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REVIEW

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GENETIC PREDICTORS OF SEVERITY AND EFFICACY OF COVID-19 PHARMACOTHERAPY

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The pandemic of the novel coronavirus infection 2019 (COVID-19) has changed many aspects of our lives and initiated numerous studies aimed at finding the factors that determine different courses of this infectious disease. The studies aimed at finding predictors of the severe course of this novel coronavirus infection, as well as the factors that determine the efficacy and safety of this disease pharmacotherapy, are acquiring special social significance.

The aim of this work is to find and summarize information on genetic predictors of severe COVID-19, as well as pharmacogenetic aspects that determine the variability of the therapeutic response to the drugs recommended for COVID-19 treatment.

Materials and methods. The article provides a review of scientific results on the research of gene polymorphism that determine a body's response to the introduction of SARS-CoV-2 infection and the effects of pharmacotherapy for this disease, obtained from open and available sources within the period of 2019 – March 2021. The search was conducted in the following electronic databases: PubMed, Cochrane Library, ClinicalTrials.gov; Elibrary, Scopus. The main search inquiries were: "predictors + severe course + COVID-19", "genetic variations + COVID-19", "pharmacogenetics + COVID-19", "gene polymorphism + SARS-CoV-2", "pharmacotherapy + gene polymorphism + COVID-19" in both Russian and English.

Results and conclusion. The exploratory research detailing the mechanisms of infecting with SARS-CoV-2, the variability of the disease severity and the individual characteristics of therapeutic responses to the drugs used, are being actively carried out by scientists all over the world. However, most of their scientific projects are diverse, and the possible predictors of a severe course of COVID-19 found in them, have not been confirmed or investigated in subsequent studies. A generalization of the individual studies results of the genetic predictors concerning COVID-19 severity and effectiveness of its pharmacotherapy, can become the basis for further search and increase the reliability of the data obtained in order to develop a strategy for preventing the spread of COVID-19 infection, to identify potential targets of the treatment, and develop the protocols for optimizing this disease pharmacotherapy.

Keywords: COVID-19; pharmacotherapy; gene polymorphism; pharmacogenetics; SARS-CoV-2; predictors

Abbreviations: COVID-19 – coronavirus disease-2019; SARS-CoV-2 – severe acute respiratory syndrome–associated coronavirus disease, COVID-19 etiology; WHO – World Health Organization; GWAS – Genome-Wide Association Studies / полногеномный поиск ассоциаций; ACE – angiotension-converting enzyme; AGTR1/2 Angiotensin II receptor Type 1 and Type 2; OR – odd ratio; TMPRSS2 – Transmembrane protease, serine 2; T NF-κB – Transcriptional nuclear factor-kB; DPP4 – Dipeptidyl-peptidase 4; MERS-CoV – coronavirus, Middle East respiratory syndrome etiology; TLR – Toll-like receptor; RNA – ribonucleic acid; IRF – Interferon Regulatory Factor; INF – interferon; IL – Interleukin; HLA – Human Leukocyte Antigens; HIV – human immunodeficiency virus; TNF – Tumor necrosis factor; TGF – Transforming growth factor; CYP – Cytochrome P450; GCSs – glucocorticosteroids; ARDS – acute respiratory distress syndrome; ATP – adenosine triphosphate; CI – confidence interval; RR – relative risk; Ig – immunoglobulin

ГЕНЕТИЧЕСКИЕ ПРЕДИКТОРЫ ТЯЖЕСТИ ТЕЧЕНИЯ И ЭФФЕКТИВНОСТИ ФАРМАКОТЕРАПИИ COVID-19

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Пандемия новой коронавирусной инфекции COVID-19 изменила многие аспекты нашей жизни и инициировала многочисленные исследования, направленные на поиск факторов, определяющих различное течение этого инфекционного заболевания. Особую социальную значимость приобретают исследования, направленные на поиск предикторов тяжелого течения новой коронавирусной инфекции, а также факторов, определяющих эффективность и безопасность фармакотерапии этого заболевания.

Цель. Целью настоящей работы является поиск и обобщение информации о генетических предикторах тяжелого течения COVID-19, а также фармакогенетических аспектах, определяющих вариабельность терапевтического ответа на рекомендованные лекарственные препараты для лечения COVID-19.

Материалы и методы. В статье представлен обзор результатов научных исследований по изучению полиморфизма генов, определяющих ответ организма на внедрение SARS-CoV-2 инфекции и эффектов фармакотерапии данного заболевания, полученных из открытых и доступных источников за период 2019- март 2021 гг. Поиск проводился в электронных базах данных: PubMed, Cochrane Library, ClinicalTrials.gov; Elibrary, Scopus. Основные поисковые запросы: «предикторы + тяжелое течение + COVID-19», «генетические вариации+COVID-19», «фармакогенетика+COVID-19», «полиморфизм генов + SARS-CoV-2», «фармакотерапия+полиморфизм генов + COVID-19». Поисковые запросы выполнялись на русском и английском языках.

Результаты и заключение. Поисковые научные исследования, детализирующие механизмы заражения SARS-CoV-2, вариабельность тяжести течения заболевания и индивидуальные особенности терапевтического ответа на применяемые препараты, активно проводятся учеными разных стран мира. Однако большинство их научных проектов являются разнонаправленными, а найденные в них возможные предикторы тяжелого течения COVID-19 не подтверждены или не изучены в последующих исследованиях. Генетически обусловленная гетерогенность иммунного ответа организма на SARS-CoV-2 инфекцию требует дальнейшего изучения, что во многом связано с отсутствием однозначного мнения о ведущем механизме, определяющем тяжесть этого заболевания. Обобщение результатов отдельных исследований генетических предикторов тяжести течения и эффективности фармакотерапии COVID-19 может стать основой для дальнейшего поиска и повышения достоверности полученных данных с целью разработки стратегии предупреждения распространения инфекции COVID-19, определения потенциальных мишеней таргетной терапии, а также разработки протоколов оптимизации фармакотерапии этого заболевания.

Ключевые слова: COVID-19; фармакотерапия; полиморфизм генов; фармакогенетика; SARS-CoV-2; предикторы

Список сокращений: COVID-19 – коронавирусная инфекция 2019 года; SARS-CoV-2 – коронавирус, этиология COVID-19; ВОЗ – Всемирная организация здравоохранения; GWAS – Genome-Wide Association Studies / полногеномный поиск ассоциаций; АПФ – ангиотензин-превращающий фермент; AGTR1/2 Angiotensin II receptor type 1 and type 2 / Рецептор ангиотензина II типа 1 и 2; ОШ – отношение шансов; TMPRSS2 – Transmembrane Serine Protease 2 / трансмембранная сериновая протеаза типа 2; NF-κB – Nuclear factor-κB / транскрипционный ядерный фактор κB; DPP4 – Dipeptidyl-peptidase 4 / дипептидилпептидаза 4; MERS-CoV – короновирус, этиология ближневосточного респираторного синдрома; TLR – Toll-подобные рецепторы; РНК – рибонуклеиновая кислота; IRF – Interferon Regulatory Factor / регуляторный фактор интерферона; ИНФ – интерферон; IL – Interleukin / интерлейкин; HLA – Human Leukocyte Antigens / главный комплекс гистосовместимости; ВИЧ – вирус иммунодефицита человека; TNF – Tumor necrosis factor / фактор некроза опухоли; TGF – Transforming growth factor / трансформирующий фактор роста; CYP – cytochrome P450 / ферменты цитохрома; ГКС – глюкокортикостероиды; ОРДС – острый респираторный дистресс-синдром; АТФ – аденозинтрифосфат; ДИ – доверительный интервал; OR – относительный риск; Ig – иммуноглобулины

INTRODUCTION

The pandemic of the novel coronavirus infection COVID-19 has changed many aspects of our lives and initiated numerous studies aimed at finding the factors that determine different courses of this infectious disease. It is known that more than 40% of people convey SARS-CoV-2 asymptomatically; in addition, a part of the population has a natural resistance to it and does not manifest the disease even with a high viral load [1]. The other pole of the individual reactivity is patients with a severe course of infection who are hospitalized in the intensive care unit with symptoms of acute respiratory distress syndrome and a multiple organ failure. In this group, the mortality rate is over 40% [2]. According to WHO dated March 31, 2021, 2,769,696 confirmed deaths from COVID-19 for 126,372,442 cases were registered in the world[1]. A lot of modern studies are aimed at finding predictors of the severe course of this novel coronavirus infection. Some of them are devoted to clinical factors: for example, a male gender, an old age and comorbid backgrounds are more often associated with a severer course of the disease [3, 4]. Other studies are looking for individual genetic variations that determine differences in the immune response to the SARS-CoV-2 infection and can explain the clinical course of COVID-19, as well as differences in morbidity and mortality from the SARS-CoV-2 infection in different countries [5, 6].

The correlation between the COVID-19 severity and certain allelic genes variants responsible for the immune response, is very important since it can be used to identify a population with a predisposition to a severer course of infection and to determine a vaccine prevention strategy. This data can be used to develop targeted therapeutic approaches, as well as to select specialists to work with COVID-19 patients in case of a high probability of mild and asymptomatic courses.

[1] WHO report on the current situation with COVID-19 in the world (as of 31 March 2021) Available from: https:// www.who.int/publications/m/item/weekly-epidemiological-update-on-covid-19---31-march-2021

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THE AIM of this work is to find and summarize information on genetic predictors of severe COVID-19, as well as pharmacogenetic aspects that determine the variability of the therapeutic response to the drugs recommended for COVID-19 treatment.

MATERIALS AND METHODS

The article provides a review of scientific results on the research of gene polymorphism that determine a body's response to the introduction of SARS-CoV-2 infection and the effects of pharmacotherapy for this disease, obtained from open and available sources within the period of 2019 – March 2021. The search was conducted in the following electronic databases: PubMed, Cochrane Library, ClinicalTrials.gov; Elibrary, Scopus. The main search inquiries were: "predictors + severe course + COVID-19", "genetic variations + COVID-19", "pharmacogenetics + COVID-19", "gene polymorphism + SARS-CoV-2", "pharmacotherapy + gene polymorphism + COVID-19" in both Russian and English.

RESULTS AND DISCUSSION

Genetic characteristics of human body response to establishment of SARS-CoV-2 infection

The genome-wide association search (GWAS) study where 8,582,968 single nucleotide polymorphisms were analyzed in 1,980 patients with severe COVID-19 in Italy and Spain has been widely publicized [5]. The control group included 2,205 healthy volunteers. According to the results of the study, no definite correlations were found between severe COVID-19 and the development of a respiratory failure and a single gene polymorphism. However, the determinants of a severe course associated with several genes have been identified. They are located in the fragment of chromosome 3 – locus 3p21.31, including the genes SLC6A20, LZTFL1, CCR9, FYCO1, CXCR6 and XCR1. Among these genes, the following ones are distinguished as the most significant in the pathogenesis of the COVID-19 development: LZTFL1 (expressed in the lungs, it determines the production of the protein that regulates a ciliary function); SLC6A20 (the gene encoding the synthesis of the corresponding transporter protein involved in the transmembrane transport of sodium and chlorine ions, it also presumably affects the interaction between SARS-CoV-2 with ACE2 receptors); CCR9 (the gene encoding the synthesis of the membrane protein of the same name, is a part of G-protein-mediated receptors, and it is also a receptor for chemokines that control the migration of effector cells to the inflammation focus); CXCR6 (expressed in the lymphoid tissue and on activated T-lymphocytes, regulating their activity; including the effect on the immune response during inhalation of viral pathogens). The presence of the GA allele in the single nucleotide sequence rs11385942 was associated with a decrease in the CXCR6 expression and an increase in the SLC6A20 expression. According to the results of the meta-analysis, the occurrence frequency of the risk allele was approximately 1.5 times higher in the group of the hospitalized with a respiratory failure receiving a respiratory support compared with the group receiving only an oxygen inhalation (OR 1.77, 95% confidence interval 1, 48–2.11; $P=3.30\times10^{-4}$). Another result of this study was the establishment of the correlation between the severe course of the disease and the 9q34.2 locus, which, in this cohort of patients, determines a blood group according to the ABO system. A meta-analysis showed that the group of patients with an A blood type was 1.5 times more likely to have a severe course compared with the other blood types (OR 1.45; 95% CI, 1.20–1.75; $P=1.48\times10^{-4}$), and a protective effect was also found in the carriers of the 0 blood type (OR 0.65; 95% CI, 0.53–0.79; $P = 1.06\times10^{-5}$). More reliable data could be obtained if the control group included people with an asymptomatic form and a mild course of SARS-CoV-2 infection, and not healthy people who were not infected with this virus.

Angiotensin-converting enzyme and transmembrane serine protease

Another direction in the search for genetically determined predictors of severe COVID-19 is to study the interaction of SARS-CoV-2 with host cell proteins during the virus introduction into the human body, which may explain the differences in viral loads. The penetration of SARS-CoV-2 into the cell is realized with the help of surface S-proteins, which interact with type 2 angiotensin-converting enzyme (ACE 2) in the place with a protease activity. The virus infects epithelial cells of the respiratory and gastrointestinal tracts and a number of other organs. There is a high expression of ACE 2 by type II alveolar pneumocytes, which determines the tropism of SARS-CoV-2 to the lung tissue. To activate the viral S-protein, the TMPRSS2 enzyme is required. It facilitates the penetration of the virus into the host cell [7]. TMPRSS2 can be considered a potential therapeutic target in the COVID-19 treatment.

TMPRSS2 inhibitors currently used in Japan and approved for the treatment of several forms of prostate cancer and pancreatitis are potential candidates for the treatment of SARS-CoV infection [8]. The TMPRSS2 gene is localized on chromosome 21q22.3; its expression level is subject to genetic polymorphism, which can determine susceptibility, a viral load, and risks of severe lung damage in the SARS-CoV-2 infection [9]. Single nucleotide polymorphisms TMPRSS2 rs383510 and rs464397 showed the highest expression in the lungs of

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patients with homozygous TT genotype, rs2070788 – GG genotype and rs469390 with AA genotype. Accordingly, rs383510, rs464397 heterozygous CT genotype and rs469390 with AG genotype showed an intermediate level of TMPRSS2 expression, and homozygous CC genotype (rs383510, rs464397), GG (rs469390) and AG and AA genotypes (rs2070788) had the lowest expression. The prevalence of the TMPRSS2 genetic polymorphism differs in the population. For example, the population of East Asia has a lower frequency of genotypes with a high expression compared to the American and European communities [10].

After a high affinity connection of the virus with ACE 2, the fusion with the host cell occurs, the penetration into it and the multiplication of the virus. The synthesis of ACE 2 is associated with a polymorphism in the gene encoding this protein. A point mutation in the ACE 2 gene (Leu584Ala) increases the penetrating ability of SARSCoV-2. It is interesting to report that several amino acid sequences fundamentally alter the interaction between the viral S1 protein and the ACE 2 receptor, changing the viral load. A total of 13 polymorphisms (rs1434130600, RS1395878099, RS142984500, RS756231991, RS1244687367, RS73635825, RS778500138, RS867318181, RS763395248, rs4646116, rs778030746, rs1199100713 and rs781255386) determine the rapid and effective interaction of ACE 2/S1, which contributes to the development of the infection. In contrast, the other 18 SNPs (rs143936283, rs961360700, rs1569243690, RS751572714, RS1348114695, RS1263424292, RS766996587, RS760159085, RS1016409802, RS146676783, RS1352194082, rs755691167, rs1325542104, rs759579097, rs762890235, rs1192tnqh_9; 192618, rs370610075 and rs1256007252) impede the interaction between ACE 2 and S1, thereby reducing the level of infecting [11]. At the same time, a high activity of ACE 2 has a protective effect on the pulmonary function. The SARS-CoV-2 infection likely decreases the regulatory function of ACE 2. A decrease in the activity of ACE 2 triggered by the virus, and an increase in the level of angiotensin II because of it, leads to the synthesis of proinflammatory cytokines and chemokines through the interaction with the AGTR1 and AGTR2 receptors with a subsequent activation of NF-κB. This contributes to damaging the alveolocytes and endothelial cells, the development of interstitial edema and infiltration of the lung tissue [11].

Another gene that can potentially influence the COVID-19 severity, is AGTR2 which encodes Angiotensin II receptor Type2 (AGTR2). Thus, it can be assumed that it is binding of SARSCoV-2 with AGTR2 directly and / or indirectly through the ACE 2 receptor, that leads to the imbalance of the renin-angiotensin system, the excessive accumulation of angiotensin II and, as a consequence, to severer forms of the disease [12].

Dipeptidyl peptidase 4

Dipeptidyl peptidase 4 is an intramembrane glycoprotein and serine exopeptidase. This enzyme is involved in the degradation of a wide range of substrates, including chemokines, neuropeptides, and incretins (e.g., glucagon-like peptide-1). DPP4 is a surface antigen also known as CD26. DPP. It is expressed in many organs and tissues, including the lungs, intestines, placenta, kidneys, and immune cells. Previously, it was found that DPP4 plays a role in the priming of glycoprotein S at the moment of MERS-CoV penetration into host cells [13]; its role in the penetration of SARS-CoV-2 is currently being considered [14]. A single nucleotide polymorphism of the DPP4 gene (rs13015258 – C allele) was found, which is associated with a very high expression and increased mortality among COVID-19 patients with type 2 diabetes mellitus [9].

Toll-like receptors

One of the most important functions of innate immunity is the recognition of microbial components by cells. It determines the launch of the first line of human body defense against the pathogens invasion. TLRs play a key role in the recognition and activation of the immune response. In humans, 10 subtypes of these receptors have been identified, each of which is responsible for the identification of various structural components of microbes. The SARS-CoV-2 virus enters the cells, binds to the endosomal TLRs of types 3 and 7, and cytoplasmic RNA receptors. These structures play an essential role in the recognition of viral RNA and the initiation of interferon genesis as one of the main components of innate immunity and antiviral defense. The cascade of reactions occurs due to the activation of the NF-κB and IRF pathways. In the literature, there are limited data on a low expression of the X-chromosome gene encoding TLR7 synthesis, and, accordingly, a reduced activity of interferons (types I and II) which was accompanied by the development of severe COVID-19 in young men [15]. In addition to finding a genetic link that could open up all sorts of new opportunities for exploring potential treatments, this study can also explain the observed trend towards higher death rate from COVID-19 in men than in women. A number of genes and regulatory elements associated with the innate and adaptive immune response have been found in the X chromosome [16].

Interferon status

The synthesis of endogenous interferon is a universal evolutionarily fixed defense mechanism against

viral infection. Delayed stimulation of the expression of genes responsible for interferon genesis and antiviral response is associated with the severity of clinical manifestations of infectious diseases. In deceased patients with MERS-CoV infection, the level of the endogenous interferon synthesis was significantly lower than in survivors [17]. The activation of this pathway is associated with the induction of the expression of several hundred genes that affect the suppression of viral replication. Gene variations that determine the low functional activity of the type 1 interferon response are characterized by the development of immunodeficiency states and the life-threatening course of viral infections.

A number of works have demonstrated the relationship of severe COVID-19 with a low activity of this pathway. Having analyzed the genome of 659 patients with severe SARS-CoV-2 infection, Zang [18] identified 13 candidate genes responsible for the implementation of the type 1 interferon response. In 23 patients out of 659 (3.5%), mutations realized by reduced activation of the interferon pathway and characterized by a more severe course of the disease, had been found. These patients had a high viral load. Previously, it was shown that the single nucleotide polymorphism rs12252C / C in the IFITM3 gene (encodes the interferon-induced transmembrane protein 3) is a risk factor for severe influenza [19]; this polymorphism, rs12252C / C, was also found in a patient with severe COVID-19 [20].

Another possible cause for severe SARS-CoV-2 infection can be the production of neutralizing autoantibodies [21]. Autoantibodies aimed at blocking regulatory proteins, in particular INF-α and INF-ω, were found in 101 out of 987 patients (10.2%; 94% of which are mostly men over 65 years old) with life-threatening conditions in the current pandemic. The production of neutralizing autoantibodies correlated with low plasma concentrations of INF-α. In addition, autoantibodies to type 1 INF proteins were not detected in 663 patients with asymptomatic or mild COVID-19, and in the population of healthy individuals not infected with SARS-CoV-2, autoantibodies were detected in 0.33% of cases (4 / 1227 people). The production of autoantibodies to type 2 INF, IL-6 and IL-17 has been detected in healthy people, patients with autoimmune diseases and opportunistic infections, but their role in determining the severity of diseases is not yet fully understood [22]. The literature provides rare cases of hereditary conditions with an autoimmune mechanism or immunodeficiency, which were accompanied by overproduction or deficiency of type 1 interferon response proteins, respectively. However, some cases have been notified that in conventionally healthy people with a low expression of the genes that determine this response, a clinically asymptomatic carrier state of these genomic variations is possible until the moment of contact with certain viruses. This may explain the cause for the severe SARS-CoV-2 in the patients without a history of immunodeficiency in anamnesis [15]. The determination of autoantibodies to type 1-interferon response proteins can be useful in determining a therapeutic strategy for patient management. In the presence of autoantibodies, recombinant INF-β preparations will not be effective, and these patients should not be considered as plasma donors. If this is a variant with a low expression of genes responsible for type 1-interferon response, then, on the contrary, therapy with recombinant interferon preparations is advisable.

HLA system

The genes of the HLA (Human Leukocyte Antigen) major histocompatibility complex system encode molecules of the same name on the cell surface. These protein structures carry out the presentation of various antigens, including causative agents of viral infections, and determine the severity of many diseases. This is the most polymorphic human genetic system (more than 9000 alleles), and it is located on the short arm of chromosome 6 [23]. Considering the role of the HLA system in the formation of the immune response, polymorphism of the genes of the main histocompatibility complex can determine the predisposition and variants of the course for infectious diseases. Thus, it is known that a severer course of H1N1 influenza is associated with the genotypes HLA-A*11, HLA-B*35 and HLA-DRB1*10; and with HIV-1, carriers of HLA-A* 02: 05 have a reduced risk of seroconversion [24]. There is a theory that the HLA gene polymorphism was formed during epidemics of infectious diseases, with the selection of alleles with a different peptide-binding ability. Moreover, heterozygotes with different HLA molecules are more adapted to the formation of an immune response as compared to homozygotes [25]. In 2003, during the SARS-CoV epidemic, a correlation was shown between the HLA gene polymorphism and a severer course of infection in carriers of HLA-B * 46:01. Based on these data, an *in silico* analysis of the 145 HLA genotypes affinity for the protein structures of SARS-CoV-2 was performed [26]. It has been shown that the HLA-B*46:01 genotype has the lowest binding capacity for SARS-CoV-2 proteins, which may be a predictor of a severer course of this disease. A similar response was predicted for genotypes HLA-A*25:01 and HLA-C*01:02. In contrast, the genotypes HLA-B*15:03, HLA-A*02:02 and HLA-C*12:03 showed a high activity in the presentation of SARS-CoV-2 antigens, which suggests good protective immunity.

In another study, Tomita Y. et al. performed an *in silico* analysis based on the prevalence of HLA gene

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polymorphisms and associations of the most frequent alleles in the countries with high mortality rates from COVID-19 [27]. The authors found possible the associations between the HLA-A*02:01 genotype, which determines a relatively low binding capacity for SARS-CoV-2 antigens, compared to individuals with the HLA-A*11:01 or HLA-A*24:01 genotype developing a more efficient T-cell mediated antiviral response to infection. The most common HLA genotypes in humans around the world have been studied and it has been shown that the variants HLA-A*02:01, HLA-C*07:01, HLA-DPB1*04:01, HLA-DQPB1*03:01 are found in more than half of the world's population. Then 19 countries were selected and divided into two groups: the first – where these allelic variants are most often found, the second – with a low frequency of these genotypes. Then, a correlation analysis was carried out between the frequency of these genotypes prevalence and the total number of confirmed cases and deaths from COVID-19 per 1 million population. A carrier state of HLA-C, HLA-DPB1, HLA-DQPB1 genotypes did not show fundamental differences between the countries in terms of the analyzed indicators. At the same time, in the countries where the HLA-A*02:01 genotype was more common, there was a statistically significant higher incidence of COVID-19 (1842 cases / 1 million population dated April 24, 2020, and 5795/1 million population dated August 15, 2020) compared with the countries where the genotypes HLA-A*24:02 and HLA-A*11:01 prevailed (97 cases / 1 million population in April 2020, and 419 cases / 1 million population in August 2020), as well as the mortality – 98 vs. 2.5 cases in April 2020 and 488 vs. 6.1 cases in August 2020, respectively. To determine the risk group, the authors of the two presented studies propose to conduct HLA typing and COVID-19 testing, as well as to vaccinate primarily high-risk individuals in accordance with genetic research data simultaneously.

Cytokine status

Cytokines are low-molecular-weight proteins that are signaling molecules and through specific receptors carry out cooperation between different cells and systems under normal conditions, as well as in the event of pathological processes. Cytokines are key mediators of the inflammatory response and are important for protecting humans from a wide range of viruses, participating in the regulation of both the innate immune system and inflammatory processes. Individual cytokine levels are highly variable, and genetic factors contribute significantly to the personal profile. Numerous studies have shown that the polymorphisms in genes encoding cytokines can affect their transcriptional activity, and, accordingly, the level of production [28]. In some cases, when exposed to infectious pathogens, autoimmune mechanisms and neoplastic syndrome, hypercytokinemia, i.e. uncontrolled release of inflammatory mediators, which is accompanied by an immune dysfunction, a systemic inflammatory process with damage to its own tissues and the development of a multiple organ failure, is possible [29]. Among the possible stimuli for the initiation of a cytokine storm, the role of coronaviruses, and in particular SARS-CoV-2, has been established. In the COVID-19 patients and a cytokine storm signs, changes in the production of many cytokines have been established: IL-1, IL-2, IL-4, IL-6, IL-8, IL-10, IFN-γ, TNF-α and TGF-α β1. Among them, the most typical overproduction is of IL-6, IL-1β, IL-10 and TNFα. The role of genes polymorphism encoding a cytokine production had been proven for many infectious diseases, including malaria, influenza, meningococcal infection and sepsis in previous studies [30,31]. Considering that, variants of proinflammatory cytokine genes were also studied in SARS-CoV- 2 infections. However, none of the previously identified polymorphisms of proinflammatory cytokine genes associated with more severe diseases, have been replicated in COVID-19 studies. Thus, in a published case-control study in SARS-CoV patients, no relationship was found out between the course of the disease and the TNF-α gene polymorphism [32], and no correlation was found out between the COVID-19 severity and the genetic variants of this gene in 900 SARS-CoV-2 patients in a modern study. [33].

IL-6 is one of the pro-inflammatory cytokines, the level of which increases dramatically in COVID-19 patients [34]. In addition, its level is considered a severity predictor of this disease. Higher levels of circulating IL-6 are observed in patients with respiratory dysfunction, suggesting that SARS-CoV-2 triggers a cytokine-mediated mechanism of the lung injury; these patients were significantly more likely to have indications for respiratory support [35]. Genetic variations that determine the IL-6 production, are considered potential determinants of the host cell's response to the SARS-CoV-2 invasion [36]. The previous studies have shown that mutations in the IL-6 gene (rs1800797 and rs1800795) are associated with the progression of cardiovascular pathology [37]; and combined polymorphisms of the IL-6 (rs1800797), IL-10 (rs1800872) and C-reactive protein genes (rs1205) correlated with the severity and prognosis in the patients with community-acquired pneumonia [38]. However, among the data available for the analysis of the role of genetic predisposition to the synthesis of IL-6 in COVID-19, only one study showed that the carrier status of the IL-6 -174C allele is associated with a higher level of the IL-6 production and severer forms of pneumonia in general. This result does not reflect a direct relationship between the disease severity and the gene polymor-

phism, but confirms that IL-6 plays a key role in the progression of the novel coronavirus pneumonia [39]. Thus, more detailed information on the relationship between the polymorphism of cytokine genes that are important in SARS-CoV-2 infection, and the COVID-19 severity, requires further scientific research.

Pharmacogenetic markers of efficacy and safety of COVID-19 therapy

Genetic variations in COVID-19 patients can affect not only the nature of the infection course and the severity of clinical manifestations, but also determine the individual response to the pharmacological drugs used. This review provides data on the drugs recommended for the COVID-19 treatment and possible changes in the efficacy and safety of the therapy associated with patient gene polymorphisms. Although there are currently no data from pharmacogenetic studies in COVID-19patients, there are plausible mechanisms by which important genetic determinants can be anticipated.

Hydroxychloroquine

Hydroxychloroquine is an antimalarial 4-aminoquinoline derivative. Due to its anti-inflammatory and immunosuppressive action and in addition to the treatment and prevention of malaria, it is included in the clinical guidelines for pharmacotherapy of rheumatoid arthritis and systemic lupus erythematosus. Hydroxychloroquine is one of the first etiotropic drugs included in COVID-19 treatment protocols. The mechanism of the hydroxychloroquine antiviral action in COVID-19 is not yet clear. Presumably, the drug prevents the penetration of the virus into the cell, disrupting the processes of endocytosis. In addition, hydroxychloroquine can directly affect the interaction between SARS-CoV-2 and ACE 2 by reducing the glycosylation of ACE 2 [40]. The immunosuppressive effect is manifested by a decrease in the production of pro-inflammatory cytokines, which may also have a beneficial effect on the hyperimmune response in COVID-19. The efficacy and safety of hydroxychloroquine is related to the pharmacokinetics of the drug. The drug is metabolized in the liver by the CYP P450 system with the participation of the enzymes CYP2D6, CYP2C8, CYP1A1 and CYP3A4. The genetic polymorphisms of enzymes affect the metabolic rate and, accordingly, the pharmacological response. In the previous studies, alleles CYP2C8*2, CYP2C8*3, CYP2C8*4 reduced the activity and capacity of the enzymes *in vitro* compared to the wild-type CYP2C8*1A allele, which led to a delayed formation of active metabolites of the drug and a decrease in the therapeutic response [42]. CYP2D6 polymorphism (rs1135840 andrs1065852) induces the metabolism of hydroxychloroquine in a patient with systemic lupus erythematosus [41]. Single nucleotide polymorphisms of the gene encoding the synthesis of glucose-6-phosphate dehydrogenase (rs5030868, rs1050828, and rs1050829) are associated with a decrease in the enzyme activity and an increased risk of hemolysis [43].

Remdesivir

Remdesivir is an antiviral drug that is an adenosine nucleotide prodrug metabolized in the cells of the body to form an active metabolite of nucleoside triphosphate. Remdesivir triphosphate acts as an analogue of ATP and competes with the natural ATP substrate for incorporation into the forming RNA chains using the RNA-dependent RNA polymerase of the SARS-CoV-2 virus, which leads to a delayed chain termination during the viral RNA replication [44]. Remdesivir undergoes serial metabolism mediated by intracellular esterases and phosphoamidase, which leads to the formation of the main metabolite of remdesivir. Pharmacogenetic studies of remdesivir have not been published to date, but *in vitro* studies show that it is a substrate for the enzymes CYP2C8, CYP2D6, and CYP3A4, as well as a substrate for the transporters OATP1B1 and P-glycoprotein [45]. Thus, the known polymorphisms of these genes can theoretically influence the pharmacokinetics of remdesivir [46].

Favipiravir

Favipiravir was developed and approved in Japan, 2014, for the treatment and prevention of influenza and is currently being investigated for its effectiveness in COVID-19. There are no published studies on the pharmacogenetics of favipiravir. Possible ways of changing its efficacy may be associated with the competitive metabolism of the aldehyde oxidase pathway, which is the main pathway for its deactivation [47].

Interferon β-1b

The use of interferon preparations, in particular IFN-β1b, has manifested its efficacy in the treatment of SARS and / or MERS coronavirus infection, and is currently being studied in COVID-19 [48]. The changes in the efficacy and increased risk of the side effects of IFNβ-1b drugs associated with pharmacogenetic factors, have not been established. However, in the cohort of Swedish patients with multiple sclerosis who had received INF-β1b, the risk of developing biologically significant neutralizing antibodies was higher in the patients with the HLA-DRB1*04 allele (OR: 3.53, 95% CI: 1.64-7.61) and lower with HLA-DRB1 * 15 (OR: 0.33, 95% CI: 0.16–0.71) [49].

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Tocilizumab

Tocilizumab, an inhibitor of IL-6 receptors, is actively used as rheumatoid arthritis biological therapy. Considering the proven role of IL-6 overproduction in the pathogenesis of severe COVID-19, the use of drugs that block the effects of IL-6, is justified. Genetic biomarkers of the tocilizumab efficacy in rheumatoid arthritis, including variations FCGR3A, IL6R, CD69, GALNT1845–47, have been previously reported. 87 patients with rheumatoid arthritis treated with tocilizumab and FCGR3Ars396991TT genotype, showed a better response in 12 months (vs. GT; OR: 5.1; 95% CI: 1.2–21.3; p = 0.03). This variant can affect the affinity of the Fc-fragment of the IgG receptor for tocilizumab and alter its systemic clearance [50].

Now, there is limited evidence that pharmacogenomic biomarkers can help with determining the response to tocilizumab therapy in COVID-19, and the translation of these data into the course of COVID-19 is incorrect. There are no studies on the pharmacogenetics of tocilizumab in the patients with cytokine overproduction syndrome that could be close to the COVID-19 pathophysiology, either. Herewith, the identification of the response markers to tocilizumab in SARS-CoV-2 can make it possible to carry out individual targeted therapy and not to use immunosuppressants to treat a viral disease in a number of patients with predictors of a therapy failure. Another important aspect of the need for these studies is the economic factor.

Janus kinase inhibitors

Tofacitinib and baricitinib are other biologically active drugs that block the hypercytokine response and are approved for the use in COVID-19. Currently, no data on the pharmacogenetics of these drugs have been published. However, their pharmacokinetic parameters include several potentially important candidate genes. The both drugs are substrates for CYP3A4. Tofacitinib is also partially metabolized by CYP2C19. The both genes of metabolic enzymes are susceptible to genetic polymorphisms, which can alter the drugs activity [51].

Systemic glucocorticosteroids

Glucorticosteroids (GCS) are powerful nonspecific anti-inflammatory and immunosuppressive drugs. In the treatment of patients infected with COVID-19, they are used in the treatment of acute respiratory distress syndrome (ARDS). Pharmacogenetic predictors of the systemic corticosteroids effectiveness in ARDS have not been identified. However, possible variants of the pharmacological response may be associated with the receptor activity, as well as pharmacokinetic pathways through the activity of metabolic enzymes and transporter proteins [52]. GCS metabolism is carried out in the liver with the participation of isoenzymes CYP3A4 and CYP3A7, as well as in the lung tissue under the influence of CYP3A5 and CYP3A7 [53]. CYP3A4*22 polymorphism of the gene encoding CYP3A4 can change the activity of the isoenzyme and, accordingly, affect the therapy effectiveness. Thus, in previous studies, it was shown that in heterozygotes with the CYP3A4*22T(C/T) genotype, the effectiveness of glucocorticosteroid therapy was higher compared to the C/C genotype [54].

Another pharmacokinetic factor that can alter the efficacy and safety of glucocorticosteroid therapy is the expression of P-glycoprotein. Its level of activity is associated with genetic polymorphism of the multidrug resistance gene MDR1 (ABCB1), which can affect the therapeutic response [55]. The expression of glucocorticoid receptors is encoded by the NR3C1 gene. A number of scientific studies are devoted to the study of the polymorphism of this gene; about 40 gene variants are known that encode the synthesis of these receptors [56]. The individual variability may determine the efficacy and safety of glucocorticosteroid therapy [57]. In addition, the affinity of glucocorticoid receptors can be altered by proinflammatory cytokines, IL-1 and TNF-α [58], which can possibly be an effectiveness factor in the of COVID-19 therapy with these drugs.

CONCLUSION

Thanks to joint efforts and concerted actions in the struggle against the novel coronavirus infection, a global access to vaccines, modern diagnostics and effective medicines is ensured for all people who need them. However, the struggle against COVID-19 is going on. Scientific studies detailing the mechanisms of infection with SARS-CoV-2, the variability of the severity of the course of the disease and the individual characteristics of the therapeutic response to the drugs used, do not lose their relevance and have great social significance. The genetically determined heterogeneity of the immune response to SARS-CoV-2 requires further study, since there is no clear opinion about the leading mechanism that determines the severity of the disease. The results of studies on the search for genetic predictors of the disease severity and the effectiveness of COVID-19 pharmacotherapy, can form the basis for further search, as well as be used to develop strategies for preventing the infection, analyze potential targets of targeted therapy and develop protocols for optimizing this disease pharmacotherapy.

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Ivan N. Shishimorov – planning and editing the review; Olga V. Magnitskaya – material search and review editing; Yulia V. Ponomareva – material search and review writing.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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STANDARDIZATION PROBLEMS OF MEDICINAL PREPARATIONS FROM *RHODIOLA ROSEA* L.

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Rhodiola rosea L. rhizomes and roots are pharmacopoeial raw materials, which are used in official medicine for obtaining medicines with adaptogenic activity. One of the most common problems in the production of medicines from *Rhodiola rosea* L. rhizomes and roots is the use of poor quality medicinal plant materials, which leads to the absence of biologically significant compounds in the preparations. One of the possible reasons is the shortcomings in the existing approaches to the standardization of *Rhodiola rosea* L. raw materials and preparations.

The aim of the study is the improvement of approaches to the standardization of medicinal preparations from *Rhodiola rosea* L. rhizomes and roots.

Materials and methods. Experimental and industrial samples of liquid extract from *Rhodiola rosea* L. roots, as well as reference samples of rosavin and salidroside, were used as materials of the research. The HPLC analysis was carried out using a Milichrom-6 chromatograph (NPAO Nauchpribor) under the following conditions of reversed-phase chromatography in an isocratic mode: a steel column KAKH-6-80-4 (2 mm x 80 mm; Separon-C18 7 μm), a mobile phase – acetonitrile: 1% solution of acetic acid in water in the ratio of 14:86, the elution rate was 100 μL/min, the eluent volume was 2000 μL. The constituents were detected at the wavelength of 252 nm (rosavin) and 278 nm (salidroside).

Results. An assay of rosavin and salidroside in the liquid extract of *Rhodiola rosea* L. was developed using the HPLC method. It was determined that the content of rosavin in the samples of the liquid extracts obtained from *Rhodiola rosea* L. rhizomes and roots of the pharmacopoeial quality, varied from 0.21%±0.03% to 0.32%±0.04%, salidroside – from 1.13% ±0.05% to 2.71%±0.12%, respectively. The results of statistical processing indicate that the relative error of the average result for the determination of rosavin and salidroside in the preparations of *Rhodiola rose*a L. with a confidence level of 95% does not exceed ±6.0%.

Conclusion. Thus, methodological approaches to the analysis of medicinal preparations from *Rhodiola rosea* L. rhizomes and roots have been substantiated. These methodological approaches consist of the quantitative determination of the dominant and diagnostically significant biologically active compounds – rosavin and salidroside.

Keywords: *Rhodiola rosea* L.; rhizomes and roots; liquid extract; rosavin; salidroside; high performance liquid chromatography

Abbreviations: HPLC – high performance liquid chromatography; TLC – thin-layer chromatography; UV – ultraviolet; PM – pharmacopoeial monograph; GPM – general pharmacopoeial monograph; NMR – nuclear magnetic resonance

ВОПРОСЫ СТАНДАРТИЗАЦИИ ЛЕКАРСТВЕННЫХ ПРЕПАРАТОВ РОДИОЛЫ РОЗОВОЙ

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RESEARCH ARTICLE

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Родиола розовая (*Rhodiola rosea* L.), фармакопейным сырьем которой являются корневища и корни, применяется в официальной медицине для получения лекарственных препаратов с адаптогенной активностью. Одной из распространенных проблем при производстве лекарственных препаратов из корневищ и корней родиолы розовой является использование недоброкачественного лекарственного растительного сырья, что приводит к отсутствию значимых биологически активных соединений в препаратах. Одной из возможных причин являются недостатки в существующих подходах к стандартизации сырья и препаратов родиолы розовой.

Цель. Совершенствование существующих подходов к стандартизации лекарственных препаратов корневищ и корней родиолы розовой.

Материалы и методы. В качестве объектов исследования использовали экспериментальные и промышленные образцы экстракта жидкого корневищ и корней родиолы розовой, а также стандартные образцы розавина и салидрозида. ВЭЖХ-анализ осуществляли с использованием хроматографа «Милихром-6» (НПАО «Научприбор») в следующих условиях: метод – обращенно-фазовая хроматография в изократическом режиме (стальная колонка «КАХ-6-80-4», размер 2 мм х 80 мм; Сепарон-С18 7 мкм); подвижная фаза – ацетонитрил : 1% раствор уксусной кислоты в воде в соотношении 14:86; скорость элюирования – 100 мкл/мин; объем элюента – 2000 мкл. Детекцию веществ осуществляли при длине волны 252 нм (розавин) и 278 нм (салидрозид).

Результаты. С использованием метода ВЭЖХ разработана методика количественного определения розавина и салидрозида в жидком экстракте родиолы розовой. Определено, что содержание розавина в образцах жидких экстрактов, полученных из корневищ и корней родиолы розовой фармакопейного качества, варьирует от 0,21%±0,03% до 0,32%±0,04%; салидрозида – от 1,13%±0,05% до 2,71%±0,12% соответственно. Результаты статистической обработки свидетельствуют о том, что относительная ошибка среднего результата определения розавина и салидрозида в препаратах родиолы розовой с доверительной вероятностью 95% не превышает ±6,0%.

Заключение. Таким образом, в работе обосновываются методологические подходы к анализу лекарственных препаратов корневищ и корней родиолы розовой (*Rhodiola rosea* L.), заключающиеся в количественном определении доминирующих и диагностически значимых биологически активных соединений – розавина и салидрозида.

Ключевые слова: родиола розовая; *Rhodiola rosea* L.; корневища и корни; жидкий экстракт; розавин; салидрозид; высокоэффективная жидкостная хроматография

Список сокращений: ВЭЖХ – высокоэффективная жидкостная хроматография; ТСХ – тонкослойная хроматография; УФ – ультрафиолетовая область; ФС – фармакопейная статья; ОФС – общая фармакопейная статья; ЯМР – ядерный магнитный резонанс

INTRODUCTION

Rhodiola rosea L. rhizomes and roots are pharmacopoeial raw materials used in official medicine to obtain pharmaceuticals with adaptogenic activity [1–6]. The species of the *Rhodiola* L. genus have been used for a long time as adaptogens in Russia and northern Europe countries. Recently, a number of new pharmacological properties have also been detected in *Rhodiola rosea* L. preparations, i.e. antioxidant, anxiolytic, nootropic, antidepressant, and immunomodulatory activities [7–13]. It was reported that *Rhodiola rosea* L. preparations increase physical endurance, reduce fatigue and have a therapeutic effect in disorders of the gastrointestinal tract, cardiovascular system and central nervous system. Some studies have shown that *Rhodiola rosea* L. preparations inhibit the growth of malignant neoplasms. At the same time, the range of medicinal products on the basis of *Rhodiola rosea* L. rhizomes and roots, approved for use in the Russian Federation, is represented by only liquid extracts from different manufacturers[1]. *Rhodiola rosea* L. liquid extracts have been approved for use since 1975; they are recommended as an adaptogenic and tonic agent that is not inferior in its activity to ginseng [14].

Phytochemical studies have shown that the biological activity of *Rhodiola rosea* L. materials and preparations is due to six classes of compounds: phenylpropanoids, flavonoids, phenolic alcohols, phenolic acids, monoterpenes and sterols. The main biologically active compounds that determine the pharmacological activity of *Rhodiola rosea* L. raw materials and preparations are phenylpropanoids (rosavin, rosin, rosarin) and phenolic alcohols (tyrosol, salidroside) [15–20]. Phenylpropanoids are known for antioxidant, neurostimulating, adaptogenic activities. The adaptogenic activity of *Rhodiola rosea* L. raw materials and preparations, is also associated with the presence of phenolic alcohols.

In the State Pharmacopoeia of the Russian Federation of the XIV edition (PM.2.5.0036.15 “Rhodiola rosea L. rhizomes and roots” and PM.3.4.0008.18 “Rhodiola rosea L. rhizomes and roots extract liquid”), standardization of *Rhodiola rosea* L. raw materials and preparations provides for the quantitative determination of the salidroside content and the total amount of cinnamic alcohol glycosides calculated on rosavin[2,3] [21]. The analysis has been carried out by high performance liquid chromatography (HPLC) with an UV detection (at 219 nm – determination of salidroside, at 250 nm – determination of the total amount of cinnamic alcohol glycosides calculated on rosavin). The procedure has been carried out using a column 250×4.0 mm, endcapped octadecylsilyl (C18) silica gel for chromatography, 5 µm), for a mobile phase – acetonitrile: phosphate buffer (pH 7.0), the elution in a gradient mode with an increase of acetonitrile concentration from 11% to 60%, the elution rate

[1] The State Register of Medicines. Available from: http://grls.rosminzdrav.ru/grls.aspx.

[2] PM.2.5.0036.15 “Rhodiola rosea rhizomes and roots”

[3] PM.3.4.0008.18 “Rhodiola rosea rhizomes and roots extract liquid”

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– 1.0 ml/min, the volume of the injected sample – 10 µl, the run time – 35 min.

At the same time, the expediency of the determination of the amount of cinnamic alcohol glycosides calculated on rosavin, causes doubt. Rosavin is the most labile compound, which, in comparison with other cinnamic alcohol glycosides, is more sensitive to the conditions for harvesting and storing raw materials due to the possibility of enzymatic destruction under the influence of the enzyme vicianosidase [1, 2]. Vicianosidase promotes the cleavage of vicianose, a carbohydrate fragment of the rosavin molecule, and exhibits a maximum activity in the temperature range of 40–60°C, which had been previously recommended for *Rhodiola rosea* L. raw materials drying. Storage of undried rhizomes, extraction of fresh raw materials with ethanol at room temperature also contribute to the destructive effect of enzymes on the composition of biologically active components. The destruction of rosavin leads to the formation of biologically inactive cinnamic alcohol, and accordingly, the pharmacological activity of *Rhodiola rosea* L. raw materials and preparations decreases [1, 2].

In this regard, in order to standardize the plant raw materials, a more conceptually correct approach, in the authors' opinion, is to quantify not the total cinnamic alcohol glycosides, but the most labile component – rosavin. The level of its content reliably reflects the correct storage and drying conditions for *Rhodiola rosea* L. rhizomes and roots [1, 2].

These assumptions are confirmed by the results of the selective quality control of *Rhodiola rosea* L. raw materials and preparations (extracts, granular powders) [22–26]. Booker A. et al. have analyzed 40 commercial *Rhodiola rosea* L. products from various suppliers on the European Union market and have found that approximately one fifth of these products did not contain rosavin, one of the main components of *Rhodiola rosea* L. Moreover, some products did not contain salidroside, the component typical of *Rhodiola* sp. In about 80% of the remaining commercial products, the rosavin content was lower than declared, and it was assumed that they had been obtained from other species of the *Rhodiola* genus [23].

Therefore, rosavin is precisely the marker that makes it possible to reliably assess the quality of *Rhodiola rosea* L. raw materials and preparations, and the problem of a proper quality control of *Rhodiola rosea* L. raw materials and preparations is of a worldwide meaning.

It should be also notified that the quantitative determination methods included in the monographs, provide for the HPLC analysis in the gradient elution mode [21]. It is known that when using the gradient mode, the correction of conditions is more critical, it can lead to incorrect identification of peaks, their overlap or shifts, at which the analytes can leave before or after the specified time of the chromatogram registration. In the authors' opinion, the selection of conditions for the analysis in the isocratic mode will increase the reproducibility of the technique[4] [21]. In addition, the 219 nm wavelength used in the pharmacopoeial method for the detection of salidroside is less specific with respect to the accompanying components in comparison with the other absorption maximum of this compound – 278 nm [2].

THE AIM of the study was to improve the existing approaches to the standardization of medicinal products of *Rhodiola rosea* L. raw materials and preparations, adopted in the State Pharmacopoeia of the Russian Federation of the XIV edition.

MATERIALS AND METHODS

Research materials

Experimental and industrial samples of the liquid extract of *Rhodiola rosea* L. rhizomes and roots were used as research materials. The experimental samples were obtained by the method of modified maceration from the medicinal plant materials, harvested in 2016–2018 (Altai region). A reference sample of rosavin that meets the requirements of PM 42-0071-01, was obtained by the authors of the article from *Rhodiola rosea* L. rhizomes and roots using silica gel column chromatography and subsequent recrystallization from 95% ethyl alcohol.

A reference sample of salidroside was obtained by the authors from *Rhodiola rosea* L. rhizomes and roots using silica gel column chromatography, rechromatography on polyamide and subsequent recrystallization from a mixture of chloroform and 95% ethyl alcohol. It was identified by means of TLC, UV-, NMR-spectroscopy and had a melting point of 162–164°C, the purity not less than 98.0% and corresponded to the requirements of the PM draft.

Acetonitrile (ZAO "Component-reagent", "For high performance liquid chromatography"); the water obtained while using a system for obtaining deionized water by a multistage purification system (adsorption, reverse osmosis, membrane filtration) and checked for purity under the conditions of chromatographic analysis, were used in the work. The rest of the reagents were of analytical reagent grade or of chemically pure grade.

Preparation of a salidroside standard sample solution. About 0.025 g (accurately weighed) of the state standard sample of salidroside (the content of the main substance ≥98%) is placed in a volumetric flask with a capacity of 50 ml, dissolved in a small amount of 95% ethanol, brought to the mark with ethanol 95%, and mixed.

Preparation of rosavin standard solution. About 0.025 g (accurately weighed) of the state standard sample of rosavin (content of the main substance ≥98%) is placed in a volumetric flask with a capacity of 50 ml, dissolved in a small amount of 95% ethanol when heated in a boiling water bath, brought to the mark with ethanol 95%, mixed.

[4] GPM.1.2.1.2.0001.15 Chromatography.

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Conditions for chromatographic separation

HPLC analysis was carried out using a Milichrom-6 chromatograph (NPAO Nauchpribor) under the following conditions of reverse-phase chromatography in an isocratic mode: steel column KAH-6-80-4 (2 mm×80 mm; Separon-C18, 7 µm), a mobile phase – acetonitrile: a 1% solution of acetic acid in water in the ratio of 14:86, the elution rate was 100 µL/min, the eluent volume was 2000 µL. The compounds were detected at the wavelength of 252 nm (rosavin) and 278 nm (salidroside). The volumes of the injected samples were: 3 µl (the reference solutions of rosavin and salidroside) and 5 µl (the experimental samples of *Rhodiola rosea* L. liquid extracts).

System suitability assessment

The suitability of the chromatographic system was evaluated in accordance with the General Pharmacopoeia Monograph 1.2.1.2.0001.15 "Chromatography". The indicators of the chromatographic system suitability (column efficiency, resolution between peaks, asymmetry factor) were calculated based on the results of a 5-fold analysis of 5 µl of the *Rhodiola rosea* L. rhizomes and roots liquid extract solution.

The results of evaluating the suitability of the system confirm the suitability of the chromatographic system for the quantitative determination of salidroside and rosavin in *Rhodiola rosea* L. raw materials and preparations (Table 1).

Methods for the simultaneous quantitative determination of rosavin and salidroside in Rhodiola rosea L. rhizomes and roots liquid extracts

1 ml of *Rhodiola rosea* L. rhizomes and roots liquid extract is placed in a 25 ml volumetric flask, diluted to the mark with purified water. Before the analysis, an aliquot of the sample is additionally filtered through a Milipore membrane filter (0.45 µm) (the test solution).

5 µl of the test solution is injected into a Milichrom-6 liquid chromatograph with an UV detector. Chromatography is carried out under the conditions of reverse phase chromatography in an isocratic mode: steel column "KAH-6-80-4" (2 mm x 80 mm; Separon-C18, 7 µm), a mobile phase – acetonitrile: a 1% solution of acetic acid in water in the ratio of 14:86, the elution rate was 100 µl/min, the eluent volume was 2000 µl.

The compounds were detected at the wavelengths of 252 nm (rosavin) and 278 nm (salidroside).

In parallel, 3 µl of solutions of reference salidroside and rosavin samples are injected into the chromatograph and chromatographed as described above. The height of the salidroside peak on the chromatogram is determined at the wavelength of 278 nm and the area of the rosavin peak on the chromatogram at the wavelength of 252 nm. The average values based on the results of three parallel determinations, are calculated.

The salidroside content (X in percent) is calculated by the formula:

$$X, \% = \frac{H_i \times m_{st} \times V_1 \times V \times 100}{H_{st} \times V_{st} \times V_2 \times V_{al}},$$

where: H_i is the height of the salidroside peak on the test solution chromatogram, absorbance units; H_{st} is the height of the salidroside peak in the reference solution chromatogram, absorbance units; m_{st} is the exact weight of salidroside reference sample, g; V_{st} is the volume of the prepared salidroside reference solution, ml; V_1 is the volume of the injected sample of the reference solution, µl; V_2 is the volume of the injected test solution, µl; V is the volume of a volumetric flask in which an aliquot of *Rhodiola rosea* L. rhizomes and roots liquid extracts was diluted, ml; V_{al} is the volume of an aliquot of *Rhodiola rosea* L. rhizomes and roots liquid extracts, ml.

The content of rosavin (X in percent) is calculated by the formula:

$$X, \% = \frac{S_i \times m_{st} \times V_1 \times V \times 100}{S_{st} \times V_{st} \times V_2 \times V_{al}},$$

where: S_i is the area of the rosavin peak on the test solution chromatogram; S_{st} is the area of the rosavin peak in the reference solution chromatogram; m_{st} is the exact weight of rosavin reference sample, g; V_{st} is the volume of the prepared rosavin reference solution, ml; V_1 is the volume of the injected sample of the reference solution, µl; V_2 is the volume of the injected test solution, µl; V is the volume of a volumetric flask in which an aliquot of *Rhodiola rosea* L. rhizomes and roots liquid extracts was diluted, ml; V_{al} is the volume of an aliquot of *Rhodiola rosea* L. rhizomes and roots liquid extracts, ml.

Validation of methods

The validation assessment of the developed methodology was carried out according to the following indicators: specificity, linearity, accuracy (recovery), precision. The specificity of the methods was determined by the correspondence of retention times of the salidroside and rosavin peaks on the HPLC chromatograms of the reference solutions and the peaks corresponding to these standards on the HPLC chromatogram of the test solution, as well as by the resolution between the closest peaks and the asymmetry factor of the peaks of salidroside and rosavin.

The determination of linearity was carried out at five concentration levels of reference sample solutions (with concentrations ranging from 0.1467 to 1.4667 mg/ml for salidroside and from 0.1200 to 0.9600 mg/ml for rosavin). Based on the data obtained, a graph was built in the coordinates "concentration, mg/ml – peak height" or "concentration, mg/ml – peak area" and there were calculated the linear regression equation ($Y = ax + b$), the value of the coefficient of the determination (r^2), a standard deviation using Microsoft Excel 2013 software.

The accuracy of the method was tested by introducing an exact amount of reference samples of rosavin and salidroside in the range of 80% to 120% of the initial content, into the aliquot of *Rhodiola rosea* L. preparation.

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Table 1 – Determination of the chromatographic system suitability

Chromatographic system parameter	Value	Limit
Column efficiency (salidroside)	5.100	At least 5000 theoretical plates
Column efficiency (rosavin)	5.201	
Resolution between closest peaks (salidroside)	1.6	Not less than 1.5
Resolution between closest peaks (rosavin)	2.3	
Asymmetry factor (salidroside)	1.47	Not more than 1.5
Asymmetry factor (rosavin)	1.21	

Table 2 – Metrological characteristics of the method for the quantitative determination of salidroside and rosavin in *Rhodiola rosea* L. liquid extracts

Analyte	f	$\overline{X}$,%	S	P,%	t (P,f)	$\Delta\overline{X}$	$\overline{\varepsilon}$,%
Salidroside	10	2.02	0.160114	95	2.23	±0.11	±5.33
Rosavin	10	0.22	0.014460	95	2.23	±0.04	±4.43

Note: f – degrees of freedom; $\overline{X}$ – average; S – standard deviation; P – confidential probability; t – Student's t-test; $\Delta\overline{X}$ – half-width of the confidence interval of the mean result; $\overline{\varepsilon}$ – mean relative error.

Table 3 – Results of determining the accuracy of the analytical procedure (salidroside)

Initial content of salidroside, mg in 1 ml of water-alcohol extract	Added salidroside mg/ml	Salidroside content, mg/ml		Error	
		Estimated	Found	Absolute, mg/ml	Relative,%
20.18	16.00	36.18	37.00	0.82	2.27
20.18	20.00	40.18	39.50	–0.68	–1.69
20.18	25.00	45.18	45.00	–0.18	–0.40

Table 4 – Results of determining the accuracy of the analytical procedure (rosavin)

Initial content of rosavin, mg in 1 ml of water-alcohol extract	Added rosavin mg/ml	Rosavin content, mg/ml		Error	
		Estimated	Found	Absolute, mg/ml	Relative,%
2.19	1.75	3.94	4.00	0.06	1.52
2.19	2.20	4.39	4.25	–0.14	–3.19
2.19	2.60	4.79	4.61	–0.18	–3.76

Table 5 – The content of rosavin and salidroside in experimental and industrial samples of liquid extracts from *Rhodiola rosea* L. rhizomes and roots

No.	Sample	Salidroside content,%	Rosavin content,%
1.	Experimental sample No. 1 (obtained from raw materials harvested in 2016)	2.13% ±0.05%	0.21% ±0.03%
2.	Experimental sample No. 2 (obtained from raw materials harvested in 2018)	2.71% ±0.12%	0.32% ±0.04%
3.	Industrial sample No. 1	1.62% ± 0.05%	Not found
4.	Industrial sample No. 2	2.75% ± 0.08%	Not found
5.	Industrial sample No. 3	2.55% ± 0.07%	Not found
6.	Industrial sample No. 4	1.20% ± 0.04%	Not found
7.	Industrial sample No. 5	1.12% ± 0.06%	Not found
8.	Industrial sample No. 6	0.96% ± 0.04%	Not found

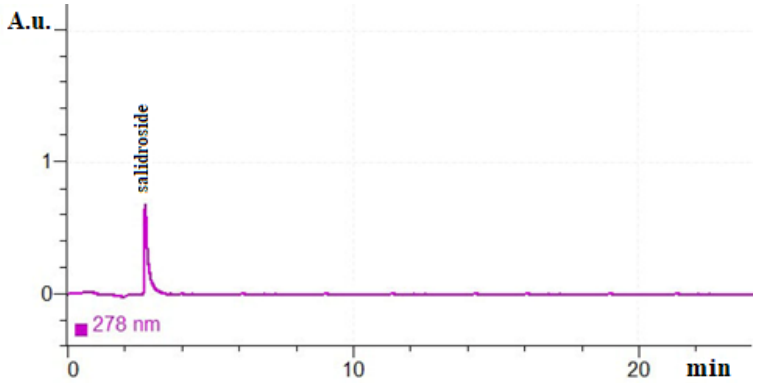

Figure 1 – HPLC chromatogram of salidroside reference sample solution, 0.88 mg/ml
Note: detection at the wavelength of 278 nm

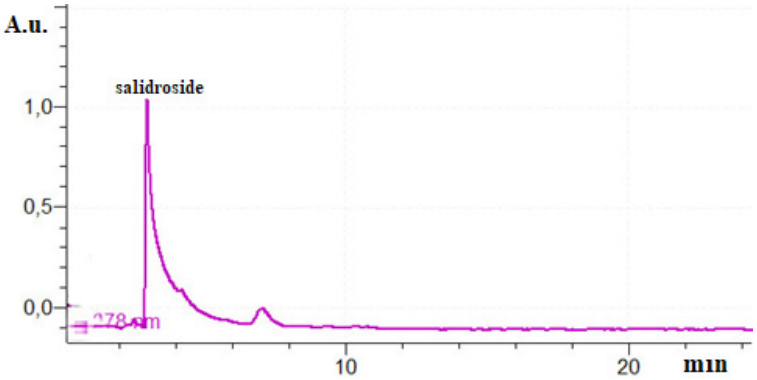

Figure 2 – HPLC chromatogram of the experimental sample of liquid extract from *Rhodiola rosea* L. rhizomes and roots
Note: detection at the wavelength of 278 nm

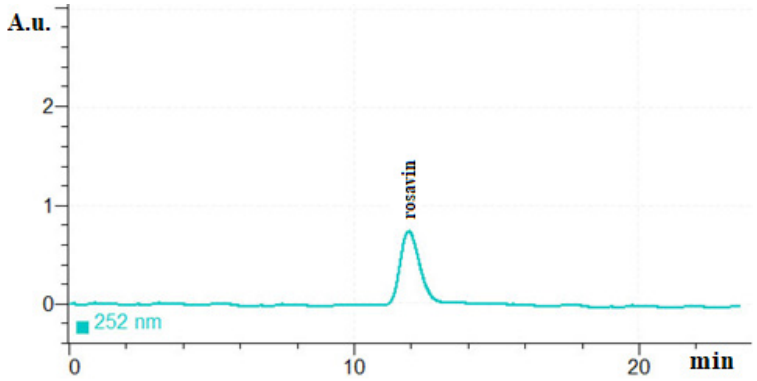

Figure 3 – HPLC chromatogram of rosavin reference sample solution, 0.60 mg/ml
Note: detection at the wavelength of 252 nm

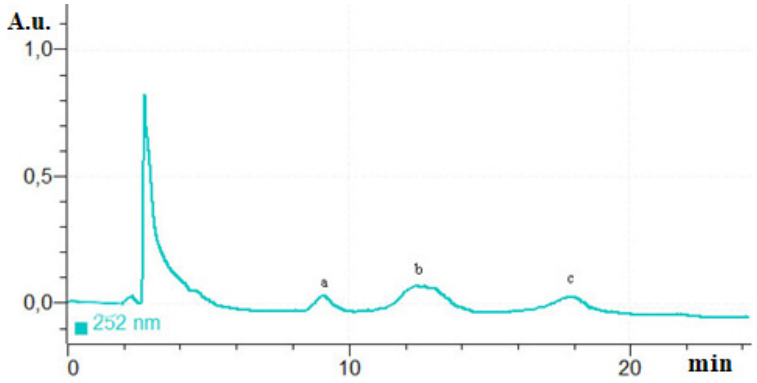

Figure 4 – HPLC chromatogram of the experimental sample of liquid extract from *Rhodiola rosea* L. rhizomes and roots
Note: a – rosarin; b – rosavin; c – rosin; detection at the wavelength of 252 nm

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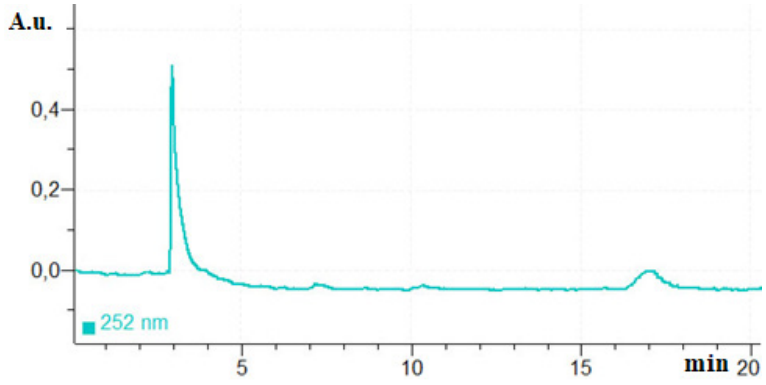

Figure 5 – Representative HPLC chromatogram of industrial samples of liquid extract from *Rhodiola rosea* L. rhizomes and roots
Note: detection at the wavelength of 252 nm

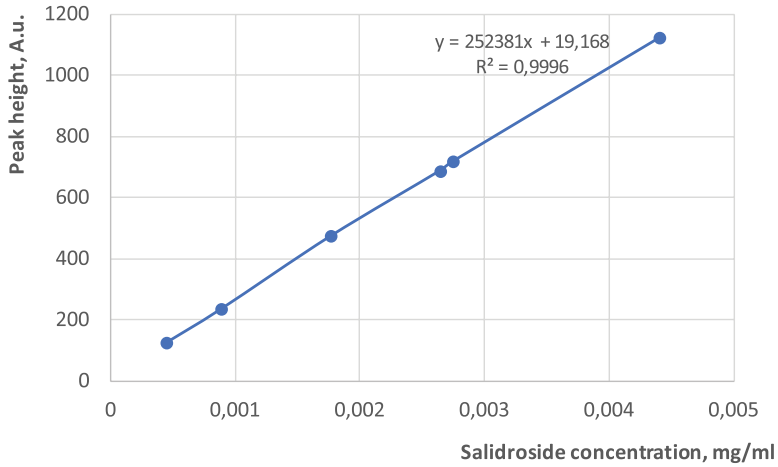

Figure 6 – Graph of the dependence of the peak height on the concentration of salidroside in the sample and the linear regression equation

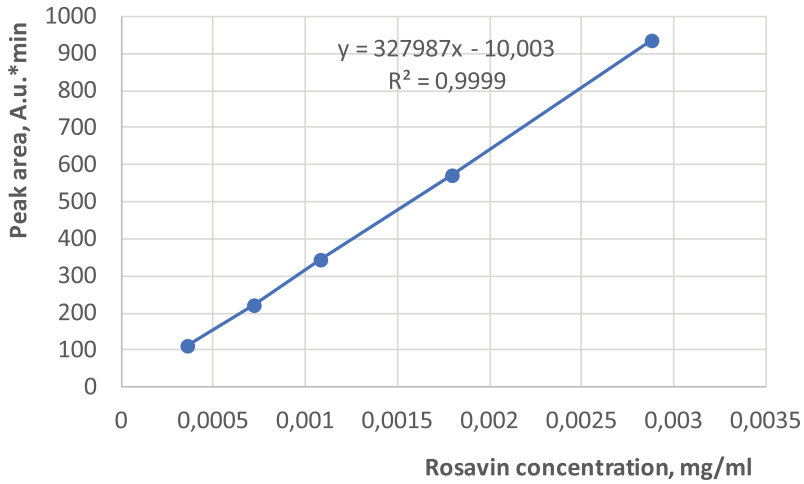

Figure 7 – Graph of the dependence of the peak area on the concentration of rosavin in the sample and the linear regression equation

RESULTS AND DISCUSSION

At the preliminary stage, under the conditions of chromatographic separation described above, the content of rosavin and salidroside in *Rhodiola rosea* L. rhizomes and roots used to obtain experimental samples of the liquid extract, was analyzed. It was determined that the content of rosavin in *Rhodiola rosea* L. rhizomes and roots varied from 1.17%±0.04% to 1.41% ± 0.06% and salidroside – from 1.63%±0.05% to 2.88%±0.12%, respectively. In the sample harvested in 2020, rosavin was not detected, although other glycosides of cinnamic alcohol were present, which indicates improper storage conditions of the raw materials.

Under the proposed conditions of HPLC analysis, the retention times of salidroside peaks on the chromatograms of the salidroside reference solution, the aqueous-alcoholic extracts from *Rhodiola rosea* L. raw materials and the solutions obtained as a result of dilution of the experimental samples of *Rhodiola rosea* L. liquid extracts, were (2.780 ± 0.077), (2.979 ± 0.070) and (2.790±0.087) min, respectively (Fig. 1 and 2). For rosavin, the corresponding values were (12.424±0.080), (12.824±0.070) and (12.429±0.070) min (Fig. 3 and 4). Rosavin was not detected in any of the 6 analyzed industrial samples.

The dependence of the height and area of the chromatographic peak on the salidroside concentration was described by a linear regression model in the concentration range from 0.1467 to 1.4667 mg/ml (Fig. 6). However, the correlation coefficient for the dependence of the peak height on the salidroside concentration was 0.9996, for the dependence of the peak area from the concentration it was 0.9888. In this regard, the calculation of the salidroside content in the test samples was carried out using the peak height.

For the dependence of the height and area of the peak on the concentration of rosavin in the concentration range from 0.1200 to 0.9600 mg/ml, the correlation coefficients were 0.9973 and 0.9999 (Fig. 7). Therefore, the determination of the content of rosavin was carried out using the peak area.

The indicated concentration ranges of salidroside and rosavin can be considered as the range of the analytical procedures.

The metrological characteristics of the proposed HPLC procedure indicate that the error in determining the average result of the salidroside content in the liquid extract of *Rhodiola rosea* L. rhizomes and roots with a confidence level of 95% is ±5.33%, rosavin – ±4.43% (Table 2). The accuracy of the method was determined by adding solutions of salidroside and rosavin with the known concentration (80%, 100%, and 120%) to the aliquot of the experimental drug. At the same time, the average percentage of the recovery was 100.06% and 98.19%, respectively (Tables 3 and 4). The errors in the determination of salidroside and rosavin in the samples with additives of the reference samples were within the error of a single determination, which indicates the absence of a systematic error.

The study of the technique repeatability indicates the convergence of the obtained concentrations of salidroside and rosavin: the relative error of the average result of determining the content of salidroside in *Rhodiola rosea* L. rhizomes and roots with a confidence level of 95% is ±5.61% and ±4.70%, respectively. The one of a single determination is ±17,7% and ±14.7%, respectively. When evaluating the intra-laboratory precision, satisfactory results were also shown, since the relative error in the determination of rosavin and salidroside on the first and second days of the analysis was in the range from 0.90 to 1.09.

It was determined that the content of rosavin in experimental samples of the liquid extracts obtained from *Rhodiola rosea* L. rhizomes and roots of the pharmacopoeial quality varies from 0.21%±0.03% to 0.32%±0.04% and the one of salidroside – from 2.13%±0.05% to 2.71%±0.12%, respectively (Table 5). In the analyzed industrial samples of two Russian manufacturers, the salidroside content varied from 0.96%±0.04% to 2.75%±0.08%. Rosavin was not found in any of the samples tested.

Therefore, the absence of rosavin in the preparations of *Rhodiola rosea* L., according to our data and other published results [22–26], is a common problem. Possible reasons are the use of other species of the *Rhodiola* L. genus for the preparation of drugs or improper conditions for harvesting, drying and storage of the medicinal plant raw materials, as well as their processing.

It is known that salidroside is present in almost all types of *Rhodiola* sp., it is not subject to enzymatic or thermal degradation, and its content in rhizomes and roots does not depend on its habitat [27]. Rosavin, unlike salidroside and other phenylpropanoids, is found only in *Rhodiola rosea* L. rhizomes, and it is the most labile of its components, since it is subject to the selective enzymatic degradation. Drying the rhizomes of this plant at the temperature of 50–60°C leads to the greatest selective enzymatic degradation of rosavin to aglycone – cinnamic alcohol. The temperature range of 70–80°C is recommended as the optimal drying conditions for *Rhodiola rosea* L. rhizomes [1].

The variability of the rosavin content depending on its habitat, was revealed. The maximum content of rosavin and salidroside was notified in *Rhodiola rosea* L. rhizomes of the Altai origin [27]. For the rhizomes of the Mongolian population of *Rhodiola rosea* L., the peculiarity of its chemical composition is a high content of flavonoids (approximately 200 times more than in other samples of the raw materials), especially in herbacetin derivatives [28].

It was also determined that rosavin appears in the rhizomes of the plant only in the second year of life and reaches its maximum value in the fourth year of life. Salidroside begins to accumulate in plants in the first year of life, reaching its maximum, just as in the case of rosavin, in the fourth year of life [2]. Taking into account the fact that the phytomass of *Rhodiola rosea* L. rhizomes actively grows in the 5th and 6th years of plant life against the background of maintaining a high content of rosavin, recommendations for harvesting the raw materials for 5–6-year-old plants are justified [2].

Therefore, the presence of rosavin is a reliable indicator of a good quality of *Rhodiola rosea* L. raw materials and preparations.

Taking into account the obtained data, it is possible to recommend a lower limit of the rosavin content in *Rhodiola rosea* L. liquid extracts – 0.1%, salidroside – 0.8%.

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CONCLUSION

Thus, the article proposes HPLC procedures for the simultaneous assays of the two most significant biologically active compounds of *Rhodiola rosea* L. rhizomes and roots in medicines obtained on their basis under the conditions of an isocratic elution mode. The error in determining the average result of the rosavin and salidroside content in the raw material of *Rhodiola rosea* L. did not exceed 6.0%.

Methodological approaches to the development of procedures for the pharmacopoeial analysis of *Rhodiola rosea* L. preparations have been considered. The quality of medicinal preparations from *Rhodiola rosea* L. rhizomes and roots is directly related to the quality of medicinal plant raw materials. Herewith, the most labile biologically active component, which is the most susceptible to the enzymatic degradation when the conditions for drying, storage of raw materials and their processing are violated, is rosavin. In this regard, it is this phenylpropanoid that is the marker of the quality of *Rhodiola rosea* L. raw materials and preparations which should be first of all paid attention to.

FUNDING

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

V.A. Kurkin – planning of the study, participation in the development of the concept and design of the study, final approval of the manuscript for publication, processing the results obtained, verification of critical intellectual content; T.K. Ryazanova – data collecting, experiment conducting, analyzing and interpreting the data obtained, preparing a manuscript draft, literature analyzing, manuscript writing.

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EFFECT OF SOLID DISPERSIONS ON THE SOLUBILITY OF METRONIDAZOLE

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The aim of the work is to study the effect of solid dispersions using polyethylene glycols of various molecular weights on the solubility of metronidazole in water. Metronidazole is an antimicrobial and antiprotozoal drug. Its low solubility in water limits the use of metronidazole, causing technological difficulties and reducing its bioavailability. The solubility and release of the active substance from dosage forms can be increased using the solid dispersion methods. Solid dispersions are bi- or multi-component systems consisting of an active substance and a carrier (a highly dispersed solid phase of the active substance or molecular-dispersed solid solutions) with a partial formation of complexes of variable compositions with the carrier material.
Materials and methods. The substance of metronidazole used in the experiment, was manufactured by Hubei Hongyuan Pharmaceutical Technology Co., Ltd. (China). To obtain solid dispersions, polyethylene glycols of various molar masses – 1500, 2000 and 3000 g/mol – were used. The solid dispersions were prepared by "the solvent removal method": metronidazole and the polymer were dissolved in a minimum volume of 96% ethyl alcohol (puriss. p.a./analytical grade) at 65±2°C, and then the solvent was evaporated under vacuum to the constant weight. A vacuum pump and a water bath were used at the temperature of 40±2°C. The dissolution of the samples was studied using a magnetic stirrer with heating, and a thermostatting device. The concentration of metronidazole was determined on a spectrophotometer using quartz cuvettes at the wavelength of 318±2 nm. To filter the solutions, syringe nozzles were used, the pores were 0.45 μm, the filter was nylon. Microcrystalloscopy was performed using a microscope with a digital camera. The optical properties of the solutions were investigated using a quartz cuvette and a mirror camera (the image exposure – 20 sec).
Results. Obtaining solid dispersions increases the completeness and rate of the metronidazole dissolution. The solubility of metronidazole from solid dispersions increases by 14–17% in comparison with the original substance. The complex of physical-chemical methods of the analysis, including UV spectrophotometry, microcrystalloscopy and the study of the optical properties of the obtained solutions, makes it possible to suggest the following. The increase in the solubility of metronidazole from solid dispersions is explained by the loss of crystallinity and the formation of a solid solution of the active substance and the solubilizing effect of the polymer with the formation of colloidal solutions of metronidazole at subsequent dissolution of the solid dispersion in water.
Conclusion. The preparation of solid dispersions with polyethylene glycols improves the dissolution of metronidazole in water. The results obtained are planned to be used in the development of rapidly dissolving solid dosage forms of metronidazole with an accelerated release and an increased bioavailability.
Keywords: solid dispersion; solubility; metronidazole; polyethylene glycol
Abbreviations: SD – solid dispersion; PEG – polyethylene glycol; R&D – research & development

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ВЛИЯНИЕ ТВЁРДЫХ ДИСПЕРСИЙ НА РАСТВОРИМОСТЬ МЕТРОНИДАЗОЛА

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Цель. В работе изучено влияние твёрдых дисперсий с применением полиэтиленгликолей различных молекулярных масс на растворимость метронидазола в воде. Метронидазол – противомикробное и противопротозойное лекарственное средство. Малая растворимость в воде ограничивает применение метронидазола, обуславливая технологические трудности и снижая биодоступность. Повысить растворимость и высвобождение действующего вещества из лекарственных форм можно с помощью метода твёрдых дисперсий. Твердые дисперсии – это би- или многокомпонентные системы, состоящие из действующего вещества и носителя (высокодиспергированная твёрдая фаза действующего вещества или молекулярно-дисперсные твёрдые растворы) с частичным образованием комплексов переменного состава с материалом носителя.

Материалы и методы. В работе использовали субстанцию метронидазола производства. Для получения твёрдых дисперсий применяли полиэтиленгликоли различных молярных масс: 1500, 2000 и 3000 г/моль. Твердые дисперсии готовили методом «удаления растворителя»: метронидазол и полимер растворяли в минимальном объёме спирта этилового 96% (ч.д.а.) при 65±2°С, затем растворитель выпаривали под вакуумом до постоянной массы. Использовали вакуумный насос и водяную баню, температура 40±2°С. Растворение образцов изучали, используя магнитную мешалку с подогревом и устройством термостатирования. Концентрацию метронидазола определяли на спектрофотометре, используя кварцевые кюветы, при длине волны 318±2 нм. Для фильтрования растворов использовали шприцевые насадки, поры – 0,45 мкм, фильтр – нейлон. Микрокристаллоскопию проводили на микроскопе с цифровой камерой. Оптические свойства растворов исследовали, используя кварцевую кювету и зеркальную камеру (экспозиция снимка 20 сек.).

Результаты. Получение твердых дисперсий увеличивает полноту и скорость растворения метронидазола. Растворимость метронидазола из твердых дисперсий повышается на 14–17% в сравнении с исходной субстанцией. Комплекс физико-химических методов анализа, включающий: УФ-спектрофотометрию, микрокристаллоскопию и изучение оптические свойства полученных растворов, позволяет утверждать, что повышение растворимости метронидазола из твердых дисперсий объясняется потерей кристалличности и образованием твёрдого раствора действующего вещества и солюбилизирующим действием полимера с образованием коллоидных растворов метронидазола при последующем растворении твердой дисперсии в воде.

Заключение. Получение твердых дисперсий с полиэтиленгликолями улучшает растворение метронидазола в воде. Полученные результаты планируется использовать при разработке быстрорастворимых твёрдых лекарственных форм метронидазола с ускоренным высвобождением и повышенной биодоступностью.

Ключевые слова: твёрдая дисперсия; растворимость; метронидазол; полиэтиленгликоль

Список сокращений: ТД – твёрдая дисперсия; ПЭГ – полиэтиленгликоль; НИР – научно-исследовательская работа

INTRODUCTION

This work continues the promising scientific area of “solid dispersions in medicine and pharmacy”.

At the moment, the study of solid dispersions (SDs) is being carried out at I.M. Sechenov First Moscow State Medical University on the basis of the departments of the Institute of Pharmacy n. a. A.P. Nelyubin: “pharmaceutical technology” and “analytical, physical and colloidal chemistry”. The work is carried out within the framework of research & development (R&D): “Increasing the bioavailability of drugs using solid dispersions.” The expected social-and-economic effect of R&D is the production of innovative drugs with an increased bioavailability at minimal economic costs, as well as an active import substitution.

Within the framework of this scientific area, solid

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dispersions of more than 30 poorly soluble medicinal substances from different pharmacological groups were obtained and studied over the past 20 years on the basis of the First Moscow State Medical University n. a. I.M. Sechenov. These medicinal substances are: albendazole, amoxicillin trihydrate, ampicillin trihydrate, anestezin, acetomepregenol, acyclovir, benzonal, diclofenac (the acid form), indomethacinic acid, quverethacin, methyluracil, naftifine hydrochloride, nifidepine, nozepam, parmidin, prothionamide, riboflavin, rifampicin, rutin, synthomycin, streptocid, sulfadimethoxin, phenazepam, furazolidone, furacilin, erythromycin, etc. [1–10].

Metronidazole is an antimicrobial and antiprotozoal drug that has been successfully used in therapy for over 60 years for the treatment of infectious diseases caused by anaerobic bacteria, as well as for the treatment of protozoal infections (amoebiasis, giardiasis, trichomoniasis) [11]. As a typical representative of the group of imidazole derivatives (1,3-diazole), metronidazole is of particular interest for this study. As an antibacterial agent, metronidazole is active against gram-negative anaerobes *Bacteroides spp.: B. fragilis, B. ovatus, B. distasonis, B. vulgatus B. thetaiotaomicron); Fusobacterium spp*. and a number of gram-positive anaerobes (*Eubacterium spp*.; *Peptococcus niger; Clostridium spp.; Peptostreptococcus spp.* The minimum inhibitory concentration for these strains is 6.250–0.125 μg/ml.

A separate area of metronidazole application is the eradication of *Helicobacter pylori* in duodenal ulcer and/or stomach ulcer. Metronidazole is used in triple therapy: bismuth-based drugs; the drugs that block H2 receptors; the drugs that inhibit the proton pump. In cases a patient has an intolerance to clarithromycin or amoxicillin, Helicobacter pylori therapy is carried out using metronidazole as a substitute for these antibiotics (0.5 g 2–3 times a day for 7 days) [11, 12].

In dentistry, metronidazole is used for various localized infections caused by anaerobes in periodontal diseases and maxillofacial inflammations. A gel, which includes a combination of chlorhexidine and metronidazole, is used in dentistry. Its indications are: infectious and inflammatory diseases of the oral mucosa and parodontium – acute and chronic – gingivitis, periodontitis, necrotising ulcerative Vincent's gingivitis, postextraction alveolitis, aphthous stomatitis. Metronidazole is used in dentistry for systemic pharmacotherapy.

In dermatology, metronidazole is used to treat rosacea [11–13]. The widespread use of metronidazole in gastroenterology, dentistry, dermatology, gynecology, etc. was the cause of the emergence of various dosage forms. Therefore, on the Russian pharmaceutical market, metronidazole is presented in the form of tablets, solutions, creams; is included in gels and suppositories along with other active ingredients. The substance of metronidazole (Fig. 1) is a crystalline powder of light yellow or white; it is slightly soluble in water, acetone and ethanol (1 : 100), which can limit its use in some cases, causes difficulties of a technological nature in the creation of new drugs, and reduces their bioavailability.

It is possible to increase the solubility and accelerate the release of substances from the dosage form by "the method of solid dispersions" (SDs) [1–10; 14–17]. SDs are either multicomponent systems that include an active substance and a carrier (a solid phase of a drug dispersed in a polymer), or solid solutions of a drug in a carrier. In some cases, the formation of complexes of various natures of the active substance with the carrier material can be observed [1, 2, 13]. Various polymeric substances are used in the role of the SD carrier [17–19].

THE AIM of the work is to study the effect of solid dispersions using polyethylene glycols of various molecular weights on the solubility of metronidazole in water.

MATERIALS AND METHODS

The substance of metronidazole used in the experiment, was manufactured by Hubei Hongyuan Pharmaceutical Technology Co., Ltd. (China). It corresponds to the Product specification file (State Pharmacopoeia, Russia, XIVth ed., Pharmacopeial monograph.2.1.0136.18). To obtain SDs, PEGs of various molar masses – 1500, 2000, and 3000 g/mol – were used as carriers (Merck, Germany).

Technology for preparing solid dispersions with PEG

The literature analysis and the accumulated actual experience make it possible to assert that, in case of PEG, the optimal technology for obtaining SDs is "solvent removal" [3, 5–7, 10, 20, 21–23]. The calculated amounts of metronidazole and polymer were dissolved in a minimum volume of 96% ethyl alcohol (analytical grade) by heating to 65±2°C, then the solvent was evaporated under vacuum to the constant weight. A UED-Lab 115 vacuum pump (China) and a UT-4301E water bath (Ulab, China) were used at the temperature 40±2°C [1, 2, 16, 18, 22].

Study of metronidazole dissolution

Carried out according to the technique described in the works of Krasnyuk I.I. et al. [1] and Beliatskaya A.V. et al. [2]. The main problem was the impossibility of using the methods according to General Pharmacopoeia Monograph 1.4.2.0014.15 "Dissolution for solid dosage forms". This is associated with the preparation of saturated solutions of metronidazole under study. The SDs obtained in the work are very sticky, thick white masses or powders of soft consistency, prone to sticking together. The conditions described in GPM 1.4.2.0014.15 for studying the dissolution of these objects, are not always acceptable. In this regard, a modified technique was used during the work. Preliminary studies [6–8] prove that the dissolution test on the "rotating basket" device

presents results similar to those obtained by the modified methods.

Thus, the dissolution of the samples was studied using a heated magnetic stirrer equipped with an RCT BASIC thermostatting device (IKA, Germany). The samples for dissolution were selected in such a way that a saturated solution of metronidazole would be achieved. The temperature of the dissolution medium was 37±1°C. The samples were immersed in 150 ml of purified water; they were continuously stirred (200 rpm).

To study the dynamics of the metronidazole dissolution, the samples (5 ml) were thieved at the intervals of 5, 10, 15, 20, 30, 40, 50, 60 min. The medium was replenished up to 150 ml with purified water. The samples were filtered.

Measurement of metronidazole concentration

In the experiment, a UNICO2800 spectrophotometer (Unitedproducts & instruments, USA) and quartz cuvettes (the absorbing layer of 10 mm) were used. If necessary, the samples were diluted with purified water, the optical density of the resulting solution was measured at the wavelength of 318±2 nm (the maximum absorption of metronidazole). The results are presented in Table 1, Fig. 2, 3.

Filtration

The filtration was carried out using syringe nozzles with Minisart® filters (Sartorius, Germany) with a pore diameter of 0.45 μm, the filter was nylon.

Microcrystalloscopy

A Levenhuk D50LNG microscope (PRC for Levenhuk, Inc., USA) with a digital camera was used. The study was carried out according to the methodology [1, 2, 6, 7, 9]. In case of the metronidazole substance, the powder was placed on a glass slide, mixed with a drop of vaseline oil, covered with a cover glass, and microscoped. In case of SD, a drop of the solution of metronidazole and PEG (in the proportions corresponding to SD) in 96% ethyl alcohol was applied to a glass slide, the solvent was completely removed and microscoped.

Separately, PEGs were studied in a similar way. A drop of the PEG solution was applied to a glass slide, the solvent was completely removed, and the PEG was solidified and microscoped.

The recrystallized substance of metronidazole was additionally investigated after the removal of alcohol. A drop of the metronidazole solution in 96% ethyl alcohol was placed on a glass slide, microscoped after a complete removal of the solvent. The micrographs of the studied samples with the microscopic condition are shown in Fig. 4.

Study of the optical properties of solutions

A quartz cuvette (the layer of 50.0 mm) was used. The cuvette was filled with a filtered solution of the studied sample. An opaque partition with a hole (1 mm in the diameter) was placed between the cell wall and the light source. A thin beam of white light was directed through the hole onto the cuvette. In the darkened room, the digital images of the Faraday-Tyndall phenomenon were filmed. A Canon 5D MarkII SLR camera (the image exposure of 20 sec) was used. The results are shown in Fig. 5.

Statistical processing

Statistical processing of the values of metronidazole concentrations in solutions was carried out in accordance with General Pharmacopoeia Monograph 1.1.0013.15 (SP RFXIV): n = 5, p = 95%.

RESULTS AND DISCUSSION

Polyethylene glycols (PEGs) are promising carriers of solid dispersions [10]. PEGs are tasteless and odorfree, readily soluble in water and alcohol, chemically stable, biologically harmless, resistant to high temperatures during sterilization. PEGs are insensitive to fluctuations in pH and the presence of electrolytes; they are resistant to the action of microorganisms due to the presence of primary hydroxyl groups in the molecule [3, 21]. The PEG consistency depends on the molecular weight. Up to 400 g/mol, PEGs are viscous colorless liquids; with a mass of more than 400 to 1000 g/mol; they are substances with the consistency of soft wax; PEGs with a mass of 1500 g/mol and more are solid. In view of the fact that the obtained data are later planned to be used in the development of solid, rapidly dissolving dosage forms of metronidazole, PEGs of a solid consistency with weights of 1500, 2000, and 3000 g/mol were chosen as the actual objects of research. The selected polymers are often used as auxiliary substances in the production of tablets and granules [5].

The use of preparations based on SD and PEG is promising due to the bioadhesive qualities of PEGs (as high molecular weight compounds). Upon contact with mucous membranes or skin, PEG macromolecules are adsorbed and, as a rule, increase the permeability of cell membranes, promoting active transmucosal transfer of the active substance. The analysis of patent and scientific literature did not reveal information on the use of PEGs in the technology of solid dosage forms as carrier polymers for the preparation of SDs with metronidazole in order to increase its solubility in water.

Based on the analysis of the scientific literature and preliminary actual research, the range of optimal metronidazole:PEGs were determined: from 1:1 to no more than 1:5 (by weight) [1-9]. Taking into account the physicochemical properties of PEG (as an excipient) and its effect on the technological characteristics of solid dosage forms (for example, on their strength), this range of ratios is optimal for the future inclusion of metronidazole SD with PEG in the composition of solid dosage forms.

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Table 1 – Changes in the concentration of metronidazole solutions and solid dispersions over time

№	Sample composition	Sample weight (g)	Average value of metronidazole concentration (mg/ml) in the sample solution from the dissolution beginning; n = 5							
			Sampling time (min.)							
			5	10	15	20	30	40	50	60
1	Metronidazole – substance	3.0	7.767	7.944	8.221	8.400	8.734	8.846	8.950	9.003
2	SD metronidazole: PEG-1500 (1:1)	2.0:2.0	8.342	10.473	12.885	11.773	10.661	9.789	9.444	9.402
3	SD metronidazole: PEG-1500 (1:3)	2.0:6.0	8.156	9.267	9.590	10.083	10.343	10.602	10.423	10.244
4	SD metronidazole: PEG-1500 (1:5)	2.0:10.0	2.971	4.180	5.408	6.383	7.875	9.112	10.303	10.534
5	SD metronidazole: PEG-2000 (1: 1)	2.0:2.0	8.165	9.272	10.114	9.415	9.721	10.026	10.214	10.402
6	SD metronidazole: PEG-2000 (1: 5)	2.0:10.0	5.969	6.980	7.711	8.667	9.144	9.499	9.683	9.686
7	SD metronidazole: PEG-3000 (1:1)	2.0:2.0	4.757	6.984	8.156	8.601	8.717	8.973	9.457	9.639
8	SD metronidazole: PEG-3000 (1:5)	4.0:20.0	4.460	7.698	9.127	8.950	9.190	9.370	9.549	9.751

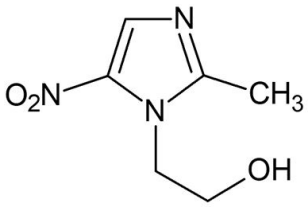

Figure 1 – Structural formula of metronidazole $C_6H_9N_3O_3$, 2-(2-Methyl-5-nitro-1H-imidazol-1-yl)ethanol, (171.15 g/mol)

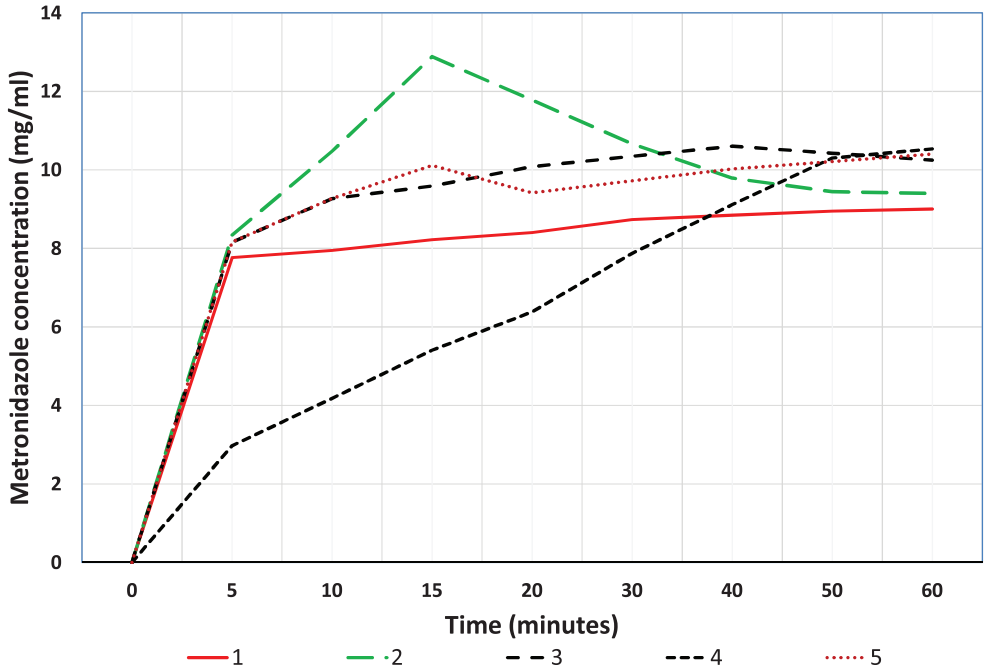

Figure 2 – Changes in concentrations of metronidazole and SD solutions with PEG-1500 and PEG-2000 over time

Note: 1 – metronidazole (substance); 2 – SD metronidazole : PEG-1500 (1: 1); 3 – SD metronidazole:PEG-1500 (1: 3); 4 – SD metronidazole:PEG-1500 (1: 5); 5 – SD metronidazole:PEG-2000 (1: 1).

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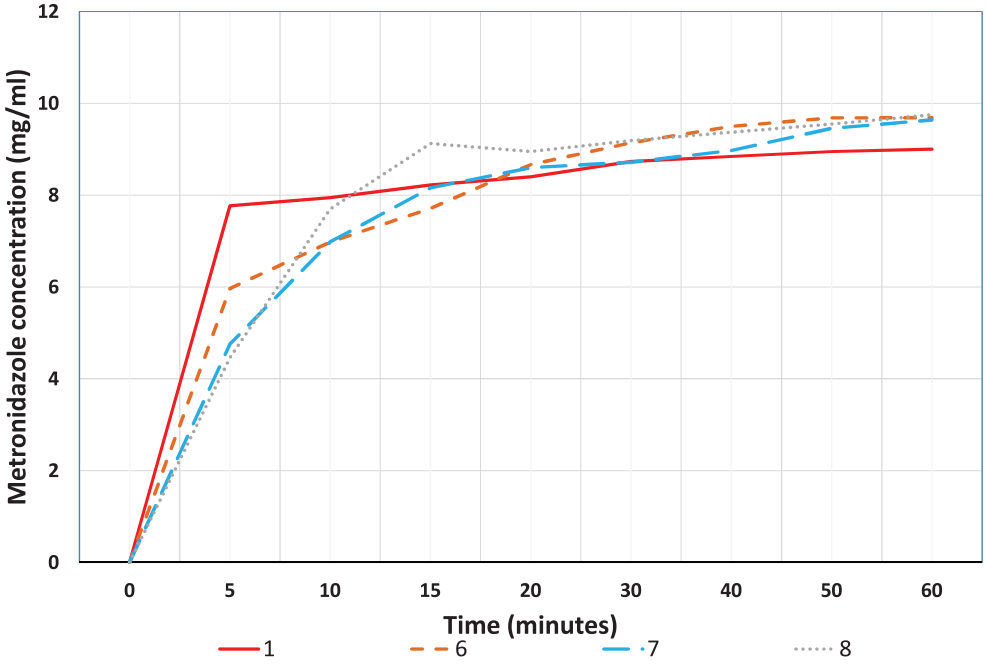

Figure 3 – Changes in concentrations of metronidazole and SD solutions with PEG-2000 and PEG-3000 over time

Note: 1 – metronidazole (substance); 6 – SD metronidazole: PEG-2000 (1:5); 7 – SD metronidazole: PEG-3000 (1:1); SD metronidazole: PEG-3000 (1:5)

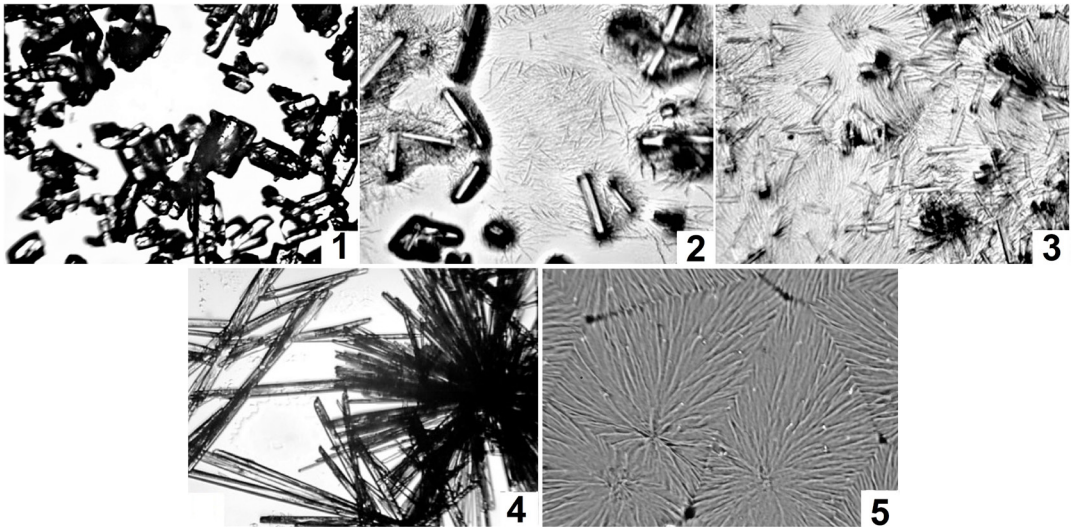

Figure 4 – Microcrystalloscopic analysis (magnification × 64)

Note: 1 – metronidazole (substance); 2 – SD metronidazole: PEG-1500 (1:1); 3 – SD metronidazole: PEG-1500 (1:3); 4 – recrystallized metronidazole substance; 5 – PEG-1500 after solvent removal

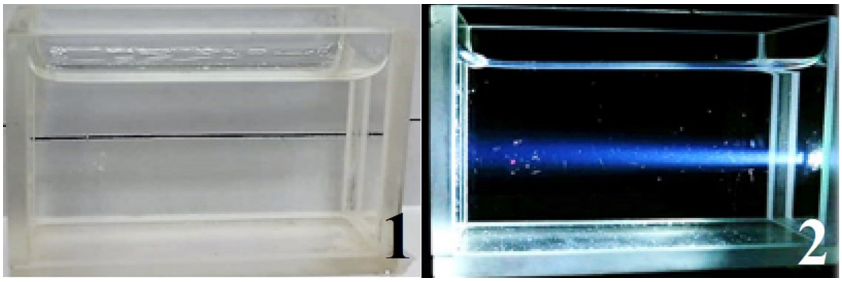

Figure 5 – Optical properties of SD metronidazole solutions

Note: PEG-1500 (1:1): 1 – appearance of the solution in daylight; 2 – the same solution, observation of Faraday-Tyndall cone

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To study the dissolution of weighed portions of the studied samples, the SD was taken in excess with respect to the solvent (purified water). The relative error for the average concentration values is ≈4.79%. The change in the solubility was calculated as the ratio of the concentration of a saturated solution of the studied sample to the concentration of a saturated solution of the metronidazole substance 60 min after the beginning of the dissolution process. By the end of the experiment, the solutions of all studied samples were cloudy and saturated. The original substance of metronidazole dissolves rather slowly (Fig. 2). After 5 minutes from the beginning of the experiment, the concentration of metronidazole reaches 7.767 mg/ml and then slightly increases, reaching an almost constant value of ≈9 mg/ml by 30 minutes.

Based on the data obtained (Table 1; Fig. 2, 3), it can be seen that in some cases metronidazole dissolves better from SD, and its solubility depends on the selected polymer and on the mass ratio of metronidazole:PEG in SD.

When using PEG-1500, the greatest increase in the solubility of metronidazole is observed in the case of SD metronidazole: PEG-1500, obtained in the mass ratios of 1:3 and 1:5 – by 14–17%. The concentration of metronidazole in the solutions of these SDs by the end of the experiment reaches 10.244 mg/ml and 10.534 mg/ml, respectively. When using PEG-2000, the greatest effect on the dissolution of metronidazole is observed in the case of SD metronidazole: PEG-2000, obtained in the mass ratio of 1: 1. The concentration of metronidazole in the solution of this SD by the time of 60 minutes reaches 10.402 mg/ml, which is 16% higher than that of the substance solution at the same time. An increase in the content of PEG-2000 in SD does not provide any pronounced increase in the solubility of metronidazole.

Thus, for SD metronidazole PEG-2000 (1:5), the solubility of metronidazole is 9.686 mg/ml, exceeding the substance solubility by 7.6% (Fig. 3). The use of PEG-3000 to obtain SD both in the ratios of 1:1 and 1:5, similarly slightly increases the solubility of metronidazole – up to 9.639 and 9.751 mg/ml, respectively (by 7.1 and 8.3%). The use of SD does not increase the dissolution rate of metronidazole in all cases. In this case, the dissolution rate of metronidazole can both increase and decrease in the first 20–30 minutes.

For solutions of some SDs, the phenomenon of supersaturation is observed. Upon dissolution of SD metronidazole: PEG-1500 (1:1) and SD metronidazole: PEG-2000 (1:1), the concentration of metronidazole sharply increases to the maximum value during the first 15 minutes. Then, probably as a result of recrystallization, the concentration decreases, with the output of the values “on the plateau” (50-60 minutes). Thus, the greatest increase in the rate of metronidazole dissolution is observed from SD with PEG-1500 (1:1). At the moment of 15 minutes from the beginning of dissolution, the concentration of metronidazole in the solution of this SD reaches its highest value – 12.885 mg/ml, which is 57% higher than the value of the concentration in the solution of the substance at the similar point of time. However, further on, by the 40th min, the concentration level of metronidazole in the SD solution decreases to the value of ≈9.8 g/ml. In the authors’ opinion, the above-described fluctuations in the metronidazole concentration of the SD solutions are associated with a number of mutually opposite processes. On the one hand, these are the processes of the metronidazole release and the PEG matrix upon dissolution of SD, and its transition into an aqueous medium in a molecular colloidal form. In this case, PEG plays the functions of a solubilizer (with a low content in SD) and/or colloidal protection, stabilizing the previously achieved high level of the metronidazole concentration. On the other hand, the processes of metronidazole recrystallization, coagulation of its colloidal particles occur, and the salting-out effect of PEG may affect it. This is especially noticeable in the case of SD with a high polymer content. The balance of these processes and their result in achieving a certain level of metronidazole concentration in the solution of its SD is difficult to describe and is a topic for the SD research.

Based on the results of the microscopy (Fig. 4), the initial substance of metronidazole is particles of the substance with a clearly pronounced crystalline structure. The fragments of crystals are colorless, transparent, oblong, layered, and in most cases, they are of the same type. Regular parallel faces in the form of a rectangular parallelepiped are traced. Presumably, the powder of the substance had not previously undergone intensive micronization. Recrystallized metronidazole differs from the initial substance and has the form of pronounced needle-like, transparent crystals. The edges are even, sometimes collected in stellate clusters. Polymer carrier (PEG) is a colorless, transparent mass located on the surface of a slide with a film without an internal structure. The surface is folded. With a high degree of probability, it can be argued that this is a non-crystalline, amorphous structure. SD with PEG are heterogeneous systems consisting of at least 3-4 phases. Some structures have a partially needle-like architecture- probably, a recrystallized substance. Very small (presumably amorphous), difficult to identify objects were notified. They represent either a stopped initial stage of recrystallization of the substance in a viscous polymer, or (possibly) its polymorphic modification, or a product of complexation with PEG. Considering the fact that the content of metronidazole in SD is from 30 to 50% by weight, the transparent background is most likely to be a solid solution of metronidazole in PEG.

Thus, SD metronidazole: PEG is a complex microcrystalline pattern that combines the features of the initial substance of metronidazole (crystalline and amorphous in nature), PEG, their solid solution, and, possibly, complexation products. When studying SD, thermo-methods are very common. With regard to SD, they are based

on the fact that melting or the thermal destruction of the active substance molecule incorporated into the polymer matrix, occurs during or after the thermal destruction of the carrier polymer. The main criterion for the formation of the complex is the disappearance of the thermal effects typical of the active substance, as an individual phase. In this case, when examining SD of metronidazole, the use of, for example, differential scanning calorimetry may be of an auxiliary nature. However, the information obtained by microscopy, in the authors' opinion, is quite sufficient to make an assumption about the effect of the crystallinity of metronidazole in SD on the increase in the solubility of the active substance from the PEG polymer matrix.

In a number of works devoted to the preparation and study of SDs, the method of IR spectroscopy is often used. Based on the analysis of the characteristic bands shift of the active substance in SD, it suggests the formation of hydrogen bonds in the complex between the carrier and drug molecules. The preceding investigation of the authors' suggest that this research method is not always possible to use when studying such multicomponent systems as SD [1–10]. First of all, this is due to the pronounced shielding effect of polymers (in this case, PEG), due to which it is often impossible to obtain a true picture of the SD components interaction. The IR spectrum of SD with PEG is almost completely identical to the IR spectrum of the studied polymer and contains almost no characteristic bands of the active substance itself. However, in the absolute majority of cases, even if the interaction with PEG was observed, it was not of a covalent nature; it was a weak interaction at the level of the occurrence of hydrogen bonds. The absence of any interaction between the components of the studied SD is indirectly confirmed by UV spectroscopy of the studied samples.

The UV spectrum of metronidazole in SD with PEG is completely identical to the UV spectrum of the initial substance of metronidazole. Microcrystalloscopy makes it possible to conclude that one of the reasons for the increase in the solubility of metronidazole from SD with PEG, is the loss of its crystalline structure even before the SD dissolution in water. At the stage of the SD preparation, when the common solvent is removed under vacuum, metronidazole is partially dissolved in the SD matrix in the medium of the PEG carrier, to form a solid solution. Then, when dissolved in water, SD, as the polymer dissolves, releases the active ingredient in a molecular colloidal form. In this case, PEG, possibly, additionally has a solubilizing effect, stabilizing the concentration of metronidazole. In addition, according to a number of reference materials [17, 19, 20, 23–25], the formation of their colloidal solutions is an important factor contributing to an increase in the dissolution of active substances from SD.

In this regard, the optical properties of the solutions obtained in the work, were studied. For the filtered solutions of all the SDs studied in the work with PEG, the Faraday-Tyndall cone is observed – scattering of light of a bluish tint due to the colloidal-dispersed state of the dissolved metronidazole (Fig. 5).

Herewith, the solutions of the auxiliary substances (PEGs) and the saturated solutions of the metronidazole substance or its mixtures with the studied polymers, similarly prepared for the study, did not demonstrate the Faraday-Tyndall effect. The results obtained, underline the fundamental importance of obtaining SD by the method of "the solvent removal" described in this work, for increasing the solubility of metronidazole in water.

CONCLUSION

The analysis of the data obtained indicates that the improvement in the dissolution of metronidazole from SD, carried out by the method of "the solvent removal" with the use of 96% ethyl alcohol as a common solvent, is associated with a decrease in the crystallinity of metronidazole upon receipt of its SD and solubilization, as well as with the formation of metronidazole colloidal solutions stabilized with PEG when SDs are dissolved.

The optimal PEG for the SD production is PEG with a molecular weight of no more than 1500 g/mol, and the best ratio of SD components (metronidazole: PEG) is 1:1 by weight. The results obtained will be used in the development of the technology of "effervescent" tablets and granules of metronidazole containing its SD with PEG.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

Ivan I. Krasnyuk (Jr.) – general management and planning of the experiment; Savva R. Naryshkin and Ivan S. Bobrov – study of metronidazole dissolution; Ivan I. Krasnyuk– measuring metronidazole concentrations of solutions; Anastasia V. Belyatskaya – preparation of solid dispersions; Olga I. Stepanova– collecting and processing of literature data; Aleksandr N. Vorobiev – investigation of solutions optical properties; Victoria G. Yankova – analysis, processing and preparation of graphic materials; Julietta V. Rau – microcrystalloscopic examinations. All the authors participated in the discussion of the results and writing the article.

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EXPERIMENTAL STUDY OF TOXIC PROPERTIES OF VMU-2012-05 DRUG – ORIGINAL NON-NUCLEOSIDE INHIBITOR OF HIV-1 REVERSE TRANSCRIPTASE

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Antiretroviral therapy is currently the main component of treatment for HIV patients. The development of new, more effective and safer drugs is an urgent task.

The aim of the research is to study the toxic properties of the finished dosage form (FDF) VMU-2012-05, a non-nucleoside reverse transcriptase inhibitor (1-[2-(2-benzoylphenoxy)ethyl]-6-methyluracil) for the HIV-1 infection treatment in single and repeated enteral administrations.

Materials and methods. The study of toxic properties in single administrations was carried out on outbred mice; the drug was administered at the limiting dose of 2000 mg/kg (by reference to the active substance). For 90 days, in repeated daily administrations, the toxic properties were studied in rats of both sexes at the doses of 0 mg/kg (placebo), 9 mg/kg (1 HTD), 45 mg/kg (5 HTD), 90 mg/kg (10 HTD). The toxic properties were also studied in rabbits of both sexes within a 28-day administration at the doses of 0 mg/kg, 4 mg/kg (1 HTD), 20 mg/kg (5 HTD), 40 mg/kg (10 HTD); the recovery period 30 days. Clinical observations and examinations, body weight registrations, physiological and clinical laboratory studies were carried out during the experiment. At the end of the administration period (50% of animals) and at the end of the recovery period, a pathological examination was performed.

Results. The LD_{50} of the drug is more than 2000 mg/kg. In the repeated administrations, the no observed adverse effect level (NOAEL) has been established. For rats, it is 9 mg/kg (1 HTD), for rabbits – 4 mg/kg (1 HTD). According to the results of the experiments carried out on rabbits and rats, the main target organ of the drug toxic effect is the liver. According to the data obtained in the study on rats, a toxic effect on the organs of the male reproductive system has been manifested (hypoplasia of the spermatogenic epithelium). Under the conditions of the experiment, the test drug had no effect on the gastrointestinal tract.

Conclusion. The results have manifested a favorable safety profile of the drug, not inferior to the ones of a similar pharmacological group used in clinical practice; it can be considered a promising drug candidate for the HIV-1 infection treatment.

Keywords: preclinical studies; HIV-1; VMU-2012-05; 1-[2-(2-benzoylphenoxy) ethyl]-6-methyluracil; toxicity; single administration; repeated administration

Abbreviations: ALT – Alanine transaminase; JSC – joint-stock company / ZAO; HAART – Highly Active Anti-Retroviral Therapy; AST – aspartate aminotransferase; APTT – activated partial thromboplastin time; BEC – Bioethics committee; HTD – highest therapeutic dose; HIV – human immunodeficiency virus; FDF – finished dosage form; AUSS – All-Union state standard; DNA – Deoxiribonucleic Acid; GIT – gastrointestinal tract; LD_{50} – half-lethal dose / 50% lethal dose; NRTI – Nucleoside Reverse Transcriptase Inhibitor; NNRTI – Non Nucleoside Reverse Transcriptase Inhibitor; SPA – Scientific Production Association; RT – reverse transcriptase; PT – prothrombin time; RNA – ribonucleic acid; AIDS – acquired immune deficiency syndrome; HR – heart-rate; AP – alkaline phosphatase; EDTA – ethylenediaminetetraacetic acid; ECG – electrocardiography; GLP – Good Laboratory Practice; NOAEL –no-observed-adverse-effect level

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ЭКСПЕРИМЕНТАЛЬНОЕ ИЗУЧЕНИЕ ТОКСИЧЕСКИХ СВОЙСТВ ПРЕПАРАТА VMU-2012-05 – ОРИГИНАЛЬНОГО НЕНУКЛЕОЗИДНОГО ИНГИБИТОРА ОБРАТНОЙ ТРАНСКРИПТАЗЫ ВИЧ-1

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Антиретровирусная терапия в настоящее время является основным компонентом лечения больных ВИЧ-инфекцией. Разработка новых более эффективных и более безопасных препаратов, является актуальной задачей.

Цель. Изучение токсических свойств готовой лекарственной формы (ГЛФ) VMU-2012-05- ненуклеозидного ингибитора обратной транскриптазы (1-[2-(2-бензоилфенокси)этил]-6-метилурацил) для лечения ВИЧ-1 инфекции при однократном и многократном энтеральном введении.

Материалы и методы. Изучение токсических свойств при однократном введении проводили на беспородных мышах, препарат вводили в лимитирующей дозе 2000 мг/кг (по активному веществу). Токсические свойства при многократном ежедневном, в течение 90 дней, введении изучали на крысах обоего пола в дозах 0 мг/кг (плацебо), 9 мг/кг (1 ВТД), 45 мг/кг (5 ВТД), 90 мг/кг (10 ВТД) и кроликах обоего пола при 28-дневном введении в дозах 0 мг/кг, 4 мг/кг (1 ВТД), 20 мг/кг (5 ВТД), 40 мг/кг (10 ВТД), период отсроченного наблюдения – 30 дней. В ходе эксперимента проводили клинические наблюдения и осмотры, регистрацию массы тела, проводили физиологические и клинико-лабораторные исследования. По окончании периода введения (50% животных) и по окончании периода отсроченного наблюдения проводили патоморфологическое исследование.

Результаты. $ЛД_{50}$ препарата – более 2000 мг/кг. При многократном введении установлен уровень доз, не вызывающих нежелательных эффектов (NOAEL), который для крыс составил 9 мг/кг (1 ВТД), для кроликов – 4 мг/кг (1 ВТД). По результатам экспериментов, проведенных на кроликах и крысах, основной орган-мишень токсического действия препарата – печень. По данным, полученным в исследовании на крысах, показано токсическое влияние на органы мужской репродуктивной системы (гипоплазия сперматогенного эпителия). Препарат в условиях проведенного эксперимента не оказал влияния на органы ЖКТ.

Заключение. Результаты показали, что препарат обладает благоприятным профилем безопасности, не уступающим показателям применяемых в клинической практике препаратов аналогичной фармакологической группы, и может рассматриваться как перспективный лекарственный кандидат для лечения ВИЧ-1 инфекции.

Ключевые слова: доклинические исследования; ВИЧ-1; VMU-2012-05; 1-[2-(2-бензоилфенокси)этил]-6-метилурацил; токсичность; однократное введение; многократное введение

Список сокращений: АЛТ – аланинаминотрансфераза; АО – акционерное общество; ВААРТ – высоко активная антиретровирусная терапия; АСТ – аспартатаминотрансфераза; АЧТВ – активированное частичное тромбопластиновое время; БЭК – биоэтическая комиссия; ВТД – высшая терапевтическая доза; ВИЧ – вирус иммунодефицита человека; ГЛФ – готовая лекарственная форма; ГОСТ – государственный стандарт; ДНК – дезоксирибонуклеиновая кислота; ЖКТ – желудочно-кишечный тракт; $ЛД_{50}$ – полулетальная доза; НИОТ – нуклеозидные ингибиторы обратной транскриптазы; ННИОТ – ненуклеозидные ингибиторы обратной транскриптазы; НПО – научно-производственное объединение; ОТ – обратная транскриптаза; ПВ – протромбиновое время; РНК – рибонуклеиновая кислота; СПИД – синдром приобретенного иммунного дефицита; ЧСС – частота сердечных сокращений; ЩФ – щелочная фосфатаза; ЭДТА – этилендиаминтетрауксусная кислота; ЭКГ – электрокардиография; GLP – Good Laboratory Practice / надлежащая лабораторная практика; NOAEL – no-observed-adverse-effect level / уровень доз, не вызывающих нежелательных эффектов

INTRODUCTION

Since the early 1980s, great strides have been made in treating HIV-infected patients. Long-term efforts of scientists have led to the creation of drugs with different mechanisms of action with antiretroviral activity. They can be divided into several groups [1]:

1. Nucleoside reverse transcriptase inhibitors (NRTIs) of HIV, competing with natural deoxynucleotides for inclusion in the growing chain of viral DNA with a subsequent disruption of the reverse transcription process, i.e., synthesis of viral DNA on the viral RNA matrix (abacavir, emtricitabine, lamivudine; tenofovir, zidov) [2].

2. Non-nucleoside reverse transcriptase inhibitors (NNRTIs) that block HIV reverse transcriptase (RT) by di-

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rect binding to the enzyme (efavirenz, etravirine, nevirapine, rilpivirine) [3].

3. Fusion inhibitors that bind to the HIV glycoprotein gp41 and disrupt the binding, fusion and penetration of virions into the cells (enfuvirtide) [4].

4. Protease inhibitors that block proteolytic cleavage of precursor proteins that are required for the production of viral particles (atazanavir, darunavir, fosamprenavir, ritonavir, saquinavir, tipranavir) [5].

5. An antagonist of CCR5-receptors, blocking the CCR receptor on the T-cell and preventing the attachment of the virus (Maravirok) [6]

6. Post-attachment inhibitors, which are monoclonal antibodies that bind CD4, preventing the virus from entering the cell (ibalizumab) [8].

7. Integrase inhibitors, blocking the action of the enzyme and preventing the insertion of the viral genome into the host cell DNA (dolutegravir, raltegravir, elvitegravir, bictegravir) [7].

8. Pharmacokinetic enhancers that inhibit the human CYP3A enzyme and increase the plasma concentration of other antiretroviral drugs (cobicistat) [9].

Currently, the main method of treating the HIV infection is highly active antiretroviral therapy (HAART), which implies the simultaneous use of several substances aimed at different stages of the HIV life cycle [10]. The use of a combination of different agents provides a synergistic antiviral effect, thereby increasing the efficiency of suppressing viral replication. Correctly selected HAART significantly increases the duration and quality of patients' life [11]. In 1981–1982, when the first cases of the HIV infection were recorded, the average life expectancy of a person with established AIDS, was 1–2 years [12, 13]. Today, for a HIV-infected person in their 20s and older who receives HAART, the projected life expectancy is about 53 years [14]. According to the recent reports from the Joint United Nations Program On HIV / AIDS (UNAIDS), 19.5 million people are now receiving life-saving HAART (accounting for 53% of all the people living with HIV in the world), and AIDS deaths have halved since 2005 year[1].

Over the past four decades, the introduction of these treatments has faced a number of problems associated with drug toxicity, inconsistent adherence to complex treatment regimens, drug resistance, low patient adherence to the prescribed treatment, and inadequate access to drugs in certain population groups [15, 16]. However, the main limitations of the existing compounds use are the formation of mutant, resistant strains of the virus in the course of therapy, which makes a constant change of drugs necessary, and the side effects, in some cases leading to premature discontinuation of treatment [17–19]. Thus, the search for new compounds with an antiretroviral activity against both the wild strain and resistant virus isolates, is an extremely important area of modern virology and medicinal chemistry.

With the emergence of new NNRTIs classes of pyrimidine nature, containing structurally complex binuclear aromatic substituents, there may be prospects for the creation of new original drugs for the treatment of the HIV-1 infection. Some representatives of pyrimidine derivatives with fragments of diphenyl ether, diphenylmethane, or benzophenone in the side chain, have demonstrated activity against wild and mutant HIV-1 strains in the nanomolar range [20]. It has been shown that pyrimidine derivatives of benzophenone exhibit the anti-HIV-1 activity *in vitro*, superior to that of nevirapine and not inferior to that of efavirenz [21]. In terms of the level of the antiviral action *in vitro*, the representatives of this class of compounds are on average 5–10 times higher than the most active analogous compounds in the absence of cytotoxic properties in new substances in the entire range of the studied concentrations (0.001–100 μM) [22].

Previous studies have shown that a representative of this class of compounds, 1-[2-(2-benzoylphenoxy)ethyl]-6-methyluracil, is highly active against HIV-1 in *in vitro* studies: it is 2.5 times higher than nevirapine and not inferior to efavirenz. The study of the acute toxicity of the substance in rats and mice showed that the LD_{50} of the compound is more than 2000 mg/kg in the oral administration. In conjunction with the previously obtained data on the effectiveness of the substance against HIV-1, it indicates the promising use of this compound in HIV therapy. A finished dosage form containing 1-[2-(2-benzoylphenoxy)ethyl]-6-methyluracil as an active substance, was developed for the oral administration.

One of the main stages in the drug candidate development after the establishment and proof of its pharmacological efficacy and mechanism of action is the assessment of its safety. In this regard, the study of the toxic properties of the finished dosage form of the drug based on the pyrimidine derivative of benzophenone in single and multiple administrations was carried out. In accordance with modern requirements for preclinical safety[2] studies, the experiments were carried out on several types of laboratory animals with the route of administration similar to that planned for clinical practice.

THE AIM of the research is to study the toxic properties of the VMU-2012-05 finished dosage

[1] UNAIDS. Press release. 2017. Available from: http://www.unaids.org/en/resources/presscentre/pressreleaseandstatementarchive/2017/july/20170720_PR_Global_AIDS_Update_2017

[2] National standard of the Russian Federation GOST R 56701-2015 dated 01.07.2016 "Medicines for medical use. Guidelines for planning preclinical safety studies for the purpose of subsequent clinical trials and drug registration". Available from: https://docs.cntd.ru/document/1200126923.

form (FDF), a non-nucleoside reverse transcriptase inhibitor (1-[2-(2-benzoylphenoxy)ethyl]-6-methyluracil) for the HIV-1 infection treatment in single and repeated enteral administrations to mice, rats and rabbits.

MATERIALS AND METHODS

Animals

The experiments on the study of the toxic properties of the FDF VMU-2012-05 in single administrations were carried out on sexually mature male and female outbred mice aged 9–11 weeks (from the nursery of «RMC «HOME OF PHARMACY» JSC). The study of the toxic properties in multiple administrations was carried out on sexually mature males and females of outbred rats aged 10–12 weeks (from the nursery of «RMC «HOME OF PHARMACY» JSC) and on sexually mature males and females of rabbits of the "White Giant" breed aged 2.5–3.5 months (from the peasant farm "Nera"). Before the beginning of every experiment, a meeting of the Bioethics Committee (BEC) of «RMC «HOME OF PHARMACY» JSC) was held. The experiments were unanimously approved by the members of the Committee (protocols of BEC No. 5.3 / 18 dated January 17, 2018 and BEC No. 6.3 / 18 dated January 17, 2018).

To exclude the influence of the researcher's preferences on the formation of the experimental groups, the selection of animals by the method of modified block randomization was carried out. The animals were kept in standard conditions in accordance with Directive 2010/63 / EU of the European Parliament and of the Council of the European Union dated September 22, 2010, on the protection of animals used for scientific purposes and in accordance with the sanitary and epidemiological rules of the structural unit 2.2.1.3218-14 "Sanitary epidemiological requirements for the design, equipment and maintenance of experimental biological clinics (vivariums)" (Resolution of the Chief State Sanitary Doctor of the Russian Federation dated August 29, 2014 No. 51). The animals were kept under controlled environmental conditions. The light regime was 12 hours of light and 12 hours of darkness.

The animals received food for keeping laboratory animals, prepared in accordance with GOST R50258-92 "Complete feed for laboratory animals. Technical conditions". The food and water were given *ad libitum.*

Study design

Planning and implementation of all the work was carried out in strict accordance with the requirements of the Ministry of Health of the Russian Federation and international standards in the field of preclinical studies of the safety of new pharmacological agents – the GLP (Good Laboratory Practice) system[3,4].

In all of the studies described below, the following FDF formulation was used:

Active substance: 1-[2-(2-benzoylphenoxy)ethyl]-6-methyluracil – 50 mg

Excipients: povidone – 200 mg; lactose monohydrate – 59 mg; microcrystalline cellulose – 29 mg; crospovidone – 40 mg; sodium carboxymethyl starch – 16 mg; colloidal anhydrous silicon dioxide – 4 mg; magnesium stearate – 2 mg.

The control animals received placebo (excipients only).

Single dose toxicity study in mice

The results of the earlier studies on the acute toxicity of a drug pharmaceutical substance based on a pyrimidine derivative of benzophenone showed that the LD_{50} of the substance when administered intragastrically to rats and mice is more than 2000 mg/kg. When administered at the dose of 2000 mg/kg, the death of rats and mice was not recorded, no pronounced signs of intoxication were notified either. Taking into account the available data on the low toxicity of the substance, in accordance with the recommendations[5,6], as well as in accordance with the principles of "3Rs", the acute toxicity of FDF of the test objects (tablets for oral administration, 50 mg) was studied when administered at the limiting dose of 2000 mg/kg in mice. The mice (5 males and 5 females) received a suspension (in a 1% starch solution) of the FDF preparation intragastrically at the dose of 2000 mg/kg (by reference to the active substance) through a ball tip needle. 5 males and 5 females received a placebo suspension of the test object (the control group). The total volume of the administration was 1.6 ml per animal weighing 20 g. Since the total volume exceeded the allowable for a single intragastrical administration to mice[7], the suspensions were injected fractionally (0.4 ml per animal weighing 20 g), with the intervals between the injections of at least 30 min.

[3] Interstate Standard of the Russian Federation GOST 33044-2014 "Principles of Good Laboratory Practice". Available from: https://docs.cntd.ru/document/1200115791. Russian

[4] Interstate standard GOST 32296-2013 "Test methods for the effects of chemical products on the human body. Basic Requirements for Tests to Assess Acute Toxicity in Intragastric Intake by a Fixed Dose Method. Available from: https://docs.cntd.ru/document/1200111000. Russian

[5] Decision of the EEC Council of November 3, 2016 N 81 "On Approval of the Rules of Good Laboratory Practice of the Eurasian Economic Union in the Sphere of Circulation of Medicines". Available from: https://www.alta.ru/tamdoc/16sr0081/. Russian

[6] Guideline for testing of chemicals. Acute Oral Toxicity – Fixed Dose Procedure No 420. OECD (2001). Available from: https://www.oecd-ilibrary.org/environment/test-no-420-acute-oral-toxicity-fixed-dose-procedure_9789264070943-en

[7] Guidelines for preclinical studies of drugs."NTsEMSP". Edited by A.N. Mironov. Volume. 1.2012.942 p. Russian

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The total observation period over the animals was 14 days. During the experiment, in order to register the signs of intoxication, the clinical observation was carried out within 4 hours after the drugs administration, then daily and weekly, a detailed clinical examination / weighing the mice was carried out immediately before the administration, one day after the administration, on the 7th and 15th days of the experiment.

To register a possible delayed effect of the drug on the locomotor and orientation-exploratory activity of the animals on the 14th day of the experiment, "Open Field" test was carried out. Euthanasia was performed on the 15th day using a CO_2 chamber. A subsequent pathological examination included necropsy, macroscopic examination, and weighing of internal organs. A study of the state of the chest and abdominal cavity and a macroscopic examination of the internal organs were carried out. Weighing of the heart, thymus, liver, spleen, kidneys, brain, testes was carried out.

Repeated dose toxicity studies in rats and rabbits

According to the draft instructions for the medical use of VMU-2012-05, developed on the basis of the study of its pharmacological activity, the mechanism of the action and the experience of the clinical use of the drugs of a similar pharmacological group and a similar mechanism of action, the clinical highest therapeutic dose (HTD) of the drug is assumed to be 100 mg per day. For a person with a body weight of 60 kg it is 1.7 mg/kg. Taking into account the coefficients of interspecies conversion of doses[8], the HTD for a rat will be 9 mg/kg, for a rabbit – 4 mg/kg. In this study, the test object was administered to the rats intragastrically at three doses: 9 mg/kg (1 HTD); 45 mg/kg (5 HTD); 90 mg/kg (10 HTD). Immediately before the injections, a suspension of the drug was prepared in a 1% starch solution with an active substance concentration of 0.94 mg/ml, 4.69 mg/ml, 9.38 mg/ml, respectively. The volume of a single injection was 2.4 ml per rat weighing 250 g. To the rabbits, the drug was administered orally at the doses of 4 mg/kg (1 HTD), 20 mg/kg (5 HTD), 40 mg/kg (10 HTD). Immediately before the injections, a suspension of FDF was prepared in a 1% starch solution with an active substance concentration of 2.79 mg/ml, 13.95 mg/ml, 27.91 mg/ml, respectively. The suspension was injected in the volume of 1.45 ml per 1 kg of the body weight. The control animals received placebo in the volume corresponding to the volume of the administrated suspensions of the test object. Each of the 4 groups consisted of 16 male and 16 female rats and 8 male and 8 female rabbits. The period of administration to rats was 90 days, to rabbits – 28 days. On the 91st day of the experiment (rats) or on the 29th day (rabbits), 50% of the animals of each group (the main groups) were euthanized, the remaining animals were euthanized after 30 days of the delayed observation (recovery groups).

During the experiment, a clinical observation was carried out daily for 1 hour after the administration of the drug and during the recovery period. A detailed clinical examination was carried out weekly, the body weight of the animals was recorded. In rats, to assess the possible effect of the drug on locomotor and orientation-exploratory activity on the 30th and 90th days of the experiment (for the animals of the main groups) and on the 120th day (for the animals of the recovery groups), "Open Field" test was performed. On the 30th and 90th days (for the animals of the main groups) and on the 120th day (for animals of the recovery groups), a clinical blood test was carried out, and the indicators of the hemostasis system were assessed: prothrombin time (PT) and activated partial thromboplastin time (APTT). The blood was taken from the rabbits twice: on the 28th day (from all animals) and on the 56th day (recovery groups). The blood was collected from the tail vein (rats) or the marginal ear vein (rabbits). The blood for a clinical analysis was collected in tubes with EDTA. Using the hematological analyzer "ABACUS JuniorVet" (Austria), an RBC count, an Hb level, hematocrit a WBC count, a platelet count, leukogram were determined in whole blood. For the determination of the hemostatic profile, the blood was taken into tubes with sodium citrate, and then centrifuged for 15 min to obtain plasma. The plasma was transferred to secondary tubes. The hemostatic profile was determined using an APG4-02-P coagulometer. Prothrombin time (PT) was determined using a set of reagents "Tekhplastin-test", Tekhnologiya-standard, Russia. APTT was determined using a set of reagents "APTV-El-test", Tekhnologiya-standard, Russia. The biochemical parameters of blood in rats were determined on the 30th and 90th days (for the animals of the main groups) and on the 120th day (for the animals of the recovery groups). Blood was taken from the rabbits twice: on the 28th day (from all the animals) and on the 56th day (from the animals of the recovery groups). For the study, the blood which had been collected in test tubes without an anticoagulant, was used. To obtain plasma, the blood was centri-

[8] Guidelines for preclinical studies of drugs."NTsEMSP".

fuged for 15 min at 3000 rpm. The resulting plasma was transferred to secondary tubes. The samples were analyzed with the help of an A-25 analyzer (Spain) using reagents from BioSystems (Spain) and in accordance with the manufacturer's instructions. The parameters evaluated were: aminotransferases (ALT and AST), creatinine, urea, albumine, alkaline phosphatase (AP), total protein, triglycerides, cholesterol, albumins / globulins ratio (calculated values), total bilirubin, globulin (calculated values), glucose.

The ECG registration in rats was carried out on the 29th and 89th days in the animals of the main groups, and on the 119th day in the animals of the recovery group; in rabbits – on the 26th day (50% of the animals) and on the 54th day (in the animals of the recovery groups). To register the ECG, the animal was preliminarily anesthetized with a mixture of Zoletila® + Xyly® at the doses of 25 mg/kg + 5 mg/kg, intramuscularly (rats) and 5 mg/kg + 2 mg/kg, intravenously (rabbits), then fixed on the operating table. The ECG registration was performed using a computer electrocardiograph for veterinary medicine "Poly-spectrum-8V". The ECG was recorded in lead II. The following indicators were assessed: heart rate (HR), RR interval (ms), P (ms), PQ (ms), QRS (ms), QT (ms). The study of the physical-chemical composition of urine was carried out using diagnostic test strips "LabStripurinalysis REF ANA-9910" and the analyzer "DocURader 2" in rats on the 28th and 88th days (in main groups) and on the 118th day (in recovery groups). In rabbits, it took place on the 27th day (in 50% of the animals, main groups) and on the 55th day (recovery groups). Urine was collected using metabolic cells, where the animal had been placed for 4 hours. Before being placed in a metabolic cage, the rats received drinking water intragastrically in the volume of 10 ml/kg. The rabbits were placed in metabolic cages without preliminary water loading. The determined parameters were: glucose, pH, bilirubin; urobilinogen, protein, urine specific gravity (USG), leukocytes, erythrocytes, ketones, nitrites.

Euthanasia of rats was carried out on the 91st day (the animals of the main groups) and on the 121st day (recovery groups) using a CO_2 camera. The rabbits were euthanized on the 29th day (50% of the animals) and on the 57th day. Euthanasia of the rabbits was carried out by means of an anesthetic overdose (Zoletil®; 25 mg/kg; intravenous). After euthanasia, the animals were carefully examined for external pathological signs. The organs extracted by necropsy were weighed (heart, thymus, liver, spleen, lungs with trachea, kidneys, adrenal glands, brain, testes / ovaries). The relative weight of the organs was calculated (the ratio of the organ mass to the body mass, expressed as a percentage).

A histological examination of the following organs was carried out: aorta, heart, trachea, lungs with bronchi, thymus, stomach, small intestine, large intestine, pancreas, liver, spleen, kidneys, urinary bladder, testes (males), ovaries (females), mandibular lymph nodes, thyroid gland, brain.

To assess the local irritating effect of the drugs during the necropsy procedure, deviations in the appearance of the organs of the gastrointestinal tract (GIT) were visually assessed, as well as their histological evaluation was carried out.

Data analysis

Descriptive statistics was applied to all data: the data were checked against the normal distribution using the Shapiro-Wilk test. Between group differences were analyzed by parametric or nonparametric methods, depending on the type of distribution. A one-way analysis of variance (ANOVA) was used to assess the normally distributed with the signs of normal distribution, followed by post-hoc Tukey's test. In case of not normally distributed data, the Kruskal-Wallis test was used with a further application of the nonparametric method of mean ranks for multiple comparisons in case of a significant influence of the factor under study. The differences were determined at the 0.05 significance level. The statistical analysis was performed using Statistica 10.0 Software. (StatSoft, USA).

RESULTS

Single dose toxicity study in mice

The death of the animals was not registered when they had been administrated with the drug at the dose of 2000 mg/kg.

At the beginning of the experiment, the body weight of female mice was 19–21 g, of males – 21–23 g. Within 14 days after the administration, there was a positive dynamics of the body weight in both the control group and in the groups of males and females administrated with VMU-2012-05. Diarrhea was observed in all animals after the administration of the last dose, of both the test object and the placebo. After 5 hours, the condition of the animals returned to normal, and then, within 14 days of the observation, no deviations from the norm were recorded.

The dose of 2000 mg/kg can be considered maximum tolerable, since the death of the animals and / or pronounced signs of intoxication were not observed when this dose was administered.

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Repeated dose toxicity studies. Influence on general habitus, results of functional tests

Throughout the experiment, three death cases of male rats were recorded. One case took place in the group administrated with the test drug at the dose of 45 mg/kg (on the 51st day of the experiment), and two – at the dose of 90 mg/kg (on the 36th and 48th days). 3–4 days before death, these animals looked depressed, their hair was rumpled; the day before the death, shortness of breath and a decrease in the muscle tone were added to the observed changes. The dead animals showed edema and hemorrhagic impregnation of the lung tissue, edema and moderate plethora of cerebral vessels. On the basis of these factors, it was recognized that an acute heart failure was the immediate cause for the animals' death. During both the administration period and the recovery period, the general condition and behavioral reactions of the remaining animals administrated with the drug, did not differ from those of the control group. The absence of the drug effect on the animals' general habitus was also confirmed by "Open Field" test: there were no changes in the individual behavior of the animals administrated with the drug, compared with the control group. In rabbits, there were no deviations from the norm according to the results of clinical examinations and observations during the entire experiment, either.

At the beginning of the experiment, the body weight of the rats was 190-200 g (males) and 178–185 g (females), the body weight of rabbits was 2800–3200 g (males) and 2300–2600 g (females). A slight slowdown in the positive dynamics of the body weight by the end of the administration period was observed only in high dose male group.

By the 91st day, the body weight of this group animals was statistically significantly reduced in comparison with the control group, while the decrease was not more than 10% of the control (Table 1). In female rats, as well as in rabbits, during the entire period of the experiment, the effect of the drug on the body weight dynamics was not revealed (Tables 2 and 3).

When assessing the individual behavior of rats in the open field test, the effect of the tested drug on the evaluated parameters was not found either on the 30th, 90th (Tables 4 and 5), or on the 120th day of the experiment.

In the course of the experiment, the functional state of the cardiovascular system was assessed according to the ECG data. Neither rats nor rabbits treated with the drug, showed any changes in the ECG parameters compared with the control group (Tables 6, 7 and 8).

Results of clinical and laboratory studies

The results of the analysis of the urine physicochemical properties showed that in the group of female rabbits administrated with the test drug at the maximum investigated dose, there was a decrease in urine pH (up to 5.5) compared to the intralaboratory norms (pH from 7 to 9)[9]. After a recovery period, a decrease in urine pH (up to 6.3) compared with the physiological norm was observed in the groups of female rabbits administrated with the test drug in medium and maximum doses. In the remaining groups of rabbits and rats, no deviations from the norm were found, either after the course of administration or after the recovery period.

According to the results of the clinical blood test on the 30th and 90th days (Table 9) of the experiment in the groups of female rats administrated with the drug at the doses of 45 mg/kg and 90 mg/kg, there was a significant increase in the number of platelets. On the 90th day, there was an increase in the number of leukocytes in these groups compared with the control group (Table 9). In the group administrated with the maximum dose, there was a shift in the leukocyte formula towards a decrease in the percentage of granulocytes and an increase in lymphocytes (compared to the control). At the same time, all the revealed changes in leukocytes and leukocyte formula did not go beyond the established intralaboratory standards for female outbred rats (leukocytes 5.5–18.0×10^9/l, the percentage of lymphocytes is 59–87%, the percentage of granulocytes is 13.5–37.6%, the platelet count is 348–950×109/l). In female rabbits of the groups administrated with medium and maximum doses, there had been an increase in the percentage of lymphocytes in relation to the control group by the end of the drug administration and a tendency to a decrease in the percentage of granulocytes (Table 10). The changes did not go beyond the intra-laboratory norms for rabbits (the percentage of lymphocytes was 30–70%, of granulocytes – 20–58%). No other differences from the control group or deviations from the physiological norm have been found out either in rats or in rabbits over the entire period of the drug administration. No delayed effects have been found out either.

According to the analysis results of the hemostasis system parameters, no clinically significant effects of the drug on PT and APTT in rabbits and rats have been established (Tables 11–14).

[9] Directory. Physiological, biochemical and biometric indicators of the norm of experimental animals / Under. ed. Makarova V.G. and Makarova M.N. SPb, 2013:116 p. Russian

Table 1 – VMU-2012-05 effect on body weight of male rats within 90-day drug administration, M ± SEM, g

Study day	Control	VMU-2012-05		
		9 mg/kg	45 mg/kg	90 mg/kg
1st	193.3±3.05 n=16	193.3±3.35 n=16	193,3±3,23 n=16	193.2±3.91 n=16
7th	222.3±3.38* n=16	223.6±3.77* n=16	220.3±4.56* n=16	211.8±4.95 n=16
14th	256.1±4.15* n=16	251.6±3.59* n=16	248.3±4.08* n=16	243.1±4.49* n=16
21st	286.0±4.91* n=16	275.9±3.62* n=16	271.9±3.77* n=16	267.8±4.33* n=16
28th	320.6±5.65* n=16	303.0±4.44* n=16	306.7±5.05* n=16	305.7±4.71* n=16
35th	302.1±4.59* n=16	286.7±5.73* n=16	284.2±5.45* n=16	281.6±4.27* n=16
42nd	337.1±6.99* n=16	316.1±6.58* n=16	309.3±5.63* n=16	310.9±5.48* n=15
49th	373.2±9.09* n=16	348.2±7.04* n=16	343.3±6.95* n=16	344.1±6.10* n=14
56th	372.7±8.61* n=16	345.7±7.07* n=16	339.2±6.81* n=15	344.6±6.25* n=14
63rd	380.3±9.03* n=16	351.2±6.90* n=16	346.3±7.63* n=15	355.4±6.88* n=14
70th	392.6±9.78* n=16	361.1±7.13* n=16	355.9±8.52* n=15	359.0±6.97* n=14
77th	401.9±9.85* n=16	369.6±7.06* n=16	366.5±8.50* n=15	374.3±7.15* n=14
84th	407.8±10.22* n=16	374.6±6.77* n=16	369.9±8.26* n=15	379.3±6.61* n=14
91st	425.6±11.44* n=16	389.6±7.28* n=16	383.7±9.77*# n=15	385.5±6.89*# n=14

Notes: * – p <0.05, the differences are statistically significant compared with the baseline in the corresponding group, Tukey's test; # – p <0.05, the differences are statistically significant compared to the control group, Tukey's test

Table 2 – VMU-2012-05 effect on body weight of female rats within 90-day drug administration, M ± SEM, n = 16, g

Study day	Control	VMU-2012-05		
		9 mg/kg	45 mg/kg	90 mg/kg
1st	180.4±1.93	180.7±1.78	180.2±2.16	180.8±2.25
7th	188.8±3.09	191.1±1.91	192.3±2.83	194.6±2.45
14th	199.7±3.64*	202.3±2.64*	200.4±2.99*	203.0±2.96*
21st	207.1±3.76*	213.7±3.51*	212.1±3.24*	221.0±5.06*
28th	231.4±4.30*	236.3±2.84*	234.6±3.65*	225.3±6.76*
35th	219.6±4.12*	224.2±2.98*	225.2±3.73*	222.5±4.22*
42nd	225.9±4.64*	232.9±3.46*	230.3±3.49*	233.6±4.94*
49th	241.3±5.53*	245.4±4.07*	248.1±4.68*	245.9±4.78*
56th	235.9±5.00*	245.8±5.00*	247.4±4.27*	239.6±5.25*
63rd	233.1±5.04*	242.6±5.21*	243.3±4.29*	236.6±4.88*
70th	238.7±5.17*	243.9±4.75*	248.5±4.14*	242.3±4.82*
77th	242.6±5.56*	250.1±5.02*	255.9±4.92*	246.7±4.85*
84th	241.3±5.89*	253.1±5.86*	254.5±4.90*	244.6±4.94*
91st	257.1±6.26*	265.8±5.92*	269.6±5.39*	257.2±4.50*

Note: * – p <0.05, the differences are statistically significant compared to baseline in the corresponding group, Tukey's test

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Table 3 – VMU-2012-05 effect on body weight of male and female rabbits within 28-day drug administration, M ± SEM, n = 8, g

Study day	Sex	Control	VMU-2012-05		
			4 mg/kg	20 mg/kg	40 mg/kg
1st	Males	3120.6±50.54	2886.3±128.79	2843.8±93.84	2895.0±112.83
	Females	2443.1±50.87	2591.3±89.16	2516.9±56.50	2540.6±47.70
7th	Males	3248.8±59.83*	2982.5±136.27	2990.6±85.48*	2966.9±112.12
	Females	2495.6±51.05	2678.1±94.76	2568.8±51.27	2620.0±49.78
14th	Males	3268.8±52.46*	3073.1±129.90*	3088.1±105.08*	3078.8±110.82*
	Females	2524.4±48.93	2737.5±90.28*	2591.3±61.17	2677.5±49.31*
21st	Males	3324.4±51.98*	3122.5±132.78*	3144.4±104.69*	3148.8±107.20*
	Females	2657.5±35.98*	2767.5±90.48*	2626.3±57.65*	2708.1±48.91*
29th	Males	3383.1±61.68*	3311.3±141.92*	3281.3±121.92*	3277.5±102.93*
	Females	2891.9±43.31*	2917.5±91.68*	2830.0±54.18*	2817.5±54.62*

Note: * – p <0.05, the differences are statistically significant compared to the baseline in the corresponding group, Tukey's test

Table 4 – Locomotor activity of rats in the open field test on the 30th and 90th days of VMU-2012-05 repeated intragastric administrations, M ± SEM, n = 16

Groups	Dose, mg/kg	Number of quadrants visited		Number of wall-huggings	
		30th day	90th day	30th day	90th day
Control	0	27.5±1.54	27.6±2.87	12.8±0.78	9.9±1.16
VMU-2012-05	9	28.1±1.53	29.8±3.19	14.0±0.97	1.4±1.23
	45	26.8±1.16	28.6±2.24	13.1±1.05	1.9±1.50
	90	24.1±1.49	27.5±2.48$	11.8±0.91	11.0±1.14$

Note: $ – number of the animals in group n = 15

Table 5 – Activity of rats in the open field test on the 30th and 90th days of repeated VMU-2012-05 intragastric administrations, Me (Q1; Q3)

Day	Groups	Dose, mg/kg	n	Number of center square entries	Number of rearings	Number of groomings	Number of urinations	Number of defecations
30th	Control	0	16	0.5 (0.0;2.0)	0.0 (0.0;1.0)	0.5 (0.0;1.5)	0.0 (0.0;1.0)	0.5 (0.0;1.0)
	VMU-2012-05	9	16	0.5 (0.0;2.0)	0.0 (0.0;1.0)	0.0 (0.0;1.5)	0.0 (0.0;1.0)	1.0 (0.0;1.0)
		45	16	1.0 (0.0;1.5)	0.0 (0.0;1.0)	0.0 (0.0;1.5)	0.0 (0.0;1.0)	0.0 (0.0;2.0)
		90	16	0.5 (0.0;1.5)	0.0 (0.0;0.0)	1.0 (0.0;2.5)	0.0 (0.0;0.5)	1.0 (0.0;2.0)
90th	Control	0	16	0.5 (0.0;1.0)	1.0 (1.0;4.5)	1.5 (0.5;3.5)	0.5 (0.0;3.0)	0.0 (0.0;1.0)
	VMU-2012-05	9	16	1.0 (0.0;2.5)	1.0 (0.0;3.5)	1.0 (0.0;2.5)	2.0 (0.0;3.5)	0.0 (0.0;0.0)
		45	16	0.5 (0.0;1.0)	1.0 (0.5;3.0)	0.0 (0.0;2.5)	1.0 (0.0;6.5)	0.0 (0.0;0.0)
		90	15	0.0 (0.0;1.0)	1.0 (0.0;3.0)	1.0 (0.0;1.0)	2.0 (0.0;2.0)	0.0 (0.0;0.0)

Table 6 – VMU-2012-05 effect on the rat ECG parameteres on the 29th day of the experiment, M±SEM, n = 8

Groups	Dose, mg/kg	Sex	Indicators					
			HR, beat/min	RR, ms	P, ms	PQ, ms	QRS, ms	QT, ms
Control	0	Males	254.9±9.2	237,1±8,7	45,8±2,7	57,0±2,4	63,0±2,5	136,8±6,6
		Females	234.4±14.1	263.8±18.6	42.3±0.9	57.3±3.5	65.1±2.9	131.0±8.2
VMU-2012-05	9	Males	280.3±6.2	214.9±4.8	41.9±0.4	53.9±2.2	69.5±2.4	142.9±9.9
		Females	264.8±13.4	219.6±10.7	37.6±1.9	49.0±1.4	75.1±2.9	149.0±8.5
	45	Males	280.3±15.9	215.1±11.3	39.9±0.8	50.4±2.6	63.4±2.2	146.0±11.0
		Females	235.5±5.1	255.5±5.5	42.3±0.7	53.4±1.9	67.1±4.1	125.8±3.1
	90	Males	242.4±11.1	242.3±15.2	45.0±4.4	57.8±4.5	63.8±1.5	128.1±4.9
		Females	248.5±8.4	243.6±8.3	42.4±1.5	50.6±1.5	64.0±1.4	152.6±8.9

Table 7 – VMU-2012-05 effect on the rat ECG parameteres on the 89th day of the experiment, M±SEM

Groups	Dose, mg/kg	Sex	n	Indicators					
				HR, beat/min	RR, ms	P, ms	PQ, ms	QRS, ms	QT, ms
Control	0	Males	8	278.3±5.1	215.3±3.4	41.8±0.8	53.1±2.1	62.1±6.5	139.9±9.9
		Females	8	304.8±7.9	198.1±4.9	47.3±2.6	56.0±2.4	65.4±2.9	154.4±9.3
VMU-2012-05	9	Males	8	294.8±5.8	204.3±3.8	42.0±1.0	52.1±2.5	64.5±3.0	139.4±6.7
		Females	8	306.5±5.7	196.3±3.6	43.9±1.9	51.5±2.6	73.5±4.6	146.0±7.6
	45	Males	8	297.8±5.9	202.1±4.3	39.0±2.0	52.3±1.2	58.4±3.3	134.4±10.2
		Females	8	298.8±10.6	202.5±6.3	40.8±0.9	50.1±0.7	67.4±4.1	155.5±9.9
	90	Males	7	284.6±14.2	215.0±13.3	48.6±4.2	57.3±2.9	63.0±4.1	136.4±7.4
		Females	8	296.1±6.7	203.4±4.5	49.0±5.2	59.9±5.1	71.8±4.4	133.8±7.7

Table 8 – VMU-2012-05 effect on the rabbit ECG parameteres on the 26th day of the experiment, M±SEM, n=4

Groups	Dose, mg/kg	Sex	Indicators					
			HR, beat/min	RR, ms	P, ms	PQ, ms	QRS, ms	QT, ms
Control	0	Males	274.8±21.3	222.3±16.2	45.5±4.8	71.3±6.2	142.5±4.1	170.8±11.1
		Females	187.5±10.5	322.5±18.7	59.0±6.4	77.0±2.5	158.8±2.0	156.8±8.1
VMU-2012-05	4	Males	300.0±9.3	200.8±5.9	55.8±7.7	70.3±7.5	139.0±7.7	163.3±11.0
		Females	208.8±6.1	288.5±8.7	51.5±2.5	82.0±4.7	146.0±12.2	192.3±14.4
	20	Males	251.3±24.9	246.3±25.2	46.5±10.1	70.8±6.2	168.8±20.4	229.5±27.6
		Females	200.3±9.7	302.3±14.9	49.5±1.0	73.0±2.6	159.0±5.2	190.5±10.5
	40	Males	267.3±7.0	225.0±6.2	52.0±3.7	66.3±4.3	166.8±3.3	190.0±8.8
		Females	182.3±6.9	330.0±12.1	49.3±2.3	79.0±2.9	156.5±3.7	206.3±23.5

Table 9 – Effect of tested drug on haemotology of the female rats on the 30th day of the experiment, M±SEM, n = 8

Investigated indicators	Control	VMU-2012-05		
		9 mg/kg	45 mg/kg	90 mg/kg
WBC, Leukocytes, 10^9/l	8.6±0.48	9.6±0.40	13.1±1.30*	12.3±1.18*
LYM, Lymphocytes, %	70.9±2.54	74.2±1.41	73.1±1.84	79.3±2.28*
MON, Monocytes, %	5.0±0.49	4.8±0.28	6.9±0.44	6.3±0.83
GRA, Granulocytes, %	24.1±2.18	20.9±1.18	20.1±1.98	14.4±1.68*
RBC, Erythrocyte, 10^{12}/l	7.6±0.21	7.4±0.08	7.6±0.12	7.0±0.41
HGB, Hemoglobin, g/l	153.5±1.95	148.1±1.36	149.1±2.81	137.9±8.18
HCT, Hematocrit, %	42.0±0.60	40.4±0.35	41.3±0.55	38.5±1.79
PLT, Platelets, 10^9/l	748.0±30.84	865.3±47.25	969.9±40.58*	989.5±57.61*

Note: * – p <0.05, statistically significant differences from the control group (one-way ANOVA, Tukey's test)

Table 10 – Effect of tested drug on haemotology of the female rabbits on the 28th day of the experiment, M±SEM, n = 8

Investigated indicators	Control	VMU-2012-05		
		4 mg/kg	20 mg/kg	40 mg/kg
WBC, Leukocytes, 10^9/l	7.2±0.29	7.6±0.60	6.5±0.51	7.9±0.46
LYM, Lymphocytes, %	61.1±1.54	61.1±2.19	67.6±1.72	67.9±2.54*
MON, Monocytes, %	3.1±0.25	3.2±0.17	2.6±0.18	3.1±0.15
GRA, Granulocytes, %	35.9±1.61	35.7±2.19	29.5±1.48	29.2±2.50
RBC, Erythrocyte, 10^{12}/l	6.1±0.15	6.2±0.25	5.9±0.21	6.0±0.11
HGB Hemoglobin, g/l	130.9±2.33	133.4±5.98	126.9±3.42	127.6±2.38
HCT Hematocrit, %	43.5±0.64	43.2±1.41	43.0±1.04	43.3±0.82
PLT Platelets, 10^9/l	302.5±17.76	320.3±30.74	289.0±22.75	304.4±32.31

Note: * – p <0.05, statistically significant differences from the control group (one-way ANOVA, Tukey's test)

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Table 11 – Results of prothrombin time (PT) determination in rats, s, M ± SEM

Groups	Dose, mg/kg	30th day of experiment		90th day of experiment		120th day of experiment	
		Males	Females	Males	Females	Males	Females
Control	0	21.6±0.37 n=8	22.7±0.16 n=8	18.4±0.25 n=8	20.0±0.35 n=8	17.3±0.27 n=8	17.6±0.27 n=8
VMU-2012-05	9	21.8±0.19 n=8	22.6±0.31 n=8	17.7±0.41 n=8	18.8±0.45 n=8	18.0±0.50 n=8	16.9±0.68 n=8
	45	21.4±0.50 n=8	22.3±0.51 n=8	17.4±0.73 n=8	19.0±0.28 n=8	18.3±0.86 n=7	16.0±0.40 n=8
	90	22.5±0.19 n=8	20.0±0.96* n=8	17.3±0.54 n=7	19.8±0.41 n=8	20.1±0.72* n=7	16.1±0.43 n=8

Note: * – statistically significant differences from the control group, Tukey's test, p <0.05

Table 12 – Results of prothrombin time (PT) determination in rabbits, s, M ± SEM

Groups	Dose, mg/kg	28th day of experiment		56th day of experiment	
		Males	Females	Males	Females
		n=8	n=8	n=4	n=4
Control	0	8.3±0.17	8.7±0.37	8.6±0.25	8.5±0.08
VMU-2012-05	4	8.1±0.20	7.9±0.26	8.0±0.18	8.0±0.16
	20	7.4±0.17*	7.5±0.14	8.3±0.20	8.1±0.19
	40	7.5±0.13*	7.2±0.42*	7.9±0.05	7.7±0.12*

Note: * – statistically significant differences from the control group, Tukey's test, p <0.05

Table 13 – Results of determination of activated partial thromboplastin time (APTT) in rats, s, M ± SEM

Groups	Dose, mg/kg	30th day of experiment		90th day of experiment		120th day of experiment	
		Males	Females	Males	Females	Males	Females
Control	0	16.2±0.67 n=8	17.6±0.89 n=8	12.8±0.42 n=8	13.3±0.94 n=8	12.2±0.33 n=8	14.2±1.06 n=8
VMU-2012-05	9	13.8±0.66 n=8	15.5±1.65 n=8	12.7±0.47 n=8	16.2±0.51* n=8	13.7±1.05 n=8	12.3±0.50 n=8
	45	13.8±0.43 n=8	18.8±0.85 n=8	12.8±0.65 n=8	14.5±0.72 n=8	13.5±0.76 n=8	12.2±0.30 n=8
	90	16.9±0.75 n=8	17.7±0.62 n=8	15.4±0.92* n=7	16.2±0.33* n=8	13.0±0.62 n=7	12.1±0.57 n=8

Note: * – statistically significant differences from the control group, Tukey's test, p <0.05

Table 14 – Results of determination of activated partial thromboplastin time (APTT) in rabbits, s, M ± SEM

Groups	Dose, mg/kg	28th day of experiment		56th day of experiment	
		Males	Females	Males	Females
		n=8	n=8	n=4	n=4
Groups	0	15.2±0.35	15.6±0.44	18.5±0.69	18.4±0.47
VMU-2012-05	4	15.1±0.72	16.8±0.61	16.9±1.16	17.4±0.51
	20	16.1±0.72	17.2±1.04	17.7±1.36	15.7±0.63*
	40	17.1±0.87	17.0±0.78	16.8±1.08	15.7±0.59*

Note: * – statistically significant differences from the control group, Tukey's test, p <0.05.

Table 15 – VMU-2012-05 effect on biochemical parameters of rat blood, M±SEM, n = 8 #

Sex	Dose, mg/kg	ALT, u/l			AST, u/l			AP, u/l		
		Day of experiment								
		30	91	121	30	91	121	30	91	121
Males	0	42±3.4	66±3.3	57±2.5	94±7.9	103±8.0	128±8.7	124±14.8	152±11.3	113±9,4
Females	0	36±5.1	57±5.0	53±2.9	86±2.2	115±4.0	139±5.6	96±7.1	134±12.0	100±4,6
Males	9	42±4.2	67±2.7	61±4.1	83,9±8.07	107±6.8	135±6.5	111±14.8	153±12.6	120±9,1
Females	9	32±2.0	56±3.8	55±7.5	95±3.6	112±5.5	135±7.3	92±12.3	125±19.3	92±9,9
Males	45	43±6.5	62±5.3	60±5.7	98±10.4	108±6.7	134±7.5	106±7.2	159±12.9	139±11,0
Females	45	31±2.5	62±5.0	50±4.6	91±4.8	98±4.8	131±10.8	117±13.1	144±12.4	91±7,7
Males	90	40±4.3	52±2.7	57±2.9	76±3.3	107±7.5	134±9.9	116±8.7	103±12.7*	122±7,0
Females	90	39±5.4	65±4.0	52±3.3	118±14.0*	112±4.6	148±4.9	103±13.5	155±14.6	87±4,5

Note: * – p <0.05, statistically significant differences from the control group, (one-way analysis of variance, Tukey's test); # – n=7 (males, 90 mg/kg group, 91st and 121st days; males, 45 mg/kg group, 121st day)

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Table 16 – VMU-2012-05 effect on biochemical parameters of rabbit blood, M±SEM

Sex	Dose, mg/kg	ALT, u/l		AST, u/l		AP, u/l	
		Day of experiment					
		28	56	28	56	28	56
		n=8	n=4	n=8	n=4	n=8	n=4
Males	0	61±4.7	50±6.3	34±3.6	24±1.4	120±7.0	106±14.8
Females	0	50±6.1	54±8.9	34±4.4	34±14.0	126±5.0	130±4.0
Males	4	56±6.7	59±6.2	33±8.3	40±11.7	123±7.3	102±10.0
Females	4	47±4.5	54±10.2	38±9.5	46±10.1	131±5.7	109±6.9
Males	20	60±5.0	57±12.0	45±7.0	29±7.5	131±3.6	115±8.4
Females	20	49±4.9	56±3.7	34±4.5	35±7.9	142±4.7	116±8.5
Males	40	64±5.0	53±4.8	53±7.0	24±2.9	139±7.4	108±13.4
Females	40	49±4.6	52±2.9	32±7.1	67±26.7	152±5.1*	130±9.9

Note: * – p <0.05, statistically significant differences from the control group, (one-way analysis of variance, Tukey's test)

Table 17 – VMU-2012-05 effect on the ralative organ weight in male rats, 91st day of the experiment, % of body weight, M±SEM

Investigated indicators	Control	VMU-2012-05		
		9 mg/kg	45 mg/kg	90 mg/kg
	n=8	n=8	n=8	n=7
Liver	3.65±0.154	3.61±0.082	3.38±0.168	3.14±0.071*
Kidneys	0.76±0.029	0.82±0.020	0.73±0.031	0.65±0.010*
Testes	1.01±0.064	1.1±0.034	0.97±0.044	0.73±0.072*

Note: * – p <0.05, statistically significant differences from the control group (one-way analysis of variance, Tukey's test)

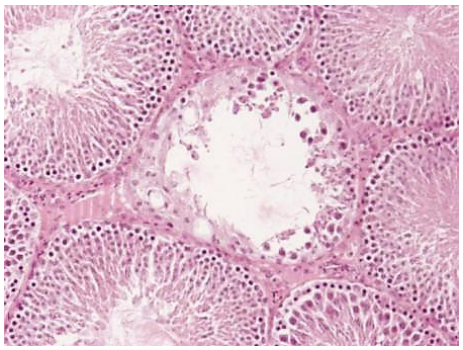

Figure 1 – Section of the testis of a male rat of the group receiving the drug at a dose of 45 mg/kg, 91st day of the experiment

Note: hypoplasia of spermatogenic epithelium. Coloring – hematoxylin-eosin, magnification×100

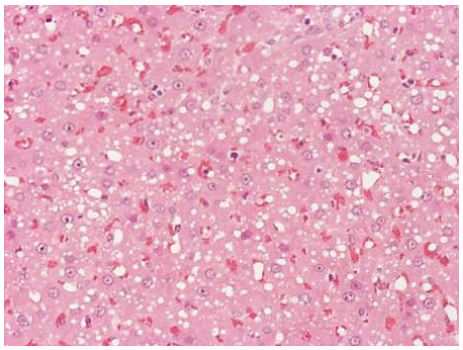

Figure 2 – Liver cross section of a male rat from the group administrated with the drug at the dose of 90 mg/kg, 91st day of the experiment

Note: Small-drop adipose degeneration of hepatocytes. Coloring – hematoxylin-eosin, magnification×200

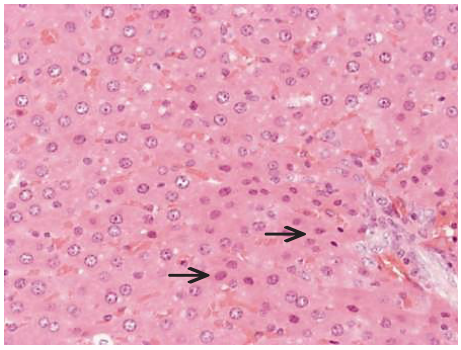

Figure 3 – Liver cross section of a female rat of the group administrated with the drug at the dose of 90 mg/kg, recovery period, 121st day of the experiment

Note: presumably apoptotic changes in hepatocytes (arrows). Coloring – hematoxylin-eosin, magnification×200

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The results of the biochemical blood test showed that against the background of the test drug administration to female rats at the maximum dose, on the 30th day of the experiment, the level of the AST activity increased (by 40% compared with the control group; the tendency that did not reach its statistical significance) (Table 15). A similar tendency (not statistically significant) was observed in male rabbits on the 28th day of the experiment: the AST activity level exceeded the indicators of the control group by 60% (Table 16). On the 28th day of the experiment, an increased level of alkaline phosphatase activity was notified in female rabbits administrated with the drug in medium and maximum doses, in comparison with the intralaboratory norms (15–140 u/l) and / or with the control group. Within the recovery period, the tested drug had no effect on the biochemical parameters of the laboratory animals' blood.

The results of the pathomorphological examination

Edema and hemorrhagic impregnation of the lung tissue, edema and moderate plethora of the cerebral vessels, as well as plethora of internal organs were recorded in necropsy of 3 male rats that had died before the planned euthanasia. An acute heart failure was the immediate cause of death in this group of animals.

In the process of histological examination, immediately after the end of the administration period at the maximum studied dose of 90 mg/kg, alveolar histiocytosis and encapsulated foci with foreign bodies in the lung tissue were revealed in two rats. A similar pathological change was found in one case after a recovery period in the medium dose group. The detected changes were probably due to the ingress of drug microparticles into the lung tissue, and, accordingly, were not directly related to its toxic effect.

On the 91st day of the experiment, hypoplasia of the spermatogenic epithelium was found out in two male rats from the groups administrated with VMU-2012-05 at the doses of 45 mg/kg and 90 mg/kg. In the same groups, the similar changes were found out in two males after a delayed observation period (Fig. 1).

In the group of rats administrated with the maximum dose, immediately after the end of the administration period, one case of small-drop hepatic steatosis was found out (Fig. 2). At the end of the recovery period, one female of the group administrated with VMU-2012-05 at the dose of 90 mg/kg showed presumably apoptotic changes in hepatocytes (Fig. 3). Small-drop hepatic steatosis was found out in three animals of this group.

In male rats, on the 91st day, immediately after the end of the administration period, a statistically significant decrease in the relative weight of the liver, kidneys and testes was revealed in the group administrated with the test drug at the dose of 90 mg/kg, compared with the control group (Table 17). After a recovery period, no differences from the control group were observed.

In female rats and in rabbits of both sexes, the effect of the test preparation on the relative organ weight, was not recorded.

While assessing the local irritant effect according to the results of histological examination on the 91st day of the experiment, one case of catarrhal gastritis was revealed in the group of male rats administrated with the test drug at the dose of 90 mg/kg. No pathological changes in the gastrointestinal tract were revealed in rabbits. At the end of the recovery period, no signs of local irritation were found in either rats or rabbits.

DISCUSSION

The study results of the toxic VMU-2012-05 properties in its single and repeated enteral administrations in mice, rats and rabbits, showed the following.

After the intragastric administration, the acute toxicity (LD_{50}) was > 2000 mg/kg. It should be notified that according to preclinical studies, the drugs of a similar pharmacological group (NNRTI) used in clinical practice, have also moderate toxicity in single enteral administrations. As for efavirenz, the minimum lethal dose for female rats ranged from 250 to 500 mg/kg, for males it was 1000 mg/kg[10]. In the experiments with nevirapine, the animal mortality (mice, rats, dogs, monkeys) was not reported in single enteral administrations in the dose range from 50 to 450 mg/kg[11].

In repeated administrations, the toxic properties of VMU-2012-05 were studied on two animal species – rats and rabbits. During the experiment on rats, three rat males died: two from the group of the maximum dose (10 HTD), one from the intermediate dose (5 HTD). The percentage of deaths was 4.7% of the total number of the animals administrated with the drug at these doses. Necropsy showed that acute heart failure had been the cause for the animals' death.

[10] Product monograph. AURO-EFAVIRENZ. Efavirenzю Tablets, 600 mg. Auro Pharma Inc. August 11, 2017. Available from: https://pdf.hres.ca/dpd_pm/00040742.PDF

[11] Product monograph. VIRAMUNE® (nevirapine). Immediate-Release Tablets 200 mg. Extended-Release Tablets 400 mg. Control Number: 167894.2013. Boehringer Ingelheim (Canada) Ltd. Available from: https://www.boehringer-ingelheim.ca/sites/ca/files/documents/viramunexrpmen.pdf

Within the periods of VMU-2012-05 administration and recovery period, the state of all other animals was characterized as satisfactory. In rabbits, no cases of death were recorded during the experiment. Therefore, it is not possible to unequivocally assert that the cause for the animals' death is directly related to the drug effect.

The results of clinical and laboratory studies revealed a slight decrease in urine pH in the female rabbits of the maximum dose group (40 mg/kg). In the rats of the maximum dose group, a decrease (no more than 15% of the control group indicators) was notified in the relative weight of the kidneys. No other changes that could indicate a violation of the urinary system functioning of rabbits and rats, were found out. These observations are not considered essential for predicting the clinical safety profile of the drug. During preclinical studies, for a number of drugs NNRTI a negative effect on the kidneys was revealed. In studies of rilpivirine, kidney toxicity has been found out in mice and dogs[12]. Nephrotoxicity was one of the main toxic effects of efavirenz in rats, with necrosis of the renal cortex, dilatation and degeneration of the tubules leading to the development of renal failure. At high doses (more than 500 mg/kg), the animals' death was caused by acute necrosis of the renal tubules. Meanwhile, no toxic effect of efavirenz on the kidneys was revealed in monkeys, despite the achievement of a systemic exposure exceeding that in rats. Later, it was shown that nephrotoxicity detected in rats is a consequence of the formation of glutathione conjugate efavirenz in this animal species, which made it possible to consider this effect as species-specific[13]. According to the results of the toxic properties study, the kidneys are not the target of nevirapine toxic action[14]. According to the results of the study carried out on two types of laboratory animals, it is also justified for VMU-2012-05.

Evaluation of the results of a clinical blood test showed a moderate effect on the leukogram and platelet count of the rats. It should be notified that, according to the results of preclinical studies, the hematopoietic system is the target of the toxic effect of nevirapine is used in clinical practice[15]. In preclinical studies of rilpivirine, a toxic effect on the hematopoietic system in mice, rats and dogs has also been established [16]. In clinical practice, among the side effects of nevirapine (according to the post-registration studies), there is a drug reaction with eosinophilia and systemic symptoms such as rash, fever, arthralgia, myalgia, etc.[17]. As a result of intaking etravirine, the following side effects can be observed: thrombocytopenia, anemia, and a decrease in the number of neutrophils [18]. In preclinical VMU-2012-05 studies, moderate changes based on the results of a clinical blood test were revealed, but taking into account the experience of clinical use of drugs with a similar spectrum of action, when planning further preclinical and clinical studies, it is necessary to take into account the potential impact on hematological parameters.

VMU-2012-05 had an effect on the functional activity of the liver (an increase in AST activity, a decrease in the relative weight of the liver in male rats of the maximum dose group, the presence of small-drop hepatic steatosis in 13% of rats in the maximum dose group, in one female (3.3% of the total number of animals) revealed presumably apoptotic changes in hepatocytes.

The experience of clinical use has shown that all NRTIs can provoke an increase in the level of transaminases, which may not be accompanied by clinical manifestations of hepatitis [23]. For example, against the background of efavirenz, an increase in AST and ALT activities more than 5 times higher than the upper limit of the norm was observed in 3% of 1008 patients taking efavirenz at the dose of 600 mg per day (5-8% with long-term antiretroviral therapy with efavirenz). A similar increase was observed in the control group (5% with long-term antiretroviral therapy without efavirenz)[19]. In preclinical studies of efavirenz, an increase in the incidence of fibrosis of the liver bile ducts in rats in multiple administrations at the doses of 500 mg/kg (about 10 HTD) and higher was found out, which was often associated with hyperplasia of the bile ducts. Changes

[12] Product monograph including patient medication information Predurant® Rilpivirine as rilpivirine hydrochloride Tablets, 25 mg Oral. Janssen Inc. Submission Control No: 223865. 2019. Available from: https://pdf.hres.ca/dpd_pm/00050300.PDF

[13] Productmonograph. AURO-EFAVIRENZ. Efavirenz Tablets, 600 mg.

[14] Product monograph. VIRAMUNE® (nevirapine).

[15] Product monograph. VIRAMUNE® (nevirapine).

[16] Product monograph including patient medication information Predurant® Rilpivirine as Rilpivirine hydrochloride.

[17] Nevirapine. Instructions for medical use. LP-005197-191118. Available from: https://grls.rosminzdrav.ru/Grls_View_v2.aspx?routingGuid=1167f0bc-0600-499f-8b2f-9ab9366bdf5f&t. Russian

[18] Intellens. Instructions for medical use. LP-006200-120520. Available from: https://grls.rosminzdrav.ru/Grls_View_v2.aspx?routingGuid=2a18740e-fe4c-4b6e-9680-a6a696993281&t. Russian

[19] Efavirenz. Instructions for medical use LP-005142-251018. Available from: https://grls.rosminzdrav.ru/Grls_View_v2.aspx?routingGuid=71eecdb2-b233-4f11-8cf2-27c5245b9479&t. Russian

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in the bile ducts were also found out in monkeys (at the doses of 150 mg/kg), while no biochemical signs of cholestasis were observed. Preclinical studies in rats and dogs have shown that the liver is a target organ for the toxic effects of nevirapine[20]. According to the experience of clinical use from the NNRTI group, it is for this drug that hepatotoxicity is most characteristic. The toxic effect on the liver can develop throughout the course of therapy and is usually most pronounced in the patients infected with hepatitis viruses in addition to HIV [23]. Considering the moderate effect of VMU-2012-05 on the functional activity of the liver, revealed in preclinical studies, the appearance of side effects in relation to the liver function cannot be excluded in case of a prolonged clinical administration of the drug. Herewith, the available experimental data do not make it possible to predict a greater severity of such effects relative to the effects of NNRTI already used in clinical practice.

When studying the toxic properties of VMU-2012-05, the presence of hypoplasia of the spermatogenic epithelium was found out in 12–14% of males from the groups administrated with the drug at medium and maximum doses. In male rats, on the 91st day, immediately after the end of the administration period, a decrease in the relative weight of the testes in the group with the maximum dose was manifested. In rabbits, no effect on the organs of the male reproductive system was found out, but it should be borne in mind that the duration of VMU-2012-05 administration to the rabbits was 28 days, while the rats were administrated with the drug for 90 days. Although the detected pathological change can occur spontaneously in rats [24], the absence of pathological changes in the control group and in the groups administrated with the drug at the lowest dose suggests that the revealed deviation may be due to the influence of VMU-2012-05. It is possible that this change is a species-specific reaction of rats to the drug. Further research is needed to confirm (or refute) this assumption. Since antiretroviral therapy is prescribed in the clinic for a long time or even permanently, the next step to study its toxic properties is to conduct studies of a longer duration (6 months in rodents, 9 months in non-rodents)[21].

[20] Product monograph. AURO-EFAVIRENZ.

[21] National standard of the Russian Federation GOST R 56701-2015 dated 01.07.2016 “Medicines for medical use. Guidelines for the planning of preclinical safety studies for the subsequent conduct of clinical trials and drug registration”.

An obligatory requirement for assessing the toxic properties of a drug is an assessment of its local tolerance. In case of the enteral administration, the effect of the drug on the organs and tissues of the gastrointestinal tract that are in direct contact with the drug, is considered. When assessing the local irritant effect according to the results of histological examination on the 91st day of the experiment, one case of catarrhal gastritis was found out in the group of the male rats administrated with the test drug at the dose of 90 mg/kg. With respect to the low frequency of occurrence (3.3% of the total number of animals) of this pathological change in the conditions of the experiment, as well as the fact that such changes in the tissues of the stomach can be considered a background pathology associated with the route of administration [25], it can be concluded that that the drug in the studied dose range did not have any local irritant effect. No pathological changes in the gastrointestinal tract have been revealed in rabbits. The data obtained suggest that the drug is relatively safe for the gastrointestinal tract when taken up to 3 months.

CONCLUSION

A preclinical study of the toxic properties of the finished dosage form based on a pyrimidine derivative of benzophenone for the treatment of the HIV-1 infection in single and repeated administrations, was carried out. The LD_{50} of FDF in the intragastric administration is more than 2000 mg/kg. In the course of the study of the toxic properties in repeated administrations, the level of the doses that do not cause undesirable effects (NOAEL) has been established. For rats it is 9 mg/kg (1 HTD), for rabbits – 4 mg/kg (1 HTD). The drug influenced the liver functional activity. A study on rats showed a toxic effect on the organs of the male reproductive system (hypoplasia of the spermatogenic epithelium). The assessment of the local irritant effect has not reveal any negative effect on the gastrointestinal tract. In general, VMU-2012-05 has shown a favorable safety profile, not inferior to the drugs of a similar pharmacological group used in the clinic, and can be considered a promising drug candidate for the treatment of the HIV-1 infection. Since antiretroviral therapy is prescribed in clinical practice for a long time or permanently, the next step for a more detailed and complete study of toxic properties is to conduct preclinical studies of toxic properties with a longer period of the drug administration (6 months in rodents, 9 months in non-rodents).

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHORS' CONTRIBUTION

Valeria A. Vavilova – experiments conducting, data collecting, data analyzing, a draft manuscript preparing; Elena V. Shekunova – experiment planning, data analysis, literature analysis; participation in manuscript writing; Ekaterina A. Jain (Korsakova) – results interpretation, literature analysis, participation in manuscript writing; Vadim Yu. Balabanyan – participation in the concept development, study design, results discussion; Alexander A. Ozerov – development of the concept and research design, results discussion; Marina N. Makarova – discussion of the results obtained, participation in manuscript writing and its final approval for publication; Valery G. Makarov – discussion of the results obtained, final approval of the manuscript for publication.

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RESEARCH ARTICLE

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SYNTHESIS, ANTIAGGREGATION AND ANTITROMBOTIC ACTIVITIES OF NEW DERIVATIVES OF HYDROXYBENZOIC ACIDS WITH TAURIC FRAGMENT

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A high prevalence of thrombotic disorders, insufficient effectiveness or safety of antithrombotic therapy is an urgent problem of modern healthcare. The main means of preventing thrombosis is acetylsalicylic acid. Despite its long history, aspirin attracts researchers in the fields of medicinal chemistry, biology, and medicine. The development of new antiplatelet agents, including chemical modification of the acetylsalicylic acid molecule, remains relevant. Modification of the acetylsalicylic acid molecule using amino acids and obtaining their salt forms makes it possible to maintain antiplatelet or antithrombotic properties, as well as to impart additional pharmacodynamic effects. In modern science, a lot of attention is paid to the sulfur-containing amino acid taurine. An analysis of modern scientific literature revealed the protective effect of taurine in diabetes mellitus and cardiovascular diseases, liver dysfunction, gastrointestinal tract, and kidney diseases.

The aim of the article is to study synthesis of new compounds, determination of their physical characteristics and assessment of their antiplatelet and antithrombotic activities *in vitro* and *in vivo*.

Materials and methods. To confirm the structure of the synthesized new derivatives of hydroxybenzoic acids with a taurine fragment by the acelation method, thin layer chromatography and NMR spectra were used. *In vitro* studies were carried out on the model of ADP-induced platelet aggregation according to the Born G. methods modified by V.A. Gabbasov. *In vivo*, the studies were carried out on the model of arterial thrombosis induced by the application of iron chloride in the following groups of animals: intact, with experimental diabetes mellitus and three-year-olds; the rate of bleeding from the tail vein was also evaluated.

Results. New compounds – derivatives of ortho-, meta- and para-hydroxybenzoic acids with a taurine residue – were synthesized. A procedure for the preparation of N-hydroxybenzoyl taurine compounds and their salt forms have been described; their spectral characteristics and melting points have been determined. The synthesized compounds are superior to acetylsalicylic acid in solubility and are not inferior to it in antiplatelet and antithrombotic activities. The results of the *in vitro* antiplatelet activity assessment in a wide concentration range from 10^{-4}M to 10^{-8}M, are presented. It has been revealed that the dipotassium salt of N-(2-hydroxybenzoyl)taurine exhibits a less antiplatelet activity than the dipotassium salt of N-(3-hydroxybenzoyl)taurine. The most pronounced antiplatelet activity is exhibited by the compound N-(4-hydroxybenzoyl) taurine. In *in vivo* experiments on the model of arterial thrombosis in 3-year-olds or animals with experimental diabetes mellitus, carotid artery thrombosis occurred faster than in young or intact animals. A single preliminary oral administration of the test compounds prolonged the time of the thrombus formation, which makes it possible to conclude that they have an antithrombotic effect. In this study, the dipotassium salt of N-(3-hydroxybenzoyl)taurine exhibits a more pronounced activity than that of acetylsalicylic acid.

Conclusion. Against the background of the modeled pathologies, the studied drugs showed the expected antithrombotic activity, in terms of the severity not inferior to that found in acetylsalicylic acid.

Keywords: antiplatelet agents; antiplatelet activity; antithrombotic activity; acetylsalicylic acid; platelet aggregation; taurine

Abbreviations: ESC – European Society of Cardiology; DMSO-d_6 – dimethyl sulfoxide-d6; GAPDH – glyceraldehyde-3-phosphate dehydrogenase (glyceraldehyde-3-phosphate dehydrogenase); HUVECs – Human Umbilical Vein Endothelial Cells (human endothelial cells); ADP – adenosine diphosphoric acid; ASA – acetylsalicylic acid; ROS – reactive oxygen species; NSAIDs – non-steroidal anti-inflammatory drugs; DM – diabetes mellitus; CVD – cardiovascular disease; COX – cyclooxygenase; GI tract – gastrointestinal tract

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СИНТЕЗ, АНТИАГРЕГАЦИОННАЯ И АНТИТРОМБОТИЧЕСКАЯ АКТИВНОСТИ НОВЫХ ПРОИЗВОДНЫХ ГИДРОКСИБЕНЗОЙНЫХ КИСЛОТ С ТАУРИНОВЫМ ФРАГМЕНТОМ

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Высокая распространенность тромботических нарушений, недостаточная эффективность или безопасность антитромботической терапии является актуальной проблемой современного здравоохранения. Основным средством профилактики тромбоза является ацетилсалициловая кислот. Несмотря на многолетнюю историю аспирин привлекает исследователей в области медицинской химии, биологии и медицины. Разработка новых антиагрегантов, в том числе и химической модификацией молекулы ацетилсалициловой кислоты остается актуальной. Модификация молекулы ацетилсалициловая кислот с использованием аминокислот и получением их солевых форм, позволяет сохранять антиагрегантные или антитромботические свойства, а также сообщить дополнительные фармакодинамические эффекты. В современной науке уделяется немало внимания серосодержащей аминокислоте таурин. При анализе современной научной литературы обнаружено протективное действие таурина при сахарном диабете и сердечно-сосудистых заболеваниях, дисфункции печени, желудочно-кишечного тракта, заболеваниях почек.

Цель. Синтез новых соединений, определение их физических характеристик и оценка антиагрегантной и антитромботической активности *in vitro* и *in vivo.*

Материалы и методы. Для подтверждения структуры, синтезированных новых производных гидроксибензойных кислот с тауриновым фрагментом методом ацелирования, использовали тонкослойню хроматографию, ЯМР спектры. Исследования *in vitro* проводили на модели АДФ-индуцированной агрегации тромбоцитов по методике Born G. в модификации Габбасова В.А. Исследования *in vivo* проводили на модели артериального тромбоза, индуцированного аппликацией хлоридом железа на следующих группах животных: интактные, с экспериментальным сахарным диабетом и трех годовалые, так же была проведена оценена скорость кровотечения из хвостовой вены.

Результаты. Были синтезированы новые соединения, представляющие собой производные орто-, мета- и пара-гидроксибензойных кислот с остатком таурина. Описана методика получения соединений N-гидроксибензоил таурина и их солевых форм, определены спектральные характеристики и температура плавления. Синтезированные соединения по растворимости превосходят ацетилсалициловую кислоту, не уступают ей в антиагрегантной и антитромботической активности. Представлены результаты оценки антиагрегантной активности *in vitro* в широком диапазоне концентраций от 10^{-4}М до 10^{-8}М. Выявлено, что дикалиевая соль N-(2-гидроксибензоил)таурина проявляет меньшую антиагрегантную активность, чем дикалиевая соль N-(3-гидроксибензоил)таурина. Наиболее выраженную антиагрегантную активность проявляет соединение N-(4-гидроксибензоил)таурин. В экспериментах *in vivo* на модели артериального тромбоза у 3-летних или животных с экспериментальным сахарным диабетом, тромбоз сонной артерии происходил быстрее, чем у молодых или интактных. Однократное предварительное пероральное введение исследуемых соединений пролонгировало время образования тромба, что позволяет сделать заключение о наличии у них антитромботического действия. Дикалиевая соль N-(3-гидроксибензоил)таурина в проведённом исследовании проявляет более выраженную чем у ацетилсалициловой кислоты активность.

Заключение. На фоне моделируемых патологий, исследуемые препараты проявили ожидаемую антитромботическую активность, по выраженности не уступающую выявленной у ацетилсалициловой кислоты.

Ключевые слова: антиагреганты; антиагрегантная активность; антитромботическая активность; ацетилсалициловая кислота; агрегация тромбоцитов; таурин

Список сокращений: ESC – европейская ассоциация кардиологии; DMSO-d_6 – диметилсульфоксид-d6; GAPDH – глицеральдегид-3-фосфатдегидрогеназа; HUVECs – эндотелиальные клетки человека; АДФ – аденозинди дифосфорная кислота; АСК – ацетилсалициловая кислота; АФК – активные формы кислорода; НПВП – нестероидные противовоспалительные препараты; СД – сахарный диабет; ЭСД – экспериментальный сахарный диабет; ССЗ – сердечно-сосудистые заболевания; ЦОГ – циклооксигеназа; ЖКТ – желудочно-кишечный тракт

INTRODUCTION

Cardiovascular diseases occupy a leading position in mortality statistics in most developed countries [1–4] and are the main cause of death in most European countries [5]. For 70 years, the main aim of the European Society of Cardiology (ESC) has been to improve the standards of diagnosis and treatment of cardiovascular diseases (CVDs), including the optimization of antiplatelet and

antithrombotic therapy [6]. First of all, this is a decrease in the number of thromboses, arising from the imbalance in the hemostasis system, being the main cause of strokes, myocardial infarction and limb amputations [7, 8].

Cardiovascular diseases are the cause of mortality for more than 50% of people in middle-income countries and <30% of people in high-income countries [9]. The observed difference arose due to the effective implementation of preventive measures and high-tech medical care, optimization of the use and improvement of the drugs quality, which in conjunction led to a decrease in mortality from CVDs [10].

The key factor determining the prognosis of most diseases of the cardiovascular system is the problem of the low efficiency of preventing arterial thrombosis. Timely implementation of preventive measures increases the life expectancy, improves its quality, and reduces the cost of treatment and rehabilitation of patients.

Antiplatelet agents, suppressing the functional activity of platelets, prevent intravascular hemocoagulation; their use has been proven to reduce the risk of thromboembolism in many socially significant diseases [11].

Attempts to improve the pharmacodynamic or pharmacokinetic properties of the known drugs are a common approach to the development of new ones.

Innovative pharmaceuticals should inhibit the functional activity of platelets more effectively and safely than the known antiplatelet agents and, at the same time, exert pleiotropic effects. Acetylsalicylic acid, blockers of ADP – P2Y12 receptors (ticlopidine, clopidogrel, prasugrel, ticangrelor, cangrelor), antagonists of glycoproteins IIb/IIIa have high efficacy and safety rates [12], but at the same time, the problem of thrombotic complications has not been fully resolved.

Acetylsalicylic acid continues to be the most often prescribed antiplatelet agent. A number of common diseases such as diabetes mellitus, metabolic syndrome, obesity are considered as independent factors of a high risk of cardiovascular complications. In the latest recommendations [13] on the primary prevention of atherosclerotic complications of diabetes mellitus (DM), low doses of acetylsalicylic acid are indicated as a means of basic therapy. This indicates the undeniable recognition of this drug merits, its therapeutic margin and availability. Chemical modification of molecules with a pronounced pharmacological activity continues to be one of the methods of drug development. Derivatization of the ASA molecule and the addition of amino acid taurine to its structure, in conjunction with the production of salt forms, makes it possible to enhance the antiplatelet effect or reduce the severity of side effects, provide an additional pharmacological action, increase the efficiency of synthesis and/or facilitate its production [14–16]. Throughout the history of the acetylsalicylic acid use, and especially intensively in the last two decades, chemists have been trying to modify its molecule in order to impart new, mainly pharmacodynamic effects to it, such as vasodilating, or the ability to generate reactive oxygen species (ROS) in tumor cells, exhibit antibacterial and antiproliferative and/or antitumor activities. So, anhydride conjugates of aspirin with fatty acids pass through cell membranes more easily, and cause a more pronounced dose-dependent platelet aggregation. Aspirin-lipid conjugates act by inhibiting the cyclooxygenase (COX)-thromboxane synthase (TXAS) pathway. All conjugates are hydrolyzed up to the parent aspirin and fatty acid molecules in a controlled manner. Aspirin-fatty acid anhydrides have a greater bioavailability (the free carboxyl group in aspirin remains an ionized species at physiological pH and is poorly absorbed through cell membranes) and an antiplatelet activity (one of the reasons for creating a hybrid "codrug" is that the prodrug aspirin-fatty acid- anhydride is hydrolyzed with the release of not one, but two active molecules, which independently inhibit COX), and are less ulcerogenic [17].

Acetylsalicylic acid and analogs of short chain fatty acids such as butyryl salicylic acid, exhibit a pronounced antimicrobial activity against *Salmonella Typhimurium.* [18].

The number of works revealing the antitumor activity of non-steroidal anti-inflammatory drugs (NSAIDs) and, in particular, ASA, continues to increase [19, 20]. Thus, aspirin derivatives based on cinnamaldehyde, are being studied as a potential agents for the treatment of colorectal cancer [21–23].

The inclusion of a metal ion in the acetylsalicylic acid molecule can impart additional pharmacodynamic properties to it. That makes it possible to retain the ability to inhibit COX. However, the ability to generate ROS by the metal part of the conjugate appears, can help overcome the resistance of tumor cells or microorganisms. The development of ASA organometallic derivatives is one of the areas of bioorganic chemistry and has become a powerful alternative to traditional approaches in the development of bioactive compounds [24]. For example, new derivatives of aspirin, which include nitric oxide (NO-aspirins) in their molecule, are safer in relation to the mucous membrane of the gastrointestinal (GI) tract, and have pronounced cytotoxic effects in relation to lung cancer [25]. IPA/NO-aspirins are prodrugs that are safer and have more pronounced pharmacodynamic effects, probably due to the improved cellular uptake and delivery. The aspirin compounds containing nitric oxide, were non-toxic to normal endothelial cells

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(HUVECs; do not affect viability up to 100 μM), but were toxic to some cancer cell lines, indicating cancer-specific sensitivity that holds promise for chemotherapy or chemoprophylaxis. The selective cytotoxicity of prodrugs based on NO-aspirin, may be associated with their effect on the activity of the glycolytic protein GAPDH (the thiol group of GAPDH is suppressed by HNO donors, which are formed during the hydrolysis of IPA/NO-aspirin in the body), the activity of which determines the rate of glycolysis in tumor cells. The use of aspirin compounds containing nitric oxide (IPA/NO-aspirin) increased the function of murine cardiomyocytes *in vivo*. This confirms that HNO donors are positive inotropic/lusitropic agents and increase transients in the Ca^{2+} channels of cardiomyocytes. Thus, prolonged forms of drugs containing aspirin and HNO or NO can have a wide therapeutic use as anti-inflammatory, antitumor, and cardioprotective agents [26].

Taurine is an organic osmolyte involved in the regulation of cell metabolism and provides a substrate for the formation of bile salts. It plays an important role in modulating the concentration of intracellular free calcium, and although it is one of the amino acids not included in proteins, taurine is one of the most abundant amino acids in the brain, retina, muscle tissue and organs [27]. A taurine derivative stimulates the formation of less toxic amyloid-β fibrils, which leads to the prevention of cognitive deficits in an acute experimental model of Alzheimer's disease in mice [28]. Taurine improves insulin secretion and decreases insulin resistance. Taurine treatment reduced the severity of oxidative stress in the brain, diabetic hepatotoxicity, the severity of vascular diseases and heart traumas in diabetes [29, 30]. The literature describes the effect of taurine supplementation on insulin resistance; the balance of iron, zinc and copper; parameters of oxidative stress in control animals and rats with a high-fat diet [31].

Thus, the synthesis and preclinical study of new derivatives of hydroxybenzoic acids is a promising and urgent problem for modern pharmacology.

THE AIM of the article is to synthesize new compounds, determine their physical characteristics and evaluate antiplatelet and antithrombotic activities *in vitro* and *in vivo.*

MATERIALS AND METHODS

Synthesis and determination of physical characteristics

The general procedure for the synthesis of dipotassium salts of N-(hydroxybenzoyl)taurines had been described in detail by the authors before [32, 33]. A solution of 2-aminoethanesulfonic acid(taurine) in 25.00 ml of water was placed in a reactor equipped with a stirrer, and 6 N. sodium hydroxide solution. Hydroxybenzoic acid chloride was added dropwise to the solution for 1.5 h under cooling. Then the reaction mixture was stirred for another 1.5 h (under cooling), controlling the pH of the medium (pH>7). The resulting mixture was poured into ice and acidified with hydrochloric acid to pH = 5, the precipitated crystals were recrystallized from isopropanol, filtered and dried. The characteristics of the compounds are presented in Table 1. Then, 100 mmol of potassium ethylate, 100.00 ml of benzene and 50 mmol of N-(hydroxybenzoyl) taurine were loaded into a 3-necked reactor equipped with a stirrer, a reflux condenser and a thermometer, and stirred at the temperature of 100°C for 30 minutes. After cooling, the product was separated by filtration, washed with a small amount of an alcoholic alkali solution and dried.

Melting points were determined by the capillary method on a Stuart SMP-30 device (Great Britain) at the heating rate of 10°C/min. The purity and individuality of the compounds were confirmed by thin-layer chromatography on Silufol UV-254 plates, the mobile phase was in an n-butanol:ethanol:water ratio of 5:2:1, the development was in iodine vapor and UV light.

^{1}H-NMR spectra of derivatives in DMSO-d_6 were recorded on a Bruker DRX500 spectrometer (Bruker, Germany) with the internal standard of hexamethyldisiloxane 500 MHz. The spectra were interpreted using a licensed software product from Advanced Chemistry Development Inc. by the trade name of ACD/HNMR Predictor Pro v. 3.

The studied N-derivatives of taurine were synthesized by the acylation reaction of taurine with an equimolar amount of 2-, 3-, or 4-hydroxybenzoic acid chloride. Then hydroxybenzoyltaurines were converted into a water-soluble form by obtaining dipotassium salts (Fig. 1).

In vitro studies

The study of the effect of substances on the functional activity of platelets *in vitro* was carried out according to the Born G. methods modified by V.A. Gabbasov (1989) [34] on a two-channel laser analyzer of platelet aggregation "Biola" 220LA (Russia). The studies were carried out on platelet-rich rat plasma according to the method described by V.A. Lyusov, Yu.B. Belousov (1971) [35]. The blood was obtained from anesthetized (chloral hydrate, 400 mg/kg, i.p., Organic, Russia) animals from the abdominal aorta [36], stabilized with a 3.8% sodium citrate solution (Reakhim, Russia) in the ratio of 9:1, then centrifuged for 10 min at 1000 rpm on a CM-6m centrifuge (ELMI, Latvia). The device was calibrated using distilled water, according to the instructions, the light transmission of distilled water was taken as 100%.

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To obtain a control sample, 300 µL of platelet-rich plasma was added to the glass cuvette of the aggregometer; after the recording of the aggregatogram had been turned on, at the 10th second of the registration process, ADP (Sigma Aldrich, USA) was added to the cuvette, at the final concentration of 5 µM [37].

To study the antiplatelet activity of the compounds under study, 30 µl of the solution of the test sample at a certain concentration was added to the cuvette with 270 µl of platelet-rich plasma. The sample was incubated in thermostated cells of the aggregometer (at 37°C for 3 minutes), after which the sample was transferred to a recording cell and the aggregatogram was recorded for 5 minutes.

Hydroxybenzoic acids derivatives and acetylsalicylic acid (the reference drug) were studied in the concentration range of 10^{-4}–10^{-8}M.

In vivo studies

The compounds were administered intragastrically once via a gastric tube. 60 minutes after the administration, the animals were anesthetized (chloral hydrate at the dose of 400 mg/kg, intraperitoneally), the left common carotid artery was isolated, and the model of intravascular thrombosis was simulated [38]. Parafilm was placed under the carotid artery. The blood flow velocity in the carotid artery had been recorded by ultrasound Doppler until it stopped completely as a result of thrombosis, initiated by applying a cotton swab moistened with a 50% solution of iron (III) chloride on the vessel.

To determine the bleeding time, the animal was cut off 5 mm from the tip of the tail, which was then placed in a flask with saline (t = 37°C) and the time until the end of the bleeding was recorded [37, 39].

Study design

Pharmacological studies of compounds in *in vitro* and *in vivo* models were performed according to the study design (Fig. 2).

Statistical processing of results

Statistical analysis of the data obtained was performed using the Microsoft Excel statistical software package and the Prism 6.0 software (Graph Pad Software Inc., USA). The data were presented as the arithmetic mean and its mean error. The comparison of the mean data of the independent samples with a normal distribution of the variant in the data set (sample), was calculated using the Student's t-test. When the variant distribution in the sample was different from the normal, the Mann-Whitney U-test (when comparing two groups) and the Kruskal-Wallis test (when comparing more than two groups) were used. A significant level of differences was considered a probability of at least 95% ($p < 0.05$).

Compliance with ethical standards

The experiments were carried out in accordance with the methodological guidelines and regulatory documents GOST ISO/IEC 17025-2009, GOST R ISO 5725-2002 and the rules of laboratory practice for preclinical studies in the Russian Federation in accordance with the Principles of Good Laboratory Practice (GOST R 33044-2014, 2015) and "On the approval of the rules of good laboratory practice" (Ministry of Health of the Russian Federation, Order No. 199n dated April 1, 2016), in compliance with Directive 2010/63 / EU of the European Parliament and the Council of the European Union dated September 22, 2010 on the protection of animals used in scientific purposes.

On compliance with ethical standards, an expert opinion from the Local Ethics Committee of Volgograd State Medical University of the Ministry of Health of the Russian Federation (registration number IRB 00005839 IORG 0004900 (OHRP)) was received.

The euthanasia of the animals was carried out in compliance with the requirements set out in the "International Recommendations for Biomedical Research Using Animals" (1997). For 24 hours before the start of the experiments, all the animals were in complete food deprivation with a free access to water.

RESULTS AND DISCUSSION

The addition of compound C-60 (dipotassium salt of N-(3-hydroxybenzoyl)taurine) to the blood plasma of the laboratory animals at the concentrations of 10^{-7} and 10^{-8} M significantly reduces the degree of platelet aggregation by 38% and 37% in comparison with the control group of the animals, and by 19% and 26% compared with the reference drug. The introduction of compound C-61 (dipotassium salt of N-(4-hydroxybenzoyl)taurine) into the platelet-rich blood plasma of the laboratory animals in all the studied concentrations, reduces the degree of platelet aggregation. At the concentrations of 10^{-4} and 10^{-5} M, the degree of aggregation decreased by 46% and 44% in comparison with the control group. When the test compound was added to the platelet-rich plasma at the concentrations of 10^{-6} and 10^{-7} M, the degree of aggregation decreased by about 10 times. At the concentration of 10^{-8}M, no statistically significant decrease in aggregation was observed.

A more pronounced antiplatelet effect of the studied compounds in comparison with ASA may be associated with the formation of a covalent bond of taurine with thiol and thioether groups of atoms or disulfide bridges in molecular targets. That had been justified for chloraminic and chlorimine derivatives of taurine [40]. The compound under the laboratory code of C-59-N-(2-hydroxybenzoyl)taurine showed its antiplatelet activity at the level of the reference drug.

The addition of compounds C-60 and C-61 at the concentrations of 10^{-5}–10^{-7} M to the blood plasma of healthy laboratory animals to the maximum and more pronounced than the reference preparation ASA, limited the development of platelet aggregation. The addition

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of ASA had an antiplatelet effect only at the concentrations of 10^{-5} and 10^{-6} M, which is confirmed by the results of numerous studies of this drug.

Compounds C-59, C-60, and C-61 are, like ASA, derivatives of hydroxybenzoic acids; therefore, there is a reason to believe that the main mechanism of the antiplatelet action is realized due to the irreversible inactivation of cyclooxygenase-1 after acetylation of the serine residue in the area of the active site. As a result, the synthesis of thromboxane A_2 is blocked, and thus the secondary activation of platelets is prevented [40].

The compounds under study are the salt form of hydroxybenzoic acids derivatives and contain potassium, which does not only improve their solubility in water, but also exhibits its own antiplatelet properties. These data are consistent with the literature data [41].

When analyzing the data obtained (Fig. 3) in the conducted experiments, it can be concluded that the most pronounced antiplatelet effect in *in vitro* studies is revealed by compound C-61, significantly superior to the similar effect of acetylsalicylic acid.

One of the important factors determining the outcomes of the cardiovascular system diseases, is the problem of arterial thrombosis [42], which is inextricably linked with the state of the hemostasis system. Thrombosis is often the cause of sudden death, myocardial infarction, vascular complications of diabetes mellitus, and limbs amputation. Therefore, the search and study of the atrombogenic effect of potential antithrombotic agents is relevant. Preclinical studies of the antithrombotic effect of new potential molecules should be evaluated not only in healthy and young animals, but also in those simulated in the state as close as possible to the clinical conditions of the antiplatelet administration. In the work by JP Garner, the following was concluded: if scientists want animal models to correspond to the pathophysiology of human diseases and be equally susceptible to the effect of the tested drugs, then the experiments should be performed on animals as if they were carried out on humans [43]. In the present work, the studies of the antithrombotic effect of new derivatives of hydroxybenzoic acids were carried out on young and healthy animals, elderly and healthy, elderly and with diabetes mellitus, which corresponds to a frequent clinical situation in which therapy with such drugs is carried out. The study of the antithrombotic activity of new derivatives of hydroxybenzoic acids was carried out on a model of arterial thrombosis induced by the application of a 50% solution of iron (III) chloride to the carotid artery of healthy animals, three-year-old (elderly) rats, and the animals with experimental pathology (diabetes mellitus, study of the bleeding time from the tail vein). The study of the antithrombotic activity of new derivatives of hydroxybenzoic acids in a model of arterial thrombosis induced by the application of a 50% solution of iron (III) chloride in healthy animals, was carried out.

In the control group of the animals, administrated with a solvent (saline), the average time of the complete occlusion of the carotid artery was 14.8±0.77 minutes.

The reference drug, acetylsalicylic acid at the dose of 36 mg/kg, increased the occlusion time by 69% ($p<0.05$).

In comparison with the control group, the compounds under study with a taurine residue under the laboratory code of C-59 at the equimolar concentration with the reference drug, prolonged the time of thrombus formation by 61%. In this experimental model of thrombosis, compound C-60 demonstrated an antithrombogenic activity and exceeded the reference drug by 3% in the time of complete occlusion and in relation to the control group, by 73% ($p<0.05$). The investigated compound C-61 increased the time of vascular occlusion twice compared with the control group ($p<0.05$), the data with the reference drug ASA did not differ. The results are presented (Fig. 4).

Study of antithrombotic effect of hydroxybenzoic acids new derivatives in model of arterial thrombosis induced by application of 50% ferric chloride solution in 3-year-old (elderly) animals

In 3-year-old (elderly) animals, the antithrombotic effect of the compounds under study was assessed in a single intragastric administration of the substances shown in Fig. 5, to the rats.

The average time of complete occlusion of the carotid artery in the animals of the control group was 16.50±0.89 minutes. The reference drug, acetylsalicylic acid, in an effective therapeutic dose increased the occlusion time to 20.14±0.55 minutes, which was 22% longer than the control group of animals. The data are statistically significant.

The compound C-60 at the dose of 18 mg/kg prolonged the time of thrombus formation to 20.67±1.20 minutes ($p<0.05$), which was 25% slower than in the group that had received saline, and 3% slower than in the group of the animals that had been administrated with the reference drug. When the compound C-61 was administered at the dose of 23 mg/kg, the time of complete occlusion was 18.17±1.56 minutes, which was 10% slower compared to the control group, and 10% slower than the group administered with the reference drug.

Thus, in the model of arterial thrombosis of the carotid artery of 3-year-old (elderly) rats, compound C-60 demonstrated the antithrombotic effect; complete occlusion of the carotid artery proceeded slower than effected by acetylsalicylic acid.

Study of platelet effect of leading compounds in model of arterial thrombosis in animals with experimental diabetes mellitus

The antithrombogenic effect of the most active compounds (C-60, C-61) was studied in the animals with streptozotocin-nicotinamide-induced diabetes mellitus.

As a result of the experiment, it was revealed that in the control group of animals, thrombus formation occurred at the 10.3th±0.78 minutes (Fig. 6).

Table 1 – Characteristics of obtained N-(hydroxybenzoyl)-substituted taurines N-(4-hydroxybenzoyl)taurine

Compound	X	Efficiency, %	Melting point, °C	Rf*	1H-NMR spectra (DMSO-d6), δ, ppm
N-(2-hydroxybenzoyl)taurine	2-OH	63.5	158–160	0.664	6.88–7.78 (4H, м, C_6H_4), 8.03–8.05 (1H, м, NH), 10.70 (1H, с, OH and SO_2OH), 2.78–3.60 (4 H, м, C_2H_4)
N-(3-hydroxybenzoyl)taurine	3-OH	63.8	199–201	0.636	6.95–7.33 (4H, м, C_6H_4), 7.50–7.83 (1H, м, NH), 9.81 (1H, с, OH and SO_2OH), 2.67–3.02 (4 H, м, C_2H_4)
N-(4-hydroxybenzoyl)taurine	4-OH	64.2	204–206	0.753	6.76–7.75 (4H, м, C_6H_4), 7.96–8.23 (1H, м, NH), 10.19 (1H, с, OH and SO_2OH), 2.23–3.34 (4 H, м, C_2H_4)

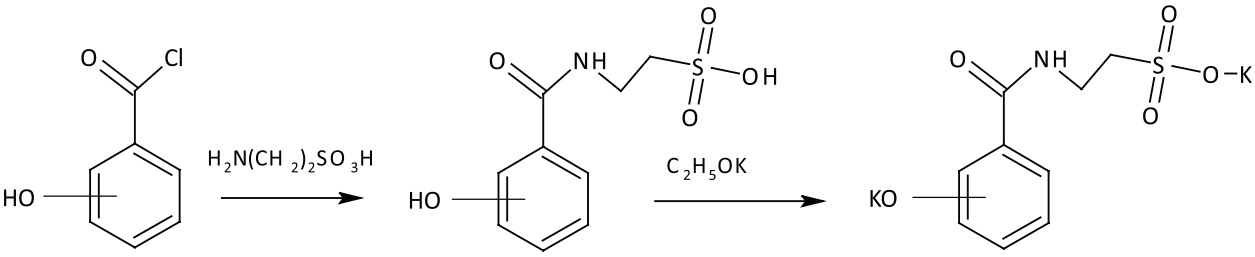

Figure 1 – Taurine acylation reaction

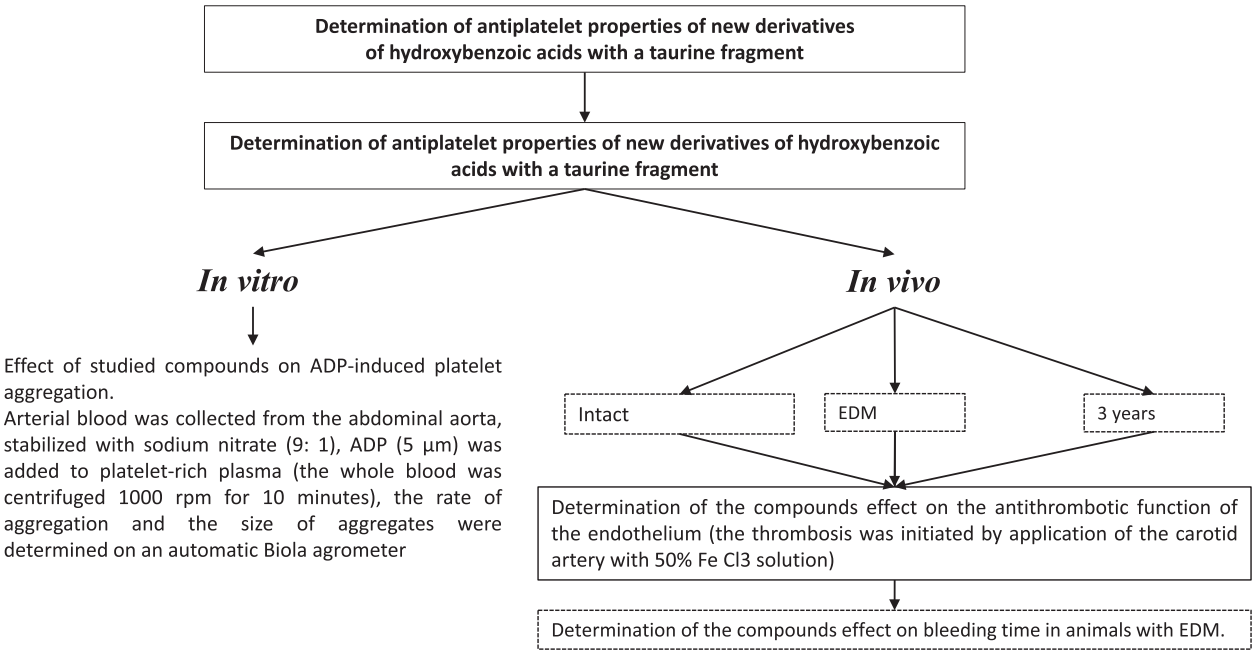

Figure 2 – Study design

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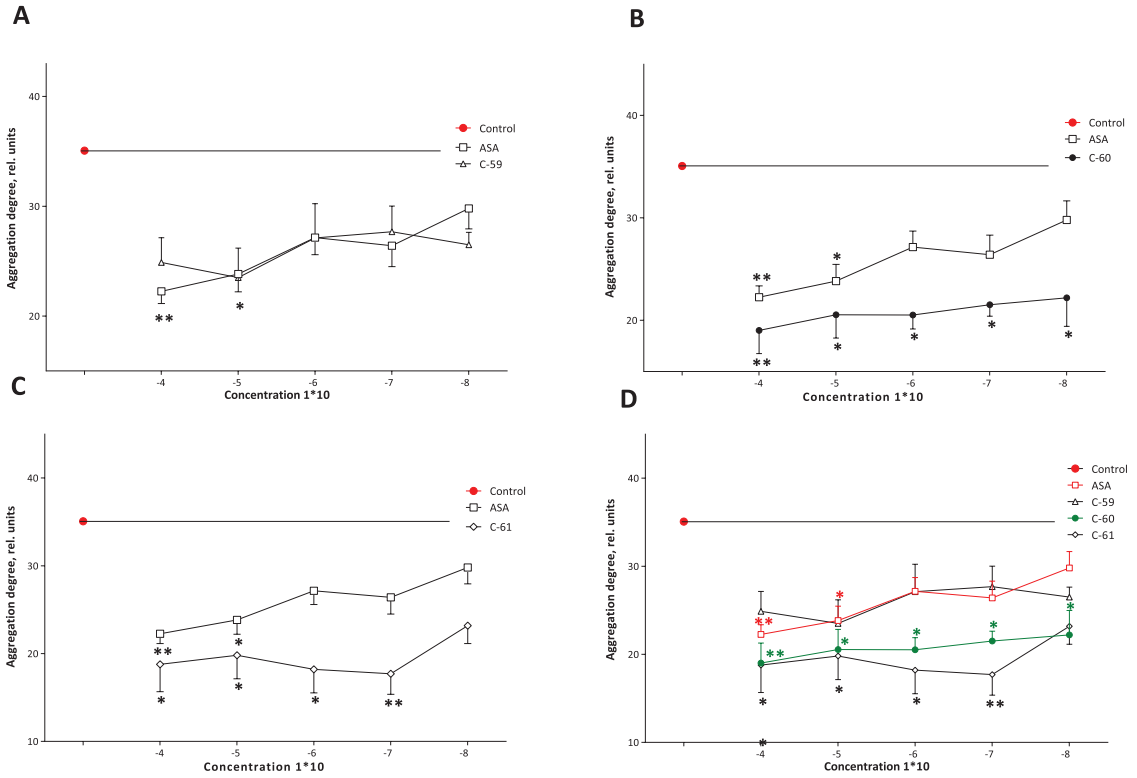

Figure 3 – Antiplatelet activity of compounds in the model of ADP-induced platelet aggregation *in vitro*

Note: A – antiplatelet activity of compound C-59; B – antiplatelet activity of compound C-60; C – antiplatelet activity of compound C-61; D – the combined scheme of the investigated compounds, reference preparation and control; * – p<0.05; ** – p<0.01 changes are statistically significant in relation to the control Student's test with Bonferroni correction.

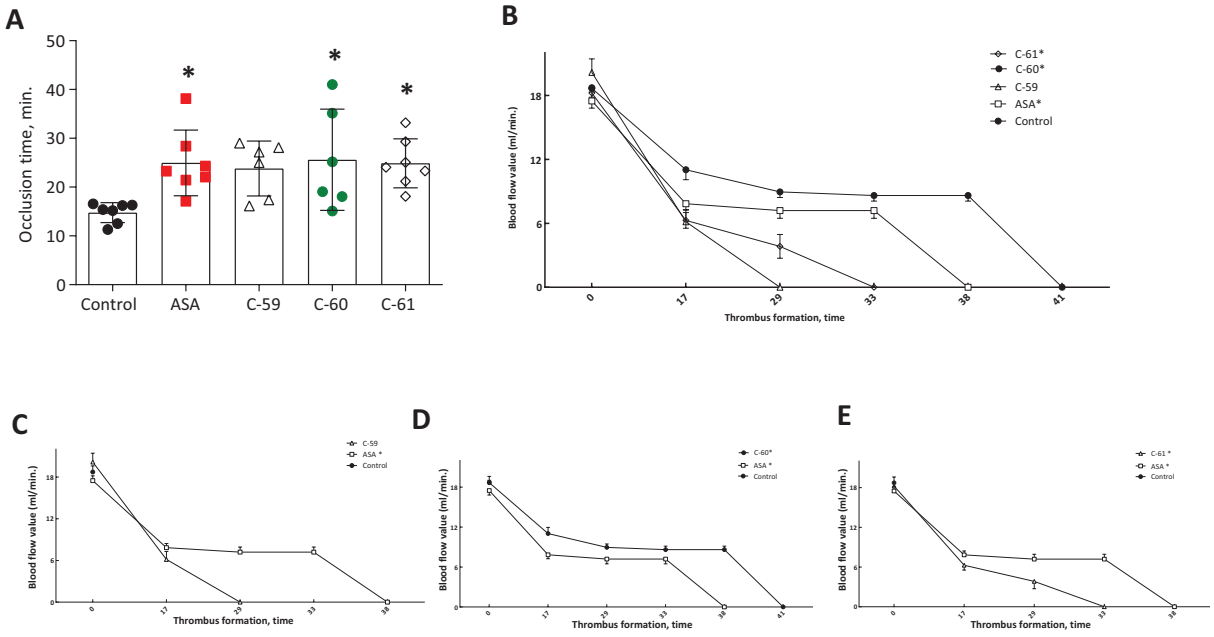

Figure 4 – Time of thrombus formation after application of 50% solution of iron chloride to carotid artery of healthy animals

Note: A – combined scheme of dynamics of thrombus formation of studied compounds, reference and control drugs; B – time of vessels complete occlusion of the studied compounds, reference and control drugs; C – dynamics of thrombus formation of the C-59 compound; D – dynamics of thrombus formation of the C-60 compound; E – dynamics of thrombus formation of the C-61 compound; * – p <0.05 changes are statistically significant in relation to the control Student's test with Bonferroni correction

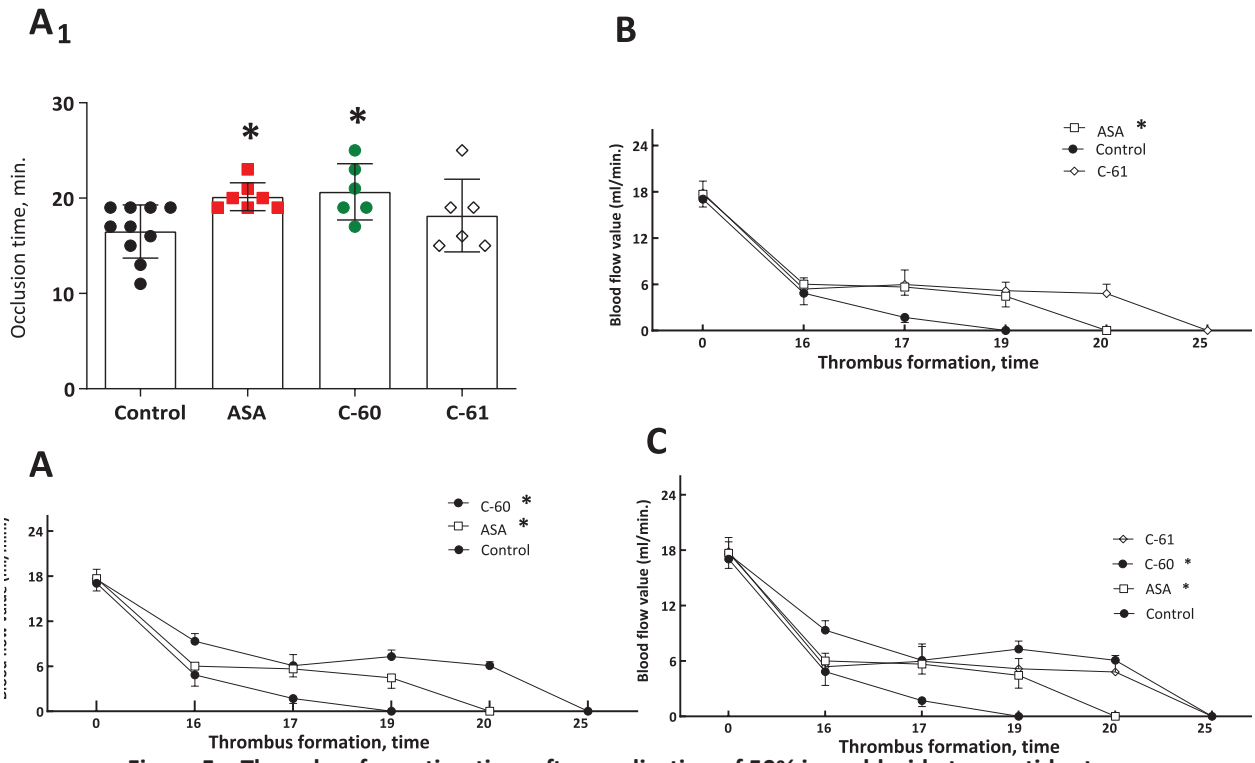

Figure 5 – Thrombus formation time after application of 50% iron chloride to carotid artery in 3-year-old (elderly) animals

Note: A – dynamics of thrombus formation of the C-60 compound; A1 is the time of complete occlusion of vessels of test compounds, reference and control drugs; B – dynamics of thrombus formation of C-61 compound; C – combined scheme of dynamics of thrombus formation of studied compounds, reference and control drugs; * – p <0.05 changes are statistically significant in relation to the control Student's test with Bonferroni's correction

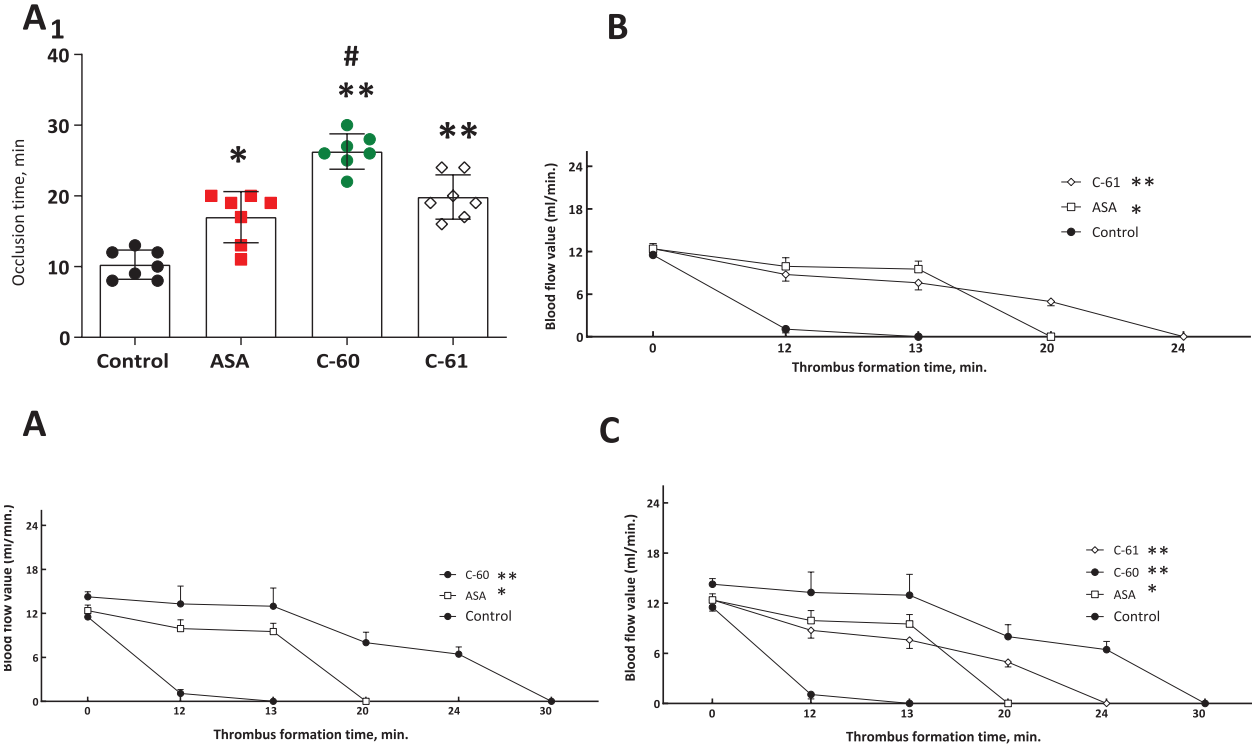

Figure 6 – Thrombus formation time after application of 50% iron chloride to the carotid artery of animals with diabetes

Note: A – dynamics of thrombus formation of C-60 compound; A1 is the time of complete occlusion of vessels of test compounds, reference and control drugs; B – dynamics of thrombus formation of C-61 compound; C – combined scheme of dynamics of thrombus formation of studied compounds, reference and control drugs; * – p <0.05, ** – p <0.01 changes are statistically significant in relation to control Student's test with Bonferroni's correction, # – p <0.05 changes are statistically significant in relation to effect of reference drug, Student's test with Bonferroni's correction

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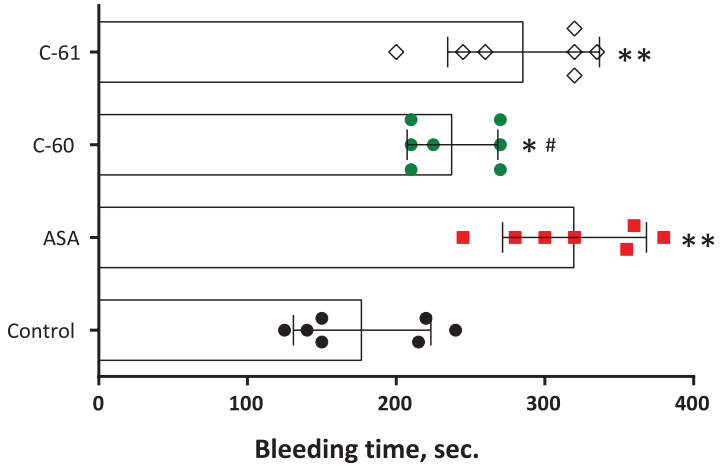

Figure 7 – Study of bleeding time in animals with diabetes mellitus
Note: * – p<0.05, ** – p<0.01 changes are statistically significant in relation to control Student's test with Bonferroni correction, # – p <0.05 changes are statistically significant in relation to effect of reference drug, Student's test with Bonferroni's correction

In an effective therapeutic dose, acetylsalicylic acid prolonged the time of complete occlusion to 17.0±1.36 minutes, which exceeded the indicators of the control group by 65%.

Compound C-60 at the dose of 18 mg/kg significantly prolonged the time of thrombus formation to 26.3±0.94 min, which was 156% slower relative to the control values and 55% slower relative to the group of the animals administered with the reference drug (p <0,01).

In the rats' group, which were administered with compound C-61 at the dose of 23 mg/kg per os, the time of complete occlusion of the carotid artery was 19.9 ± 1.18 minutes, which was 93% slower than in the control group (p <0.01) and 17% slower than in the group of the animals administered with the reference drug.

Thus, in the single oral administration to the animals with experimental diabetes mellitus in the doses taken at equimolar concentrations, the compounds under study have a pronounced antithrombotic effect and are superior to the reference drug, acetylsalicylic acid.

Investigation of leading compounds and acetylsalicylic acid effect on bleeding time from tail vein of animal with diabetes mellitus

In the control group of the animals, the bleeding time was 177.1±17.45 seconds. The studied derivatives in the doses taken in equimolar concentrations, prolonged the bleeding time. The reference drug increased the bleeding time by 81%; it was 320.0±18.29 seconds.

In the group of the animals administered with the compound under the laboratory code of C-60, the bleeding time was prolonged up to 237.9±11.54 seconds, which was 34% slower than in the control group, and 26% faster than in the group of the animals administered with the reference drug.

Compound C-61 increased bleeding time up to 285.7±19.29 seconds; which was 61% slower than in the animals administered with saline, and 11% faster than in the group of the animals administered with acetylsalicylic acid. The results are shown in Fig. 7.

CONCLUSION

According to the results of the study *(in vitro),* it was found out that the compound under the laboratory code of C-60 exhibits a pronounced antiplatelet activity, in *in vivo* studies it prolongs a thrombus formation. Compound C-60 has a more pronounced antiplatelet and antithrombotic effect than the reference drug. The safety of new derivatives of hydroxybenzoic amino acids, in terms of the effect on the bleeding rate, is comparable to the ASA indicator.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

CONTRIBUTION OF AUTHORS

A.K. Brel, Y.N. Budaeva, S.V. Lisin – methodology for synthesis of compounds, interpretation of results, text writing; N.V. Atapina, S.S. Tsaruk – research methodology, statistical processing of results, interpretation of results, text writing; D.V. Kurkin – work on the concept and design of research, research methodology, interpretation and of results visualization, text writing; I.N. Tyurenkov – work on the concept and design of research, interpretation and visualization of the results.

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ECONOMIC ASPECTS OF APPLICATION OF THE RUSSIAN BIOSIMILAR OMALIZUMAB IN PATIENTS WITH ATOPIC BRONCHIAL ASTHMA OF MODERATE TO SEVERE CLINICAL COURSES

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A certain success in the treatment of bronchial asthma is associated with the introduction of monoclonal antibodies into the treatment process. They made it possible to improve the control of the disease. A number of original genetically engineered biological drugs, such as benralizumab, reslizumab, dupilumab, mepolizumab and omalizumab, are currently registered in Russia. In 2020, this list was supplemented by the first Russian biosimilar drug omalizumab – Genolar® (JSC Generium, Russia). High rates of the development of modern medicine are closely related to the use of biosimilars. The prescription of biosimilars today often makes it possible to provide a larger number of patients with modern drugs at lower costs.

The aim of the study was a comprehensive pharmacoeconomic assessment of the application of the domestic biosimilar drug omalizumab in the treatment of patients suffering from moderate and severe atopic bronchial asthma.

Materials and methods. At the first stage, an information search in the available databases (Cochrane Library, MedLine, Embase, eLIBRARY) was carried out. According to the results obtained, a meta-analysis (Agache I. et al.) was found out; within its framework, the efficacy and safety of the use of several monoclonal antibodies was assessed. Dupilumab was chosen as the reference drug. Pharmacoeconomic analyses were carried out using a "Cost-Minimization Analysis" (CMA) and a "Budget Impact Analysis" (BIA). Taking into account various options of bronchial asthma, the developed algorithm for providing medical care to adult patients with atopic asthma made it possible to assess the costs, including direct medical and indirect costs.

Results. The cost analysis demonstrated the advantage of using the Russian biosimilar omalizumab in patients with atopic asthma compared to dupilumab due to financial savings of up to 40%. The Budget Impact Analysis showed that the use of the domestic biosimilar omalizumab, even taking into account the annual increase in the number of patients (8%), will save up to 109,641,409.64 rubles (or 3%) compared to the current practice.

Conclusion. The use of the domestic biosimilar omalizumab in patients with moderate to severe atopic bronchial asthma is a clinically effective and economically justified approach to organizing medical care for adult patients in Russia.

Keywords: bronchial asthma; biosimilar; omalizumab; dupilumab; costs; pharmacoeconomic analysis

Abbreviations: BA – bronchial asthma; BIA – Budget Impact Analysis; IGCSs – inhaled glucocorticosteroids; OGCSs – oral glucocorticosteroids; GEBDs – genetically engineered biologic drugs; VEDs – vital and essential drugs; GETE – Global Evaluation of Treatment Effectiveness; CMA – cost minimization analysis; A – Ambulance / EMS – Emergency medical services; RICU – resuscitation and intensive care unit; ALV – artificial lung ventilation; CHI – compulsory health insurance; GDP – gross domestic product; VAT – value added tax; PSGs – Programme on State Guarantees; DRG – diagnostic related groups; IIC – input intensity coefficient; INN – international non-proprietary name; TN – tradename

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ЭКОНОМИЧЕСКИЕ АСПЕКТЫ ПРИМЕНЕНИЯ РОССИЙСКОГО БИОАНАЛОГА ОМАЛИЗУМАБА У ПАЦИЕНТОВ С АТОПИЧЕСКОЙ БРОНХИАЛЬНОЙ АСТМОЙ СРЕДНЕТЯЖЕЛОГО И ТЯЖЕЛОГО ТЕЧЕНИЯ

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Определённый успех в лечении бронхиальной астмы связан с внедрением в лечебный процесс моноклональных антител, которые позволили улучшить контроль заболевания. На территории России в настоящее время зарегистрирован целый ряд оригинальных генно-инженерных биологических препаратов, таких как бенрализумаб, реслизумаб, дупилумаб, меполизумаб и омализумаб. В 2020 году этот список пополнил первый российский биоаналог препарата омализумаб – Генолар® (АО «Генериум», Россия). Высокие темпы развития современной медицины тесно связаны с применением биоаналогов. Назначение биоаналогов сегодня зачастую дает возможность обеспечить большее количество пациентов современными препаратами за счет более низкой стоимости.

Цель. Проведение комплексной фармакоэкономической оценки применения отечественного биоаналогичного препарата омализумаба при лечении пациентов, страдающих атопической бронхиальной астмой среднетяжелого и тяжелого течения.

Материалы и методы. На первом этапе был проведен информационный поиск в доступных базах данных (Cochrane Library, MedLine, Embase, eLIBRARY), по результатам которого был обнаружен мета-анализ Agache I., с соавторами, 2020 г., в рамках которого проводилась оценка эффективности и безопасности применения нескольких моноклональных антител. В качестве препарата сравнения был выбран дупилумаб. Фармакоэкономический анализ проводился с применением метода «минимизации затрат» и анализа влияния на бюджет. Разработанный алгоритм оказания медицинской помощи взрослым пациентам с атопической бронхиальной астмой, учитывающий ее различные варианты, позволил провести оценку затрат, включающую прямые медицинские и непрямые затраты.

Результаты. Анализ затрат продемонстрировал преимущество применения российского биоаналога омализумаба у пациентов с атопической бронхиальной астмой по сравнению с дупилумабом в связи с экономией финансовых средств до 40%. Анализ влияния на бюджет показал, что применение отечественного биоаналога омализумаба даже с учетом ежегодного прироста числа пациентов (8%) позволит сэкономить до 109 641 409,64 руб. (или 3%) по сравнению с текущей практикой.

Заключение. Применение отечественного биоаналога омализумаба у пациентов с атопической бронхиальной астмой среднетяжелого и тяжелого течения является клинически эффективным и экономически выгодным подходом к организации медицинской помощи взрослым пациентам на территории России.

Ключевые слова: бронхиальная астма; биоаналог; омализумаб; дупилумаб; затраты; клинико-экономический анализ

Список сокращений: БА – бронхиальная астма; АВБ – анализ влияния на бюджет; иГКС – ингаляционные глюкокортикостероиды; пГКС – пероральные глюкокортикостероиды, ГИБП – генно-инженерные биологические препараты, ЖНВЛП – жизненно необходимые и важнейшие лекарственные препараты; GETE – глобальная оценка эффективности лечения (Global Evaluation of Treatment Effectiveness); СМА – анализ «минимизации затрат» (cost-minimization analysis); СМП – скорая медицинская помощь; ОРИТ – отделения реанимации и интенсивной терапии; ИВЛ – искусственная вентиляция легких; ОМС – обязательное медицинское страхование; ФФОМС – Федеральный фонд обязательного медицинского страхования; ВВП – валовый внутренний продукт; НДС – налог на добавленную стоимость; ПГГ – программа государственных гарантий; КСГ – клинико-статистические группы; КЗ – коэффициент затратоемкости; МНН – международное непатентованное наименование; ТН – торговое наименование

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INTRODUCTION

Bronchial asthma (BA) is a serious medical and social problem that occurs in almost 262 million people [1]. Regardless of age, when treated insufficiently, patients with this disease are subject to various restrictions of daily life, a decrease in its quality, or even, in extreme cases, death[1]. Recently, there has been a higher increase in the prevalence of BA and the mortality rate from it in developing countries [2]. As evidenced by official statistics[2,3] and the results of epidemiological studies, an increase in the number of patients with this pathology is also typical of the domestic population [3–5].

A high prevalence, a long-term chronic course of the disease, the need for constant drug therapy – all these factors determine a high social significance of BA [6, 7]. Asthma is a significant socioeconomic burden for low- and middle-income countries [3]. The term "a socio-economic burden" refers not only to high costs of treatment (direct medical costs), but also to the costs associated with both temporary and permanent kinds of disability (direct non-medical costs), limitation of physical and social activities, and, as a consequence, a decrease in the quality of life of patients and their families (indirect costs) [8]. As defined by the World Health Organization, the global burden of disease is measured in terms of the number of years of life lost as a result of disability. This concept combines the years of life lost due to a state of health that does not meet the criteria for full health, and the years of life lost due to premature mortality [9].

According to the data published in 2019, among all diseases, BA was placed high in terms of the socio-economic burden: among the children aged 0 to 9 years (the 19th place); among the adults aged 50 to 74 years (the 28th place); in the group of 75 years and older (the 24th place). In the overall picture of the global burden of all the diseases, BA amounted to 0.85% of all life years lost as a result of disability (Disability Adjusted Life years, DALY) [10]. In 2019, the uncontrolled asthma caused the loss of 752 thousand years of quality-adjusted life years (QALYs) in the United States [11]. Medical expenses for the struggle against BA in the United States in the period from 2008 to 2013 only amounted to about 50.3 billion US dollars [12], in Greece in 2019 the economic losses due to BA amounted to 727 million euros [13].

Asthma is a common cause of absenteeism from school and work days. A retrospective analysis showed that in the United States, school-age children with BA are 1.54 times more likely to miss classes. That leads to a more frequent absenteeism on the part of their parents – they miss their work 1.16 times as often. This results in an annual loss of about 7 million school days [14]. This indicator also correlates with the severity of asthma. So in the work by Song H.J. et al. in 2020 [15] it was reported that for a year, 1 patient with mild, moderate and severe BA accounts for 0.76, 2.31 and 7.19 lost school and work days, respectively. In terms of indirect costs, it leads to additional expenses per person in the amount of 106, 321 and 1000 US dollars per year. A severe course of asthma deserves special attention, since in this case the focus should be not only on significant economic costs, but also on a significant decrease in the quality of life and an increase in the risk of death [16, 17]. For example, in Turkey, about 4.4 thousand US dollars is spent annually per patient with severe asthma [18]. A retrospective analysis for the period of June–November, 2016 in Spain showed that severe asthma results in an average loss of 9.1 working days per patient per year, while the average direct costs are about 7.5 thousand euros per patient per year. Taking into account indirect costs, the amount increases to 8.6 thousand euros per year [19].

According to American researchers, the socioeconomic burden of uncontrolled asthma in adolescents and adults over the next 20 years will be about US dollars 963.5 billion and 15.46 million lost QALYs [11]. It should be notified that all these are related to preventable losses, since they are associated with insufficient control of the disease.

A disease control is the most important criterion for the effectiveness of BA therapy; it implies a stable state with minimum symptoms of BA or their absence. That leads to a significant decrease in the likelihood of exacerbation and, accordingly, hospitalization, which reduces the burden of disease for both patients and the healthcare system. [20, 21].

Therefore, this problem is considered not only as a medical one, but also as an important problem of the organization and economics of the health care system of the Russian Federation [21]. In the general structure of the disease, about 5–10% [22] of patients can be attributed to suffering from severe BA. Such patients do not answer well the standard medical therapy or achieve control over the disease only with the use of inhaled glucocorticosteroids (IGCSs) in high doses and / or oral corticosteroids (OGCSs)[4]. As a rule, severe BA is associated with an increase in the frequency of using resources of

[1] Global Initiative for Asthma. Global Strategy for Asthma Management and Prevention, 2021. Available from: http://www.ginasthma.org/.

[2] Aleksandrova G.A., Golubev N.A., Tyurina E.M., Oskov Yu.I., Shelepova E.A., Polikarpov A.V., Kadulina N.A., Belyaeva I.M., Gladkikh T E.E., Shcherbakova G.A., Semenova T.A. The incidence of the entire population of Russia in 2019 with a diagnosis established for the first time in life. Statistical materials. Part I. M .: Department of Monitoring, Analysis and Strategic Development of Health Care of the Ministry of Health of the Russian Federation, Federal State Budgetary Institution "Central Research Institute for Organization and Informatization of Health Care" of the Ministry of Health of the Russian Federation. 2020. Available from: https://mednet.ru/napravleniya/mediczinskaya-statistika. Russian

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[4] Global Initiative for Asthma. Global Strategy for Asthma Management and Prevention, 2021.

the health care system due to more frequent exacerbations and, accordingly, more frequent visits to medical institutions at various levels [23].

Progress in the treatment of severe asthma was achieved against the background of the introduction of monoclonal antibodies into the treatment process. The development of genetically engineered biological drugs (GEBDs) has made it possible to achieve disease control in the most difficult to treat group of patients [24].

A number of GEBDs, such as benralizumab, reslizumab, dupilumab, mepolizumab, and omalizumab, are currently registered in the Russian Federation [25]. The efficacy and safety of these drugs have been proven in numerous clinical studies; they are included in the list of vital and essential drugs (VEDs)[5] and federal clinical guidelines[6].

Given the significant pace of development, modern medicine is currently closely related to the use of biosimilars. Prescribing biosimilars today often makes it possible to provide more patients with vital and essential drugs at lower costs. The market analysis demonstrates the possibility to achieve significant budget savings after the release of biosimilar drugs [26]. The development and research of biosimilars in the Russian Federation are carried out in accordance with international requirements in order to prove their comparability in terms of quality, safety and efficacy to the original drug [27].

In 2020, a domestic biosimilar of the drug omalizumab, TN Genolar® (JSC Generium, Russia)[7], was registered in Russia. Its efficacy, safety and immunogenicity were demonstrated in a double-blind, parallel-group, randomized, phase III study conducted from June 2018 to December 2019 (NCT04607629 Clinicaltrials.gov Database). On the basis of 25 clinical centers, 191 patients took part in the study. They were randomized into 2 groups in the 2:1 ratio: the 1st (n = 127) was treated with TN Genolar® for 52 weeks ± 3 days; and the 2nd (n = 64) was treated with TN Xolar® for 26 weeks ± 3 days [28].

The main criterion for the effectiveness of therapy in this study was the proportion of patients with a research physician rating "excellent" or "good" on the scale of the Global Evaluation of Treatment Effectiveness (GETE) after 26 weeks of therapy. In both studied populations (PP (per protocol) and FAS (full analyzes set)), no statistically significant differences were found out (p> 0.05); according to the GETE scale, in the 1st group it was 57.4%, in the 2nd – 45.2% (p = 0.132). The calculated one-sided 95% confidence interval in order to test the statistical hypothesis of the research that the studied drug is "not worse" than the reference drug in the PP-population was –0.5–25.0% (p = 0.116) and –1, 1–24.2% (p = 0.134) – in the FAS population. The safety analysis showed the comparability of the analyzed drugs in terms of the incidence of adverse events. Based on the results of the detection frequency analysis of common antidrug antibodies to omalizumab, the absence of the antibody production in response to the administration of the studied drugs was shown.

Based on the results of the clinical study [28], it can be concluded that the drugs TNs Genolar® (JSC Generium, Russia) and Xolar® (Novartis Pharma AG, Switzerland) are clinically comparable in patients with moderate to severe atopic BA.

Thus, a high prevalence of asthma, as well as the difficulties associated with achieving control over the disease, a low adherence of patients to long-term multicomponent therapy necessitate the search for new ways to solve the problem of drug provision. However, a high cost of drugs demonstrates the importance of proper allocation of financial resources when choosing targeted BA therapy. The appearance of the first biosimilar omalizumab on the domestic pharmaceutical market will gradually increase the provision of patients with effective and modern drug therapy while saving financial resources of the Russian Federation healthcare system.

THE AIM of the study was a comprehensive pharmacoeconomic assessment of the application of the domestic biosimilar drug omalizumab in the treatment of patients suffering from moderate to severe atopic bronchial asthma (BA).

The research objectives were as follows:

1. conduct a search and analysis of scientific publications on the clinical efficacy and safety of omalizumab in atopic asthma in adult patients;
2. determine the population of patients suffering from atopic BA and requiring the prescription of immunobiological drugs, based on the published data on the incidence and prevalence of BA in the world and in Russia;
3. develop a scheme for providing care to adult patients with atopic asthma, taking into account the application of the analyzed approaches to drug therapy;
4. analyze direct and indirect costs of therapy for patients with atopic BA;
5. conduct a comparative clinical and economic analysis of the use of the analyzed drugs in patients with atopic BA;
6. analyze the impact of prescribing immunobiological drugs to patients with atopic BA, on the budget taking into account the current and simulated practice;
7. analyze the sensitivity of the results obtained to changes in the initial parameters.

The research hypothesis is the following: the application of the domestic biosimilar omalizumab in adult patients with atopic asthma is a clinically effective and economically justifiable strategy for organizing patient care in the Russian healthcare system.

[5] Order of the Government of the Russian Federation of October 12, 2019 No. 2406-r "On approval of the list of vital and essential drugs, as well as lists of drugs for medical use and the minimum range of drugs required for the provision of medical care" (as amended by Government orders RF from 26.04.2020 N 1142-r, from 12.10.2020 N 2626-r, from 23.11.2020 N 3073-r). Russian

[6] Clinical guidelines of the Ministry of Health of the Russian Federation. Bronchial asthma. Russian Respiratory Society. 2019. Available from: https://spulmo.ru/upload/kr_bronhastma_2019.pdf. Russian

[7] Instructions for the use of the medicinal product for medical use Genolar®. [Electronic resource]. Available from: http://grls.rosminzdrav.ru. Russian

ОРИГИНАЛЬНАЯ СТАТЬЯ
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MATERIALS AND METHODS

Study design

The pharmacoeconomic methods "Cost-Minimization Analysis" (CMA) and a "Budget Impact Analysis" (BIA) were used.

At the first stage of the study, an information search in the available databases (Cochrane Library, MedLine and Embase, Russian information and analytical system eLIBRARY) was carried out. That was the search for the data on the efficacy and safety of the application of various types of GEBDs as targeted therapy in patients with atopic BA.

According to the results obtained, a systematic review with a meta-analysis by Agache I. et al. 2020 [29] was found out. Within its framework, the efficacy and safety of the use of three genetically engineered biologic drugs – benralizumab, dupilumab, omalizumab – was assessed. Based on the hypothesis of the study, within the framework of this investigation, a comprehensive pharmacoeconomic assessment of the use of various GEBDs in this indication was carried out. The drug dupilumab was chosen as the reference drug, since in the published indirect comparative analysis by Bateman E.D. et al., 2020 [30], there was no statistically significant difference between omalizumab and dupilumab in terms of reducing the exacerbations frequency.

The "cost-minimization analysis" (CMA) is a special case of the "cost-efficacy analysis" in which two or more technologies that have identical efficacy and safety but different costs, are compared [31]. The "cost minimization" is calculated according to the formula:

$$CMD = Cost1 – Cost2,$$

where: CMD (cost-minimization difference) is a cost difference indicator, Cost1 and Cost2 are direct and indirect costs for the use of the 1st and 2nd technologies.

To assess the level of costs, in the course of the clinical and economic analysis, an algorithm for the provision of medical care to adult patients with atopic BA was developed. Since this form of asthma is characterized by frequent exacerbations, the resources of the health care system associated with their management, were assessed in the study. Their average annual frequency was calculated as the product of the frequency of exacerbations against the background of the absence of GEBDs therapy [32, 33] and the Incidence Rate Ratio (IRR), obtained on the basis of the meta-analysis by Agache I. et al., 2020 [29]. The tactics of management of exacerbations (the conditions for the provision of medical care) was determined on the basis of the previously published data [34, 35]. According to this algorithm, the provision of medical care to 1 adult patient with atopic BA was taken into account. The time horizon of the study was 1 year. The general research scheme is shown in Fig. 1.

Description of model assumption

1. Within the framework of the study, only patients with atopic asthma receiving one of the analyzed variants of GEBDs, were considered.

2. Outside of the exacerbation (a controlled course), patients with atopic BA receive basic therapy and GEBDs; to control the therapy course, they are observed by a doctor in an outpatient clinic.

3. In case of an exacerbation, a patient with atopic BA, in addition to the emergency medical care (EMC), can receive medical care in inpatient conditions, if necessary, with assistance in the conditions of the resuscitation intensive care unit (RICU) and artificial lung ventilation (ALV).

To conduct a pharmacoeconomic analysis, the position of "state" was chosen. In this regard, the analysis estimated direct medical costs paid from the budget funds and / or the funds from the compulsory health insurance (CHI) system and indirect costs.

Within the framework of the study, the costs of providing care to patients with atopic asthma included several types.

Direct medical costs are targeted drugs (GEBDs); basic drug therapy; monitoring therapy (outpatient supervision); ambulance / EMC; treatment of exacerbation in a day and round-the-clock hospital, assistance in RICU conditions.

Indirect costs are in the form of loss of gross domestic product (GDP) associated with premature mortality in patients with atopic asthma.

At the first stage, direct medical costs of GEBDs were determined, analyzed within the framework of this study. The dosage regimen of drugs was determined taking into account the official instructions for use[8,9,10], the costs of drug therapy were determined per 1 patient for one year. Based on the data of the Register of Patients with Severe Bronchial Asthma (as at December 31, 2020)[11], the average monthly doses of the analyzed drugs were determined: for omalizumab it was 383 mg, for dupilumab – 567 mg.

[8] Instructions for the use of the medicinal product for medical use Genolar®. [Electronic resource]. Available from: http://grls.rosminzdrav.ru. Russian

[9] Instructions for the use of a medicinal product for medical use Ksolar® [Electronic resource]. Available from: http://grls.rosminzdrav.ru. Russian

[10] Instructions for the use of a medicinal product for medical use Dupixent® [Electronic resource]. Available from: http://grls.rosminzdrav.ru. Russian

[11] IPO "Russian Respiratory Society". Register of patients with severe bronchial asthma (as of 31.12.20). [Electronic resource] (materials provided by JSC "Generium"). Russian

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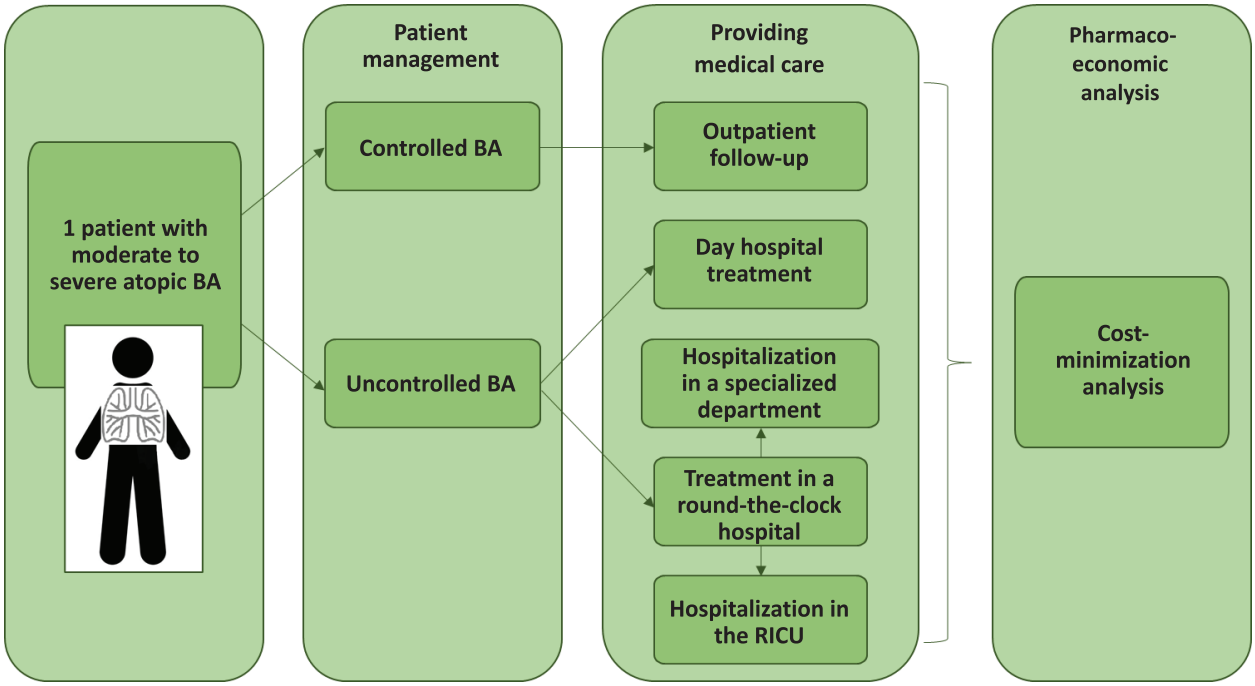

Figure 1 – General research scheme

Table 1 – Direct medical non-drug costs

Medical service	Cost, rub.	Reference source
Calling the ambulance brigade	2 713.40	Programme on State Guarantees, 2021
Disease treatment in the provision of medical care on an outpatient basis	1 505.10	Programme on State Guarantees, 2021
Basic rate for a round-the-clock hospital hospital	37 382.3	Programme on State Guarantees, 2021
Basic rate for a day hospital	22 261,5	Programme on State Guarantees, 2021
Bronchial asthma, adults (DRGs st23.005; IIC = 1.1)	26 971.33	Methodological recommendations on methods of paying for medical care at the expense of the compulsory health insurance fund, 2021
Diseases of the respiratory system (DRGs ds23.001; IIC = 0.9)	12 021.,21	Methodological recommendations on methods of paying for medical care at the expense of the compulsory health insurance fund, 2021
Influenza and pneumonia with organs dysfunction syndrome (DRGs st12.013; IIC = 4.4)	106 913.38	Methodological recommendations on methods of paying for medical care at the expense of the compulsory health insurance fund, 2021

Table 2 – Results of the indicators analysis of the effectiveness of the considered GEBDs per 1 patient suffering from atopic moderate to severe BA, within 1 year

Indicator	Dupilumab	Omalizumab
Weighted average frequency of exacerbations per year, number of cases	0.50	0.49
Hospitalization rate, number of cases	0.13	0.13
Frequency of hospitalizations in RICU, number of cases	0.005	0.005
Death rate, number of cases	0.00003	0.00003

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Table 3 – Results of the costs analysis of drug therapy with GEBDs per patient with atopic BA per year

INN	TN	Dosage form / dosage / packaging	Dosage regimen	Per month costs of therapy, rub.	Per year costs of therapy, rub.
Dupilumab	Dupixent	Solution for subcutaneous administration, 175 mg / ml, 1.14 ml (2)	Initial dose – 400 mg (2 injections of 200 mg), then – 200 mg every 2 weeks	80 534.19	966 410.34
		Solution for subcutaneous administration, 150 mg / ml, 2 ml – syringe with a needle protection system (2)	Initial dose – 600 mg (2 injections of 300 mg), then – 300 mg every 2 weeks		
Omalizumab	Genolar	Lyophilisate for preparation of a solution for subcutaneous administration, 150 mg (1)	From 75 to 600 mg, once every 2 or 4 weeks	46 586.37	559 036.47

Table 4 – Results of direct medical and indirect costs analysis for the management per patient with atopic BA per year

Indicator	Dupilumab	Omalizumab
Direct medical costs, rub.		
Costs for GEBDs therapy, rub.	966 410.34	559 036.47
Basic therapy costs, rub.	15 959.00	15 959.00
Costs for control over injections, rub.	18 061.20	18 061.20
Exacerbation costs without hospitalization, rub.	4 354.31	4 324.56
Exacerbation costs with hospitalization (excluding RICU), rub.	3 468.85	3 445.15
Exacerbation costs with hospitalization (including RICU), rub.	572.93	569.02
Costs for EMS, rub.	1 346.36	1 337.16
Total, rub.	1 010 172.99	602 732.56
Indirect costs, rub.		
Lost GDP resulting from premature deaths, rub.	59,50	59.10
Total, rub.	59,50	59.10
Total costs, rub.	1 010 232,49	602 791.65

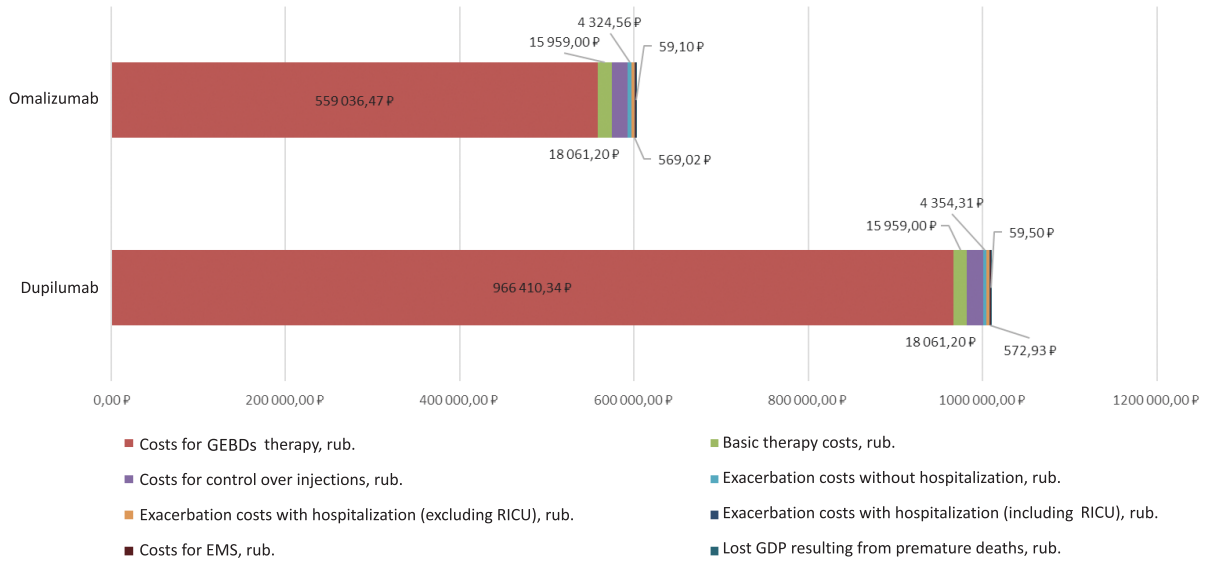

Figure 2 – Results of assessing costs for providing medical care per patient with atopic BA per year

Table 5 – Impact of different variants of GEBDs use in patients with atopic asthma on the budget: results of analysis

Indicator	Current practice	Share,%	Simulated practice 1	Share,%	Simulated practice 2	Share,%
			Omalizumab			
Medicinal product	TN Xolair®		TN Xolair®+TN Genolar®.		TN Genolar®.	
Number of patients, persons	5608	93%	6040	93%	6040	93%
Costs, rub.	3 783 840 662,93		3 858 091 061,99		3 640 861 581,16	
Medicinal product			Dupilumab			
Number of patients, persons	422	7%	455	7%	455	7%
Costs, rub.	426 318 110,36		459 655 782,50		459 655 782,50	

The analyzed medicinal products are included in the VED list. The cost of 1 package for them was calculated based on the price registered in the State Register of maximum selling prices[12], taking into account the weighted average size of the maximum wholesale markup approved in various regions of the Russian Federation (the population was also taken into account) and the added tax Cost (VAT)[13,14,15]. For pharmaceuticals in the Russian Federation, VAT is 10% (preferential taxation); the maximum wholesale markup is 11.39%.

Basic calculations were made on the basis of the following prices for medicines (excluding VAT and the maximum wholesale markup) for 1 package:

1. Omalizumab (Genolar®), lyophilisate for the preparation of a solution for a subcutaneous administration, 150 mg – 14,890.61 rubles;

2. Dupilumab (Dupixent®), solution for a subcutaneous administration, 150 mg/ml, 2 ml (2) – 69,552 rubles;

3. Dupilumab (Dupixent®), solution for a subcutaneous administration, 175 mg/ml, 1.14 ml (2) – 46,368 rubles.

Based on the determined cost of 1 package of the drug, the cost of 1 mg of the active substance was calculated to determine the costs of one-month therapy, taking into account the average monthly doses, then the costs of the annual course of therapy (12 months) was calculated.

The costs of basic therapy for a patient with atopic asthma were determined on the basis of the previously published studies [36]; they amounted to 15,959 rubles per patient per year.

Taking into account the form of release for GEBDs, the costs of monitoring therapy were also taken into account, since according to the instructions for medical use, this group of drugs should be administered under the supervision of medical personnel, however, for the drug dupilumab, the possibility of self-administration by the patients is provided.

It was assumed that for all the drugs under consideration, the frequency of visits for medical care in an outpatient setting should be once per month. The costs of monitoring therapy were calculated on the basis of the standard of financial costs for 1 treatment for a disease on an outpatient basis in accordance with the Programme on State Guarantees (PSGs) for the provision of free medical care to citizens for 2021[16].

At the next stage, the costs associated with the management of patients during the period of exacerbation of atopic asthma, were determined. In BA exacerbations, the study included the provision of several types of medical care: in the event of an exacerbation, the patient made an emergency call, after which he could undergo treatment either in a day hospital or in a round-the-clock hospital. When providing medical care in a round-the-clock hospital, if necessary, the patient received assistance in an RICU with artificial lung ventilation.

The costs for emergency medical care and inpatient treatment were determined on the basis of the standard of financial costs at the expense of the compulsory medical insurance funds according to the PSGs for the provision of free medical care to citizens for 2021. Herewith, the Methodological recommendations on methods of paying for medical care at the expense of the compulsory medical insurance funds[17] of the Federal

[12] State register of maximum selling prices of the Russian Federation. [Electronic resource]. Available from: http://grls.rosminzdrav.ru/PriceLims.aspx. Russian

[13] Information on the maximum tax of wholesale mark-ups and the maximum tax of retail mark-ups to prices for vital and essential medicines established in the constituent entities of the Russian Federation (data as of 19.02.2021). The site of the Federal Antimonopoly Service of Russia. [Electronic resource] Available from: https://fas.gov.ru/documents/687611. Russian

[14] Federal State Statistics Service. Estimated resident population as of January 1, 2021. Available from: https://www.gks.ru/folder/12781. Russian

[15] Tax Code of the Russian Federation (part two) of 08/05/2000 No. 117-FZ. Article 164. Tax rates. P. 2. Available from: http://www.consultant.ru/document/cons_doc_LAW_28165/35cc6698564adc4507baa31c9cfdbb4f2516d068/. Russian

[16] Decree of the Government of the Russian Federation of December 28, 2020 No. 2299 "On the program of state guarantees for free provision of medical care to citizens for 2021 and for the planning period of 2022 and 2023." [Electronic resource] Available from: http://government.ru/news/41272/. Russian

[17] Methodical recommendations on methods of payment for medical care at the expense of compulsory medical insurance funds. Minutes of the meeting in absentia of the members of the working group dated December 29, 2020 No. 06/11/8 [Electronic resource] Official website of the Federal Compulsory Medical Insurance Fund. Available from: http://www.ffoms.ru/. Russian

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Compulsory Medical Insurance Fund and Methodological recommendations of the Federal State Budgetary Institution "Centre of expertise and quality control of medical care" were taken into account[18]. It was assumed that the number of emergency calls was equal to the number of exacerbations of atopic asthma within 1 year. All cases of inpatient treatment were compared with the corresponding diagnostic related groups (DRGs), for which the input intensity coefficients (IIC) had been determined. All calculations for inpatient care (round-the-clock and day hospitals) were carried out according to the following formula:

$$AC = AS \times IIC \times CC,$$

where: AC is the average cost of a completed hospitalization case included in the DRGs in medical organizations (their structural units) providing medical care in inpatient conditions at the expense of compulsory medical insurance funds; AS is the average standard of financial costs for 1 case of hospitalization in medical organizations (their structural subdivisions) providing medical care in inpatient conditions at the expense of compulsory medical insurance; IIC is the input intensity coefficient of DRGs, to which the given case of hospitalization is attributed; CC is the correction coefficient reflecting a lower level of the base rate from the standard of financial costs established by the PSGs (for a day hospital it is 0.6, for a round-the-clock hospital – 0.65).

General characteristics of direct medical costs included in the analysis (excluding drug therapy) are presented in Table 1.

Indirect costs were calculated per patient per year. The lost state GDP due to the losses of premature mortality was calculated based on the probability of deaths in patients with exacerbations of atopic asthma who are on ALV, multiplied by the amount of the lost GDP per day, equal to 6208.23 rubles per day[19,20].

Based on the calculations carried out, the BIA was further carried out, which is a part of a comprehensive drugs assessment and is aimed at assessing the financial consequences of the use of certain drugs or medical devices[21].

Taking into account the fact that BA is a chronic disease that requires constant and long-term treatment, the time horizon for BIA was also 1 year. The main characteristics of the population in which the use of the analyzed medical technology (domestic biosimilar omalizumab) is expected, are as follows: patients aged 18 years and older suffering from moderate to severe atopic bronchial asthma, who are to be prescribed immunobiological drugs.

According to the Department of Health Monitoring, Analysis and Strategic Development of the Federal State Budgetary Institution "Central Research Institute for Organization and Informatization of Health Care" of the Russia Ministry of Health, the number of BA diagnoses determined for the first time in patient lives in 2019, amounted to 122,897 cases, or 83.7 cases per 100 thousand people. In 2019, the total number of asthma registered cases was 1,592,596 people. Among the general population of patients with asthma, about 68% have atopic asthma [37]; about 5–10% have a severe form (for modeling the population, it was assumed to be 8%) [38, 39], thus the number of patients with severe atopic asthma in the Russian Federation is about 86,637. According to the "Register of patients with severe bronchial asthma in the Russian Federation," about 8% of patients with severe asthma receive therapy with GEBDs [40, 41]. The proportion of the patients receiving GEBDs omalizumab and dupilumab (INNs) is about 87%, while the correlation between these drugs is 93% and 7%[22], respectively. Based on the analyzed data, it can be established that the population of patients with severe atopic asthma receiving therapy with GEBDs omalizumab and dupilumab (INNs) can be about 6,030 people.

At the final stage of the study, a sensitivity analysis was carried out. Its aim was to reveal the sensitivity of the study results to the changes in the initial parameters.

RESULTS AND DISCUSSION

In the review, it was revealed that so far there had been no direct comparative clinical studies for the analyzed drugs.

In the systematic review by Agache I. et al., 2020 [29], the efficacy and safety of treatment for severe al-

[18] Omelyanovskiy VV, Avksentyeva MV, Sura MV, et al. Guidelines for the comparative clinical and economic assessment of a medicinal product (new edition). Approved by order of the Federal State Budgetary Institution "CEKKMP" of the Ministry of Health of Russia dated December 29, 2018 No. 242-od. Moscow, 2018 46 p. Available from: https://rosmedex.ru/wp-content/uploads/2019/06/MRKE%60I_novaya-redaktsiya_2018-g..pdf. Russian

[19] Omelyanovskiy VV, Avksentyeva MV, Sura MV, et al. Methodological recommendations for calculating costs when conducting clinical and economic studies of drugs. Approved by order of the Federal State Budgetary Institution "CEKKMP" of the Ministry of Health of Russia dated December 29, 2017 No. 185-od. Moscow, 2017, 24 p. Available from: https://rosmedex.ru/wp-content/uploads/2018/02/Metodicheskie-rekomendatsii-po-raschetu-zatrat-pri-provedenii-kliniko-e%60konomicheskih-issledovaniy-lekarstvennyih-preparatov-2017.pdf. Russian

[20] On the approval of the Methodology for calculating economic losses from mortality, morbidity and disability of the population [Electronic resource] Order of the Ministry of Economic Development of Russia No. 192, Ministry of Health and Social Development of Russia No. 323n, Ministry of Finance of Russia No. 45n, Rosstat No. 113 dated 10.04.2012 (registered with the Ministry of Justice of Russia 28.04.2012 No. 23983). Available on the reference legal system "ConsultantPlus" Russian

[21] Omelyanovskiy VV, Avksentyeva MV, Sura MV, et al. Methodological recommendations for assessing the impact on the budget in the framework of the program of state guarantees of free provision of medical care to citizens.

[22] IPO "Russian Respiratory Society". Register of patients with severe bronchial asthma (as of 31.12.20). [Electronic resource] (materials provided by JSC "Generium"). Russian

lergic asthma with the use of benralizumab, dupilumab, and omalizumab have been considered. The review included 28 studies (3 randomized controlled clinical trials (RCCTs) on benralizumab, 1 RCCT on dupilumab, and 24 RCCTs on omalizumab) in the patients aged 12 to 75 years (excluding the patient population in the omalizumab studies, the age of patients in these studies was also 6–11 years old), receiving therapy for 12–56 weeks.

The exacerbation rates for dupilumab were assessed in 1 RCCT and for omalizumab in 6 RCCTs. It was shown that all the analyzed GEBDs reduce the frequency of exacerbations with a high reliability of evidence: for benralizumab, the incidence rate (IR) was 0.63 (95% CI 0.50–0.81), for dupilumab it was 0.58 (95% CI 0, 47–0.73), for omalizumab – 0.56 (95% CI 0.45–0.69). According to the results of a systematic review, the use of benralizumab, dupilumab and omalizumab leads to a statistically significant improvement in asthma control. At the same time, the use of omalizumab and benralizumab is associated with an improvement in the quality of patient life. It has also been shown that the use of omalizumab leads to a decrease in the required dose of IGCSs and OGCSs.

To carry out the "cost minimization" analysis, a selection of criteria for efficacy affecting the level of costs was carried out. For this study, the rate of exacerbation was chosen a criterion for efficacy affecting the overall level of costs and the use of resources of the health care system.

According to the RCCTs data of Hanania N.A. et al., 2011 [32], the frequency of exacerbations in patients with atopic asthma who do not receive GEBDs, is 0.88 per year; relative to this indicator, the number of exacerbations was determined against the background of omalizumab therapy, taking into account the data obtained in the meta-analysis carried out by Agache I. et al., 2020 [29].

According to the RCCTs data by Corren J. et al., 2019 [33], the frequency of exacerbations in patients with atopic asthma who do not receive GEBDs, is 0.736 in one group and 0.975 in the other group per year. Relative to these values, the average number of exacerbations was determined against the background of dupilumab therapy, taking into account the data obtained in the framework of meta-analysis by Agache I [29].

The number of hospitalizations was determined on the basis of the number of exacerbations, taking into account the frequency of hospitalizations equal to 27% [37], while about 4% of patients require hospitalization in the RICU. About 5% of all patients with BA exacerbation require tracheal intubation and ALV; in case of ALV, the lethality of patients can reach 9.8% [34]. The details are presented in Table 2.

The results of the costs analysis of drug therapy with the use of GEBDs per patient with atopic BA per year are presented in Table 3. The difference in the costs for drug therapy with GEBDs is 407,373.87 rubles, or 42% in favor of the domestic biosimilar omalizumab.

The lowest total costs are associated with the provision of medical care to patients with atopic BA when using the domestic biosimilar omalizumab (602, 791.65 rubles), the highest – when using dupilumab (1,010,232.49 rubles) (Table 4 and Fig. 2). At the same time, the difference in favor of the domestic biosimilar (TN Genolar®) was 407,440.84 rubles, or 40% per year per patient. For all analyzed GEBDs, the largest share in the structure of direct medical costs is accounted for the drugs themselves, the share of costs reaches a maximum of 96% (for dupilumab).

Thus, the costs analysis demonstrated the advantage of using the domestic biosimilar omalizumab in patients with atopic asthma compared to dupilumab, since its use is associated with the lowest costs per patient with moderate to severe atopic asthma per year. The difference was 40%.

As a part of the BIA, the approximate size of the target population was calculated (6030 people). However, the data obtained may not fully reflect the real picture of the number of patients with atopic asthma who need GEBDs therapy. There is still a cohort of patients who have comorbid conditions (i.e., atopic dermatitis, rhinosinusitis polyposa, or idiopathic urticarial) in which GEBDs are also required. In this regard, it is difficult to determine the exact size of the patient population.

To assess the level of costs within the framework of the analysis of real and simulated practice, the costs of treating patients with atopic asthma have been calculated. The results are presented in Table 5. Within the framework of the BIA, it was assumed that 2 options would be considered for the simulated practice. Currently, the main share of INN omalizumab is the original drug TN Xolar®. After the appearance of the biosimilar by TN Genolar® on the domestic pharmaceutical market, its application structure will undergo changes. Based on this assumption, several options were considered – the use of INN omalizumab preparations in a 1:1 ratio and the use of only the domestic biosimilar by TN of Genolar®. At the same time, taking into account the modeling horizon of 1 year, it was taken into consideration that the annual increase in patients would be about 8% according to official statistics, which would lead to an increase in the population to 6495 patients.

BIA demonstrated that the costs for the current practice of providing medical care to patients requiring therapy with the use of GEBDs by INNs omalizumab and dupilumab amounted to 4,210,158,773.29 rubles. For the 1st option (TN Xolar® + TN Genolar® 50/50) of the simulated practice, the costs amounted to 4,317,746,844.48 rubles, for the 2nd option (TN Genolar® 100%) – to 4,100,517,363.65 rubles. Thus, the BIA demonstrated that the use of the domestic biosimilar omalizumab, even taking into account the annual increase in the number of patients (by 8%), will save up to 109,641,409.64 rubles, or 3%, compared to the current practice.

To assess the stability of the model, the values of the efficacy criteria for omalizumab and dupilumab, the cost of drugs omalizumab and dupilumab, as well as the size of the target population, were changed in the directions of decreasing and increasing. The sensitivity analysis was carried out taking into account the fact that in real prac-

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tice, the indicators of the therapy efficacy may differ due to the individual characteristics of the patient, the level of adherence to therapy, etc. The cost of drugs may also differ in different regions of the Russian Federation, because each region has its own level of wholesale markups. The sensitivity analysis demonstrated the robustness of the present model to changes in the initial parameters.

Thus, this study is the first experience in conducting a comprehensive pharmacoeconomic assessment of the use of the domestic biosimilar omalizumab in patients with moderate to severe atopic BA. A review of the domestic literature showed that several pharmacoeconomic studies have already been carried out on the application of the original drug omalizumab.

So in the work by Kolbin A.S. et al., 2016 [42], the issues of the omalizumab use in children with severe uncontrolled asthma were considered. However, in the course of the work, there was only a comparison with standard therapy; no comparison of several GEBDs was carried out. It was shown that, within a 5-year horizon of modeling, the use of omalizumab would lead to additional costs in the amount of 39,821 rubles per hospitalization averted [42]. The BIA demonstrated the comparability of the total costs for 100 children with BA, 7 of which would receive omalizumab, and for 105 children with BA without any use of targeted therapy. Prior to this, in the work by Kolbin AS et al., 2015 [43], similar aspects of the use of omalizumab in relation to adult patients were considered.

In another work by Kulikov A.Yu. et al., 2018 [44], a comparative assessment of the use of several targeted drugs, omalizumab and reslizumab, was carried out. The results of the work showed that in the treatment of severe bronchial asthma with eosinophilic type of inflammation, the use of reslizumab is more cost-effective, while the study used a "cost-effectiveness" analysis. In the work by Tolkushin A.G. et al., 2019 [45], the pharmacoeconomic analysis was carried out from the position that there was no statistically significant difference in efficacy and safety between the drugs omalizumab and mepolizumab. The total direct medical costs for mepolizumab were 870 130 rubles, and for omalizumab – 1 852 063 rubles. The obtained differences in the annual costs of therapy with omalizumab in comparison with the results in the present work, can be justified by different approaches to modeling and taking into account the cost of the original drug, in calculating. The comparison of the results obtained with the annual costs of mepolizumab therapy manifests financial savings in favor of the domestic biosimilar omalizumab. A comparative pharmacoeconomic analysis conducted by Zyryanov S.K. et al., 2019 [46] already included, in addition to omalizumab and mepolizumab, reslizumab for the treatment of patients with uncontrolled atopic moderate to severe BA. The analysis demonstrated the cost-effectiveness of omalizumab compared to other drugs included in the study.

In the course of a few interesting studies, a comparative pharmacoeconomic evaluation of the application of omalizumab and dupilumab was carried out [35, 47], in the framework of which the economic advantage of using dupilumab in patients with severe asthma was demonstrated. So, in the publication by Salasyuk A.S. et al., 2019 [37], the results of their own indirect comparative analysis of the omalizumab and dupilumab effectiveness were presented, during which differences were shown in relation to the incidence of exacerbations. The hypothesis of this study was based on the indirect comparative analysis by Bateman E.D. et al., 2020 [30]. The emergence of newer information regarding a comparative efficacy, as well as the entry into the domestic pharmaceutical market of biosimilars, prompted a new study. In the course of this work, the calculations of the authors' own on the frequency of exacerbations based on the data of reliable randomized controlled clinical trials and a meta-analysis by Agache I. et al., 2020 [29], have been presented.

Thus, at present, a large amount of data on various economic aspects of the use of GEBDs in domestic conditions has been accumulated. Within the framework of all the studied publications, the issues of the original drugs use have been considered. At the same time, the use of various approaches to modeling and the choice of information sources for calculations have led to some heterogeneity of the data. In the course of the present work, the issues of using the domestic biosimilar omalizumab have been considered. Using these GEBDs will make it possible to reduce the average annual cost of BA therapy.

CONCLUSION

Based on the results of the conducted pharmacoeconomic analysis, it was found out that the use of the domestic biosimilar omalizumab in patients with moderate to severe atopic BA is a clinically effective and economically justified approach to organizing medical care for adult patients in the Russian Federation.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHORS' CONTRIBUTION

Vera S. Krysanova – concept development and study design, analysis and interpretation of the research results, text writing; Alina D. Ermolaeva – material collecting and processing; Tatyana N. Ermolaeva – development of the research concept; Maria V. Davydovskaya – scientific consulting; Konstantin A. Kokushkin – scientific consulting.

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