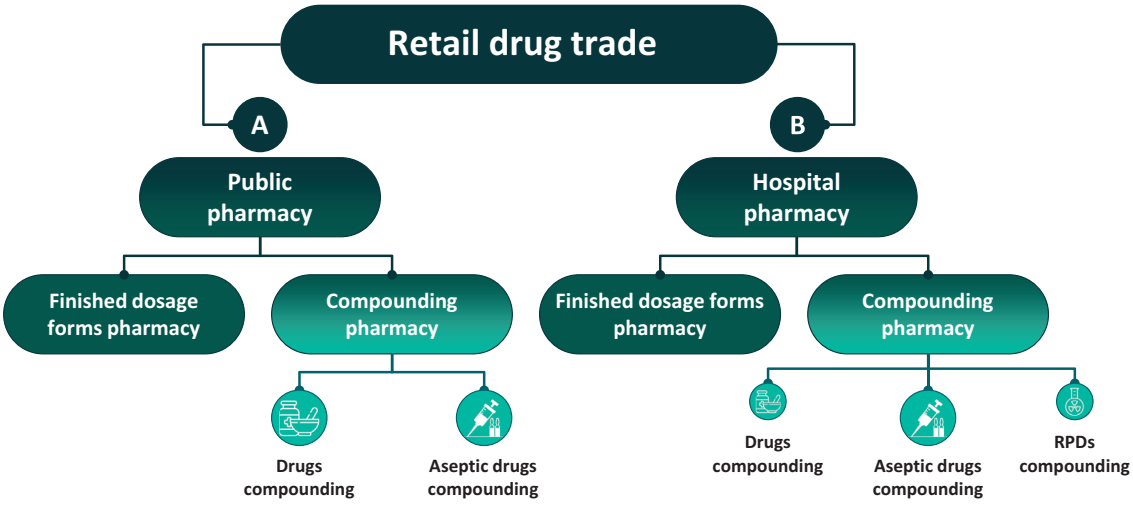

Figure 3 – Types of pharmacy organizations in the Russian Federation

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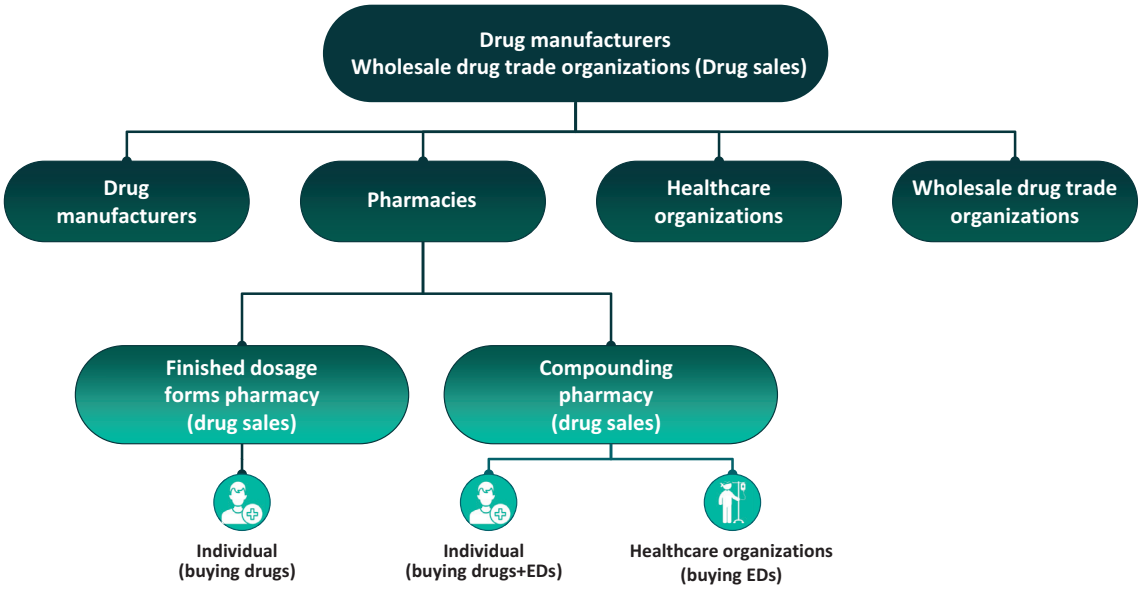

Figure 4 – Drug sales channels in the Russian Federation

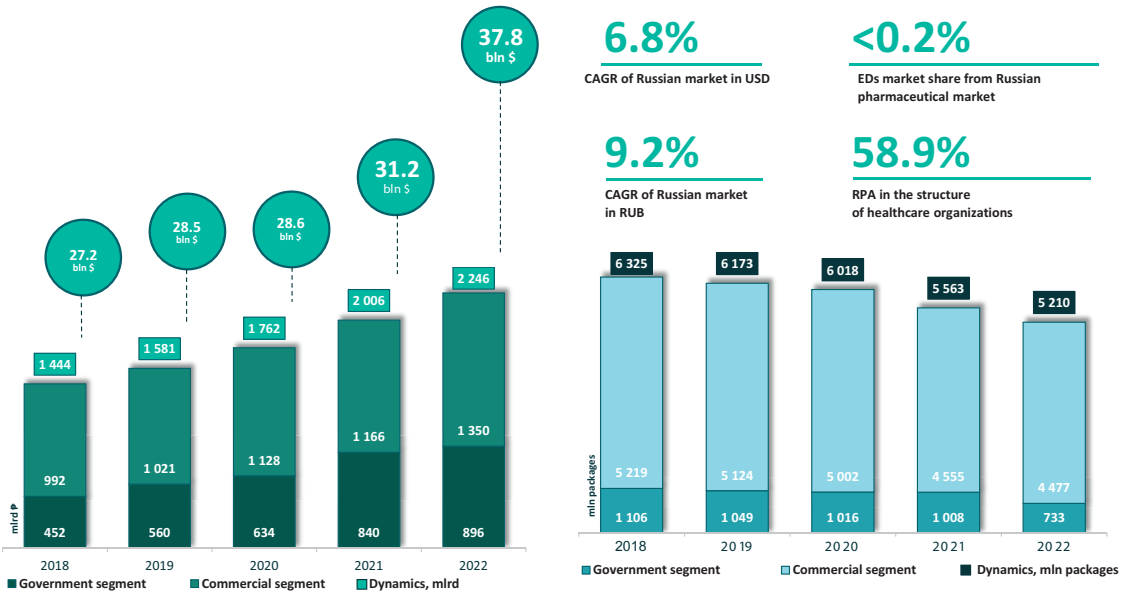

Figure 5 – Capacity of Russian pharmaceutical market

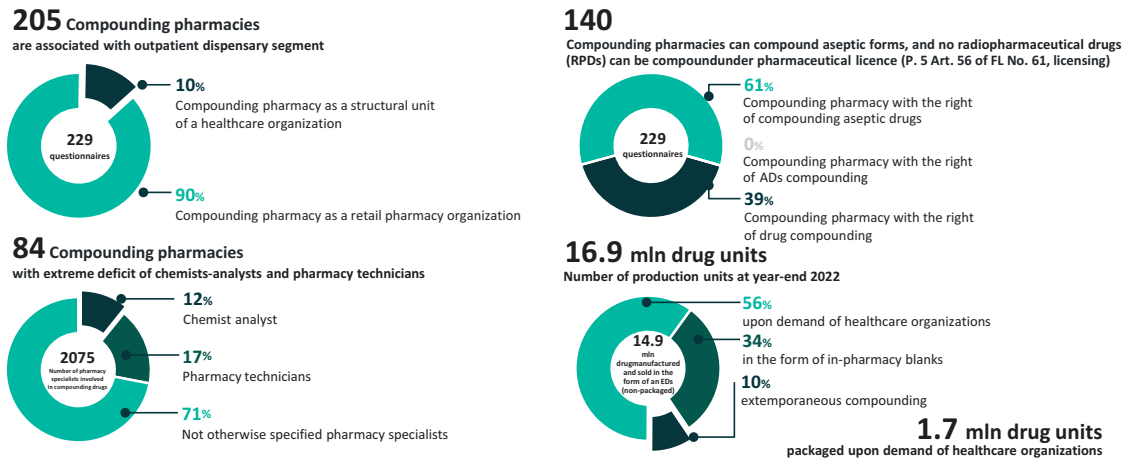

Figure 6 – Consolidated results of monitoring compounding pharmacies in the Russian Federation

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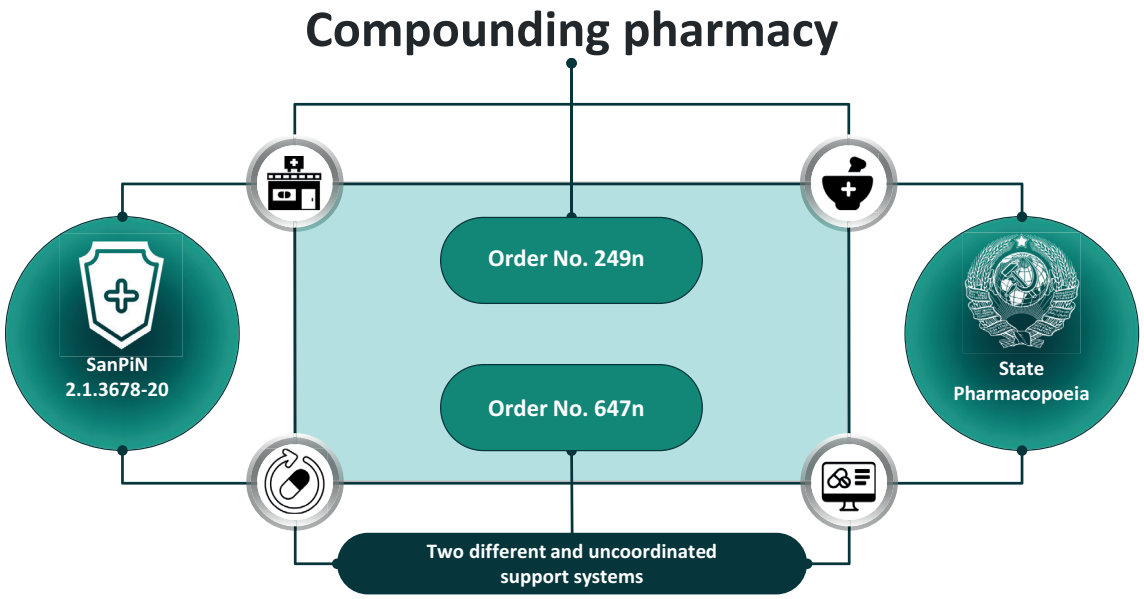

Figure 7 – Key regulatory standards of compounding pharmacies

Table 1 – Requirements for microbiological purity of air during drugs compounding

EAEU GDP Rules		SanPiN 2.1.3678-20		
Cleanliness class	Total number of microorganisms in 1 m³ of air (CFU/m³)	Cleanliness class	Total number of microorganisms in 1 m³ of air (CFU/m³)	
			in equipped condition	in operating condition
A	<1	A	200	500
B	10	B	500	750
C	100	–	–	–
D	200	–	–	–

1. Pharmacy carrying out retail drug trade (dispensing) to the population:

– ADs;

– Compounding pharmacy with the right to drugs compounding;

– Compounding pharmacy with the right to aseptic drugs compounding.

2. Pharmacy as a structural unit of the Moscow Region:

– ADs;

– Compounding pharmacy with the right to drugs compounding;

– Compounding pharmacy with the right to aseptic drugs compounding;

– Compounding pharmacy with the right to compound RPDs.

Thus, the activities of manufacturing drugs relate to the retail drug trade, are equivalent to retail medical supplies stores, and are not associated with healthcare institutions. That differs significantly from the approaches of developed healthcare systems, where POs are full-fledged participants in the provision of medical care to the population and the drug supply system, performing the functions of providing pharmaceutical assistance to the population in accordance with the established types of pharmaceutical services and work [1, 23].

The requirements for POs segregated compounding areas are described within the framework of the Resolution of Russia's Chief Public Health Officer No. 44 dated December 24, 2020 (hereinafter referred to as SanPiN 2.1.3678-20)[15]. The procedure for prescribing and dispensing drugs (including the rules for filling prescriptions) in the Russian Federation is carried out in accordance with Order of the Ministry of Health of Russia No. 1094n[16] dated November 24, 2021 [24, 25], dispensing drugs (sale) to the population in accordance

[15] Resolution of the Chief State Sanitary Doctor of the Russian Federation dated December 24, 2020 No. 44 "On approval of sanitary rules SP 2.1.3678-20 "Sanitary and epidemiological requirements for the operation of premises, buildings, structures, equipment and transport, as well as the conditions of activity of business entities carrying out sale of goods, performance of work or provision of services." Available from: https://docs.cntd.ru/document/573275590

[16] Order of the Ministry of Health of Russia dated November 24, 2021 No. 1094n On approval of the Procedure for prescribing drugs, forms of prescription forms for drugs, the Procedure for registration of these forms, their accounting and storage, forms of prescription forms containing the prescription of narcotic drugs or psychotropic substances, the Procedure for their production , distribution, registration, accounting and storage, as well as Rules for the preparation of prescription forms, including in the form of electronic documents. Available from: https://docs.cntd.ru/document/727251258

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with Order of the Ministry of Health of Russia No. 1093n[17] dated November 24, 2021. [26].

Trade channels, dispensing and sale of drugs by subjects of the drug circulation in the Russian Federation are presented in Figure 4.

The quality of drugs must comply with the requirements of the State Pharmacopoeia of the Russian Federation, which is a set of general pharmacopoeial monographs that describe the general requirements for the drug, as well as methods and techniques for monitoring the drugs quality, and pharmacopoeial monographs containing the requirements for the quality of a specific drug.

EDs sales volume

The right of citizens to the medication provision is the prerogative of every citizen of the Russian Federation for health protection, enshrined in Art. 41 of the Constitution of the Russian Federation [27, 28]. In the Russian Federation, nowadays, medical care to the population is provided by a system of government institutions [29], which are financed using different levels of a budgetary support of the Russian Federation. According to the general principle, the drug provision to the population of the Russian Federation when providing free medical care, is carried out within the framework of the List of Essential Medicines (EMs), which is annually approved by the Government of the Russian Federation[18] [30]. The availability of the medications at no cost to the population is fixed in accordance with the basic compulsory medical insurance program, in accordance with Art. 80 federal Law No. 323-FZ dated November 21, 2011 (hereinafter – FL No. 323)[19]. Each citizen of the Russian Federation deducts 5.1% of their salary to finance the Compulsory Medical Insurance Fund [31, 32], from which the above mentioned program is financed (Chapter 34 of the Tax Code of the Russian Federation), while tax payments are carried out by the employers. The latter led to the division of the Russian pharmaceutical market into a commercial segment (Pos sales) [33] and a government segment (purchases of healthcare organizations in accordance with Federal Law No. 44-FZ dated April 5, 2013 (hereinafter – FL No. 44)[20] Dynamics of the pharmaceutical Russian market is shown in Figure 5[21].

According to the previously mentioned study [9], in 2022, it was determined that the total number of the drugs compounded in pharmacies amounted to 16.9 million units. According to the requirements of healthcare organizations, in the structure of compounded drugs, the demand for services (jobs) for the production of drugs and in-pharmacy packaging of approval drugs prevails: 34% of the total number of compounded and dispensed units are compounded drugs in the form of in-pharmacy blanks – pre-compounded drugs intended for dispensing according to the most frequently received prescriptions for drugs or requirements of healthcare organizations (Fig. 6).

At the moment, according to the expert assessment of the authors of the article, the total market for the EDs circulation in the Russian Federation is about 3.5–4.0 billion rubles (38.0–44.0 million USD at the date of this study publication), herewith, it is represented with outdated formulations, where almost 95% falls on the outpatient segment of dispensing in the retail segment. It is worth noting that the insufficient supply to the healthcare community for the use of modern formulations and the lack of systemic demand from the healthcare system, both in the segments of regional and federal benefits, and from the healthcare organizations participating in the compulsory health insurance system, the volume of the EDs market retains its small share, but has a significant potential and prerequisites for development. A limited demand is primarily associated with the outdated infrastructure of the POs and its systemic stagnation, a bias towards a retail trade of approval drugs, which is generally caused by the current provisions of the law. Despite the annual increase in the need for EDs, their share in the structure of the total circulation of approval drugs is less than 0.4% [9].

[17] Order of the Ministry of Health of Russia dated November 24, 2021 No. 1093n "On approval of the Rules for the dispensing of drugs for medical use by pharmacy organizations, individual entrepreneurs licensed to carry out pharmaceutical activities, medical organizations licensed to carry out pharmaceutical activities, and their separate divisions (outpatient clinics , paramedic and paramedic-midwife stations, centers (departments) of general medical (family) practice) located in rural settlements in which there are no pharmacies, as well as the Rules for the dispensing of narcotic drugs and psychotropic substances registered as drugs for medical use, drugs for medical use containing narcotic drugs and psychotropic substances, including the Procedure for the dispensing of immunobiological drugs by pharmacies." Available from: https://docs.cntd.ru/document/727251237

[18] Order of the Government of the Russian Federation dated October 12, 2019 No. 2406-r "On approval of the list of vital and essential drugs for medical use for 2020, the list of drugs for medical use, including drugs for medical use prescribed by decision of doctors commissions of medical organizations, a list of medications intended to provide people with hemophilia, cystic fibrosis, pituitary dwarfism, Gaucher disease, malignant neoplasms of lymphoid, hematopoietic and related tissues, multiple sclerosis, hemolytic-uremic syndrome, juvenile arthritis with systemic onset, mucopolysaccharidosis I , II and VI types, persons after organ and (or) tissue transplantation and the minimum range of medications necessary to provide medical care." Available from: https://docs.cntd.ru/document/563469457

[19] Federal Law of November 21, 2011 No. 323-FZ "On the fundamentals of protecting the health of citizens in the Russian Federation." Available from: https://docs.cntd.ru/document/902312609

[20] Federal Law of April 5, 2013 No. 44-FZ "On the contract system in the field of procurement of goods, works, services to meet state and municipal needs." Available from: https://docs.cntd.ru/document/499011838

[21] DSM Group reports. Available from: https://dsm.ru/news-reports/?category=13

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Retrospective of the regulatory impact on the pharmaceutical compounding segment

Today, precision and translational kinds of medicine are considered a new healthcare paradigm. However, an individual approach to the treatment of diseases, taking into account all factors of the health status of a particular person, is not new for Russia and is reflected in the works by great Russian doctors and pharmacists of the past [34, 35]. There are many examples of drugs with different pharmacokinetic profiles between children and adults, highlighting the importance of understanding pediatric physiology and the potential impact on drug concentrations [36]. Dose adjustments are made to ensure an appropriate internal exposure and pharmacodynamic effects. However, these parameters depend on the specific properties of the drug and the ontogenesis of the corresponding physiological processes in the patient. A recent review [37] of approval clinical trials in children reported that the pharmacokinetic data had been collected in only 24% of all ongoing trials, with the majority being conducted in children over 2 years of age in North America. The need of a number of population groups for individual dosages of drugs in each state makes their industrial manufacturing impossible due to a low profitability. At the same time, the pharmacoeconomic advantages of pharmacy compounding drugs in highly specialized, high-cost nosological units of diseases (orphan, oncological ones, etc.) are telling of one thing – pharmacy compounding drugs and their industrial manufacturing complement each other, must be developed and improved in parallel, while pharmacy compounding drugs is a universal tool for every doctor in the pharmacotherapy of patients. The latter thesis is clear to all market participants and can be traced throughout the scientific literature, starting from the USSR and ending with our time [1, 3].

Over the past 20 years, the pharmaceutical compounding segment has undergone a global transformation in the USA and EU countries. Currently, it is impossible to consider drug compounding activities in isolation from the principles and recommendations of GMP. Thus, in the USA, the organizations involved in the drugs compounding are divided into two types: pharmacies (type 503A) and outsourcing facilities (type 503B), where the former must have a license for pharmaceutical activities and comply with the requirements of chapters 795 and 797 of the US Pharmacopoeia, and the latter must be certified for compliance with cGMP rules. Herewith, Chapters 795 and 797, among other things, contain the implementation of the provisions of these rules. In the EU, there is a ResAP (2011) 1 on quality and safety assurance requirements for drugs prepared in pharmacies for the special needs of patients, according to which the Guide to Good Manufacturing Practice for drugs (PIC/S) is recommended to be used as a reference book in the manufacture of the group of “hazardous drugs”, and the Guide to Good Manufacturing Practice for drugs (PIC/S) for the manufacture of “low-risk medicinal drugs”. The latter is an adaptation of the GMP rules for the activity of drug compounding and includes 9 main chapters of the rules of Good Manufacturing Practice [1, 2, 11, 38].

Within the framework of the USSR policy, the concept of drug production quality was based on stage-by-stage (operational) and final quality control of finished products. Herewith, the main priority of the chemical and pharmaceutical industry of the USSR was the volume of products produced. In such an iteration, taking into account the interpretation of the GMP concept as “no more than the modernization of technical means of production (buildings and equipment)” [39] in the absence of a policy for exporting drugs (where compliance with GMP rules is a condition for importing drugs into the EU), on the part of domestic manufacturers and regulators put forward the thesis that the costs of implementing GMP requirements in the USSR were too high. In the Russian Federation, the following standards were successively adopted: OST 42-510-98[22], GOST R 52249-2004[23], GOST R 52249-2009[24], which are a compilation of various GMP rules (WHO, USA, EU). The final transition of the domestic pharmaceutical industry to GMP rules took place in 2013 through the adoption of Order No. 916[25] of the Ministry of Industry and Trade of Russia dated June 14, 2013, which was generally consistent with European GMPs. The requirement to comply with these rules has become mandatory since 2014 [39–42]. From 2021, Russian drug manufacturers must comply with the EAEU GMP Rules, while the issuance of national certificates has been discontinued [43].

In the Soviet Union, the described problems of transition to GMP requirements affected the pharmaceutical production of drugs in the Russian Federation. Until 1997, there were regulatory legal requirements for the implementation of this type of activity, developed in the USSR and, accordingly, containing outdated approaches to

[22] Order of the Ministry of Health of Russia and the Ministry of Economy of Russia dated December 3, 1999 No. 432/512 “On the implementation of the Industry Standard OST 42-510-98 “Rules for the organization of production and quality control of drugs (GMP).” Available from: https://dokipedia.ru/document/5180069

[23] National standard of the Russian Federation GOST R 52249-2004 “Rules for the production and quality control of drugs.” Available from: https://docs.cntd.ru/document/1200071754

[24] National standard of the Russian Federation GOST R 52249-2009 “Rules for the production and quality control of drugs.” Available from: https://docs.cntd.ru/document/1200036160

[25] Order of the Ministry of Industry and Trade of Russia dated June 14, 2013 No. 916 “Rules for the production and quality control of drugs.” Available from: https://docs.cntd.ru/document/499029882

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both drug compounding technologies themselves, and to methods and techniques for monitoring their quality. The regulatory documents adopted in 1997 describing the quality control of EDPs[26] compounding, standards of deviations in the EDPs[27], technology for the production of liquid dosage forms[28], the sanitary regime of compounding parmacies[29], qualitatively and quantitatively repeated the regulatory legal documents that had been in force in the USSR, creating additional discrepancies in some of their provisions, which can be traced as a result of the historical and technical analysis of the development of regulation and changes in legislation in the field of drug compounding [1].

In the next two decades, Fl No. 61 was adopted, one of the main objectives of which was the harmonization of the Russian legal regulation with international principles and standards adopted in relation to the circulation of drugs [44]. Since 2010, the process of transition to GxP began, which is currently reflected at the following levels:

– preclinical studies, which are regulated by the Rules of Good Laboratory Practice (Art. 11 of the FL No.61) [45, 46];

– production of drugs, which is regulated by the EAEU GMP Rules (Art.45 of the FL No. 1);

– wholesale trade, which is regulated by the EAEU GMP Rules (Art. 54 of the FL No.61);

– retail drug trade, which is regulated by the Rules of Good Pharmacy Practice (Order No. 647n, Art. 55 of the FL No. 61).

In this concept of a new regulation of the drug circulation market, the professional community expected a further implementation of GxP principles in the pharmaceutical compounding segment of drugs. However, in 2015, the Rules for Good Manufacturing Practice for drugs were adopted, approved by Order of the Ministry of Health of Russia No. 751n dated October 26, 2015 (hereinafter referred – Order No. 751n) [30], where, on the one hand, an attempt was made to collect previously existing orders, methodological recommendations and instructions regarding the compounding of drugs in POs, and on the other hand, the existing world practice and approaches to the processes of pharmaceutical compounding, a quality control, and studying the stability of EDs were left without attention [1].

Since 2010, Art. 56 of the FL No. 61 contains a ban on the compounding of AD, which significantly limited the activities of POs and led to a reduction in the number of compounding pharmacies in all constituent entities of the Russian Federation. After the adoption of the FL No. 61, Roszdravnadzor published a letter[31] regarding the norms of Art. 56, which indicated the limited ability of the POs to ensure the appropriate level of the quality for compounded drugs, which was the main reason for introducing restrictions on the compounding of AD. However, the results, once again confirmed and obtained over the past decade, allow us to say with confidence that EDs are an integral element of providing medical care to the population, and the level of development of technological and engineering systems allows us to ensure an appropriate level of the ED quality, comparable to the requirements of GxP and processes pharmaceutical facilities [1, 2].

Current state of legal and regulatory framework of pharmaceutical drugs compounding in the Russian Federation

In 2019, a group of deputies led by Ayrat Zakievich Farrakhov introduced draft Federal Law No. 798952-7 "On Amendments to Part 2 of Art. 56 of the Federal Law "On drug circulation" (hereinafter – Draft Law No. 798952-7)[32], which expanded the powers of compounding parmacies, allowing the compounding of drugs from ADs, and also eliminated the ban on the compounding of the latter. The proposal of Draft Law No. 798952-7 to eliminate this limitation was determined by the need to satisfy the requirements of patients for individual dosages of drugs, incl. ultra-small quantities, to meet the needs of pediatric practice, and the drugs approval in the SRMRs; but temporarily absent from the pharmaceutical market of the Russian Federation, through their compounding in POs. The explanatory note to Draft Law No. 798952-7 also led to a significant reduction in the range and quantity of compounded drugs, including the massive closure of compounding pharmacies in all regions of the Russian Federation. Draft Law No. 798952-7 was adopted on December 5, 2022 in the form of Federal Law No. 502-FZ dated December 5, 2022 "On Amendments to Art. 56 of the Federal Law "On Drug Circulation" (hereinafter – FL No. 502)[33] with

[26] Order of the Ministry of Health of Russia dated July 16, 1997 No. 214 "On quality control of drug compounding in pharmacy organizations (pharmacies)." Available from: https://docs.cntd.ru/document/902062371

[27] Order of the Ministry of Health of Russia dated October 16, 1997 No. 305 "On the norms of deviations permissible in the drug compounding and packaging of industrial products in pharmacies." Available from: https://docs.cntd.ru/document/901701705

[28] Order of the Ministry of Health of Russia dated October 21, 1997 No. 308 "On approval of instructions for the production of liquid dosage forms in pharmacies." Available from: https://docs.cntd.ru/document/901702358

[29] Order of the Ministry of Health of Russia dated October 21, 1997 No. 309 "On approval of the Instructions for the sanitary regime of pharmacy organizations (pharmacies)." Available from: https://docs.cntd.ru/document/901701706

[30] Order of the Ministry of Health of Russia dated October 26, 2015 No. 751n "On approval of the rules for the compounding and dispensing of drugs for medical use by pharmacy organizations and individual entrepreneurs with a license for pharmaceutical activities." Available from: https://docs.cntd.ru/document/420313316

[31] Letter of Roszdravnadzor dated June 1, 2010 No. 04I-516/10 "On the quality of injection and infusion solutions of compounding pharmacies ." Available from: https://docs.cntd.ru/document/902218497

[32] Materials for bill No. 798952-7 "On amendments to Part 2 of Article 56 of the Federal Law "On the Circulation of Drugs". Available from: https://sozd.duma.gov.ru/bill/798952-7

[33] Federal Law of December 5, 2022 No. 502-FZ "On Amendments to Article 56 of the Federal Law "On the Circulation of Drugs". – Available from: https://docs.cntd.ru/document/1300131660

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the starting date of coming into force on September 1, 2023. However, the provisions that would have lifted the ban on the compounding of ADs were excluded from it [1].

In January 2023, a specialized Working Group was created to form a unified system of legal and regulatory framework of activities in the field of drug compounding under the State Duma Committee on Health Protection (hereinafter – Working Group), whose activities are aimed at accelerating and preparing for the implementation of the norms of FL No. 502 in terms of drugs compounding and making necessary amendments to the delegated legislation and legal and regulatory framework.

During the period from March 28 to April 7, 2023, in accordance with paragraph 4 of the first meeting protocol No. 1 of the Working Group dated January 26, 2023, monitoring of compoundings pharmaciesactivities in Russia was carried out [9]. It was aimed at identifying key infrastructural, technological and personnel characteristics of the compounding pharmacies segment. As of March 28, 2023, 1 019 legal entities and individual entrepreneurs operating at 1 378 addresses, had the right to drug compounding. The study was conducted within the framework of a sample presented by the State Duma, the structure of which included 643 addresses at the place of pharmaceutical activities, which in general accounted for 46.7% of the total number of addresses for the activities of compounding and dispensing drugs.

Based on the results of the POs survey, it was found that a part of the compounding pharmacies – 17 out of 47 (7.3%) ceased their activities in the period from 2015 to 2019. Most of these organizations were in the Far Eastern Federal District (35.3%), the Central Federal District (26.9%) and in the North Caucasus (22.2%). The respondents noted that the main factors that had influenced this decision were:

– lack of demand for compounded drugs within the framework of regional state guarantee programs, both at the expense of compulsory health insurance and preferential drug provision;

– outdated infrastructure, lack of proper equipment with technological, analytical, engineering equipment and lack of financial measures of state support;

– problems in concluding and executing contracts for the provision of services (work) for the drugs compounding and in-pharmacy packaging of approval drugs within the legal and regulatory framework of the contract system in the field of procurement (FL No. 44);

– almost complete nomenclature (physical) and price unavailability of substances and excipients in small packages, including the lack of a number of necessary raw materials.

As a result of monitoring, it was established that the total area of all pharmacies surveyed was 36 282 m^2; 8 149 m^2 of that area was in the "segregated compounding area" and 4 760 m^2 – in the "clean rooms". The extrapolation of the results showed that the total number of production facilities that needed reconstruction is more than 140 000 m^2. This study also provides statistics regarding the classification of pharmacies into retail entities and hospital pharmacies. It shows the distribution according to the list of services (jobs) provided that constitute pharmaceutical activities with the right to compounding and dispense drugs, and touches upon the issue of the of pharmacy specialists' structure. That revealed an acute shortage of chemists-analysts and (or) pharmacy technicians, which indicates a high risk of suspension of activities at any time. The results of the study demonstrated and confirmed the "traditional" [1, 2, 9–11] problems of compounding pharmacies, accumulated over a long period of time, i.e., since the formation of the Russian Federation.

In the framework of this study, one cannot help dwelling on the requirements for compounding pharmacies. In accordance with SanPiN 2.1.3678-20, they are:

– a pharmacy must be located in an isolated block of premises in apartment buildings, public buildings or in separate buildings;

– pharmacy premises must have natural and artificial lighting. Natural lighting may be absent in warehouses (without a permanent workplace), storerooms, toilets, dressing rooms, showers, household and auxiliary premises;

– the premises of the aseptic unit are equipped with a ventilation system with a predominance of inflow over exhaust. The supply of clean air is carried out by laminar airflows;

– pharmacy premises must be subjected to daily wet cleaning using detergents and disinfectants. Pharmacy must be provided with a 3-day supply of detergents and disinfectants, which is calculated taking into account the area of surfaces to be treated, the amount of equipment to be processed, and the availability of household equipment to ensure sanitary conditions;

– cleaning of all premises with the treatment of walls, floors, equipment, implements, lamps using detergents and disinfectants should be carried out at least once a month, and in premises for the drug compounding under aseptic conditions – weekly.

In addition, compounding pharmacies have established requirements for the microbiological purity of air. At the same time, there are no standards regulating the content of the maximum permissible amount of particles in the air, while class A of microbiological purity SanPiN 2.1.3678-20 is equal to class D of the EAEU GDP Rules (Table 1).

The final scheme of subordination of compounding pharmacies to the main governing documents is presented in Figure 7.

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In May 2023, Order No. 249n was signed, where, on the one hand, it was possible to partially reflect and lay the foundations for the development of the POs quality assurance system, but on the other hand, it was not possible to solve the problem of improving the operation of compounding pharmacies at the level of developed healthcare systems, where the number of significant restrictions of Order No. 751n has not been eliminated, i.e.:

– list principle of nomenclature formation, which limits the fulfilment possibility of new formulations development by compounding pharmacies;

– absence of the POs possibility to independently determine expiration dates by conducting studies of the EDs stability;

– text of the Order describes significant general technological limitations associated with direct indications of a specific technology for the compounding of dosage forms or the use of specific, often unqualified, equipment, which limits the opportunities for independent development of compounding technologies and methods for the quality EDP control by compoundings pharmacies;

– lack of principles, methods and validation tools, which does not allow compoundings pharmacies to carry out research and development work, thereby eliminating cooperation with research and educational organizations, including the implementation of the results of research and development obtained by them (a technology transfer);

– Order contains excessive requirements for a "100% quality inspection" of compounded drugs at all stages of the compounding process, which does not correspond to international regulatory practice, experience in implementing the principles of good pharmacy practices, and in general will be a key factor in negative profitability in pharmacy compounding drugs;

– Order includes quality control requirements higher than for drug manufacturers. So, for example, when producing one injection or infusion solution of the same dosage and packaging, in the amount of 2 (two) units (doses), a compounding pharmacies must conduct an aseptic study and test for pyrogenicity or bacterial endotoxins, which in total will cost more than 6,000 rubles (80 USD on the date the manuscript was submitted for printing); at the same time, the compounding pharmacies will be made to produce a third unit of solution, which will be sent for the analysis.

To date, for the implementation of activities for the diluting (reconstitution) of drugs [47–49], no requirements or rules have been established, which is implemented in healthcare organizations without a licensing procedure. The only mention of this activity is the requirement[34] for healthcare institutions providing medical care in the "Oncology" profile to have a laminar airflow workbench for an aseptic diluting (reconstitution) of drugs or a class 2 biological safety cabinet, which differs significantly from the approaches of healthcare institutions to working with highly hazardous substances in developed healthcare systems [1], and also increases the risks of toxic effects on medical and pharmaceutical specialists.

In the framework of the 3rd meeting of the Working Group, held on June 29, 2023, the relevance and demand for EDs in the segments of oncology, pediatrics, orphan and other diseases were noted. The importance of developing the activities of compoundings pharmacies in terms of intra-pharmacy packaging of approval drugs was especially emphasized, as well as the feasibility of transitioning from the rules for compounding and dispensing of drugs (the Soviet regulatory system) to the rules of Good Manufacturing Practice for MPs (the modern regulatory system).

In August 2023, the work on the preparation of the State Pharmacopoeia of the Russian Federation (XV edition) was completed, as well as the development of the necessary general pharmacopoeial monographs in the field of the drug production within the time frame agreed with the Russian Ministry of Health. In order to increase the efficiency of the processes of their preparation and adoption, a separate expert section for the standardization of pharmaceutical preparations was created at the Institute of Pharmacopoeia and Standardization in the sphere of drug circulation of the Federal State Budgetary Institution "Scientific Center for Evaluation of Medical Products" of the Ministry of Health of the Russian Federation (hereinafter – SCEMPs) [50], subordinate to the Ministry of Health of Russia.

From the point of view of the government budgetary policy, the development of modern pharmacy infrastructure in the field of drug compounding in the Russian Federation will help improve the efficiency of costs at all levels of the healthcare system. The goal of optimizing drug costs is to compensate for the actual volume of the drugs required for a specific patient per unit of time. Medical and economic standards and calculations for the provision of medical care, both within the framework of the program of state guarantees of free medical care provision to citizens, and at the expense of citizens' own funds, should be guided by the methods of cost accounting accepted in international practice within the framework of the course, daily or annual need for drugs [1, 11].

Compounding pharmacies, as an element of the healthcare infrastructure, are also of key importance for optimizing budget costs in terms of reducing the level of drug inventory. In particular, the data analysis results of the federal project implementation of "Combating Cardiovascular Diseases", carried out by

[34] Order of the Ministry of Health of Russia dated February 19, 2021 No. 116n "On approval of the Procedure for providing medical care to the adult population for cancer." Available from: https://docs.cntd.ru/document/573956757

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the Accounts Chamber of the Russian Federation at the end of 2022, show a high level of inventory balances of drugs intended to provide people who have suffered a stroke or heart attack[35]. Thus, as of January 1, 2022, in 54 regions, the level of such balances for a number of drugs exceeded 24 months (with a 2–3 year shelf life for the specified category of patients), the report indicates the risk of potential write-off of drugs totaling 4 671.6 million rubles due to the expiration of their shelf life. The latter became a reality; from the beginning of 2022 to May 2023, the State Budgetary Institution of Higher Education "Center for the Procurement in the Healthcare Sector of the Vladimir Region" wrote off drugs for a total amount of 58.6 million rubles due to expiration dates, 32.1 million rubles of which had been spent on the drugs purchased as a part of the regional project "Combating Cardiovascular Diseases"[36]. The described indicates the need to increase the efficiency of using budget funds at any level allocated for the purchase of drugs; in resolving this issue, the development of the activities of compounding pharmacies in the field of individual in-pharmacy packaging of approval drugs will be of particular importance, the implementation of which will ensure the modernization of the accounting system from packages to the accounting of course doses, will eliminate budget overspending within the current drug supply system.

CONCLUSION

In order to provide conditions for the development of a competitive, sustainable and structurally balanced industry in Russia, in 2014, the Government of the Russian Federation approved the state program "Development of Pharmaceutical and Medical Industry", which over the next 10 years, made it possible to create a necessary level of the material and technical base for the implementation of the stages of the ADs production up to 82% within the List of EDs. The Strategy for the Development of the Pharmaceutical Industry in the Russian Federation for the period until 2030 (hereinafter – Strategy), approved by the Decree of the Government of the Russian Federation No. 1495-r dated June 7, 2023, especially emphasizes a close relationship between manufacturers and compounding pharmacies, which consists in the unity of principles based on meeting the needs of the healthcare system to the greatest possible extent and ensure an uninterrupted access for the citizens of the Russian Federation to the required range of drugs. In particular, Section 3 of the Strategy establishes the priorities for its implementation, which include (including, but not being limited to): the development of gene and targeted therapy technologies, new treatment methods, including the use of biomedical cell products; development, implementation and use of new medical technologies and drugs in accordance with the Strategy for the development of healthcare in the Russian Federation.

One of the main directions for the implementation of the Strategy, set out in section 4 of the Strategy "Main directions for the implementation of the Strategy", is the creation of prerequisites for the development of the personalized therapy segment, new treatment methods, stimulating the development of conditions for localizing the production of in-demand drugs in case of a limited supply at the national pharmaceutical market, as well as building stable supply chains in order to ensure the physical and economic accessibility of drugs. From the point of view of training scientific, technological and production personnel for the Russian compounding pharmacies, subsection 9 of section 4 of the Strategy "Main directions for the implementation of the Strategy" also notes the need to implement measures aimed at further developing competencies in the field of development of drugs intended for the treatment of socially significant diseases that prevail in the structure of morbidity and mortality of the population of the Russian Federation, as well as the diseases that pose a danger to others, including in pharmacies, using semi-industrial equipment and production packaging of APIs in small doses. The basis for the further development of the pharmaceutical industry, including the development of compounding pharmacies, and the introduction of personalized medicine methods, is the expansion of an access provision of the pharmaceutical infrastructure to raw materials – API (especially in small packages), pharmaceutical grade excipients, reagents, packaging, closures and other consumables materials that are used both in the manufacture of drugs and in the compounding of drugs.

Taking into account the existing prerequisites in the development of the pharmaceutical compounding segment of drugs, i.e., the adoption of FL No. 502, the Strategy, the creation of a specialized Working Group and a separate expert section for the standardization of pharmaceutical drugs at the SCEMPs, and also understanding that the need for personification of pharmacotherapy is unlikely to decrease in the near future, with a simultaneous permanent increase in the financial burden on all budgets of the healthcare system, the next most important step in the development of compounding pharmacies will be the formation of a unified harmonized system of legal regulation of the EDP circulation. Taking into account the experience of global healthcare systems, the basic concept of the necessary

[35] Accounts Chamber of the Russian Federation. Appendix No. 4 to the report on the work of the Accounts Chamber of the Russian Federation in 2022 "Report on the work of the audit of healthcare and sports of the Accounts Chamber of the Russian Federation in 2022." Available from: https://ach.gov.ru/reports/report_2022

[36] Federal Project "Combating Cardiovascular Diseases". Available from: https://minzdrav.gov.ru/poleznye-resursy/natsproektzdravoohranenie/bssz

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measures for the development of modern pharmacy infrastructure in the Russian Federation assumes that the current system of regulation of compounding pharmacies will be fundamentally rethought, improved and finalized, where the key legislative initiatives at the federal level should be:

1. The changes made to FL No. 323 in terms of expanding the possibilities of using EDPs and including them in clinical recommendations, as well as to FL No. 326-FZ dated November 29, 2010 "On Compulsory Health Insurance in the Russian Federation" on the tariff structure of the basic program compulsory health insurance, by supplementing it with regulations on the use of medical services (jobs) for the production of drugs and packaging of approval drugs by compounding pharmacies.

2. The changes made to FL No. 61 (including, but not limited to) in terms of the transition from the rules of good manufacturing and dispensing of drugs to the rules of good manufacturing and dispensing practices for drugs, thereby achieving the goals of completing the GxP concept in the legislation on the circulation of drugs in the Russian Federation , as well as to ensure the quality, safety and effectiveness of electronic drugs, including the processes of in-pharmacy packaging of approval drugs.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

All authors made equivalent and equal contributions to the preparation of the publication. All authors made a substantial contribution to the conception of the work, acquisition, analysis, interpretation of data for the work, drafting and revising the work, final approval of the version to be published and agree to be accountable for all aspects of the work.

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ОБЗОРЫ

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Overview of drugs approved by the FDA in 2022

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The aim of the work was to conduct a review of drugs approved by the FDA in 2022.

Materials and methods. In searching for the materials to write this review article, bibliographic databases including PubMed, Google Scholar and e-library.ru were utilized. The search was conducted for the publications spanning the period from 2008 to 2023. Herewith, the following keywords and word combinations were used: new drug approval, NDA, drug authorization, approval package, breakthrough medicine.

Results. The discovery and development of drugs are among the most crucial scientific processes in healthcare. Developing a new drug is a highly intricate, expensive, and time-consuming process. Nowadays, the problem of costs reduction and the process of expedited discovering of new medications are particularly pertinent. To optimize the search for active compounds, virtual and high-throughput screenings, machine learning, artificial intelligence, cryo-electron microscopy, and drug repurposing are employed. Simultaneously, the search for original molecules to serve as the basis for innovative drugs continues. This article presents a review of medications approved by the FDA in 2022 for the treatment of various pathologies.

Conclusion. A drug development is a complex and resource-intensive process, with only a small fraction of candidates advancing to clinical trials. A drug design evolves in tandem with societal needs, and this review highlights some of the drugs approved by the FDA in 2022. Technological advancements are expected to expedite drug development, potentially reducing the time to the market. Biotechnology, including cell therapy, holds significant prospects, and achievements in genetic mapping and chip technologies will enhance the accessibility of personalized pharmacology.

Keywords: FDA; biopharmaceuticals; monoclonal antibodies; drug design trends

Abbreviations: ADMET – absorption, distribution, metabolism, excretion, and toxicity; AI – artificial intelligence; Ang2 – angiopoietin 2; ANN – artificial neural networks; CALD – cerebral adrenoleukodystrophy; CNN – convolutional neural networks; cryo-EM – cryogenic electron microscopy; CVDs – cardiovascular diseases; DL – deep learning; EMA – European Medicines Agency; FBS – fragment-based screening; HTS – high throughput screening; FDA – Food and Drug Administration; GIP – gastric inhibitory polypeptide; GLP-1 – glucagon-like peptide-1; IL – interleukin; ISMC – International Symposium on Medicinal Chemistry; JAK1 – Janus kinase 1; LDA – linear discriminant analysis; MHRA – The Medicines and Healthcare products Regulatory Agency; ML – machine learning; MLP – multilayer perceptron; NME – New Molecular Entity; NYHA – New York Heart Association; PD-1 – programmed cell death 1 receptor; PDB – Protein Data Bank; QSAR – quantitative structure-activity relationship; RF – random forest; RNN – recurrent neural networks; SBDD – structure-based drug discovery; SVM – support vector machine; TSLP – thymic stromal lymphopoietin; VEGF – vascular endothelial growth factor; VLCFAs – very long chain fatty acids; R&D – research and development; CNS – central nervous system; CT – clinical trials.

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Обзор лекарственных средств, одобренных FDA в 2022 году

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Цель. Анализ актуальных тенденций зарубежной практики в области разработки и регистрации лекарственных препаратов.

Материалы и методы. При поиске материала для написания обзорной статьи использовали реферативные базы данных PubMed, Google Scholar и e-library.ru. Поиск осуществляли по публикациям за период с 2008 по 2023 год, с использованием следующих ключевых слов: «new drug approval», «NDA», «drug authorization», «approval package», «breakthrough medicine».

Результаты. Открытие и разработка лекарственных средств являются одними из наиболее важных научных направлений в здравоохранении. Разработка нового препарата – очень сложный, дорогой и длительный процесс. Как снизить затраты и ускорить открытие новых лекарств? Этот вопрос является особенно актуальным на сегодняшний день. Для оптимизации процесса поиска активных соединений используются виртуальный и высокопроизводительный скрининг, машинное обучение, искусственный интеллект, криоэлектронная микроскопия, а также перепрофилирование существующих лекарственных средств. В то же время продолжается поиск оригинальных молекул для разработки на их основе инновационных препаратов. В данной статье представлен обзор лекарственных средств, одобренных в 2022 году Food and Drug Administration (FDA), для лечения различных патологий.

Заключение. Разработка лекарств представляет собой сложный и ресурсоемкий процесс, при котором лишь небольшая часть кандидатов доходит до клинических испытаний. Дизайн лекарств развивается вместе с потребностями общества, и в этом обзоре освещаются некоторые из препаратов, одобренных FDA в 2022 году. Ожидается, что технологические достижения ускорят разработку лекарств, потенциально сокращая время их выхода на рынок. Биотехнология, включая клеточную терапию, имеет большие перспективы, а достижения в области генетического картирования и технологий чипов повысят доступность персонализированной фармакологии.

Ключевые слова: Food and Drug Administration; FDA; биофармацевтика; моноклональные антитела; тенденции лекарственного дизайна

Список сокращений: ADMET – абсорбция, распределение, метаболизм, экскреция и токсичность; AI – искусственный интеллект; Ang2 – ангиопоэтин 2; ANN – искусственные нейронные сети; CALD – церебральная адренолейкодистрофия; CNN – сверточные нейронные сети; cryo-EM – криоэлектронная микроскопия; ССЗ – сердечно-сосудистые заболевания; DL – глубокое обучение; EMA – Европейское агентство по лекарственным средствам; FBS – фрагментарный скрининг; HTS – высокопроизводительный скрининг; FDA – Управление по контролю за продуктами и лекарствами; ГИП – глюкозозависимый инсулинотропный полипептид; ГПП-1 – глюкагоноподобный пептид-1; IL – интерлейкин; ISMC – Международный симпозиум по медицинской химии; JAK1 – янус-киназа-1; LDA – линейный дискриминантный анализ; MHRA – Агентство по регулированию лекарственных средств и товаров медицинского назначения; ML – машинное обучение; MLP – сеть многослойного персептрона; NME – новые молекулярные соединения; NYHA – Нью-Йоркская кардиологическая ассоциация; PD-1 – рецептор программируемой смерти клеток 1; PDB – Банк данных белков; QSAR – количественное соотношение структура-активность; RF – метод случайного леса; RNN – рекуррентные нейронные сети; SBDD – структура-зависимое исследование лекарственных препаратов; SVM – метод опорных векторов; TSLP – стромальный лимфопоэтин тимуса; VEGF – фактор роста эндотелия сосудов; VLCFAs – жирные кислоты с очень длинной цепью; ЛС – лекарственное средство; НИОКР – научно-исследовательские и опытно-конструкторские работы; ЦНС – центральная нервная система; КИ – клинические исследования.

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INTRODUCTION

The search for new drugs is a long and complex process, which can be roughly divided into four main phases: (i) target identification and validation; (ii) compound screening and optimisation of hit structures; (iii) pre-clinical studies; and (iv) clinical trials [1]. Completion of a typical drug development cycle from a target identification to an FDA-approved drug takes up to 14 years at an estimated cost of $800 million [2–4]. For this reason, large corporations are continually searching for new methods to accelerate this process, as well as monitoring new technologies from other areas of research [5]. Various approaches are used to optimise the process of active compounds searching: virtual screening [4], machine learning [6], artificial intelligence [1], high throughput screening [7]. Cryo-electron microscopy is a rapidly developing tool for investigating drugs based on their chemical structures [8].

Furthermore, the protracted timeline for the emergence of drug candidates has opened avenues for the repurposing and repositioning of existing medications. Drug repurposing entails the utilization of drugs previously sanctioned for treating established pathologies, as authorized by regulatory agencies such as the Food and Drug Administration (FDA), the European Medicines Agency (EMA), and the Medicines and Healthcare products Regulatory Agency (MHRA) [9]. This paper provides a selective overview of the strides made within the global pharmaceutical industry, with a specific focus on market leaders and enterprises committing substantial financial resources to the research and development of pharmaceuticals and healthcare products.

THE AIM of this study is to analyze current trends in the development and registration of pharmaceutical drugs.

MATERIALS AND METHODS

The literature search (including results of clinical studies, literature reviews, and, in some cases, information on preclinical studies) was based on the information available on the official websites of the FDA and EMA related to the issuance of registration certificates for new pharmaceutical drugs, as well as in bibliographic databases like PubMed, Google Scholar and e-library.ru. The search encompassed publications from 2008 to 2023. The list of keywords included (but was not limited to) the following ones: new drug approval, NDA, drug authorization, approval package, breakthrough medicine. A total of 410 sources were analyzed. The exclusion criteria comprised earlier publications, non-English language articles, retracted articles, and the articles not directly relevant to the review topic. Furthermore, after a systematic categorization, 37 articles with duplicative information were removed. Following thorough the examination, 82 sources were deemed suitable for an inclusion in the review.

RESULTS AND DISCUSSION

Challenges of drug development

There has been an incredible increase in the amount of scientific information available on a wide range of areas of biology, including new findings on human physiology and the pathophysiology of human diseases. Research and development in the pharmaceutical industry is estimated to the cost over US$100 billion annually [10]. Clinical trials (CTs) of drugs account for up to 63% of this amount, while the cost of preclinical studies is only estimated at 32% [11]. In 2011, out of 5408 clinically investigational drugs worldwide, around 2000 were in phase I and phase II, and around 850 were in phase III [10]. It is worth pointing out that many medicinal products that have reached the phase are indicated for more than one treatment application [12].

In recent years, significant attention has been dedicated to the advancement of genomic and bioinformatic approaches for the discovery of novel disease biomarkers or potential drug targets [13]. The revolution in genomics and proteomics has resulted in the emergence of thousands of new drug targets [14]. These biomarkers can be regarded as indicators for measuring and evaluating normal biological processes or for influencing biological systems by certain agents, such as therapeutics, biological, or physical agents [15]. The utilization of biomarkers for patient selection in phase I/II studies may expedite the development of anticancer drugs [14, 16].

In spite of the favourable profile of pre-clinical studies, of course, drugs can fail in CTs or reach the market with delays as a result of serious side-effects [17]. Although the best efforts have been made, failures in CTs have increased dramatically over the past 20 years, with drop-out rates increasing between 1990 and 2010 for phase I from 33 to 46%, for phase II from 43 to 66% and for phase III from 20 to 30% [10]. The current reasons for phase II failures are: lack of efficacy (51%), safety issues (19%), strategic issues (29%) and pharmacokinetic/bioavailability issues (1%) [18]. The reasons for phase III failures are: lack of efficacy (66%), safety problems (21%),

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financial or commercial difficulties (7%) and other aspects (6%). Overall, the performance of new drugs in phase II is around 20% (51% ineffectiveness and 19% safety concerns) and it is around 50% in phase III (66% ineffectiveness and 21% safety concerns) [19]. Thus, although success in the execution of research and development (R&D) is important at each stage, the right choice of strategy at the earliest stage remains crucial.

Drug development trends by therapeutic areas

During the inaugural "International Symposium on Medicinal Chemistry" (ISMC) held in the 1970s, infectious diseases constituted a substantial focal point, comprising 30% of the proceedings in both 1970 and 1972 [20, 21]. However, the significance of this domain steadily declined, reaching less than 15% until 2016, followed by a resurgence in 2018. Meanwhile, deliberations related to central nervous system (CNS) disorders took precedence at ISMCs until the 2000s, accounting for 20.8% in the 1980s, 16.6% in the 1990s, and 16.8% in the 2000s. However, their prominence waned to third place in the 2010s (9.9%). This overall decline can be attributed to a dwindling number of CNS drug candidates in clinical development since the 1990s, indicative of waning pharmaceutical industry interest in CNS conditions [22]. In CNS-related research, the focus is gradually transitioning from psychiatry, which dominated the landscape until the late 1980s, toward neurology.

In the 1970s, cardiovascular diseases (CVDs) occupied the third position, commanding a notable share of 30% in 1974. Subsequently, CVDs ascended to the second spot in the 1980s (13.1%) and the 1990s (14.5%). Nevertheless, the number of CVD-related presentations plummeted significantly in the late 1990s, relegating this domain to the lower echelons with only 3.6% in the 2000s and 3.9% in the 2010s [20]. This corresponds to the diminishing focus on the pharmaceutical research and development in this area, leading to its stagnation spanning two decades [23].

The most conspicuous transformation has occurred in oncology. While this therapeutic realm was scarcely represented in the 1970s (2.7%), it experienced a remarkable surge in contributions by the 2000s and has dominated the ISMC program, occupying 21.5% in the 2010s. This evolution aligns with the consistent rise in the FDA-approved New Molecular Entities (NMEs) designated for cancer treatment and the preeminence of anti-tumour drug candidates, constituting 36.7% of the overall pharmaceutical R&D landscape [21].

In contrast, other therapeutic domains make more modest contributions to contemporary symposium programs [20]. Immunology and metabolic disorders each account for up to 10%, while analgesia issues contribute up to 7%, and respiratory diseases – only 1%. Several therapeutic areas have made sporadic appearances in ISMC curricula, including musculoskeletal diseases, gastrointestinal diseases, otorhinolaryngology, reproduction, sleep disorders, urology/nephrology, dermatology, and ophthalmology.

Strategies for drug design

Over the last 30 years, the targeted drug discovery has made it possible to significantly expand the list of chemotypes and pharmacophores for their development. New techniques such as high throughput screening (HTS), fragment-based screening (FBS), crystallography combined with molecular modelling, and combinatorial and parallel chemistry have made the generation of a considerable variety of chemical hit structures possible [24]. Moreover, this plethora of chemotypes can now be used as a source of compounds-tools to explore the undiscovered biological space and search for new drug targets or for phenotypic screening using systematic approaches to identify drug candidates in an agnostic manner.

Artificial Intelligence (AI) encompasses multiple domains of techniques, including reasoning, knowledge representation, decision mining, and machine learning (ML) [25]. ML employs algorithms capable of identifying patterns within datasets, which are subsequently classified [1]. Deep learning (DL), a subset of ML, involves artificial neural networks (ANN) [26], which consist of interconnected computational elements resembling "perceptrons", akin to biological neurons, replicating electrical impulse transmission in the human brain [27]. ANNs comprise nodes receiving distinct inputs, ultimately producing output signals, either single or multi-connected, through algorithmic processes to solve problems [28]. ANNs encompass various types, including multilayer perceptron networks (MLP), recurrent neural networks (RNNs), and convolutional neural networks (CNNs), all employing supervised or unsupervised learning procedures [29, 30]. Despite its merits, AI confronts substantial data challenges such as scale, growth, diversity, and data uncertainty. Within pharmaceutical drug development, datasets may encompass millions of compounds, posing challenges for traditional machine learning tools [31]. Quantitative structure-activity relationship (QSAR)-based computational models can swiftly generate numerous compounds or predict simple physicochemical parameters like logP or logD. However, these models fall short in predicting complex

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biological properties, including compound efficacy and side effects [32]. They also grapple with issues like small training datasets and experimental data errors. To surmount these challenges, recent AI approaches, including DL and modeling studies, have emerged for assessing drug molecule safety and efficacy through a big data analysis [33]. In 2012, Merck sponsored the QSAR ML task, evaluating DL's utility in pharmaceutical industry drug development [34, 35]. DL models have demonstrated superior predictive capabilities compared to traditional ML approaches across 15 candidate drug absorption, distribution, metabolism, excretion, and toxicity (ADMET) datasets [33, 36].

Virtual screening (VS) operates within the expansive virtual chemical space, presenting a spatial representation of molecules and their properties. VS endeavors to identify biologically active compounds within this space, facilitating the selection of molecules for a further evaluation [37]. Several publicly accessible chemical spaces, including PubChem, ChemBank, DrugBank, and ChemDB, support these efforts.

Diverse *in silico* methods for virtual screening, employing both structure- and ligand-based approaches, offer an enhanced profile analysis, an expedited removal of non-promising compounds, and a cost-effective selection of drug candidates [38]. Drug design algorithms, such as Coulomb matrices and a molecular fingerprint recognition, consider physical, chemical, and toxicological profiles to identify lead compounds [1].

Various strategies, encompassing predictive models, molecular similarity assessments, molecular generation processes, and *in silico* techniques, facilitate the prediction of desired compound chemical structures [39, 40]. Pereira et al. introduced the DeepVS system, capable of docking 40 receptors and 2 950 ligands with an exceptional performance in screening 95 000 ligands across these receptors [41]. Another approach employed a multi-criteria automated replacement algorithm to optimize the activity profile of a cyclin-dependent kinase-2 inhibitor, assessing their form similarity, biochemical activity, and physicochemical properties [42].

Table 1 – Current trends in FDA approved drugs by indication

Group of disorders	Number of approved drugs, absolute value (%)	Indications
Cancer	17 (21%)	Angiofibroma, hepatocellular carcinoma, melanoma, myelofibrosis, multiple myeloma, non-small cell lung cancer, acute myeloid leukemia, prostate cancer, ovarian cancer, follicular lymphoma, cholangiocarcinoma
CNS diseases	10 (12%)	Insomnia, Alzheimer's disease, major depressive disorder, multiple sclerosis, attention deficit hyperactivity disorder, seizures of various genes, anxiety disorder, cerebral adrenoleukodystrophy
Dermatological diseases	8 (10%)	Atopic dermatitis, plaque psoriasis, generalized pustular psoriasis, skin burns, rosacea
Infectious diseases	6 (7%)	Vaginal fungal infection, HIV infection, prevention of COVID-19, prevention of measles, mumps, rubella, prevention of recurrent *Clostridioides difficile* infection, Helicobacter infection
Metabolic disorders	6 (7%)	Acid sphingomyelinase deficiency, pyruvate kinase deficiency, urea cycle disorders, type 2 diabetes, transthyretin amyloidosis
Complications of anticancer therapy	5 (6%)	Chemotherapy-related neutropenia, prevention of cisplatin-induced ototoxicity
Diagnoses and examinations	5 (6%)	Not applicable
Ophthalmological disorders	5 (6%)	Open-angle glaucoma, macular degeneration, yellow spot oedema, glaucoma/intraocular hypertension, superficial anesthesia
Musculoskeletal disorders	4 (5%)	Amyotrophic lateral sclerosis, spasticity
Cardiological diseases	3 (4%)	Hypertention, coronary heart disease, angina pectoris, heart failure, hypertrophic cardiomyopathy
Haematological diseases	3 (4%)	Beta-thalassaemia, cold agglutinin disease, haemophilia B

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Table 2 – Drugs approved by FDA in 2022

No.	Date of approval	INN	Trade name	Manufacturer	Indications for use	Drug class / mechanism of action	Dosage form
1	07.12.22	Daridorexant	Quviviq®	Idorsia Ltd.	Insomnia	Dual orexin receptor antagonist (DORA)	Tablets
2	13.01.22	Mometasone Furoate And Olopatadine Hydrochloride	Ryaltris	Glenmark Pharmaceuticals, Inc.	Seasonal allergic rhinitis (SAR)	Combination of corticosteroids and antihistamines	Nasal spray
3	14.01.22	Abrocitinib	Cibinqo®	Pfizer Inc.	Atopic dermatitis	Janus kinase inhibitor (JAK) 1	Tablets
4	25.01.22	Tebentafusp	Kimmtrak®	Immunocore	Uveal melanoma	GP100-HLA bispecific peptide- directed activator of CD3 T cells	Solution for injection
5	28.01.22	Faricimab	Vabysmo	Genentech	Macular degeneration, diabetic macular edema	Bispecific antibody targeting (VEGF) and angiopoietin 2 (ANG- 2) pathways	Intravitreal Injection
6	31.01.22	COVID-19 vaccine	Spikevax®	Moderna, Inc.	COVID-19 prevention	mRNA-vaccine	Solution for injection
7	04.02.22	Sumimlimab	Enjaymo®	Sanofi	Cold agglutinin disease	Classical complement inhibitor	Solution for injection
8	04.02.22	Baclofen	Fleqsuvy	Azurity Pharmaceuticals, Inc.	Spasticity	GABA-derived skeletal muscle relaxants	Oral suspension
9	17.02.22	Mitapiwat	Pyrukynd®	Agios Pharmaceuticals, Inc.	Pyruvate kinase deficiency	Pyruvate kinase activator	Tablets
10	22.02.22	Technetium tc 99m succimer	NephroScan	–	Diagnosis	Radioactive diagnostic agent	Injection kit
11	24.02.22	Amlodipine besylate	Norliqva®	CMP Pharma, Inc.	High blood pressure, coronary heart disease, angina pectoris	Calcium channel blocker	Oral solution
12	25.02.22	Filgrastim	Releuko®	Kashiv BioSciences, LLC	Chemotherapy-related neutropenia	Recombinant human granulocyte colony-stimulating factor	Solution for injection
13	28.02.22	Ciltacabtagen autoleucel	Carvykti®	Janssen Pharmaceutical Companies	Multiple myeloma	BCMA-directed immunotherapy CAR-T	Intravenous suspension
14	28.02.22	Pacritinib	Vonjo®	CTI BioPharma Corp.	Myelofibrosis	JAK2/FLT3 multi-kinase inhibitor	Capsules
15	11.03.22	Donepezil	Adlarity®	Corium, Inc.	Alzheimer's	Acetylcholinesterase inhibitor	Transdermal system

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Continuation of table 2

No.	Date of approval	INN	Trade name	Manufacturer	Indications for use	Drug class / mechanism of action	Dosage form
16	17.03.22	Mometasone furoate monohydrate	Nasonex 24HR Allergy	Perrigo Company pic	Allergic rhinitis	Corticosteroid	Nasal spray
17	18.03.22	Ganaxolone	Ztalmy®	Marinus Pharmaceuticals, Inc.	Seizures associated with CDKL5 deficiency	Neuroactive steroid, positive modulator of the GABA receptor	Oral suspension
18	18.03.22	Nivolumab/ Relatlimab	Opdualag	Bristol Myers Squibb	Melanoma	A combination of antibodies blocking programmed death receptor-1 (PD-1) and antibodies blocking lymphocyte activation gene-3 (LAG-3)	Solution for injection
19	22.03.22	Dextroamphetamine	Xelstrym	Noven Pharmaceuticals, Inc.	Attention deficit hyperactivity disorder (ADHD)	CNS stimulant	Transdermal System
20	22.03.22	Sirolimus	Hyftor®	Nobelpharma America, LLC	Facial angiofibroma associated with tuberous sclerosis	mTOR inhibitor immunosuppressant	Topical gel
21	23.03.22	Gallium ga 68 gosetotide	Locametz®	Novartis Pharmaceuticals Corporation	Positron emission tomography	Radioactive diagnostic agent	Solution for injection
22	23.03.22	Lutetium lu 177 vipivotide tetraxetan	Pluvicto®	Novartis	Prostate cancer	Radioligand therapeutic agent	Solution for injection
23	28.03.22	Testosterone	Tlando®	Antares Pharma, Inc.	Hypogonadism, male	Testosterone replacement therapy	Capsules
24	05.04.22	Alpelisib	Vijoice®	Novartis	The spectrum of overgrowth associated with PIK3CA	Kinase inhibitor	Tablets
25	05.04.22	Dexmedetomidine	Igalmi	BioXcel Therapeutics, Inc.	Alarm	Alpha2-adrenoceptor agonist	Sublingual form
26	13.04.22	Bevacizumab	Alymsys®	Amneal Pharmaceuticals, Inc.	Colorectal cancer, non-small cell lung cancer, glioblastoma multiforme, renal cell cancer, cervical cancer, ovarian cancer, fallopian tube cancer, peritoneal cancer	Vascular endothelial growth factor inhibitor	Solution for injection
27	22.04.22	Benzoyl peroxide	Epsolay®	Sol-Gel Technologies, Ltd.	Rosacea	Oxidising agent for topical use	Cream

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Continuation of table 2

No.	Date of approval	INN	Trade name	Manufacturer	Indications for use	Drug class / mechanism of action	Dosage form
28	26.04.22	Oteconazole	Vivjoa®	Mycovia Pharmaceuticals, Inc.	Vaginal fungal infection	Oral antifungal azole	Capsules
29	28.04.22	Mavakamten	Camzyos®	Bristol Myers Squibb	Hypertrophic cardiomyopathy	A first-in-class cardiac myosin inhibitor	Capsules
30	28.04.22	Trientine tetrahydrochlorid	Cuvrior	Orphalan SA	Wilson's disease	Copper chelator	Tablets
31	03.05.22	Amoxicillin, Clarithromycin and Vonoprazan	Voquezna® Triple Pak®	Phathom Pharmaceuticals, Inc.	Helicobacter infection	Amoxicillin (penicillin class antibiotic), clarithromycin (macrolide antimicrobial), vonoprazan (potassium-competitive acid blocker (PCAB))	Capsules+pills
32	12.05.22	Edaravon	Radicava ORS®	Mitsubishi Tanabe Pharma Corporation	Amyotrophic lateral sclerosis	Free radical scavenger	Oral suspension
33	13.05.22	Tyrzepatid	Mounjaro	Eli Lilly and Company	Type 2 diabetes mellitus	Glucose-dependent insulinotropic polypeptide (GIP) and glucagonlike peptide-1 (GLP-1) receptor agonist	Solution for injection
34	23.05.22	Tapinarof	Vtama®	Dermavant Sciences	Plaque psoriasis	An agent that modulates the local arylhydrocarbon receptor (AHR)	Cream
35	26.05.22	Pegfilgrastim	Fylnetra	Amneal Pharmaceuticals, Inc.	Chemotherapy-related neutropenia	White blood cell growth factor	Solution for injection
36	03.06.22	Live measles, mumps and rubella vaccine	Priorix®	GSK	Prevention of measles, prevention of mumps, prevention of rubella	Live attenuated vaccine	Solution for injection
37	13.06.22	Wuthrisiran	Amvuttra®	Alnylam Pharmaceuticals, Inc.	Transthyretin amyloidosis	Rnai therapeutic	Solution for injection
38	08.07.22	Sodium indigotindisulphonate	Bludigo	Provepharm	Urological and gynaecological diagnostics and examinations	Diagnostic dye	Solution for injection
39	15.07.22	Zonisamide	Zonisade®	Azurity Pharmaceuticals, Inc.	Cramps	Gabaergic blocker of potential- dependent sodium and calcium channels	Oral suspension
40	27.07.22	Undecanoate Testosterone	Kyzatrex	Marius Pharmaceuticals	Male hypogonadism	Testosterone replacement therapy	Capsules

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Continuation of table 2

No.	Date of approval	INN	Trade name	Manufacturer	Indications for use	Drug class / mechanism of action	Dosage form
41	29.07.22	Roflumilast	Zoryve	Arcutis Biotherapeutics, Inc.	Plaque psoriasis	Local phosphodiesterase 4 (PDE4) inhibitor	Cream
42	02.08.22	Ranibizumab	Cimerli	Coherus BioSciences, Inc.	Yellow spot degeneration, yellow spot oedema, diabetic yellow spot oedema, diabetic retinopathy, myopic chorioidal neovascularisation	(VEGF) inhibitor	Intravitreal injection
43	17.08.22	Betabeglogen autotemcel	Zynteglo®	Bluebird Bio, Inc.	Beta-thalassaemia	Gene therapy based on autologous haematopoietic stem cells	Intravenous suspension
44	18.08.22	Dextromethorphan and Bupropion	Auvelity	Axsome Therapeutics, Inc.	Major depressive disorder	NMDA receptor antagonist	Extended-release tablets
45	30.08.22	Omeprazole and sodium bicarbonate	Konvomep	Azurity Pharmaceuticals, Inc.	Gastric ulcer, gastrointestinal bleeding	Proton pump inhibitor (PPI) combination of omeprazole and sodium bicarbonate	Oral powder
46	31.08.22	Olipudase alfa	Xenpozyme	Sanofi	Acid sphingomyelinase deficiency	Hydrolytic lysosomal sphingomyelin-specific enzyme	Lyophilised powder for injection
47	01.09.22	Spesolimab	Spevigo®	Boehringer Ingelheim	Generalised pustular psoriasis	Interleukin-36 receptor antagonist	Solution for injection
48	01.09.22	Pegfilgrastim	Stimufend®	Fresenius Kabi	Chemotherapy-related neutropenia	White blood cell growth factor	Solution for injection
49	07.09.22	Daxitulinotoxin A	Daxxify®	Revance Therapeutics, Inc.	Glabellar lines	Acetylcholine release inhibitor and neuromuscular blocker	Lyophilised powder for injection
50	09.09.22	Deukravacitinib	Sotyktu	Bristol Myers Squibb	Plaque psoriasis	Tyrosine kinase inhibitor 2 (TYK2)	Tablets
51	09.09.22	Eflapegrastim	Rolvedon®	Spectrum Pharmaceuticals, Inc.	Chemotherapy-related neutropenia	White blood cell growth factor	Solution for injection
52	14.09.22	Terlipressin	Terlivaz®	Mallinckrodt pic	Hepatorenal syndrome	Vasopressin receptor agonist	Lyophilised powder for injection
53	16.09.22	Aprepitant	Aponvie	Heron Therapeutics, Inc.	Nausea/vomiting in the postoperative period	P/neurokinin-1 receptor antagonist (NK1)	Solution for injection

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Continuation of table 2

No.	Date of approval	INN	Trade name	Manufacturer	Indications for use	Drug class / mechanism of action	Dosage form
54	16.09.22	Elivaldogen autotemsel	Skysona®	Bluebird bio, Inc.	Cerebral adrenoleukodystrophy	Functional copies of the ABCD1 gene added to the patient's stem cells and created using the patient's own blood stem cells	Intravenous suspension
55	20.09.22	Sodium thiosulphate	Pedmark®	Fennec Pharmaceuticals Inc.	Prevention of cisplatin-induced ototoxicity	Cisplatin-neutralising agent	Solution for injection
56	21.09.22	Gadopilenol	Elucirem	Guerbet	Paramagnetic contrast agent for magnetic resonance imaging	Macrocyclic gadolinium-based contrast agent (GBCA)	Solution for injection
57	22.09.22	Omidenepag isopropyl	Omlonti®	Santen Inc.	Glaucoma/intraocular hypertension	A relatively selective prostaglandin E2 receptor agonist (EP2)	Ophthalmic solution
58	27.09.22	Chloroprocaine hydrochloride	Iheezo	Harrow	Superficial eye anaesthesia	Ether anaesthetic	Ophthalmic gel
59	27.09.22	Bevacizumab	Vegzelma®	Celltrion USA	Colorectal cancer, non-small cell lung cancer, glioblastoma multiforme, renal cell cancer, cervical cancer, ovarian cancer, fallopian tube cancer, peritoneal cancer	Vascular endothelial growth factor (VEGF) inhibitor	Solution for injection
60	29.09.22	Sodium phenylbutyrate and taurursodiol	Relyvrio	Amylyx Pharmaceuticals, Inc.	Amyotrophic lateral sclerosis	Oral fixed-dose combination therapy for the treatment of adults with bass	Oral powder
61	30.09.22	Futibatinib	Lytgobi®	Taiho Oncology, Inc.	Cholangiocarcinoma	Irreversible tyrosine kinase inhibitor FGFR1, 2, 3 and 4	Tablets
62	07.10.22	Furosemide	Furoscix®	scPharmaceuticals, Inc.	Heart failure	Loop diuretic	Solution for injection
63	21.10.22	Tremelimumab	Imjudo®	AstraZeneca	Hepatocellular carcinoma	Antibody blocking cytotoxic t- lymphocyte-associated antigen 4 (CTLA-41)	Solution for injection
64	25.10.22	Teclistamab	Tecvayli®	Janssen Pharmaceutical Companies of Johnson & Johnson	Multiple myeloma	Bispecific B-cell maturation antigen (BCMA) targeting CD3 T-cells	Solution for injection
65	14.11.22	Mirvetuximab soravtansine	Elahere	ImmunoGen, Inc.	Ovarian cancer, fallopian tube cancer, peritoneal cancer	Antibody conjugate against folic acid receptor alpha (fra) and microtubule inhibitor	Solution for injection

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Continuation of table 2

No.	Date of approval	INN	Trade name	Manufacturer	Indications for use	Drug class / mechanism of action	Dosage form
66	17.11.22	Teplizumab	Tzield®	Provention Bio, Inc.	Delaying the onset of type 1 diabetes at stage 3	CD3-directed antibody	Solution for injection
67	17.11.22	Sodium phenobarbital	Sezaby	Sun Pharmaceutical Industries Limited	Neonatal seizures	GABA mimetic	Injection powder
68	22.11.22	Etranacogene dezaparvovec	Hemgenix®	CSL	Haemophilia B	Adeno-associated virus vector-based gene therapy	Intravenous suspension
69	30.11.22	Faecal microbiota, live	Rebyota®	Ferring Pharmaceuticals Inc.	Prevention of recurrent clostridioides difficile infection	Live biotherapeutic based on microbiota	Rectal suspension
70	01.12.22	Olutazidenib	Rezlidhia	Forma Therapeutics	Acute myeloid leukaemia	Isocitrate dehydrogenase inhibitor-1 (IDH1)	Capsules
71	12.12.22	Adagracib	Krazati®	Mirati Therapeutics, Inc.	Non-small cell lung cancer	Low molecular weight inhibitor KRAS g12c	Tablets
72	13.12.22	Latanoprost	Iyuzeh	Thea Pharma, Inc.	Intraocular hypertension, open- angle glaucoma,	Prostaglandin F2α analogue	Ophthalmic solution
73	13.12.22	Adalimumab	Idacio®	Fresenius Kabi	Rheumatoid arthritis, juvenile idiopathic arthritis, psoriatic arthritis, Bechterew disease, Crohn's disease, ulcerative colitis, plaque psoriasis	Tumour necrosis factor (TNF) blocker	Solution for injection
74	16.12.22	Nadofaragen firaden	Adstiladrin®	Ferring Pharmaceuticals	Bladder cancer	Gene therapy based on a non-replicating adenovirus vector	Suspension for intravesicular use
75	22.12.22	Lenacapavir	Sunlenca®	Gilead Sciences, Inc.	HIV infection	Long-acting HIV-1 capsid inhibitor	Injectable solution and tablets
76	22.12.22	Mosunetuzumab	Lunsumio	Genentech	Follicular lymphoma	Bispecific CD20-directed activator of CD3 T cells	Solution for injection
77	22.12.22	Sodium phenylbutyrate	Olpruva	Acer Therapeutics Inc.	Urea cycle disorders	Nitrogen-binding agent	Oral suspension
78	23.12.22	Xe 129 hyperpolarised	Xenoview	Polarean Imaging plc	Diagnosis	Hyperpolarised contrast agent	Inhalation agent
79	28.12.22	Ublituximab	Briumvi	TG Therapeutics, Inc.	Multiple sclerosis	CD20-directed cytolytic antibody	Solution for injection
80	28.12.22	Anacaulase	NexoBrid®	MediWound Ltd.	Skin burns	Proteolytic enzyme concentrate	Topical gel

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Table 3 – Combinations of chemotherapeutic drugs with evacizumab to treat different types of cancer.

Indication	Drug administered in combination with bevacizumab
Metastatic colorectal cancer	Fluorouracil (1st–2nd line) Fluoropyrimidine+irinotecan or Fluoropyrimidine+oxaliplatin (2nd line)
Non-small cell lung cancer	Carboplatin+paclitaxel (1st line)
Metastatic renal cell cancer	Interferon alpha
Persistent, recurrent or metastatic cervical cancer	Paclitaxel+cisplatin or Paclitaxel+topotecan
Epithelial cancer of the ovaries, fallopian tubes or primary peritoneal cancer	Paclitaxel

QSAR modeling tools have evolved into AI-based QSAR approaches, such as a linear discriminant analysis (LDA), support vector machines (SVMs), random forest (RF), and decision trees, accelerating a QSAR analysis [43–45].

Cryogenic electron microscopy. Until 2014, cryogenic electron microscopy (cryo-EM) rarely provided the resolution below 4.0 Å, often necessary for a structure-based drug discovery (SBDD) [46]. However, the tremendous advances in the methodology over the latest few years have led to a higher availability of high-resolution structural data. "The quantum leap" of cryo-EM is due to many advances, such as direct electron detectors for image recording, improved computational methods and a hardware parallelisation for processing large datasets [47]. Furthermore, the nature of cryo-EM as a direct imaging technique allows a rapid diagnosis of biochemical problems such as aggregation and instability of samples, leading to the rapid improvement through genetic and biochemical modifications [48]. As a result, the number of cryo-EM structures deposited in the Protein Data Bank (PDB) with a resolution of 4.0 Å or higher increased from 16 before 2014 to 1753 new structures deposited in 2020 alone. The proportion of newly deposited structures with resolutions better than 4.0 and 3.5 Å increased from 36 and 12% in 2015 to 75 and 50% in 2020 [8]. Perhaps most impressively, the proportion of deposited structures above 3.0 and 2.5 Å resolution in 2020, previously almost non-existent, is now a significant 18 and 3% respectively [8, 49].

Drug repositioning. Drug repositioning has a number of interrelated benefits [50]. Essentially, they include the simplification of regulatory procedures to bring a previously approved drug to the market, especially in some countries such as the US [51]. This procedure considers previously obtained data, in particular on the safety and toxicity of the drug, which can significantly speed up the initial stages of development of a new drug [52] and hence make it cheaper (by more than 80% according to Naylor) [53], and increase the chances of it reaching the market. One important consideration, however, is that because the level of safety required for a medicine is highly dependent on its indication, the side effects of a medicine will be proportionately less acceptable if it is repositioned to treat a less serious disease than its original indication [54, 55]. Any change in the formulation, dosage or route of administration would require a reassessment of the safety profile of the drug in this new setting, as it would be a new pharmaceutical product.

Current state of FDA-approved drugs

According to the analysis of recent developments, drugs with an anticancer activity (21%), CNS disorders (12%), and dermatological conditions (8%) are most frequently approved (Table 1). It is worth pointing out that about 22% of the approved drugs are biotech products, which may indicate current trends in the drug design (Table 2).

Spikevax® is an mRNA vaccine that can be used for an active immunization against COVID-19 in persons aged 12 years and older. The FDA-approved vaccine Spikevax® (monovalent) and the emergency-approved (EUA) vaccine Moderna COVID19 (monovalent) contain the same mRNA component of the original SARS-CoV-2 strain, but when used with the FDA approval, the vaccine is labeled Spikevax® and when used under the EUA – Moderna COVID19 vaccine. Moderna COVID19, a bivalent vaccine, differs from the original Moderna COVID19 (monovalent) vaccine and Spikevax® because it contains two SARS-CoV-2 mRNA components. The efficacy of the vaccine has been confirmed by numerous studies [56–58].

Opdualag® (nivolumab and relatlimab) is an antibody combination indicated for the treatment of unresectable or metastatic melanoma in adults and children from 12 years of age [59]. Nivolumab is an antibody that blocks programmed death-1 receptor (PD-1), first approved under the brand name of Opdivo for the treatment of unresectable or metastatic melanoma in 2014. Lymphocyte activation gene-3

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(LAG-3) is a cell surface molecule expressed on effector T cells and regulatory T cells, and it is associated with the T cell depletion and resistance to immunotherapies such as antibodies that block PD-1 [60]. Relatlimab is an LAG-3 blocking antibody that binds to LAG-3 on T cells, thereby restoring the effector function of depleted T cells and potentially promoting an anti-tumour response [61]. The combination of nivolumab and relatlimab leads to an increased T-cell activation compared to the activity of either antibody alone. The FDA approval of Opdualag® is based on the results of the phase 2/3 RELATIVITY-047 trial, in which a fixed combination of relatlumab and nivolumab demonstrated a statistically significant and clinically relevant progression-free survival benefit compared to nivolumab monotherapy [62].

Alymsys® (bevacizumab) is a vascular endothelial growth factor (VEGF) inhibitor intended to treat several types of cancer, including metastatic colorectal cancer; non-small cell lung cancer; glioblastoma; metastatic renal cell carcinoma; cervical cancer; epithelial ovarian, fallopian tube or primary peritoneal cancer [63–66] (Table 3). Instead of directly targeting cancer cells, bevacizumab affects the tumour microenvironment, characterised by complex interactions between cancer cells, normal cells and the extracellular matrix. Moreover, VEGF plays additional roles independent of angiogenesis in the complex tumour microenvironment, including a modulation of anticancer immune response. Since the initial approval of bevacizumab, a number of targeted cancer therapies have become available, changing the treatment landscape for many solid tumour indications and providing opportunities for new approaches to combination therapy. Notably, the approval of bevacizumab in combination with immune checkpoint inhibitors for non-small cell lung cancer treatment has recently occurred, with additional CTs demonstrating clinical benefits in renal cell cancer patients in combination with PARP inhibitors for ovarian cancer treatment [67].

Camzyos® (mavacamten) is a first-in-class cardiac myosin inhibitor for the treatment of adults with symptomatic obstructive hypertrophic cardiomyopathy of class II-III according to the New York Heart Association (NYHA) classification [68]. Hypertrophic cardiomyopathy is a type of a heart disease characterised by thickening of the heart muscle and the left ventricular stiffness. The obstruction occurs when the thickened septum causes a narrowing that can block or reduce a blood flow from the left ventricle to the aorta, making it difficult for the heart to expand normally and fill with blood. Camzyos® is an allosteric, reversible and selective inhibitor of cardiac myosin [69]. It is thought to work by reducing the cardiac muscle contractility by inhibiting an excessive formation of myosin-actin cross-links. The drug has an embryo-fetal toxicity.

Fleqsuvy is an oral baclofen suspension for the treatment of spasticity caused by multiple sclerosis, especially for the relief of flexor spasms and the associated pain, clonus and muscle stiffness. Fleqsuvy may also be useful for patients with a spinal cord disease, including trauma. It is increasingly used off-label for the treatment of skeletal muscle pain, a gastroesophageal reflux disease and alcohol dependence [70–72].

Mounjaro (tirzepatide) is a glucose-dependent insulinotropic polypeptide (IGP) receptor and a glucagon-like peptide-1 (GFP-1) receptor agonist shown as a supplement to diet and exercise to improve a glycaemic control in adults with type 2 diabetes. Mounjaro works by activating the body's receptors for GIP and GFP-1, which are naturally occurring incretin hormones [73–75]. The drug is administered subcutaneously once a week. Patients with diabetic retinopathy should take the drug with caution as taking Mounjaro may exacerbate the condition.

Cibinqo® (abrocitinib) is indicated for the treatment of adults and children from 12 years of age with moderate to severe refractory atopic dermatitis refractory to other treatments. Cibinqo® selectively inhibits Janus kinase-1 (JAK1) [76]. The inhibition of JAK1 is thought to modulate several cytokines involved in the pathophysiology of atopic dermatitis, including interleukin IL-4, IL-13, IL-31, IL-22 and thymic stromal lymphopoietin (TSLP) [77]. The study published in The Lancet assessed the effectiveness and safety of abrocitinib in comparison to dupilumab [78]. Abrocitinib, administered at a daily dose of 200 mg, demonstrated a superior efficacy to dupilumab in adults with moderate to severe atopic dermatitis who were receiving topical therapy, resulting in rapid alleviation of itching and improvement in atopic dermatitis symptoms. Cibinqo® is administered orally once daily.

Adstiladrin® (nadofaragen firadenovec) is a non-replicative gene-based adenoviral vector designed to treat adult patients with Bacillus Calmette-Guerin (BCG) unresponsive non-muscle invasive bladder cancer with or without papillary tumours [79]. Adstiladrin® acts by delivering the interferon alfa-2b gene into bladder wall cells, resulting in the increased secretion of interferon alfa-2b protein, a native cancer-fighting agent [80]. Adstiladrin® is injected into the bladder once every three months.

Skysona® (elivaldogen autotemcel) is a single-dose gene therapy administered intravenously to treat the underlying cause of cerebral adrenoleukodystrophy (CALD). Skysona® is indicated for slowing the progression of a neurological dysfunction in boys aged

4–17 years with early active CALD. This indication was approved in a fast-track procedure after the evidence of a 24-month survival rate without major functional impairments [68].

CALD is a genetic disorder caused by mutations in the ABCD1 gene that lead to an accumulation of very long chain fatty acids (VLCFAs) in the brain. VLCFAs can destroy the myelin coating of nerve cells and cause brain damage [81]. Skysona® is made specifically for each patient using the patient's own blood stem cells. Functional copies of the ABCD1 gene are added to the patient's stem cells, which can then help the body to degrade VLCFAs to slow the progression of brain damage and slow the decline in a neurological function [82]. Skysona packets are administered intravenously over less than 60 min each.

CONCLUSION

Drug development is obviously a very time-consuming and labour-intensive process requiring a huge number of resources. A small proportion of pharmaceuticals undergoing the preclinical research cycle reach the stage of CTs. Drug design trends are not static and changing according to the demands of the society. This review presents only a few of the most promising drugs approved by the FDA in 2022. As the technology advances, the speed of drug development will increase, resulting in a shorter time for the drugs to reach the market. Biotechnology-based pharmaceuticals, particularly cell-based therapies, have great a potential and demand, while genetic mapping and improved chipping technologies (cell / tissue / organ on a chip) will increase the availability of personalized treatment strategies.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHORS CONTRIBUTION

Denis V. Kurkin – conception and planning of the work's content; Dmitry A. Bakulin, Yuliya V. Gorbunova, Yury A. Kolosov, Marina A. Dzhavakhyan – data collection and drafting of the manuscript; Evgeniy I. Morkovin, Andrey V. Strygin – data collection, editing the final version of the manuscript; Igor E. Makarenko, Roman V. Drai, Andrew V. Zaborovsky, Olga V. Shatalova, Vladimir I. Petrov, Anatoliy P. Pleten, Aleksei A. Prokopov, Tatiana Yu. Tatarenko-Kozmina – consulting, editing, and approval of the final version of the manuscript. All authors made a substantial contribution to the conception of the work, acquisition, analysis, interpretation of data for the work, drafting and revising the work, final approval of the version to be published and agree to be accountable for all aspects of the work.

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ОРИГИНАЛЬНАЯ СТАТЬЯ

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Neuroprotective properties of GABA and its derivatives in diabetic encephalopathy in old animals

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The aim of the work was to evaluate the GABA neuroprotective properties and its structural analogues in old animals after seven months of hyperglycemia.

Materials and methods. Diabetes mellitus was modeled in white outbred male rats (12 months old) by the administration of a streptozotocin (65 mg/kg) and nicotinamide (230 mg/kg) combination. After 6 months, the animals with a postprandial glycemia level between 11 and 18 mmol/l were selected for the study. After the groups had been formed, the animals were administrated with GABA and GABAergic compounds (Compositions MPBA and PPC), respectively, for 1 month, the control group animals were administrated with saline. After the treatment, an oral glucose tolerance test and a set of behavioral tests aimed at studying sensory-motor (Open Field, Adhesion test, Rotarod) and cognitive functions (New Object Recognition and Morris Water Maze), as well as the functional state evaluation of the endothelium were performed. Further on, sampling of blood and brain tissues for a biochemical and enzyme immunoassay (the level of glucagon-like peptide-1 (GLP-1) and TNF-α in serum and the level of Klotho protein, BDNF, Nrf2, NF-κB and malondialdehyd (MDA) in brain homogenates), as well as a morphological analysis of changes in CA1 and CA3 neurons of the hippocampus and somatosensory cortex, was carried out.

Results. GABA and compositions with its derivatives had a pronounced neuroprotective effect in old animals with prolonged hyperglycemia. The hypoglycemic effect of the studied compositions was accompanied by an increase in the production of GLP-1. In the animals with DM, after 6 weeks of the test substances administration, higher rates of sensory-motor and cognitive functions and a less structural damage to the sensory-motor cortex and the brain hippocampus were recorded. These effects may be due to higher levels of the Klotho proteins, Nrf2 and BDNF, as well as lower levels of NF-κB, which may underlie the suppression of the oxidative stress, the reduction of MDA and inflammation (TNF-α).

Conclusion. After 6 weeks of the administration, GABA and its compositions in old animals (19 months old) significantly improved sensory-motor and cognitive functions, reduced negative structural changes in the hippocampus and somatosensory cerebral cortex.

Keywords: GABA; Klotho protein; diabetes mellitus; streptozotocin; NF-kB; Nrf2; GLP-1; endothelium; hippocampus

Abbreviations: BDNF – Brain-Derived Neurotrophic Factor; eNOS – endothelial nitric oxide synthase; NF-κB – nuclear factor-kB; Nrf2 – nuclear factor erythroid 2-related factor 2; STZ-NA – the streptozotocin-nicotinamide diabetes model; TNF α – tumour necrosis factor alpha; vWF – von Willebrand's factor; KP – Klotho protein; GABA – gamma-amino-butyric acid; GLP-1 – glucagon-like peptide-1; DI – Discrimination index (New Object Recognition test); ELISA – enzyme-linked immunosorbent assay; CBF – cerebral blood flow; OF – Open Field; OGTT – oral glucose tolerance test; NOR – New Object Recognition; DM – diabetes mellitus; CEC – circulating endothelial cells.

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Нейропротективные свойства ГАМК и её производных при диабетической энцефалопатии у старых животных

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Цель. Оценить нейропротекторные свойства ГАМК и её структурных аналогов на старых животных после продолжительной гипергликемии (7 мес).

Материалы и методы. Сахарный диабет моделировали на белых аутбредных крысах-самцах в возрасте 12 мес посредством введения комбинации стрептозотоцина (65 мг/кг) и никотинамида (230 мг/кг). Через 6 мес для исследования были отобраны животные с уровнем постпрандиальной гликемии между 11 и 18 ммоль/л. После формирования групп, в течение 1 мес животные соответственно получали ГАМК и ГАМК-ергические соединения (композиция МФБА и ФПС), контрольная группа получала физиологический раствор. После лечения был выполнен пероральный тест на толерантность к глюкозе и комплекс поведенческих тестов, направленных на изучение сенсорно-моторных (Открытое поле, Адгезивный тест, Ротарод) и когнитивных функций (Распознавание нового объекта и Водный лабиринт Морриса), а также проведена оценка функционального состояния эндотелия. Далее был произведен забор образцов крови и тканей головного мозга (ГМ) для биохимического и иммуноферментного (уровень глюкагоноподобного пептида-1 (ГПП-1) и фактора некроза опухоли альфа (ФНО-α) в сыворотке и уровень белка Клото, BDNF, Nrf2, NF-κB и малонового диальдегида (МДА) в гомогенатах ГМ), а также для морфологического анализа изменений нейронов CA1 и CA3 зон гиппокампа и соматосенсорной коры.

Результаты. ГАМК и композиции с её производными оказали выраженное нейропротекторное действие у старых животных с продолжительной гипергликемией. Гипогликемическое действие исследуемых композиций сопровождалось повышением продукции ГПП-1. У животных с СД после 6 нед введения исследуемых веществ регистрировали более высокие показатели сенсорно-моторных и когнитивных функций и меньшие структурные повреждения сенсорно-моторной коры и гиппокампа ГМ. Эти эффекты могут быть обусловлены более высоким уровнем белка Клото, Nrf2 и BDNF, а также более низким уровнем NF-κB, что может лежать в основе подавления окислительного стресса, снижения МДА и воспаления (TNF-α).

Заключение. ГАМК и её композиции у старых животных (19 мес) после 6 нед введения значительно улучшали сенсорно-моторные и когнитивные функции, уменьшали негативные структурные изменения в гиппокампе и соматосенсорный коре ГМ.

Ключевые слова: ГАМК; белок Клото; сахарный диабет; стрептозотоцин; NF-kB; Nrf2; ГПП-1; эндотелий; гиппокамп

Список сокращений: BDNF – нейротрофический фактор мозга; eNOS – эндотелиальная синтаза оксида азота; ГМ – головной мозг; NF-κB – ядерный фактор каппа B; Nrf2 – ядерный фактор, родственный эритроидному фактору 2; STZ-NA – модель стрептозотоцин-никотинамидного диабета; ФНО-α – фактор некроза опухоли альфа; vWF – фактор Виллибранта; БК – белок Клото; ГАМК – гамма-аминомасляная кислота; ГПП-1 – глюкагоноподобный пептид-1; ИД – индекс дискриминации в тесте Распознавания нового объекта; ИФА – иммуноферментный анализ; МК – мозговой кровоток; ОП – открытое поле; ПТТГ – пероральный тест на толерантность к глюкозе; РНО – тест Распознавание нового объекта; СД – сахарный диабет; ЦЭК – циркулирующие эндотелиальные клетки.

INTRODUCTION

Diabetes mellitus (DM) is the most common and progressive endocrine pathology. According to the International Diabetes Federation (IDF), among the world's population aged 20–79 years, there are currently 537 million people with diabetes, and by 2030, the number will reach 643, and by 2045 – 783 million [1]. With an increase in the number of patients with diabetes, the prevalence of its complications, which lead to an early disability and premature death, is steadily increasing, as well as the cost of their treatment [2, 3].

Hyperglycemia provokes and exacerbates many pathological conditions in almost all organs and tissues. Prolonged hyperglycemia plays a significant role in the development of encephalopathy affecting psycho-

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emotional, sensory-motor and cognitive functions, especially in adults and the elderly [4–6]. It has been established that patients with diabetes are more prone to cognitive impairment [7–9]. Xue M. et al. (2019), based on a meta-analysis of 122 studies, showed that DM increases the risk of developing cognitive impairment and dementia by 1.25–1.9 times compared with people without DM [7]. Therefore, important tasks in the treatment of DM are not only the normalization of glycemia, but also the prevention and treatment of its complications.

Prolonged hyperglycemia in DM provokes not only gluco- and lipotoxicity, but also enhances an oxidative and nitrosative stress, inflammation and other pathological processes, which can lead to accelerated apoptosis and death of intensively functioning cells in various organs and tissues, as well as accelerate the aging process. Among all the organs, the most sensitive and vulnerable to these pathological factors is the brain, especially such structures as the hippocampus and cortex [10]. Based on the above, the authors concluded that it is necessary to investigate the encephalopathic effects associated with hyperglycemia, while focusing on the hippocampus and somatosensory cortex.

In recent years, understanding of the physiological role of the GABAergic system, which was originally considered as the main inhibitory system playing a decisive role in ensuring various brain functions, has radically changed. Currently, GABA receptors and the GABA-synthesizing enzyme (glutamate decarboxylase) are found in the tissues of the cardiovascular and respiratory systems, in immunocompetent cells, the gastrointestinal tract, etc. [11, 12]. In pancreatic β cells, the density of GABA receptors is comparable to that in the brain [13]. Numerous publications also reflect the effect of GABAergic substances on the pancreatic function [11, 12, 14]. GABA derivatives are widely used in the clinic as drugs with neuro- and psychotropic effects and continue to be actively studied in experimental pharmacology. Thus, since DM is associated with a decrease in the cognitive function and structural changes in the brain [4–6], it seems promising to search for agents for the correction of diabetic encephalopathy caused by long-term hyperglycemia in a number of substances with a GABAergic effect.

During the preliminary screening, among the derivatives of linear and cyclic GABA, the substances with a pancreatic protective effect were isolated (Fig. 1). The results obtained formed the basis for the selection of these compositions to study their effect on the function and structure of the brain in old animals with long-term hyperglycemia.

THE AIM of the work was to evaluate the GABA neuroprotective properties and its structural analogues in old animals after seven months of hyperglycemia.

MATERIALS AND METHODS

Model objects

All the experiments were performed in accordance with the legislation of the Russian Federation and the technical standards of the Eurasian Economic Union for good laboratory practice (GOST R 53434-2009, GOST R 51000.4-2011). The study design and protocol were approved by the local ethical committee of Volgograd State Medical University, Protocol No. 2022/116 dated March 4, 2022 (registration number IRB 00005839 IORG 0004900 [OHRP]).

The experimental study was carried out on 50 outbred laboratory rats obtained from the nursery of "Stolbovaya" (Moscow region, Russia) at the age of 4–5 months. Animals were kept in the vivarium of the Scientific Center for Innovative Medicines of Volgograd State Medical University at the temperature of 20–22°C, the air humidity of 40–60%, the light regime of 12/12 h and a free access to drinking water and food (GOST R 51849-2001) (LLC Laboratorkorm, Moscow, Russia). By the beginning of the experiment, at the time of the administration of streptozotocin, the animals had reached the age of 12 months.

Test compounds

Composition MPBA (a composition of a linear GABA derivative with L-arginine) exhibits pronounced endothelioprotective properties [15, 16]. This also indicates the expediency of its study as a means for the prevention of DM vascular complications CD (Fig. 1).

Composition PPC (a composition of a 2-pyrrolidone derivative with succinic acid) has a nootropic, neuro-, cardioprotective effect [17, 18].

The both compositions were selected among various GABA derivatives in preliminary screening studies to research their pancreoprotective effect on the alloxan model of DM in animal survival tests, a preservation of β-cell mass, an ability to stimulate the production of glucagon-like peptide-1 (GLP-1) and improve a glucose utilization.

GABA was chosen as a reference substance for comparison of pancreatic protective properties, which are well reflected in the literature [12, 14].

Pathology modeling

DM was modeled by an intraperitoneal (ip) administration of streptozotocin – 65 mg/kg with a preliminary (15 min before) administration of nicotinamide (230 mg/kg, i.p.). Streptozotocin was diluted in cold citrate buffer (1 mM, pH 4.5); nicotinamide was diluted in a NaCl solution (0.9%). After 3 days, blood glucose levels were determined in all animals, and 50 individuals with a postprandial glycemia level of more than 11 and less than 18 mmol/l were selected for the experiment. Glucose levels were measured using

a Contour TS glucometer and appropriate test strips (Bayer). The blood for measurements was taken by puncture of the hyoid vein.

Experiment design

Subsequently, 50 selected animals were under constant observation for 6 months (after modeling DM) in the vivarium of the Scientific Center for Innovative Medicines of Volgograd State Medical University. Every 4 weeks, the level of glycemia was measured. During this observation period, prolonged DM caused the death of 6 (12%) animals, and 4 (8%) had a significant increase in postprandial glycemia (>18 mmol/l) and they were excluded from the study before the distribution into groups. After 6 months, fasting glycemia (4 h after food intake) was evaluated in rats and an oral glucose tolerance test (OGTT) was performed, in which glucose was administered at the dose of 4 g/kg *(per os).* Next, the rats were randomly distributed into 4 equal groups (*n*=10) with an average comparable level of glycemia (from 7.5 to 9.5 mmol/l after 4 h of fasting). After that, GABA, MPBA, and PPC were administered daily and continuously at the dose of 1000, 20, and 50 mg/kg *(per os),* respectively, for 30 days and then for another 2 weeks, during which the condition of the animals was evaluated. The negative control group (DM+0.9% NaCl) was similarly treated with saline (0.9% NaCl) in the volume of 0.1 ml/100 g (*n*=10). For the positive control, the rats without DM (intact) of the same age from the same animal importation lot (*n*=10) were used.

After 30 days of the test substances administration, the following manipulations were performed in turn for 12 days: on the 1st day an OGTT with the determination of glucose and GLP-1 was carried out: on the 2nd–3rd days, a sensory-motor function was assessed in the Open Field (OP) tests, an Adhesion test, and a motor coordination in the Rotarod test. On the 4th day, a NOR test was performed; on the 5–9th days, a Morris water maze test took place; on the 10–12th day after an endothelial function testing, euthanasia was performed with blood and brain sampling for a morphological study and an enzyme immunoassay of homogenates.

Based on the studied parameters totality, a comprehensive assessment of the morphofunctional state of the brain in the old animals with prolonged hyperglycemia, which had received GABA derivatives for 6 weeks, was made.

The developed study design is shown in Fig. 2.

Assessment of psychoneurological deficit

The motor and exploratory activity of the animals was assessed in the OF test. The installation "Open Field", (Research and manufacturing complex "Open Science", Russia). The animal was placed in the center of the arena and, using a webcam, its behavior was observed for 3 min, fixing the number of crossed squares (the distance traveled) as an indicator of a motor activity (MA). Herewith, the sum of "racks" acts and the number of the examined holes – minks – were evaluated as an exploratory activity (EA) of the animals.

An Adhesion test was used to assess a sensory function and fine motor skills. As a foreign object, stickers were placed on the volar surface of the forepaws of the animal – square pieces of an adhesive tape on a tissue basis (6×6 mm, Veropharm, Russia), and then for 3 min., the detection time (a sensory function) and the removal time of the sticker (a motor function) were recorded.

Movement coordination was assessed in the Rotarod test (a device manufactured by Neurobotics LLC, Russia), in which the total duration of keeping the animal on a rotating rod (25 rpm) was recorded in 3 attempts.

The New Object Recognition Test (NOR) is based on the natural need of animals to explore new objects and does not require the presence of external motivation. It is used to assess a cognitive function (the ability to identify and compare the information stored in short-term memory) and was performed in 2 stages: the 1st stage was a familiarization in a home cage (545×395×200 mm) with 2 identical objects (A1 and A2) for 3 min. Then, 60 min after the familiarization, the 2nd stage was performed, when the animal was placed in the same cage for 3 min, but one of the old (studied) objects (A2) was replaced with a new one (object B). Based on the test results, the discrimination index (DI) – the time spent on the study of a new object (B) minus the time spent on the study of the old object (A) in the second landing, was calculated.

After the treatment course, learning and retention of long-term spatial memory were assessed in the Morris Water Maze. The installation of "Water maze (Morris test)" (RMC "Open Science", Russia). The animals were trained for 5 days (4 attempts per day) to search for a flooded platform using landmarks on the wall of the setup, placing the rat at different starting points relative to the platform. In this study, the duration of the search for a flooded platform during the first landing was recorded for 5 days of the experiment.

Evaluation of functional state of cerebral vessels endothelium

The functional state of the endothelium was tested to determine the endothelium-dependent vasodilation and an antithrombotic function, by the number of desquamated endotheliocytes, by the level of the von Willebrand factor (vWF) in blood serum.

An endothelium-dependent vasodilation was assessed in the anesthetized animals (chloral hydrate 400 mg/kg) by relative changes in the flow level of the cerebral blood in response to the intravenous

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administration of acetylcholine (0.01 mg/kg, Acros Organics, USA; eNOS activator). The administration of acetylcholine caused an increase in the NO production, the endothelium-dependent vasodilation of blood vessels and an increase in the cerebral blood flow, which was determined in the projection of the middle cerebral artery by Doppler ultrasound Minimax-Doppler_K (LLC SP MINIMAX, Russia).

An antithrombotic function was assessed by the time of a complete thrombosis development, i.e. the blood flow cessation through the carotid artery when applying an iron (III) chloride solution to its adventitia.

The number of descaminated (circulating) endothelial cells (CECs) in the blood was determined by the method of Hladovec J [19]. An increase in the number of CECs makes it possible to judge the severity of an endothelial dysfunction, the degree of damage to the endothelium and its reparative activity.

The Von Willebrand factor (vWF), which is produced by the endothelium, was determined in the blood serum by enzyme-linked immunosorbent assay (ELISA) as a factor, with an increase in which one can judge endothelial damage and an endothelial dysfunction. An elevated vWF level is observed in DM and is a predictor of mortality from cardiovascular diseases [20].

The brain samples were homogenized in the lysis buffer (1 ml lysis buffer per 50 mg tissue sample) in a glass homogenizer on ice. The resulting suspension had been sonicated with an ultrasonic disperser until it became clear, then the solutions were centrifuged for 5 min at 10000g. The resulting supernatant was used for an enzyme immunoassay and a biochemical analysis.

Carrying out biochemical and enzyme immunoassay

The concentration of malonic dialdehyde (MDA) in the homogenates was determined using the reaction with thiobarbituric acid.

An ELISA was performed using ready-made kits (Cloud-Clone Corp., USA) in accordance with the manufacturer's instructions. The supernatant of the brain homogenates and blood serum were used for the analysis. The optical density was measured using a microplate analyzer SPECTROstar Nano (BMGLabtech, Germany) at the wavelength of 450 nm.

Morphological study

For morphological studies, the brain was fixed for 24 h in 10% neutral buffered formalin (pH 7.4) at 22–24°C. Then the samples were dehydrated in a battery of ascending strength alcohols, clarified in chloroform using a Cytadel 2000 histoprocessor (Shendon, UK), and embedded in a Histomix paraffin medium (Biovitrum, Russia). Paraffin blocks were cut on a rotary microtome HM34OE (MICROM, Germany), the sections 5 μm thick were obtained and mounted on the glass treated with poly-L-lysine (Menzel, Germany). The staining was carried out with thionin according to the Nissl method.

The structural changes in the brain cortex, a specific number of hyperchromic neurons (a reversible damage) and hyperchromic shriveled neurons (an irreversible damage) were evaluated.

Histological sections were photographed with an AxioCam 305 color digital camera (Carl Zeiss Microscopy GmbH, Germany) based on an AxioImager A2 microscope (Carl Zeiss Microscopy GmbH, Germany).

Statistical processing

Statistical processing of the obtained results was carried out by methods of descriptive and analytical statistics using Prism 6 Software (GraphPad Software Inc., USA). The distribution of quantitative indicators was assessed using the Shapiro–Wilk test. The intergroup differences were assessed using the Kruskal–Wallis test and the Dunn's post hoc test. The numerical values were presented as the arithmetic mean and the standard error of the arithmetic mean (M±m). To express a specific quantity of neurons in the pyramidal layer of the hippocampus, the interquartile range Me (Q1; Q3) was indicated, where Me is the median, Q1 is a 25 percentile, Q3 is a 75 percentile. The differences were considered significant at $p < 0.05$.

RESULTS

Assessment of psychoneurological deficit

It is assumed that hyperglycemia in DM is the main pathogenetic factor contributing to the deterioration of cognitive functions, neurodegeneration, the progress of aging, brain atrophy, and dementia [21]. The test suite used was chosen to capture abnormalities consistent with the clinical consequences for the brain as viewed from long-term uncontrolled hyperglycemia.

In animals aged 19 months with prolonged hyperglycemia (7 months) in the RD (relative density) test, a lower motor activity and a significantly reduced exploratory behavior were recorded in intact animals of the same age. In all experimental groups receiving treatment, higher rates of exploratory activity were recorded. In the group that received the composition of 2-pyrrolidone derivative (PPC), a motor activity was also significantly higher (Fig. 3A).

In the Adhesion test, in the animals with chronic hyperglycemia without treatment, a sensory dysfunction and a pronounced deterioration in fine motor skills were observed compared with the animals of the intact group. In the group treated with PPC, the time of detection and disposal of a foreign object was significantly less than in the animals of the control group (Fig. 3B).

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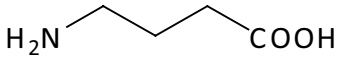
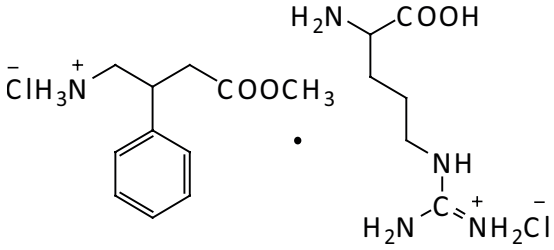


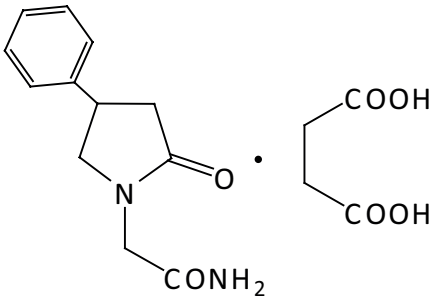
1

4-Aminobutanoic acid (GABA)
(Trivial nomenclature: γ-Aminobutyric acid or GABA)



2

DL-4-amino-3-phenylbutanoic acid methyl ester hydrochloride and L-2-amino-5-guanidinepentanoic acid monohydrochloride (L-arginine monohydrochloride) in a 1:1 ratio
(Substances code: MPBA)



3

2-(2-Oxo-4-phenylpyrrolidin-1-yl)acetamide and butanedioic (succinic) acid in ratio 2:1
(Substances code: PPC)

Figure 1 – Test compounds

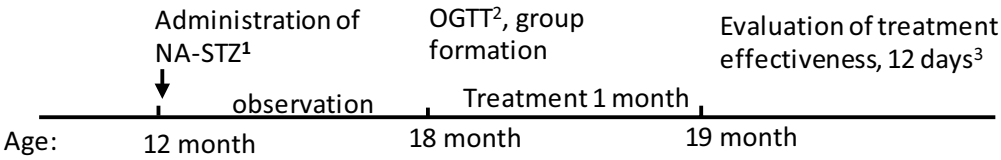


Figure 2 – Experiment design

Note: 1 – 3 days after the administration of streptozotocin with nicotinamide (NA-STZ), animals with postprandial glycemia levels >11 and <18 mmol/l were selected; 2 – after 6 months, an oral glucose tolerance test (OGTT) was performed and 4 groups were formed, comparable in terms of glycemia; 3 – after the treatment, the following indicators were assessed:
Day 1. Assessment of carbohydrate metabolism (OGTT) and determination of GLP-1.
Day 2. Motor and exploratory activity (Open field), sensory-motor disorders (Adhesion test).
Day 3. Coordination disorders (Rotarod).
Day 4. Assessment of short-term memory (a New Object Recognition).
Day 5–9. Long-term memory assessment (Morris Water Maze).
Day 10–12. Assessment of an endothelium-dependent vasodilation and an antithrombotic function of the endothelium, euthanasia, sampling of blood and brain for the morphological and enzyme immunoassay (ELISA).

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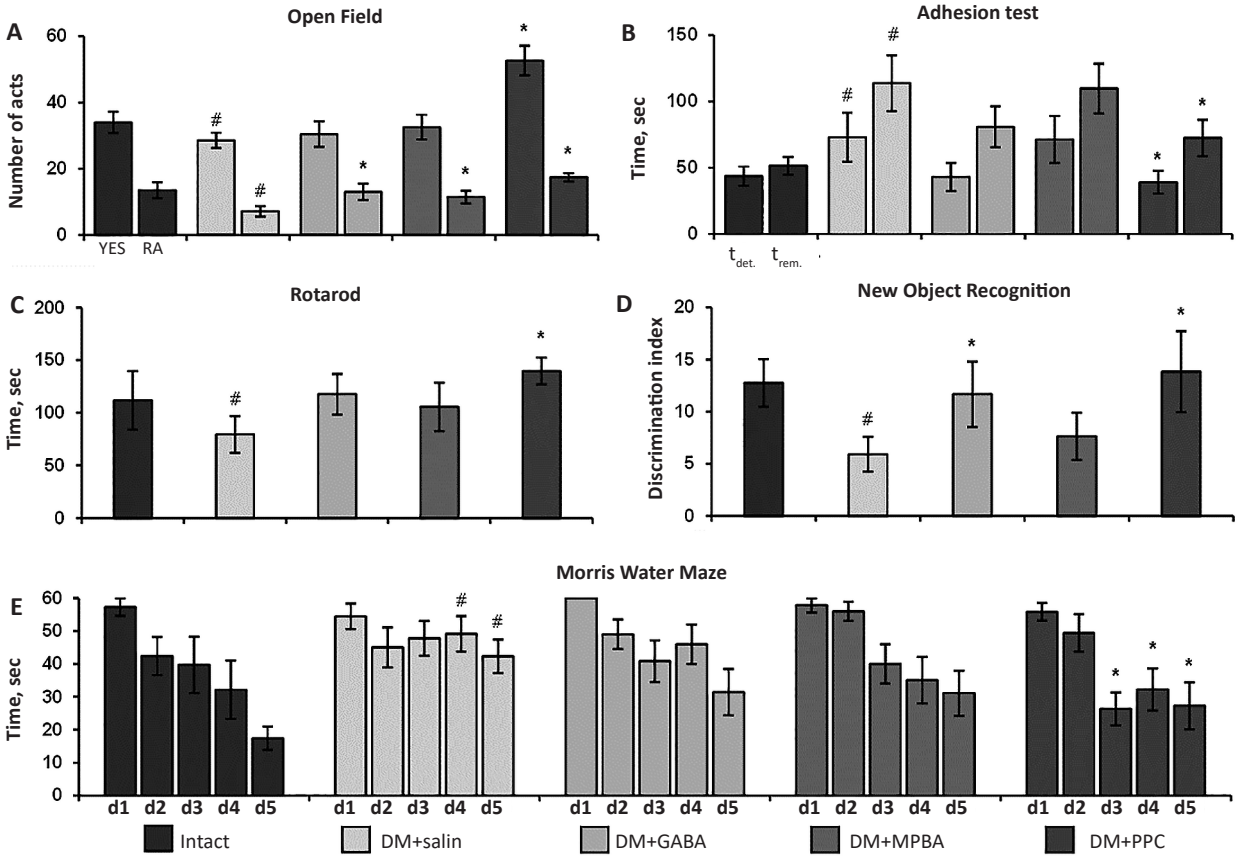

Figure 3 – Motor and exploratory activities in the Open Field test (A), average time of detection and removal of the sticker in the Adhesion test (B), retention time on a rotating rod in the Rotarod test (C), Discrimination index in the New Object Recognition test (D) and the duration of the search for a flooded platform in the Morris Water Maze test (E)

Note: # – differences are statistically significant in comparison with the animals of the "Intact" group (p <0.05); * – differences are statistically significant in comparison with the animals of the "DM+salin" group (p <0.05) (Kruskal–Wallis and Dunn test); the data are presented as M±m. In the Open field test: YES – motor activity (left columns), the number of sectors crossed in 3 minutes, RA – research activity (right columns), the sum of research acts. In the Adhesion test: $t_{det.}$ – average time of sticker detection on the palmar surface of the forepaws (left columns), $t_{rem.}$ – average sticker removal time (right columns); in the Morris Water Maze test, the duration of the search for a flooded platform during the first landing during 5 days of the experiment is shown.

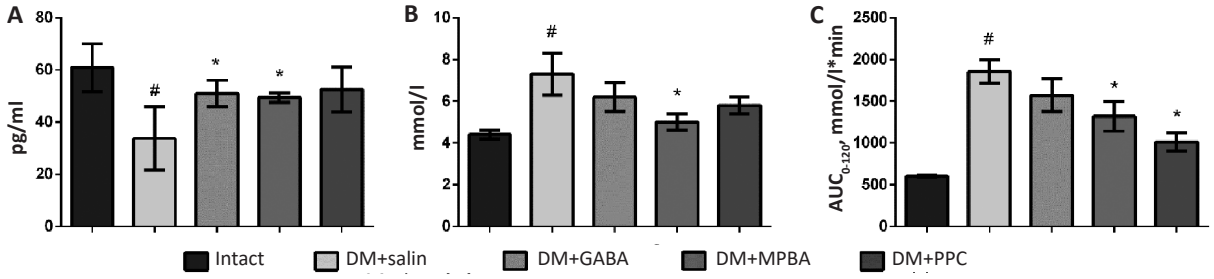

Figure 4 – Serum GLP-1 level (A), fasting blood glucose level (B) and area under glucose level–time curve (C)

Note: # – differences are statistically significant in comparison with the animals of the "Intact" group (p <0.05); * – differences are statistically significant in comparison with the animals of the "DM+salin" group (p <0.05; Kruskal–Wallis and Dunn test); data are presented as M±m.

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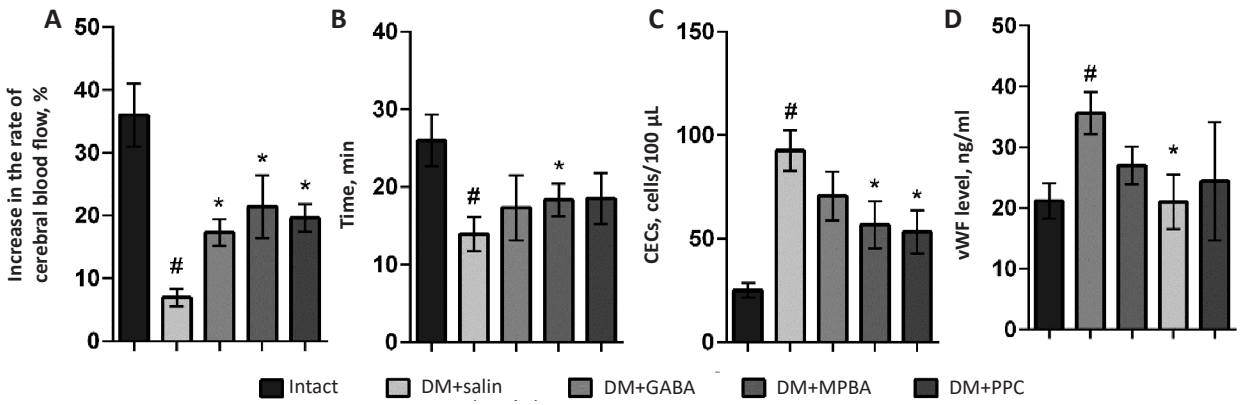

Figure 5 – Relative increase in the rate of cerebral blood flow after the administration of acetylcholine (A), the duration of thrombus formation in the carotid artery with the application of iron (III) chloride (B), the number of circulating endothelial cells (CECs) in plasma (C), the serum level of von Willebrand factor (vWF; D)

Note: # – the differences are statistically significant in comparison with the animals of the "Intact" group (p <0.05); * – the differences are statistically significant in comparison with the animals of the "DM+salin" group (p <0.05) (Kruskal–Wallis and Dunn test); the data are presented as M±m; MDA is malonic dialdehyde.

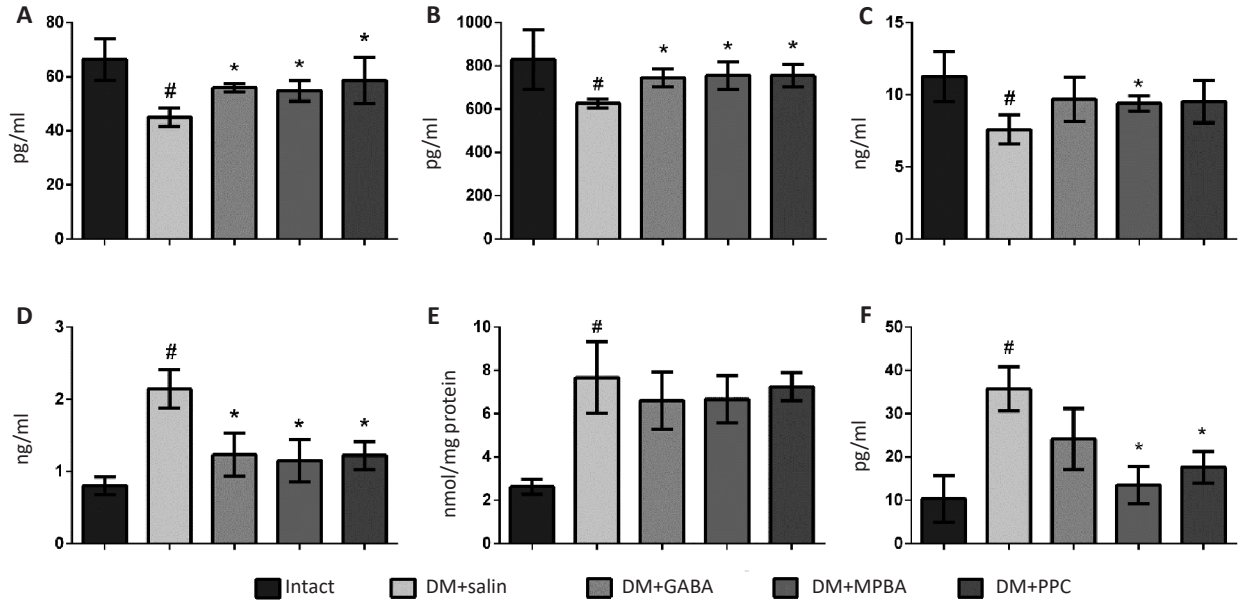

Figure 6 – The concentration of Klotho protein (A), BDNF (B), Nrf2 (C), Nf-kB (D), MDA (D) in brain tissues and TNF-α in blood serum (F) in animals with experimental DM

Note: # – the differences are statistically significant in comparison with the animals of the "Intact" group (p <0.05); * – the differences are statistically significant in comparison with the animals of the "DM+salin" group (p <0.05) (Kruskal–Wallis and Dunn test); the data are presented as M±m; MDA is malonic dialdehyde.

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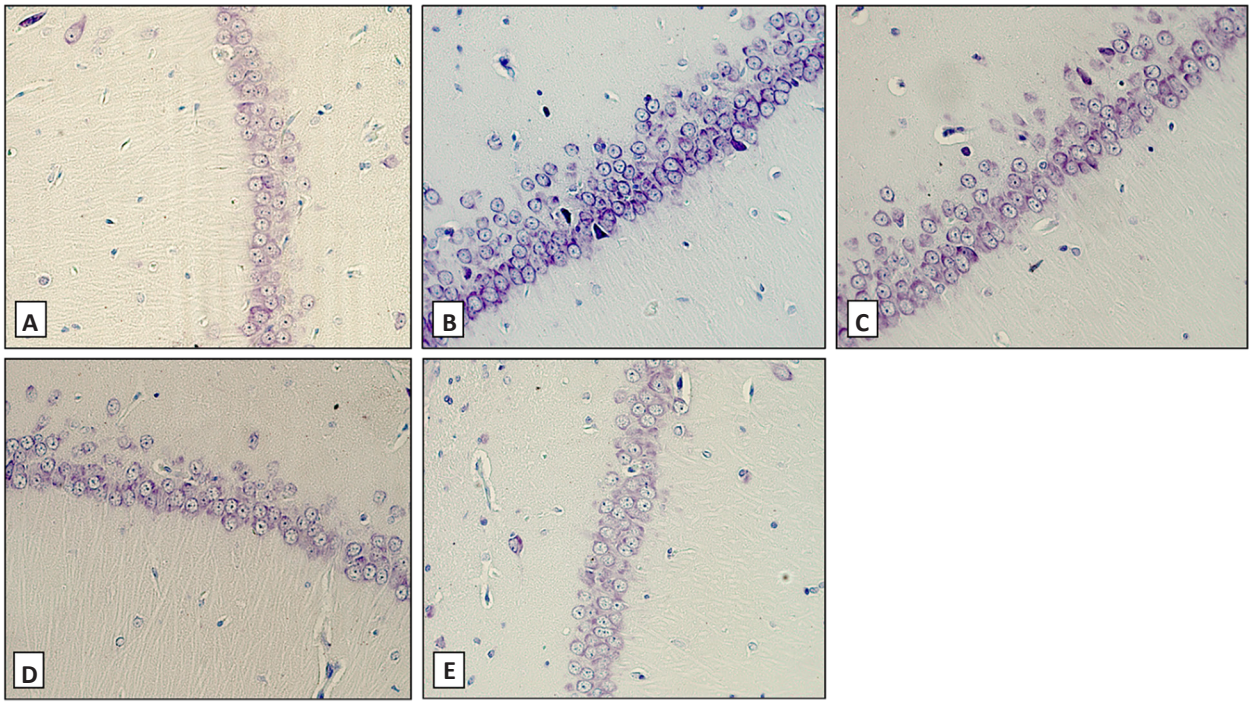

Figure 7 – Morphological changes in pyramidal layer CA1 of hippocampus

Note (here and in Fig. 8): A – Intact; B – DM+salin; C – DM+GABA; D – DM+MPBA; E – DM+PPC. Stained with thionine according to the Nissl method. Magn. ×400.

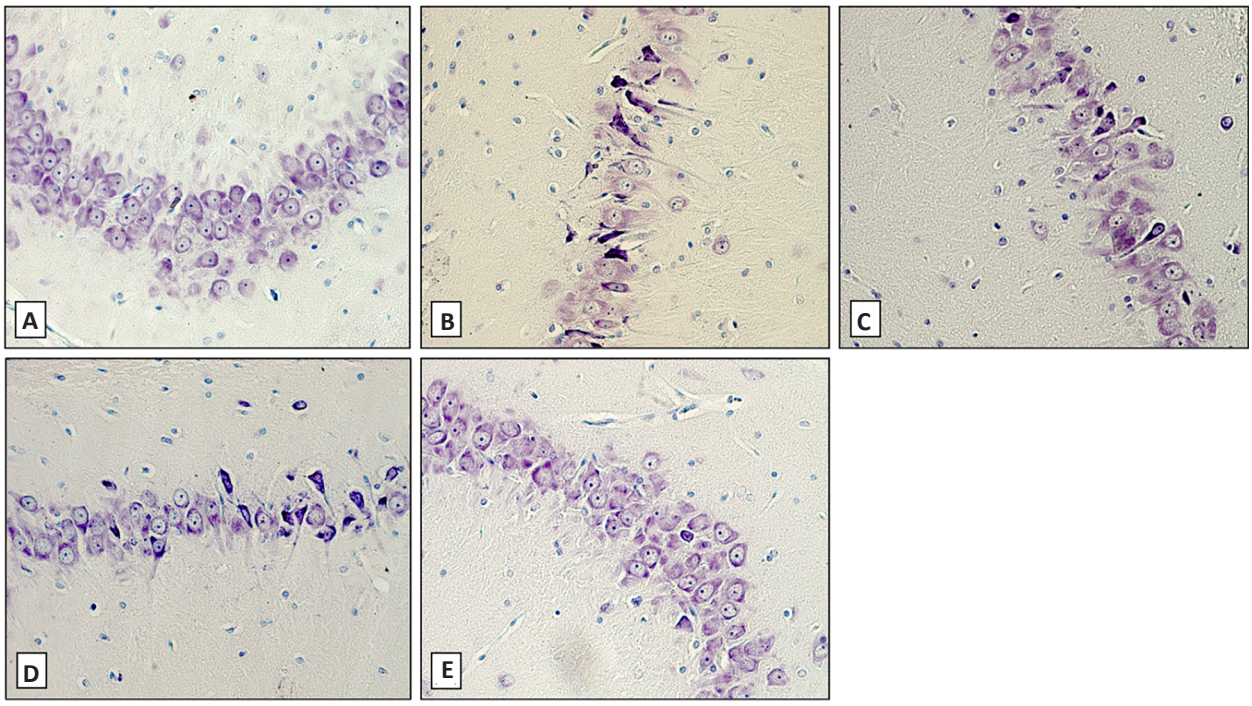

Figure 8 – Morphological changes in pyramidal layer CA3 of hippocampus

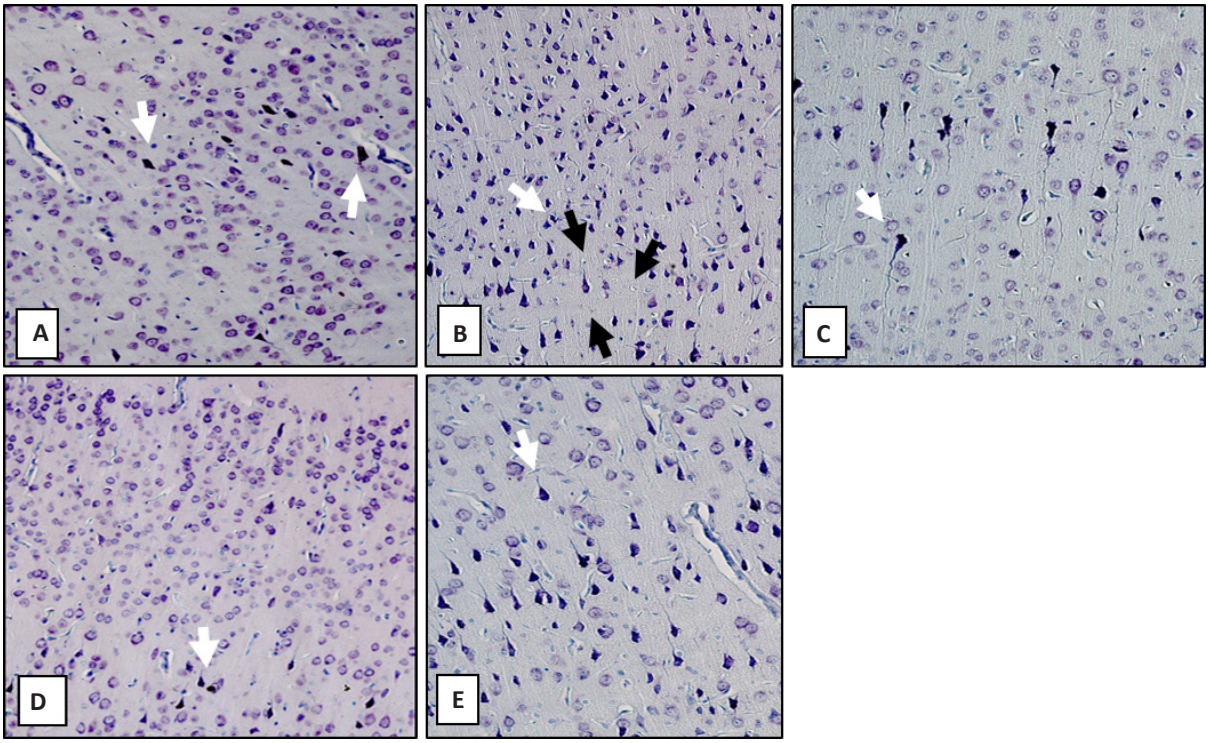

Figure 9 – Histological structure of the primary sensorimotor cortex of the studied animals

Note: A – Intact (the presence of single hyperchromic and hyperchromic shriveled neurons in all layers); B – DM+salin (pronounced hyperchromatosis in all layers, the presence of zones of "neurons loss"); C – DM+GABA (hyperchromatosis in the inner pyramidal layer); D – DM+MPBA (mild hyperchromatosis); E – DM+PPC (presence in the outer granular, outer pyramidal and inner pyramidal layers of a significant number of shriveled neurons with hyperchromatosis of the cytoplasm). White arrows are hyperchromic shriveled neurons. Black arrows are zones of neurons "loss". Stained with thionine according to the Nissl method. Magn.×200.

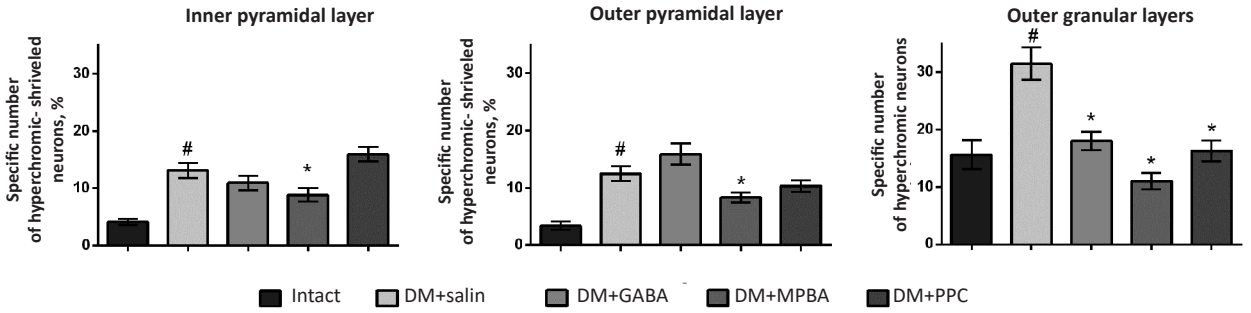

Figure 10 – Specific number of hyperchromic, hyperchromic-shriveled neurons in the primary somatosensory cortex of rats' brain

Note: # – the differences are statistically significant in comparison with the animals of the "Intact" group (p <0.05); * – the differences are statistically significant in comparison with the animals of the "DM+salin" group (p <0.05) (Kruskal-Wallis and Dunn test). The data are presented as M±m.

Table 1 – Specific number of neurons in pyramidal layer of hippocampus with hyperchromatosis and shriveled neurons with hyperchromatosis

Hyperchromatic neurons Me (Q1-Q3), %					
Hippocampus zone	Intact	DM+salin	DM+GABA	DM+MPBA	DM+PPC
CA1	0.8 (0–4.7)	11.6 (3.1–16.9) #	0 (0–0)*	0 (0–0)*	0 (0–0)*
CA3	0 (0–0)	17.5 (2.8–27.9) #	2.6 (0–18.8)	0 (0–5.9) *	0 (0–25.6)
Hyperchromic shriveled neurons Me (Q1-Q3), %					
Hippocampus zone	Intact	DM	DM+GABA	DM+PPC	DM+MPBA
CA1	1.8 (0–4.4)	3.6 (0–9.2)	3.1 (1.9–6.9)	5.5 (0–18.4)	2 (0–10.9)
CA3	4.4 (0–11.5)	2.3 (0–21.9)	0 (0–7.8)	2.4 (0–31)	0 (0–4.2)

Note: # – the differences are statistically significant in comparison with the animals of the "Intact" group (p <0.05); * – the differences are statistically significant in comparison with the animals of the "DM+ salin" group (p <0.05) (Kruskal–Wallis and Dunn test); GABA – gamma-aminobutyric acid; DM – diabetes mellitus.

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In the Rotarod test, in the animals of the negative control group, the retention time was statistically significantly less than in the intact animals, which indicated an impaired coordination of movements. The animals treated with GABA, MPBA, and especially PPC, stayed longer on the rotating rod (Fig. 3C).

In the New Object Recognition test, a significant decrease in the DI (impaired short-term memory) was noted in the group of the animals with DM. A significantly longer examination time of a new object was recorded compared to the old object, i.e. the animals were distinguished by a better ability to identify and compare the information stored (for 60 min) about a previously studied subject (Fig. 3E).

In the Morris Water Maze test, the preservation of long-term spatial memory was assessed by the time it took to search for a submerged platform for 5 days. In the group of the animals with chronic hyperglycemia without treatment, a violation of long-term memory was noted: every day of the experiment, on the first landing, they searched for a flooded platform longer than the intact ones (Fig. 3E). In the groups treated with GABA, MPBA, and especially PPC from the 3rd day in each first test session, the animals found the flooded platform faster than in the control group, i.e. the preservation of long-term spatial memory was better.

Thus, in the animals with DM, in comparison with the intact animals, there were especially pronounced impairments in a cognitive function (New Object Recognition, the Morris Water Maze, and an EA in the OF) and fine motor skills (Adhesion test). A decrease in the motor activity (OF), deterioration in coordination (Rotarod) and a sensory function of the forelimbs (Adhesion test) were also observed.

A course administration of GABA, its linear derivative (MPBA), and especially its cyclic derivative (PPC) contributed to the improvement of the functional state of the brain, reducing the severity of cognitive, sensory and motor impairments in the animals with prolonged hyperglycemia.

Effect of GABA derivatives on carbohydrate metabolism

At the time of assessing the functional state of the brain, in the animals with DM, the level of GLP-1 in the control group was significantly lower than in the intact animals of the same age. In the animals treated with the test substances, the level of GLP-1 in the blood serum was comparably on average by 50% higher than in the animals of the control group (Fig. 4A).

During the OGTT, against the background of the course administration of MPBA and PPC compositions, a normalization of carbohydrate metabolism was noted in the animals. It was manifested in a decrease in the level of glycemia (after a 4-hour fast) and the area under the glucose level-time curve (Fig. 4B and 4C).

Effect of test substances on endothelial dysfunction

DM significantly increases the risk of developing cardiovascular diseases, and the latter increase the likelihood of developing vascular dementia [6, 22, 23]. At the same time, an endothelial dysfunction plays a significant role in pathogenesis.

In the present work, after assessing the neurological deficit in these animals, a study of the functional state of the endothelium was carried out. In the animals with DM without treatment, a pronounced decrease in endothelium-dependent vasodilation was noted: a slight increase in the level of the cerebral blood flow in response to the administration of acetylcholine compared with the animals of the intact group (Fig. 5A). In the animals that were injected with GABA, PPC, and especially MPBA, a significantly more pronounced increase in the level of the cerebral blood flow than in the control group of animals, was observed in response to the administration of acetylcholine.

When evaluating the antithrombotic function of the endothelium, it was found that when iron (III) chloride was applied to the adventitia of the common carotid artery, the time to stop a blood flow in the animals of the control group was almost twice shorter than in the intact ones (Fig. 5B). In the animals treated with MPBA, the time of a thrombus formation was significantly longer than in the animals of the control group.

To assess an endothelial dysfunction in the DM animals, two more indicators were used: the number of CECs and the von Willebrand factor (vWF). The amount of CEC in control animals with DM was 4 times higher than in intact animals, and in animals treated with GABA derivatives (MPBA and PPC), the amount of CEC was significantly lower than in the control group of animals (Fig. 5C).

The level of vWF in the DM animals without treatment was 68% higher than in the intact animals (Fig. 5D). In the animals treated with the composition of MPBA, the content of vWF in the blood serum was significantly lower than in the animals of the negative control group.

Thus, against the background of prolonged hyperglycemia, a pronounced endothelial dysfunction is formed, which may underlie structural and functional changes in the brain. The studied GABA derivatives (MPBA and PPC) improved the vasodilating and antithrombotic function of the endothelium, which may play an important role in adaptation and ensuring an adequate blood flow in intensively functioning structures.

Assessment of Klotho protein, BDNF, Nrf2, NF-κB, TNF-α and MDA levels

The literature data [12, 24] indicate that GABA increases the production of Klotho protein (KP),

which affects a cognitive function [25, 26]. It has been established that the main effects of KP are associated with its influence on the expression of nuclear transcription factors Nrf2 and NF-κB, which play an important role in the development of DM and its complications. In DM, along with a decrease in KP, there is also a decrease in the expression of a brain-derived neurotrophic factor (BDNF), which is expressed and synthesized not only in the brain but also in the pancreas, intestines, and other tissues, where it plays an important role in cytoprotection [27, 28].

Compared with the intact animals, the untreated DM animals had significantly lower levels of KP, BDNF, and transcription factor Nrf2, higher levels of transcription factor NF-κB, MDA, and higher serum levels of TNF-α (Fig. 6). Against the background of a course administration of GABA, PPC, and especially MPBA, there was a normalization of the content of the noted markers observed: KP, BDNF and transcription factors, as well as the pro-inflammatory cytokine TNF-α and the main product of lipid peroxidation, MDA.

Morphology of hippocampus

SD leads to the formation of structural and functional changes in the brain. Some areas of the brain, primarily the hippocampus, are particularly sensitive to prolonged hyperglycemia, which is one of the causes of a cognitive decline [29, 30]. At the same time, a relationship has been proven between the degree and duration of hyperglycemia and the risk of developing dementia in people with DM [4].

In the CA1 zone of the old intact rats' hippocampus, most neurons in the pyramidal layer were characterized by a close to rounded perikaryon with a centrally located rounded light nucleus and, as a rule, a well-defined nucleolus. There were sporadic areas of neuron loss, neurons with focal chromatolysis, and shriveled hyperchromic cells (Fig. 7A). In CA3, the pyramidal layer neurons were more dispersed than in CA1, and had a polygonal shape with a clearly visualized nucleus and one nucleolus; single hyperchromic neurons, shriveled neurons with hyperchromia, neurons with focal chromatolysis, and single areas of neuronal loss were found (Fig. 8A).

In the group of DM animals without treatment, in CA1 of the pyramidal layer compared with intact, some animals showed areas of neuronal loss, and in the terminal sections of the CA1 pyramidal layer, neurons with focal chromatolysis, hyperchromic and hyperchromic shriveled neurons were found, which were located in a group (Fig. 7B). In CA3 of the pyramidal layer, neurons were located more loosely than in CA1 (Fig. 8B). In all the animals there were areas of loss of neurons, focal chromatolysis. Most animals showed neurons with hyperchromic cytoplasm and hyperchromic neurons with shriveled perikaryons. There was a significant increase in the specific number of hyperchromic neurons by 10.8% in CA1 (p <0.05) and by 17.5% in CA3 of the hippocampus (p <0.05), compared with intact animals (Table 1). At the same time, there were no significant differences in the change in the specific number of hyperchromic shriveled neurons with CA1 and CA3 (p > 0.05) (Table 1).

In old DM rats, with a pharmacological correction of GABA, PPC, and MPBA, most neurons in the pyramidal layer were characterized by normochromic cytoplasm. In some animals, neurons with cytoplasmic hyperchromatosis and hyperchromic shriveled neurons were found; these neurons were located in a group in the terminal CA1 sections, and areas of neurocytes loss were observed (Fig. 7C–7E; Fig. 8–8E).

During statistical processing of the data in the CA1 zone of the hippocampus with the use of GABA, PPC and MPBA, a significant decrease in the specific number of hyperchromic neurons (p <0.05), compared with the DM group was found (Table 1). The use of MPBA also demonstrated statistically significant differences in CA3, a specific number of hyperchromic neurons decreased by 17.5% (p <0.05) (Table 1).

Morphology of somatosensory cortex

Histological examination of the intact rats' primary somatosensory cortex (Fig. 9) revealed single hyperchromic and hyperchromic-shriveled neurons in all layers. However, in some animals, loci of pronounced hyperchromatosis were found in layers 2nd, 3rd, and 5th, where, along with unchanged neurons, there were the neurons, the perikarya of which had an irregular shriveled, elongated or twisted shape, the nucleolus was not visualized. The pathological changes found in the intact rats' cortex, are associate with the age of the studied rats.

In experimental modeling of DM in aging rats (aged 12–19 months), the most pronounced signs of damage were found in neurons of layers 2nd, 3rd, and 5th of the primary somatosensory cortex. Herewith, an increase in the areas of neurons loss perikarya, revealed in layers 4th, 5th of the primary somatosensory cortex, indicates the development of atrophic changes in the cortex of the rats' brain, which is confirmed by a decrease in cognitive functions.

After treatment in aging rats (at the age of 12–19 months), the least pronounced pathological changes were found in neurons of layers 2nd, 3rd, 5th of the primary somatosensory cortex in the MPBA correction group; which indicates a protective effect of the composition on the structure of the cortex of the rats' brain.

A morphological study of the somatosensory cortex of DM rats' brain without treatment showed a significantly higher relative number of hyperchromic shriveled neurons compared to the animals without DM (Fig. 7).

In the MPBA pharmacocorrection group, a statistically significant decrease in the specific number of

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hyperchromic-shriveled neurons in the inner pyramidal, outer pyramidal, and outer granular layers was found compared to the DM group without treatment.

In DM rats treated with GABA and PPC, a significant decrease in the specific number of hyperchromic neurons in the outer granular layer was found compared to DM without treatment.

DISCUSSION

Under conditions of prolonged hyperglycemia, Nrf2 is considered as a key factor counteracting an oxidative stress, which activates the expression of genes responsible for the expression of antioxidant defense enzymes [31]. Therefore, the search for ways to activate the Nrf2 system is considered a potential cytoprotective strategy for the prevention and treatment of diseases with the pathogenesis based on an oxidative stress, including neurological pathologies [32–34].

The Nrf2 system and the NF-κB system are in an antagonistic relationship. For example, when Nrf2 is deficient, there is an increased NF-κB activity, and the Nrf2 activation has anti-inflammatory effects in many rodent models of inflammation. It has been shown that Nrf2 suppresses the expression of pro-inflammatory cytokine genes and has the ability to negatively affect NF-κB by inducing an antioxidant response [35].

In order to increase the translational potential, the present study was carried out on old animals with experimental DM, which already have pronounced functional and structural disorders in the brain. The vast majority of studies aimed at studying antidiabetogenic effects and diabetic complications, are performed on young animals at the age of 4–6 months, and the duration of modeled diabetes is limited to 12–16 weeks. Despite this fact, the development of age-related changes is significantly aggravated by prolonged hyperglycemia and an associated inflammation, glucose and lipotoxicity, oxidative and nitrosative stress.

The study was of a complex nature with the assessment of pathogenetic changes based on the morphofunctional approach. It showed that in old animals (19 months) with long-term (7 months) hyperglycemia, there were pronounced impairments of sensory and motor functions, coordination of movements, as well as a pronounced decrease in short-term, long-term and spatial memory. These cognitive impairments are of great importance in the clinic, as they reduce the quality of life of the patient, and in case of profound impairments, they become a burden for the family and society. The mitigation of cognitive impairments in patients with DM remains an unresolved task, even with the normalization of blood glucose levels.

Functional disorders are basically consistent with the structural disorders noted at the next stage. In old DM rats, the most pronounced signs of damage were found in pyramidal neurons in the CA1 and CA3 zones of the hippocampus, the areas of neuron loss, a significant increase in the specific number of neurons with signs of reversible changes (neurons with hyperchromia of the perikaryon cytoplasm without shriveling), an increase in the number of shriveled hyperchromic neurons. These are consistent with the data on progressive atrophic changes, ultrastructural damage to neurons and hippocampal synapses, which are accompanied by an increased oxidative stress, neuroinflammation, neuronal apoptosis, as well as cognitive deficits, learning and memory impairments in DM [21, 36, 37].

In the present article, the use of GABA derivatives, PPC and especially MFBA for pharmacological corrections, contributed to a significant decrease in signs of reversible disorders in pyramidal neurons CA1 and CA3 in the hippocampus zones, a decrease in the specific number of hyperchromic neurons compared to the DM group without treatment. At the same time, the drugs used did not have a significant effect on the level of neurons with signs of irreversible damage (hyperchromic shriveled neurons) in the pyramidal layer in the CA1 and CA3 zones of the hippocampus.

A comprehensive analysis of structural changes in the primary sensorimotor cortex and hippocampus of old rats with experimental DM showed the predominance of signs of irreversible and reversible neuronal damage. These changes were more pronounced in the outer granular, outer pyramidal and inner pyramidal layers. The damage to the neurons of the pyramidal layer of the CA1 and CA3 zones of the hippocampus was accompanied by the appearance of areas of neurons loss and a decrease in the absolute and relative areas of the perikarya of neurons in the primary sensorimotor cortex of the brain, which indicates the progression of atrophic processes.

Impairments of cognitive functions (an exploratory activity, short-term and long-term spatial memory) in rats with diabetes compared to intact animals of the same age (19 months) were accompanied by the development of morphological signs of neuronal damage and atrophic changes in the primary sensorimotor cortex and hippocampus. The use of GABA derivatives as a pharmacological correction for 1 month contributed to a decrease in the severity of sensory-motor and cognitive impairments, a decrease in morphological signs of neuronal damage and atrophic changes, which may be based on the normalization of glucose levels, improvement of the vasodilating and antithrombotic functions of the endothelium. They are considered leading pathogenetic factors in the development of diabetic encephalopathy and angiopathy [21].

Damage to neurons and glia contributes to the

combined effect of an increased oxidative stress, neuroinflammation, neurotransmitter abnormalities, respectively, the use of drugs that normalize the above processes, promotes neuroprotection, reducing neurodegenerative changes in the hippocampus and cerebral cortex during the progression of diabetic encephalopathy [38]. Therefore, close attention to GABA, which stimulates the production of PK, is paid. In this study, it was noted that the level of Klotho protein in the brain increased under the influence of both GABA and its derivatives, which had been used in much lower doses. At the same time, KP is considered a reasonable therapeutic target due to its ability to increase the activity of various body defense systems under the influence of a number of damaging factors: oxidative and nitrosative stress, inflammation, mitochondrial dysfunction, apoptosis and cell death, and to prevent early aging processes [12, 39].

Particularly noteworthy is the fact that the detected multiple heterogeneous signs of damage in the cerebral cortex and hippocampus arose with a combination of the implementation of age-dependent factors in conditions of hyperglycemia in DM. Accordingly, the use of drugs that have multimodal neuro-, geroprotective, hypoglycemic effects is becoming the preferred therapy strategy. Thus, it has been shown that aging and DM are accompanied by a decrease in the production of KP, expressed in the kidneys, brain, pancreatic beta cells and other tissues [24, 40, 41], and the use of GABA leads to pancreatic protective effects, increases the level of circulating Klotho protein. GABA and KP inhibit the activation of the NF-κB protein, which promotes the stimulation of inflammatory reactions that trigger beta-cell apoptosis [12, 39], which makes it possible to consider GABA derivatives as a promising group for studying their effects in the treatment of diabetic encephalopathy in the elderly.

What underlies the neuroprotective action of GABA and its derivatives (MPBA and PPC)? The answer to this question is of fundamental importance. All of them had a unidirectional effect, improving sensory-motor, cognitive, and endothelial function in DM rats. This may indicate a similar mechanism of action. Given the analogy of the action of GABA and KP on pancreatic β-cells. It has been shown that the cytoprotective effect of GABA is associated with an increase in the production and level of KP [12]. In animals with KP knockout, the pancreatic protective effect of GABA was significantly reduced. Therefore, in this work, after a 30-day administration of GABA, the content of KP in the brain was determined. In DM animals that did not receive the test substances, the content of KP in the brain was significantly lower than in intact animals. In the animals treated with GABA derivatives, the level of KP was statistically significantly higher than in the animals of the control group. This action of the studied substances deserves special attention, because its content in the brain, fluid media, blood serum, urine, etc. can serve as a marker of diabetic complications, aging, prognosis of dementia and cardiovascular risks, as well as the assessment of the severity course of various diseases, when its level decreases [42]. KP deficiency plays an important role in the pathogenesis of cognitive impairments [26] and cardiovascular diseases [43].

At present, a wide spectrum of biological activity of the anti-aging Klotho protein is presented in the literature [12, 43, 44]. It has been established that in many pathologies (cardiovascular diseases, DM, kidney pathology, aging), its level decreases. Therefore, the search and development of substances that stimulate the production of KP is an important task. In relation to the interpretation of the results obtained, its effect on the expression of nuclear factors Nrf2 and NF-κB, which play a key role in the development of complications of DM, should be noted.

CONCLUSION

In old animals (aged 19 months) with long-term (7-month) hyperglycemia, disturbances in sensory-motor functions, coordination of movements, a pronounced decrease in short-term and long-term memory, and a significant deterioration in the vasodilating function of the cerebral vascular endothelium were found. These functional disorders were accompanied by the development of morphological signs of neuronal damage and atrophic changes in the primary sensorimotor cortex and hippocampus. Compared with intact animals, untreated DM animals had significantly lower levels of Klotho protein, BDNF, and transcription factor Nrf2, as well as higher levels of transcription factor Nf-kB, MDA, and TNF-α in brain homogenates.

A course GABA administration, its linear and cyclic derivatives (MPBA and PPC) contributed to an increase in the GLP-1 production and normalization of carbohydrate metabolism, a decrease in the severity of cognitive and sensory-motor disorders, an improvement in the vasodilating and antithrombotic function of the endothelium, which was accompanied by a decrease in morphological signs of neuronal damage.

The neuroprotective properties of GABA and its derivatives in diabetic encephalopathy in old animals can be explained by an increase in the levels of the anti-aging PK, brain-derived neurotrophic factor (BDNF), the level of the transcription factor Nrf2, which regulates the activity of antioxidant defense enzymes, as well as a decrease in the level of nuclear transcription factor NfkB and TNF-α, which are responsible for the formation and maintenance of inflammatory processes that underlie the development of diabetes complications.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

Ivan N. Tyurenkov – idea and planning of the study, writing a draft of the article, approval of the final version of the manuscript; Dmitry A. Bakulin – modeling and control of pathology, design of the final version of the manuscript; Aleksey V. Smirnov, Maria R. Ekova, Aislu I. Bisinbekova, Grigory L. Snigur, Yulia I. Velikorodnaya – histochemical staining and assessment of morphological changes in the hippocampus and sensorimotor cortex of the brain, analysis and description of the results; Evgeniy I. Morkovin – assessment of neuropsychiatric deficit; Dmitry V. Verkholyak – assessment of cerebral blood flow and the functional state of the endothelium of cerebral vessels; Olga S. Vasilyeva – development of the studied compounds. All authors made a substantial contribution to the conception of the work, acquisition, analysis, interpretation of data for the work, drafting and revising the work, final approval of the version to be published and agree to be accountable for all aspects of the work.

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RESEARCH ARTICLE

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Model for predicting risk of developing drug-induced liver injury during remdesivir therapy: observational prospective open case-control study

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Remdesivir is a drug widely used for the etiotropic treatment of COVID-19. According to a number of studies, the incidence of adverse reactions during remdesivir therapy reaches 66%, with the most common is an increase in liver function tests.

The aim of the work was to study the influence of clinical, demographic and pharmacogenetic factors on the development of drug-induced liver damage during remdesivir therapy in COVID-19 patients.

Materials and methods. The study comprised 100 hospitalized patients treated with remdesivir. The patients were divided into two groups: group 1 (n=32) – remdesivir therapy, developed an increase in the level of liver transaminases; group 2 (control, n=68) – did not develop this adverse reaction. The patients in both groups underwent a pharmacogenetic study, and a retrospective analysis of medical records was performed. Based on the data obtained, the association of clinical, laboratory, pharmacological and pharmacogenetic parameters with the development of drug-induced liver damage during remdesivir therapy was studied.

Results. In the group of patients with the development of drug-induced liver damage, people with a high body mass index were significantly more likely than in the control group (30.7±4.2 kg/m^2 in group 1 *vs.* 27.3±5.5 kg/m^2 in group 2, p=0.003), with a history of diabetes mellitus (odds ratio (OR)=2.647, 95% confidence interval (CI)=1.092–6.414, χ^2=4.785, p=0.029), with higher levels of ferritin in the blood (724.03±432.27 and 553.19±358.48 mg/mol, respectively, p=0.040), receiving therapy with angiotensin-converting enzyme inhibitors (OR=5.440, 95% CI=2.160–13.699, χ^2=14.027, p=0.000), statins (OR=3.148, 95% CI=1.307–7.581, χ^2=6.795, p=0.009), and also being heterozygous for the polymorphic marker *rs776746* of the *CYP3A5* gene (OR=3.961, 95% CI=1.343–11.686, χ^2=6.772, p=0.009).

Conclusion. A high body mass index, a history of diabetes mellitus, high levels of ferritin in the blood, concomitant therapy with angiotensin-converting enzyme inhibitors and statins, as well as a carriage of the AG genotype for the polymorphic marker *rs776746* of the *CYP3A5* gene increase the likelihood of developing drug-induced liver damage during remdesivir therapy. In this regard, it is necessary to consider these factors when prescribing remdesivir therapy, conduct a more careful monitoring of clinical and laboratory indicators of liver damage, and develop personalized approaches to the treatment of COVID-19 patients.

Keywords: COVID-19; remdesivir; hepatotoxicity; adverse reactions; predictors of adverse reactions; pharmacogenetic study; clinical trial

Abbreviations: CES1 – carboxyl esterase 1; ALT – alanine aminotransferase; AST – aspartate aminotransferase; ACEI – angiotensin-converting-enzyme; angiotensin-converting-enzyme inhibitors – ACE inhibitors; IL-6 – interleukin-6; BMI – body mass index; DILI – drug-induced liver injury; NSAIDs – non-steroidal anti-inflammatory drugs; ICD – International Classification of Diseases; AR – adverse reaction; PCR – polymerase chain reaction; GFR – glomerular filtration rate.

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Модель прогнозирования риска развития лекарственного поражения печени на фоне терапии ремдесивиром: обсервационное проспективное открытое контролируемое исследование

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Ремдесивир является препаратом, широко используемым для этиотропной терапии COVID-19. Согласно данным ряда исследований, частота развития нежелательных реакций при терапии ремдесивиром достигает 66%, при этом наиболее частой из них является повышение показателей печеночных проб.

Цель. Изучение влияния клинико-демографических и фармакогенетических факторов на развитие лекарственного поражения печени при терапии ремдесивиром у пациентов с COVID-19.

Материалы и методы. В исследование было включено 100 госпитализированных пациентов, получавших лечение препаратом редмесивир. Пациенты были разделены на две группы: группа 1 (*n*=32), у которой на фоне терапии ремдесивиром развилось повышение уровня печеночных трансаминаз; группа 2 (контроль, *n*=68), у которых не было выявлено развития упомянутой нежелательной реакции. Пациентам обеих групп было проведено фармакогенетическое исследование, а также был проведен ретроспективный анализ историй болезни. На основании полученных данных изучена ассоциация клинических, лабораторных, фармакологических и фармакогенетических показателей с развитием лекарственного поражения печени при терапии ремдесивиром.

Результаты. В группе пациентов с развитием лекарственного поражения печени достоверно чаще, чем в группе контроля, встречались лица с высоким индексом массы тела (30,7±4,2 кг/м^2 в группе 1 против 27,3±5,5 кг/м^2 в группе 2, *p*=0,003), имеющие сахарный диабет в анамнезе (отношение шансов (ОШ)=2,647, 95% доверительный интервал (ДИ)=1,092–6,414, χ^2=4,785, *p*=0,029), более высокий уровень ферритина в крови (724,03±432,27 и 553,19±358,48 мг/моль соответственно, *p*=0,040), получавшие терапию ингибиторами ангиотензин-превращающего фермента (ОШ=5,440, 95% ДИ=2,160–13,699, χ^2=14,027, *p*=0,000), статинами (ОШ=3,148, 95% ДИ=1,307–7,581, χ^2=6,795, *p*=0,009), а также являющиеся гетерозиготой по полиморфному маркеру *rs776746* гена *CYP3A5* (ОШ=3,961, 95% ДИ=1,343–11,686, χ^2=6,772, *p*=0,009).

Заключение. Высокий индекс массы тела, сахарный диабет в анамнезе, высокий уровень ферритина в крови, сопутствующая терапия ингибиторами ангиотензин-превращающего фермента и статинами, а также носительство генотипа AG по полиморфному маркеру *rs776746* гена *CYP3A5* повышают вероятность развития лекарственного поражения печени при терапии ремдесивиром. В связи с этим, необходимо учитывать эти факторы при назначении терапии ремдесивиром, проводить более тщательный мониторинг клинических и лабораторных показателей поражения печени и разрабатывать персонализированные подходы к лечению пациентов с COVID-19.

Ключевые слова: COVID-19; ремдесивир; гепатотоксичность; нежелательные реакции; предикторы нежелательных реакций; фармакогенетическое исследование; клиническое исследование

Список сокращений: CES1 – карбоксиэстераза 1; АЛТ – аланинаминотрансфераза; АСТ – аспартатаминотрансфераза; АПФ – ангиотензинпревращающий фермент; иАПФ – ингибиторы АПФ; ИЛ-6 – интерлейкин-6; ИМТ – индекс массы тела; ЛПП – лекарственное поражение печени; НПВП – нестероидные противовоспалительные препараты; НР – нежелательная реакция; МКБ – Международная классификация болезней; ПЦР – полимеразная цепная реакция; СКФ – скорость клубочковой фильтрации.

INTRODUCTION

In the context of the COVID-19 pandemic, there is a need to search for more effective antiviral drugs. Remdesivir is an antiviral drug approved for the treatment of a mild to moderate coronavirus infection. Remdesivir has become widely used in clinical practice, but there are limited data on its safety, pharmacokinetic properties and drug interactions in the treatment of COVID-19.

There are studies on the development of adverse reactions (ARs) during remdesivir therapy. Thus, in a retrospective observational study conducted using the data from the FDA's Adverse Event Reporting System (FAERS), reports of ARs with the use of remdesivir were analyzed for the period from 2019 to 2021. As a result, it was determined that one of the most common ARs was an increase in the liver function tests, the incidence of which was 14.28% [1]. Another retrospective study conducted from September 2020 to February 2021 also found out that the most common AR was elevated liver function tests, with an incidence of 12.9% [2].

The widespread prevalence of drug-induced liver injury (DILI) during remdesivir therapy indicates the need for a further study of its safety, as well as developing methods for a personalized approach patients treatment.

Remdesivir is a prodrug. There is an evidence that 80% of its metabolism occurs under the action of carboxylesterase 1 (*CES1*), which is its main metabolic enzyme, as well as 10% by cathepsin A and 10% by *CYP3A* [3, 4]. In this regard, polymorphism of the genes encoding these enzymes may affect the pharmacokinetics of remdesivir.

Cytochrome P450 of family 3 subfamily A *(CYP3A)* accounts for about 30% of the total content of CYP450 enzymes in the human liver; *CYP3A* enzymes are involved in the metabolism of approximately 50% of drugs [5, 6]. According to the PharmGKB resource [7], some drugs are metabolized with the participation of *CES1*. These two factors indicate that when treating with remdesivir, it is necessary to take into account both the genetic characteristics of patients and concomitant drug therapy.

THE AIM of the work was to study the influence of clinical, demographic and pharmacogenetic factors on the development of drug-induced liver damage during remdesivir therapy in COVID-19 patients.

MATERIALS AND METHODS

Study design

The study was a prospective observational open "case-control" type. The study comprised men and women (*n*=100) aged 18 years and older, hospitalized with a confirmed new coronavirus infection (COVID-19) (U07.1; U07.2 according to the ICD), meeting the inclusion criteria and not meeting the exclusion criteria.

The study was conducted at the city of Moscow Municipal Clinical Hospital No. 15 n.a. O.M. Filatov (Moscow, Russia).

Inclusion criteria for the study were as follows: the established diagnosis of a new coronavirus infection (COVID-19) (U07.1; U07.2 according to the ICD); signed voluntary informed consent to participate in the study; the duration of hospitalization >48 h; the use of remdesivir as etiotropic therapy.

Non-inclusion criteria for the study were as follows: glomerular filtration rate (GFR) less than 30 ml/min/1.73 m^2, pregnancy, breastfeeding, increased alanine aminotransferase (ALT) levels above 5 upper limits of normal ones, a severe liver failure (class C according to Child-Pugh).

Ethical approval

The study complied with the requirements of the World Medical Association's Declaration of Helsinki and was approved by the local ethics committee of the Russian Medical Academy of Continuing Professional Education (RMA CPE) (Protocol No. 15 dated October 16, 2021). A informed consent to participate in this study was obtained from all patients or their legal representatives.

Study duration

The study was conducted between November 2021 and February 2022.

The study comprised 100 hospitalized patients. The age of all patients ranged from 44 to 96 years (the mean age was 73.0±12.5 years). Of these, 31 (31%) were the men, whose average age was 72.91±12.62 years, and 69 (69%) were the women, whose average age was 73.0±12.5 years.

Research methodology

Remdesivir was used in the standard dose: 200 mg IV on the first day, then 100 mg once a day for 5–10 days. The investigator had no influence on the choice of an antiviral drug or a therapy duration.

Subsequently, taking into account the aim of the study, the patients were divided into 2 groups. Group 1 (main group, *n*=32) – patients who, during remdesivir therapy, experienced an increase in transaminase levels, 19 (59%) were the women averagely aged 68.6±12.2 years, as well as 13 (41% of the men) whose average age was 68.5±12.3 years. Group 2 (control group, *n*=68) patients who did not develop DILI during remdesivir therapy, 50 (74%) were the women averagely aged 75.1±12.2 years and 18 (26% of the men) averagely aged 75.0±12.4 years.

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Based on the retrospective analysis of medical histories, it was established that these groups were comparable in gender, anamnesis data – the time of the onset of the disease, the results of the objective examination, the condition severity, concomitant diseases, laboratory parameters, such as a general blood test, a biochemical blood test, including the determination of the total levels of bilirubin, glucose, creatinine, lactate dehydrogenase and indicators of the systemic inflammatory response syndrome: C-reactive protein (CRP), procalcitonin and interleukin-6. The study groups were also comparable in terms of the lung damage degree according to the chest computed tomography data and the duration of hospitalization. At the same time, the groups differed in age, body mass index (BMI), ferritin and D-dimer levels in the blood.

Molecular and genetic research

10 ml of venous blood was collected from the patients using a Vacuette® vacuum system (Greiner Bio-One, Austria) into the tubes with ethylenediaminetetraacetate (EDTA). The whole blood and extracted DNA were stored at –80°C and transported at –20°C. Genotyping was carried out on the basis of the Research Institute of Molecular and Personalized Medicine, the Russian Medical Academy of Continuing Professional Education (Moscow, Russia). The isolation of the genomic DNA from the whole blood was carried out using a set of S-Sorb reagents for the DNA isolation on a silicon sorbent (Syntol LLC, Russia). The concentration of the extracted DNA was determined using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA). The determination of carriage of the single nucleotide polymorphism C>T of the *CYP3A4*22* gene (*rs35599367*) was carried out by allele-specific polymerase chain reaction (PCR) in real time on a CFX96 Touch Real Time System device with a CFX Manager software version 3.0 (BioRad, USA) using the commercial "TaqMan® SNP" kit Genotyping Assays" and "TaqMan Universal Master Mix II" (Applied Biosystems, USA). The carriage of the polymorphic marker A>G of the *CYP3A5*3* gene (*rs776746*) was determined using a commercial reagent kit (Synthol LLC, Russia). The determination of single-nucleotide genetic polymorphisms was carried out by allele-specific PCR in real time on a CFX96 Touch Real Time System device with CFX Manager Software version 3.0 (BioRad, USA). The carriage of the polymorphic marker *rs2244613* (A>C) of the *CES1* gene was detected by a real-time polymerase chain reaction using GenTest *CES1* reagent kits (Nomotek LLC, Russia) on a Real-Time CFX96 Touch amplifier (Bio-Rad Laboratories Inc., USA).

Statistical processing

When statistically processing the results obtained, the standard application package StatSoft Statistica 10.0 (StatSoft, USA) was used. To assess the normality of the quantitative data distribution, graphical (frequency histogram) and calculation methods of Kolmogorov-Smirnov and Shapiro-Wilk were used. Considering the normal distribution of the quantitative data, they were expressed as the arithmetic mean and standard deviation (M±SD); the Student's *t*-test was used to analyze intergroup differences in the quantitative characteristics.

Qualitative indicators are presented as absolute values (*n*) and percentages (%). To identify intergroup differences in the frequencies of qualitative parameters and assess their statistical significance, the χ^2 test (Pearson chi-square) was used. For a small number of observations, Fisher's exact test was calculated. To assess the correlation between the studied parameters, the odds ratio (OR) of the event development with a 95% confidence interval (CI) was calculated. A multivariate regression analysis was also conducted to identify the predictors associated with the development of drug-induced liver injury during remdesivir therapy. The distribution of genotype frequencies of all studied polymorphic markers corresponded to the Hardy-Weinberg equilibrium. The significance of the identified differences and correlations in all types of analysis was accepted at the level of $p < 0.05$.

RESULTS

Gender and age characteristics

The analysis of the demographic data showed that the patients' age of the studied groups was statistically significantly different (p=0.015). In the group of patients with DILI, the average age was 68.6±12.2 years compared to the patients in the control group, whose average age was 75.1±12.2 years. Moreover, the groups were comparable by gender (χ^2=2.038, p=0.153).

Clinical characteristics

When analyzing clinical characteristics, it was revealed that a high BMI was 30.7±4.2 kg/m^2 in the group with DILI *vs.* 27.3±5.5 kg/m^2 in the control group, statistically significantly increases the likelihood of developing DILI during remdesivir therapy (p=0.003). The presence of diabetes mellitus in a patient also increases the likelihood of developing DILI (Table 1).

The analysis of the laboratory data before the start of remdesivir therapy showed that the groups were comparable in terms of general and biochemical blood tests, with a statistically significant difference in ferritin and D-dimer levels. The level of D-dimer was twice higher in the control group (Table 2).

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Table 1 – Comparison of clinical characteristics

Characteristics		Number of patients, *n*		*p*, χ^2	OR (95% CI)
		Main group (group 1) *n*=32	Control group (group 2) *n*=68		
Age, years		68.6±12,2	75.1±12.2	0.015 –	–
Body mass index, kg/m^2		30.7±4,2	27.3±5.5	0.003 –	–
History of adverse reactions		8	15	0.744 0.106	1.178 (0.440–3.152)
Severity of illness	Moderate	22	43	0.793* –	–
	Major	6	13		
	Extreme	4	12		
Comorbidity		28	61	0.742 0.108	0.803 (0.217–2.96)
Cardiovascular diseases		26	59	0.471 0.519	0.661 (0.213–2.049)
Cardiac ischemia		11	30	0.355 0.854	0.663 (0.277–1.588)
Chronic heart failure		4	13	0.411* –	0.663 (0.180–2.026)
Arterial hypertension		28	58	0.767 0.088	1.207 (0.348–4.188)
Diabetes mellitus		15	17	0.029** 4.785	2.647 (1.092–6.414)
Chronic kidney disease		0	14	0.006* –	–
Active cancer (diagnosed earlier than 6 months before study entry)		0	8	0.043* –	–
Encephalopathy		17	44	0.268 1.227	0.618 (0.263–1.452)

Note: OR – odds ratio; CI – confidence interval; *p* – significance level; χ^2 – Pearson's test. **p*-value was comparable to Fisher's exact test. ** Differences are statistically significant.

Table 2 – Laboratory data comparison of main and control groups

Indicators of general and biochemical blood tests	Main group (group 1), *n*=32	Control group (group 2), *n*=68	*p*
Leukocyte count, 10^9/l	6.7±3.8	7.1±3.6	0.652
Absolute neutrophil count, 10^9/l	5.2±3.3	6.9±12.0	0.417
Absolute lymphocyte count, 10^9/l	1.0±0.5	1.1±0.8	0.686
Alanine transaminase, IU/l	33.7±20.4	35.7±38.8	0.791
Aspartic transaminase, IU/l	49.7±22.5	46.1±31.6	0.564
De Ritis coefficient	1.7±0.9	1.8±0.9	0.848
Glucose, mmol/l	7.5±3.8	7.4±3.3	0.851
Creatinine, µmol/l	94.4±16.0	99.3±38.8	0.497
Lactate dehydrogenase, IU/l	432.0±179.3	402.0±180.3	0.438
Ferritin, mg/mol	724.0±432.2	553.1±358.5	0.040**
Interleukin-6, pg/ml	159.3±329.9	100.6±208.1	0.282
Procalcitonin, ng/ml	0.2±0.2	1.6±7.0	0.323
C-reactive protein, mg/l	108.4±75.3	100.5±79.3	0.640
D-dimer, ng/ml	1124.9±1109.0	2225.5±2429.5	0.016**

Note: *p* – significance level. ** Differences are statistically significant.

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Table 3 – Comparison of pathogenetic drug therapy for COVID-19 patients

Drugs	Number of patients, *n*		*p*, χ^2	OR (95% CI)
	Main group (group 1), *n*=32	Control group (group 2), *n*=68		
Glucocorticosteroids	8	15	0.744 0.106	1.178 (0.440–3.152)
Janus kinase inhibitors	30	52	0.036** 4.402	4.615 (0.992–21.467)
Interleukin inhibitors	32	59	0.031** 4.654	–
Repeated administration of interleukin inhibitors	9	15	0.508 0.439	1.383 (0.529–3.613)
Enoxaparin sodium	30	64	0.942 0.005	0.938 (0.163–5.405)

Note: OR – odds ratio; CI—confidence interval; *p*—significance level; χ^2—Pearson's test. ** Differences are statistically significant.

Table 4 – Comparison of drug therapy for concomitant diseases

Drugs	Number of patients, *n*		*p*, χ^2	OR (95% CI)
	Main group (group 1), *n*=32	Control group (group 2), *n*=68		
Antibacterial drugs	14	36	0.391 0.735	0.691 (0.297–1.610)
Antifungal drugs (azoles)	2	2	0.431* –	2,200 (0.296–16.369)
Statins	17	18	0.009** 6.795	3,148 (1.307–7.581)
Beta blockers	9	29	0,163 1,948	0,526 (0.212–1.305)
Calcium channel blockers	7	21	0.349 0.876	0.627 (0.234–1.675)
ACE inhibitors	18	13	0.000** 14.027	5.440 (2.160–13.699)
Angiotensin II receptor blockers (sartans)	2	13	0.093* –	0.282 0.060–1.334
Diuretics	11	23	0.957 0.003	1.025 (0.423–2.485)
Nonsteroidal anti-inflammatory drugs	8	23	0.373 0.792	0.652 (0.254–1.678)
Antipsychotics	4	11	0.631* –	0.740 (0.216–2.534)
Prokinetics	3	12	0.280* –	0.483 (0.126–1.848)
Proton pump inhibitors	31	67	0.581 0.304	0.463 (0.028–7.642)
Biguanides	3	2	0.168* –	3.414 (0.541–21.532)
Salicylates	3	5	0.728* –	1.303 (0.292–5.827)

Note: ACE – angiotensin-converting enzyme; OR – odds ratio; CI – confidence interval; *p* – significance level; χ^2 – Pearson's test. * *p* corresponds to Fisher's exact test. ** Differences are statistically significant.

Table 5 – Genetic data

Gene	Genotype	Number of patients, *n*		*p*, χ^2	OR (95% CI)
		Main group (group 1), *n*=32	Control group (group 2), *n*=68		
CYP3A5 (*rs776746*) A>G	AA	22	61	0.009** 6.772	0.252 (0.086–0.745)
	AG	10	7	0.009** 6.772	3.961 (1.343–11.686)
	GG	0	0	–	–
CYP3A4 (*rs35599367*) C>T	CC	31	65	0.759 0.094	1.431 (0.143–14.317)
	CT	1	3	0.759* –	0.699 (0.070–6.994)
	TT	0	0	–	–
CES1 (*rs2244613*) A>C	AA	25	43	0.136 2.217	2.076 (0.785–5.490)
	AC	4	21	0.048* –	0.320 (0.100–1.027)
	CC	3	4	0.523* –	1.655 (0.348–7.876)

Note: OR – odds ratio; CI – confidence interval; *p* – significance level; χ^2 – Pearson's test. * *p* corresponds to Fisher's exact test. ** Differences are statistically significant.

Table 6 – Risk prediction of drug-induced liver damage during remdesivir therapy

Parameter	Regression coefficient, B±SE	95% CI	OR	*p*
Intercept	–7.195±1.782	[-10.688; -3.702]	–	<0.001
Body mass index	0.183±0.055	[0.075; 0.29]	1.2 [1.08; 1.34]	<0.001
ACE inhibitors	2.215±0.577	[1.083; 3.346]	9.16 [2.95; 28.39]	<0.001
CYP3A5 AG	1.567±0.662	[0.269; 2.864]	4.79 [1.31; 17.54]	0.018

Note: OR – odds ratio; CI – confidence interval; ACE – angiotensin-converting enzyme; *p* – significance level.

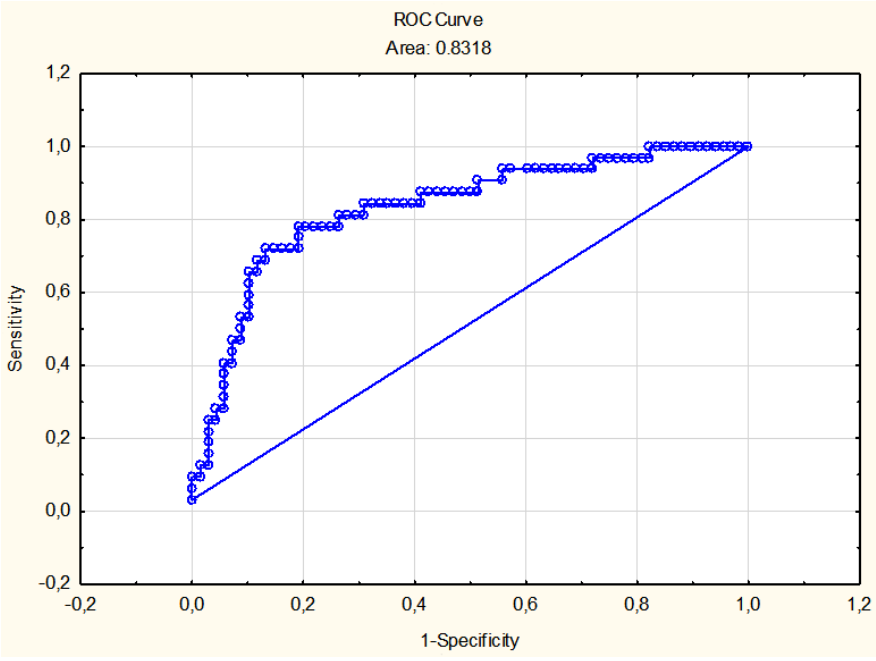

Figure 1 – ROC curve of logistic regression model

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Drug therapy

When analyzing pathogenetic drug therapy for COVID-19, it was revealed that the co-prescription of interleukin inhibitors significantly increases the likelihood of DILI. However, in group 1, the number of patients in whom interleukin inhibitors were used corresponded to 100%, which requires a further study of the effect of pathogenetic drug therapy for COVID-19 on development of DILI during remdesivir therapy in a larger sample. Statistically significant differences in the studied groups for the joint therapy with Janus kinase inhibitors were identified; however, the OR (95% CI) did not reach the level of statistical significance (Table 3).

An analysis of the drug therapy used to treat concomitant diseases showed that the combined use of the drugs from the group of HMG-CoA reductase inhibitors (statins), as well as the group of angiotensin-converting enzyme inhibitors (iACE), significantly increases the chance of developing DILI during remdesivir therapy, the probability increases on average by 3.14 and 5.44 times, respectively (Table 4).

Genetic data

The patients who are heterozygous for the *rs776746* polymorphic marker of the *CYP3A5* gene had a statistically significant likelihood of developing DILI during remdesivir therapy on average 3.96 times higher, while the carriers of the "wild" genotype were significantly less likely to be in the group of patients with DILI (Table 5).

Clinical outcomes

The average duration of hospitalization in group 1 was 12.5±6.9 bed-days, in group 2 – 13.0±10.9 bed-days. There was no statistically significant difference in the duration of hospitalization in the studied groups (p=0.813).

Multivariate logistic regression analysis

As a result of a multivariate logistic regression modeling, an ROC curve was obtained for risk predicting of developing drug-induced liver damage during remdesivir therapy (Fig. 1, Table 6). The modeling was performed with a stepwise elimination based on the Wald Chi-square statistics. The resulting model generalizes 38.9% of the variance in the predicted outcome and allows us to predict the risk of developing DILI in the patients receiving remdesivir, with an accuracy of 83.2%. Moreover, the forecast fully corresponds to the actual data (Hosmer-Lemeshow test, p=0.831).

It was found out that high BMI increases the risk of developing DILI remdesivir therapy by an average of 20% per unit of indicator (p <0.001).

A concomitant use of drugs from the group of ACE inhibitors increases the risk of developing DILI during remdesivir therapy by an average of 9.16 times (p <0.001).

Carriage of the AG genotype for the *rs776746* polymorphic marker of the *CYP3A5* gene increases the risk of developing DILI during remdesivir therapy by an average of 4.79 times compared to other genotypes (p=0.018).

DISCUSSION

According to various studies, the prevalence of ARs during remdesivir therapy ranges from 12[1] to 66% [8]. Moreover, one of the most common ARs is an increase in the activity of ALT and AST, which indicates liver damage. More serious and potentially fatal side effects, including bradycardia and renal failure, have been reported in the literature [9, 10]. Therefore, there is a need to develop a personalized approach for a timely prediction of the complications development when using remdesivir to treat COVID-19 patients. The available scientific works in this area are few.

According to the clinical guidelines, predisposing factors to the development of idiosyncratic DILI as age, gender, pregnancy, malnutrition, obesity and diabetes mellitus, as well as a DILI history[2].

In one of the observational studies, no significant relationship was found between age and the occurrence of ARs during COVID-19 therapy [11]. In the present study, it has been found out that patients who experienced DILI were on average younger in age compared to the group in which patients did not develop this AR. At the same time, no influence of gender on the increased risk of developing DILI has been identified.

In the present study, an association between the development of DILI during remdesivir therapy and an increase in BMI and the presence of diabetes mellitus has been revealed. At the same time, an increase in BMI for each unit of the indicator increases the risk of DILI by an average of 20%.

The course of COVID-19 is associated with actively occurring inflammatory processes [12]. As is known, the factors that cause this inflammation, in particular

[1] U.S. Food and Drug Administration. Gilead Sciences, Inc. VEKLURY® (remdesivir) for injection, for intravenous use. Available from: https://www.accessdata.fda.gov/drugsatfda_docs/label/2022/214787Orig1s010Lbl.pdf

[2] Ministry of Health of the Russian Federation. Clinical recommendations. Drug-induced liver injury (DILI) in adults, 2022. Available from: https://cr.minzdrav.gov.ru/schema/747_1?ysclid=lm9n3chyjg835121911

IL-6, reduce the activity and expression of enzymes of the cytochrome P450 system in the liver [13, 14], which may increase the risk of ARs during COVID-19 pharmacotherapy.

There is an assumption that a key pathogenetic molecular step in the course of COVID-19 is an attack on hemoglobin, leading to the dissociation of porphyrins from iron and the release of iron into the blood circulation. Thus, hemoglobin loses its ability to bind with oxygen and prevents its delivery to the main organs, which is accompanied by a rapid development of multiple organ failure. In addition, the free iron released into the bloodstream can lead to the iron overload, causing oxidative damage to the lungs and other organs. All this dictates an increased absorption and storage of the iron in iron-binding proteins. This is supported by the increased concentration of ferritin in the blood of COVID-19 patients. A high iron load leads to an increased blood viscosity with recurrent and diffuse macro- and microcirculatory thrombosis [15]. Considering this fact, the higher identified ferritin level in the group with DILI may indicate a possible connection between the ferritin level and the risk of developing DILI during remdesivir therapy. A trend towards higher levels of IL-6 in DILI patients has also been identified, but it did not reach a statistically significant difference.

According to the clinical guidelines for DILI in adults, the drugs with a risk of DILI include the following ones: antibacterial drugs, systemic antifungals belonging (azole group), statins, NSAIDs, acetylsalicylic acid, antihypertensive drugs, iACE and calcium channel blockers[3]. No clinical drug interaction studies have been fixed with remdesivir[4].

According to the clinical studies, the most common adverse event with the use of interleukin inhibitors was the liver damage, manifested by an increase in the activity of hepatic transaminases (ALT, AST), with an incidence of 3.7 to 35.8% [16–23]. The STOP-COVID study [24] showed that the highest incidence of liver enzyme elevations was observed in Janus kinase inhibitor therapy (4.2%).

In this regard, when remdesivir is co-administered with these drugs, the risk of liver toxicity theoretically increases. It has been found out that the concomitant therapy with iACE, statins and interleukin inhibitors increase the risk of developing DILI. In treatment with iACE, this risk increases by an average of 9.16 times. Statistically significant differences in the studied groups for joint therapy with Janus kinase inhibitors with an increase in the likelihood of DILI during joint therapy with this group of drugs have also been identified, but the OR (95% CI) did not reach the statistical significance level.

Remdesivir is extensively metabolized to GS-443902, a pharmacologically active nucleoside triphosphate analogue. Remdesivir is initially hydrolyzed by esterases to form the intermediate metabolite GS-704277. Carboxylesterase 1 and cathepsin A are responsible for 80 and 10% of the remdesivir metabolism, respectively, and *CYP3A* is responsible for the remaining 10%. Phosphoramidate cleavage of GS-704277 and a further phosphorylation of the resulting monophosphate nucleoside analogue leads to the formation of GS-443902. Dephosphorylation of all phosphorylated metabolites can lead to the formation of the nucleoside analogue GS-441524 [25].

*CYP3A5*3,* defined by an intronic variant (NM_000777.5: c.219-237A>G; *rs776746*), is associated with poor metabolism (historically also known as a non-expressor phenotype) [5]. As a result of the study, it was revealed that carriage of the AG genotype for the polymorphic marker *rs776746* of the *CYP3A5* gene is associated with an increase in the risk of developing drug-induced liver damage during remdesivir therapy by an average of 4.79 times relative to other genotypes.

Study limitations

The limitations of the present study were as follows: a small sample size, so some possible clinically significant associations between the factors could not be proven by statistical methods; a limited number of candidate genes and allelic variants in the analysis, and a limited follow-up period. The study was a “case-control” design and has inherent limitations.

CONCLUSION

The analysis results of the relationship between the development of DILI during remdesivir therapy with gender and age, clinical, anamnestic and laboratory parameters and concomitant drug therapy showed that a high BMI, a history of diabetes mellitus, a high level of ferritin in the blood, joint therapy with iACE and statins, and also carriage of the AG genotype for the *rs776746* polymorphic marker of the *CYP3A5* gene, was significantly more common in patients with DILI. The results obtained indicate that when prescribing remdesivir therapy, it is necessary to take these factors into account with more careful monitoring of clinical and laboratory signs of liver damage in these groups of patients, and in the future, to develop a personalized approach to pharmacotherapy of COVID-19 patients.

[3] Ibid.
[4] U.S. Food and Drug Administration. Gilead Sciences, Inc. VEKLURY® (remdesivir) for injection, for intravenous use, 2022

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS’ CONTRIBUTION

Yulia V. Shevchuk – idea and concept of the study, conducting the study, systematizing literature data, writing and editing the text of the manuscript, formulating conclusions; Alexander V. Kryukov – idea and development of the manuscript concept, systematization of literary data, text editing, formulation of conclusions, approval of the final version of the manuscript for publication; Ilyas I. Temirbulatov – analysis and interpretation of the literature data, participation in the research, analysis and discussion of the results obtained; Ivan V. Sychev – statistical data processing, editing the manuscript text, formulation of conclusions; Karin B. Mirzaev – development of the research concept, critical revision of the content and results of the work, editing the text of the manuscript; Natalya P. Denisenko – analysis and interpretation of the literature data, editing the manuscript, participation in the study; Sherzod P. Abdullaev – analysis of the literature data, editing of the manuscript text; Svetlana N. Tuchkova – laboratory processing of materials; Valery I. Vechorko – critical revision of the manuscript, approval of the final version of the manuscript sections; Oleg V. Averkov – participation in the development of the manuscript concept, editing the manuscript sections; Dmitry A. Sychev – development of the research concept, critical analysis of the results obtained, approval of the final version of the manuscript for publication. All the authors confirm that their authorship meets the international ICMJE criteria (all the authors have made a significant contribution to the development of the concept, conduct of the study and preparation of the article, read and approved the final version before the publication).

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RESEARCH ARTICLE

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Study of biotransformation of new selective carbonic anhydrase II inhibitor 4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonamide

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The aim of the study was to determine biotransformation products of a new selective carbonic anhydrase II inhibitor – 4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonamide.

Materials and methods. The study was conducted on 3 Wistar rats and 3 rabbits of the Soviet Chinchilla breed. The suspension of the drug was administered intraperitoneally to rats at a dosage of 20 mg/kg, to rabbits - at a dosage of 1.6 mg/kg. The animal blood samples were collected before the administration and 1, 2, 4, 24 h after. Urine sampling was also performed in the rats before the administration and in the intervals of 0–4, 4–8, 8–24 h after. The identification of metabolites in blood, urine and plasma was carried out using HPLC-MS/MS. Poroshell 120 C 18 column (50×3.0 mm, 2.7 µm) with a Zorbax Eclipse Plus C18 pre-column (12.5×2.1 mm, 5.0 µm) was used for the chromatographic separation. The assumed metabolites were synthesized, their structure was confirmed by the NMR spectroscopy method and a high-resolution mass spectrometry. The obtained substances were compared with the substances identified in biological fluids by retention time, the main MRM-transitions and mass spectra.

Results. The N-hydroxymetabolite was revealed in the analyses of plasma, blood and urine samples which had been formed by the addition of an oxygen atom to the drug molecule. Chromatographic peaks of this compound were identified at the MRM-transitions of 255→159, 255→117, 255→89 m/z at the 7.2nd min of the analysis. The N-oxide of 4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonamide and N-hydroxy-4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonamide were synthesized; potentially, they could have been obtained during the biotransformation. During the confirmatory HPLC-MS/MS tests based on the coincidence of the retention times, the main MRM transitions and mass spectra, the ratio of the peak areas at the identified metabolite it was established that an N-hydroxy derivative. Chromatographic peaks of the N-oxide detected in the analysis of the model mixtures of the standard substance at the MRM-transitions of 255→175, 255→133, 255→89 m/z at the retention time of 5.43 min, were absent in the animal samples.

Conclusion. The studied drug is metabolized to form a single metabolite of N-hydroxy-4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonamide. This compound was found in freshly collected samples of biological fluids of both animal species. The structure of the metabolite was confirmed by the HPLC-MS/MS-method by comparison with the synthesized standard substance.

Keywords: biotransformation; metabolite identification; selective carbonic anhydrase II inhibitor; HPLC-MS/MS; N-hydroxysulfonamide

Abbreviations: OXSA – 4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonamide; HPLC-MS/MS – high-performance liquid chromatography with tandem mass spectrometric detection; ESI – electrospray ionization; MRM – multiple reaction monitoring mode; MS2 – mode for obtaining the mass spectrum of a molecular ion; t_R – retention time; DMSO-D6 – deuterated dimethyl sulfoxide; DIPEA – diisopropylethylamine.

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Изучение биотрансформации нового селективного ингибитора карбоангидразы II 4-(2-метил-1,3-оксазол-5-ил)-бензолсульфонамида

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Цель. Определение продуктов биотрансформации нового селективного ингибитора карбоангидразы II – 4-(2-метил-1,3-оксазол-5-ил)-бензолсульфонамида.
Материалы и методы. Исследование проводили на 3 крысах-самцах породы Wistar и 3 кроликах породы Советская шиншилла. Суспензию препарата вводили внутрибрюшинным способом крысам в дозировке 20 мг/кг, кроликам – в дозировке 1,6 мг/кг. Образцы крови животных отбирали до введения и через 1, 2, 4 и 24 ч после. У крыс также выполняли забор мочи до введения и в промежутки 0–4, 4–8 и 8–24 ч после. Идентификацию метаболитов в крови, моче и плазме осуществляли с помощью метода ВЭЖХ-МС/МС. Для хроматографического разделения использовали колонку Poroshell 120EC-C18 (50×3,0 мм, 2,7 мкм) с предколонкой Zorbax Eclipse Plus C18 (12,5×2,1 мм, 5,0 мкм). Предполагаемые метаболиты синтезировали, подтверждали их структуру методом ЯМР-спектроскопии и масс-спектрометрии высокого разрешения. Полученные вещества сопоставляли с идентифицированными в биологических жидкостях веществами по времени удерживания, основным MRM-переходам и масс-спектрам.
Результаты. При анализе проб плазмы, крови и мочи обнаружен N-гидроксиметаболит, образовавшийся путём присоединения атома кислорода к молекуле препарата. Хроматографические пики данного соединения были идентифицированы на MRM-переходах 255→159, 255→117, 255→89 m/z на 7,2 мин анализа. Были синтезированы N-оксид 4-(2-метил-1,3-оксазол-5-ил)-бензолсульфонамида и N-гидрокси-4-(2-метил-1,3-оксазол-5-ил)-бензолсульфонамид, которые потенциально могли быть получены в процессе биотрансформации. В ходе подтверждающих ВЭЖХ-МС/МС-испытаний по совпадению времён удерживания, соотношения площадей пиков на основных MRM-переходах и масс-спектров установлено, что идентифицированный метаболит – N-гидроксипроизводное препарата. Хроматографические пики N-оксида, обнаруженные при анализе модельных смесей стандартного образца на MRM-переходах 255→175, 255→133, 255→89 m/z при времени удерживания 5,43 мин, отсутствовали в образцах животных.
Заключение. Изучаемый препарат метаболизируется с образованием единственного метаболита N-гидрокси-4-(2-метил-1,3-оксазол-5-ил)-бензолсульфонамида. Данное соединение обнаружено в свежеотобранных пробах биологических жидкостей обоих видов животных. Структура метаболита подтверждена методом ВЭЖХ-МС/МС путём сравнения с синтезированным стандартным образцом.
Ключевые слова: биотрансформация; идентификация метаболитов; селективный ингибитор карбоангидразы II; ВЭЖХ-МС/МС; N-гидроксисульфонамид
Список сокращений: OXSA – 4-(2-метил-1,3-оксазол-5-ил)-бензолсульфонамид; ВЭЖХ-МС/МС – высокоэффективная жидкостная хроматография с тандемным масс-спектрометрическим детектированием; ESI – ионизация электрораспылением; MRM – режим мониторинга множественных реакций; MS2 – режим получения масс-спектра молекулярного иона; t_R – время удерживания; ДМСО-D6 – дейтерированный диметилсульфоксид; DIPEA – диизопропилэтиламин.

INTRODUCTION

The selective carbonic anhydrase II inhibitors are widely used for the treatment of open-angle glaucoma. A benzenesulfonamide derivative 4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonamide (OXSA) is a new agent of this pharmacological group (Fig. 1). This compound exceeds the previously developed dorzolamide, brinzolamide and acetazolamide in terms of the efficiency of the intraocular pressure reduction and duration of the action in experimental models [1–4]. Thus, a further investigation of OXSA is perspective.

The identification of metabolic products is an important and mandatory part of the preclinical study of each drug[1]. The quantification of these compounds in biological media is necessary for a complete pharmacokinetic investigation of a drug including the

[1] Guidelines for conducting preclinical studies of medicines. Vol. 1. Moscow: Polygraph – Plus; 2012, 944 p. Russian

elimination process. Besides, detected metabolites may have a greater pharmacological activity than the basic substance [5]. There are two main experimental methods for the drug metabolites determination. The first method is an *in vitro* experiment with the use of microsomes [6–8], S9 fraction [9] or human or animal hepatocyte cell cultures [10–13]. The second method consists in the administration of the studied substance to the laboratory animals followed with biological sampling at certain intervals. Rodents often act as experimental subjects; in this case these are rats [14–17] and mice [18].

The most universal analytical method for the identification of metabolites is HPLC-MS/MS. Hybrid high-resolution mass spectrometric detectors based on «Orbitrap» [7, 10, 15, 16] or time-of-flight analyzers [8, 9, 17] and various types of ion traps [6, 13, 18] are used for these purposes. Triple quadrupole mass spectrometers are also applied which is most common for pharmacokinetic studies [11, 9, 20]. The identification of metabolites can be performed in the MRM-mode using the predicted MRM-transitions in this case: the m/z value of the predicted modification of the molecule is added or subtracted to the m/z values of the molecular ion and product ions of the drug [19, 20]. This method provides a higher sensitivity compared to the full scan mode [21]. The synthesis of the proposed metabolites and a confirmatory analysis are carried out by comparing the retention time of the analytes, their mass spectra, the ratio of MRM transitions and other parameters after the preliminary mass spectrometric determination of the structure.

The OXSA biotransformation process has not been studied before [1–4]. Widely used selective carbonic anhydrase II inhibitors, dorzolamide and brinzolamide, are metabolized by dealkylation. Thus, dorzolamide is subjected to N-deethylation [22], and brinzolamide is subjected to O-demethylation and N-deethylation [23, 24]. The previously developed acetazolamide is not exposed to a biotransformation [25]. A structurally similar derivative of aryl sulfonamide, hydrochlorothiazide, is also eliminated unchanged [26, 27]. The main ways of metabolism of sulfonamide antibacterial drugs are N-acetylation and N-hydroxylation of the aromatic aminogroup, as well as methylation and hydroxylation of the substituents, the sulfonamide group and the amino group [28, 29]. The formation of N-oxides by aromatic nitrogen atoms is also known [21]. Thus, N-oxidation of the 1,3-oxazole nitrogen atom and hydroxylation of the methyl group of the 2-methyl-1,3-oxazole residue are possible during metabolism of 4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonamide. There are no examples of modifications of the sulfonamide group in the published data.

THE AIM of the study was to determine biotransformation products of a new selective carbonic anhydrase II inhibitor – 4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonamide.

MATERIALS AND METHODS

Bioanalysis

The identification of biotransformation products was carried out using an HPLC-MS/MS-system, including a tandem mass spectrometric detector «AB Sciex QTRAP5500» (AB Sciex, Singapore) and chromatograph «Agilent 1260 Infinity» (Agilent Technologies, USA), consisting of pump G1312B, autosampler G1329B with thermostat G1330B, column thermostat G1316A (a control of the device was carried out by Software «Analyst 1.6.2» (AB Sciex, Singapore), processing chromatograms – «MultiQuant 3.0.5» (AB Sciex, Singapore), a prediction of metabolites and creation of MRM methods for identifying metabolites – «LightSight 2.3» (AB Sciex, Singapore).

The chromatographic separation of the prepared samples was performed on Poroshell 120EC-C18 (50×3.0 mm, 2.7 µm) chromatographic column with a Zorbax Eclipse Plus C18 pre-column (12.5×2.1 mm, 5.0 µm). A mobile phase (Table 1) based on a 0.1% aqueous solution of formic acid and methanol was used for a gradient elution. The column thermostat temperature was 40°C. The parameters of the mass spectrometric detector are presented in Table 2.

A 200 µl of methanol was added to 50 µl of blood, plasma or urine for preparing samples. The mixture was shaken and centrifuged for 5 min at 10000 rpm (Heraeus Multifuge X3R, Thermo Fisher Scientific, USA). Then 1 µl of the supraplastic fluid was injected into the HPLC-MS/MS system.

The signal-to-noise ratio of the OXSA chromatographic peak at the MRM-transition of 239→159 m/z (the control MRM-transition for the assessment of the system suitability and controlling the administration of the drug) at a concentration of 1 ng/ml in plasma, blood and urine samples was at least 50:1 in the above conditions.

2.5 µl of metabolites methanol solution at the concentrations of 10 µg/ml (for the concentration of 500 ng/ml) and 200 µg/ml (for the concentration of N-hydroxymetabolite in plasma of 10000 ng/ml for the stability study) were added to 47.5 µl of the biological fluid to prepare model mixtures.

Design of animal experiment

The study of biotransformation of 4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonamide was carried out on 3 Wistar rats and 3 rabbits of the Soviet Chinchilla breed obtained from the bio-nursery «SMK Stezar» LLC (Russia). The OXSA suspension was administered intraperitoneally to the rats at the dose of 20 mg/kg and to the rabbits at the dose of 1.6 mg/kg. The blood samples had been taken before the drug administration and 1, 2, 4 and 24 h after the administration in the volume of 0.2 ml in tubes containing K_3EDTA. The rats had been pre-catheterized into the right jugular vein. In the rabbits, the samples had been collected from the ear

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vein using an insulin syringe (Beijing Fornurse Medical Equipment Co., Ltd, China).

After sampling, 50 µL of the blood was immediately sampled and analyzed. The remaining blood in the tube (≈150 µl) was centrifuged for 10 min at 2500 rpm. At the temperature +4°C to obtain plasma. The urine was also sampled from the rats (the entire volume of rat urine over a specified period of time) using a metabolic cage before the administration (a blank sample) and during the intervals from the moment of the administration up to 4, from 4 to 8, from 8 to 24 h. The urine was also immediately processed and analyzed.

Thus, at the first stage, 5 samples of plasma and blood were obtained from each animal (4 experimental and 1 control), and 4 urine samples from the rat (3 experimental and 1 control). After the synthesis of the N-hydroxy metabolite, the experiment was repeated 1 month later to confirm the structure due to its instability in the biological samples and to check for the presence of a new possible sulfonic acid-derived metabolite. As a result, in 2 stages, 30 plasma samples (including 6 control samples) and 30 blood samples of both animal species (including 6 control samples), as well as 24 rat urine samples (including 6 control samples) were analyzed, which is sufficient for a reliable identification of all possible biotransformation products. A quantitative determination of the drug and its metabolites concentrations, as well as the calculation of their pharmacokinetic parameters, is not carried out at this stage of the study. Therefore, the number of animals of each species was reduced to 3 individuals below the standard sample size based on humane considerations[2].

The study was approved by the Ethics Committee of Yaroslavl State Medical University n.a. K.D. Ushinsky (Protocol No. 1 dated 10.06.2023).

Synthesis of OXSA metabolites

N-oxide and N-hydroxymetabolite of 4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonamide were synthesized after determining the possible structure of the metabolite by changes in the MRM transitions of the analyte.

For the synthesis of metabolites, organic, inorganic reagents and solvents were obtained from the following commercial sources: diisopropylethylamine (≥98%, Sigma-Aldrich, Germany), acetonitrile (chemical pure, Vecton, Russian Federation), 50% hydrogen peroxide (technical, Ekros group, Russian Federation), glacial acetic acid (chemical pure, Vecton, Russian Federation), hydroxylamine hydrochloride (≥99%, Vecton, Russian Federation). These reagents were used without any additional purification.

The reactions were controlled by a thin-layer chromatography on SilufolUV aluminum plates (10×20 cm, Merck Millipore, Germany). 5 µl of a 0.5% solution of the initial reagent in acetone and the reaction mixture were applied to the start line. The plate was dried and then placed in a chamber. The separation was carried out using a mixture of ethyl acetate: petroleum ether as an eluent in a ratio of 1:1 (v/v). The plate was removed from the chamber and dried in air when the solvent front reached a height of 10 cm. The detection was performed at a wavelength of 254 nm. The reaction had been carried out until the spot corresponding to the initial reagent completely disappeared in the reaction mixture.

NMR spectra were recorded on the "Varian UNITY Plus – 400" (Varian LLC, USA) device for DMSO-d6 solutions at 25°C. The signals of residual solvent protons in ^{1}H-NMR (δH 2.50 ppm) or ^{13}C-NMR (δC 39.5 ppm) were chosen as a reference for counting chemical shifts. The signal designation forms are: s – singlet, d – doublet, t – triplet, q – quartet, d.d – doublet of doublets, d.t. – triplet of doublets, m – multiplet. The melting point was measured on the device for determining the melting and boiling points «Buchi M-560» (Büchi Labortechnik AG, Switzerland). High-resolution mass spectra were obtained using a Bruker Daltonics MicrOTOF–II» (Bruker Daltonics GmbH, USA) mass spectrometer by ionization method (ESI).

Statistical processing

Statistical calculations were performed using Microsoft Excel 2016 (Microsoft Corporation, USA). The analytical signal in the animal samples and model mixtures of the synthesized compound was compared by retention time (t_R), by peak area ratios at the main MRM-transitions. Herewith, the ratio of the arithmetic mean of the parameters in the subjects and standard samples was calculated. The maximum deviation of t_R of the synthesized substance should be within ±1% of the t_R metabolite in the animal samples, the maximum deviation in the ratio of peak areas at the MRM-transitions should be within ±20% of the ratio in the animal samples. These criteria are established in accordance with the requirements of the Russian State Pharmacopoeia of the the XVth edition), which it used to confirm the identity of the drug by HPLC and mass spectrometry due to the absence of other special requirements for biotransformation studies[3,4]. The standard deviation value (SD) is shown in the tables as a measure of the dispersion of the obtained data. The percentage of coincidence of the mass spectra of the standard sample and the metabolite registered in the MS2-mode was also calculated ("Analyst 1.6.2", AB Sciex, USA).

[2] Ibid.

[3] Russian State Pharmacopoeia. XV edition. GPhM.1.2.1.2.0001 Chromatography. Available from: https://pharmacopoeia.regmed.ru/pharmacopoeia/izdanie-15/1/1-2/1-2-1/1-2-1-2-khromatograficheskie-metody-analiza/khromatografiya/. Russian

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Table 1 – Parameters of gradient elution method for OXSA metabolites identification

Time, min	A, %	B, %
0.00	90	10
0.50	90	10
10.00	10	90
15.00	10	90
15.10	90	10
20.00	90	10

Note: A – 0.1% aqueous solution of formic acid, B – 0.1% aqueous solution of methanol.

Table 2 – Mass spectrometry detection parameters of OXSA and its metabolites

Parameter	Value
Ionization mode	Electrospray ionization (ESI)
ESI voltage	+5500 V
Curtain gas	30 psi (Nitrogen)
CAD-Gas (collision-activated dissociation)	High (Nitrogen)
Ion source temperature	700°C
Gas 1 (heating up gas)	55 psi (Air)
Gas 2 (nebulizer gas)	55 psi (Air)

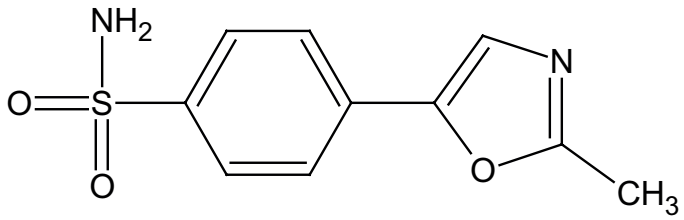

Figure 1 – Structure of 4-(2-methyl-1,3-oxazol-5-yl)-benzenesulfonamide

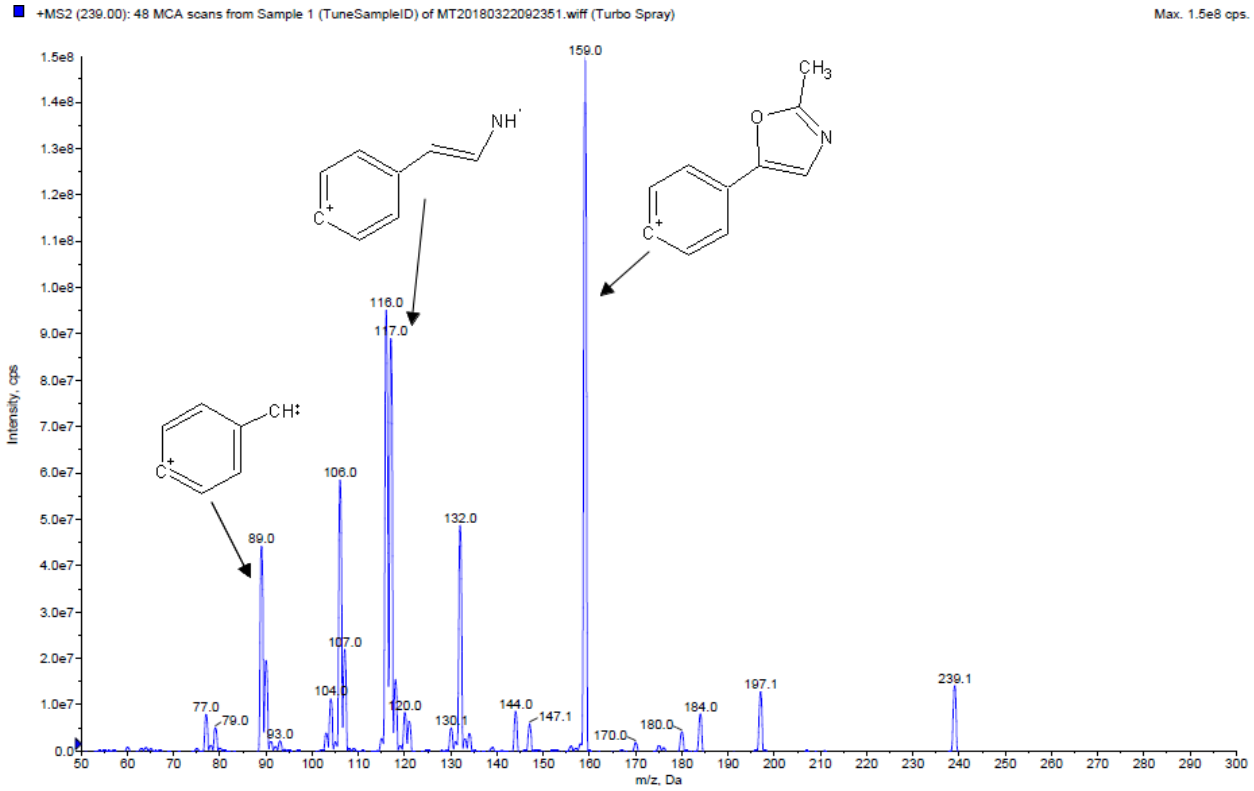

Figure 2 – Mass-spectrum of OXSA (MS2-mode; positive polarity; CE=40eV)

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Table 3 – MRM-transitions for identification of possible metabolites

Modification	M/z difference	Predicted MRM-transitions, m/z
Control (OXSA)	–	239→159 m/z
Hydroxylation / N-oxidation (+OH/→O)	+16	255→159 m/z; 255→117 m/z; 255→ 89 m/z; 255→175 m/z; 255→133 m/z; 255→105 m/z
Methylation (+CH_3)	+14	253→159 m/z; 253→117 m/z; 253→89 m/z; 253→173 m/z; 253→131 m/z; 253→103 m/z
Hydroxylation +N-oxidation (+OH+→O)	+32	271→159 m/z; 271→117 m/z; 271→ 89 m/z; 271→175 m/z; 271→133 m/z; 271→105 m/z; 271→191 m/z; 271→149 m/z; 271→121 m/z
Glucuronidation*	+176	415→159 m/z; 415→117 m/z; 415→89 m/z
Acetylation*	+42	281→159 m/z; 281→117 m/z; 281→89 m/z
Sulfonation*	+80	319→159 m/z; 319→117 m/z; 319→89 m/z
Formation of sulfonic acid -SO_2NH_2→ -SO_3**	+1	240→159 m/z; 240→117 m/z; 240→89 m/z

Note: * There was no addition of m/z difference to the product ion due to the sulfonamide group elimination in the CAD-fragmentation process.
** Analysis was performed only during confirmatory tests.

Table 4 – The results of identification of hydroxylated / N-oxidated metabolite

Samples		Time points	Retention time of metabolite, min (Mean±SD)[5]					
			255→159 m/z	255→117 m/z	255→89 m/z	255→ 175 m/z	255→ 133 m/z	255→ 105 m/z
Rat samples	Plasma	0	N/A	N/A	N/A	N/A	N/A	N/A
		1	7.20± 0.01	7.21± 0.01	7.20±0.01	N/A	N/A	N/A
		2	7.20± 0.01	7.21± 0.01	7.20±0.01	N/A	N/A	N/A
		4	7.20± 0.01	7.21± 0.01	7.21±0.01	N/A	N/A	N/A
		24	7.20± 0.02	N/A *	N/A*	N/A	N/A	N/A
	Blood	0	N/A	N/A	N/A	N/A	N/A	N/A
		1	7.18± 0.01	7.19± 0.01	7.19± 0.01	N/A	N/A	N/A
		2	7.19± 0.01	7.19± 0.01	7.19± 0.01	N/A	N/A	N/A
		4	7.20± 0.01	7.19± 0.01	7.19± 0.01	N/A	N/A	N/A
		24	7.19± 0.01	7.19± 0.01	7.19± 0.01	N/A	N/A	N/A
	Urine	0	N/A	N/A	N/A	N/A	N/A	N/A
		0–4	7.19± 0.01	7.19± 0.01	7.19± 0.01	N/A	N/A	N/A
		4–8	7.20± 0.01	7.19± 0.01	7.20± 0.01	N/A	N/A	N/A
		8–24	7.19± 0.01	7.19± 0.01	7.19± 0.01	N/A	N/A	N/A
Rabbit samples	Plasma	0	N/A	N/A	N/A	N/A	N/A	N/A
		1	7.21± 0.02	7.21± 0.02	7.20± 0.01	N/A	N/A	N/A
		2	7.19± 0.02	7.19± 0.02	7.20± 0.01	N/A	N/A	N/A
		4	7.20± 0.01	7.20± 0.01	7.21± 0.01	N/A	N/A	N/A
		24	7.20± 0.01	N/A *	N/A*	N/A	N/A	N/A
	Blood	0	N/A	N/A	N/A	N/A	N/A	N/A
		1	7.20± 0.01	7.19± 0.01	7.19± 0.01	N/A	N/A	N/A
		2	7.20± 0.02	7.20± 0.01	7.19± 0.01	N/A	N/A	N/A
		4	7.19± 0.01	7.19± 0.01	7.19± 0.01	N/A	N/A	N/A
		24	7.20± 0.01	7.21± 0.01	7.20± 0.01	N/A	N/A	N/A
N-oxide OXSA	Blood		N/A	N/A	5.43±0.01	5.43±0.01	5.43±0.01	N/A
	Plasma		N/A	N/A	5.42±0.01	5.42±0.01	5.42±0.01	N/A
	Urine		N/A	N/A	5.43±0.01	5.43±0.01	5.43±0.01	N/A
N-hydroxy-OXSA	Blood		7,20±0,01	7.20±0.01	7.20±0.01	N/A	N/A	N/A
	Plasma		7,20±0,01	7.20±0.01	7.20±0.01	N/A	N/A	N/A
	Urine		7,22±0,01	7.21±0.01	7.21±0.01	N/A	N/A	N/A

Note:* Analytical signal was absent due to low OXSA-M1 concentration, N/A was not detected.

[5] The average tR value obtained after analyzing of 3 samples at each time point is shown in each cell of the table.

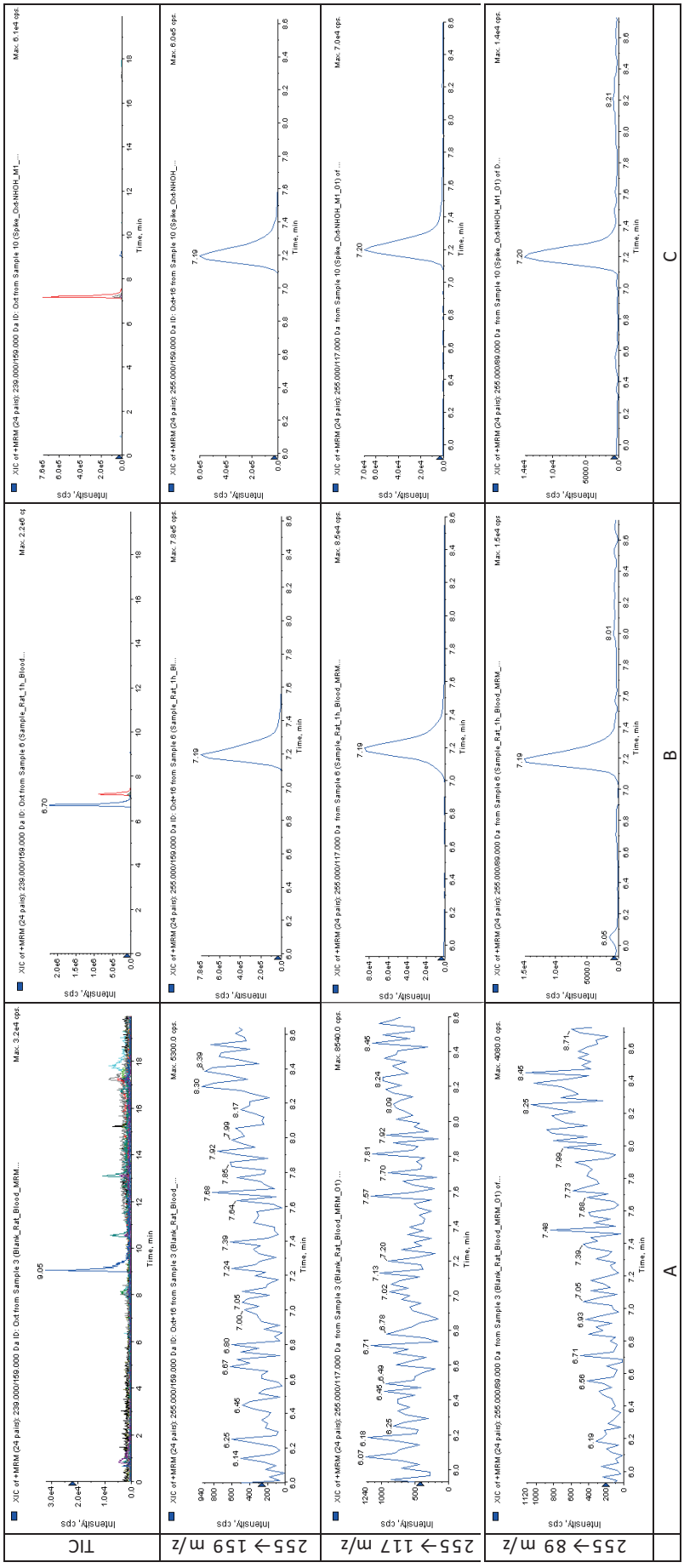

Figure 3 – Examples of blank sample chromatograms (A – rat's blood), rat's sample (B – rat's blood at 1 h point), blood sample spiked by N-hydroxy-4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonamide (C – analyte spiked blood sample of rat at concentration of 500 ng/ml)
Note: TIC – chromatograms of all MRM transitions (Table 3).

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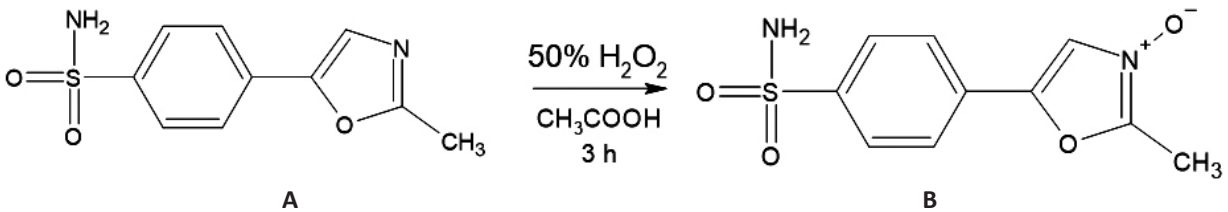
Table 5 – MRM- transitions for identification of possible conjugates of hydroxylated metabolite

Modification	M/z difference	Predicted MRM-transitions
Glucuronidation	+176	431→159 m/z; 431→117 m/z; 431→89 m/z; 431→335 m/z; 431→293 m/z; 431→265 m/z
Acetylation	+42	297→159 m/z; 297→117 m/z; 297→89 m/z; 297→201 m/z; 297→131m/z
Sulfonation	+80	335→159 m/z; 335→117 m/z; 335→89 m/z; 335→239 m/z; 335→197 m/z; 335→169 m/z
Methylation	+14	269→159 m/z; 269→117 m/z; 269→89 m/z; 269→173 m/z; 269→131 m/z; 269→103 m/z

Table 6 – The results of confirmation of structure of main metabolite of OXSA

Parameters		t_R, min (*n*=3)	Peak area ratios 255→117 / 255→159 m/z	Peak area ratios 255→89 / 255→159 m/z	Coincidence of MS2 mass spectra** (min–max, %)
Rat plasma samples	Collected samples at points of 1, 2, 4 h (*n*=9)	7.16±0.01	0.1181±0.0090	0.0231±0.0015	89–95
	Spiked samples (*n*=3)	7.15±0.01	0.1146±0.0109	0.0220±0.0016	
	% of coincidence*	100.08	103.05	105.16	–
Rat blood samples	Collected samples at point of 1, 2, 4 h (*n*=9)	7.16±0.01	0.1287±0.0112	0.0224±0.0016	95–98
	Spiked samples (*n*=3)	7.15±0.01	0.1260±0.0082	0.0231±0.0015	
	% of coincidence	100.12	102.14	97.06	–
Rat urine samples	Collected samples at intervals of 0–4, 4–8 h (*n*=6)	7.15±0.01	0.1304±0.0146	0.0229±0.0015	91–95
	Spiked samples (*n*=3)	7.16±0.01	0.1244±0.0081	0.0228±0.0015	
	% of coincidence	99.88	104.81	100.51	–
Rabbit plasma samples	Collected samples at point of 1, 2, 4 h (*n*=9)	7.16±0.01	0.1232±0.0116	0.0229±0.0015	90–93
	Spiked samples (*n*=3)	7.16±0.01	0.1250±0.0145	0.0227±0.0017	
	% of coincidence	100.09	98.53	100.54	–
Rabbit blood samples	Collected samples at point of 1, 2, 4 h (*n*=9)	7.16±0.01	0.1244±0.0105	0.0225±0.0012	93–97
	Spiked samples (*n*=3)	7.15±0.02	0.1287±0.0119	0.0245±0.0012	
	% of coincidence	99.80	96.65	91.75	–

Note: * Ratio of mean values. ** Point 1 h and 0–4 h period after administration (*n*=3).



A B

Figure 4 – Synthesis of 4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonamide N-oxide (B) by using 4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonamide (A)

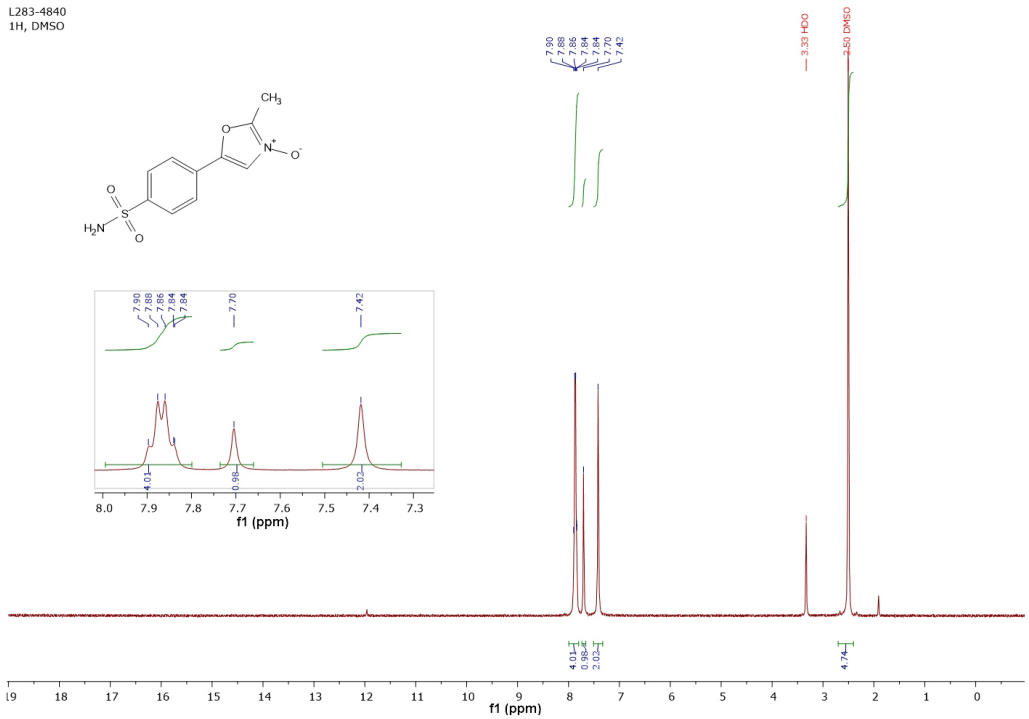


Figure 5 – ^{1}H-NMR-spectrum of N-oxide of 4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonamide in DMSO-D$_6$

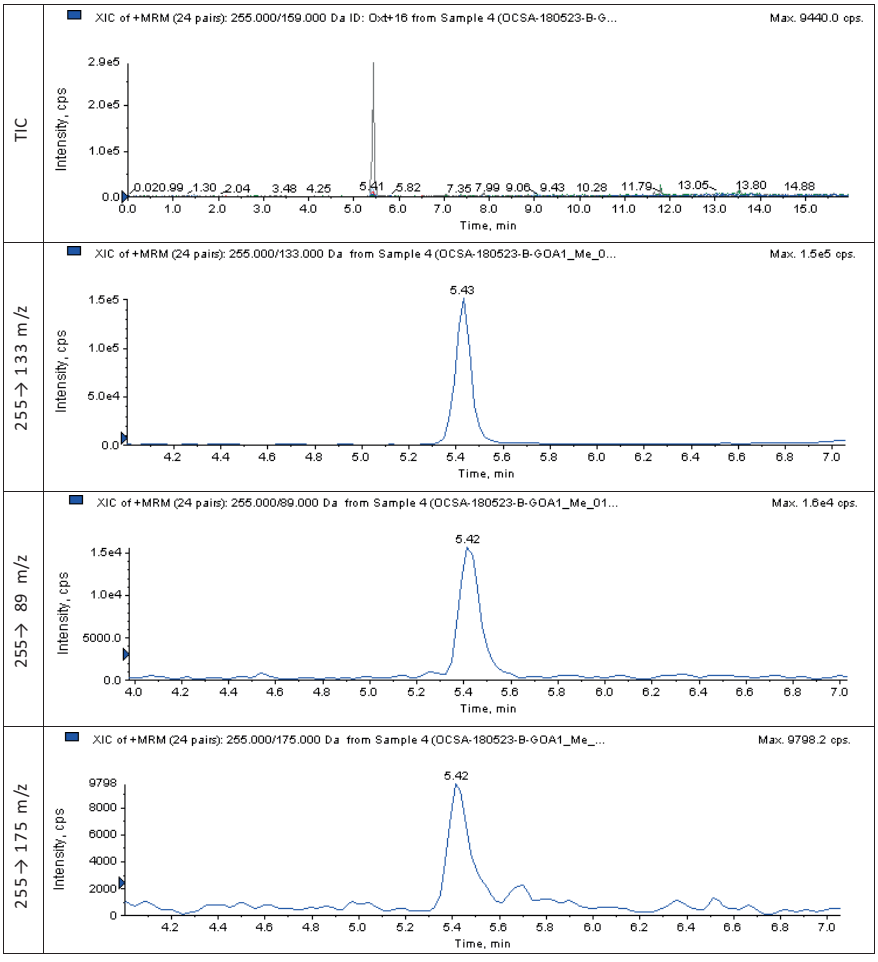


Figure 6 – Example of MRM-chromatogram of N-oxide OXSA in spiked blood sample in concentration of 500 ng/ml

Note: TIC – chromatograms of all MRM transitions (Table 3).

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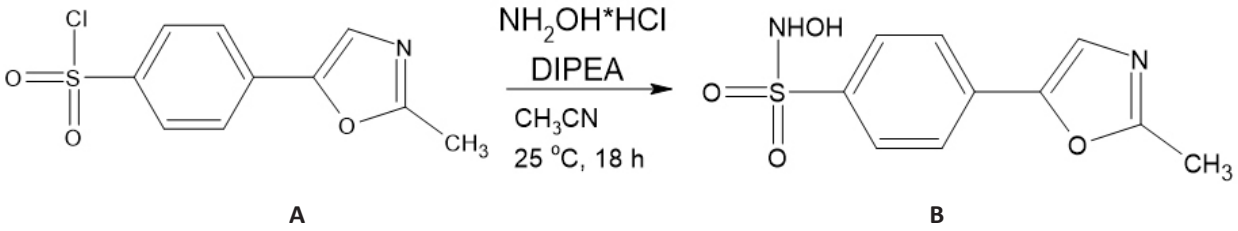

A B

Figure 7 – Synthesis of N-hydroxy-4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonamide
Note: DIPEA – diisopropylethylamine.

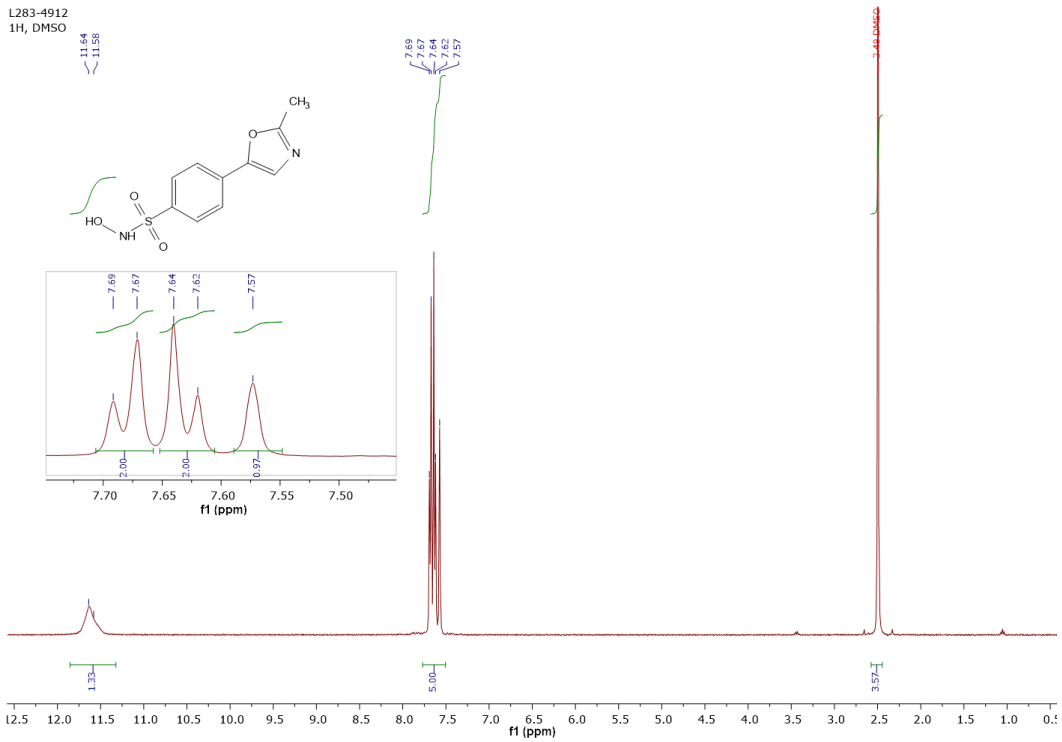

Figure 8 – ^{1}H-NMR-spectrum of N-hydroxy-4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonamide in DMSO-D_6

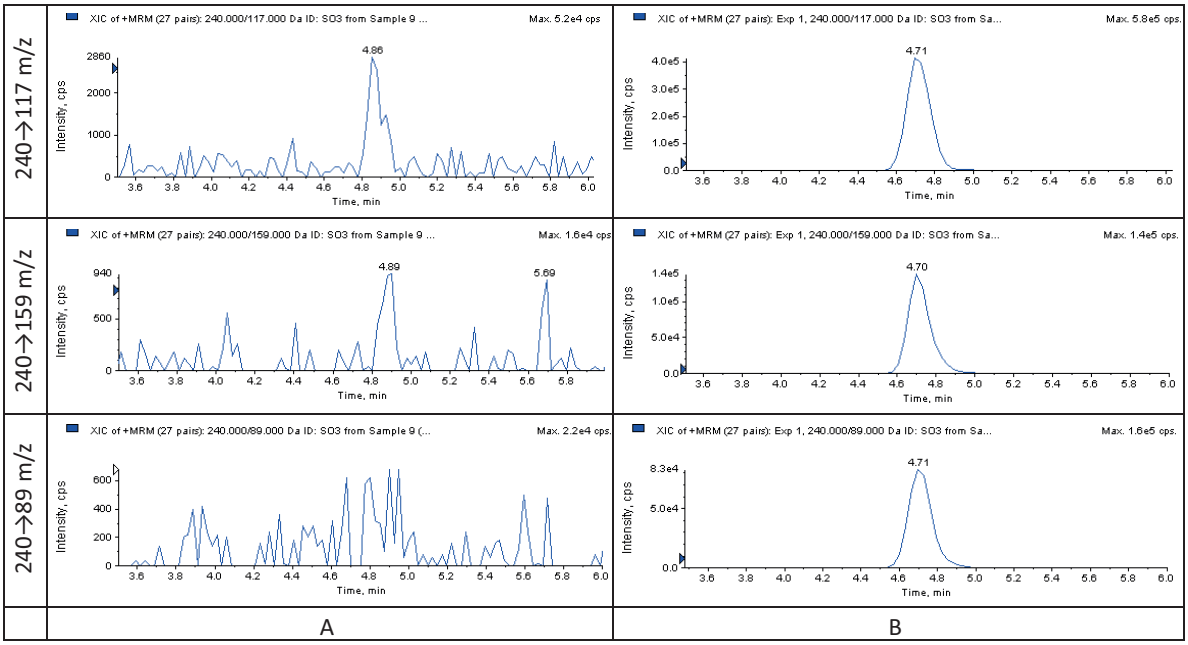

Figure 9 – MRM-chromatogram examples of plasma sample spiked by metabolite after preparation (A) and spiked metabolite sample after 48 h of storage at room temperature (B)

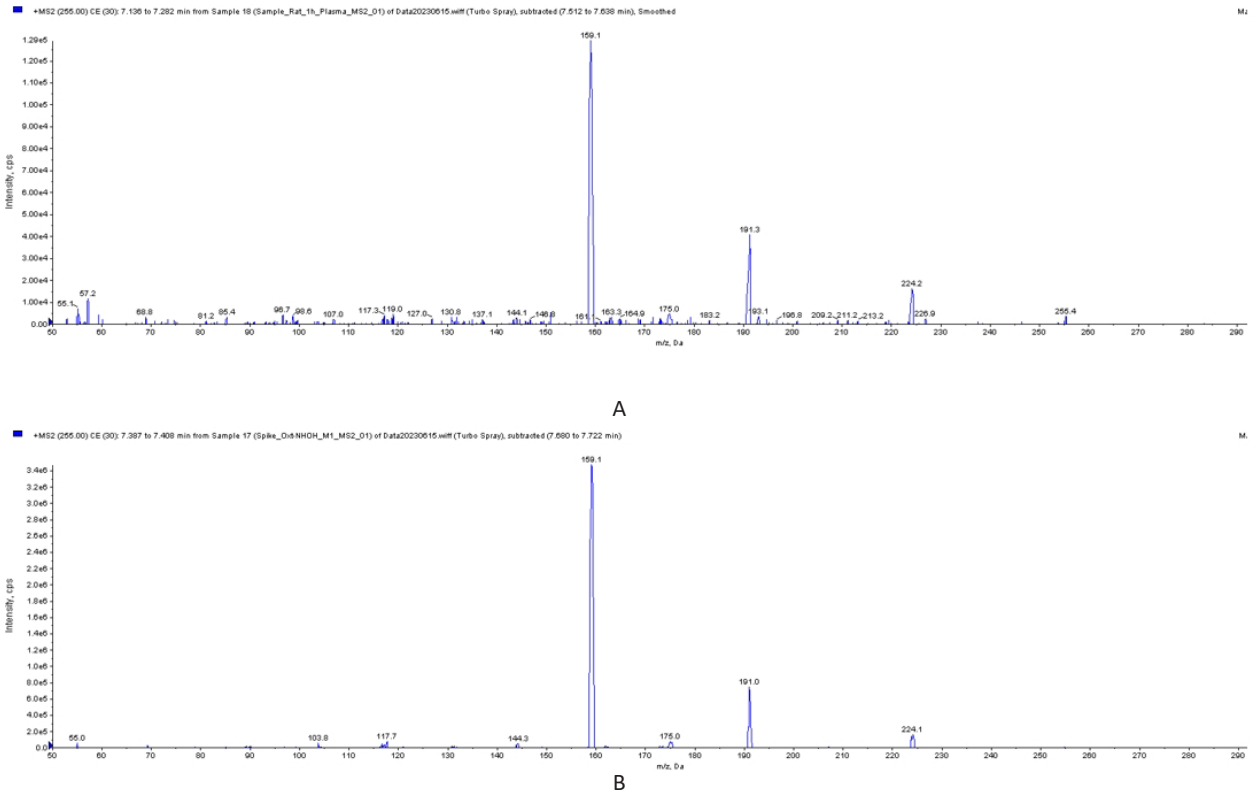

Figure 10 – Mass-spectra examples of N-hydroxy-4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonamide in rat plasma sample (1 h point) (A) and in spiked plasma sample at analyte concentration of 500 ng/ml (B)

RESULTS AND DISCUSSION

The mass spectrum of the molecular ion (MS2 mode) of 4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonamide 239 m/z was obtained at the initial stage of the study (Fig. 2). Product ions 89, 117 and 159 m/z were selected to create the MRM screening method which it had the highest signal intensity and fully reflected OXSA structure fragments with a potential addition of functional groups. Using the LightSight 2.3 Software, a possible hydroxylation of the methyl radical of a 2-methyl-1,3-oxazole, N-oxidation fragment of the oxazole nitrogen atom, as well as methylation, acetylation and sulfonation of the sulfonamide group, were established during the prediction of possible metabolites. An increase of m/z in the case of each modification was added to the m/z of the molecular ion and the selected values of m/z of OXSA product ions (Table 3). MRM-transitions with unchanged values of 89, 117 and 159 m/z were also created.

After analyzing the chromatograms of plasma, blood and urine samples, chromatographic peaks were detected on the MRM-transitions of 255→159, 255→117, 255→89 m/z with t_R=7.20 min (Table 4) which were absented on the chromatograms of the blank samples of these objects. These signals with Δm/z=16 indicate the addition of an oxygen atom to the molecule and possible hydroxylation or formation of N-oxide. Chromatographic peaks at the MRM-transitions of other modifications have not been identified (Fig. 3).

An increase of m/z of 159, 117, 89 product ions by 16 m/z was not observed. Most likely, the detected metabolite can be obtained as a result of N-oxidation of the 1,3-oxazole nitrogen atom, and there is no growth of the m/z due to the destruction of the weak bond as a result of CAD-fragmentation [19]. The process of hydroxylation of the sulfonamide group is also possible because it is eliminated at the selected MRM-transitions.

Due to the possible hydroxylation of the OXSA molecule, an additional MRM method that takes into account the possible conjugation of this metabolite was created (Table 5). The prepared samples were reanalyzed. However, the chromatographic peaks of metabolites in comparison with the blank samples were not observed on the obtained chromatograms.

The synthesis of possible OXSA metabolites was performed in the course of the study. N-oxide of OXSA was obtained the first because the N-hydroxylation examples of the sulfonamide group of drugs during biotransformation had not been previously published.

The substance of 4-(2-Methyl-1,3-oxazole-5-yl)-benzenesulfonamide (Fig. 4-A) (0.20 g, 0.84 mmol, 1 eq) obtained by the method [1] was suspended in 5 ml of glacial acetic acid. A 50% aqueous solution of hydrogen peroxide (0.170 g, 2.25 mmol, 3 eq) was added to the mixture and stirred for 3 h at the temperature of 50°C. The reaction mixture was cooled to the room temperature, the precipitate was filtered and washed with 1 ml of glacial acetic acid. The 0.1 g (50%) of OXSA N-oxide isolated during the experiment as a white solid

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was obtained. The melting point of this substance was 227–228°C. The structure confirmation results were:

^{1}H-NMR-spectroscopy (400 MHz, DMSO-D_6) δ, ppm: 7.87 (q, *J*=8,0 Hz, 4H, Ar), 7.70 (s, 1H, Het), 7.42 (s, 2H, NH_2), 2,52 (s, 3H, CH_3) (Fig. 5).

^{13}C-NMR – spectroscopy (100 MHz, DMSO-D_6) δ, ppm: 168.31, 143.82, 133.51, 128.56, 128.19, 127.91, 123.42, 10.63.

Mass-spectrometry: the m/z value of the molecular ion $[M+H]^+$: 255,0431 m/z; Δm/z=-1,18 ppm (calculated for the theoretical value $C_{10}H_{10}N_2O_4S$+:255.0434 m/z).

Model mixtures in plasma, blood and urine at the concentration of 500 ng/ml were prepared and analyzed to verify the compliance of the prepared N-oxide of 4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonamide to the structure of the OXSA metabolite. The retention time, as well as the main MRM-transitions of the obtained substance differed from the characteristics of the compound detected in the biological fluids (Table 4, Fig. 6). Its t_R was 5.43 min, which was 1.8 min smaller than the OXSA metabolite. The m/z value of the OXSA N-oxide product ions 175 and 133 m/z containing an oxazole nitrogen atom is 16 Da higher than the values of the product ions of the drug 159 and 117 m/z. Thus, the studied metabolite was not N-oxide.

Next, N-hydroxy-4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonamide was synthesized by the nucleophilic substitution reaction of hydroxylamine and 4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonyl chloride (Fig.7).

The initial 4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonyl chloride (Fig. 7) was obtained by a well-known method [1]. Diisopropylethylamine (DIPEA) (0.75 g, 5.82 mmol, 3 eq.) was added to the cooled aqueous solution of hydroxylamine hydrochloride (0.2 g in terms of a pure substance, 2.9 mmol, 1.5 eq.). Then, a solution of 4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonyl chloride in 2 ml of acetonitrile (0.5 g, 1.64 mmol, 1 eq.) was slowly to the mixture during cooling and it was stirred for 18 h at room temperature. The reaction mixture was diluted with cold purified water. The precipitate was filtered and washed with purified water. 0.29 g of N-hydroxy-4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonamide was obtained in the form of a white precipitate with an output of 60%. The decomposition temperature of this substance was 300°C. The structure confirmation results were:

^{1}H-NMR-spectroscopy (400 MHz, DMSO-D_6) δ, ppm was: 11.61 (d, *J*=23.5 Hz, 2H, OH, NH), 7.68 (d, *J*=8.1 Hz, 2H, Ar), 7.63 (d, *J*=8,2 Гц, 2H, Ar), 7,57 (s, 1H, Het), 2.49 (s, 3H, CH_3) (Fig. 8).

^{13}C-NMR-spectroscopy (100 MHz, DMSO-D_6) δ, ppm was: 161.95, 150.74, 148.39, 128.18, 126.97, 123.80, 122.91, 14.31.

Mass-spectrometry was: the m/z value of the molecular ion $[M+H]^+$ was 255,0433 m/z; Δm/z=0,39 ppm (calculated for the theoretical value $C_{10}H_{10}N_2O_4S^+$: 255.0434 m/z).

Next, plasma, blood and urine model mixtures were prepared at the concentration of 500 ng/ml with an addition of the N-hydroxy derivative OXSA. The analysis of these samples showed that the retention times and the main MRM transitions of the synthesized and detected substances coincide (Table 4, Fig. 3C). Chromatographic peaks of the metabolite were not detected during the confirmatory testing by a repeated analysis of previously collected samples of animal biological fluids, which indicated its decomposition. The chromatographic peaks were detected at MRM-transitions of 240→159, 240→117, 240→89 m/z with increased m/z of the molecular ion on 1 Da and the retention time of 4.7 min (Fig. 9). These signals were also identified in the old animal samples after a subsequent optimizing the parameters of the mass spectrometric detector. This suggests that the obtained compound decomposes to form sulfonic acid.

The drug was re-administered to the animals 1 month later in the dosages described above to confirm the structure of the OXSA metabolite and to check the presence in fresh samples of the second possible metabolite – 4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonic acid. The samples were taken at the same time intervals. Herewith, the ratios of the chromatographic peak areas of the MRM-transitions of 255→117 m/z to 255→159 m/z and 255→89 m/z to 255→159 m/z were estimated. The late time points of 24 h for plasma and blood as well as the time range of 8–24 h for urine were not used for the comparison with the synthesized standard sample due to low concentrations of the metabolite.

Chromatographic peaks at the MRM-transitions of 240→159, 240→117, 240→89 m/z during the retention time of 4.7 min were not detected on the obtained chromatograms of freshly collected samples. Consequently, either the OXSA-sulfonic acid content in the samples was below the detection limit of the method or this compound is not formed in the body during metabolism. Therefore, 4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonic acid was not synthesized and its structure was not confirmed when compared with the standard substance.

The results of the retention time comparison, the ratio of chromatographic peak areas at MRM-transitions of OXSA N-hydroxymetabolite are presented in Table 6. The ratio of the average t_R values between the test samples and the model mixtures of the metabolite for blood, plasma, urine fell within the permissible range of 99.0–101.0%, and the ratio of peak areas – in the range of 80.0–120.0%.

The coincidence percentage of the mass spectra of the 255 m/z molecular ion obtained in the MS2 mode was estimated additionally. The comparison of the obtained data was performed using the software

Analyst 1.6.2 at the point of 1 h for blood and plasma and in the period of 0–4 h for urine. The mass spectra in biological samples coincided with the mass spectra in standard model mixtures by at least 89%, which confirms the metabolite structure. The examples of MS2-mass spectra of the analyte in the prepared plasma samples are shown in Figure 10.

Thus, the identified metabolite is an N-hydroxy derivative of OXSA by the sulfonamide group. Previously, similar biotransformation examples of drugs containing this functional group have not been founded in scientific publications [25–29]. It may be due to the chemical decomposition of these compounds in biological fluids during the storage to sulfonic acids.

During pharmacokinetic studies, for an accurate quantification of the OXSA metabolite, it will be necessary in future to add stabilizers to the samples immediately after sampling to prevent their degradation.

CONCLUSION

It was found that the studied drug is metabolized by formation the main metabolite N-hydroxy-4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonamide. This compound has been identified in plasma, blood and urine of laboratory animals. The structure of the metabolite was confirmed by comparing the retention time, the ratio of the areas of chromatographic peaks at the main MRM-transitions, as well as mass spectra with its synthesized standard. The complete pharmacokinetic study of the drug will be conducted using the synthesized substance of the identified compound, and its pharmacological activity will also be studied in the future.

FUNDING

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The synthesis of the medicinal substance and its metabolites has been performed at the expense of the grant of the Ministry of Education of the Russian Federation «Development of an innovative drug for the treatment of open-angle glaucoma by selective inhibition of carbonic anhydrase II» No. 073-00077-21-02 (register No. 730000F.99.1.BV10AA00006).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

Alexander L. Khokhlov – formulation and development of the key aim and objectives; Ilya I. Yaichkov – concept development, design development of biotransformation study, bioanalytical method, analysis of blood, plasma and urine samples, analysis and interpretation of the obtained data, writing of the paper; Anton A. Shetnev – development of synthesis technology of the drug and its metabolites, analysis and interpretation of the obtained data, writing of the paper (synthesis part); Sergey A. Ivanovskiy – pharmaceutical analysis and characterization of the drug structure and its metabolites; Mikhail K. Korsakov – formulation and development of the key aim and objectives, development of synthesis technology of the drug and its metabolites, analysis and interpretation of the obtained data; Olga A. Gasilina – synthesis of the drug and its metabolites; Nikita N. Volkhin – blood, plasma and urine sample collection; Sergey S. Petukhov -– blood, plasma and urine sample collection. All authors confirm that their authorship complies with the international ICMJE criteria (all authors have made a significant contribution to the development of the concept, research and preparation of the article, read and approved the final version before the publication).

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Russian development for drug independence in endocrinology: comparative analysis of bioequivalence, safety and tolerability of the first domestic liraglutide

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Liraglutide is one of the analogues of the incretin hormone human glucagon-like peptide-1 (GLP-1) and is currently a priority treatment for diseases such as type 2 diabetes mellitus (mono- and combination therapy), obesity and overweight in the presence of at least one concomitant disease.

The aim of the work was to assess the bioequivalence and comparability of the safety and tolerability profile of the drug Enligria® (liraglutide 6 mg/ml, Promomed RUS LLC, Russia) and the drug Saxenda® (liraglutide 6 mg/ml, Novo Nordisk AS, Denmark) after a single dose in healthy volunteers.

Materials and methods. This study was an open-label, randomized, crossover comparative study to evaluate pharmacokinetic parameters, safety, tolerability and immunogenicity. The study comprised 26 healthy volunteers, 26 of whom were included in the bioequivalence assessment population. The study consisted of 2 periods, in each of which the volunteers received either the test drug (liraglutide at a single dose of 0.6 mg) or the reference drug (liraglutide at a single dose of 0.6 mg) once. The washout period between each dose was 7 days. Blood plasma samples were taken to determine the concentration of liraglutide in the range from 0 to 72 hours in each study period. Liraglutide concentrations were determined using a previously validated enzyme-linked immunosorbent assay (ELISA) method. A quantitative determination of antibodies to liraglutide in the blood serum samples was carried out using a microplate photometer and ready-made ELISA kits pre-validated by the manufacturer. The conclusion about the equivalence of the compared drugs was made based on the ratio of the parameters C_{max}, $AUC_{0\to t}$ and $AUC_{0\to t}$ of the studied drug in relation to the reference one.

Results. The pharmacokinetic parameters of the drugs were comparable to each other. The resulting 90% confidence intervals for the ratio of the values of C_{max}, $AUC_{0\text{-}t}$ and $AUC_{0\text{-}\infty}$ of the Russian test and reference drug were 87.18–110.46, 84.40–104.11 and 86.69–103.22% respectively, which satisfied the criteria for assessing bioequivalence. The tolerability of the drugs in the volunteers was notified as good. The incidence of adverse events was comparable for the test and reference drugs. No

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serious adverse events were reported throughout the study. According to the results of the immunogenicity analysis, no antibodies to russian produced liraglutide were detected in the blood serum of the volunteers, which indicated the lack of the drug immunogenicity.

Conclusion. During the study, the pharmacokinetic equivalence of the test and reference drugs was confirmed. The Russian drug Enligria® (liraglutide 6 mg/ml, Promomed RUS LLC, Russia) in comparison with a foreign drug Saxenda® (liraglutide 6 mg/ml, Novo Nordisk AS, Denmark)

Keywords: glucagon-like peptide-1; bioequivalence; pharmacokinetics; liraglutide, obesity, type 2 diabetes mellitus, Enligria

Abbreviations: T2DM – type 2 diabetes mellitus; GLP-1 – glucagon-like peptide-1; GIP – glucose-dependent insulinotropic polypeptide; CVDs – cardiovascular diseases; ASCVDs – atherosclerotic cardiovascular diseases; HbAlc – glycated hemoglobin; ELISA – enzyme-linked immunosorbent assay; BMI – body mass index; DPP-4 – dipeptidyl peptidase-4; API – active pharmaceutical substance; DNA – deoxyribonucleic acid; RNA – ribonucleic acid; GI tract – gastrointestinal tract; ARVI – acute respiratory viral infection; PCR – polymerase chain reaction; BAS – biologically active supplement; BP – blood pressure; HR – heart rate; RR – respiratory rate; ECG – electrocardiography; LDL-C – low-density lipoprotein cholesterol; HDL-C – high-density lipoprotein cholesterol; AE – adverse event; SAE – serious adverse event; CI – confidence interval; POMC – pro-opiomelanocortin, CART – cocaine-amphetamine-regulated transcript; NPY – neuropeptide Y; AgLP – agouti-like protein; GABA – gamma-aminobutyric acid, CHF – chronic heart failure; CKD – chronic kidney disease.

Российская разработка для лекарственной независимости в эндокринологии: сравнительный анализ биоэквивалентности, безопасности и переносимости первого отечественного лираглутида

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ОРИГИНАЛЬНАЯ СТАТЬЯ

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Лираглутид является одним из аналогов инкретинового гормона человеческого глюкагоноподобного пептида-1 (ГПП-1) и в настоящее время является приоритетным средством для лечения таких заболеваний, как сахарный диабет 2-го типа (в моно- и комбинированной терапии), ожирение и избыточная масса тела при наличии хотя бы одного сопутствующего заболевания.

Цель. Оценить биоэквивалентность и сопоставимость профиля безопасности и переносимости лекарственного препарата Энлигрия® (лираглутид 6 мг/мл, ООО «ПРОМОМЕД РУС», Россия) и лекарственного препарата Саксенда® (лираглутид 6 мг/мл, Ново Нордиск А/С, Дания) при однократном применении здоровыми добровольцами.

Материалы и методы. Данное исследование представляло собой открытое рандомизированное перекрестное сравнительное исследование по оценке фармакокинетических параметров, безопасности, переносимости и иммуногенности. В исследование были включены 26 здоровых добровольцев, из них в популяцию для оценки биоэквивалентности вошли все 26 участников. Оно включало 2 периода, в каждом из которых добровольцы получали либо исследуемый препарат (лираглутид в дозе 0,6 мг), либо референтный препарат (лираглутид в дозе 0,6 мг) однократно. Отмывочный период между каждым из приемов составлял 7 сут. Отбор образцов плазмы крови для определения концентрации лираглутида производили в диапазоне от 0 до 72 ч в каждом из периодов исследования. Концентрацию лираглутида определяли с помощью предварительно валидированного метода иммуноферментного анализа (ИФА). Количественное определение антител к лираглутиду в образцах сыворотки крови проводили с помощью фотометра для микропланшетов с использованием готовых предварительно валидированных производителем ИФА-наборов. Вывод об эквивалентности сравниваемых препаратов делали по отношению параметров C_{max}, $AUC_{0\to t}$ и $AUC_{0\to\infty}$ исследуемого лекарственного препарата по отношению к референтному.

Результаты. Фармакокинетические параметры препаратов были сопоставимы между собой. Полученные 90%-ные доверительные интервалы для отношения значений C_{max}, AUC_{0-t} и $AUC_{0-\infty}$ исследуемого российского и референтного препарата составили 87,18–110,46, 84,40–104,11 и 86,69–103,22% соответственно, что удовлетворяло критериям оценки биоэквивалентности. Переносимость препаратов у добровольцев была отмечена как хорошая. Частота нежелательных явлений была сопоставима для исследуемого и референтного препаратов. В течение всего исследования не было зарегистрировано ни одного серьёзного нежелательного явления. По результатам анализа иммуногенности у добровольцев не были выявлены антитела к лираглутиду российского производства в сыворотке крови, что свидетельствовало об отсутствии иммуногенности препарата.

Заключение. В ходе проведенного исследования была подтверждена фармакокинетическая эквивалентность исследуемого и референтного препаратов. Был продемонстрирован высокий профиль безопасности и отсутствие иммуногенности у российского препарата Энлигрия® (лираглутид 6 мг/мл, ООО «ПРОМОМЕД РУС», Россия) в сравнении с зарубежным препаратом Саксенда® (лираглутид 6 мг/мл, Ново Нордиск А/С, Дания).

Ключевые слова: глюкагоноподобный пептид-1; биоэквивалентность; фармакокинетика; лираглутид; ожирение; сахарный диабет 2 типа; Энлигрия

Список сокращений: СД 2 – сахарный диабет 2 типа; ГПП-1 – глюкагоноподобный пептид-1; ГИП – глюкозозависимый инсулинотропный полипептид; ССЗ – сердечно-сосудистые заболевания; АССЗ – атеросклеротические сердечно-сосудистые заболевания; HbAlc – гликированный гемоглобин; ИФА – иммуноферментный анализ; ИМТ – индекс массы тела; ДПП-4 – дипептидилпептидаза-4; АФС – активная фармацевтическая субстанция; ДНК – дезоксирибонуклеиновая кислота; РНК – рибонуклеиновая кислота; ЖКТ – желудочно-кишечный тракт; ОРВИ – острая респираторная вирусная инфекция; ПЦР – полимеразная цепная реакция; БАД – биологически активная добавка; АД – артериальное давление; ЧСС – частота сердечных сокращений; ЧДД – частота дыхательных движений; ЭКГ – электрокардиография; ХС ЛПНП – холестерин липопротеидов низкой плотности; ЛПВП ХС – холестерин липопротеидов высокой плотности; НЯ – нежелательное явление; СНЯ – серьёзное нежелательное явление; ДИ – доверительный интервал; ПОМК – проопиомеланокортин, КАРТ – кокаин-амфетамин-регулируемый транскрипт; НПУ – нейропептид Y; АПБ – агутиподобный белок; ГАМК – гамма-аминомасляная кислота., ХСН – хроническая сердечная недостаточность; ХБП – хроническая болезнь почек.

INTRODUCTION

An increased body weight is associated with metabolic disorders and is a pressing problem in modern medicine, as it leads то the development of a number of chronic diseases, including cardiovascular diseases (CVDs), type 2 diabetes mellitus (T2DM) and also has a serious impact on mental health[1,2]. T2DM is a disorder of carbohydrate metabolism caused primarily by insulin resistance and relative insulin deficiency or directly by an impaired insulin secretion[3].

The analysis of clinical practice data shows that patients often have two diseases at once: obesity and T2DM [1]. Moreover, people with T2DM have more difficulty losing weight than people without the disease. This is due to the fact that in an insulin-resistant state, skeletal muscles and the liver are the main organs responsible for glucose utilization. Hyperinsulinemia

[1] WHO European Regional Obesity Report 2022. Copenhagen: WHO Regional Office for Europe; 2022. Available from: https://www.who.int/europe/publications/i/item/9789289057738#:~:text=Overweight%20and%20obesity%20affect%20almost,in%20the%20WHO%20European%20Region.

[2] Clinical guidelines of the Ministry of Health of the Russian Federation "Obesity", 2020. Available from: https://cr.minzdrav.gov.ru/schema/28_2. Russian

[3] Clinical guidelines Type 2 diabetes mellitus in adults, 2022. Available from: https://cr.minzdrav.gov.ru/schema/290_2. Russian

promotes the synthesis and accumulation of triglycerides, while inhibiting lipolysis in adipocytes; all these lead to an increase in the volume of adipose tissue [1, 2]. The result of a compensatory response to metabolic and hormonal changes that accompany an initial weight loss is an increase in the synthesis of orexigenic hormones responsible for stimulating appetite [1]. Hypoglycemic drugs, such as sulfonylureas, thiazolidinediones, and insulin, used in the treatment of patients with T2DM have a number of side effects, such as hypoglycemia, weight gain, a congestive heart failure, and osteoporosis, which prevent many patients from achieving key therapeutic goals[4].

In recent decades, the role of incretin hormones in the regulation of carbohydrate metabolism in the human body and their effect on β-cells have been actively studied. Glucagon-like peptide-1 (GLP-1) is one of the most important incretins, responsible for the production of insulin after meals, stimulating glucose-dependent insulin secretion. In addition, GLP-1 suppresses an excessively increased secretion of glucagon, slows down gastric emptying, reduces an appetite and energy consumption, and as a result, it reduces a body weight [3, 4][5,6]. The therapeutic potential of native GLP-1 is limited due to its rapid degradation by the enzyme dipeptidyl peptidase-4 (DPP-4) and short half-life (1–2 min). In this regard, liraglutide was developed – the first analogue of human GLP-1, demonstrating a persistent improvement in glycated hemoglobin (HbAlc) levels and normalization of a β-cell function in patients with T2DM, as well as reducing the body weight of overweight or obese patients regardless of the presence or absence of T2DM [3, 5]. Due to the unique structure of liraglutide, the drug half-life from plasma increases to 13 h compared to 2 min for native GLP-1. A prolonged action is ensured by three mechanisms: 1) oligomerization into heptamers through the interaction between hydrophobic palmitate residues on each liraglutide molecule - the replacement of one amino acid residue (arginine with lysine) at position 34 and an addition at position 26 to lysine of the side chain of C_{16} palmitic acid, as a result of which a slow absorption of the drug occurs; 2) binding to serum albumin in subcutaneous tissue, leading to a longer half-life after a subcutaneous administration (13 h). Taking into account the maximum concentration of the drug in the blood, which is observed after 10–14 h, the duration of the liraglutide action is 24 h[7] [3–5].

The GLP-1 receptor agonist liraglutide, like native GLP-1, has beneficial metabolic effects that include a glucose-dependent stimulation of an insulin secretion, decreased gastric emptying due to a direct effect on the hypothalamus, inhibition of food intake leading to a weight loss, increased natriuresis and diuresis, lowering total cholesterol and systolic / diastolic blood pressure[8,9] [3].

The original drug liraglutide was approved for a medical use in 2009 and is used in clinical practice under the trade names of Victoza® (Novo Nordisk AS, Denmark) and Saxenda® (Novo Nordisk AS, Denmark). In addition, the drugs based on liraglutide are currently registered in the USA, Japan and some European countries, including Russia. Phase II studies on the determination of the optimal dose have demonstrated that liraglutide has all the expected properties of GLP-1 in humans: its administration provides a glucose control throughout the day, a low incidence of hypoglycemia, and a weight loss in most patients[10].

At the doses of 1.2 and 1.8 mg/day, liraglutide has been successfully used in clinical practice for the treatment of patients with T2DM since 2010. The results of a meta-analysis of the 6-th phase of III LEAD (Liraglutide Effect and Action in Diabetes) studies demonstrated that liraglutide compared with other glucose-lowering drugs, ensures a more effective achievement of therapeutic parameters of a metabolic control HbA1c[11] [6].

Liraglutide showed benefit in the secondary prevention of atherosclerotic CVDs. The LEADER (Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results) study investigated long-term cardiovascular outcomes during a long-term liraglutide use (the median of 3.5 years) in patients with T2DM and high cardiovascular risks. The study demonstrated the reduction in the likelihood of developing serious adverse events (SAEs) when using the drug at a dose of 1.8 mg compared to placebo [7].

[4] Ibid.
[5] Register of Drugs of Russia. Instructions for medical use of the drug Enligria. Available from: https://www.rlsnet.ru/drugs/enligriya-89718. Russian
[6] Assessment report EMA/143005/2015. Saxenda, 2015. Committee for Medicinal Products for Human Use (CHMP). Russianhttps://www.ema.europa.eu/en/documents/assessment-report/saxenda-epar-public-assessment-report_en.pdf
[7] Register of Drugs of Russia. Instructions for medical use of the drug Enligria. Russian
[8] Ibid.
[9] Assessment report EMA/143005/2015. Saxenda, 2015.
[10] Questions and answers on generic medicines. EMEA document. EMEA/393905/2006. London, UK: European Medicines Agency, 2007. Available from: www.emea.europa.eu/pdfs/human/pcwp/39390506en.pdf
[11] Ibid.

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For the treatment of obesity, liraglutide 3 mg (Saxenda®, Novo Nordisk AS, Denmark) was registered in 2014. The significant superiority of liraglutide (3 mg) over placebo in its effect on the body weight was confirmed in a series of randomized, double-blind, placebo-controlled studies that were part of the SCALE program (Satiety and Clinical Adiposity – Liraglutide Evidence in nondiabetic and diabetic individuals)[12] [8–12].

Other clinical studies have also shown that liraglutide has a unique therapeutic potential due to its combined effects on both body weight and glycemic control [13–15]. Liraglutide is one of the hypoglycemic drugs that are successfully used in patients with T2DM, including patients with cardiovascular pathology. In the SCALE Diabetes study, the proportion of patients who achieved an level of HbA1c <7% during the treatment with liraglutide (3 mg) was 69.2 *vs.* 27.2% (placebo). In the SCALE Obesity and Prediabetes study, the prevalence of prediabetes among patients diagnosed at screening after 56 weeks decreased to 30.8%, while in the placebo group in the same category of patients it decreased to 67.3% [10, 11]. During the SCALE research program, it was noted that therapy with liraglutide (3 mg) is accompanied by a decrease in the systolic blood pressure, waist circumference, total cholesterol and low-density lipoprotein cholesterol (LDL-C), and an increase in high-density lipoprotein cholesterol (HDL-C), which also proves that liraglutide therapy helps reduce a cardiometabolic risk even in patients with CVD [16, 17].

A study examining the effect of liraglutide therapy on the body weight in adolescents 12 years of age and older demonstrated that liraglutide was superior to placebo in reducing the standard deviation of BMI (95% confidence interval [CI] -0.37 to -0.08; *p*=0.002), no additional risks were identified regarding the safety of the drug [18].

At the present stage, liraglutide is included in Russian clinical guidelines for the treatment of T2DM in adults, the treatment of obesity in adults and children, the treatment of chronic kidney disease (CKD) to reduce the risk of progression in patients with CKD and T2DM, the treatment of lipid metabolism disorders in patients with T2DM and CVD, having a very high and high cardiovascular possibility to reduce the risk of both new cardiovascular complications (CVD) and the death[13] [5], which determines its demand in the Russian Federation. Accordingly, the import substitution of foreign drugs with Russian analogues and the localization of the full production cycle from the substance to the finished dosage form for liraglutide drugs is of particular relevance and importance from the point of view of ensuring the country's medicinal independence.

Liraglutide was presented on the pharmaceutical market only as a biotechnologically produced compound. However, taking into account the amino acid structure of this peptide, its lack of tertiary structure[14,15], and a number of limitations known for recombinant drugs, it is advisable to produce the active pharmaceutical substance (API) liraglutide through chemical synthesis [19]. Moreover, the production possibilities of biotechnological drugs are limited by a low productivity of the strains used, which may prevent the production of the required amount of the substance, that can be critical given the demand for this group of drugs in patients. This factor also indicates the feasibility of obtaining such drugs by chemical synthesis.

Thus, it is of interest to develop, analyze and produce synthetic liraglutide, as well as compare its physicochemical and biological properties relative to a biotechnologically produced molecule. Chemical synthesis is a high-throughput, scalable, commercially viable process [20–22]. The production of liraglutide by this method makes it possible to eliminate the spontaneous replacement of amino acids in the final product characteristic of the vital activity of microorganisms, and to obtain a product of high purity, with a minimum amount of predictably identified impurities, and a high yield [23–25]. Moreover, such a product is unchanged, homogeneous and does not contain residual impurities of producer cells, such as proteins, enzymes, DNA and RNA fragments, which improves the safety profile and reduces the risk of immunogenicity, and, consequently, the risk of a treatment failure [25].

There is a certain pool of studies to prove the effectiveness and safety of the drug. According to the FDA[16] guidelines, alpha-amino acid polymers,

[12] Register of Drugs of Russia. Instructions for medical use of the drug Saxenda. Available from: https://www.rlsnet.ru/drugs/saksenda-75258

[13] Clinical guidelines Type 2 diabetes mellitus in adults, 2022.

[14] Liraglutide (Compound). Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Liraglutide#section=3D-Status

[15] Ibid.

[16] U.S. Food and Drug Administration. ANDAs for Certain Highly Purified Synthetic Peptide Drug Products That Refer to Listed Drugs of rDNA Origin Guidance for Industry, 2021. Available from: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/andas-certain-highly-purified-synthetic-peptide-drug-products-refer-listed-drugs-rdna-origin

such as glucagon, liraglutide, etc., containing up to 40 amino acid residues, are considered not protein molecules, but peptides[17]. According to the FDA, to confirm the equivalence of a synthetic peptide and the biotechnologically derived liraglutide contained in the precursor drug, it is sufficient to demonstrate the structural identity of the API[18] using modern analytical methods. The Promomed RUS LLC company has developed its own technology for the production of API using methods of chemical synthesis and isolation of liraglutide and a finished dosage form for the treatment of both obesity and T2DM. For an additional assessment of the quality and safety of the developed drugs, its registration in our country in accordance with the Russian regulatory requirements, in addition to physicochemical methods of analysis and preclinical studies, a study of the pharmacokinetics, safety and immunogenicity of the drug Enligria® (liraglutide 6 mg/ml) was conducted in comparison with a foreign predecessor drug.

THE AIM of the work was to assess the bioequivalence and comparability of the safety and tolerability profile of the drug Enligria® (liraglutide 6 mg/ml, Promomed RUS LLC, Russia) and the drug Saxenda® (liraglutide 6 mg/ml, Novo Nordisk AS, Denmark) after a single dose in healthy volunteers.

MATERIALS AND METHODS

Study drugs

The compositions of the study drugs were identical, the composition of the domestically produced liraglutide Enligria® (Promomed RUS LLC, Russia), the solution for the subcutaneous administration, 6 mg/ml (hereinafter referred to as Russian liraglutide, test drug) corresponded to the composition of the reference drug liraglutide Saxenda® (Novo Nordisk AS, Denmark), and the solution for the subcutaneous administration of 6 mg/ml (hereinafter referred to as foreign liraglutide, reference drug).

Physical and chemical research

Spectrophotomery in the ultraviolet region (200–400 nm). When comparing the absorption spectra in the ultraviolet region of the Russian and foreign drugs of liraglutide, Russia, the test solutions of each drug was diluted with water for injection to the concentration of liraglutide in the solution of 0.03 mg/ml. The analysis was carried out on a Shimadzu UV-1800 Spectrophotometer (Shimadzu, Japan), with a spectral wave range of 190–1100 nm.

Size exclusion-high-performance liquid chromatography. Size exclusion chromatography method was used to determine the quantitative content of high molecular compounds in the original foreign drug and a synthetic Russian analogue of liraglutide. The analysis was carried out on a liquid chromatograph with a UV detection Agilent 1260 Infinity LC (Agilent Technologies, USA) using a Tosoh TSK-gel G 2000 SWXL, 7.8×300 mm, 5 µm column. The study was carried out at a wavelength of 276 nm.

Reversed-phase high-performance liquid chromatography. A reverse-phase chromatography method was used to determine the quantitative content of liraglutide, its impurities and phenol in the foreign drug and a synthetic Russian analogue, as well as a confirmation of the authenticity of the active substance (liraglutide) and preservative (phenol). The analysis was carried out using a liquid chromatograph with a UV detection Prominence (Shimadzu, Japan), at a wavelength of 215 nm. For the analysis, Jupiter 4 µm Protea 90A (Phenomenex, 250×4.6 mm, 4 µm, 90 A) and Luna RP C8 (2) (Phenomenex, 4.6×50 мм, 5 µm) columns were used.

Verification of amino acid sequence and determination of intact mass using gas chromatography-mass spectrometry (LC-MS). The confirmation of the authenticity of the target component in the original foreign drug and the Russian analogue of liraglutide was carried out using the tandem mass method – high-resolution spectrometry on a quadrupole-time-of-flight mass spectrometer maXis 4G ETD (Bruker, USA). The Amino acid sequence verification was carried out by a peptide mapping with a peptide identification by HPLC/MS/MS with an electrospray ionization (ESI) and a secondary collision-initiated ionization (CID). The peptide identification was performed by precise monoisotopic mass using high-performance liquid chromatography-high-resolution mass spectrometry (HPLC/MS) with an electrospray ionization (ESI).

Study of biological activity *in vitro*

The biological activity of the studied drugs was assessed *in vitro* on the CHO-K1/GLP-1R cell culture

[17] Ibid.
[18] Ibid.

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(GenScript, USA). This cell line has receptors for GLP-1, to which the active ingredient of the drugs, liraglutide, binds.

The cultivation of the cell line was carried out using the RPMI culture medium (PanEco, Russia) with the addition of a penicillin / streptomycin solution (1%) and fetal bovine serum (10%), under standard conditions (temperature – 37±1°C, CO_2 content – 5±1%), for 2 days. The resulting suspension was diluted to the concentration of 2.5×105 cells/ml, transferred into 96-well plates (5×103 cells/well), and incubated.

Upon the incubation completion, the medium was removed from the plates and 7.5 µl of the test samples were added, after which the plates were mixed for 30 sec and incubated at room temperature for 20 min. Then, 7.5 µl of the lysis buffer was added to the plates and incubated at room temperature for 15 min with continuous stirring.

The results were assessed using the cAMP-GloTM Assay kit (Promega, USA) in accordance with the instructions for the kit.

Assessment of bioequivalence, safety profile, tolerability and immunogenicity

This phase I clinical trial No. LIR-062022 was an open-label, randomized, crossover, two-period comparative study in healthy volunteers. The study design is presented in Figure 1.

Study conditions and duration

The study was conducted from January 23 to April 25, 2023 at the research center of the Yaroslavl Region Clinical Hospital No. 3 (Yaroslavl, Russia).

Ethical approval

The study complied with the ethical principles set forth in the Declaration of Helsinki, as recently revised, the rules of Good Clinical Practice of the Eurasian Economic Union, the Rules of Good Clinical Practice of the International Council for Harmonization (ICH E6 GCP R2), as well as other legislation applicable to this study. The clinical trial protocol was approved by the Ministry of Health of Russia (Permission No. 725 dated December 26, 2022) and the Ethics Council of the Ministry of Health (extract from Protocol No. 335 of the meeting dated May 30, 2023), as well as the local ethics committee at the research center of the state budgetary healthcare institution of the Yaroslavl region “Clinical Hospital No. 3” (extract from Protocol No. 165 of the meeting dated September 30, 2022).

Study objects and eligibility criteria

A total of 26 healthy volunteers, men aged 18 to 45 years (32.42±7.78 years), were included in the study. All participants signed an informed consent form and expressed their ability and willingness to comply with all requirements of the study Protocol. In addition, the main inclusion criteria were: body weight >50 kg; BMI 18.5–26 kg/m^2 inclusive; a verified diagnosis “healthy” according to standard clinical, laboratory and instrumental examination methods; negative results of tests for the use of alcohol, psychotropic and narcotic substances and willingness to stop drinking alcohol during the participation in the study. The participants were warned to use reliable methods of contraception and to abstain from sperm donation throughout the study and for 3 months after the end of the study.

The main non-inclusion criteria were: the presence of chronic diseases of various organ systems; mental illness; hypersensitivity to study drugs; administration of liraglutide or other analogues of human GLP-1 in past history, taking medications that have a pronounced effect on hemodynamics and/or a liver function for less than 2 months before screening; taking illicit drugs less than 4 weeks before screening; inability to perform subcutaneous injections; any history of difficulty with blood collection or any vasovagal seizures during blood collections; history of surgical interventions on the gastrointestinal tract (except appendectomy). The volunteers were not allowed to take part in the study if you had the following diseases and conditions: a history of medullary thyroid cancer, including a family history; a history of multiple endocrine neoplasia type 2; severe depression; suicidal thoughts or behavior, including a history; acute infectious diseases or ARVI symptoms for less than 4 weeks before screening; presence of a positive PCR test for SARS-CoV-2. The volunteers were excluded from the study if they refused to participate in the clinical trial, if they were taking drugs for prohibited therapy and if they were tested positive for the use of alcohol, psychotropic and/or narcotic substances, if there were gross violations of the requirements and procedures of the Protocol, if adverse events occurred, or if a volunteer had any diseases or conditions that made his further participation in the study impossible during the study. The study physician had a right to arrive at the decision to exclude a volunteer in the best interests of the volunteer.

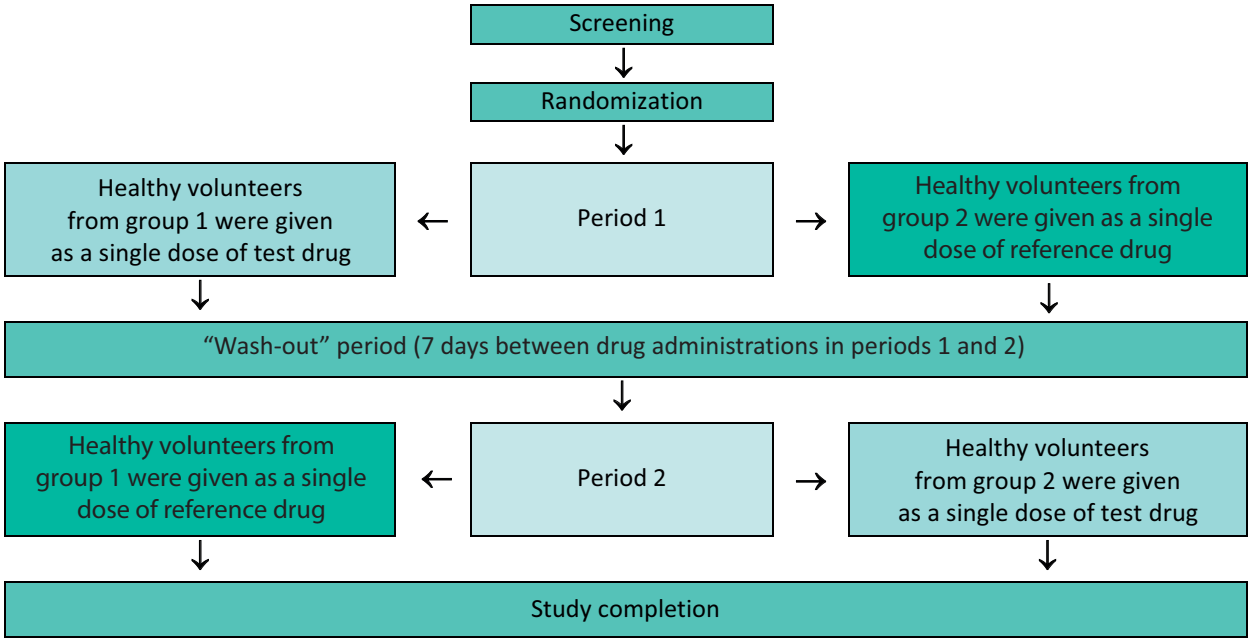

Figure 1 – Study design

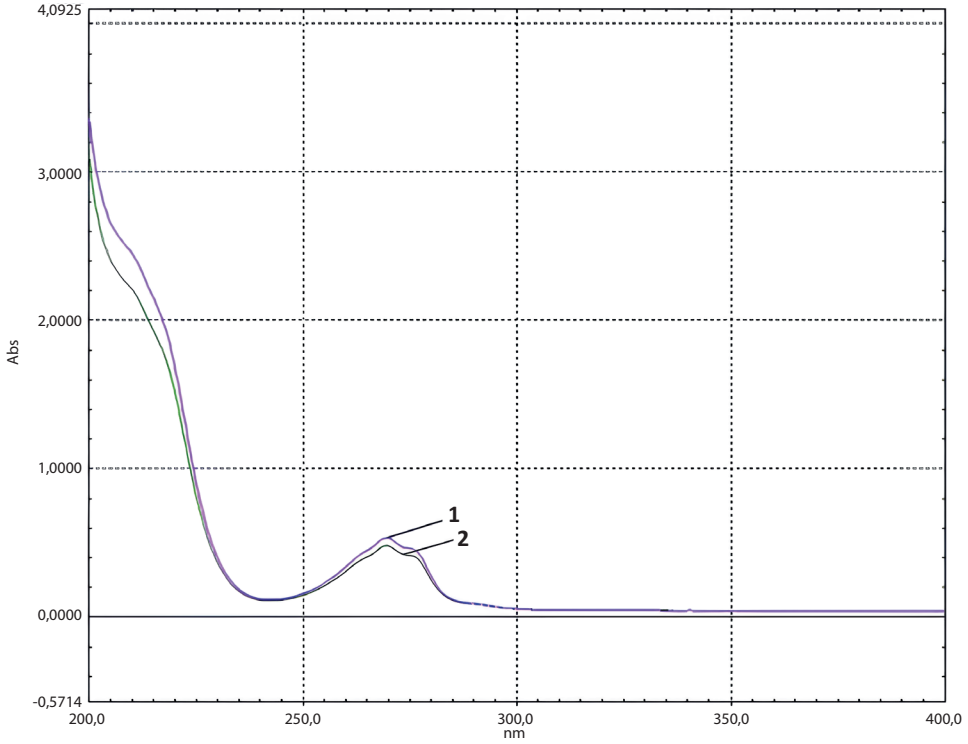

Figure 2 – Absorption spectrum of liraglutide

Note (here and in Fig. 3–6): 1 – synthesized Russian liraglutide; 2 – foreign liraglutide, an original drug.

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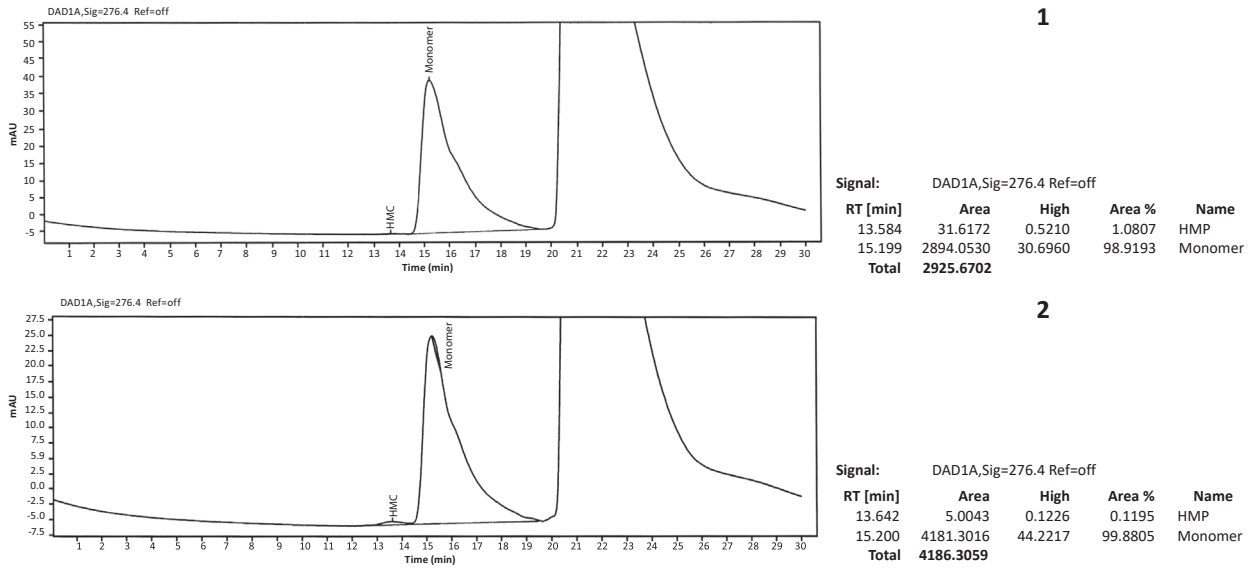


Figure 3 – Chromatograms for determining the content of high-molecular compounds in the drugs of liraglutide, a solution for subcutaneous administration of 6 mg/ml

Table 1 – Chromatographic analyses results of liraglutide drugs

Index	Saxenda®, solution for subcutaneous administration 6 mg/ml, Novo Nordisk AS, Denmark	Enligria®, solution for subcutaneous administration 6 mg/ml, Promomed RUS LLC, Russia
Quantitative determination of liraglutide, mg/ml	6.4	6.3
Amount of impurities, %	2.367	0.904
Hydrophilic impurities,%	0.164	0.072
Impurity A, %	0.614	0.515
Impurity B, %	0.877	0.241
Impurity C, %	0.346	0.076
Hydrophobic impurities,%	0.366	None
Phenol, mg/ml	5.51	5.4

Table 2 – Average values of pharmacokinetic parameters after administration of the study / reference drug

Parameter	Results, Mean±SD	
	Saxenda®, solution for subcutaneous administration 6 mg/ml, Novo Nordisk AS, Denmark	Enligria®, solution for subcutaneous administration 6 mg/ml, Promomed RUS LLC, Russia
C_{max} (ng/ml)	53.68±21.65	54.70±21.91
T_{max} (h)	11.93±4.60	13.60±4.94
$AUC_{0\to t}$ (ng*h/ml)	2313.96±671.11	2468.50±904.96
$AUC_{0\to\infty}$ (ng*h/ml)	2695.30±677.48	2849.24±905.32
$AUC_{0\to t}/AUC_{0\to\infty}$ (%)	85.84±4.57	86.63±5.00
K_{el} (h^{-1})	0.04±0.007	0.042±0.011
$T_{1/2}$ (h)	17.45±3.19	16.54±3.35
V_d (l)	5.60±2.21	5.03±2.51
$AUC_{(t-\infty)}$ (%)	13.35±4.56	12.23±5.00

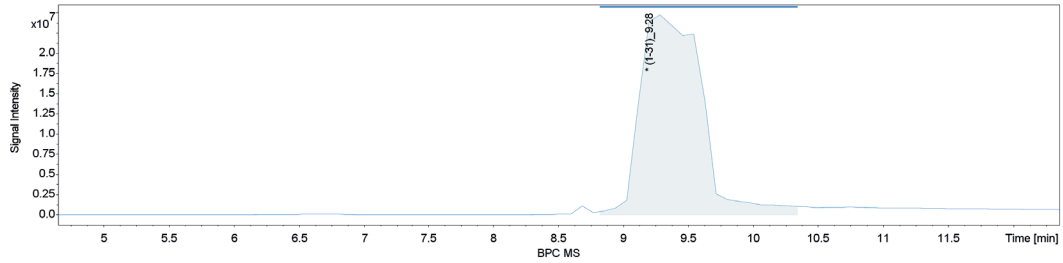
Note: C_{max} – maximum plasma concentration; T_{max} – time to reach C_{max}; $AUC_{0\to t}$ – is the area under the plasma concentration-time curve from the administration moment to the last determined concentration at time point t; $AUC_{0\to\infty}$ – area under the plasma concentration-time curve from the moment of taking the drug to infinity; K_{el} – terminal elimination rate constant; V_d – apparent volume of distribution.

Table 3 – Values of calculated 90% confidence intervals for indicators of relative liraglutide bioavailability after Russian and foreign drugs administration

Indicator	Ratio of geometric means	Calculated values 90% CI	$CV_{intra,}$ %
f'' C_{max} (T)/ C_{max} (R)	98,13±34,24	87,18–110,46	25,33
f' AUC_{0-t} (T)/ AUC_{0-t} (R)	93,74±26,73	84,40–104,11	22,38
f $AUC_{0\to\infty}$ (T)/ $AUC_{0\to\infty}$ (R)	94,60±21,78	86,69–103,22	18,55

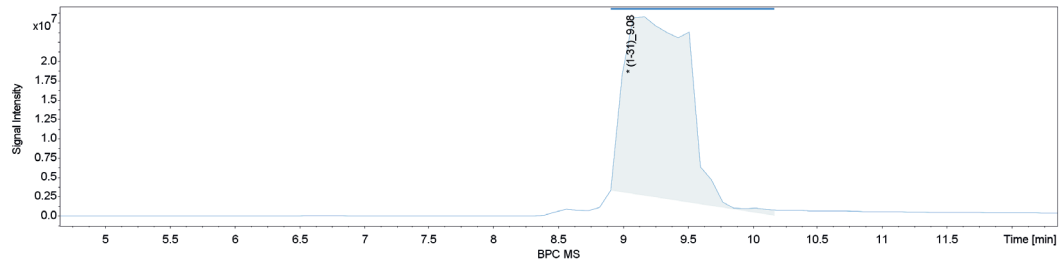
Note: C_{max} – maximum plasma concentration; $AUC_{0\to t}$ – area under the "plasma concentration – time" curve from the moment of administration to the last determined concentration at time point t; $AUC_{0\to\infty}$ is the area under the "plasma concentration – time" curve from the moment of taking the drug to infinity ; T – test drug; R – reference drug.

1



Cmpd.	m/z meas.	z	Δ m/z [ppm]	Δ m/z [Da]	Rt [min]	Score	No. of Cmpds.	P	Peptide	Sequence	Modification	Mod. Ratio [%]	Range
4	850.3762	2	0.32	0.0003	5.44	26.7	1	15		-.HAEGTFTSDVSSYLEG.Q		100.0	1-16
94	1160.6108	3	2.34	0.0027	10.14	18.4	1	27		-.HAEGTFTSDVSSYLEGQAAKEFIAWLVR.G	Gamma-(E)_Palmitoate: 20	100.0	1-28
97	1179.615	3	-0.20	-0.0002	10.23	24.5	1	28		-.HAEGTFTSDVSSYLEGQAAKEFIAWLVRG.R	Gamma-(E)_Palmitoate: 20	100.0	1-29
57	1250.6552	3	-0.72	-0.0009	9.37	62.7	3	30		-.HAEGTFTSDVSSYLEGQAAKEFIAWLVRGRG.-	Gamma-(E)_Palmitoate: 20	100.0	1-31
76	1204.969	3	-0.66	-0.0008	9.71	68.6	1	29		H.AEGTFTSDVSSYLEGQAAKEFIAWLVRGRG.-	Gamma-(E)_Palmitoate: 19	100.0	2-31
79	1181.2899	3	-0.72	-0.0009	9.71	65.3	1	28		A.EGTFTSDVSSYLEGQAAKEFIAWLVRGRG.-	Gamma-(E)_Palmitoate: 18	100.0	3-31
81	1119.2687	3	-0.63	-0.0007	9.71	57.3	1	26		G.TFTSDVSSYLEGQAAKEFIAWLVRGRG.-	Gamma-(E)_Palmitoate: 16	100.0	5-31
89	973.8689	3	-1.95	-0.0019	9.97	48.9	1	22		S.DVSSYLEGQAAKEFIAWLVRGRG.-	Gamma-(E)_Palmitoate: 12	100.0	9-31

2



Cmpd.	m/z meas.	z	Δ m/z [ppm]	Δ m/z [Da]	Rt [min]	Score	No. of Cmpds.	P	Peptide	Sequence	Modification	Mod. Ratio [%]	Range
69	1160.6098	3	1.48	0.0017	10.11	40.5	1	27		-.HAEGTFTSDVSSYLEGQAAKEFIAWLVR.G	Gamma-(E)_Palmitoate: 20	100.0	1-28
71	1179.6175	3	1.92	0.0023	10.19	33.5	1	28		-.HAEGTFTSDVSSYLEGQAAKEFIAWLVRG.R	Gamma-(E)_Palmitoate: 20	100.0	1-29
48	1231.6495	3	0.45	0.0006	9.51	61.4	2	29		-.HAEGTFTSDVSSYLEGQAAKEFIAWLVRGR.G	Gamma-(E)_Palmitoate: 20	100.0	1-30
38	1250.6567	3	0.48	0.0006	9.25	72.8	3	30		-.HAEGTFTSDVSSYLEGQAAKEFIAWLVRGRG.-	Gamma-(E)_Palmitoate: 20	97.6	1-31
25	942.2424	4	-0.23	-0.0002	8.90	50.4	1	30		-.HAEGTFTSDVSSYLEGQAAKEFIAWLVRGRG.-	Gamma-(E)_Palmitoate: 20; Oxidation: 25	2.4	1-31
51	1204.9692	3	-0.49	-0.0006	9.68	60.2	1	29		H.AEGTFTSDVSSYLEGQAAKEFIAWLVRGRG.-	Gamma-(E)_Palmitoate: 19	100.0	2-31
53	1181.2907	3	-0.05	-0.0001	9.68	54.8	1	28		A.EGTFTSDVSSYLEGQAAKEFIAWLVRGRG.-	Gamma-(E)_Palmitoate: 18	100.0	3-31
52	1002.881	3	-0.48	-0.0005	9.59	52.4	1	23		T.SDVSSYLEGQAAKEFIAWLVRGRG.-	Gamma-(E)_Palmitoate: 13	100.0	8-31
65	973.8703	3	-0.52	-0.0005	10.02	46.3	1	22		S.DVSSYLEGQAAKEFIAWLVRGRG.-	Gamma-(E)_Palmitoate: 12	100.0	9-31

Figure 4 – Verification results of the amino acid sequence of liraglutide drugs samples, solution for subcutaneous administration 6 mg/ml

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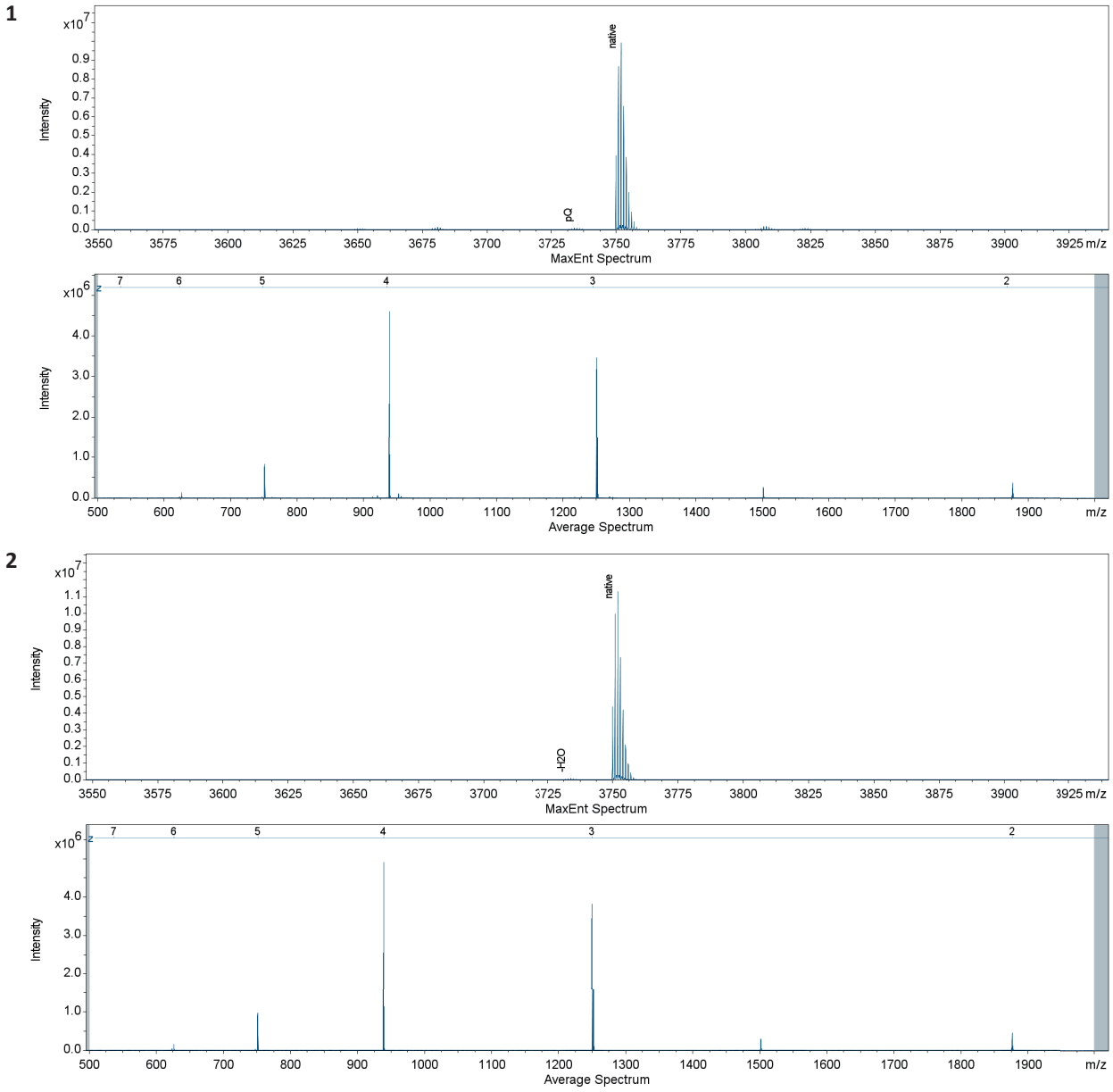

Figure 5 – Identification results of protein masses in samples of liraglutide, solution for subcutaneous administration 6 mg/ml (after deconvolution)

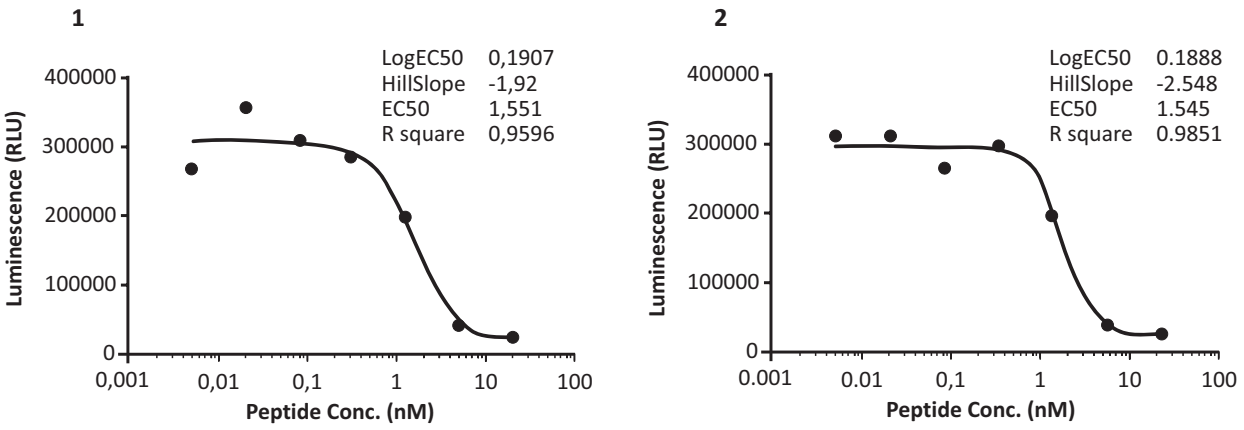

Figure 6 – Dependence graphs of RLU *vs* protein concentration, obtained as a study part of the connection with GLP-1 receptors of the active substance of liraglutide samples

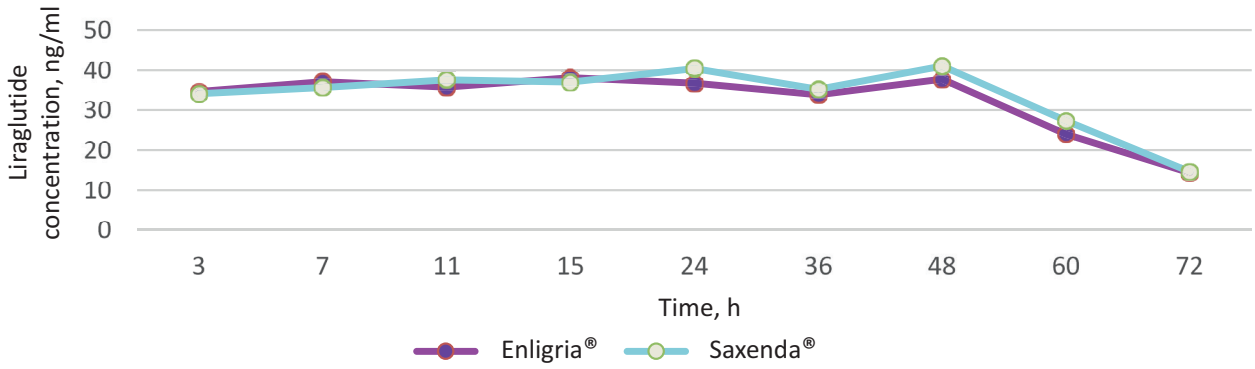

Figure 7 – Mean values (Geom Mean) of drug concentrations in linear transformation without standard deviations

The exclusion criteria were: a refusal of the volunteer to participate in the clinical trial; the research physician decided that the volunteer should be excluded in the interests of the volunteer himself; an erroneous inclusion of a volunteer who does not meet the inclusion criteria and/or meets the non-inclusion criteria; a positive test for the use of alcohol, psychotropic and/or narcotic substances; being late to the clinic for hospitalization by more than 1 h; skipping 2 or more blood samples to determine pharmacokinetic parameters in a row during one study period or three blood samples to determine pharmacokinetic parameters during one study period; a volunteer's administration of vitamins, BAS, medications, including herbal and/or homeopathic, with the exception of the test / reference drug; if a volunteer develops any diseases or conditions that make it impossible for him to further participate in the study; if a volunteer refuses to cooperate, is not disciplined, and does not comply with the rules of participation in the study.

Concomitant medications and exclusion criteria were assessed throughout the volunteer's participation in the study. The total duration of the study for each volunteer was not more than 25 days.

Randomization procedure

Each volunteer who met all the inclusion criteria and did not meet any of the non-inclusion criteria was assigned a randomization number in accordance with the randomization plan prepared for this study in the WinPepi 11.65 program (ETCETERA 3.26 module) using the random number generation method. The randomization number of the volunteer was entered by the study physician into the Screening / Randomization of Study Subjects Log. If a volunteer left the study prematurely, their randomization number was not reused and the volunteer could not subsequently return to the study.

Drugs administration

The test drug was Enligria® (liraglutide, a solution for the subcutaneous administration, 6 mg/ml, Promomed RUS LLC, Russia. The reference drug was Saxenda® (liraglutide, a solution for the subcutaneous administration, 6 mg/ml, Novo Nordisk AS, Denmark). The volunteers who had met the inclusion criteria and those who had not met the non-inclusion criteria, were randomized into 2 groups in a 1:1 ratio. Group I (*n*=13) received Russian liraglutide in the first period of the study, and a reference drug, foreign liraglutide, in the second period of the study. Group II (*n*=13) received the reference drug in the first period of the study, and the test drug in the second period. The reference / test drug was administered at a single dose of 0.6 mg subcutaneously in the abdominal area. The choice of a dose is based on the Russian and international regulatory requirements for the ethics and safety of the drugs administration in healthy volunteers[19],[20], as well as taking into account the requirements of Good Clinical Practice, within the framework of which, before the start of the study, an assessment of the ratio of foreseeable (predictable) risks and inconvenience (in in this case, the effect of the active substance on the glycemic profile and insulin production) with the expected benefit for the study subject and society, in healthy volunteers. It should be also noted that for the selected dose there is the experience in the clinical administration of the drugs biosimilar to liraglutide in healthy volunteers[21,22] [4].

[19] WMA declaration of Helsinki – Ethical principles for medical research involving human subjects. Edinburgh, Oct. 2000, 50 p.
[20] Decision of the Council of the Eurasian Economic Commission dated November 3, 2016 No. 85 (as amended on February 15, 2023) "On approval of the Rules for conducting bioequivalence studies of medicinal products within the framework of the Eurasian Economic Union". Available from: https://www.consultant.ru/document/cons_doc_LAW_207405/. Russian
[21] Register of Medicines of Russia. Instructions for medical use of the drug Enligria.
[22] Assessment report EMA/143005/2015. Saxenda, 2015.

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It is worth noting that the research results show linearity and proportionality of the kinetics with increasing doses according to the dosage regimen of liraglutide in accordance with the instructions for a medical administration, which also confirms the validity of the chosen dose in healthy volunteers and confirms the consistency of the results of pharmacokinetic parameters and safety parameters comparison regardless of the dose increase [4, 26, 27]. The washout period was 7 days and began immediately after the test / reference drug had been administered to the volunteer during period I. According to the literature, the half-life of liraglutide is about 13 h [4]. Thus, to minimize the risks of the first dose influence of drugs, the washout period should be at least 5 half-lives, i.e. at least 65 h.

To administer the test / reference drug, the volunteers were admitted to the hospital the evening before and at least 10 h before the drug administration. During the period of their stay in the hospital, the volunteers complied with the rules of their stay. The duration of hospitalization was no more than 4 days. During the study, the administration of vitamins, dietary supplements and/or medications, including herbal and homeopathic drugs, was prohibited, except as provided for in this Protocol. During the entire study, from the start of the screening examination until the completion of the final examination, the volunteers abstained from foods and drinks that may affect a circulatory function, a gastrointestinal function, a liver or kidney function, and an alcohol intake.

Preparation and sampling

After the randomization and before the administration of the test / reference drug, the volunteers were placed in a cubital heparinized catheter for no more than 16.5 h. After the removal of the catheter, the blood was collected from the volunteers by venipuncture. The blood samples were taken to determine pharmacokinetic parameters at the following time points: 10–15 min before the administration of the test / reference drug (the initial (0) sample) and then after 1, 3, 5, 7, 8, 9, 10, 11 , 12, 13, 14, 15, 16, 24, 36, 48, 60 and 72 h after the liraglutide administration. The original (0) sample was also used to assess immunogenicity. Thus, in all periods of the study, 19 blood samples were taken for each volunteer (6 ml each) for pharmacokinetic studies.

Blood samples were collected to assess the immunogenicity of the test / reference drug 10–15 min before the administration of the test / reference drug in periods I and II of the study. During screening, a blood volume of no more than 18 ml was taken for clinical, biochemical, serological tests and the determination of blood glucose levels using a glucometer. At the end of the II period of the study, the blood was also collected for clinical, biochemical tests and the determination of blood glucose levels using a glucometer (12 ml). Blood samples were collected in test tubes to obtain serum with a coagulation activator. After a clot formation, the tubes were centrifuged, and the resulting serum was carefully transferred into pre-labeled cryovials, dividing the serum into two aliquots: one for analysis (aliquot A), the other for repeat tests (aliquot B). The serum samples were frozen immediately after the receipt, transferred to cryovials and stored at the temperature not exceeding -70°C.

Analytical method

Pharmacokinetics was assessed by the concentration of liraglutide in the blood plasma and antibodies to it in the blood serum of each volunteer after the subcutaneous administration of the test / reference drug. A quantitative determination of liraglutide in serum samples was carried out using a HiPo MPP-96 microplate photometer (Biosan, Latvia). The calculation of liraglutide concentrations was carried out using the Quant Assay v0.8.2.6 Software, and the concentrations of antibodies to liraglutide – using GraphPad Prism 8.4.3. The determination of liraglutide in blood serum samples was carried out using a previously validated enzyme-linked immunosorbent assay (ELISA) method using a commercially available Enzyme-linked Immunosorbent Assay Kit For Liraglutide (LRT) Organism Species: Pan-species (General) (CEV769Ge 96 Tests)"; antibodies to liraglutide – using the KRIBIOLISATM Anti-Liraglutide ELISA kit. The sensitivity of the method was 4.64 ng/ml, the detection range was 12.35–1000.00 ng/ml. The preparation of calibration samples from the kit was carried out by diluting the standard sample. The analytical range was selected in accordance with the instructions for the kit.

Safety and tolerability assessment

During the study, a clinical observation of volunteers was carried out with the assessment of physical examination data, including a survey about the volunteer's complaints, basic vital signs (BP, HR, RR, body temperature), 12-lead ECG, laboratory parameters of clinical, biochemical blood tests, general urine analyses, determining blood glucose levels using a glucometer. Safety assessment criteria included the frequency and severity of AEs recorded based on abnormal laboratory test results, physical examinations, vital signs, and ECG; a number of cases of early participations' termination in the study due to the development of adverse events (AEs)

and/or serious adverse events (SAEs), including those related to the test / reference drug; the frequency of volunteers with detected antibodies to liraglutide; the assessment of the overall tolerability of the test / reference drug on a Likert Scale. The safety and tolerability of liraglutide were assessed for all volunteers. The identification of AEs occurred from the moment of the administration of the study drugs until the end of the volunteer's participation in the study.

Pharmacokinetic parameters: a maximum concentration of the substance in the blood serum (C_{max}); the time to reach C_{max} (T_{max}); the area under the concentration-time curve from the moment of the drug administration to the last determined concentration at the time point t ($AUC_{0\to t}$); the area under the pharmacokinetic curve, starting from the zero time value to infinity ($AUC_{0\to\infty}$); the ratio of $AUC_{0\to t}$ values to $AUC_{0\to\infty}$ ($AUC_{0\to t}/AUC_{0\to\infty}$); a terminal elimination rate constant (Ke_l); half-life ($T_{1/2}$); volume of distribution (V_d); residual (extrapolated) area under the curve, determined by the formula $AUC_{0\to\infty}–AUC_{0\to t}/AUC_{0\to\infty}$ ($AUC_{(t-\infty)}$%); the indicators of relative bioavailability and a relative degree of absorption ($f=AUC_{0\to\infty}$ (T)/$AUC_{0\to\infty}$ (R); $f'=AUC_{0\text{-}t}$ (T)/$AUC_{0\text{-}t}$ (R); $f''=C_{max}$ (T)/C_{max} (R)).

Statistical analysis

To calculate the number of participants, the data on the coefficients of intra-individual variability (CV_{intra}) of the C_{max}, $AUC_{0\text{-}t}$ and $AUC_{0\text{-}\infty}$ parameters of liraglutide were used. According to the literature sources, the intraindividual coefficient of variation for C_{max}, $AUC_{0\text{-}t}$ и $AUC_{0\text{-}\infty}$ of liraglutide does not exceed 21% [7]. In a crossover design, taking into account that the 90% CI was 80.00–125.00%, CV_{intra}=21%, α=0.05, a study power – 80%, a geometric mean ratio – 0.95, it was necessary to include at least 21 healthy volunteers (22 volunteers, taking into account an equal distribution in the study groups), who had completed the study and were included in the statistical analysis. Taking into account dropouts, the study planned to randomize 26 healthy volunteers.

For pharmacokinetic calculations, the actual time of blood sampling was used. The calculation of pharmacokinetic parameters, a statistical analysis of safety indicators and the presentation of results were carried out using statistical packages StatSoft Statistica version 10.0/13.3, IBM SPSS Statistics 22 and using the R Project program (version 3.5.1, GPL-2/GPL-3 license) with the extension *bear*, version 2.8.3-2. The indicators used to evaluate the pharmacokinetics of liraglutide are presented in Table 1.

For all pharmacokinetic parameters, the following statistical parameters were calculated: an arithmetic mean, a geometric mean, a standard deviation of the mean, a variation coefficient, median, minimum and maximum values, scatter.

A statistical analysis was carried out based on the assumption of a log-normal distribution of $AUC_{0\text{–}t}$, $AUC_{0\text{-}\infty}$, C_{max} and a normal distribution of other pharmacokinetic parameters with the exception of T_{max}. After a logarithmic transformation, these parameters were analyzed using the analysis of variance (ANOVA), with a standard significance level of α=0.05. The analysis of variance was used to test hypotheses about the statistical significance of the contribution to the observed variability of the following factors: differences between drugs, differences between healthy volunteers, sequence drug administration, study periods.

The conclusion about the equivalence of the compared drugs was made using an approach based on the assessment of 90% CI for the ratios of the geometric mean values of the C_{max}, $AUC_{0\text{–}t}$, $AUC_{0\text{-}\infty}$ liraglutide parameters.

For all the safety and tolerability indicators collected during the study, the descriptive statistics data are presented. For the analysis of frequencies, proportions, a two-sided version of Fisher's exact test or the χ^2 test was carried out. To compare quantitative continuous indicators, the Student's t-test (in the case of a normal distribution) or the Mann-Whitney test (in the case of a non-normal distribution) were used. The differences were considered statistically significant at p <0.05.

RESULTS

Physical and chemical research

Spectrophotomery in ultraviolet spectrum

The results obtained (Fig. 2) demonstrated the similarity of the absorption spectra in the UV of foreign liraglutide (min=242.4 nm; max=269.6 nm) and domestic liraglutide (min=242.2 nm; max=269, 4 nm).

Size exclusion-high-performance liquid chromatography

Typical chromatograms obtained during the determination of high-molecular compounds are shown in Figure 3.

The results obtained demonstrate the comparability of the retention time of high-molecular compounds and liraglutide, as well as the comparability of the quantitative content of impurities of high-molecular compounds in the preparation of foreign (0.131%) and Russian (0.052%) liraglutide.

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Reversed-phase high-performance liquid chromatography

Based on the results obtained (Table 1), it is shown that the retention time of the chromatographic peaks of liraglutide in the reference drug and in the test drug was about 14.7 min, the chromatogram peak profiles for liraglutide and impurities were similar. Moreover, at the time of the analysis, the content of impurities in Russian liraglutide was 3.5 times lower than in a foreign-made drug, which may be due to the technology for its production.

Moreover, the Russian liraglutide, which contains a synthetic molecule, did not contain hydrophobic impurities.

The retention time of the chromatographic peaks of phenol in the drugs was about 1.3 min, which corresponded to the retention time of phenol in the reference solution. The data obtained indicate that the phenol content in the drugs of foreign and Russian liraglutides is almost equivalent.

Verification of the amino acid sequence and determination of intact mass using gas chromatography-mass spectrometry (LC-MS)

The results of verification of the amino acid sequence of a domestically produced liraglutide sample with a chemically synthesized active substance and foreign liraglutide are presented in Figure 4.

The results of the protein masses identification taking into account possible isoforms of the sample with chemically synthesized Russian and foreign liraglutides are presented in Figure 5.

As a result of the amino acid sequence verification, a 100% coverage was obtained for all samples. The amino acid sequence of the samples fully corresponded to the declared one. As a result of the identification based on the exact mass of the protein, taking into account possible isoforms, it was found that the exact monoisotopic mass of all samples corresponded to the declared structural formula.

Biological activity *in vitro*

Dependence graph of RLU vs protein concentration are presented in Figure 6.

The results obtained demonstrate the comparability of the Russian drug (EC_{50}=1.545) biological activity with a chemically synthesized active substance and the original foreign liraglutide (EC_{50}=1.551). The range of activity of both study drugs is from 80 to 120% relative to the standard sample.

Bioequivalence and comparability of safety and tolerability profile

Population

A total of 26 male volunteers were included in the study. All the volunteers were included in the population for the safety assessment, pharmacokinetic analysis and bioequivalence assessment. The average age of the volunteers in the population was 32.42±7.78 years, the average body weight was 78.61±5.27 kg, the average height was 178.62±4.99 cm, the average body mass index (BMI) was 24.62±0.94 kg/m^2. Demographic and baseline characteristics (gender, age, race, weight, height) of the volunteers did not differ between the groups.

Pharmacokinetics and bioequivalence

The average values of the main and additional pharmacokinetic parameters after administration of the test and reference drugs are presented in Table 2.

A graph of the dynamics of average liraglutide concentrations during the administration of the test / reference drug is shown in Figure 7.

As it follows from the presented data, the mean values of both main and additional pharmacokinetic parameters obtained after the use of the test and reference drugs were comparable to each other.

According to the results of the statistical analysis, the obtained 90% CI for the ratio of the values of C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ of the studied Russian and foreign drugs were 87.18–110.46, 84.40–104.11 and 86.69–103.22%, respectively. The intra-individual coefficients of variation calculated based on the ANOVA analysis were 25.33% for the C_{max} value, 22.38% for the AUC_{0-t} value and 18.55% for the $AUC_{0-\infty}$ value.

Thus, the intervals obtained during the study fully complied with the equivalence limit of 80.00–125.00% for C_{max}, AUC_{0-t} and $AUC_{0-\infty}$, clearly demonstrating the bioequivalence of the study and the reference drugs (Table 3).

The results of the ANOVA showed that the differences in the mean values of the main pharmacokinetic parameters were not statistically significant and had not been caused by the differences between the compared drugs for the factors “Sequence of the administration”, “Period” and “Drug” for the pharmacokinetic parameters of C_{max}, AUC_{0-t} and $AUC_{0-\infty}$.

Safety

All volunteers completed the study entirely in accordance with the approved study protocol. During the study, no AEs were recorded in the volunteers. In 100% (26) cases, the tolerability among the volunteers

was rated as "good." No SAEs were identified in the volunteers during the study or after its completion. No deaths were observed. There were no cases of pregnancy of the sexual partner of a study participant during the study or after its completion. No abnormalities were found in the results of clinical and biochemical blood tests, the determination of blood glucose levels, general urinalyses, vital signs, physical examinations and ECGs.

Immunogenicity assessment

According to the results of the immunogenicity parameters analysis, no antibodies to liraglutide were detected in the blood serum of the volunteers, which indicated the absence of immunogenicity of the drug. No unexpected results were noted during the study.

Thus, the test drug Enligria® and the reference drug Saxenda® had a similar safety profile. At the same time, no cases of immunogenicity were observed for the domestic drug, which confirms a high safety profile and reduces the risk of ineffective therapy.

DISCUSSION

A wide range of liraglutide benefits makes it a strategically advantageous and attractive product for manufacturing. Based on this, the authors searched for ways to obtain this compound and analyzed the advantages and disadvantages of each method in order to fully form the evidence base in favor of this compound.

Biotechnological method for the production of liraglutide

Liraglutide, developed by Novo Nordisk (US7572884B2 [28], US7273921B2 [29]), was first introduced to the Russian market in 2010 as a hypoglycemic agent called Victoza®, a solution for the subcutaneous administration, 6 mg/ml [30]. The peptide precursor of liraglutide was obtained by the genetic recombination technology [31] using *Saccharomyces cerevisiae* to express the Arg34GLP-1 molecule (7–37), the structure of which was designed to be 97% homologous to native human GLP-1 by amino acid substitution arginine to lysine at position 34 [32, 33]. After that, liraglutide itself was obtained by adding C_{16} palmitic acid through a glutamine spacer to the ε-amino group of lysine located at position 26 [32].

Saccharomyces cerevisiae is a biologically safe strain with simple genetic manipulations and a clear mechanism for regulating gene expression [34]. Compared with the complete chemical synthesis of liraglutide, semichemical synthesis, including expression of the precursor peptide in a genetically engineered protein expression system and a further chemical modification of them, is more economical and environmentally friendly [33].

However, such eukaryotic systems have a number of disadvantages. It is known that the expression level of heterolytic proteins in *Saccharomyces cerevisiae,* especially GLP-1 polypeptides, is highly dependent on the protease during fermentation. That is why researchers attempted to knock out various protease and glycosylase genes in *S. cerevisiae* to create a protease-deficient strain in order to increase the expression and reduce the degradation of the target product [34].

Currently, the expression of foreign genes can be carried out using not only eukaryotic systems, but also prokaryotic ones [35]. *Escherichia coli (E. coli)* is one of the preferred expression systems (US10851146B2 [31], CN114807205A [36], US2020024321A1 [37]), since its genetic background and regulatory mechanism are well studied [35], it is able of reproduction and does not require expensive equipment [35, 38, 39].

Despite all the advantages of using eukaryotic and prokaryotic expression systems to produce the liraglutide precursor peptide, a biotechnological production has a number of significant disadvantages. The main challenge is to ensure a genetic stability and an adequate product yield, since the expression systems used may produce proteins with imperfect structures. When producing liraglutide using recombinant technology, it is also necessary to prove that the product is not contaminated with microorganisms and does not contain their metabolic products [40]. Based on this, there is a need to study and develop methods for obtaining liraglutide via an alternative route.

Chemical synthesis

There are two standard approaches to the chemical synthesis of GLP-1 agonists, i.e., liquid-phase peptide synthesis and solid-phase peptide synthesis. In addition, hybrid approaches can be used, as described in patents WO 2019069274 [40] and CN104650219 [41], in which the fragments are first synthesized by one of the above-mentioned methods and then are condensed together [42]. The main disadvantage of this synthesis is that the condensation reaction requires an excessive amount of peptide fragments, which in turn leads to serious losses, the formation of a large number of impurities, and as a result, complicates the purification of liraglutide, thereby preventing the obtaining of a pure target product [43]. It is possible to increase the yield of the peptide and obtain a product of a greater purity using

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a hybrid approach through the process of ultrasonic radiation and binding of fractionated peptides using an ionic liquid and a eutectic solvent [44].

The main challenge in the synthesis of a compound such as liraglutide is the introduction of a lipophilic group into the Lys^{20} lysine side chain. The creation of this branched structure can be achieved by directly introducing a lipidated dipeptide intermediate into the growing peptide chain or by using an orthogonally protected lysine. In the first case, to obtain a lipidated building block, orthogonally protected lysine is chosen as the starting material in order to selectively form a peptide bond between the ε-amino group of lysine and the Y-carboxylic group of glutamic acid [24]. For example, patents US11066439B2 [22] and WO2013/037266 [45] describe the solid-phase synthesis of liraglutide using lysine containing in its structure an allyloxycarbonyl protecting group (Alloc), the removal of which requires a metal catalyst - tetrakis(triphenylphosphine) palladium $Pd(PPh_3)_4$ [22, 43]. However, this method cannot be used for a large-scale production due to technological difficulties and high costs. In addition, the proposed $Pd(PPh_3)_4$ catalyst is sensitive to moisture, therefore, the reaction must be carried out under strictly controlled conditions, and when obtaining the final product, the content of heavy metals must be taken into account [22].

The use of copper (II) lysinate can greatly simplify the preparation of the palmitoylated intermediate, since copper complexes of trifunctional amino acids such as Lys, Asp or Glu can provide a temporary protection to selectively introduce protecting groups into the side chain. This method is commercially viable as it is widely used in the production of protected amino acids as raw materials for industrial peptide synthesis. Moreover, the procedures for storing and disposing of copper-containing waste are well known and do not pose any particular problems due to the low toxicity of copper salts [24].

In the drug Enligria®, the active substance liraglutide is obtained as a result of chemical synthesis. This method has a number of advantages in the production of peptides, ensuring the stable production of a clearly defined peptide structure, which is associated with a predictable and controllable effect and the exclusion of foreign impurities of producers (proteins and biomolecules) from entering the finished form, which ensures a high degree of purity and eliminates the risks of changes in the properties of the resulting substance; it also reduces the risk of AEs and immunogenicity [22, 44, 46, 47].

Key benefits of liraglutide

As stated earlier, liraglutide is a human GLP-1 analogue that stimulates glucose-dependent insulin secretion. However, the mechanism of a liraglutide action has a number of differences from the effects of native GLP-1. As a result of the experimental work on the animals, it was found that the drug has a predominantly central effect, exerting an effect in the arcuate nuclei of the hypothalamus. When administered peripherally, liraglutide, by binding to GLP-1 receptors, activates a pool of anorexigenic pro-opiomelanocortini and cocaine-amphetamine-regulated transcript, or otherwise POMC/CART-producing neurons. At the same time, a decrease in the orexigenic neurons activity producing neuropeptide Y (NPY) and agouti-like protein (AgLP) occurs indirectly, by inhibiting the GABA production [48, 49]. According to the studies conducted in humans, the administration of liraglutide statistically significantly increased the feeling of fullness after eating, and decreased the severity of hunger compared to placebo. At the same time, a slowdown in gastric emptying was observed only during the first hour after the drug administration; after 5 h, the difference compared to placebo, was not traced [50].

The studies have shown that liraglutide (1.8 mg), without taking into consideration other GLP-1 analogues, is one of the priority drugs in patients with T2DM and with indications of a high risk of CVD or existing CVD, CHF, CKD. Liraglutide (3 mg) was the only drug from the GLP-1 group with the indication (in Russia) of “the correction of body weight in adults and adolescents with obesity or overweight in the presence of concomitant diseases”[23]. In the Russian Federation, foreign-made liraglutide drugs have recently become commercially unavailable due to supply restrictions from the manufacturing company.

In 2023, liraglutide was included in the list of drugs that are in defect or for which there is a risk of its occurrence[24]. The company Promomed RUS LLC has developed the drug Enligria® based on liraglutide. The phase I comparative clinical study was conducted in accordance with the current legislation of the Russian Federation and the EAEU.

On September 14, 2023, the state registration of the first domestic liraglutide drug Enligria® (LP 008822) was

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carried out, which returns the opportunity to personalize therapy for patients with obesity and overweight.

Despite some limitations of the study, in particular, the participation of only healthy male volunteers, the study was conducted in accordance with GCP standards and Russian and international recommendations developed for this group of drugs.

During the administration of the drug Enligria®, no AEs were identified in the study participants, and a good tolerability was noted. There were also no SAEs identified. Separately, it is worth noting that none of the participants had antibodies to liraglutide (Enligria®). In obese or overweight patients with at least one comorbid condition receiving the reference drug liraglutide 3.0 mg, the most commonly reported side effects were mild to moderate gastrointestinal disturbances. In this group, 2.5% of patients developed antibodies to liraglutide, which did not lead to a decrease in the effectiveness of the drug[25] [8, 9–12]. The main factors influencing the likelihood of an immune response[26] include the manufacturing process, formulation, and stability characteristics of the drug[27] [51]. The reference drug of liraglutide uses a biotechnological method of recombinant DNA in *Saccharomyces cerevisiae*[28]. With this production method, there is a potential danger of inducing an immune reaction due to the structural transformation of the active substance protein and the presence of impurities, for example, fragments of producer cells or reaction products with excipients. Adverse reactions can range from clinically insignificant, for example, the development of antibodies that do not affect the effectiveness of therapy and the severity of side effects, to serious adverse reactions when antibodies neutralize the protein of the active substance up to a complete loss of a biological activity[29] [51, 52]. The chemical synthesis-based technology used for the production of Russiam liraglutide eliminates the presence of impurities from the producer cells and, therefore, provides a high safety profile and a reduction in the above-described risks of developing immunogenicity[30,31] [51–53].

CONCLUSION

As a result of the studies, a sufficient amount of data was collected confirming the similarity of the physicochemical and biological properties of the drug with the chemically synthesized active substance liraglutide Enligria® (solution for the subcutaneous administration, 6 mg/ml, Promomed RUS LLC, Russia) with the reference drug Saxenda® (solution for the subcutaneous administration6 6 mg/ml, Novo Nordisk AS, Denmark). Based on this, it can be concluded that the quality, safety and effectiveness of the drug with a synthetic analogue of the active substance are similar to the reference drug, and in a number of parameters they even surpass them.

The reduction of medical and social damage caused by the increasing prevalence of obesity is one of the priority areas for the development of the Russian healthcare system. The inclusion of modern, high-quality, effective and safe drugs in treatment regimens for this disease is of particular importance. The entry of Russian GLP-1 receptor agonist drugs onto the market will allow patients to receive therapy that meets modern requirements. In an open, randomized, crossover comparative study assessing pharmacokinetic parameters, safety and tolerability in healthy volunteers, the equivalence of the study drug Enligria® and the reference drug Saxenda® was confirmed, and a high safety profile, tolerability of the study drug and lack of immunogenicity were demonstrated. Based on the data obtained, the drug Enligria® was registered in the Russian Federation.

The use of a chemical synthesis method in the production of the drug determines the identity of the active substance to the original product and a low risk of adverse immune reactions. It is advisable to conduct further clinical studies to assess the effectiveness and safety of therapy in patients with obesity and overweight, as well as to identify potential new possibilities for therapy with GLP-1 agonists, including the Russian analogue of liraglutide.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

Alexander S. Ametov – development of a clinical trial concept, analysis and description of results, text correction; Igor E. Shokhin – organization and conduct of physical and chemical research, interpretation of results; Ekaterina A. Rogozhina – organization and conduct of physicochemical and biological properties research, discussion of the design and results of the study; Tatyana G. Bodrova – analysis and selection of literary sources, writing the text of the article, organizing and conducting physical and chemical studies, interpreting the results; Maria E. Nevretdinova – analysis and selection of literary sources, interpretation of results, writing the text of the article; Petr A. Bely – implementation of the research design, processing of research data; Kira Ya. Zaslavskaya – development of the design and concept of the study, writing the text of the article; Denis V. Kurkin – analysis and description of results; Ksenia N. Koryanova – analysis and description of results, search and analysis of literary sources; Ekaterina S. Mishchenko – analysis and description of the results; Sergey M. Noskov – development of the design and concept of a clinical trial.
All authors made a substantial contribution to the conception of the work, acquisition, analysis, interpretation of data for the work, drafting and revising the work, final approval of the version to be published and agree to be accountable for all aspects of the work.

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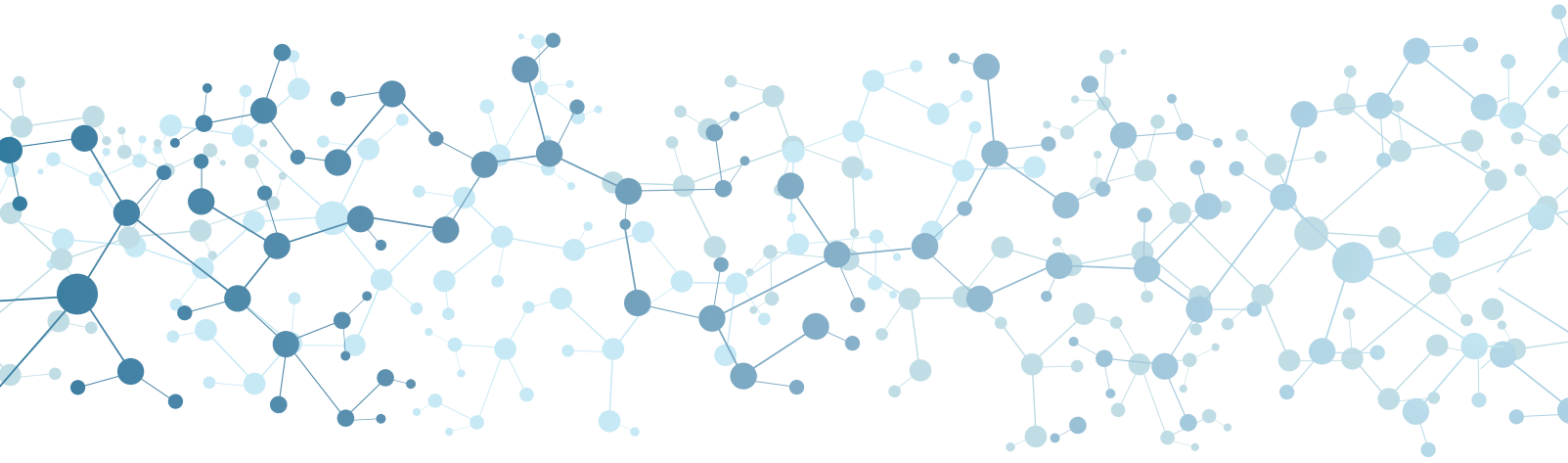


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