Научно-практический журнал
Scientific and Practical Journal

ISSN 2307-9266
e-ISSN 2413-2241

# ФАРМАЦИЯ И ФАРМАКОЛОГИЯ

## PHARMACY & PHARMACOLOGY

2022 Том / Volume X

№ 4

**Обзоры, лекции**
Reviews, Lectures

**Фармакогнозия, ботаника**
Pharmacognosy, Botany

**Фармацевтическая технология и биотехнология**
Pharmaceutical Technology and Biotechnology

**Фармацевтическая и токсикологическая химия**
Pharmaceutical and Toxicological Chemistry

**Фармакология и клиническая фармакология**
Pharmacology and Clinical Pharmacology

**Информационные технологии в фармации**
Information Technologies in Pharmacy

**Организация и экономика фармацевтического дела**
Organization and Economy of Pharmacy

**Экономика и менеджмент медицины**
Economy and Management of Medicine

**Фармацевтическое образование**
Pharmaceutical Education

**Дискуссии, рецензии, юбилеи, научные школы, история фармации и фармакологии**
Discussions, Referee Reports, Anniversaries, Schools of Thought, History of Pharmacy and Pharmacology

www.pmedpharm.ru www.pharmpharm.ru

ПМФИ
Филиал ВолгГМУ

Scientific and Practical Journal

# PHARMACY & PHARMACOLOGY

Scientific and practical journal

**Volume X, Issue 4, 2022**

The mass media registration certificate:
ПИ №ФС77–67428 от 13.10.2016

**ISSN 2307-9266 e-ISSN 2413-2241**



Manuscripts presented in sections **Reviews, Lectures / Pharmaceutical Education / Brief Reports / Discussions, Referee Reports, Anniversaries, School of Thought, History of Pharmacy and Pharmacology** can be considered by any members of the editorial board.



*Founder: Volgograd State Medical University. 1, Pavshikh Bortsov Sq., Volgograd, Russia, 400131*
*Editors office address: 11, Kalinin ave., Pyatigorsk, Russia, 357532*
*Pyatigorsk Medical and Pharmaceutical Institute – branch of Volgograd State Medical University*
*Phone number: +7(8793) 32-44-74. E-mail: pharmjournal@mail.ru*
*www.pharmpharm.ru*
*Union catalogue. Russian Press / Newspapers an journals. Code 94183*
A4 size, 1000 issues circulation. Price free

**Journal "Pharmacy & Pharmacology" is recommended International Comittee Of Medical Journal Editors and included in Higher Attestation Commission, Scopus, Web of Science (ESCI), Russian citation database, eLibrary, ARISTI (All-Russian Institute of Scientific and Technical Information), RSL (Russian State Library), CyberLeninka, Socionet, EMBASE, Chemical Abstracts (CAS), Directory of Open Access Journals (DOAJ), EBSCO Discovery Service, RNMJ, University of CAMBRIDGE, Ulrich'sWeb, Google Scholar, Biefeld Academic Search Engine (BASE), Directory of Open Access Scholarly Resources (ROAD), Research Bible, Open Archives Initiative, Academic Keys, JournalTOCs, WorldCat, OpenAIRE, University of Oxford, The British Library, Universitait Gent, Université de Montréal, University of Saskatchewan.**

*Printed in the LLC "Amirit" in accord with provided materials, 410004, Saratov, 88, Chernishevsky Str.*

Научно-практический журнал

# ФАРМАЦИЯ И ФАРМАКОЛОГИЯ

Периодичность 6 номеров в год

**Том 10, Выпуск 4, 2022**

Свидетельство регистрации СМИ:
ПИ №ФС77–67428 от 13.10.2016 г.

**ISSN 2307-9266 e-ISSN 2413-2241**



Учредитель: Федеральное государственное бюджетное образовательное учреждение высшего образования «Волгоградский государственный медицинский университет» Минздрава России.
400131, Россия, г. Волгоград, площадь Павших Борцов, д. 1
Адрес издательства: 357532, г. Пятигорск, пр-кт Калинина, 11.
Пятигорский медико-фармацевтический институт – филиал ФГБОУ ВО ВолгГМУ Минздрава России
Телефон: +7 (8793) 32-44-74. E-mail: pharmjournal@mail.ru
www.pharmpharm.ru
Объединенный каталог. Пресса России . Газеты и журналы. Индекс 94183
Формат А4, тираж 1000 экз. Цена свободная.

**Журнал «Фармация и фармакология» включен в перечень рецензируемых научных изданий, входящих в международные реферативные базы данных и системы цитирования, и в соответствии с пунктом 5 правил формирования перечня рецензируемых научных изданий, в которых должны быть опубликованы основные научные результаты диссертаций на соискание ученой степени кандидата наук, на соискание ученой степени доктора наук (Перечень ВАК), Scopus, Web of Science (ESCI), РИНЦ, eLibrary, ВИНИТИ, РГБ, Киберленинка, Соционет, EMBASE, Chemical Abstracts (CAS), Directory of Open Access Journals (DOAJ), EBSCO Discovery Service, RNMJ, University of CAMBRIDGE, Ulrich'sWeb, Google Scholar, Biefeld Academic Search Engine (BASE), Directory of Open Access Scholarly Resources (ROAD), Research Bible, Open Archives Initiative, Academic Keys, JournalTOCs, WorldCat, OpenAIRE, University of Oxford, The British Library, Universitait Gent, Université de Montréal, University of Saskatchewan.**

*Отпечатано в соответствии с предоставленными материалами в ООО «Амирит», 410004, г. Саратов, ул. Чернышевского, 88.*

ISSN 2307-9266 e-ISSN 2413-2241

Scientific and Practical Journal
PHARMACY & PHARMACOLOGY

# CONTENS / СОДЕРЖАНИЕ

## REVIEW / ОБЗОР



## RESEARCH ARTICLE / ОРИГИНАЛЬНЫЕ СТАТЬИ

### Pharmaceutical Technology and Biotechnology / Фармацевтическая технология и биотехнология



### Pharmacology and Clinical Pharmacology / Фармакология и клиническая фармакология



## Editorial / Редакционная статья

*REVIEWS, LECTURES*

*ISSN 2307-9266 e-ISSN 2413-2241*

Scientific and Practical Journal
PHARMACY & PHARMACOLOGY



# EVALUATION OF PLANT-BASED UV FILTERS POTENTIAL IN MODERN CONCEPT VIEW OF SKIN PHOTOPROTECTION

**O.D. Nemyatykh[1], I.I. Terninko[1,2], A.S. Sabitov[3], A.I. Lyashko[1], Z.B. Sakipova[3]**

[1] Saint-Petersburg State Chemical and Pharmaceutical University,
14, Prof. Popov Str., Saint-Petersburg, Russia, 197376
[2] Testing Laboratory (Medicines Quality Control Center)
of Saint-Petersburg State Chemical and Pharmaceutical University,
Bld. A, 14, Prof. Popov Str., Saint-Petersburg, Russia, 197376
[3] Asfendiyarov Kazakh National Medical University,
Bld. 4, 88, Tole Bi Str., Almaty, Republic of Kazakhstan, 050000

E-mail: oksana.nemyatyh@pharminnotech.com

Received 04 May 2022 After peer review 20 July 2022 Accepted 25 Aug 2022

A therapeutic plants potential is based on the pharmacological effects due to their phytochemical profile. Today, scientific interest in botanicals is increasing as a result of recent research that looks at the prospect of using these raw materials for the cosmetic industry as a means to protect the skin from the harmful effects of UV rays.

**The aim** of the study was to evaluate a potential of plant-based UV-filters in modern concept view of skin photoprotection.

**Materials and methods.** A systematic literature search was carried out using the electronic information arrays PubMed, Scopus, Google Scholar, eLibrary. The search depth was 10 years (the period from 2010 to 2021). The search was carried out by the following keywords: antioxidants, cosmetics, photoprotection, chemical composition, pharmacological action.

**Results.** In the paper, modern principles of skin photoprotection based on the use of chemical or physical UV-filters are considered and scientifically substantiated A trend for the use of plant-based materials and their components in the formulation of photoprotectors was notified. That is associated with a wide activity spectrum, the absence of a xenobiotic effect, and a high bioavailability of organic plant compounds.

**Conclusion.** The data analysis from scientific publications demonstrated a potential photoprotective activity of plant-based biologically active substances due to antioxidant, anti-inflammatory and anti-radical effects. The results of the study are a theoretical basis for a further comprehensive experimental study of plant objects in order to obtain a pool of evidence in the field of photoprotection in *in vivo* experiments.

**Keywords:** plant-based UV filters; photoprotection; concept of modern skin photoprotection

**Abbreviations:** IPD – Immediate Pigment Darkening; IPF – Immune Protection Factor; NADH – nicotinamide adenine dinucleotide; NADPH – nicotinamide adenine dinucleotide phosphate; PPD – Persistent Pigment Darkening; SPF – Sun Protection Factor; UVA – ultraviolet A rays; UVB – ultraviolet B rays; UVC – ultraviolet C rays; ROS – reactive oxygen species; BASs – biologically active substances; WHO – World Health Organization; DNA – deoxyribonucleic acid; UV rays – ultraviolet rays.

# ОЦЕНКА ПОТЕНЦИАЛА РАСТИТЕЛЬНЫХ UV-ФИЛЬТРОВ В СВЕТЕ СОВРЕМЕННОЙ КОНЦЕПЦИИ ФОТОЗАЩИТЫ КОЖИ

**О.Д. Немятых[1], И.И. Тернинко[1,2], А.С. Сабитов[3], А.И. Ляшко[1], З.Б. Сакипова[3]**

[1] Федеральное государственное бюджетное образовательное учреждение высшего образования
«Санкт-Петербургский государственный химико-фармацевтический университет»
Министерства здравоохранения Российской Федерации,
197376, Россия, г. Санкт-Петербург, ул. Проф. Попова, д. 14, лит. А

**For citation:** O.D. Nemyatykh, I.I. Terninko, A.S. Sabitov, A.I. Lyashko, Z.B. Sakipova. Evaluation of plant-based UV filters potential in modern concept view of skin photoprotection. *Pharmacy & Pharmacology.* 2022;10(4): 308-319. **DOI:** 10.19163/2307-9266-2022-10-4-308-319



**Для цитирования:** О.Д. Немятых, И.И. Тернинко, А.С. Сабитов, А.И. Ляшко, З.Б. Сакипова. Оценка потенциала растительных UV-фильтров в свете современной концепции фотозащиты кожи. *Фармация и фармакология.* 2022;10(4):308-319. **DOI:** 10.19163/2307-9266-2022-10-4-308-319

Научно-практический журнал
ФАРМАЦИЯ И ФАРМАКОЛОГИЯ

ОБЗОРЫ, ЛЕКЦИИ

DOI: 10.19163/2307-9266-2022-10-4-308-319

[2] Испытательная лаборатория (Центр контроля качества лекарственных средств)
федерального государственного бюджетного образовательного учреждения высшего образования
«Санкт-Петербургский государственный химико-фармацевтический университет»
Министерства здравоохранения Российской Федерации,
197376, Россия, г. Санкт-Петербург, ул. Проф. Попова, д. 14, лит. А
[3] Казахский национальный медицинский университет имени С.Д. Асфендиярова,
050000, Республика Казахстан, г. Алматы, ул. Толе Би, д. 88, корп. 4

E-mail: oksana.nemyatyh@pharminnotech.com

Получена 04.05.2022 После рецензирования 20.07.2022 Принята к печати 25.08.2022

Терапевтический потенциал растений основан на фармакологических эффектах, обусловленных их фитохимическим профилем. Сегодня научный интерес к растительным объектам возрастает в результате последних исследований, в которых рассматривается перспектива применения данного сырья для косметической отрасли в качестве средств для защиты кожи от пагубного воздействия УФ-лучей.

**Цель.** Оценка потенциала растительных UV-фильтров в свете современной концепции фотозащиты кожи.

**Материалы и методы.** Систематический поиск литературы проводился с помощью электронных информационных массивов PubMed, Scopus, Google Scholar, eLibrary. Глубина поиска составила 10 лет (период с 2010 по 2021 гг.). Поиск проводили по ключевым словам: антиоксиданты, косметические средства, фотозащита, химический состав, фармакологическое действие.

**Результаты.** В работе рассмотрены и научно обоснованы современные принципы фотозащиты кожи, базирующиеся на применении химических или физических UV-фильтров. Отмечена тенденция использования растительного сырья и его компонентов в рецептуре фотопротекторов, что связано с широким спектром активности, отсутствием ксенобиотического эффекта и высокой биодоступностью органических растительных соединений.

**Заключение.** Анализ данных научных публикаций продемонстрировал потенциальную фотопротекторную активность биологически активных веществ растений, обусловленную антиоксидантным, противовоспалительным и антирадикальным эффектами. Результаты исследования являются теоретическим базисом для дальнейшего всестороннего экспериментального изучения растительных объектов с целью получения пула доказательных данных в области фотопротекции в опытах *in vivo*.

**Ключевые слова:** растительные UV-фильтры; фотопротекция; концепция современной фотозащиты кожи

**Список сокращений:** IPD – быстрое пигментационное потемнение; IPF – фактор защиты иммунитета; NADH – никотинамидадениндинуклеотид восстановленный; NADPH – никотинамидадениндинуклеотидфосфат; PPD – постоянное пигментационное потемнение; SPF – солнцезащитный фактор; UVA – ультрафиолетовые лучи A; UVB – ультрафиолетовые лучи B; UVC – ультрафиолетовые лучи C; АФК – активная форма кислорода; БАВ – биологически активные вещества; ВОЗ – Всемирная организация здравоохранения; ДНК – дезоксирибонуклеиновая кислота; УФ-лучи – ультрафиолетовые лучи.

## INTRODUCTION

Despite the fact that sunlight is an initiator and catalyst of most metabolic processes, ultraviolet radiation, as one of the fragments of the sunlight spectrum, provokes photoaging with multiple exposures to the skin, and also contributes to the development of a number of pathologies, incl. photocarcinogenesis [1].

The effects of harmful UV exposure highlight the need for the skin protection across the full sunlight spectrum. A modern concept of human skin photoprotection involves the use of broad-spectrum cosmetics, which leads to the search for new natural multifunctional ingredients, including those with a photoprotective activity. Components of the plant origin, which, due to the multicomponent chemical composition, have a multi-vector biological effect, are of considerable interest. This factor is primarily due to the presence of active centers of biologically active substances (hydroxo groups of polyphenolic compounds, keto-enol tautomeric groups of ascorbic acid, systems of conjugated bonds, etc.) and contributes to the realization of natural photoprotective properties [2].

The literature [3–5] contains information on the ability of a number of representatives of various botanical classification groups, from algae and lichens to dicotyledonous plants, to exert antioxidant, anti-inflammatory, and immunomodulatory effects in the experiment. In combination, they implement a photoprotection mechanism. Thus, a number of authors [6–10] report the active components ability of *Silybum marianum* L., *Gracilariopsis longissimi* (S.G. Gmelin) M. Steentoft, *Elaeagnus angustifolia* L., *Moringa oleifera Lam., Vitis vinífera* L., *Ruta graveolens* L., *Ginkgo biloba* L., *Dirmophandra mollis Benth.,* to exhibit their photoprotective properties. The literature also presents the results of studying the chemical composition

ISSN 2307-9266 e-ISSN 2413-2241

Scientific and Practical Journal
PHARMACY & PHARMACOLOGY

[11–14] and experimentally proven photoprotective properties [15–17] of rosehip raw materials; the data on a high bioavailability of rosehip oil biomolecules have been generalized [18–21]. Given a low toxicity, the absence of a xenobiotic effect, a relative availability of phytosubstances, a study and evaluation of plant components as potential photoprotectors, are of scientific and practical importance [22].

The results of this study are based on a systematic analysis of the latest data from scientific publications and can contribute to the formation of logical-structural interrelations of this kind: "phytochemical composition – spectrum of pharmacological activity – prospects for using a plant to develop original cosmetic products with a photoprotective effect."

**THE AIM** of the study was to evaluate the potential of plant-based UV-filters in modern concept view of skin photoprotection.

## MATERIALS AND METHODS

The study was carried out by the methods of the content analysis and data aggregation. A systematic literature search was carried out using the electronic information databases PubMed, Scopus, Google Scholar, eLibrary. The search depth was 10 years (the period from 2010 to 2021). The reference (key) words for the targeted search were in Russian: photoprotection, antioxidants, cosmetics, chemical composition, pharmacological action. To search for foreign sources, similar queries were used but only in English.

## RESULTS AND DISCUSSION

### UV characteristics

To date, it seems undeniable that a human body is affected by two spectra of UV rays: medium-waves (UVB) with a wavelength range of 280–320 nm, constituting 5% of ultraviolet, and long-waves (UVA) with a wavelength range of 320–400 nm covering 95% of the UV spectrum. In recognition of the importance of the ultraviolet radiation dose affecting human skin, the World Health Organization (WHO) introduced the UV index, i.e., the index of a daily assessment of the UV rays intensity near the Earth's surface, depending on the weather conditions in a particular area. It should be notified that the intensity of the UVB radiation depends on the angle of the sun above the horizon and increases by 4–10% every 300 meters up and by 3% for every degree of latitude southward. Unlike the UVB, the effect of UVA rays is not softened by the ozone layer, they have a significant penetrating power, pass through clouds, glass, and are emitted constantly throughout the daylight hours [1, 23].

### Effect of UV radiation on human skin

Attention is drawn to the fact that human skin perceives the entire spectrum of electromagnetic waves, but reacts to them in different ways. In this case, the interaction of electromagnetic waves with skin structures depends on the energy of its photon, which is inversely proportional to the wavelength. This process is characterized by the parameters of reflection/scattering, absorption, and waves penetration depth. Visualization of the UV rays impact on the skin according to various authors is presented in Fig. 1.

In relation to visible light, the skin is an optically semitranslucent formation. Reflection, scattering, and absorption of waves occur insignificantly on its surface and massively in its thickness. The index of the intralayer reflection is one of the indicators of youthfulness of the skin, because getting older, the proportion of reflected light decreases (Fig. 2) [1, 23].

The studies by Russian and foreign authors have shown that the shorter the wavelength (the greater its frequency and energy), the more tightly it interacts with tissue and cellular formations due to the intense absorption by skin chromophores. As a consequence, such waves realize predominantly a surface action. In the *stratum corneum*, an important and massive absorbing agent is keratin, which absorbs predominantly UVB rays and, to a lesser extent, interacts with the UVA spectrum [23–25]. Along with keratin, keratinocytes, melanocytes, fibroblasts, Langerhans cells and endotheliocytes are also involved in the process of absorption of UV-wave energy. The biological effect of this interaction is accompanied by the production of biological markers, primarily reactive oxygen species (ROS), as well as nitrogen and carbon, including both free radicals and nonradical substances (Table 1) [1, 23].

It is important to note that ultraviolet radiation has a cumulative damaging effect on the skin, i.e. small daily doses accumulate into monthly/yearly ones. In this case, it is not the doses of UV rays that accumulate, but the consequences of their exposure, mainly represented by the oxidative damage to cells and the extracellular matrix. To date, a number of studies have shown that up to 80% of facial skin aging signs are associated with an exposure to UV rays [24–26]. Thus, an increase in the time of the daily skin exposure to sunlight, from 1–2 to 5 hours a day, leads to a loss of moisture in the skin, a

DOI: 10.19163/2307-9266-2022-10-4-308-319

violation of the capillary tone and an increase in the formation of fine wrinkles by 4.8 times. A daily use of a cosmetic product with a sun protection filter (SPF) 4–10 can reduce the cumulative UV dose by 50% [23].

Considering that there are absorbers for all types of waves in the cells and the extracellular matrix, the biological consequences are multi-vector. The studies of the etiology and pathogenesis of malignant skin diseases testify to the adverse effects of UV rays on the skin. The rays of UVA and UVB spectra lead to the oxidation of lysine, proline and arginine, often found in proteins in cells and matrix. That triggers an irreversible glycation process with the formation of products of completed glycolysis, cross-links of protein fibers inside and outside cells. The latter circumstance leads to pronounced melanogenesis in the epidermis and thinning of the dermis [23, 25]. It is worth emphasizing that the use of cosmetics with SPF 15 in children reduces the risk of skin cancer later in life by 78% [23].

Attention is drawn to the fact that UVB-waves realize their effect exclusively at the level of the epidermis, in which primary lesions are formed. Biologically, the most important absorber of UVB rays is the DNA of keratinocytes of the germ layer of the epidermis, and the target is the adjacent pyrimidine bases of thymine and cytosine, between which pathological covalent bonds arise under the conditions of a UVB photon absorption. As a result of the interaction, two photoproducts are formed: cyclobutane–pyrimidine dimer and a 6–4 photoproduct (Fig. 3) [1, 23]. It should be notified that the formed photoproducts with altered spatial geometry lead to a nucleotide substitution in the newly synthesized chain, and lead to UVB mutations, which occur during UVB irradiation in 85% of cases. In addition, as a result of a massive DNA damage in the epidermis, the first “burn” cells with a characteristic morphology are formed. Thus, pyrimidine dimers and “burn” cells are an early, accurate and informative indicator of the UVB exposure intensity. Immune Langerhans cells, which are extremely sensitive to the effects of UVB rays, are also a marker of the exposure intensity to UVB radiation, since they lose their processes and migrate to regional lymphatic nodes [23].

Despite the fact that UVB-rays practically do not penetrate the dermis, dermal reactions are also indicators of the UVB damage degree, incl. activation of dermal MMP-1, which destroys collagen, reddening the skin and elastolysis, which increases due to the accumulation of a large amount of immature elastin in the papillary dermis [1].

Herewith, the cumulative effect of UVA rays on the human body is manifested by more pronounced destructive changes than UVB radiation. Despite the fact that the UVB rays energy is 1000 times as much as the UVA rays energy, 90% of UVB rays are blocked by the epidermis stratum corneum, while 50–60% of UVA rays are able to penetrate deep into the dermis. It is worth notifying that it is UVA irradiation *in via* the avalanche-like production of a free radicals pool that damage the cells and extracellular structures of the dermis, which determines the manifestation of the skin photoaging picture through the elasticity loss and the wrinkles formation. An indicator of DNA damage during UVA irradiation is the oxidation of DNA in the region of the purine base of guanine with the accumulation of 8-hydroxyguanine. Along with this, an excess of ROS leads to rapid ligand-independent activation of membrane cell receptors for numerous growth factors and interleukins, distortion of gene activity, including AP-1 and NF-kB. These factors ultimately cause inhibition of procollagen synthesis processes, synthesis of products glycation and activation of metalloproteinases MMP-1, -3, and -9, which determine the destruction of dermal matrix structures (Fig. 4, Table 2) [1, 22, 23].

Physiological mechanisms of the body’s protection from UV/UVB rays are represented by the epidermis stratum corneum, able of compaction under the influence of UV/UVB rays, melanin (from Greek the *“melanos”* – “black”), which delays up to 90% of photons, as well as urocaninic acid. When irradiated, the acid transform goes over into a cis form, and in the absence of the UV radiation exposure, the reverse reaction occurs (Fig. 5) [23, 27].

Thus, a multi-vector nature and depth of morphological and functional changes in the skin exposed to the entire spectrum of UV rays, emphasize the importance of photoprotection of the largest organ of the human body.

## Characteristics and mechanism of UV protective filters effect

UV filters have a protective effect on UV rays. These are the substances designed to protect the skin from the penetration of UV radiation by absorption, reflection or scattering. The effectiveness of UV filters is measured by the Sun Protection Factor (SPF) and is the ratio of the minimum dose of UV radiation that causes redness of the protected skin to the minimum dose of UV radiation that causes redness of the unprotected skin.

*ISSN 2307-9266 e-ISSN 2413-2241*

Scientific and Practical Journal
PHARMACY & PHARMACOLOGY

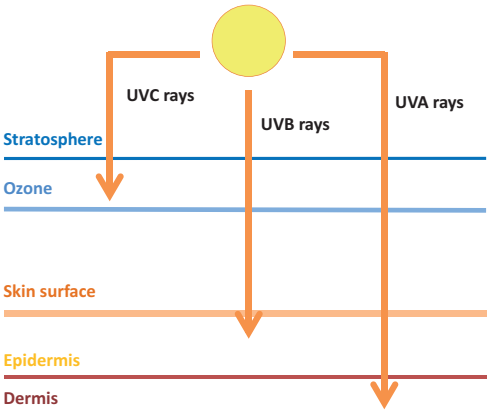



**Figure 1 – Scheme of UV rays exposure on human skin[1]**

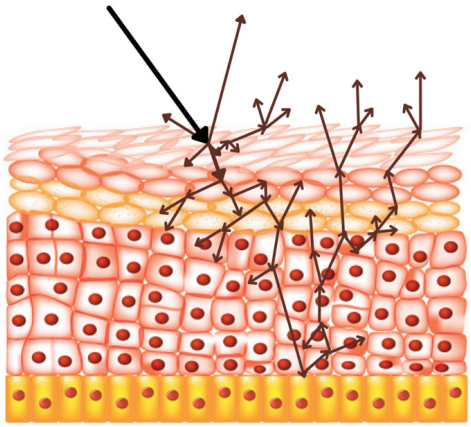


**Figure 2 – Propagation of a light wave inside the skin**

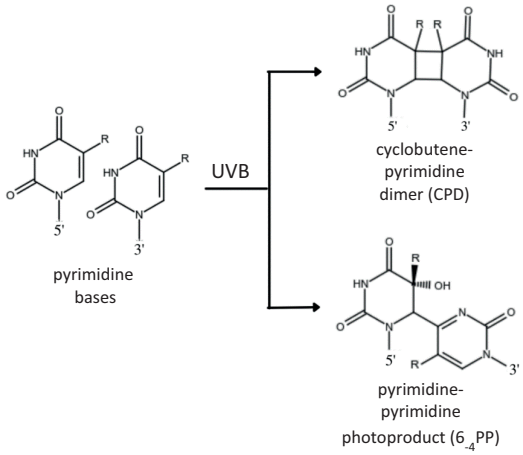



**Figure 3 – Transformation of thymine T and cytosine C when absorbing a UVB-wave photon**

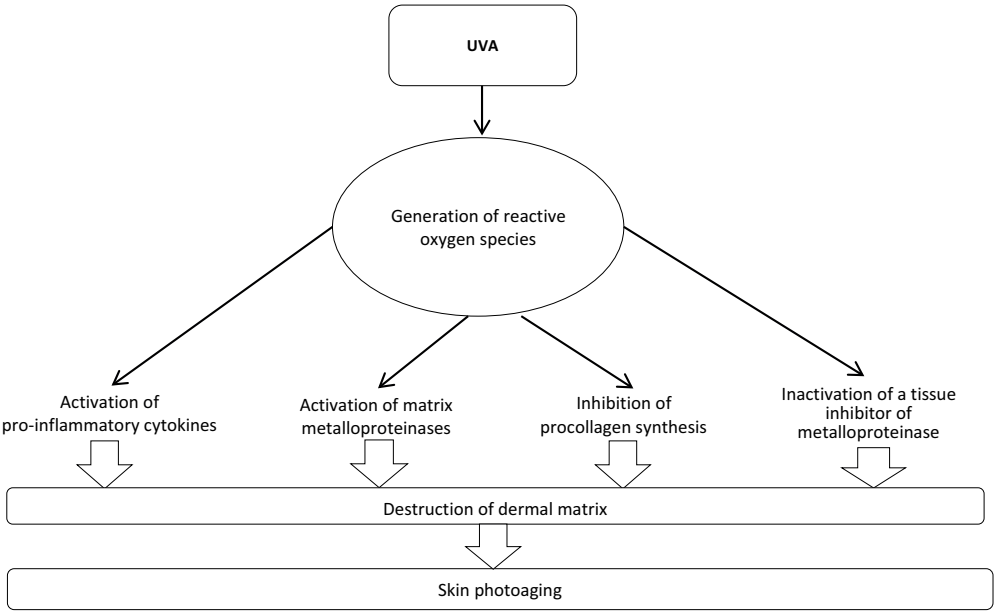



**Figure 4 – Skin exposure to UVA rays**

[1] Here and below, the figures represent the author's interpretation of references comprehensive analysis on certain aspects of the consideration problem.

DOI: 10.19163/2307-9266-2022-10-4-308-319

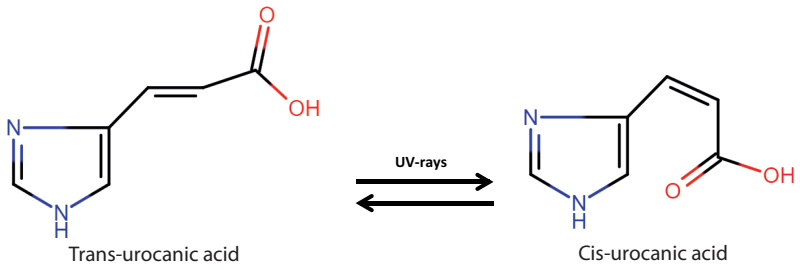



**Figure 5 – Isomerization of urocaninic acid by a quantum of light under the conditions of exposure to UV rays**

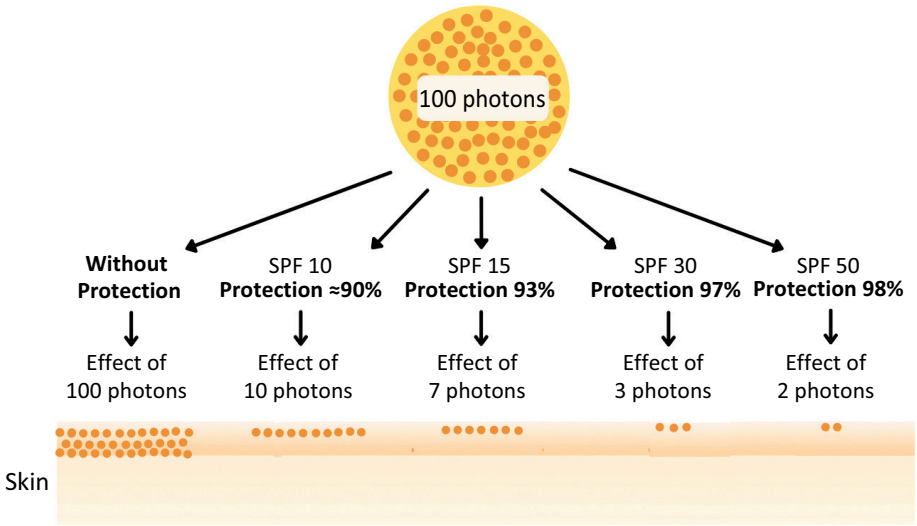



**Figure 6 – Principle of calculating SPF value**

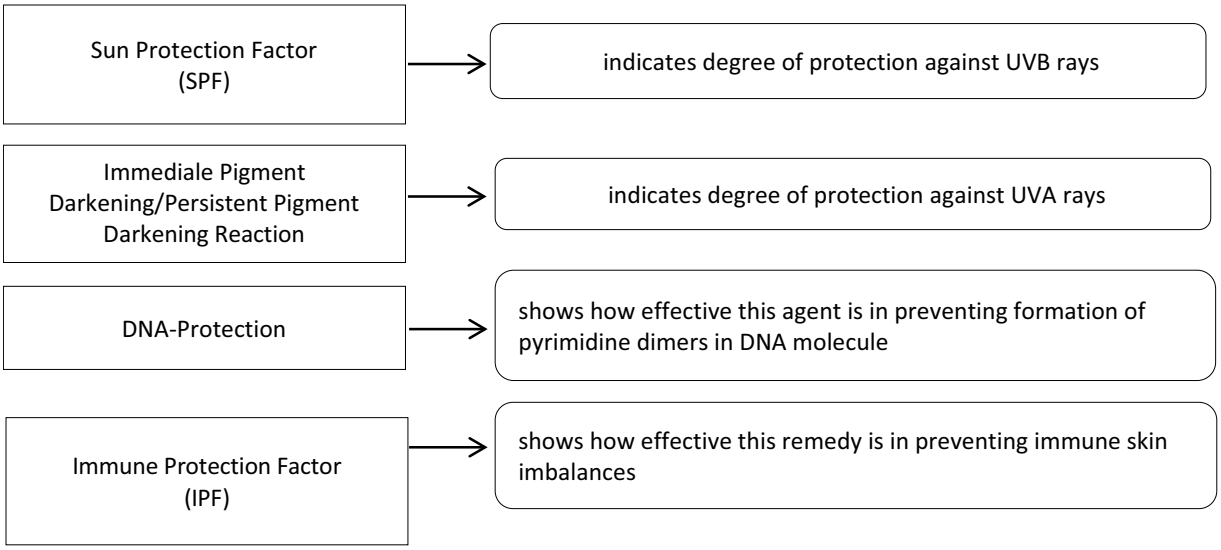



**Figure 7 – Methods for testing cellular bioprotection exposed to UV rays**

*ISSN 2307-9266 e-ISSN 2413-2241*

Scientific and Practical Journal
PHARMACY & PHARMACOLOGY

**Table 1 – Generation of toxic ROS when interacting with different sunlight spectra**

| Skin chromophores | Absorption peak, nm | ROS |
|---|---|---|
| Bilirubin | 400–600 | $H_2O_2$ |
| Collagen/elastin | 320–400 | $H_2O_2$ |
| Collagen AGE (pentosidine) | 320–400 | $\bullet O_2^-$ $H_2O_2$ •OH |
| Melanin | 230–600 | $H_2O_2$ |
| Copper/cytochrome C complex IV (mitochondrion) | 770–1400 | $\bullet O_2^-$ $H_2O_2$ •OH |
| NADH, NADPH | 290–405 | $\bullet O_2^-$ $^1O_2$ |
| 2-thioracil/4-thiouridine | 290–405 | $\bullet O_2^-$ $^1O_2$ |
| Porphyrins | 290–700 | $\bullet O_2^-$ $H_2O_2$ $^1O_2$ |
| Tryptophan | 300–400 | $\bullet O_2^-$ $H_2O_2$ $^1O_2$ |
| Riboflavin | 290–465 | $\bullet O_2^-$ $^1O_2$ |
| Urocaninic acid | 310 | $^1O_2$ |

Note: $\bullet O_2^-$ – superoxide anion radical; • OH – hydroxyl radical; $^1O_2$ – singlet oxygen; ROS – reactive oxygen species; NADH – nicotinamide adenine dinucleotide; NADPH – nicotinamide adenine dinucleotide phosphate.

**Table 2 – Depth of penetration and UVA/UVB rays effect on the skin**

| Depth of rays penetration | Type of rays | Result of exposure to the skin |
|---|---|---|
| Epidermis | UVA+UVB | – keratinocyte lesion поражение меланоцитов<br>– melanocyte lesion<br>– migration of Langerhans cells |
| Dermis | UVA | – generation of free radicals (including DNA, proteins and lipids, membranes and mitochondria are affected)<br>– decrease in synthetic activity of fibroblasts, disruption of their cell cycle and ability to migrate<br>– violation of the synthesis and destruction of collagen and elastin<br>– infiltration of the circulatory matrix by shaped blood elements |
| Hypodermis | UVA | – adipocyte damage, including impaired lipid synthesis and decreased triglyceride content<br>– reduction of adiponectin synthesis |

**Table 3 – Characteristics of photoprotectors with different SPF values**

| Degree of photoprotection | SPF value | Transmission | Absorption (%) |
|---|---|---|---|
| Low | 6 | 0.167 | 83.3 |
| Medium | 10 | 0.100 | 90.0 |
| High | 15 | 0.067 | 93.3 |
| | 20 | 0.050 | 95.0 |
| | 25 | 0.040 | 96.0 |
| Very high | 30 | 0.033 | 96.7 |
| | 50 | 0.020 | 98.0 |
| | 60 (50+) | 0.017 | 98.3 |

The solar protection index, e.g. 50, means that a 50 times more UV dose is required to produce erythema with protection than without protection (Fig. 6) [1, 23, 27].

To date, it has been proven that the transmission (transmittance without changing a direction) of UV rays under the conditions of using a photoprotector is inversely related to the absorption value. At the same time, a significant increase in the values of the SPF factor is realized only by a slight increase in the values of the absorption index (Table 3) [1, 27].

The level of photoprotection required by the skin is strictly individual and is determined by the age and characteristics of the body, the type and condition of the skin, the predicted duration of exposure to the sun, and the UV index (0-12) of the region, taking into account cloud coverage. Since UVB rays are more intense, the number on the sunscreen label indicates protection against this type of wave. Weaker UVA rays do not require a very high degree of protection, so protection from them is 1/3 of type B rays. For example, marking SPF 30 means that there will be protection

DOI: 10.19163/2307-9266-2022-10-4-308-319

**Table 4 – Classification and nomenclature of UV filters**

| Criterium | Physical UV filters | Chemical UV filters |
|---|---|---|
| Chemical nature | – Inorganic compounds (used in micronized, ultra-micronized, finely dispersed and nanoparticle forms) | – Organic compounds |
| Mechanism of action | – They are mineral screens that scatter and reflect sun rays. | – they absorb ultraviolet light and undergo isomerization under quantum of light action |
| Effect localization | – Surface of epidermis stratum corneum | – epidermis |
| Advantages | – high level of safety, inertness in relation to UV rays<br>– UVA/UVB – photoprotection<br>– price affordability | – ability to create combinations with minimal "working" concentrations and a wide protective spectrum<br>– compatibility with other recipe components |
| Disadvantages | – physical particles are removed during the day due to glands activity and other factors<br>– "whitewash effect"<br>– rather high "working" concentrations<br>– difficulties when combined with other recipe components | – likelihood of developing allergic reactions<br>– potential phototoxicity (incl. provocation of photo-contact dermatitis formulation) |
| Names of filters present in European and Russian cosmetic markets | – zinc dioxide, titanium dioxide | – UVB: p-aminobenzoic acid (PABA) and its derivatives (padimate O), salicylates (homomentyl salicylate, octisalate, trolamine salicylate), cinnamates (octinoxate), camphor derivatives, methoxycinnamic acid derivatives |
| | | – UVA: avobenzone, mexoril, neo heliopan UVA/UVB: octocrylene, benzophenone-3,4,5,8, phenylbenzotriazole sulfonic acid (ensulizol), dioxybenzone, triazines and triazones (tinosorb, juvinul) |

30 from UVB, and from UVA, it will be 1/3, i.e., 10[2] [26].

Several tests are used to accurately determine the level of UVA protection. All of them are based on transmission/absorption measurements (Fig. 7). *In vivo* methods such as Immediate Pigment Darkening (IPD) and Persistent Pigment Darkening (PPD) are based on determining the skin response to UVA irradiation (pigmentation and erythema) and then calculating the UVA protective factor, similar to the SPF calculation. Besides, additional tests have now been introduced to assess not only the protective effect against erythema, but also the protection against immunological and mutational effects caused by ultraviolet radiation[3].

The composition of sunscreen preparations includes filters that trap the sun rays (Table 4), the principle of operation of which is based on physical or chemical interaction. Chemical filters capture UV rays and convert them into thermal energy. Physical filters, such as mineral compounds of titanium or zinc, remain on the surface of the skin and block solar radiation by reflecting the rays. Physical filters almost always whiten the skin and are washed off worse, they are suitable for sensitive skin; chemical filters are transparent and invisible on the face, and can have an irritating effect. The list of UV filters approved for use in the European Union is provided in Regulation (EU) 1223/200[4]. The document contains 28 positions of the International Nomenclature of Cosmetic Ingredients (INCI) indicating the absorption area and the maximum allowable concentration. In Russia, the corresponding list is regulated by Technical regulation Commission of the Customs Union, Annex 5 009/2011[5], which also determines the conditions for the use of photoprotectors and the warnings, information about which should be brought to the consumer.

In the light of discussions about the properties of photoprotectors, it is worth notifying photostability, i.e. the ability to maintain its structure and properties under the influence of radiation, as well as the extinction coefficient, which shows how actively the drug absorbs the energy. It is important to emphasize that some

[2] Regulation (Ec) No. 1223/2009 Of The European Parliament And Of The Council of 30.11.2009 on cosmetic products. Official Journal of the European Union. 2009; L.342:59–209.

[3] Commission Recommendation of 22 September 2006 on the efficacy of sunscreen products and the claims made relating thereto. Official Journal of the European Union. 2006; L265:P. 39–42.

[4] Regulation (EC) No 1223/2009 Of The European Parliament And Of The Council of 30.11.2009 on cosmetic products, 2009.

[5] Decision of the Customs Union Commission dated September 23, 2011 No. 799 "On the adoption of the technical regulation of the Customs Union "On the safety of perfumery and cosmetic products" (together with "TR TS 009/2011. Technical regulation of the Customs Union. On the safety of perfumery and cosmetic products"). Russian

ISSN 2307-9266 e-ISSN 2413-2241
Scientific and Practical Journal
PHARMACY & PHARMACOLOGY

chemical filters undergo photolysis to a large extent. For example, 15 minutes after the exposure to the sunlight, a decrease in the activity is notified: avobenzone – up to 36%, octyl-p-methoxycinnamate – by 4.5%. The values greater than 20 (butylmethoxydibenzoylmethane – 31.0, octyldemethyl p-aminobenzoic acid – 28.4, ethylhexyl-p-methoxycinnamate – 24.2) are considered effective values of the quenching coefficient [27].

Thus, the features of the chemical structure, mechanism of action, the need to maintain stability, efficiency and safety of use determine the range of requirements for a modern photoprotector. They are: the ability to absorb rays in a wide range; photo, thermal and water resistance; low penetrating ability in relation to the epidermis stratum corneum; acceptable safety profile (lack of toxicity, as well as carcinogenic, sensitizing effects); the ability to effectively prevent visible (sunburn) and invisible (photoaging, carcinogenesis) effects of UV radiation [28, 29].

## Photoprotective properties of plant-based biologically active substances

A high level of innovation and dynamism in the pharmaceutical and cosmetics industries is reflected in the active search for new multifunctional natural ingredients. Currently, special attention is drawn to the trend of using plant-based materials and their components in the formulation of sunscreens, which is due to a wide spectrum of activity, the absence of a xenobiotic effect, and a high bioavailability of organic plant compounds[6] [30, 31].

Today, it seems undeniable that plants adapt to environmental changes [32–35], incl. the damaging effect of sunlight through the implementation of protective mechanisms, comprising production or activation of the biosynthesis processes of antioxidant molecules [36–38]. Moreover, in a number of studies [22, 24, 26, 30], hypotheses about the potential photoprotective activity of plant compounds have been scientifically substantiated.

At the same time, in a number of works [39, 40], it is notified that plant extracts exhibit a synergistic effect with chemical and physical UV filters, thereby increasing the SPF factor of herbal cosmetic compositions. Moreover, the mechanism of the photoprotective action is different: from its own absorption of UV rays up to antioxidant and anti-inflammatory effects, which indirectly implement photoprotection, leveling the negative effect of the sun.

[6] Ibid.

In the series of biologically active substances in plants, researchers attach the greatest photoprotective significance to various classes of phenolic compounds. So, Acevedo A. and his colleagues report that after the introduction of verbascoside and linarin into the composition of a photoprotective cosmetic product, its SPF factor was 24. These compounds are glycosides of caffeic acid and acacetin, which once again confirms the thesis about the pronounced antiradical activity of polyphenolic compounds [41]. Polyphenolic compounds exhibit a wide range of biological properties: antioxidant, anti-inflammatory, hepatoprotective, vasoactive, antithrombotic, antitumor, antibacterial and antiprotozoal. In the work by Velasco M.V.R. et al., the structure similarity of polyphenolic compounds with organic UV filters, for example, ethylhexylmethoxycinnamate and, as a result, the similarity of photoprotective properties, is notified [42].

Photoprotective properties have been studied for many plant phenolic complexes. Thus, the positive effect of polyphenolic compounds of the plantain lanceolate dry extract, was demonstrated in the work of Brazilian scientists [43]. It was shown that the addition of 7% dry extract to the phytocomposition increased the UVA/UVB ratio from 0.49 to 0.52. Silymarin, a complex of *Silybum marianum* L. flavolignan compounds, demonstrates optimal photoprotective properties, which, when added to cosmetic photoprotective agents, provides SPF 13–14 [44]. High photoprotective properties (SPF = 9.9) are also characteristic of the seaweeds phenolic complex [45]. The photoprotective properties of anthocyanins, a separate group of the flavonoid nature compounds, which provide red-violet shades of the aerial parts color (mainly flowers and fruits) of plants, are reported. Cefali L.C. et al showed that oil extracts of raspberry and blueberry anthocyanins exhibit SPF 37 and 54, respectively [46].

Despite the predominant focus on the evidence of the phenolic compounds photoprotective properties, other groups of plant-based biologically active substances are also characterized by a protective action from the sunlight negative effects. The authors [47–49] note a high photoprotective activity of lignin due to its ability to neutralize free radicals. The incorporation of lignin into cosmetic lotions has been notified to increase the UVA/UVB ratio in the range of 0.69–0.72. Publications [50, 51] provide the data on the study of the caffeine photoprotective activity – catechol, chlorogenic and 3,4,5-tricofeylquinic acids isolated from coffee beans. Their effectiveness in the protection of skin aging caused by the exposure to UV rays and a positive effect on damaged DNA has been shown.

DOI: 10.19163/2307-9266-2022-10-4-308-319

The literature provides an evidence base and shows the possibility of using vegetable oils as natural UV filters: avocado (SPF 4–15), coconut (SPF 2–8), macadamia (SPF 6), shea (SPF 3–6), jojoba (SPF 4). This is due to the presence of residues of polyunsaturated (mainly linoleic and linolenic) acids and their ability to neutralize free radicals, providing antioxidant protection. Plant extracts, e.g., aloe, chamomile, skullcap, grapes, etc., can be considered as complex phytocompositions without individualization of the marker component of the photoprotective action [31, 32].

It should be notified that Russian and foreign authors have studied and experimentally established the photoprotective potential expressed by the antioxidant activity of the wild rose biologically active substances [11–14]. Potential possibilities of using the oil of nuts (fruits) in the development of sunscreens due to the components of the oil – carotenoids, vitamins E and F, triglycerides of polyunsaturated (linoleic and linolenic) acids, which contribute to protection from UV radiation, have been identified. The literature [18, 19] summarizes the data on the high bioavailability of rosehip oil biomolecules, which is determined by its composition similar to the lipid layer of human skin.

The possibility of using secondary plant metabolites – phenolic compounds (including flavonoids, hydroxycinnamic acids, lignans), vitamins, terpenes – can be confirmed by a wide use of the latter in the formulation of photoprotective cosmetics and nutraceutical products [1, 22, 23]. Since the formation of an evidence base for the clinical effectiveness in the field of photoprotection of plant-based sunscreens, which represent a whole complex of biomolecules with antioxidant, anti-inflammatory and anti-radical effects, is complicated by a high labour intensity of isolating and developing the optimal amount of biologically active substances. There are no such positions on the market of cosmetic ingredients today [24].

## CONCLUSION

The data analysis from scientific publications demonstrates a potential photoprotective activity of biologically active substances in plants due to antioxidant, anti-inflammatory and anti-radical effects. The results of the study form a theoretical basis for a further comprehensive experimental study of plant objects in order to obtain a pool of evidence in the field of photoprotection in vivo.

## FUNDING

This study did not receive financial support from outside organizations.

## CONFLICT OF INTERESTS

The authors declare no conflict of interest.

## AUTHORS' CONTRIBUTION

ODN – concept and design of the work, text writing; IIT – materials collection and processing, text writing; ASS – materials collection and processing; AIL – materials collection and processing, text writing; ZBS – materials collection and processing, text writing.

## REFERENCES

1. Wolf P. UV-Filter. State of the Art [UV Filters. State of the art]. Hautarzt. 2009 Apr;60(4):285–93. DOI: 10.1007/s00105-008-1623-y. German
2. He H, Li A, Li S, Tang J, Li L, Xiong L. Natural components in sunscreens: Topical formulations with sun protection factor (SPF). Biomed Pharmacother. 2021 Feb;134:111161. DOI: 10.1016/j.biopha.2020.111161
3. Westfall A, Sigurdson GT, Giusti MM. Antioxidant, UV Protection, and Antiphotoaging Properties of Anthocyanin-Pigmented Lipstick Formulations. J Cosmet Sci. 2019 Mar/Apr;70(2):63–76.
4. Korać RR, Khambholja KM. Potential of herbs in skin protection from ultraviolet radiation. Pharmacogn Rev. 2011 Jul;5(10):164–73. DOI: 10.4103/0973-7847.91114
5. Lohézic-Le Dévéhat F, Legouin B, Couteau C, Boustie J, Coiffard L. Lichenic extracts and metabolites as UV filters. J Photochem Photobiol B. 2013 Mar 5;120:17–28. DOI: 10.1016/j.jphotobiol.2013.01.009
6. Vostálová J, Tinková E, Biedermann D, Kosina P, Ulrichová J, Rajnochová Svobodová A. Skin Protective Activity of Silymarin and its Flavonolignans. Molecules. 2019 Mar 14;24(6):1022. DOI: 10.3390/molecules24061022
7. Álvarez-Gómez F, Korbee N, Casas-Arrojo V, Abdala-Díaz RT, Figueroa FL. UV Photoprotection, Cytotoxicity and Immunology Capacity of Red Algae Extracts. Molecules. 2019 Jan 18;24(2):341. DOI: 10.3390/molecules24020341
8. Ahmady A, Amini MH, Zhakfar AM, Babak G, Sediqi MN. Sun Protective Potential and Physical Stability of Herbal Sunscreen Developed from Afghan Medicinal Plants. Turk J Pharm Sci. 2020 Jun;17(3):285–92. DOI: 10.4274/tjps.galenos.2019.15428
9. Baldisserotto A, Buso P, Radice M, Dissette V, Lampronti I, Gambari R, Manfredini S, Vertuani S. Moringa oleifera Leaf Extracts as Multifunctional Ingredients for "Natural and Organic" Sunscreens and Photoprotective Preparations. Molecules. 2018 Mar 15;23(3):664. DOI: 10.3390/molecules23030664
10. Cefali LC, Ataide JA, Fernandes AR, Sanchez-Lopez E, Sousa IMO, Figueiredo MC, Ruiz ALTG, Foglio MA, Mazzola PG, Souto EB. Evaluation of In Vitro Solar

ISSN 2307-9266 e-ISSN 2413-2241


Protection Factor (SPF), Antioxidant Activity, and Cell Viability of Mixed Vegetable Extracts from *Dirmophandra mollis* Benth, *Ginkgo biloba* L., *Ruta graveolens* L., and *Vitis vinífera* L. Plants (Basel). 2019 Oct 26;8(11):453. DOI: 10.3390/plants8110453

11. Kiralan M, Yildirim G. Rosehip (*Rosa canina* L.) Oil. Fruit Oils: Chemistry and Functionality. 2019: 803–14. DOI: 10.1007/978-3-030-12473-1_43
12. Fascella G, D'Angiolillo F, Mammano MM, Amenta M, Romeo FV, Rapisarda P, Ballistreri G. Bioactive compounds and antioxidant activity of four rose hip species from spontaneous Sicilian flora. Food Chem. 2019 Aug 15;289:56-64. DOI: 10.1016/j.foodchem.2019.02.127
13. Jiménez PP, Masson SL, Quitral RV. Chemical composition of chia seed, flaxseed and rosehip and its contribution in fatty acids omega-3. Revista Chilena De Nutricion. 2013;40(2):155–60. DOI: 10.4067/S0717-75182013000200010
14. Pashazadeh H, Özdemir N, Zannou O, Koca I. Antioxidant capacity, phytochemical compounds, and volatile compounds related to aromatic property of vinegar produced from black rosehip (*Rosa pimpinellifolia* L.) juice. Food Bioscience. 2021;44(9):101318. DOI: 10.1016/j.fbio.2021.101318
15. Dąbrowska M, Maciejczyk E, Kalemba D. Rose hip seed oil: Methods of extraction and chemical composition. European Journal of Lipid Science and Technology. 2019;121(8):1800440. DOI: 10.1002/ejlt.201800440
16. Duru N, Karadeniz F, Erge HS. Changes in bioactive compounds, antioxidant activity and HMF formation in rosehip nectars during storage. Food and Bioprocess Technology. 2012;5(7):2899–907. DOI: 10.1007/s11947-011-0657-9
17. Kayath H, Dhawan S, Nanda S. *In-vitro* Estimation of Photo-Protective Potential of Rosehip Seed Oil and QbD Based Development of a Nanoformulation. Current Nanomedicine. 2019;9(3):216–31. DOI: 10.2174/2468187309666190126112141
18. Kulaitienė J, Medveckienė B, Levickienė D, Vaitkevičienė N, Makarevičienė V, Jarienė E. Changes in Fatty Acids Content in Organic Rosehip (*Rosa* spp.) Seeds during Ripening. Plants (Basel). 2020 Dec 17;9(12):1793. DOI: 10.3390/plants9121793
19. Bhave A, Schulzova V, Chmelarova H, Mrnka L, Hajslova J. Assessment of rosehips based on the content of their biologically active compounds. J Food Drug Anal. 2017 Jul;25(3):681–90. DOI: 10.1016/j.jfda.2016.12.019
20. Dubtsova GN, Negmatulloeva RN, Bessonov VV, Baĭkov VG, Sheviakova LV, Makhova NN, Perederiaev OI, Bogachuk MN, Baĭgarin EK. [Composition and content of biologically active substances in rose hips]. Vopr Pitan. 2012;81(6):84–8. Russian
21. Chu CC, Nyam KL. Application of seed oils and its bioactive compounds in sunscreen formulations. Journal of the American Oil Chemists' Society. 2021;98(7):713–26. DOI: 10.1002/aocs.12491
22. Radice M, Manfredini S, Ziosi P, Dissette V, Buso P, Fallacara A, Vertuani S. Herbal extracts, lichens and biomolecules as natural photo-protection alternatives to synthetic UV filters. A systematic review. Fitoterapia. 2016 Oct;114:144–62. DOI: 10.1016/j.fitote.2016.09.003
23. Olisova OY, Vladimirova EV, Babushkin AM. The skin and the sun. Russian Journal of Skin and Venereal Diseases. 2012;15(6):57–62. DOI: 10.17816/dv36783. Russian
24. Sviridova AA, Ischenko AA. Sun protective materials i. classification and interaction mechanism for organic UV-filters. ChemChemTech. 2006;49(11):3–14. Russian
25. Cavinato M, Jansen-Dürr P. Molecular mechanisms of UVB-induced senescence of dermal fibroblasts and its relevance for photoaging of the human skin. Exp Gerontol. 2017 Aug;94:78–82. DOI: 10.1016/j.exger.2017.01.009
26. Wu S, Han J, Laden F, Qureshi AA. Long-term ultraviolet flux, other potential risk factors, and skin cancer risk: a cohort study. Cancer Epidemiol Biomarkers Prev. 2014 Jun;23(6):1080–9. DOI: 10.1158/1055-9965.EPI-13-0821
27. Fitzpatrick TB. The validity and practicality of sun-reactive skin types I through VI. Arch Dermatol. 1988 Jun;124(6):869–71. DOI: 10.1001/archderm.124.6.869
28. Smanalieva J, Iskakova J, Oskonbaeva Z, Wichern F, Darr D. Investigation of nutritional characteristics and free radical scavenging activity of wild apple, pear, rosehip, and barberry from the walnut-fruit forests of Kyrgyzstan. European Food Research and Technology. 2020;246(5):1095–104. DOI: 10.1007/s00217-020-03476-1
29. Ácsová A, Hojerová J, Janotková L, Bendová H, Jedličková L, Hamranová V, Martiniaková S. The real UVB photoprotective efficacy of vegetable oils: *in vitro* and *in vivo* studies. Photochem Photobiol Sci. 2021 Jan;20(1):139–51. DOI: 10.1007/s43630-020-00009-3
30. Charles Dorni AI, Amalraj A, Gopi S, Varma K, Anjana SN. Novel cosmeceuticals from plants- an industry guided review. Journal of Applied Research on Medicinal and Aromatic Plants. 2017;7:1–26. DOI: 10.1016/j.jarmap.2017.05.003
31. Baumann L. How to Use Oral and Topical Cosmeceuticals to Prevent and Treat Skin Aging. Facial Plast Surg Clin North Am. 2018 Nov;26(4):407–13. DOI: 10.1016/j.fsc.2018.06.002
32. Akbarzadeh A, Rezaei-Sadabady R, Davaran S, Joo SW, Zarghami N, Hanifehpour Y, Samiei M, Kouhi M, Nejati-Koshki K. Liposome: classification, preparation, and applications. Nanoscale Res Lett. 2013 Feb 22;8(1):102. DOI: 10.1186/1556-276X-8-102
33. Fivenson D, Sabzevari N, Qiblawi S, Blitz J, Norton B, Norton S. Sunscreens: UV filters to protect us: Part 2-Increasing awareness of UV filters and their potential toxicities to us and our environment. International Journal of Women's Dermatology. 2021;7(Issue 1. – P. 45–69. DOI: 10.1016/j.ijwd.2020.08.008
34. Takshak S, Agrawal SB. Defense potential of secondary metabolites in medicinal plants under UV-B stress. J Photochem Photobiol B: Biology. 2019;193:51–88. DOI: 10.1016/j.jphotobiol.2019.02.002
35. Shikov AN, Narkevich IA, Akamova AV, Nemyatykh OD, Flisyuk EV, Luzhanin VG, Povydysh MN, Mikhailova IV, Pozharitskaya ON. Medical Species Used in Russia for the Management of Diabetes and Related Disorders. Front Pharmacol. 2021 Jul 20;12:697411. DOI: 10.3389/fphar.2021.697411
36. Li Y, Kong D, Fu Y, Sussman MR, Wu H. The effect of developmental and environmental factors on secondary metabolites in medicinal plants. Plant Physiol Biochem. 2020 Mar;148:80–9. DOI: 10.1016/j.plaphy.2020.01.006
37. Verdaguer D, Jansen MA, Llorens L, Morales LO, Neugart S. UV-A radiation effects on higher plants: Exploring the known unknown. Plant Sci. 2017 Feb;255:72–81. DOI: 10.1016/j.plantsci.2016.11.014
38. Cefali LC, Ataide JA, Moriel P, Foglio MA, Mazzola PG.

DOI: 10.19163/2307-9266-2022-10-4-308-319

Plant-based active photoprotectants for sunscreens. Int J Cosmet Sci. 2016 Aug;38(4):346–53. DOI: 10.1111/ics.12316

39. Piovesana de Souza F, Campos GR, Packer JF. Determinação da atividade fotoprotetora e antioxidante em emulsões contendo extrato de *Malpighia glabra* L. Acerola J Basic Appl Pharm Sci. 2013;34(1):69-77.
40. Kumar N, Jose J. Current developments in the nanomediated delivery of photoprotective phytochemicals. Environ Sci Pollut Res. 2020;27(31): 38446–71. DOI: 10.1007/s11356-020-10100-y
41. Avila Acevedo JG, Castañeda CM, Benitez FJ, Durán DA, Barroso VR, Martínez CG, Muñoz LJ, Martínez CA, Romo de Vivar A. Photoprotective activity of Buddleja scordioides. Fitoterapia. 2005 Jun;76(3–4):301–9. DOI: 10.1016/j.fitote.2005.03.009
42. Velasco MVR, Balogh TS, Pedriali CA, Sarruf FD, Pinto CASO, Kaneko TM, Rolim Baby A. Association of rutin with octyl p-methoxycinnamate and benzophenone-3: in vitro evaluation of photoprotective efficacy by reflectance spectrophotometry [Associação da rutina com p-metoxicinamato de octila e benzofenona-3: avaliação *in vitro* da eficácia fotoprotetora por espectrofotometria de refletância]. Lat Am J Pharm. 2008;27(1): 23–7. Portuguese
43. Velasco MV, Sarruf FD, Salgado-Santos IM, Haroutiounian-Filho CA, Kaneko TM, Baby AR. Broad spectrum bioactive sunscreens. Int J Pharm. 2008 Nov 3;363(1–2):50–7. DOI: 10.1016/j.ijpharm.2008.06.031
44. Netto MPharm G, Jose J. Development, characterization, and evaluation of sunscreen cream containing solid lipid nanoparticles of silymarin. J Cosmet Dermatol. 2018 Dec;17(6):1073-1083. DOI: 10.1111/jocd.12470
45. Shanuja SK, Iswarya S, Sridevi J, Gnanamani A. Exploring the UVB-protective efficacy of melanin precursor extracted from marine imperfect fungus: Featuring characterization and application studies under *in vitro* conditions. Int Microbiol. 2018 Jun;21(1-2):59–71. DOI: 10.1007/s10123-018-0005-2
46. Cefali LC, Franco JG, Nicolini GF, Ataide JA, Mazzola P.G. *In vitro* antioxidant activity and solar protection factor of blackberry and raspberry extracts in topical formulation. J Cosmet Dermatol. 2019;18(2):539-44. DOI: 10.1111/jocd.12842
47. Young AR, Narbutt J, Harrison GI, Lawrence KP, Bell M, O'Connor C, Olsen P, Grys K, Baczynska KA, Rogowski-Tylman M, Wulf HC, Lesiak A, Philipsen PA. Optimal sunscreen use, during a sun holiday with a very high ultraviolet index, allows vitamin D synthesis without sunburn. Br J Dermatol. 2019 Nov;181(5):1052–62. DOI: 10.1111/bjd.17888
48. Li SX, Li MF, Bian J, Wu XF, Peng F, Ma MG. Preparation of organic acid lignin submicrometer particle as a natural broad-spectrum photo-protection agent. Int J Biologic Macromolecul. 2019;132:836–43. DOI: 10.1016/j.ijbiomac.2019.03.177
49. Cefali LC, Ataide JA, Fernandes AR, Sousa IMO, Gonçalves FCDS, Eberlin S, Dávila JL, Jozala AF, Chaud MV, Sanchez-Lopez E, Marto J, d'Ávila MA, Ribeiro HM, Foglio MA, Souto EB, Mazzola PG. Flavonoid-Enriched Plant-Extract-Loaded Emulsion: A Novel Phytocosmetic Sunscreen Formulation with Antioxidant Properties. Antioxidants (Basel). 2019 Oct 1;8(10):443. DOI: 10.3390/antiox8100443
50. Cho YH, Bahuguna A, Kim HH, Kim DI, Kim HJ, Yu JM, Jung HG, Jang JY, Kwak JH, Park GH, Kwon OJ, Cho YJ, An JY, Jo C, Kang SC, An BJ. Potential effect of compounds isolated from *Coffea arabica* against UV-B induced skin damage by protecting fibroblast cells. J Photochem Photobiol B. 2017 Sep;174:323–32. DOI: 10.1016/j.jphotobiol.2017.08.015
51. Musazzi UM, Franzè S, Minghetti P, Casiraghi A. Emulsion versus nanoemulsion: how much is the formulative shift critical for a cosmetic product? Drug Deliv Transl Res. 2018 Apr;8(2):414–21. DOI: 10.1007/s13346-017-0390-7

## AUTHORS

**Oksana D. Nemyatykh** – Doctor of Sciences (Pharmacy), Associate Professor, Professor of Management and Economics Department of Pharmacy, Saint-Petersburg State Chemical and Pharmaceutical University. ORCID ID: 0000-0001-5933-2120. E-mail: oksana.nemyatyh@pharminnotech.com

**Inna I. Terninko** – Doctor of Sciences (Pharmacy), Associate Professor, Head of the Structural Subdivision, Testing Laboratory (Medicines Quality Control Center) of Saint-Petersburg State Chemical and Pharmaceutical University. ORCID ID: 0000-0002-2942-1015. E-mail: inna.terninko@pharminnotech.com

**Askhat S. Sabitov –** teacher of the School of Pharmacy, Asfendiyarov Kazakh National Medical University, Republic of Kazakhstan. ORCID ID: 0000-0001-8101-2123. E-mail: acxam78@gmail.com

**Anastasia I. Lyashko** – Candidate of Sciences (Pharmacy), Associate Professor of Management and Economics Department of Pharmacy, Saint-Petersburg State Chemical and Pharmaceutical University. ORCID ID: 0000-0001-9534-0398 E-mail: anastasia.fitisova@pharminnotech.com

**Zuriyadda B. Sakipova** – Doctor of Sciences (Pharmacy), Professor, Dean of the School of Pharmacy, Asfendiyarov Kazakh National Medical University, Republic of Kazakhstan. ORCID ID: 0000-0003-4477-4051. E-mail: sakipova.z@kaznmu.kz

*RESEARCH ARTICLE*

*ISSN 2307-9266 e-ISSN 2413-2241*

Scientific and Practical Journal
PHARMACY & PHARMACOLOGY



# DEVELOPMENT OF MICROCAPSULES BASED ON COMBINED ANTIDIABETIC SUBSTANCE: PHARMACOLOGICAL CHARACTERISTICS

**A. Giyoszoda[1], E.F. Stepanova[2], O.F. Veselova[3], S.E. Storozhenko[3]**

[1] Non-state educational institution "Medical College" of M.S. Hamadoni district, 26, Somoni Str., R.M.C.A. Hamadoni Khatlon region, Tajikistan, 735140
[2] Pyatigorsk Medical and Pharmaceutical Institute – branch of Volgograd State Medical University, 11, Kalinin Ave., Pyatigorsk, Russia, 357532
[3] Krasnoyarsk State Medical University named after Professor V.F. Voyno-Yasenetsky, 1, Partisan Zheleznyak Str., Krasnoyarsk, Russia, 660022

E-mail: EFStepanova@yandex.ru

Received 30 April 2022 After peer review 22 June 2022 Accepted 15 Aug 2022

The comparative assessment results of the hypoglycemic activity of a combined preparation containing microcapsules with a phytocomposition consisting of *Glycyrrhiza glabra* L. extracts, a dry extract of *Galega officinalis* L., *Mentha piperita* L., and gliclazide, are discussed in the article. Methods for obtaining microcapsules with an original PEG-6000 shell are described.

**The aim** of the study was to develop an optimal technology for obtaining microcapsules with a PEG-6000 shell containing a combined antidiabetic substance, and conduct its detailed pharmacological study on the model of type 2 diabetes mellitus, to conduct a detailed comparative pharmacological study of a microencapsulated antidiabetic composition with a shell based on PEG-6000, including gliclazide and a sum of phytoextracts on the model of type 2 diabetes mellitus.

**Materials and methods.** As the main objects of the study, microcapsules with a PEG-6000 shell were obtained using methyl miristate as the base liquid. The capsules contained the amount of plant extracts in their composition: a dry extract of *Glycyrrhiza glabra* L., a dry extract of *Galega officinalis* L., a dry extract of *Mentha piperita* L., and gliclazide. The study of a hypoglycemic activity was carried out after a single administration of drugs to the animals with alloxan-induced type 2 diabetes mellitus. The cumulative effect assessment of the drugs was carried out within 14 days with a test for the resistance to oral glucose on days 7 and 14.

**Results.** Microcapsules with the original shell were obtained by dispersion in a liquid-liquid system with the adjustment of some technological stages. The effect of the drugs under study on the glycemic profile in the rats with an experimental model of type 2 diabetes mellitus was investigated. A comparative evaluation of the pharmacological effect was carried out with a separate and combined use of microencapsulated preparations.

**Conclusion.** The rationality of combining phytocomponents and a synthetic antidiabetic agent in microcapsules has been proven. The obtained results testify to the rationality of plant extracts combination and a synthetic hypoglycemic agent – gliclazide in microcapsules.

**Keywords:** microcapsules; PEG-6000, methylmiristate; diabetes mellitus; *Glycyrrhiza glabra* extracts; *Galega officinalis* L.; *Mentha piperita* L.; gliclazide

**Abbreviations:** DM – diabetes mellitus; PPAR-γ – peroxisome proliferator-activated receptor; GLUT-4 – glucose transporter type 4; AMPK – adenosine monophosphate protein kinase; PEG – polyethylene glycol; PEMC(s) – plant extract microcapsules; GMC(s) – gliclazide microcapsules; GT(s) – gliclazide tablets; PBO – placebo; HGA – hypoglycemic activity.

**For citation:** A. Giyoszoda, E.F. Stepanova, O.F. Veselova, S.E. Storozhenko. Development of microcapsules based on combined antidiabetic substance: pharmacological characteristics. *Pharmacy & Pharmacology.* 2022;10(4):320-330. **DOI:** 10.19163/2307-9266-2022-10-4-320-330



**Для цитирования:** А.Ш. Гиёзода, Э.Ф. Степанова, О.Ф. Веселова, С.Е. Стороженко. Разработка микрокапсул на базе комбинированной антидиабетической субстанции и ее фармакологическая характеристика. *Фармация и фармакология.* 2022;10(4): 320-330. **DOI:** 10.19163/2307-9266-2022-10-4-320-330

Научно-практический журнал
ФАРМАЦИЯ И ФАРМАКОЛОГИЯ

*ОРИГИНАЛЬНАЯ СТАТЬЯ*

*DOI: 10.19163/2307-9266-2022-10-4-320-330*

# РАЗРАБОТКА МИКРОКАПСУЛ НА БАЗЕ КОМБИНИРОВАННОЙ АНТИДИАБЕТИЧЕСКОЙ СУБСТАНЦИИ И ЕЕ ФАРМАКОЛОГИЧЕСКАЯ ХАРАКТЕРИСТИКА

**А.Ш. Гиёсзода[1], Э.Ф. Степанова[2], О.Ф. Веселова[3], С.Е. Стороженко[3]**

[1] Негосударственное образовательное учреждение «Медицинский колледж» района М.С. Хамадони, 735140, Таджикистан, Хатлонская область, р. М.С.А. Хамадони ул. Сомони, д. 26
[2] Пятигорский медико-фармацевтический институт – филиал федерального государственного бюджетного образовательного учреждения высшего образования «Волгоградский государственный медицинский университет» Министерства здравоохранения Российской Федерации, 3573352, Россия, г. Пятигорск, пр-т Калинина, д. 11
[3] Федеральное государственное бюджетное образовательное учреждение высшего образования «Красноярский государственный медицинский университет имени профессора В.Ф. Войно-Ясенецкого» Министерства здравоохранения Российской Федерации, 660022, Россия, г. Красноярск, ул. Партизана Железняка, д. 1

E-mail: EFStepanova@yandex.ru

Получена 30.04.2022 После рецензирования 22.06.2022 Принята к печати 15.08.2022

В статье рассматриваются результаты сравнительной оценки гипогликемической активности комбинированного препарата, содержащего микрокапсулы с фитокомпозицией, состоящей из экстрактов солодки голой, козлятника лекарственного, мяты перечной и гликлазида. Описаны способы получения микрокапсул с оригинальной оболочкой из ПЭГ-6000.

**Цель.** Разработать оптимальную технологию получения микрокапсул с оболочкой из ПЭГ-6000, содержащих комбинированную субстанцию антидиабетического действия и провести её подробное фармакологическое исследование на модели сахарного диабета 2-го типа. Провести подробное сравнительное фармакологическое исследование микрокапсулированной антидиабетической композиции с оболочкой на основе ПЭГ-6000, включающей гликлазид и сумму фитоэкстрактов на модели сахарного диабета 2-го типа.

**Материалы и методы.** В качестве основных объектов исследования были получены микрокапсулы с оболочкой из ПЭГ-6000 с использованием в качестве базовой жидкости метилмиристата. Капсулы содержали в своем составе сумму растительных экстрактов: сухой экстракт солодки голой, сухой экстракт козлятника лекарственного, сухой экстракт мяты перечной, а также гликлазид. Изучение гипогликемической активности проведено после однократного введения препаратов животным с аллоксан-индуцированным сахарным диабетом 2-го типа. Оценка накопительного эффекта препаратов проведена в течение 14 суток с проведением теста на резистентность к пероральной глюкозе на 7 и 14 сутки.

**Результаты.** Получены микрокапсулы с оригинальной оболочкой методом диспергирования в системе жидкость-жидкость с корректировкой некоторых технологических этапов. Изучено влияние исследуемых препаратов на гликемический профиль у крыс с экспериментальной моделью сахарного диабета 2-го типа. Проведена сравнительная оценка фармакологического эффекта при раздельном и совместном применении микрокапсулированных препаратов.

**Заключение.** Доказана рациональность комбинирования фитокомпонентов и синтетического средства сахароснижающего действия в микрокапсулах. Полученные результаты свидетельствуют о рациональности комбинации растительных экстрактов и синтетического сахароснижающего средства – гликлазида в микрокапсулах.

**Ключевые слова:** микрокапсулы; ПЭГ-6000, метилмиристат; сахарный диабет; экстракт солодки голой; козлятник лекарственный (галега); мята перечная; гликлазид

**Список сокращений:** СД – сахарный диабет; PPAR-γ – рецептор-активатор пролиферации пероксисом; GLUT-4 – глюкозный транспортёр тип 4; AMPK – аденозинмонофосфатная протеинкиназа; ПЭГ – полиэтиленгликоль; МКЭ – микрокапсулы с растительными экстрактами; МКГ – микрокапсулы с гликлазидом; ТГ – таблетки гликлазида; Пл – плацебо; ГА – гипогликемическая активность.

## INTRODUCTION

One of the most problematic aspects of modern medicine and pharmacy, in particular epidemiology, is diabetes mellitus (DM) which can be considered as a non-infectious epidemic of the present. The prevalence of this disease reaches more than 6% in some countries [1, 2]. At the same time, the disease is unstable; it is usually accompanied by a frequent disability [3] and lethality [4]. This has been especially acute in recent years associated with the pandemic [5, 6]. It was during this period that the problem of diabetes reached its maximum.

Interest in preventive measures (diets, dietary regimens) has grown. However, this did not affect

the need for the development and improvement of pharmacotherapy, in which the basic emphasis had also to be revised. The interest of diabetologists in medicinal plants, which occupy an important position in the treatment and prevention of DM today, has sharply increased [7–8]. The search for new antidiabetic drugs made out of medicinal plants, is an important and strategically significant task, since they contain bioactive phytochemicals that are more active and safe than conventional therapy. At the same time, it is impossible to underestimate the role of synthetic drugs that have been solving these problems for many years quite successfully – these are, first of all, metformin, gliclazide, glibenclamide, and other well-known drugs which are constantly in demand [9–11].

The main component of the proposed phytocomposition is an extract of *Glycyrrhiza glabra* L., which has a wide range of a pharmacological activity. Biologically active substances in the *Glycyrrhiza glabra* L. extract can significantly bind to gamma *peroxisome* proliferator-activated receptors (PPAR-γ), which leads to a decrease in blood glucose levels. Therefore, chalcone and amorphrutin, obtained from *Glycyrrhiza glabra* L., promote the differentiation of adipocytes and improve glucose and lipid metabolism. Amorphrutin increases the sensitivity of cells to insulin and, as a result, glucose tolerance. Glabridin prevents glucose intolerance and ensures its maximum utilization by translocation of GLUT-4 using adenosine monophosphate protein kinase (AMPK) [12]. In addition, glycyrrhizic acid, which is one of the main bioactive components of *Glycyrrhiza glabra* L., inhibits the activity of peroxidase, hemoglobin esterase and hemoglobin-mediated oxidative damage, while not affecting the ability of the protein to bind oxygen. This effect is especially important in the treatment of DM, given the complications associated with an oxidative stress [13–15]. In addition, there are studies [16] on the positive effect of the *Glycyrrhiza glabra* L extract on the intestinal microbiota, which is extremely important, since it is known that the pathogenesis of the onset and severity of DM is indirectly associated with a violation of the enzymatic and biochemical balance of the vital activity of the normal intestinal microflora.

One of the medicinal plants with a pronounced hypoglycemic effect is *Galega officinalis* L. In the 1920s, it was found out that guanidine, an active component in the herb of *Galega officinalis* L., reduces blood glucose levels, which became the basis for the synthesis of several antidiabetic compounds and, in particular, metformin [17]. The hypoglycemic effect is also associated with the alkaloid of galegin in the plant [18]. However, the study of anti-diabetic properties of *Galega officinalis* L. is ongoing. To date, a decrease in the concentration of glucose and glycosylated hemoglobin in the blood of animals against the background of the galega extract administration, as well as an increase in the cell tolerance to glucose, an increase in the content of C-peptide and insulin in blood plasma, have been proven. The *in vivo* studies confirmed the cytoprotective effect of the *Galega officinalis* L. extract on pancreatic cells, expressed in an increase in the number of Langerhans islets, their average area, diameter, volume, and the number of β-cells [19, 20]. It has been established that the *Galega officinalis* L. extract regulates disorders of the proliferation, function and apoptosis of leukocytes associated with DM, thereby having a pronounced immunocorrective effect [21].

Aromatic plants rich in essential oils, such as *Mentha piperita* L., *Melissa officinalis* L., *Cuminum cyminum* L., are among the potential new sources of drugs. These plants are very promising due to their diverse chemical composition and multiple mechanisms of action. Thus, the antidiabetic effect of *Mentha piperita* L. is associated with the inhibition of ATP-sensitive K+channels on the β-cell membrane, increased insulin exocytosis under the action of menthol. In addition, menthol increases the survival of β-cells by stimulating the expression of Bcl-2, an anti-apoptotic factor, and protects pancreatic β-cells from apoptosis in a rat model of diabetes [22, 23]. In addition, the hypoglycemic effect of the *Mentha piperita* L. extract is associated with an inhibitory effect on α-amylase and α-glucosidase, which, in turn, has a significant inhibitory effect on postprandial hyperglycemia [24].

Thus, the literature data on the spectrum of hypoglycemic activity of the *Glycyrrhiza glabra* L. extract; *Galega officinalis* L. and *Mentha piperita* L. confirm the rationality of including extracts of these medicinal plants in the proposed phytocomposition and the development of combined antidiabetic dosage forms based on them.

**THE AIM** of the study was to develop an optimal technology for obtaining microcapsules with a PEG-6000 shell containing a combined antidiabetic substance, and conduct its detailed pharmacological study on the model of type 2 diabetes mellitus, to conduct a detailed comparative pharmacological study of a microencapsulated antidiabetic composition with a shell based on PEG-6000, including gliclazide and a sum of phytoextracts on the model of type 2 diabetes mellitus.

## MATERIALS AND METHODS

### Objects of study

The main objects of the study were microcapsules containing a composition of dry extracts of *Glycyrrhiza glabra*, *Galega officinalis* L. and *Mentha piperita* L. in the ratio of 6:1:3, respectively, as well as gliclazide in a traditional dosage.

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2022-10-4-320-330

PEG-6000 with a density of 1200 kg/m$^3$, soluble in water and alcohol, non-toxic, was chosen as a film former for microcapsules. Methylmiristat is a process medium, it is a clear, colorless liquid with a low melting point. To obtain microcapsules, dry extracts of *Glycyrrhiza glabra* L., *Galega officinalis* L. and *Mentha piperita* L. obtained from the manufacturer of LLC Kazan Extract Plant (Russia), were used. The moisture content of the extracts was not more than 4.8%.

### Reference drug

For a comparative assessment of the hypoglycemic activity, the tablet form of gliclazide (60 mg, manufacturer LLC SERVIER RUS, Russia) had been chosen as the reference drug. For this purpose, model tablets with an active ingredient content of 2.5 mg were used. The drug was administered using a gastric tube, taking into account the interspecies conversion factor, in several doses to achieve the required dose of gliclazide.

### Equipment

Measurements of blood glucose concentration in the animals with an experimental model of type 2 diabetes mellitus was carried out using an Accu-Chek Performa Nano glucometer (Roche Diagnostics GmbH, Germany).

The preparation of microcapsules was carried out using an overhead stirrer US-1170D with a four-bladed propeller nozzle (ULAB, Russia). A vessel with a hemispherical bottom with a volume of 500 ml was used as a reactor for obtaining microcapsules.

A six-seater water bath LOIP LB-161 (LC LOIP, Russia) was also used.

The administration of the study drugs was carried out using an intragastric metal probe for rodents.

### Microcapsules obtaining

Taking into account the originality of the PEG-6000 microcapsule shell used for the first time, the most reliable technological method of dispersion in the liquid-liquid system was chosen. A saturated complex was sequentially delivered in a thin stream: 25.0 g of the total extracts solutions and 25.0 g of a gliclazide solution together with 100 g of the solution forming the shell of PEG-6000 microcapsules. The complex was delivered into the reactor with the base medium in the form of methyl miristat at the temperature of 40°C. Herewith, defining the technological novelty was the choice of the base solution and an active mixing device – a propeller mixer operating at the speed of 500–600 rpm. The temperature factor also changed, quickly passing from a state of heating to artificial cooling.

The drops shaped in the oil, quickly solidified, spherical particles were formed from them, and then they were separated from the methyl miristate solution and washed with 90% ethyl alcohol, making sure that the shell did not dissolve. The standardization of the finished product of microcapsules was carried out according to their flowability and the angle of repose, which was 30° and which indicated a normal technological cycle [25].

### Experimental animals

The hypoglycemic activity (GA) of the studied preparations was investigated on male rats of the Wistar line weighing 200–220 g, with alloxan-induced DM. The animals were obtained from the Novosibirsk Nursery of Laboratory Animals (State Unitary Enterprise of Laboratory Animals, the RAS Siberian branch) and kept in the vivarium of Krasnoyarsk State Medical University of the Ministry of Health of Russia. The work with laboratory animals was carried out in accordance with Directive N 2010/63/EU of the European Parliament and the Council of the European Union "On the protection of animals used for scientific purposes" and GOST 33044-2014 "Principles of good laboratory practice". The animals were kept in conventional cages with an area of 820 cm$^2$, with litter of non-coniferous sawdust, the indoor temperature of 22±2°C, a the relative air humidity of 65±5%, with a free access to water and food. The study protocol was approved by the ethics committee of Krasnoyarsk State Medical University of the Ministry of Health of Russia (protocol No. 104/2021 dated April 17, 2021).

### Setting up DM model type 2

Setting up the model of alloxan-induced DM was carried out in the classical way in accordance with the guidance by A.M. Mironov[1]. The animals were intraperitoneally injected with a 5% solution of nicotinamide at the dose of 230 mg/kg, followed by the administration of a 5% solution of alloxan at the rate of 150 mg/kg after 15 minutes. On the 4th day after the injection of alloxan, 5 groups of animals (n=8) with an average blood glucose level of more than 20 mmol/l, were formed according to the principle of paired analogs. Taking into account the previous studies, the doses of the administered drugs were selected [26, 27] and calculated based on the interspecies dose conversion factor. The studied substances and the reference drug were administrated into the stomach using a metal probe.

The administration of drugs was carried out for 14 days according to the following scheme:

Group 1 – microcapsules with plant extracts (PEMCs) – 750 mg/kg;

[1] [Guidelines for conducting preclinical studies of drugs (Part 1)]. Mironova AN, editor. M: Grif and K; 2012. 944 p. Russian

Group 2 – microcapsules with gliclazide (GMCs) – 150 mg/kg;

Group 3 – gliclazide tablets (GTs) – 60 mg/kg – reference group;

Group 4 – combination of microcapsules (PEMCs + GMCs);

Group 5 – 2.5 ml of purified water (PBO) – control group.

### Determination of hypoglycemic effect

The assessment of hypoglycemic activity (HGA) was carried out by monitoring the concentration of glucose in the blood of animals for 24 hours after a single administration of the study drugs. Taking into account the established cumulative hypoglycemic effect, characteristic of this phytocomposition and confirmed for microcapsules with a gelatin shell, the effectiveness of the developed microcapsules with a shell based on PEG-6000, was studied for a long-term use. For this purpose, tests for the resistance to oral glucose were carried out on days 7 and 14 according to the methods [26, 27]. The general study design of the hypoglycemic activity of the microencapsulated preparations is shown in Fig. 1. The scheme of the test for the glucose resistance is shown in Fig. 2.

### Statistical analysis

Statistical processing of the obtained results was carried out using a Microsoft Excel software package (2016), by a descriptive statistics method, by calculating the mean value of the studied indicator and the standard deviation (σ). The normality of the sample distribution was determined using the Shapiro-Wilk test. The Wilcoxon's t-test was used to assess differences between quantitative non-normally distributed data of related samples, the Student's t-test was used for normally distributed data of unrelated samples ($p < 0.05$).

## RESULTS AND DISCUSSION

The results of the HGA study after a single administration of microcapsules containing the composition of plant extracts, gliclazide, as well as their combination, are presented in Table1.

The comparison of the presented data in Table 1 shows that the decrease in the animals' blood plasma glucose concentration was established in all the groups after a single administration of the studied drugs, except the control group. There was no significant decrease in blood glucose levels in the animals of the control group within 24 hours after starting the experiment. Against the background of the use of microcapsules with plant extracts, a hypoglycemic activity (HGA) develops after 4 hours and is maintained within 8 hours. After the administration of GMCs, HGA manifests itself after 2 hours and continues to grow within 10 hours. However, the achievement of the physiological blood glucose level in the animals after a single administration of monopreparations is not observed within 24 hours. With a combination of PEMCs+GMCs, the pharmacological effect occurs after 2 hours, and after 6 hours, the animals' blood glucose concentration reaches its physiological level. After a single administration of the developed microcapsules and the reference drug, a stable hypoglycemic effect is observed from 6 to 12 hours. At the same time, the average animals' blood glucose level in the in this time interval was as follows: for group 1 – 18.0 ± 1.4 mmol/l; group 2 – 11.8 ± 0.9 mmol/l; group 3 – 6.6 ± 0.2 mmol/l; group 4 – 7.7 ± 0.6 mmol/l; the blood glucose level in the control animals' group was 24.7 ± 1.9 mmol/L.

According to the statistical analysis of the obtained results, the HGA combination of PEMCs+GMCs is comparable to the gliclazide tablet form. In addition, in the pharmacological activity, the combined composition of microcapsules is superior to the microcapsules containing only one component. 6 hours after the administration of the drugs, the animals' blood glucose level in group 4 decreased by 68.8% ($p < 0.05$), while the blood glucose concentration in the animals of groups 1 and 2 decreased by 22.9% ($p < 0.05$) and 51.4% ($p<0.05$), respectively. It should be also notified that 6 hours after the administration of the PEMCs+GMCs combination and the reference drug, there are significant differences in the average values of the animals' blood glucose levels of the respective groups. The glucose concentration in the blood of the animals receiving a combination of PEMCs+GMCs (group 4) is 23.3% ($p < 0.05$) higher than in the blood of the animals treated with GTs. The absence of statistically significant differences in the initial values of the animals' blood plasma glucose concentration in these groups indicates different pharmacokinetic characteristics of the dosage forms of the studied drugs, i.e., a different degree of elongation.

Analyzing the results of monitoring the level of the animals' blood plasma glucose concentration, it can be concluded that a stable hypoglycemic effect after a single administration of the developed microcapsules was not observed immediately, just as after a single administration of the reference drug, in the time range from 4 to 12 hours. However, in this time interval, a significant decrease in blood glucose was observed with the use of gliclazide tablets (group 3 of the animals), with the use of gliclazide microcapsules (group 2) and with the administration of a combination of microcapsules (group 4). At the same time, the levels of glucose in the animals' blood plasma of groups 3 and 4 are almost equivalent and slightly lower than in group 2 which may indicate the presence of potentiation of the hypoglycemic effect when the herbal components are combined with a synthetic drug.

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2022-10-4-320-330

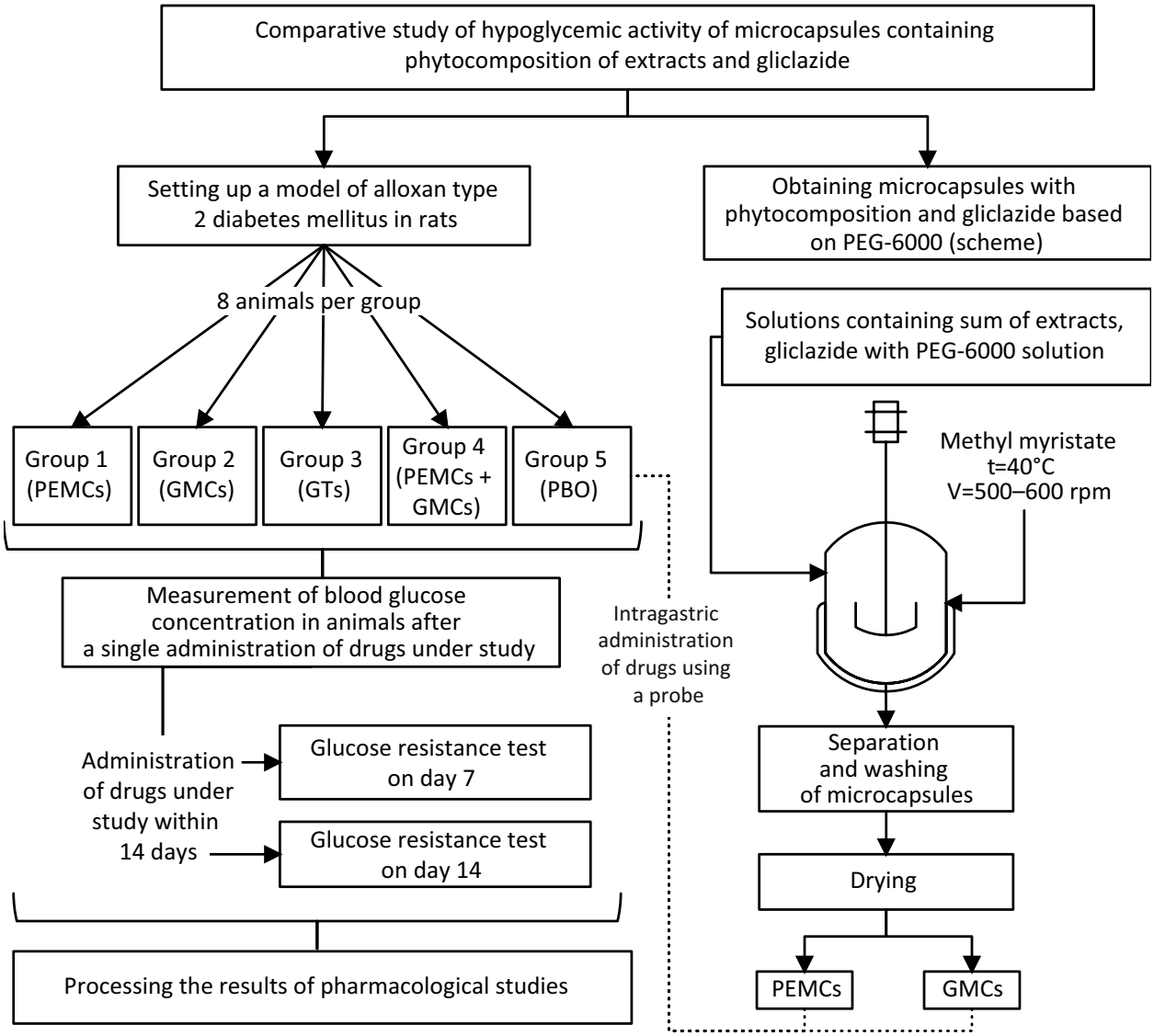



**Figure1 – Study design**

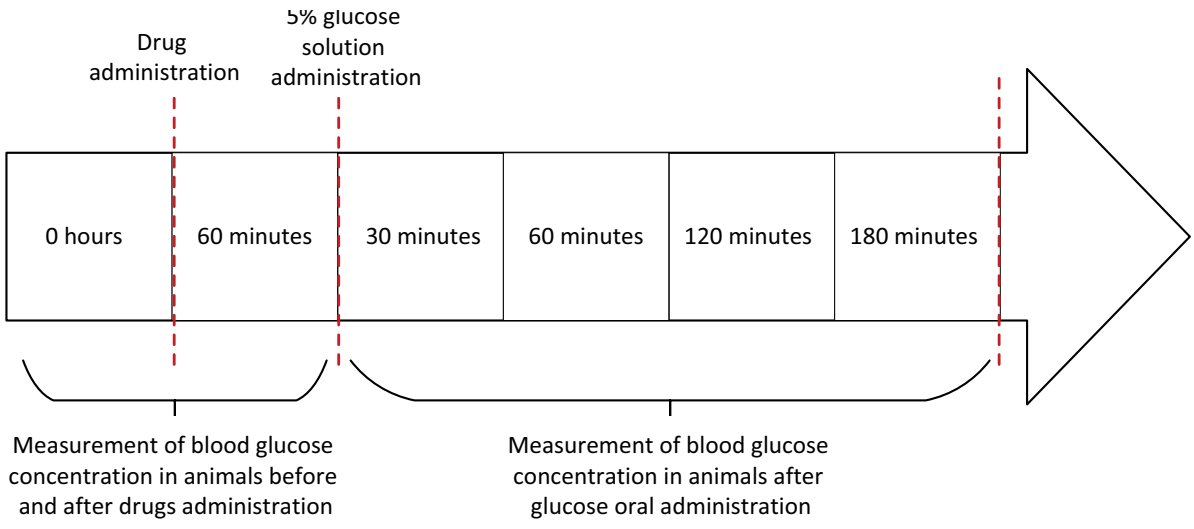



**Figure 2 – Scheme of glucose resistance test on days 7 and 14**

ISSN 2307-9266 e-ISSN 2413-2241
Scientific and Practical Journal
PHARMACY & PHARMACOLOGY

**Table 1 – Blood glucose level in animals with experimental model of type 2 diabetes mellitus after a single administration of drugs under study**

| Group | Time, hours | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 2 | 4 | 6 | 8 | 10 | 12 | 24 |
| 1 | 25.3±3.8 | 23.5±4.1# | 20.9±3.4# | 19.5±2.3*# | 19.1±4.3*# | 16.8±5.3*# | 16.8±4.5*# | 23.6±4.4# |
| 2 | 25.1±3.3 | 18.5±2.6 | 15.3±1.9*# | 12.2±1.7*# | 11.2±1.0*# | 11.0±0.7*# | 13.0±0.9*# | 24.3±4.2# |
| 3 | 27.7±2.4 | 15.1±3.8* | 8.4±2.4* | 6.6±1.1* | 6.4±0.9* | 6.5±0.7* | 6.8±0.5* | 12.9±1.8* |
| 4 | 27.6±4.0 | 17.6±3.0* | 10.2±2.2* | 8.6±2.0*# | 7.3±1.7* | 7.3±1.5* | 7.5±1.3* | 26.5±3.0# |
| 5 | 21.7±1.2 | 22.1±0.6 | 21.7±0.8 | 25.7±0.5 | 26.4±0.9 | 24.5±0.6 | 22.2±1.2 | 26.1±1.4 |

Note: results are presented as Mean±σ; * – mean blood glucose concentration values significantly different from the control group (p <0.05) and # – mean blood glucose concentration values significantly different from the comparison group (p <0.05), according to the results of the analysis by the Student's t-criterion.

**Table 2 – Study results of cumulative effect on day 7**

| Group | Mean glucose concentration, mmol/l | | | | | |
|---|---|---|---|---|---|---|
| | Drug administration | | Glucose administration | | | |
| | 0 | 60 min | 30 min | 60 min | 120 min | 180 min |
| 1 | 25.3 ± 4.4# | 24.4 ± 4.4# | 24.0 ± 4.2# | 25.8 ± 3.3# | 25.5 ± 4.6# | 24.0 ± 4.2# |
| 2 | 23.5 ± 4.8# | 20.7 ± 5.5*# | 18.0 ± 4.6*# | 16.6 ± 3.8*# | 14.4 ± 3.4*# | 13.6 ± 3.5*# |
| 3 | 6.4 ± 0.7* | 6.2 ± 0.9* | 6.9 ± 1.1* | 6.0 ± 1.1* | 5.8 ± 0.8* | 5.8 ± 0.8* |
| 4 | 19.8 ± 1.8*# | 12.4 ± 1.5*# | 9.9 ± 1.0*# | 6.8 ± 0.6* | 6.8 ± 0.5*# | 6.8 ± 0.4*# |
| 5 | 23.9 ± 3.5 | 24.9 ± 4.0 | 28.5 ± 2.9 | 28.7 ± 4.2 | 26.7 ± 2.8 | 25.4 ± 2.0 |

Note: results are presented as Mean±σ; * – mean values of blood glucose concentration significantly different from the control group (p <0.05) and # – mean values of blood glucose concentration significantly different from the comparison group (p <0.05), according to the results of the analysis by the Student's t-criterion.

**Table 3 – Study results of cumulative effect on day 14**

| Group | Mean glucose concentration, mmol/l | | | | | |
|---|---|---|---|---|---|---|
| | Drug administration | | Glucose administration | | | |
| | 0 | 60 min | 30 min | 60 min | 120 min | 180 min |
| 1 | 18.2 ± 3.2*# | 17.9 ± 3.3*# | 16.5 ± 4.2*# | 17.6 ± 4.4*# | 16.0 ± 3.4*# | 12.2 ± 2.5*# |
| 2 | 14.0 ± 2.3*# | 12.7 ± 1.4*# | 12.7 ± 1.6*# | 12.4 ± 1.5*# | 11.6 ± 0.9*# | 11.1 ± 0.7*# |
| 3 | 6.7 ± 1.0* | 5.6 ± 1.3* | 5.6 ± 1.2* | 5.9 ± 1.1* | 6.1 ± 1.3* | 6.3 ± 1.2* |
| 4 | 12.6 ± 1.4*# | 9.0 ± 0.5*# | 8.5 ± 0.5*# | 6.6 ± 0.4* | 6.5 ± 0.4* | 6.5 ± 0.4* |
| 5 | 21.7 ± 3.3 | 21.3 ± 3.0 | 27.0 ± 2.8 | 28.7 ± 1.0 | 28.3 ± 2.4 | 28.1 ± 1.4 |

Note: results are presented as mean ± standard deviation; * – mean values of blood glucose concentration significantly different from the control group (p <0.05) and # – mean values of blood glucose concentration significantly different from the comparison group (p <0.05), according to the results of the analysis by the Student's t-criterion.

Thus, after a single administration, the HGA profile of the preparations under study was investigated and confirmed. It has been reliably established that the above described hypoglycemic effect of microcapsules based on PEG-6000 and containing a combination of PEMCs+GMCs, can be characterized as more delayed and prolonged.

As it has been notified in the previous studies[2] [25], the composition of medicinal plants extracts is characterized by a cumulative hypoglycemic effect. In support of this trend, PEG-6000 based microcapsules were tested for resistance to the alimentary glucose administration on the 7th and 14th days of the study. For this purpose, one hour after the administration of the studied preparations, the animals of all experimental groups were administrated with 2.5 ml of a 5% glucose solution using an intragastric metal probe, and the blood glucose level was measured within the next 3 hours. The study results of the resistance to oral glucose on the 7th day of therapy are presented in Table 2.

According to the data presented in Table 2, it can be notified that before the administration of drugs on day 7, the blood glucose level in the animals' blood of the studied groups was higher than a physiological one in all experimental groups, except group 3. In the control group, the blood glucose level in the animals was high throughout the entire time of the experiment. It is also worth notifying that in the animals of groups 1 and 2 before the administration of drugs, the blood glucose concentration does not have significant changes compared to the control group.

The blood glucose concentration level in the animals receiving microcapsules with plant extracts (group 1) was high throughout the experiment, no statistically

[2] Ibid.

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2022-10-4-320-330

significant changes were detected (p >0.05) either against the background of the drug administration and or against the background of a glucose oral administration.

The glucose content in the animals' blood of group 2 decreased by 13.5% (p <0.05) from the initial value. This trend persisted within the group throughout the experiment, and at the end of the test, there was a decrease in the glucose content in the animals' blood by 42.1% (p <0.05), which indicates a smooth increase in the pharmacological effect. Despite the fact that the decrease in the blood glucose concentration to the physiological level was not observed, against the background of the use of microcapsules with gliclazide, the animals showed resistance to oral glucose.

In the animals receiving the comparison drug (group 3), before the administration of the drug, the blood glucose level was at the level of the physiological norm, which is associated with the manifestation of a stable hypoglycemic effect characteristic of gliclazide tablets. This trend continued throughout the experiment. Against the background of the alimentary load, an increase in the blood glucose concentration in the animals of group 2 was not observed, which also indicates the stability of the reference drug.

Before the administration of the drug, the blood glucose level in the animals receiving combined therapy with microcapsules (group 4) on day 7 was above the physiological norm. At the same time, the glucose concentration was 67.7% (p <0.05) higher than in the animals of the comparison group, but 17.2% (p <0.05) lower than in the control group. However, after the administration of the drug, the hypoglycemic effect increased, and already after 60 minutes, a statistically significant (the Wilcoxon T-test) decrease by 37.4% (p <0.05) was established in the blood glucose concentration in the animals of group 4 with the dynamics unchanged. 60 min after the glucose oral administration, the level of blood glycemia in the of animals of group 4 decreased by 65.7% (p <0.05) with the maintenance of a physiologically significant level till the end of the test, which indicates the animals' resistance to receiving a combination of microcapsules.

According to the statistical analysis results, it can be said that the administration of the microcapsules combination (group 4) contributed to the manifestation of a tolerance effect comparable to that of the reference drug (group 3) 60 minutes after the glucose oral administration.

According to the test results on the resistance to alimentary glucose on the 7$^{th}$ day of the study, therapy with a combination of PEMCs+GMCs gives a more pronounced pharmacological effect compared to the separate use of the drugs.

Table 3 presents the study results of the resistance to oral glucose on the 14$^{th}$ day of the administration of the investigated drugs.

According to the results presented in Table 3, it can be said that before the administration of drugs on the 14th day, the blood plasma glucose concentration in the animals of the studied groups was also higher than the physiological norm in all the experimental groups, except the comparison one. The blood glucose level in the animals treated with the reference drug (group 3) was at the physiological level both before and after the administration of the drug. Based on the statistical analysis results of blood glycemia in the animals of the 3rd group (the Wilcoxon T-test), it can be said that the observed changes after the alimentary load are statistically insignificant (p >0.05).

It should be notified that before the administration of drugs, the glucose level on the 14$^{th}$ day in groups 1, 2 and 4 decreased by 28.1% (p <0.05), 40.4% (p <0.05) and 36.4% ( p <0.05) compared with the values of this parameter in the respective groups on the 7$^{th}$ day.

In addition, before the administration of drugs, on day 14, the glucose concentration in groups 1, 2 and 4 was lower by 16.1% (p <0.05), 35.5% (p <0.05) and 41.9% (p <0.05) than the glucose concentration in the blood plasma of the control group. As a whole, these factors indicate the manifestation of a cumulative antidiabetic effect of the studied microcapsule preparations.

60 minutes after the administration of microcapsules, a significant change by 9.3% (p <0.05) and 28.6% (p <0.05), respectively, from the initial level, was found out in the blood glucose concentration in the animals of groups 2 and 4. This trend persisted throughout the experiment, and 60 minutes after the oral administration of glucose, group 4 showed a decrease in the glucose concentration by 47.6% (p <0.05) while maintaining a physiologically significant level. Meanwhile, in group 2, at the end of the experiment, a decrease in the animals' blood glucose content was observed only by 20.7% (p <0.05). The average blood glucose level of animals treated with microcapsules with plant extracts (group 1), had significantly decreased by 32.9% (p <0.05) from the initial level only by the end of the test. At the same time, a decrease in the blood glucose concentration of the animals in groups 1 and 2 to the normal level was not established.

In the control group, the animals' blood glucose level was high throughout the entire time of the experiment, and 30 min after the administration of oral glucose there was a significant increase in its blood concentration.

The blood glucose level in the animals' control group was high throughout the entire time of the experiment, and 30 min after the administration of oral glucose there was a significant increase in its blood concentration.

Analyzing the dynamics of changes in the blood

glucose concentration of the studied groups' animals, it is worth notifying that a significant decrease in glucose was observed in groups 2, 3 and 4 both after the treatment and against the background of the glucose administration, in comparison with the control group. However, as previously stated, a decrease in the blood glucose concentration in the animals treated with monopreparations in microcapsules up to the physiological norm was not observed, while the administration of PEMCs+GMCs combination gives a hypoglycemic effect comparable to the reference drug 2 hours after the administration, both on days 7 and 14.

A more pronounced therapeutic effect caused by the combination of plant extracts and gliclazide, is most likely associated with a potentiation of the action due to a broader focus of the plant components. This is a decrease in blood glucose associated with the interaction of biologically active substances of *Glycyrrhiza glabra* L. extract with PPAR-γ receptors and due to an increase in the utilization by the GLUT-4 transport system and an increase in the cell sensitivity to insulin [12]. The active components of *Galega officinalis* L. extract increase the number of islets of Langerhans and increase the sensitivity of cells to insulin [19, 20]. The versatile orientation of the mechanisms of the hypoglycemic action of the plant components used is of particular importance, given the hypoglycemic effect of gliclazide realized by stimulating insulin secretion. Additional inhibition of α-amylase and α-glucosidase by the active components of *Mentha piperita* L. extract [24] contributes to an increase in resistance to alimentary factors in the development of hyperglycemia. As a whole, these effects contribute to the versatile orientation of therapy, which is a positive characteristic of the proposed composition of microcapsules.

Thus, according to the results of studying the cumulative effect of the investigated drugs for 14 days, the administration of PEMCs+GMCs combination gives a more pronounced pharmacological effect compared to the separate use of the drugs. The HGA combination is comparable to the reference drug and has significant statistical differences compared to the control group of animals. A pharmacological potentiation is characteristic for the developed original antidiabetic substance, consisting of the sum of phytoextracts (dry extracts of *Glycyrrhiza glabra* L., *Galega officinalis* L., *Mentha piperita* L.) with the addition of gliclazide, i.e. a combined composition. As a dosage form and a delivery vehicle, the developed microcapsules based on PEG-6000, containing the entire complex of active substances, showed a rather original HGA profile. The combination of biologically active substances of the plant origin, given their versatile action direction, with a synthetic antidiabetic agent in one dosage form, has a number of advantages over classical hypoglycemic drugs, i.e., the range and variability of the action. The use of microcapsules also makes it possible to achieve a reduction in the irritating effect of the dosage form on the gastrointestinal tract, which is characteristic of tablets.

In addition, microcapsules have certain technological and economic advantages – this is the possibility of reducing the dose of gliclazide, and given their prolongation, the obtained microcapsules can later be used as an intermediate and preparatory stage for constructing a form – a spansule. Therefore, the pharmacological role of the developed microcapsules is obvious, and its fixation and discussion presented in this article, are necessary.

## CONCLUSION

A technology for obtaining microcapsules with a shell based on PEG-6000, has been developed and substantiated. It has been shown that microcapsules containing the studied complex – the sum of plant extracts and gliclazide – have a pronounced positive property of the gradual development of a hypoglycemic effect. The administration of a microcapsules combination causes a more pronounced pharmacological response, comparable to the reference drug in terms of the hypoglycemic activity, but not inferior to it in terms of the elongation. In addition, it causes a comparable resistance to the alimentary glucose administration, which confirms the feasibility of the chosen technology and the originality of the microcapsule shell structure.

## FUNDING

This study did not receive financial support from outside organizations.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## AUTHORS' CONTRIBUTION

AG – development of the composition and production of microcapsules with a composition of extracts and gliclazide, article writing; EFS – study design development, microcapsule composition development, general study management, article writing; OFV, SES – conducting pharmacological studies, statistical processing of the results, article writing.

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2022-10-4-320-330

## REFERENCES

1. Dedov II, Shestakova MV, Vikulova OK, Zheleznyakova AV, Isakov MA. [Epidemiological characteristics of diabetes mellitus in the Russian Federation: clinical and statistical analysis according to the federal diabetes register data of 01.01.2021]. Diabetes Mellitus. 2021;24(3):204–21. DOI: 10.14341/DM12759. Russian
2. Dedov II, Mokrysheva NG, Shestakova MV, Nikonova TV, Mayorov AYu, Galstyan GR, Shamhalova MS, Barysheva VO, Ametov AS, Antsiferov MB, Babenko AYu, Bardymova TP, Valeeva FV, Vachugova AA, Grineva EN, Demidova TYu, Kiseleva TP, Kunicyna MA, Markova TN, Mkrtumyan AM, Petunina NA, Ruyatkina LA, Saluhov VV, Suplotova LA, Hadarceva EL, Halimov YuS. [Glycemia control and choice of antihyperglycemic therapy in patients with type 2 diabetes mellitus and COVID-19: a consensus decision of the board of experts of the Russian association of endocrinologists]. Diabetes mellitus. 2022;25(1):27–49. DOI: 10.14341/DM12873. Russian
3. Luneva IE, Suponeva NA. [Cognitive impairment in diabetic patients. Effective pharmacotherapy]. 2022;18(10):38–44. DOI: 10.33978/2307-3586-2022-18-10-38-44. Russian
4. Markova TN, Ponomareva AA, Samsonova IV, Kichigin VA, Arefieva NA. [Risk factors for fatal outcome in patients with type 2 diabetes mellitus and a new coronavirus infection]. Endocrinology: News, Opinions, Training. 2022; 11 (1): 8–16. DOI: 10.33029/2304-9529-2022-11-1-8-16. Russian
5. Rogozhkina AV, Romanova ES, Startseva GYu, Filipovich OM, Pogromskaya MN, Fedunyak IP. [Comparative characteristics of patients with COVID-19 with concomitant type 2 diabetes mellitus]. Preventive and clinical medicine. 2022;2(83):72–8. DOI: 10.47843/2074-9120_2022_2_72. Russian
6. Karaseva AA, Khudyakova AD, Ragino YuI. [Metabolic disorders and the risk of COVID-19]. Siberian Scientific Medical Journal. 2022;42(1):4–12. DOI: 10.18699/SSMJ20220101. Russian
7. Yakimova TV, Nasanova ON, Vengerovsky AI. [Influence of herbal extracts on metabolic disturbances in diabetes mellitus and insulin resistance model]. Bulletin of Siberian Medicine. 2015;14(2):75–81. DOI: 10.20538/1682-0363-2015-2-75-81. Russian
8. Ismail HF, Hashim Z, Zaidel DNA, Zainol SN, Tap FM, Majid FAA, Zakaria NH. Triple-action of the standardized antidiabetic polyherbal extract; Synacinn™ through upregulation of $GLUT_4$ and inhibition of DPP(IV), α-amylase, and α-glucosidase activity. Med J Malaysia. 2022 Jul;77(Suppl 1):16–22.
9. Pavets, N.R., Javakhyan M.A. [Analysis of the range of drugs used for diabetes]. Problems of Biological, Medical and Pharmaceutical Chemistry. 2020; 23(6): 10–15. DOI 10.29296/25877313-2020-06-02. Russian
10. Eroglu TE, Jia L, Blom MT, Verkerk AO, Devalla HD, Boink GJJ, Souverein PC, de Boer A, Tan HL. Sulfonylurea antidiabetics are associated with lower risk of out-of-hospital cardiac arrest: Real-world data from a population-based study. Br J Clin Pharmacol. 2021 Sep;87(9):3588–98. DOI: 10.1111/bcp.14774
11. Naik A, Adeyemi SB, Vyas B, Krishnamurthy R. Effect of co-administration of metformin and extracts of *Costus pictus* D.Don leaves on alloxan-induced diabetes in rats. J Tradit Complement Med. 2021;12(3): 269–80. DOI: 10.1016/j.jtcme.2021.08.007
12. Hasan MK, Ara I, Mondal MSA, Kabir Y. Phytochemistry, pharmacological activity, and potential health benefits of *Glycyrrhiza glabra*. Heliyon. 2021 Jun 7;7(6):e07240. DOI: 10.1016/j.heliyon.2021.e07240
13. Jafari F, Jafari M, Moghadam AT, Emami SA, Jamialahmadi T, Mohammadpour AH, Sahebkar A. A Review of *Glycyrrhiza glabra* (Licorice) Effects on Metabolic Syndrome. Adv Exp Med Biol. 2021;1328:385–400. DOI: 10.1007/978-3-030-73234-9_25
14. Sen S, Singh R. *Glycyrrhiza glabra* alcoholic root extract ameliorates hyperglycemia, hyperlipidemia, and glycation-induced free iron-mediated oxidative reactions. J Food Biochem. 2021 Dec;45(12):e13970. DOI: 10.1111/jfbc.13970
15. Sen S. Liposome-encapsulated glycyrrhizin alleviates hyperglycemia and glycation-induced iron-catalyzed oxidative reactions in streptozotocin-induced diabetic rats. J Liposome Res. 2022 Feb 15:1–10. DOI: 10.1080/08982104.2022.2036756
16. Zhang Y, Xu Y, Zhang L, Chen Y, Wu T, Liu R, Sui W, Zhu Q, Zhang M. Licorice extract ameliorates hyperglycemia through reshaping gut microbiota structure and inhibiting TLR4/NF-κB signaling pathway in type 2 diabetic mice. Food Res. Int. 2022;153: 110945. DOI: 10.1016/j.foodres.2022.110945
17. Wang GS, Hoyte C. Review of Biguanide (Metformin) Toxicity. J Intensive Care Med. 2019 Nov-Dec;34(11-12):863–76. DOI: 10.1177/0885066618793385
18. Khezri M, Asghari Zakaria R, Zare N, Johari-Ahar M. Improving galegine production in transformed hairy roots of *Galega officinalis* L. via elicitation. AMB Express. 2022 Jun 3;12(1):65. DOI: 10.1186/s13568-022-01409-7
19. Hachkova H, Nagalievska M, Soliljak Z, Kanyuka O, Kucharska AZ, Sokół-Łętowska A, Belonovskaya E, Buko V, Sybirna N. Medicinal Plants *Galega officinalis* L. and Yacon Leaves as Potential Sources of Antidiabetic Drugs. Antioxidants (Basel). 2021 Aug 26;10(9):1362. DOI: 10.3390/antiox10091362
20. Kurylo K, Budniak L, Volska A, Zablotskyy B, Klishch I. Influence of phytocompositions on dynamics of changes in basal glycemia and glycemia in oral glucose tolerance test in rats with streptozotocin-nicotinamide-induced diabetes mellitus type 2. Georgian Med News. 2020 Mar;(300):112–16.
21. Nagalievska M, Sabadashka M, Hachkova H, Sybirna N. *Galega officinalis* extract regulate the diabetes mellitus related violations of proliferation, functions and apoptosis of leukocytes. BMC Complement Altern Med. 2018 Jan 8;18(1):4. DOI: 10.1186/s12906-017-2079-3
22. Heghes SC, Filip L, Vostinaru O, Mogosan C, Miere D, Iuga CA, Moldovan M. Essential Oil-Bearing Plants From Balkan Peninsula: Promising Sources for New Drug Candidates for the Prevention and Treatment of Diabetes Mellitus and Dyslipidemia. Front Pharmacol. 2020 Jun 30;11:989. DOI: 10.3389/fphar.2020.00989
23. Abdellatief SA, Beheiry RR, El-Mandrawy SAM. Peppermint essential oil alleviates hyperglycemia caused by streptozotocin- nicotinamide-induced type 2 diabetes in rats. Biomed Pharmacother. 2017 Nov;95:990–9. DOI: 10.1016/j.biopha.2017.09.020

*24.* Agawane SB, Gupta VS, Kulkarni MJ, Bhattacharya AK, Koratkar SS. Chemo-biological evaluation of antidiabetic activity of *Mentha arvensis* L. and its role in inhibition of advanced glycation end products. J Ayurveda Integr Med. 2019 Jul-Sep;10(3):166–70. DOI: 10.1016/j.jaim.2017.07.003

*25.* Stepanova EF, Polkovnikova YuA, Kul' I.Ya. [The influence of microencapsulation on the physical-technological characteristics microcapsules afobazol]. Advances in current natural sciences. 2011; (5): 75-7. Russian

*26.* Giyoszoda A, Stepanova EF, Ogay MA, Storozhenko SE, Veselova OF, Morozov YuA, Makieva MS, Morozova EV, Butenko LI, Slivkin AI, Belenova AV. Study of the sugar-lowering activity of phytocompositions of antidiabetic action. Bulletin of the Voronezh State University. Series: Chemistry. Biology. Pharmacy. 2021;4:96–104. Russian

*27.* Giyoszoda A, Stepanova EF, Sharopov FS, Bobizoda MG, Nazarov UA. Investigation of the phytochemical platform of a number of plants with antidiabetic effect. Storage and processing of Farm Products. 2021;(4):89–97. DOI: 10.36107/spfp.2021.267. Russian

## AUTHORS

**Giyoszoda Asomuddin** – Candidate of Sciences (Biology), Deputy Director for Science, Practice and Innovation of the Non-state educational institution "Medical College", R.M.C.A. Hamadoni Khatlon region, Tajikistan. ORCID ID: 0000-0002-7889-496X. E-mail: asom_giysov@mail.ru

**Eleonora F. Stepanova** – Doctor of Sciences (Pharmacy), Professor, Professor of the Department of Pharmaceutical Technology with a Course of Medical Biotechnology, Pyatigorsk Medical and Pharmaceutical Institute – branch of Volgograd State Medical University, ORCID ID: 0000-0002-4082-3330. E-mail: efstapanova@yandex.ru

**Olga F. Veselova** – Candidate of Sciences (Medicine), associate professor, Head of the Department of Pharmacology and Clinical Pharmacology with a Postgraduate Course, Krasnoyarsk State Medical University n.a. Prof. V.F. Voyno-Yasenetsky. ORCID ID: 0000-0002-6126-665X. E-mail: veselovaof@mail.ru

**Sergey E. Storozhenko** – Senior Lecturer of the Department of Pharmacy with a Postgraduate Course, Krasnoyarsk State Medical University n.a. Prof. V.F. Voyno-Yasenetsky. ORCID ID: 0000-0002-8058-9147. E-mail: s.e.storozhenko@yandex.ru

Научно-практический журнал
ФАРМАЦИЯ И ФАРМАКОЛОГИЯ

*ОРИГИНАЛЬНАЯ СТАТЬЯ*

*DOI: 10.19163/2307-9266-2022-10-4-331-342*



# ADAPTATION OF "DRIED BLOOD DROP" METHOD FOR THERAPEUTIC DRUG MONITORING

**V.I. Petrov[1,2], I.S. Anikeev[1,2], T.E. Zayachnikova[3], A.V. Strygin[1,2,4], A.M. Dotsenko[1,4]**

[1] Volgograd State Medical University,
1, Pavshikh Bortsov Sq., Volgograd, Russia, 400131
[2] Scientific Center of Innovative Medicines with Pilot Production, Volgograd State Medical University,
39, Novorossiyskaya Str., Volgograd, Russia, 400087
[3] Institute for Continuing Medical and Pharmaceutical Education, Volgograd State Medical University,
1, Pavshikh Bortsov Sq., Volgograd, Russia, 400131
[4] Volgograd Medical Research Center,
1, Pavshikh Bortsov Sq., Volgograd, Russia, 400131

E-mail: Anikeev.iv@gmail.com

Received 05 July 2022 After peer review 10 Aug 2022 Accepted 20 Aug 2022

To control the concentration of drugs with a narrow therapeutic range, and to conduct effective and safe treatments, Therapeutic Drug Monitoring (TDM) is carried out. However, to date, the implementation of TDM is associated with various difficulties, for the solution of which more convenient and less invasive methods for collecting biological material are being developed.

**The aim** of the study was to develop protocols for the collection and storage of "dried blood spot" (DBS) samples, as well as protocols for the validation methods for the quantitative determination of drugs in whole blood, using this technology for subsequent therapeutic drug monitoring.

**Materials and methods.** To analyze a "dried blood spot" method in detail and to identify the characteristic features of taking and storing biosamples, a collection and analysis of scientific literature over the past 10 years has been conducted. The search for literature materials has been carried out from open and accessible sources located in the scientific libraries of institutions, in electronic databases and search engines: Elibrary, PubMed, Scopus, Cyberleninka, Medline, ScienceDirect, Web of Science, Google Scholar. Primary protocols for taking, storing and analyzing samples of the "dried blood drop" have been prepared. To obtain the adequate quality samples, the developed protocols have been tested and optimized at the stages of selection and storage. By high-performance liquid chromatography with mass spectrometric detection (HPLC-MS/MS), using a "dried blood drop" as a sample preparation, drug validation protocols have been optimized to ensure that acceptable validation characteristics were achieved, and subsequent Therapeutic Drug Monitoring was performed.

**Results.** The features of the collection, storage and analysis of the "dried blood spot" samples have been revealed. Such characteristics as a spot volume effect, a hematocrit effect, a droplet uniformity, which can affect the results of a quantitative HPLC-MS/MS analysis, have been determined. For a successful use of the new methods, appropriate protocols for taking samples of "dried blood spot" from the finger of adult patients and from the heel of newborns, as well as protocols for validating methods for the quantitative determination of drugs from these samples, have been developed.

**Conclusion.** The application of the "dried blood spot" method using newly developed protocols for taking, storing and analyzing biological samples, relieves the existing constraints in conducting TDM, and can later become a promising method for conducting preclinical and clinical studies.

**Keywords:** "dried blood spot" method; therapeutic drug monitoring; bioanalysis; method validation; HPLC-MS/MS

**Abbreviations:** MP/D – medicinal preparation/drug; HPLC-MS/MS – high performance liquid chromatography with tandem mass spectrometry; TDM – Therapeutic Drug Monitoring; DBS – Dried Blood Spot; QCL – low quality con

**For citation:** V.I. Petrov, I.S. Anikeev, T.E. Zayachnikova, A.V. Strygin, A.M. Dotsenko. Adaptation of "dried blood drop" method for therapeutic drug monitoring. *Pharmacy & Pharmacology.* 2022;10(4):331-342. **DOI:** 10.19163/2307-9266-2022-10-4-331-342



**Для цитирования:** В.И. Петров, И.С. Аникеев, Т.Е. Заячникова, А.В. Стрыгин, А.М. Доценко. Адаптация метода «высушенной капли крови» для проведения терапевтического лекарственного мониторинга. *Фармация и фармакология.* 2022;10(4):331-342. **DOI:** 10.19163/2307-9266-2022-10-4-331-342

*RESEARCH ARTICLE*

*ISSN 2307-9266 e-ISSN 2413-2241*

Scientific and Practical Journal
PHARMACY & PHARMACOLOGY

# АДАПТАЦИЯ МЕТОДА «ВЫСУШЕННОЙ КАПЛИ КРОВИ» ДЛЯ ПРОВЕДЕНИЯ ТЕРАПЕВТИЧЕСКОГО ЛЕКАРСТВЕННОГО МОНИТОРИНГА

**В.И. Петров[1,2], И.С. Аникеев[1,2], Т.Е. Заячникова[3], А.В. Стрыгин[1,2,4], А.М. Доценко[1,4]**

[1] Федеральное государственное бюджетное образовательное учреждение высшего образования «Волгоградский государственный медицинский университет» Министерства здравоохранения Российской Федерации, 400131, Россия, г. Волгоград, пл. Павших Борцов, д. 1

[2] Центр инновационных лекарственных средств с опытно-промышленным производством федерального государственного бюджетного образовательного учреждения высшего образования «Волгоградский государственный медицинский университет» Министерства здравоохранения Российской Федерации, 400087, Россия, г. Волгоград, ул. Новороссийская, д. 39

[3] Институт непрерывного медицинского и фармацевтического образования федерального государственного бюджетного образовательного учреждения высшего образования «Волгоградский государственный медицинский университет» Министерства здравоохранения Российской Федерации, 400131, Россия, г. Волгоград, пл. Павших Борцов, д. 1

[4] Государственное бюджетное учреждение «Волгоградский медицинский научный центр», 400131, Россия, г. Волгоград, пл. Павших Борцов, д. 1

E-mail: Anikeev.iv@gmail.com

Получена 05.07.2022 После рецензирования 10.08.2022 Принята к печати 20.08.2022

Для контроля концентрации лекарственных препаратов, обладающих узким терапевтическим диапазоном, и проведения эффективных и безопасных методов лечения проводится терапевтический лекарственный мониторинг (ТЛМ). Однако на сегодняшний день проведение ТЛМ связано с различными затруднениями, для решения которых разрабатываются более удобные и менее инвазивные методы сбора биологического материла.

**Цель.** Разработать протоколы взятия и хранения образцов «высушенной капли крови» (Dried Blood Spot, DBS), а также протоколы валидации методов количественного определения лекарственных препаратов в цельной крови с использованием данной технологии для последующего проведения терапевтического лекарственного мониторинга.

**Материалы и методы.** Для детального анализа метода «высушенной капли крови» и выявления характерных особенностей взятия и хранения биообразцов, был проведен сбор и анализ научной литературы за последние 10 лет. Поиск литературных материалов проводился с помощью открытых и доступных источников, размещенных в научных библиотеках учреждений, в электронных базах данных и поисковых системах: Elibrary, PubMed, Scopus, КиберЛенинка, Medline, ScienceDirect, Web of Science, Google Scholar. Подготовили первичные протоколы взятия, хранения и анализа образцов «высушенной капли крови». На стадиях отбора и хранения проводили апробацию и оптимизацию разработанных протоколов для получения образцов надлежащего качества. Методом высокоэффективной жидкостной хроматографии с масс-спектрометрической детекцией (ВЭЖХ-МС/МС) с использованием в качестве пробоподготовки «высушенную каплю крови», оптимизировали протоколы валидации лекарственных препаратов для обеспечения достижения приемлемых валидационных характеристик и проведения последующего ТЛМ.

**Результаты.** Выявлены особенности сбора, хранения и анализа образцов «высушенной капли крови». Определены такие характеристики, как: эффект объёма капли, эффект гематокрита, однородность капли, которые могут оказывать влияние на результаты количественного ВЭЖХ-МС/МС анализа. Для успешного использования новой методики нами были разработаны надлежащие протоколы взятия образцов «высушенной капли крови» из пальца руки взрослых пациентов и из пятки новорожденных детей, а также протоколы валидации методов количественного определения лекарственных препаратов из данных образцов.

**Заключение.** Применение метода «высушенной капли крови» с использованием новых разработанных протоколов взятия, хранения и анализа биологических образцов снимает существующие ограничения при проведении ТЛМ, а также в последствии может стать перспективным методом для проведения доклинических и клинических исследований.

**Ключевые слова:** метод «высушенной капли крови»; терапевтический лекарственный мониторинг; биоанализ; валидация метода; ВЭЖХ-МС/МС

**Список сокращений:** ЛП – лекарственный препарат; ВЭЖХ-МС/МС – высокоэффективная жидкостная хроматография с масс-спектрометрической детекцией; ТЛМ – терапевтический лекарственный мониторинг; DBS – «высушенная капля крови»; QCL – низкий показатель контроля качества; QCM – средний показатель контроля качества; QCH – высокий показатель контроля качества; КК – контроль качества; НПКО – нижний предел количественного определения.

**Ключевые слова:** метод «высушенной капли крови»; терапевтический лекарственный мониторинг; биоанализ; валидация метода; ВЭЖХ-МС/МС

**Список сокращений:** ЛП – лекарственный препарат; ВЭЖХ-МС/МС – высокоэффективная жидкостная хроматография с масс-спектрометрической детекцией; ТЛМ – терапевтический лекарственный мониторинг; DBS – «высушенная капля крови»; QCL – низкий показатель контроля качества; QCM – средний показатель контроля качества; QCH – высокий показатель контроля качества; КК – контроль качества; НПКО – нижний предел количественного определения.

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2022-10-4-331-342

## INTRODUCTION

"Dried blood spot" (DBS) is a relatively simple sample preparation method for collecting small volumes of blood that can eliminate plasma collection and sample freezing.

The DBS method presents a wide range of possible applications that are not feasible or difficult to perform with traditional methods of biosampling [1]. One of the main options for using such a technology is therapeutic drug monitoring (TDM).

To date, for TDM and analysis of medical preparations (MPS), it is necessary to collect a large amount of biological material – plasma or blood serum. To obtain these biosamples, it is necessary to take whole blood, which is obtained by standard venipuncture methods. However, for many groups of patients, standard methods for collecting whole blood cause significant difficulties, limiting the implementation of TDM and the adjustment of dosing regimens for drugs with a narrow therapeutic range [1, 2].

A new technology for collecting whole blood samples, is a less invasive and simple DBS method, makes it possible to overcome the significant limitations associated with standard methods, and perform TDM in the clinical situations in which it was difficult or impossible in principle [3].

However, a quantitative analysis of MPs in DBS samples require the use of highly sensitive and selective analytical technologies, such as high-performance chromatography with mass spectrometric detection/ tandem mass spectrometry (HPLC-MS/MS). The use of such a modern analytical system will optimize the analysis of biosamples for TDM and expand the possibilities of using the DBS at various stages of drug development and within the framework of pharmacokinetic studies.

However, despite a great potential and advantages of this technology, its widespread introduction into the routine practice of TDM will be possible only after a reliable confirmation of the accuracy and reproducibility of the obtained analytical data on the concentration of the studied drugs. These factors will require additional development and validation of bioanalytical methods using the DBS method at the stage of taking the biomaterial and preparing it for the study.

**THE AIM** of the study was to develop protocols for the collection and storage of "dried blood spot" (DBS) samples, as well as protocols for the validation methods for the quantitative determination of drugs in whole blood, using this technology for subsequent therapeutic drug monitoring.

## MATERIALS AND METHODS

To analyze the DBS method and identify its characteristic features of taking and storing biosamples, a collection and analysis of scientific literature over the past 10 years have been carried out. The search for literature materials has been conducted using open and accessible sources located in the scientific libraries of institutions, in electronic databases and search engines: Elibrary, PubMed, Scopus, Cyberleninka, Medline, ScienceDirect, Web of Science, Google Scholar.

The following words and phrases were chosen as the parameters for the selection of literature: DBS; HPLC-MS; therapeutic drug monitoring; validation; sample preparation; bioanalysis. The search was also carried out using the English analogues of the keywords: DBS; HPLC-MS; therapeutic drug monitoring; validation; sample preparation; bioanalysis.

After analyzing the literature data, primary protocols for taking, storing and analyzing the DBS samples were developed, based on the detailed analysis and identification of the specific characteristics of this technique.

Then, using the HPLC-MS/MS method and a DBS as a sample preparation, the ivabradine samples were analyzed and the drug validation protocols were optimized to achieve acceptable validation characteristics and conduct subsequent TDM.

The basic equipment was an Agilent 1260 HPLC system with a binary pump and a temperature-controlled autosampler. A chromatographic separation of the components was carried out on a Poroshell 120 C18 column (4.6 x 50 mm x 2.7 µm). The analytes were determined using a Sciex QTRAP 5500 hybrid mass spectrometric system.

The mobile phase was represented by a water-acetonitrile mixture, the mobile phase modifier was a 0.1% formic acid, which was added to both the aqueous and organic mobile phases.

In the course of optimizing the conditions for a chromatographic separation, a gradient elution mode was chosen. The mobile phase ratio of water-acetonitrile (70:30) did not change at 0.6 ml/min until 0.5 minutes, after which there was a gradual change to the ratio of water-acetonitrile (0:100), which was reached at the 2$^{nd}$ minute. At the 3$^{rd}$ minute, the ratio was changed to the original, and, at the same time, the system was balanced up to the 5$^{th}$ minute. The mobile phase modifier was a 0.1% formic acid, which was added to both the aqueous and organic components of the mobile phase.

In this methods, ivabradine standards (Servier, France) and N-desmethylivabradine as an internal standard (Toronto Research Chemical Inc., Canada),

were used to prepare calibration standards and quality control samples.

For each analytical series, fresh standard working solutions were prepared. The final concentrations of ivabradine working solutions were 10, 20, 100, 500, 1000, 5000, 8000, 10000 ng/ml.

To obtain a calibration solution, 100 µl of whole blood was transferred into 1.5 ml microtubes and 10 µl of a working solution of the appropriate concentration was added. The concentrations of calibration solutions were 1, 2, 10, 50, 100, 500, 800, 1000 ng/ml.

Quality control (QC) samples were prepared at the following four concentration levels: 1 µg/mL (lower limit of quantitation, LLQQ), 3 µg/mL (low QC, QCL), 400 µg/mL (medium QC, QCM) and 750 mcg / ml (high QC, QCH).

Next, 20 µl of the resulting working solutions were applied to the filter paper and let them dry at room conditions for 3 hours.

To prepare the DBS samples, a disc with the diameter of 6 mm was cut from the cards with a Uni-Core marker cutter, placed in a test tube and extracted with an extraction solution. The extraction was performed with a shaker for 20 min at 25°C, then the samples were transferred to new tubes and analyzed by HPLC-MS/MS.

### Statistical processing of results

The obtained data were processed using the R 3.6.1 software-statistical environment in the RStudio 1.2 program, as well as the specialized Sciex Analyst 1.6.2 software. During the system operation, the acquired data were processed in the Analyst software as full mass spectra, single or multiple ion intensities versus time, or total ion current. For a quantitative determination, a calibration curve method with a weighting factor of 1/x2 was used. The ratio of the peak areas of the analyte and the internal standard was taken as a parameter [8, 10, 12, 23].

## RESULTS AND DISCUSSION

### General provisions on DBS method

Unlike standard whole blood collection methods, DBS involves collecting a minimal amount of capillary blood from the finger of an adult or the heel of a newborn child and applying the drop of blood to the marked area of filter paper. After this procedure, a drop of blood dries in air at room temperature for at least 4 hours in a dry place without direct sunlight. The dried samples are transported to the laboratory, where they are subjected to the manipulations related to cutting discs with a special device from 3 to 8 mm from the filter card. This disk is subsequently extracted with an organic solvent or a mixture of an aqueous and organic solvent. Further on, the obtained samples are quantitatively determined by various bioanalytical technologies [4].

Thus, the entire process of collecting DBS samples is considered as the distribution of a blood drop over a porous surface with a simultaneous penetration and spreading inside it. The distribution and wetting of substrate pores by blood is a complex process that depends on the physical and chemical properties of paper and blood, which must be further investigated before being used in wide clinical practice [4].

This whole mechanism of the DBS method determines a number of significant advantages of this technology over standard methods for collecting biomaterial, as well as some of its limitations.

### Advantages and disadvantages of DBS technology

One of the main advantages of this technology is the collection of a sufficiently smaller amount of whole blood, which makes it possible to overcome the existing limitations of TDM for various patient groups. The DBS method also involves the process of drying samples under standard laboratory conditions and their transportation to an analytical laboratory without the use of special equipment and with a minimal risk of infection [3, 5].

It is possible to carry out the procedure for taking biomaterial at home without the involvement of special personnel. This will allow more analyzes to be performed using the DBS method and to build a more accurate concentration-time curve to optimize the dosing regimen of the drugs with a narrow therapeutic range.

All of the above advantages can have significant economic benefits in the collection and analysis of DBS samples compared to standard biosampling methods. Potential home DBS sampling would also result in significant economic benefits in patients requiring TDM [7].

However, this method is not free from shortcomings. The analysis of the DBS samples imply special highly sensitive analytical equipment and the need to train medical specialists to obtain samples of the adequate quality [8, 9].

It should be also taken into account that the currently used requirements for the validation of bioanalytical methods, which are described in the manuals for traditional matrices, do not provide all the necessary aspects of the method development, analytical and clinical validation for DBS samples and their use in TDM. Such specific parameters characteristic of the new technology are as follows: the effect of hematocrit, which can lead to different blood viscosities and a droplet distribution on paper, the homogeneity

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2022-10-4-331-342

of the droplet and its size can affect the result obtained and, in turn, require additional validation [10, 11].

All these shortcomings cause the need to develop new protocols for taking and storing blood samples, as well as additional validation that would be characteristic of the DBS method and its use in TDM.

### Analysis of current literature sources

Over the past 10 years, a large number of articles describing the development, validation of a new DBS method, as well as the possibility of using it as a part of TDM for various classes of drugs (analgesics, antibiotics, antiepileptics, antidepressants, antimalarials, antifungals, antiretrovirals, diuretics, immunosuppressants and others), have been published [12].

The existing methods for the quantitative determination of MPs using HPLC-MS/MS in the DBS samples have been analyzed (Table 1). The additional validation characteristics that are unique to the new method of collecting biosamples and have a significant impact on the results of the analysis, have been taken into account in the selected studies. These characteristics are: a droplet size, the nature of the material, a map for the DBS samples, the extraction conditions, and the hematocrit level [9, 10, 13, 15, 16, 18–20, 25–29].

Such parameters as a hematocrit effect, a drop volume effect and a droplet uniformity can have a significant impact on the result of a MP analysis in the DBS samples and can vary from sample to sample. It is these characteristics that have been identified as additional parameters requiring the development of new validation protocols for the quantitative HPLC-MS/MS analysis of drugs, as well as working out practical recommendations for medical professionals on the collection and storage of these whole blood samples to obtain acceptable analytical results. [8, 9, 17, 39]

### Development of protocols for sampling, storage of samples and validation DBS

Based on the literature analysis and existing methods for collecting whole blood drops on special paper, protocols for taking DBS samples from an adult patient's finger and from a newborn's heel have developed and optimized [40–42]. The developed protocols are presented below.

**Protocol for taking and storing DBS samples from newborn's heel:**

1. Issue the informed voluntary consent of the legal representative;
2. Sign the drops with a patient's ID and date;
3. Choose the puncture location on the lateral side of the heel;
4. Warm up the foot with a warm diaper;
5. Clean hands and put on sterile gloves;
6. Place the heel below the baby's torso and hold it without sharp bending of the ankle;
7. Treat the puncture location with an antiseptic solution and let it dry;
8. Quickly pierce the skin laterally, in the place as shown in the picture (Fig. 1) using a lancet, and wipe off the first drop of blood with a sterile cotton ball;
9. Hold the puncture location down, gently pressing on the adjacent area, and take blood on a filter paper blank;
10. Hold the card without touching the marked area (Fig. 2);
11. Carefully, touching a drop of blood with a filter paper card, apply it to the card. Let the card absorb the blood until the circle is full. After the application, do not touch the marked area;
12. Let the blood spot dry in a dark place. Avoid direct sunlight for at least 4 hours. Do not heat or let dried spot samples come into contact with other surfaces during the drying process;
13. Seal the card (or parts of the card) in a gas-tight bag with a zipper. Store no more than one card per package in the refrigerator until sent to the laboratory at 2–8°C.

**Protocol for taking and storing samples of DBS from a finger:**

1. Issue the informed voluntary consent of the legal representative;
2. Sign the drops with a patient's ID and date;
3. Choose the puncture location on the index finger;
4. The phlebotamist must clean his hands and put on sterile gloves;
5. Treat the puncture location with an antiseptic solution and let it dry;
6. Quickly pierce the skin with a lancet and wipe off the first drop of blood with a sterile cotton ball;
7. Hold the puncture location down gently pressing on the adjacent area, and take blood on a filter paper blank;
8. Hold the card without touching the marked area;
9. Gently touching a drop of blood with a filter paper card, apply it to the card. Let the card absorb the blood until the circle is full. After the application, do not touch the marked area;
10. Let the blood spot dry in a dark place. Avoid direct sunlight for at least 4 hours. Do not heat or let the dried spot samples come into contact with other surfaces during the drying process;
11. Seal the card (or parts of the card) in a gas-tight bag with a zipper. Store no more than one card per package in the refrigerator until sent to the laboratory at 2–8°C.

*ISSN 2307-9266 e-ISSN 2413-2241*

Scientific and Practical Journal
PHARMACY & PHARMACOLOGY

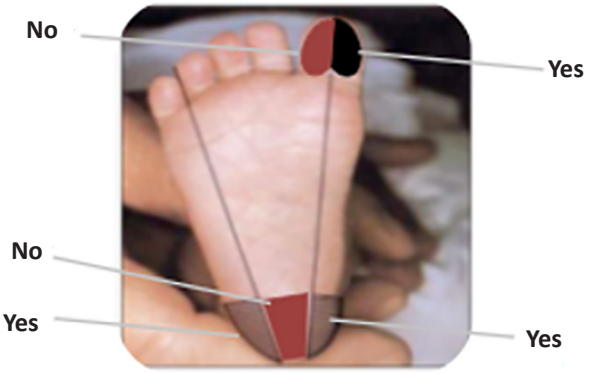



**Figure 1 – Puncture location of newborn's heel**

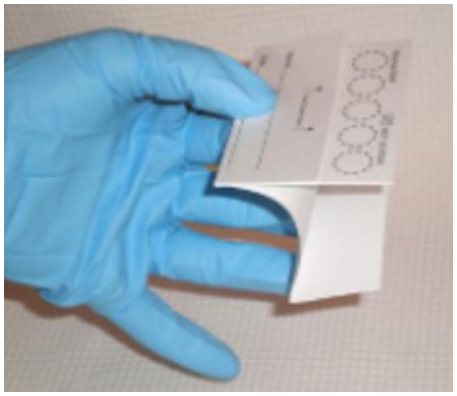


**Figure 2 – Example of a map for DBS**

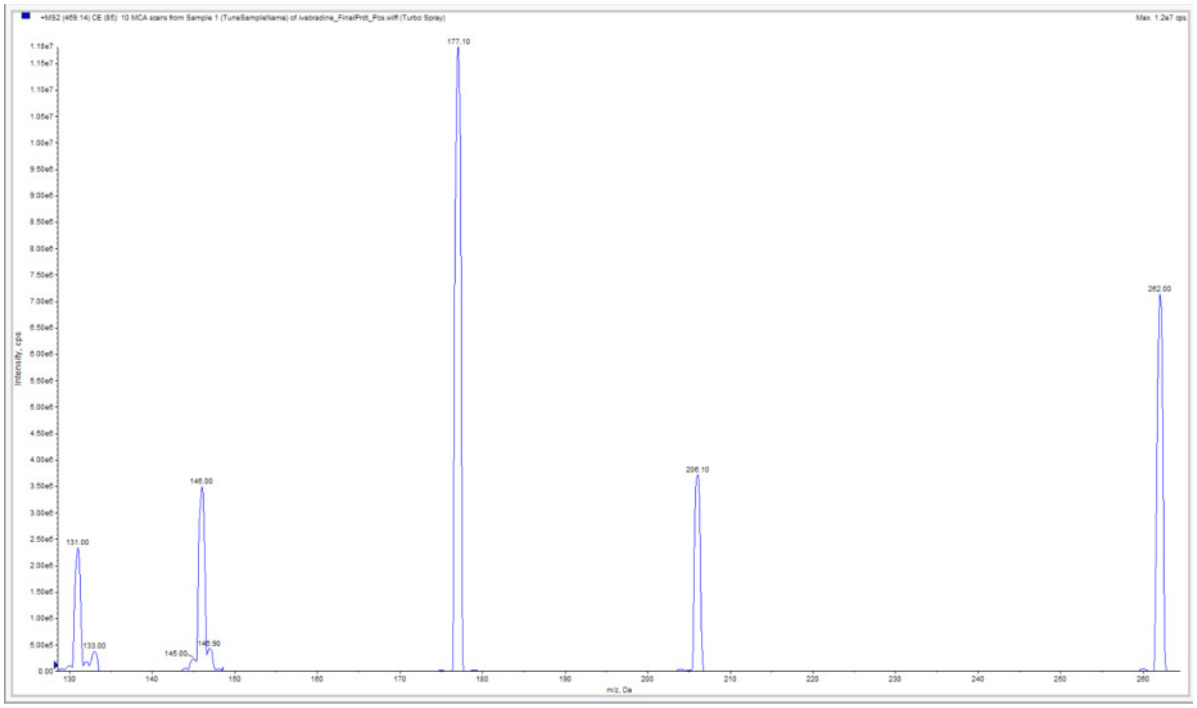



**Figure 3 – Mass spectrum of ivabradine in blood plasma**

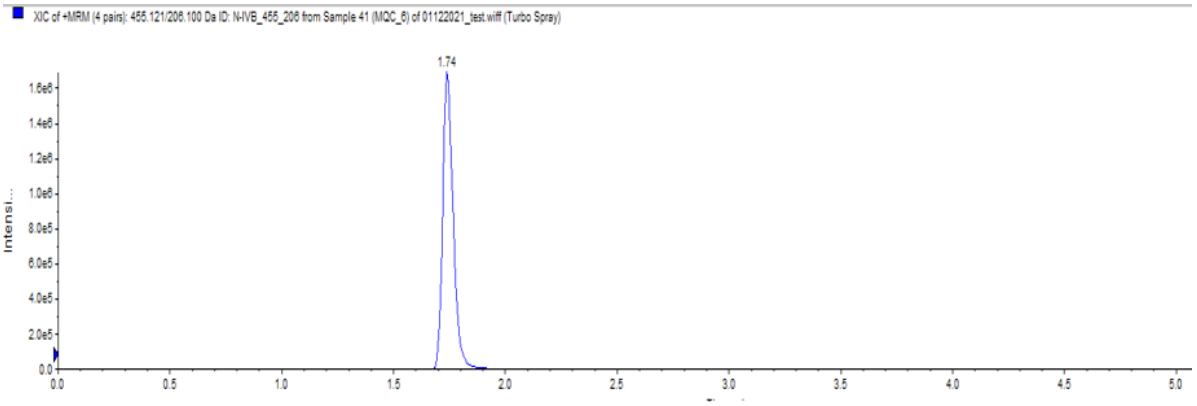



**Figure 4 – Chromato-mass spectrogram of ivabradine in blood plasma**
Note: on the abscissa – time (min), on the ordinates – signal intensity.

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2022-10-4-331-342

**Table 1 – Additional validation parameters for drug analysis by HPLC-MS/MS**

| MP | Class of MP | Additional DBS validation | References |
|---|---|---|---|
| Fluconazole | Antifungal | Drop volume effect | [32, 33] |
| Mycophenolic acid | Antitumor | – | [15, 19, 20] |
| Methotrexate | Antitumor | Hematocrit effect | [18] |
| Voriconazole | Antifungal | Drop volume effect | [14, 15] |
| Moxifloxacin | Antibiotic | Drop volume effect, paper type | [14, 21] |
| Pipyrecillin | Antibiotic | – | [18, 20] |
| Sirolimus | Immunosuppressant | Homogeneity, hematocrit effect, drop volume effect | [22–26] |
| Everolimus | Immunosuppressant | Homogeneity, hematocrit effect, drop volume | [24-26, 28, 37, 38] |
| Tazobactam | Antibiotic | – | [16, 31] |
| Vancomycin | Antibiotic | Homogeneity, hematocrit effect, drop volume effect | [16, 17] |
| Tacrolimus | Immunosuppressant | Homogeneity, hematocrit effect, drop volume effect | [22–30] |
| Cyclosporin A | Immunosuppressant | Homogeneity, hematocrit effect, drop volume effect | [26, 30, 34-36, 43] |

**Table 2 – Standard validation parameters**

| Validation parameter | Tests performed | Acceptance criteria [8, 10] |
|---|---|---|
| Linearity | 8 calibration samples + blank sample + zero sample | ≤ 15% of nominal values (≤ 20% for LLQL), not less than for 75% of samples |
| Selectivity | 6 blank samples and 6 LLQLs | ≤ 20% of LLQL |
| Accuracy | 5 QC samples at 4 concentration levels | ≤ 15% (≤ 20% for LLQL) |
| Precision | 5 QC samples at 4 concentration levels | ≤ 15% (≤ 20% for LLQL) |
| Matrix effect | 5 QC samples at 2 concentration levels at 3 different hematocrit values | ≤ 15% |
| Stability | 5 QC samples at 2 concentration levels at room temperature at three time points (2 hours, 7 days and 14 days after applying the blood drop) | ≤ 15% |

Note: QC – quality control samples; LLQL – lower limit of quantitation level.

**Table 3 – Specific validation parameters**

| Validation parameter | Tests performed | Acceptance criteria [8, 10] |
|---|---|---|
| Drop volume effect | 5 QC samples at 3 drop volumes (10, 40, 70 µl) at 3 hematocrit levels (0.3; 0.4; 0.5), at 2 concentration levels | ≤ 15% of nominal values |
| Hematocrit effect | 5 QC samples for 3 hematocrit levels, at 2 concentration levels | ≤ 15% of nominal values |
| Droplet uniformity | 5 QC samples at 2 concentration levels at 3 hematocrit levels are compared; obtained by 2 drop cut options: from the center of the drop and at the edge. | ≤ 15% of nominal values |

Note: QC – quality control samples.

**Table 4 – Validation parameters of DBS sample preparation methods**

| Parameter | | Value | | | |
|---|---|---|---|---|---|
| | | LLQL (1 ng/ml) | QCL (3 ng/ml) | QCM (400 ng/ml) | QCH (750 ng/ml) |
| Precision (CV %) | Inside the cycle | 9.4 | 8.0 | 7.5 | 11.4 |
| | Between cycles | 12.5 | 10.1 | 9.2 | 5.8 |
| Accuracy (%) | Inside the cycle | 112.3 | 110.7 | 106.1 | 107.2 |
| | Between cycles | 91.2 | 100.7 | 95.9 | 96.3 |
| Stability (%) | | – | 88.3 | – | 91.2 |
| Selectivity (%) | | 10.4 | – | – | – |
| Correlation coefficient | | 0.99 | | | |

**Table 5 – Hematocrit effect on the analysis results**

| Hematocrit, % | QC | Nominal concentration (ng/ml) | Accuracy (%) |
|---|---|---|---|
| 0.3 | QCL | 3 | 113.7 |
| | QCH | 750 | 109.6 |
| 0.4 | QCL | 3 | 99.4 |
| | QCH | 750 | 105.1 |
| 0.5 | QCL | 3 | 95.5 |
| | QCH | 750 | 94.7 |

In this case, all the obtained samples on the filter paper should be evenly distributed over the marked area and the drops should not merge with each other. In case of an incomplete distribution of blood in the marked area or the fusion of two drops, such samples are considered unacceptable and are not used for a further analysis.

## Validation protocols for quantitative HPLC-MS/MS methods of analysis

To date, such organizations as the International Association for Therapeutic Drug Monitoring and Clinical Toxicology and the Food and Drug Administration (FDA) are working at developing general guidelines for the validation of dried blood spot technology, describing not only validation parameters specific to standard sample preparation methods and traditional matrices, but also specific parameters inherent only to this new technology [10, 11, 45-48, 50].

Based on the current methods analysis for the quantitative HPLC-MS/MS determination of MPs using this sample preparation method, new validation protocols using standard (Table 2) and specific validation parameters for the DBS method, were developed (Table 3).

While developing methods for the quantitative determination of ivabradine in the DBS samples, ions-ivabradine "precursors" which corresponded to the particles with m/z 469, were found out. To develop a multiple reactions monitoring (MRM) method, ion transitions corresponding to the highest intensity of the ions-"products" were used. It was found out that in the course of dissociation in the collision chamber, the most intense ions-"products" were: m/z 262.2 and 177.1 m/z (Fig. 3).

In the process of chromatographic determination under the optimized conditions, the retention time of ivabradine in blood plasma was 1.74 min (Fig. 4).

The developed methods confirmed its linearity in the concentration range from 1 to 1000 ng/ml using a weighted coefficient $1/x^2$, while >0.99. The coefficient of variation (%) calculated when determining inter- and intraday accuracy did not exceed 15% for the main range of concentrations.

The lower limit of the procedure quantification was determined based on the data of linearity, accuracy, and precision. The minimum concentration of ivabradine in the samples of DBS in the analytical range for which it is possible to quantify ivabradine with values of the relative standard deviation of no more than 20%, was taken as the lower limit of quantification in these methods, and it was 1 ng/mL (Table 4).

For the DBS method, the influence of specific parameters of the new method on the results of the analysis was also evaluated.

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2022-10-4-331-342

The hematocrit effect was evaluated at 3 levels (0.3; 0.4; 0.5) for QCL and QCH, the concentrations obtained were in the range from 95.5 to 107.1% of the nominal (Table 5).

To validate the volume effect, 3 volumes (10, 40, 70 µl) (30, 40 and 55 µl) were analyzed at the average hematocrit level (0.4), at 2 concentration levels in 5 series. At the average volume, the relative error of the calculated concentrations did not exceed 15% of the obtained values.

When validating the droplet homogeneity, the results of the QC samples at QCL and QCH, obtained by 2 drop cut options – from the center of the droplet, and from the edge, were compared. The analysis was carried out in 5 series. In this case, the relative error in comparing the concentrations of the samples obtained from the central and edge notches did not exceed 15% of the nominal values.

The DBS samples at QCL and QCH levels were used to assess stability, and the samples were analyzed at three time points of 1, 7 and 14 days along with freshly prepared samples as a part of the same analytical series. The calculated concentrations of the samples after storage were compared with the average concentrations of freshly prepared quality control samples. At the same time, the values obtained after 14 days of storage were in the ranged of 87.2-93.8%.

Thermal stability was evaluated when storing the DBS samples for 14 days at 22°C and 45°C, as potential temperatures during their storage and transportation.

The developed protocols turned out to be suitable for validating the methods for the ivabradine quantitative determination and are acceptable for a further use in research and TDM.

## CONCLUSION

It should be noted that the DBS method is the latest method of biological sampling, which has a number of significant advantages over the standard methods.

With the use of the optimized sample preparation and the new protocols that have been developed for taking, storing, validating, and methods for the quantitative analysis of DBS samples, the technology is widely used in preclinical and clinical studies, therapeutic and toxicological drug monitoring, as well as in large epidemiological studies. At the same time, this technology can provide a more cost-effective model for the analysis of drugs, as well as provide much-needed pharmacokinetic results in a fairly efficient and reliable way.

## FUNDING

This study did not receive financial support from outside organizations.

## CONFLICT OF INTERESTS

The authors declare no conflict of interest.

## AUTHORS' CONTRIBUTION

ISA, TEZ, AMD – research concept, article planning, review of literature sources, materials collection, article writing and editing; VIP, AVS – development of the study design, editing and final approval of the article.

## REFERENCES

1. Abaimov DA, Sariev AK, Noskova TYu, Shvedkov VV, Shiryaeva MV, Styrova EYu, Prokhorov DI, Seyfulla RD. modern technologies in therapeutic drug monitoring (review). Epilepsy and paroxysmal conditions. 2013;5(2):31–41. Russian
2. Déglon J, Thomas A, Mangin P, Staub C. Direct analysis of dried blood spots coupled with mass spectrometry: concepts and biomedical applications. Anal Bioanal Chem. 2012 Mar;402(8):2485–98. DOI: 10.1007/s00216-011-5161-6
3. Demirev PA. Dried blood spots: analysis and applications. Anal Chem. 2013 Jan 15;85(2):779–89. DOI: 10.1021/ac303205m
4. Edelbroek PM, van der Heijden J, Stolk LM. Dried blood spot methods in therapeutic drug monitoring: methods, assays, and pitfalls. Ther Drug Monit. 2009 Jun;31(3):327–36. DOI: 10.1097/FTD.0b013e31819e91ce
5. Antunes MV, Charão MF, Linden R. Dried blood spots analysis with mass spectrometry: Potentials and pitfalls in therapeutic drug monitoring. Clin Biochem. 2016 Sep;49(13–14):1035–46. DOI: 10.1016/j.clinbiochem.2016.05.004
6. Amsterdam PV, Waldrop C. The application of dried blood spot sampling in global clinical trials. Bioanalysis. 2010 Nov;2(11):1783–6. DOI: 10.4155/bio.10.158
7. Jimmerson LC, Zheng JH, Bushman LR, MacBrayne CE, Anderson PL, Kiser JJ. Development and validation of a dried blood spot assay for the quantification of ribavirin using liquid chromatography coupled to mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2014 Jan 1;944:18–24. DOI: 10.1016/j.jchromb.2013.10.035

8. Timmerman P, White S, Globig S, Lüdtke S, Brunet L, Smeraglia J. EBF recommendation on the validation of bioanalytical methods for dried blood spots. Bioanalysis. 2011 Jul;3(14):1567–75. DOI: 10.4155/bio.11.132
9. Jager NG, Rosing H, Schellens JH, Beijnen JH. Procedures and practices for the validation of bioanalytical methods using dried blood spots: a review. Bioanalysis. 2014 Sep;6(18):2481–514. DOI: 10.4155/bio.14.185.
10. Capiau S, Veenhof H, Koster RA, Bergqvist Y, Boettcher M, Halmingh O, Keevil BG, Koch BCP, Linden R, Pistos C, Stolk LM, Touw DJ, Stove CP, Alffenaar JC. Official International Association for Therapeutic Drug Monitoring and Clinical Toxicology Guideline: Development and Validation of Dried Blood Spot-Based Methods for Therapeutic Drug Monitoring. Ther Drug Monit. 2019 Aug;41(4):409–30. DOI: 10.1097/FTD.0000000000000643
11. Martial LC, Aarnoutse RE, Schreuder MF, Henriet SS, Brüggemann RJ, Joore MA. Cost Evaluation of Dried Blood Spot Home Sampling as Compared to Conventional Sampling for Therapeutic Drug Monitoring in Children. PLoS One. 2016 Dec 12;11(12):e0167433. DOI: 10.1371/journal.pone.0167433
12. Barfield M. The Application of Dried Blood Spots in Toxicokinetic and Pharmacokinetic Studies. Thesis submitted in partial fulfilment of the requirements of the University of Lincoln. 2017: 188 p.
13. Vu DH, Koster RA, Alffenaar JW, Brouwers JR, Uges DR. Determination of moxifloxacin in dried blood spots using LC-MS/MS and the impact of the hematocrit and blood volume. J Chromatogr B Analyt Technol Biomed Life Sci. 2011 May 1;879(15–16):1063–70. DOI: 10.1016/j.jchromb.2011.03.017
14. Arpini J, Antunes MV, Pacheco LS, Gnatta D, Rodrigues MF, Keitel E, Linden R. Clinical evaluation of a dried blood spot method for determination of mycophenolic acid in renal transplant patients. Clin Biochem. 2013 Dec;46(18):1905–8. DOI: 10.1016/j.clinbiochem.2013.10.011
15. Barco S, Risso FM, Bruschettini M, Bandettini R, Ramenghi LA, Tripodi G, Castagnola E, Cangemi G. A validated LC-MS/MS method for the quantification of piperacillin/tazobactam on dried blood spot. Bioanalysis. 2014;6(21):2795–802. DOI: 10.4155/bio.14.205
16. Al-Ghazawi M, Khaled Daoud Noor EH, Hadidi K, Alzweiri M, Aburuz S. Determination of vancomycin content in dried blood spots for therapeutic drug monitoring. Acta Poloniae Pharmaceutica. 2021;78(1): 3–10. DOI:10.32383/appdr/132021
17. Hawwa AF, Albawab A, Rooney M, Wedderburn LR, Beresford MW, McElnay JC. A novel dried blood spot-LCMS method for the quantification of methotrexate polyglutamates as a potential marker for methotrexate use in children. PLoS One. 2014 Feb 25;9(2):e89908. DOI: 10.1371/journal.pone.0089908
18. Wilhelm AJ, den Burger JC, Chahbouni A, Vos RM, Sinjewel A. Analysis of mycophenolic acid in dried blood spots using reversed phase high performance liquid chromatography. J Chromatogr B Analyt Technol Biomed Life Sci. 2009 Nov 15;877(30):3916–9. DOI: 10.1016/j.jchromb.2009.09.037
19. Heinig K, Bucheli F, Hartenbach R, Gajate-Perez A. Determination of mycophenolic acid and its phenyl glucuronide in human plasma, ultrafiltrate, blood, DBS and dried plasma spots. Bioanalysis. 2010 Aug;2(8): 1423–35. DOI: 10.4155/bio.10.99
20. Scribel L, Zavascki AP, Matos D, Silveira F, Peralta T, Gonçalves Landgraf N, Lamb Wink P, Cezimbra da Silva AC, Bordin Andriguetti N, Loss Lisboa L, Venzon Antunes M, Linden R. Vancomycin and creatinine determination in dried blood spots: Analytical validation and clinical assessment. J Chromatogr B Analyt Technol Biomed Life Sci. 2020 Jan 15;1137:121897. DOI: 10.1016/j.jchromb.2019.121897
21. Koster RA, Alffenaar JW, Greijdanus B, Uges DR. Fast LC-MS/MS analysis of tacrolimus, sirolimus, everolimus and cyclosporin A in dried blood spots and the influence of the hematocrit and immunosuppressant concentration on recovery. Talanta. 2013 Oct 15;115:47–54. DOI: 10.1016/j.talanta.2013.04.027
22. Sadilkova K, Busby B, Dickerson JA, Rutledge JC, Jack RM. Clinical validation and implementation of a multiplexed immunosuppressant assay in dried blood spots by LC-MS/MS. Clin Chim Acta. 2013 Jun 5;421:152–6. DOI: 10.1016/j.cca.2013.02.009
23. Li Q, Cao D, Huang Y, Xu H, Yu C, Li Z. Development and validation of a sensitive LC-MS/MS method for determination of tacrolimus on dried blood spots. Biomed Chromatogr. 2013 Mar;27(3):327–34. DOI: 10.1002/bmc.2795
24. Egas AC, Van Maarseveen EM, Kwakkel-Van Erp JM. Rapid and combined measurement of cyclosporin a, tacrolimus, sirolimus and everolimus in whole blood and dried blood spot with LC-MSMS. J Heart Lung Transplant. 2014; 33 (Issue 4): S68. DOI: 10.1016/j.healun.2014.01.217
25. Koop DR, Bleyle LA, Munar M, Cherala G, Al-Uzri A. Analysis of tacrolimus and creatinine from a single dried blood spot using liquid chromatography tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2013 May 1;926:54–61. DOI: 10.1016/j.jchromb.2013.02.035
26. Shokati T, Bodenberger N, Gadpaille H, Schniedewind B, Vinks AA, Jiang W, Alloway RR, Christians U. Quantification of the Immunosuppressant Tacrolimus on Dried Blood Spots Using LC-MS/MS. J Vis Exp. 2015 Nov 8;(105):e52424. DOI: 10.3791/52424
27. Zwart TC, Gokoel SRM, van der Boog PJM, de Fijter JW, Kweekel DM, Swen JJ, Guchelaar HJ, Moes DJAR. Therapeutic drug monitoring of tacrolimus and mycophenolic acid in outpatient renal transplant recipients using a volumetric dried blood spot sampling device. Br J Clin Pharmacol. 2018 Dec;84(12):2889–902. DOI: 10.1111/bcp.13755

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2022-10-4-331-342

28. Hinchliffe E, Adaway J, Fildes J, Rowan A, Keevil BG. Therapeutic drug monitoring of ciclosporin A and tacrolimus in heart lung transplant patients using dried blood spots. Ann Clin Biochem. 2014 Jan;51(Pt 1):106–9. DOI: 10.1177/0004563213488759
29. Cohen-Wolkowiez M, Watt KM, Zhou C, Bloom BT, Poindexter B, Castro L, Gao J, Capparelli EV, Benjamin DK Jr, Smith PB. Developmental pharmacokinetics of piperacillin and tazobactam using plasma and dried blood spots from infants. Antimicrob Agents Chemother. 2014 May;58(5):2856–65. DOI: 10.1128/AAC.02139-13
30. van der Elst KC, Span LF, van Hateren K, Vermeulen KM, van der Werf TS, Greijdanus B, Kosterink JG, Uges DR, Alffenaar JW. Dried blood spot analysis suitable for therapeutic drug monitoring of voriconazole, fluconazole, and posaconazole. Antimicrob Agents Chemother. 2013 Oct;57(10):4999–5004. DOI: 10.1128/AAC.00707-13
31. Wilhelm AJ, den Burger JC, Vos RM, Chahbouni A, Sinjewel A. Analysis of cyclosporin A in dried blood spots using liquid chromatography tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2009 May 15;877(14-15):1595–8. DOI: 10.1016/j.jchromb.2009.03.024.
32. Wilhelm AJ, Klijn A, den Burger JC, Visser OJ, Veldkamp AI, Janssen JJ, Swart EL. Clinical validation of dried blood spot sampling in therapeutic drug monitoring of ciclosporin A in allogeneic stem cell transplant recipients: direct comparison between capillary and venous sampling. Ther Drug Monit. 2013 Feb;35(1):92–5. DOI: 10.1097/FTD.0b013e31827d76ce
33. Hinchliffe E, Adaway JE, Keevil BG. Simultaneous measurement of cyclosporin A and tacrolimus from dried blood spots by ultra high performance liquid chromatography tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2012 Feb 1;883–4:102-7. DOI: 10.1016/j.jchromb.2011.05.016
34. Antunes MV, Charão MF, Linden R. Dried blood spots analysis with mass spectrometry: Potentials and pitfalls in therapeutic drug monitoring. Clin Biochem. 2016 Sep;49(13–14):1035–46. DOI: 10.1016/j.clinbiochem.2016.05.004
35. Hoffman JT, Rossi SS, Espina-Quinto R, Letendre S, Capparelli EV. Determination of efavirenz in human dried blood spots by reversed-phase high-performance liquid chromatography with UV detection. Ther Drug Monit. 2013 Apr;35(2):203–8. DOI: 10.1097/FTD.0b013e31827fb72b.
36. Li W, Lee MS. Dried Blood Spots: Applications and Techniques; Wiley, 2014: 376 p.
37. Chermont AG, Falcão LF, de Souza Silva EH, de Cássia Xavier Balda R, Guinsburg R. Skin-to-skin contact and/or oral 25% dextrose for procedural pain relief for term newborn infants. Pediatrics. 2009 Dec;124(6):e1101–7. DOI: 10.1542/peds.2009-0993
38. Hummel P, Puchalski M, Creech SD, Weiss MG. Clinical reliability and validity of the N-PASS: neonatal pain, agitation and sedation scale with prolonged pain. J Perinatol. 2008 Jan;28(1):55–60. DOI: 10.1038/sj.jp.7211861
39. Kireev SS. Pain and stress in the newborns (literature review). Journal of New Medical Technologies. 2016;23(4):328–42. DOI 10.12737/issn.1609-2163
40. Streit F, William Armstrong V, Oellerich M. Rapid Liquid Chromatography–Tandem Mass Spectrometry Routine Method for Simultaneous Determination of Sirolimus, Everolimus, Tacrolimus, and Cyclosporin A in Whole Blood. Clinical Chemistry. 2002;48 (Issue 6):955–8. DOI: 10.1093/clinchem/48.6.955
41. Tsao JC, Evans S, Meldrum M, Altman T, Zeltzer LK. A Review of CAM for Procedural Pain in Infancy: Part I. Sucrose and Non-nutritive Sucking. Evid Based Complement Alternat Med. 2008 Dec;5(4):371–81. DOI: 10.1093/ecam/nem084
42. Freeman JD, Rosman LM, Ratcliff JD, Strickland PT, Graham DR, Silbergeld EK. State of the Science in Dried Blood Spots. Clin Chem. 2018 Apr;64(4):656–79. DOI: 10.1373/clinchem.2017.275966
43. Zakaria R, Allen KJ, Koplin JJ, Roche P, Greaves RF. Advantages and Challenges of Dried Blood Spot Analysis by Mass Spectrometry Across the Total Testing Process. EJIFCC. 2016 Dec 1;27(4):288–317.
44. Linder C. Possibilities of Dried Blood Spots As a Matrix in Therapeutic Drug Monitoring of Antiepileptic Drugs in Children. Karolinska Institutet, Stockholm, Sweden. 2019: 61 p.
45. Murphy SC, Daza G, Chang M, Coombs R. Laser cutting eliminates nucleic acid cross-contamination in dried-blood-spot processing. J Clin Microbiol. 2012 Dec;50(12):4128–30. DOI: 10.1128/JCM.02549-12.
46. Li Y, Henion J, Abbott R, Wang P. Dried blood spots as a sampling technique for the quantitative determination of guanfacine in clinical studies. Bioanalysis. 2011 Nov;3(22):2501–14. DOI: 10.4155/bio.11.262.
47. Klak A, Pauwels S, Vermeersch P. Preanalytical considerations in therapeutic drug monitoring of immunosuppressants with dried blood spots. Diagnosis (Berl). 2019 Mar 26;6(1):57–68. DOI: 10.1515/dx-2018-0034.
48. Knapen LM, Beera Y, Brüggemannc RM, Stolkab LM, Vries F, Vivianne CG, et al. Development and validation of an analytical method using UPLC–MS/MS to quantify everolimus in dried blood spots in the oncology setting. Journal of Pharmaceutical and Biomedical Analysis. 2018;149:106–13. DOI: 10.1016/j.jpba.2017.10.039
49. Börsch-Supan A, Börsch-Supan M, Weiss LM. Dried blood spot samples and their validation. Health and socio-economic status over the life course. 2019: 349–58. DOI:10.1515/9783110617245-036

## AUTHORS

**Vladimir I. Petrov** – Doctor of Sciences (Medicine), Professor, Academician of the Russian Academy of Sciences; Head of the Department of Clinical Pharmacology and Intensive Care, Volgograd State Medical University; director of Scientific Centre of Innovative Medicines with Pilot Production, Volgograd State Medical University; Chief freelance specialist – a clinical pharmacologist of the Ministry of Health of the Russian Federation; Honored Scientist of the Russian Federation; Honored Doctor of the Russian Federation. ORCID ID: 0000-0002-0258-4092. E-mail: brain@sprintnet.ru

**Ivan S. Anikeev** – post-graduate student, Department of Clinical Pharmacology and Intensive Care, Volgograd State Medical University; Head of the Laboratory of Pharmacokinetics, Scientific Center of Innovative Medicines with Pilot Production, Volgograd State Medical University. ORCID ID: 0000-0002-9384-4338. E-mail: anikeivan@yandex.ru

**Tatyana E. Zayachnikova** – Candidate of Sciences (Medicine), Associate Professor, Professor of the Department of Pediatrics and Neonatology, Institute of Continuous Medical and Pharmaceutical Education, Volgograd State Medical University. ORCID ID: 0000-0001-6758-4686. E-mail: guz5deti@mail.ru

**Andrey V. Strygin** – Candidate of Sciences (Medicine), Associate Professor, Head of the Department of Fundamental Medicine and Biology, Volgograd State Medical University; Deputy Director of Scientific Center of Innovative Medicines with Pilot Production, Volgograd State Medical University; Head of the Laboratory of Genomic and Proteomic Research, Volgograd Medical Research Center. ORCID ID: 0000-0002-6997-1601. E-mail: drumsav@mail.ru

**Anna M. Dotsenko** – Assistant of the Department of Fundamental Medicine and Biology, Volgograd State Medical University; Junior Researcher, Laboratory of Genomic and Proteomic Research, Volgograd Medical Research Center. ORCID ID: 0000-0003-3324-3351. E-mail: ev8278@mail.ru

Научно-практический журнал
ФАРМАЦИЯ И ФАРМАКОЛОГИЯ

*ОРИГИНАЛЬНАЯ СТАТЬЯ*

*DOI: 10.19163/2307-9266-2022-10-4-343-353*



# INTERRELATION BETWEEN MITOCHONDRIAL ENZYME ACTIVITY AND ANTIOXIDANT ACTIVITY OF SECONDARY POLYPHENOL NATURE METABOLITES IN HEMIPARASITE *VISCUM ALBUM* L. LEAVES

**S.L. Adjiakhmetova, D.I. Pozdnyakov, N.M. Chervonnaya, E.O. Kulichenko, E.T. Oganesyan**

Pyatigorsk Medical and Pharmaceutical Institute –
branch of Volgograd State Medical University,
11, Kalinin Ave., Pyatigorsk, Russia, 357532

E-mail: similla503@mail.ru

Received 20 Dec 2021 After peer review 12 June 2022 Accepted 01 July 2022

Antioxidants are widely used in practical medicine. Not only the search for new plant antioxidants, but also the study of the factors affecting their accumulation in plants, are relevant.

**The aim** is to study the interrelation between the activity of mitochondrial enzymes and the antioxidant activity of the secondary polyphenolic nature metabolites in hemiparasite *Viscum Album* L. leaves.

**Materials and methods.** The sampling material was *Viscum album* L. leaves, collected in winter from a host tree, *Malus domestica* Borkh. The extraction of biologically active substances was carried out with ethyl alcohol of various concentrations (90%, 70% and 50%), or purified water. The amount of total antioxidants was estimated by the amperometric method. The amount of total phenols was evaluated in the reaction with the Folin-Ciocalteu reagent. The concentration of total flavonoids was estimated by the change in the optical density of the rutin with aluminum (III) chloride complex. The antioxidant properties of the analyzed extracts were determined *in vitro* in the induced lipid peroxidation test. The activity of aconitase was assessed by a conjugated aconitase-isocitrate dehydrogenase reaction, citrate synthase – by changing the color intensity of the Ellman reagent solution, succinate dehydrogenase were determined in the reaction of succinate-dependent oxidation of 2,6-dichlorophenolindophenol.

**Results.** The carried out study showed that the maximum amount of total phenols (2.39±0.05%) is observed in a 50% ethanol extract from *Viscum album* L. leaves, with the content of total flavonoids equal to 1.83±0.04%, and the antioxidants equal to 0.503±0.007 mg/g (a quercetin equivalent) and 0.322±0.006 mg/g (a gallic acid equivalent). A 50% ethanol extract suppressed lipid peroxidation in the model mixture with $IC_{50}$=106.3±1.09 μg/ml. In *Viscum album* L. leaves, a high activity of aconitase which strongly correlated (r=0.88416) with changes in the concentration of flavonoids, has been notified.

**Conclusion.** The optimal extractant for obtaining extracts with a high antioxidant activity is 50% ethyl alcohol. The analysis of the mitochondrial enzymes activity showed that in *Viscum album* L. leaves collected in winter, a high activity of aconitase strongly correlated with changes in the concentration of flavonoids (r=0.88416).

**Keywords:** antioxidants; flavonoids; aconitase; succinate dehydrogenase; citrate synthase; *Viscum album* L.; correlation analysis; plants-hemiparasites; mitochondrial enzymes

**Abbreviations:** LPO – lipid peroxidation; DMSO – dimethyl sulfoxide; TBA – thiobarbituric acid; EDTA – ethylene diamine tetraacetatic acid; NADH – nicotinamide adenine dinucleotide, reduced form; NADP – nicotinamide-adenine dinucleotide phosphate; PAL – phenylalanine ammonia lyase; AU(s) – unit(s) of activity; ROS – reactive oxygen spiecies.

**For citation:** S.L. Adjiakhmetova, D.I. Pozdnyakov, N.M. Chervonnaya, E.O. Kulichenko, E.T. Oganesyan. Interrelation between mitochondrial enzyme activity and antioxidant activity of secondary polyphenol nature metabolites in hemiparasite *Viscum album* L. leaves. *Pharmacy & Pharmacology.* 2022;10(4):343-353. **DOI:** 10.19163/2307-9266-2022-10-4-343-353



**Для цитирования:** С.Л. Аджиахметова, Д.И. Поздняков, Н.М. Червонная, Е.О. Куличенко, Э.Т. Оганесян. Взаимосвязь активности митохондриальных ферментов и антиоксидантной активности вторичных метаболитов полифенольной природы листьев гемипаразита *Viscum album* L. *Фармация и фармакология.* 2022;10(4):343-353. **DOI:** 10.19163/2307-9266-2022-10-4-343-353

*RESEARCH ARTICLE*

*ISSN 2307-9266 e-ISSN 2413-2241*

Scientific and Practical Journal
PHARMACY & PHARMACOLOGY

# ВЗАИМОСВЯЗЬ АКТИВНОСТИ МИТОХОНДРИАЛЬНЫХ ФЕРМЕНТОВ И АНТИОКСИДАНТНОЙ АКТИВНОСТИ ВТОРИЧНЫХ МЕТАБОЛИТОВ ПОЛИФЕНОЛЬНОЙ ПРИРОДЫ ЛИСТЬЕВ ГЕМИПАРАЗИТА *VISCUM ALBUM* L.

**С.Л. Аджиахметова, Д.И. Поздняков, Н.М. Червонная, Е.О. Куличенко, Э.Т. Оганесян**

Пятигорский медико-фармацевтический институт – филиал федерального государственного бюджетного образовательного учреждения высшего образования «Волгоградский государственный медицинский университет» Министерства здравоохранения Российской Федерации,
357532, Россия, г. Пятигорск, пр-т Калинина, д. 11

E-mail: similla503@mail.ru

Получена 20.12.2021 После рецензирования 12.06.2022 Принята к печати 01.07.2022

Антиоксидантные средства находят широкое применение в практической медицине. Актуальным является не только поиск новых растительных антиоксидантов, но и изучение факторов, влияющих на их накопление в растениях.

**Цель.** Изучение взаимосвязи активности митохондриальных ферментов и антиоксидантной активности вторичных метаболитов полифенольной природы листьев гемипаразита *Viscum album* L.

**Материалы и методы.** В качестве исследуемого материала выступали листья омелы белой, собранные зимой с дерева-носителя – яблони обыкновенной. Экстракцию биологически активных веществ проводили спиртом этиловым различной концентрации (90%, 70% и 50%) или водой очищенной. Количество суммы антиоксидантов оценивали амперометрическим методом. Количество суммы фенолов оценивали в реакции с реактивом Фолина-Чокальтеу. Концентрацию суммы флавоноидов оценивали по изменению оптической плотности комплекса рутина с алюминия (III) хлоридом. Антиоксидантные свойства анализируемых извлечений определяли *in vitro* в тесте индуцированного перекисного окисления липидов. Активность аконитазы оценивали путем сопряженной аконитаза – изоцитратдегидрогеназной реакции; цитратсинтазы – по изменению интенсивности окраски раствора реактива Эллмана; сукцинатдегидрогеназы определяли в реакции сукцинат-зависимого окисления 2,6-дихлорфенолиндофенола.

**Результаты.** Проведенное исследование показало, что максимальное количество суммы фенолов (2,39±0,05%), наблюдается в 50% этанольном извлечении из листьев омелы белой, при содержании суммы флавоноидов 1,83±0,04% и антиоксидантов 0,503±0,007 мг/г (эквивалент кверцетина) и 0,322±0,006 мг/г (эквивалент галловой кислоты). 50% этанольное извлечение подавляло перекисное окисление липидов в модельной смеси с $IC_{50}$=106,3±1,09 мкг/мл. В листьях омелы белой отмечена высокая активность аконитазы, сильно коррелировавшая (r=0,88416) с изменением концентрации флавоноидов.

**Заключение.** Оптимальным экстрагентом для получения извлечений с высокой антиоксидантной активностью является спирт этиловый 50%. Анализ активности митохондриальных ферментов показал, что в листьях омелы белой, собранных зимой, отмечена высокая активность аконитазы, сильно коррелировавшая с изменением концентрации флавоноидов (r=0,88416).

**Ключевые слова:** антиоксиданты; флавоноиды; аконитаза; сукцинатдегидрогеназа; цитратсинтаза; *Viscum album* L.; корреляционный анализ; растения гемипаразиты; митохондриальные ферменты

**Список сокращений:** ПОЛ – перекисное окисление липидов; ДМСО – диметилсульфоксид; ТБК – тиобарбитуровая кислота; ЭДТА – этилендиаминтетраацетат натрия; НАДФН – никотинамидадениндинуклеотид фосфат восстановленный; НАДФ – никотинамидадениндинуклеотид фосфат; ФАЛ – фенилаланин-аммиак лиаза; ЕД – единицы действия; АФК – активные формы кислорода.

## INTRODUCTION

About 100 species of evergreen semi-parasitic plants belong to the Mistletoe genus, and only *Viscum album* L. and *Viscum coloratum* (Kom.) Nacai are common in Russia. *Viscum album* L. is an evergreen hemispherical forking shrub that grows on the trunks and branches of many deciduous trees as a parasitic form. The roots of *Viscum album* L. form branches that penetrate under the bark and into the wood of the host tree, forming numerous special attachment organs – haustoria in it[1,2].

According to the taxonomic position and according

[1] Plant resources of the USSR. Flowering plants, their chemical composition, use: Families *Rutaceae* – *Elaeagnaceae* / Academy of Sciences of the USSR, Botan. in-t im. V.L. Komarova; [Compiled by I.B. Sandina and others]; Rep. ed. P.D. Sokolov. – L.: Nauka: Leningrad. department, 1988: 356 p. Russian

[2] Liakh Y., Yurel D. [Mistletoe (*Viscum album*) and its ecological significance in the republic of Belarus]. Sakharov readings 2018: environmental problems of the XXI century: materials of the 18th int. scientific conf. Minsk: Information Center of the Ministry of Finance. 2018; 2: 152–54.

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2022-10-4-343-353

to the classification proposed by Takhtadzhyan A.E. and Gilyarova M.S., *Viscum album* L. is assigned to the Mistletoe genus (*Viscaceae* Miers.), the santal order *(Santalales).* According to Elenevskaya A.G. and others' classification – to the *Loranthaceae* genus (*Loranthaceae* Juss.); according to Tsitsin N.V.'s one – to the *Loranthaceae* Juss. genus[3,4].

Between *Viscum album* L. and other parasitic plants is that *Viscum album* L. does not destroy host cells to obtain nutrients, but rather binds to the vascular system of the host tree. For its vegetation, a *Viscum album* L. tree uses a significant part of the host tree's nutrients, which leads to the loss of its resources, thereby increasing its susceptibility to various diseases. The resulting morphological disturbances reduce protective functions of the host tree, which leads to hypertrophic processes, resulting in dying off a part of the tree[5].

In the structure of *Viscum album* L., there is a variety of biologically active compounds – lectins, viscotoxins, viscerin, flavonoids, nitrogen-containing substances, oleanolic and ursolic acids, alcohols, amines and other active compounds that determine its unique therapeutic properties [1, 2].

Three lectins from *Viscum album* L. have been investigated, the most studied of which is lectin ML-1, the immunomodulating protein which increases the activity of the immune system, and herewith the therapeutic effects are manifested in extremely small doses [3]. Selectively interacting with the immune cells (monocytes, lymphocytes), lectin stimulates the secretion of interferon proteins, interleukins and a tumor necrosis factor by cells. [4–6]. In addition to lectins, viscotoxins have been isolated from *Viscum album* L. extracts; their action in large doses is cytotoxic, which is a prerequisite for an anticancer activity [1, 5].

Flavonoids and phenolcarboxylic acids found out in *Viscum album* L., are marked by an antioxidant activity, they are characterized by a structural diversity, a high biological activity, and a low toxicity [6].

Some of the most effective antioxidants are: fat-soluble polyphenols (tocopherol and its derivatives, ubiquinones, some steroid hormones), phenolic compounds (flavonoids, phenolic acids). In their chemical structure and mechanism of action, these substances are close to the most active antioxidants of a synthetic origin: hydroquinone, agidol, naphthols [6–9].

Metal ions play a special role in the initiation of peroxide processes, herewith, among metals with a variable valence, iron and copper most often take part in oxidation-reduction reactions. Ionized iron is a component of the active sites of many enzymatic systems, such as aconitase, proline hydroxylase, or the antioxidant defense enzyme – catalase. A biological role of iron is associated with its ability to accept and donate electrons at a high reaction rate, transforming, respectively, into divalent ($Fe^{2+}$) or trivalent ($Fe^{3+}$) forms [6, 11].

Being secondary plant metabolites, flavonoids perform many vital biological functions, offering a valuable metabolic and genetic model for studying the transcriptional control of the gene expression. Flavonoid biosynthesis includes many well-characterized enzymatic and regulatory proteins that control flavonoid biosynthesis by activating the early stages of biosynthesis [12, 13].

**THE AIM.** To study the interrelation between the activity of mitochondrial enzymes and the antioxidant activity of the secondary polyphenolic nature metabolites in hemiparasite *Viscum Album* L. leaves.

## MATERIALS AND METHODS

### Study objects

The object under study was *Viscum album* L. leaves growing on *Malus domestica* Borkh., collected in the vicinity of Stavropol, Russia. The raw materials were collected during the winter period (01/21/2022). The choice of time for harvesting raw materials was based on the preliminary studies results of a comparative analysis of the active components accumulation in *Viscum album* L. leaves depending on the season for harvesting raw materials; the maximum content of biologically active compounds is observed in winter.

### Determination of antioxidants total content

The total content of antioxidants was determined by a amperometric method on a Tsvet Yauza 01-AA liquid chromatograph (NPO Khimavtomatika, Russia) using a calibration plot of the dependence of the output signal on the concentration of quercetin and gallic acid [14, 15].

### Obtaining extracts under study

A precisely weighed amount of the raw material dried by the air-shadow method and crushed with office scissors (1 g), was placed in a flask with a capacity of 100 ml; 30 ml of ethyl alcohol of the appropriate concentration (50%, 70% and 90%) or purified water was added, boiled in the water bath for 60 minutes. The contents of the flask were filtered through a paper filter into a 100 ml volumetric flask. The extraction by the above method was repeated twice, the filter was washed with the appropriate extractant and the volume of the filtrate was brought to the mark. If necessary, the sample was diluted [14–17].

The total content of antioxidants (mg/g) was determined by formula 1:

$$X = \frac{X_G \cdot V_n \cdot N}{m_n \cdot 1000}, \quad (1)$$

where: $X_G$ is a mass concentration of antioxidants found according to the calibration chart, mg/l; $V_n$ is an extraction volume, ml; $m_n$ is a sample of raw materials, g; $N$ is a dilution factor.

[3] Ibid.
[4] Plant resources of the USSR. Flowering plants, their chemical composition, use: Families *Rutaceae – Elaeagnaceae*, 1988.
[5] Turova AD. [Medicinal plants and their use]. M.: Medicine, 2013: 203 p. Russian

### Evaluation of antioxidant activity of extracts under study on iron (II)-induced lipid peroxidation model

The antioxidant effect of the extracts under study on the model of $Fe^{2+}$-induced lipid peroxidation (LPO) was investigated *in vitro*. Lecithin liposomes acted as a model medium for LPO. The intensity of liposomes lipid peroxidation was evidenced by a change in the concentration of the products that react with 2-thiobarbituric acid (TBA-active products). The content of lipid liposomes in the medium under study was 40 mg/ml [18–20].

The antioxidant activity was evaluated after a preliminary evaporation of the extract in the water bath and drying in the oven at the temperature not exceeding 60°C [18–22].

The reaction of $Fe^{2+}$-induced LPO was carried out in the water bath at 37°C with continuous bubbling. The studied dried extracts were preliminarily dissolved in dimethyl sulfoxide (DMSO), added to the liposome suspension and a ferrous iron solution and incubated for 15 min. Then 0.5 ml of a 20% trichloroacetic acid solution was added and centrifuged for 15 minutes at 3 thousand rpm. Next, 1 ml of a 0.5% thiobarbituric acid solution was added to the supernatant and heated in the water bath for 15 minutes at 100°C. The content of TBA-active products was measured on an SF-102 spectrophotometer (Aquilon, Russia) at 532 nm [23]. The percentage of the LPO inhibition was calculated with respect to the control sample (solvent) using formula 2 [24]:

$$AOA = \frac{\Delta A_k - \Delta A_{op}}{\Delta A_k} \cdot 100\%, \quad (2)$$

$$\Delta A_k = A_{k15} - Ak;\ \Delta A_{op} = A_{op15} - A_{op}$$

where: *AOA* is an antioxidant activity, %; $A_k$ and $A_{op}$ are optical densities before the incubation; $A_{k15}$ and $A_{op15}$ are optical densities after 15 min of the incubation.

In this work, the semi-inhibition coefficient ($IC_{50}$) of lipid peroxidation, which had been calculated by the regression analysis method [18–20], served as a criterion indicator that made it possible to determine the antioxidant activity level of the extracts under study.

### Determination of phenolic compounds total content by Folin-Ciocalteu method

In the solutions under study, the concentration of phenolic compounds in terms of gallic acid was determined according to the calibration curve, and the percentage (X) in terms of absolutely dry raw materials was calculated using formula 3:

$$X = \frac{C \cdot W_1 \cdot W_2 \cdot 100}{a \cdot V_a \cdot (100 - W)}, \quad (3)$$

where: *C* is a concentration of phenolic compounds in the extract under study, calculated according to the calibration curve, g/100 ml; *a* – sample of raw materials, g; $V_a$ – aliquot volume, ml; $W_1$, $W_2$ – volumes of volumetric flasks, ml; W is a weight loss during drying of raw materials, % [25–27].

The quantitative determination of flavonoids was carried out by the spectrophotometric method on an SPh-102 spectrophotometer. When calculating the total flavonoids content, the value of the specific absorption index of the rutin with aluminum chloride complex was used, because the extracts from *Viscum album* L. leaves in the presence of a 2% aluminum (III) chloride alcohol solution have a maximum absorption at the wavelength of 415±2 nm[6].

The total content of flavonoids in percent (X) in terms of rutin and absolutely dry raw materials is calculated by formula 4:

$$X = \frac{A \cdot 25 \cdot 100 \cdot 100}{A_{1cm}^{1\%} \cdot a \cdot 2 \cdot (100 - W)}, \quad (4)$$

where: *A* is the optical density of the test solution; $A_{1cm}^{1\%}$ is a specific absorption index of the rutin with aluminum chloride complex at the wavelength of 415±2 nm equal to 248; *a* is a weighed amount of raw materials, g; *W* is the raw material moisture content, % [35].

### Preparation of plant materials for determining activity of mitochondrial enzymes

The activity of mitochondrial enzymes was evaluated in the cytosolic fraction enriched with mitochondria, for which 1.0 g of precisely weighed crushed raw materials was placed in a 50 ml conical flask and filled with 10 ml of a buffer system (composition: 50 mM/l HEPES + 2 mM/l magnesium (II) chloride + 40 mM/l potassium chloride + 1 mM/l EDTA + 0.1% bovine serum albumin + 1% polyvinylpyrrolidone 40 000 + 10% glycerol + 50 μg/ml trypsin and potassium hydroxide up to pH 7.8). The resulting mixture was kept at the temperature of 37°C for 30 minutes. Then it was filtered through a glass filter and the filtrate was centrifuged at 1 800 g for 10 minutes. The resulting supernatant was taken into sterile Eppendorf tubes, layered with a 10% Percoll solution and centrifuged again at 18 000 g for 10 minutes. The secondary supernatant was removed for the analysis [28].

[6] Russian State Pharmacopeia XIV edition. Publisher: Ministry of Health of the Russian Federation, 2018; 2, 4. Available from: http: // http: // femb.ru/femb/pharmacopea.php

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2022-10-4-343-353

### Assessment of aconitase activity

An aconitase activity was assessed spectrophotometrically using a PROMECOLAB PE-5300V spectrophotometer (Shanghai Mapada Instruments Co., Ltd., China) in the course of a conjugated aconitase–isocitrate dehydrogenase reaction by detecting NADH at 340 nm. The course of the analysis was as follows: 0.1 mg/ml (10 µl) of a sodium citrate solution was added to the medium containing 0.03 U/l isocitrate dehydrogenase, 0.32 mg/ml NADP, 55 µl phosphate-buffered saline and 50 µl of the test biomaterial. The change in the optical density of the obtained solutions was recorded against the mixture of reagents without the biomaterial under study within 2 minutes. An aconitase activity was calculated according to the change in the absorbance using an extinction coefficient of 0.0313µM-1. The enzyme activity was expressed as U/mg protein. In the analyzed samples, the protein content was determined by the Bradford method [29].

### Assessment of citrate synthase activity

The citrate synthase activity was evaluated in the medium consisting of a 5,5'-di-thiobis-2-nitrobenzoic acid solution (Ellman's reagent) 100 mM Tris-HCl buffer with pH 7.8 and higher; acetyl-CoA 100 mm; 0.1% Triton-x 100 µl and 4 µl of the test biomaterial. The reaction was started by adding 100 µl of oxaloacetate. After 3 minutes, the change in the optical density of the medium at 412 nm was evaluated. The citrate synthase activity was also expressed as U/mg of protein. In the samples under study, the protein content was determined by the Bradford method [30].

### Assessment of succinate dehydrogenase activity

The activity of succinate dehydrogenase was evaluated spectrophotometrically (spectrophotometer PROMEKOLAB PE-5300V) in the reaction of succinate-dependent oxidation of 2,6-dichlorophenolindophenol at 600 nm. During the analysis, standard kits from Abcam were used.

### Statistical analysis

The statistical analysis was performed using the STATISTICA 6.0 Software package. The results were expressed as M ± SEM (mean ± standard error of the mean). A further analysis was performed by a one-way analysis of variance (ANOVA) with Newman-Keuls post-processing in the presence of a Gaussian distribution and the Kruskal-Wallis test in the absence of the Gaussian distribution. The distribution normality was tested using the Shapiro-Wilk test. The differences between the study groups were considered statistically significant at $p < 0.05$. The correlation analysis was performed using the Spearman's test as interpreted by the Chaddock's scale.

## RESULTS

### Estimation of antioxidants total amount in objects under study

The total content of antioxidants in hydroalcoholic and aqueous extracts from *Viscum album* L. leaves was expressed as the equivalent of quercetin and gallic acid. The results are presented in Table 1.

By the amperometric method, it was found out that the maximum amount of antioxidants in the extracts from *Viscum album* L. leaves was notified with the use of 50% ethanol as an extractant, which amounted to 0.503±0.007 mg/g in terms of quercetin and 0.322±0.006 mg/g in terms of gallic acid.

### Determination of antioxidant effect on $Fe^{2+}$-induced lipid peroxidation model

The extracts from *Viscum album* L. leaves were added to the medium under study in the form of water-alcohol and aqueous solutions in the final concentrations of two-fold dilutions: 200 µg/ml – 12.5 µg/ml. The extract from *Viscum album* L. leaves, obtained by the extraction with 50% ethanol, exhibits the highest antioxidant activity.

The maximum decrease in lipid peroxidation is observed in the extracts from *Viscum album* L. leaves (the extractant is 50% ethyl alcohol) collected in winter, at the concentration of 200 µg/ml, and it is 73.64±1.43%. A closer result for quercetin was observed at the concentration of 25 µg/ml.

The calculated $IC_{50}$ values, numerically equal to the concentration of the substances in the analyzed medium, causing a 50% decrease in the intensity of lipid peroxidation, were 19.27±0.97 µg/ml for quercetin and 106.3±1.09 µg/ml for the 50% alcohol extraction from *Viscum album* L. leaves under study.

The data obtained when determining the total content of antioxidants on a liquid chromatograph Tsvet Yauza 01-AA by the amperometric method, are consistent with the data obtained when determining the antioxidant effect of the extracts from *Viscum album* L. leaves on $Fe^{2+}$-induced LPO in the liposomal system.

### Determination of phenolic compounds amount using Folin-Ciocalteu reagent

For the quantitative determination of phenolic compounds, the equation of the calibration curve was used: y = 1746x+0.002. The results of determining the total content of phenolic compounds are presented in Fig. 1 and Table 3.

ISSN 2307-9266 e-ISSN 2413-2241
Scientific and Practical Journal
PHARMACY & PHARMACOLOGY

**Table 1 – Total amount of antioxidants in *Viscum album* L. extracts**

| Raw materials used | Extractants | Dilution ratio | Content of antioxidants, mg/g in terms of | |
|---|---|---|---|---|
| | | | Quercetin (n=6) | Gallic acid (n=6) |
| *Viscum album* L. leaves harvested in winter | ethanol 90% | – | 0.127±0.002 | 0.078±0.001 |
| | ethanol 70% | – | 0.374±0.003 | 0.244±0.003 |
| | **ethanol 50%** | **2** | **0.503±0.007** | **0.322±0.006** |
| | purified water | – | 0.261±0.003 | 0.167±0.002 |

**Table 2 – Effect of extracts from *Viscum album* L. leaves on $Fe^{2+}$-induced LPO in liposome system**

| Final concentration, μg/ml | % decrease in LPO, n=3 | | | |
|---|---|---|---|---|
| | Extractants | | | |
| | Ethanol 90% | Ethanol 70% | **Ethanol 50%** | Purified water |
| 12.5 | 8.22±1.28 | 9.05±1.05 | **12.08±1.36** | 11.28±1.27 |
| 25 | 10.16±1.89 | 17.45±1.24 | **22.48±1.23** | 22.17±2.89 |
| 50 | 29.75±1.76 | 39.53±1.49 | **41.77±2.19** | 37.04±1.54 |
| 100 | 41.17±2.35 | 51.61±2.32 | **55.71±2.04** | 44.71±2.77 |
| 200 | 56.01±1.63 | 68.71±1.77 | **73.64±1.43** | 62.26±2.69 |
| $IC_{50}$ | 160.52±2.26 | 120.07±1.94 | **106.30±1.09** | 137.02±2.16 |

Note: A 95% alcohol solution of quercetin was used as a reference, which was added in final concentrations of two-fold dilutions: 3.125 μg/ml, 6.25 μg/ml, 12.5 μg/ml, 25 μg/ml, 50 μg/ml. The decrease in the accumulation of LPO products was as follows: 13.89±1.56%, 31.04±2.41%, 47.04±2.21%, 69.91±2.07%, 88.97±1.37%.

**Table 3 – Amount of phenolic compounds from *Viscum album* L. leaves**

| Raw material name | *Viscum album* L. leaves | |
|---|---|---|
| Extractants used | Ratio of extract sample and Folin-Ciocalteu reagent | Content of phenolic compounds, % (n=6) |
| Ethanol 90% | 1:1 | 1.74±0.03 |
| Ethanol 70% | 1:2 | 2.02±0.05 |
| **Ethanol 50%** | **1:1** | **2.39±0.05** |
| Purified water | 1:1 | 1.75±0.04 |

Note: When determining the total content of phenolic compounds, $V_a$ = 0.5 ml was used for *Viscum album* L. leaves extracts.

**Table 4 – Stability of 50% ethanol extraction complexes with Folin-Ciocalteu reagent in dynamics**

| Raw material | Exposure time, min | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 5 | 10 | 15 | 20 | 25 | 30 | 35 | 40 | 45 | 50 | 55 | **60** |
| Optical density | 0.613 | 0.664 | 0.686 | 0.704 | 0.710 | 0.713 | 0.716 | 0.720 | 0.733 | 0.762 | 0.767 | **0.770** |
| Phenols content | 1.902 | 2.061 | 2.129 | 2.185 | 2.204 | 2.219 | 2.223 | 2.235 | 2.275 | 2.366 | 2.381 | **2.391** |

**Table 5 – Total content of phenolic compounds from *Viscum album* L. leaves**

| Raw material name | *Viscum album* L. leaves | |
|---|---|---|
| Extractants used | Stabilization time (min) of optical density | Content of flavonoids, % (n=6) |
| Ethanol 90% | 30 | 0.327±0.004 |
| Ethanol 70% | 35 | 1.46±0.03 |
| Ethanol 50% | 30 | 1.83±0.05 |
| Purified water | 35 | 0.282±0.003 |

Note: when determining the total content of phenolic compounds, $V_a$ = 5.0 ml was used for extracts from *Viscum album* L. leaves growing on *Malus domestica* Borkh.

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2022-10-4-343-353

**Table 6 – Study of complexes color stability of 50% ethanol extract with 2% aluminum (III) chloride alcohol solution in dynamics**

| Raw material name | Exposure time, min | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 5 | 10 | 15 | 20 | 25 | 30 | 35 | 40 | 45 |
| Optical density | 0.796 | 0.811 | 0.820 | 0.823 | 0.832 | **0.833** | 0.832 | 0.818 | 0.816 |
| Phenols content | 1.744 | 1.777 | 1.80 | 1.804 | 1.823 | **1.825** | 1.823 | 1.793 | 1.788 |

**Table 7 – Changes in mitochondrial enzymes activity in *Viscum album* L. leaves**

| Index | *Viscum album* L. leaves collected in winter |
|---|---|
| Aconitase, U/g protein | 3.36±0.14 |
| Citrate synthase, U/g protein | 0.54±0.07* |
| Succinate dehydrogenase, U/g protein | 1.12±0.06* |

Note: * – statistically significant in relation to the activity of aconitase (Kruskell-Wallis test, p <0.05).

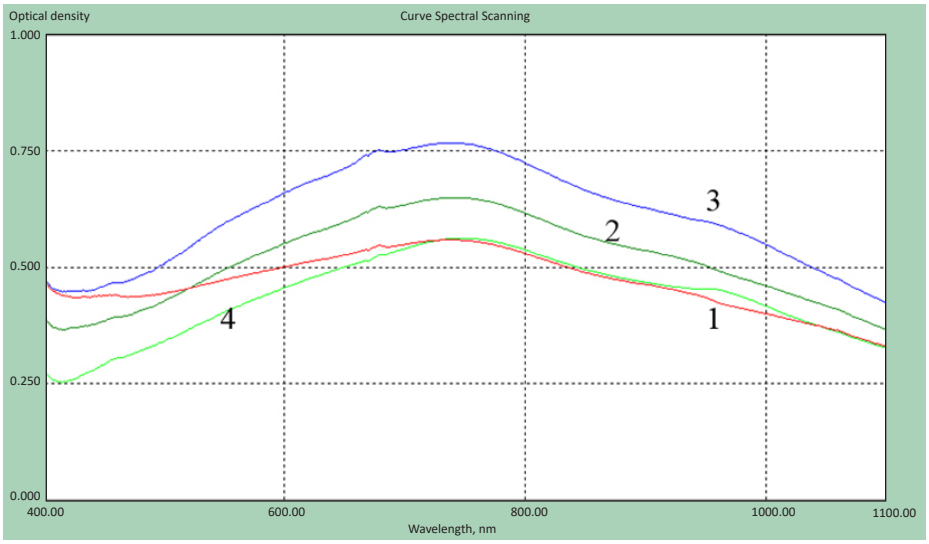



**Figure 1 – UV absorption spectra of extracts complexes from *Viscum album* L. leaves with Folin-Ciocalteu reagent**

Note: 1 – extract obtained by extraction with 90% ethanol; 2 – extract obtained by extraction with 70% ethanol; 3 – extract obtained by extraction with 50% ethanol; 4 – extract obtained by extraction with purified water.

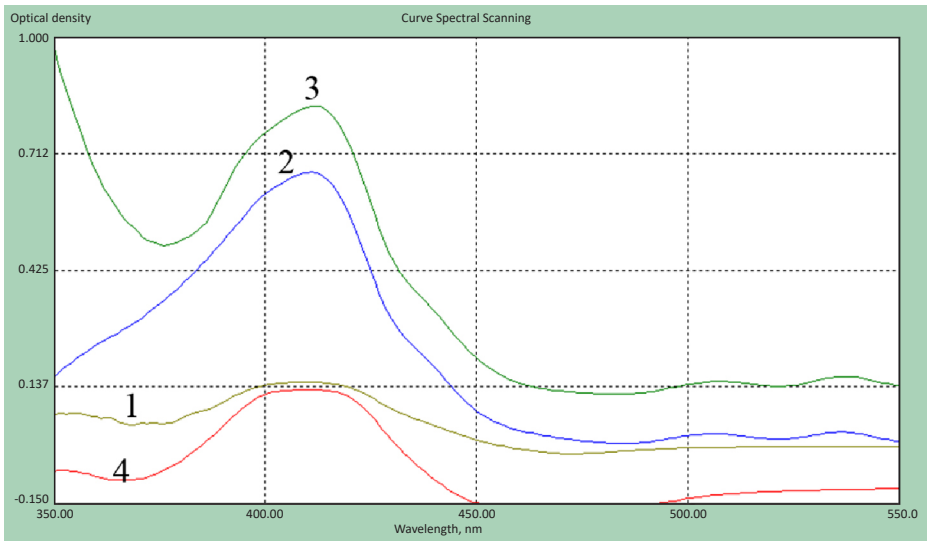



**Figure 2 – UV absorption spectra of extracts complexes from *Viscum album* L. leaves with 2% alcohol solution of aluminum (III) chloride**

Note: 1 – extract obtained by extraction with 90% ethanol; 2 – extract obtained by extraction with 70% ethanol; 3 – extract obtained by extraction with 50% ethanol; 4 – extract obtained by extraction with purified water.

ISSN 2307-9266 e-ISSN 2413-2241

Scientific and Practical Journal
PHARMACY & PHARMACOLOGY

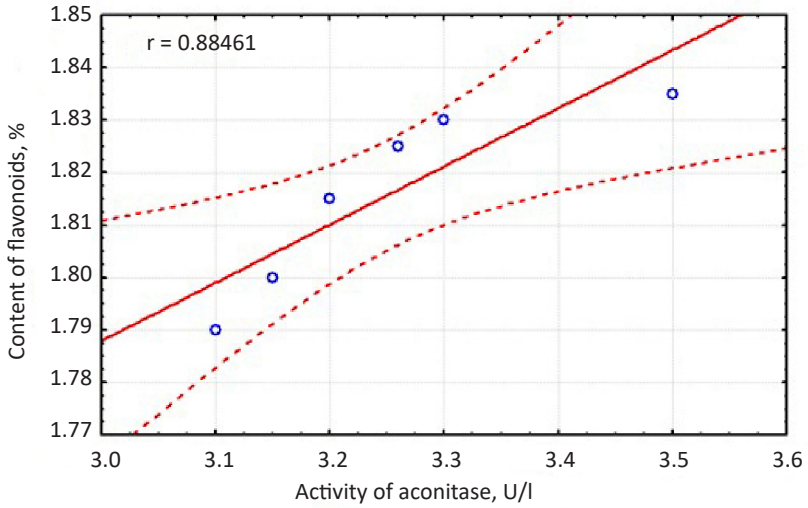



**Figure 3 – Dependence of the change in the concentration of flavonoids on the activity of aconitase in *Viscum album* L. leaves**

Note: the figure shows the determined indicators are highlighted in blue; the correlation trend line is solid; the data scatter boundaries are highlighted in dotted lines.

The maximum amount of total phenols was notified in the extract from the *Viscum album* L. leaves obtained by extraction with 50% ethyl alcohol, and it was 2.39 ± 0.05%. The optical density stabilization time was 60 min.

Due to the fact that the reaction develops in dynamics, it has been found out that the optical density value stabilizes within 60 min, which is optimal for the analysis.

### Qualitative and quantitative analyses of flavonoids

A quantitative determination of flavonoids was carried out in terms of rutin in 6 repetitions. The results of determining the total content of flavonoids are shown in Fig. 2 and Table 5.

The data presented in Table 5 show that the maximum amount of total flavonoids in the extracts from *Viscum album* L. leaves is observed during the extraction of raw materials with 50% ethyl alcohol, and it is 1.83 ± 0.04%. The stabilization time of the optical density in the analyzed solutions is from 30 to 35 min.

Due to the fact that the reaction evolves in time, it has been found out that the optical density value stabilizes within 30 min, which is optimal for the analysis [26].

### Evaluation of changes in the activity of mitochondrial enzymes in *Viscum album* L. leaves

The study of changes in the activity of enzymes of the mitochondrial origin in *Viscum album* L. leaves showed that the activity of aconitase was higher than the activity of citrate synthase and succinate dehydrogenase by 6.2 times ($p < 0.05$) and 3.0 times ($p < 0.05$), respectively.

As a result of the correlation analysis, it was found that the content of flavonoids, as the main polyphenolic antioxidants, in *Viscum album* L. leaves strongly correlates with the changes in the aconitase activity with a correlation coefficient of r=0.88416 (Fig. 2). In case of assessing the dependence of changes in the concentration of flavonoids on the activity of citrate synthase and succinate dehydrogenase, a weak correlation was notified (r=0.23681 and r=0.33984).

## DISCUSSION

Flavonoids are a class of structurally diverse secondary metabolites that perform many functions in plants. For example, the leading role of flavonoids in the growth regulation, pigment biosynthesis, transmission of intracellular signals, a plant protection from ultraviolet radiation and the action of reactive oxygen species have been established. Today, flavonoids are one of the most studied groups of natural compounds, while most of the ongoing investigations are aimed at evaluating their pharmacological activity. At the same time, the features of biosynthesis and accumulation of flavonoids are also of certain scientific interest [31].

Biosynthesis of all secondary metabolites in plants is a controlled and deterministic process based on a strictly defined sequence of enzymatic reactions. While the pathways for the synthesis of carbohydrates, fats, proteins, and nucleic acids, or simply primary metabolites, are essentially the same, there is a

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2022-10-4-343-353

wide variety of synthesis mechanisms for secondary metabolites in all living organisms. Understanding these metabolic processes is critical to regulating the biosynthesis of the desired phytochemicals and their potential use in the food or medical industry. Enzymatic control of flavonoid biosynthesis reactions implies the intensification of synthesis under certain conditions, which, as applied to a plant, depend on growing conditions and the season [32].

The majority of secondary metabolites, including flavonoids, primarily perform a protective function in plants, and their synthesis is activated under the influence of unfavorable external factors. In connection with this, in this study, the content of secondary metabolites of the polyphenolic structure in *Viscum album* L. leaves collected in winter, has been estimated, i.e. under the conditions most predisposing to their synthesis in large quantities. As a result, it was shown that the maximum extraction of flavonoids was notified when using 50% ethyl alcohol while the content of the desired substances was 1.83 ± 0.05%. A high content of these substances in *Viscum album* L leaves collected in winter, can be explained by the fact that low temperatures stimulate the expression of genes for enzymes responsible for the synthesis of flavonoids: PAL (Phenylalanine-ammonia lyase), chalcone synthase, flavanone-3-hydroxylase, dihydroflavonol- 4-reductase, anthocyanidin synthase, flavonoid-3-O-glucosyltransferase, leukoanthocyanidin reductase [12, 13], which lead to an increase in the synthesis and accumulation of flavonoids and other polyphenols. A strong correlation has also been notified between the change in the content of flavonoids and the activity of aconitase (r=0.88416) in *Viscum album* L. leaves, while the dependence in relation to citrate synthase (r=0.23681) and succinate dehydrogenase (r=0.33984) was weak.

Aconitase, also known as aconitate hydratase, is an iron-sulfur cluster protein enzyme that catalyzes the reversible isomerization of citrate to isocitrate via cis-aconitate in the tricarboxylic acid cycle. In mammals, the enzyme is found out in two-cell compartments – mitochondria and cytoplasm. The mitochondrial isoform is directly involved in the Krebs cycle, while the role of the cytoplasmic isoenzyme is reduced to the regulation of the stability of certain transcripts upon cleavage of the iron-sulfur cluster (in this case, aconitase acts as an RNA-binding protein) [33].

In plants, cytoplasmic and mitochondrial enzymes have similar characteristics and cannot be differentiated with a high degree of selectivity. Plant aconitase is involved in the reactions of the glyoxylate cycle and sucrose metabolism, as well as in several important physiological processes, including the inactivation of peroxides and nitroxides. Recent studies have shown an important role of aconitase in the biosynthesis of secondary metabolites, including flavonoids. Thus, some hybrids demonstrating a reduced concentration of flavonoids compared to the maternal line, have a deficient aconitase phenotype [34].

Citrate synthase also plays a significant role in the synthesis of secondary plant metabolites. Zhao H. et al., showed that mitochondrial citrate synthase is a key regulator of anthocyanin synthesis in *Petunia hybrida hort. ex E. Vilm* [35]. In its turn, succinate dehydrogenase is responsible for the synthesis of organic acids in plant cells [36]. Apparently, different roles of mitochondrial enzymes in the biosynthesis of secondary plant metabolites can explain their interrelation established in this study.

Thus, it can be assumed that, under conditions of a temperature stress, protective enzymatic and non-enzymatic mechanisms (aconitase and increased synthesis of flavonoids, respectively) are activated in *Viscum album* L. leaves, the action of which is aimed at maintaining cell viability. It has been established that the impact of low temperatures as a strong stressor on a plant cell leads to the accumulation of reactive oxygen species (ROS): hydroxyl radicals, superoxide anion, singlet oxygen and hydrogen peroxide. High levels of ROS have a detrimental effect on cell structures, leading to the DNA damage, lipid peroxidation, protein denaturation, reduced photosynthesis, an impaired enzyme activity, and a cell death. Therefore, maintaining a moderate level of ROIS is necessary for the protection against various abiotic and biotic stresses. It is the activation of oxidative stress that can be associated with an increased expression of aconitase from *Viscum album* L. leaves and the synthesis intensification of secondary metabolites [37].

## CONCLUSION

The content of antioxidants, phenols, flavonoids in the leaves of *Viscum album* L. growing on *Malus domestica* Borkh., has been established. The optimal extractant is 50% ethyl alcohol. The analysis of the mitochondrial enzymes activity showed that in *Viscum album* L. leaves collected in winter, a high activity of aconitase which strongly correlated with the changes in the concentration of flavonoids (r = 0.88416), was notified, while there was no interrelation between the activity of succinate dehydrogenase, citrate synthase and the content of flavonoids in *Viscum album* L. leaves.

## FUNDING

This study did not receive financial support from outside organizations.

## CONFLICT OF INTERESTS

The authors declare no conflict of interest.

## AUTHORS' CONTRIBUTION

SLA – development of the study concept, obtaining analyzed extracts, conducting an *in vitro* study, preparing the final version of the manuscript; DIP – development of the study concept, assessment of the mitochondrial enzymes activity, statistical processing of the study results, preparing the final version of the manuscript; NMCh – development of the study concept, obtaining analyzed extracts, conducting an *in vitro* study, preparing the final version of the manuscript; EOK – determination of the antioxidant effect, analysis of the literature; ETO – development of the study concept, analysis of the literature, preparing the final version of the manuscript.

## REFERENCES

1. Korman DB. [Antitumor properties of mistletoe lectins]. Problems of Oncology. 2011;57(6):689–98. Russian
2. Sayakova GM, Khamitova AE, Olatayeva ZN. [Creation of new dosage forms from domestic plant raw materials sophora colonic (Sophóra pachycárpa) and white mistletoe (*Viscum album*) as promising sources of biologically active substances]. Vestnik KazNMU. 2018; 4: 217–220. Russian
3. Franz H. Mistletoe lectins and their A and B chains. Oncology. 1986;43 Suppl 1:23–34. DOI: 10.1159/000226417
4. Steuer-Vogt MK, Bonkowsky V, Ambrosch P, Scholz M, Neiss A, Strutz J, Hennig M, Lenarz T, Arnold W. The effect of an adjuvant mistletoe treatment programme in resected head and neck cancer patients: a randomised controlled clinical trial. Eur J Cancer. 2001 Jan;37(1): 23–31. DOI: 10.1016/s0959-8049(00)00360-9
5. Hajtó T, Fodor K, Perjési P, Németh P. Difficulties and perspectives of immunomodulatory therapy with mistletoe lectins and standardized mistletoe extracts in evidence-based medicine. Evid Based Complement Alternat Med. 2011;2011:298972. DOI: 10.1093/ecam/nep191
6. Jäger S, Beffert M, Hoppe K, Nadberezny D, Frank B, Scheffler A. Preparation of herbal tea as infusion or by maceration at room temperature using mistletoe tea as an example. Sci Pharm. 2011 Jan-Mar;79(1):145–55. DOI: 10.3797/scipharm.1006-06
7. Uzbekov MG. LIPID Peroxidation and antioxidant systems in mental disorders. Part I. Social and clinical psychiatry. 2014; 24 (4): 97–103. Russian
8. Uzbekov MG. LIPID Peroxidation and antioxidant systems in mental disorders. Part II. Social and clinical psychiatry. 2015; 25 (4): 92–101. Russian
9. Jomova K, Valko M. Advances in metal-induced oxidative stress and human disease. Toxicology. 2011 May 10;283 (2-3):65–87. DOI: 10.1016/j.tox.2011.03.001
10. Minotti G, Aust SD. The requirement for iron (III) in the initiation of lipid peroxidation by iron (II) and hydrogen peroxide. J Biol Chem. 1987 Jan 25;262(3):1098–104.
11. Vashchenko G, MacGillivray RT. Multi-copper oxidases and human iron metabolism. Nutrients. 2013 Jun 27;5(7):2289–313. DOI: 10.3390/nu5072289
12. Li S. Transcriptional control of flavonoid biosynthesis: fine-tuning of the MYB-bHLH-WD40 (MBW) complex. Plant Signal Behav. 2014;9(1):e27522. DOI: 10.4161/psb.27522
13. Catala R, Ouyang J, Abreu IA, Hu Y, Seo H, Zhang X, Chua NH. The Arabidopsis E3 SUMO ligase SIZ1 regulates plant growth and drought responses. Plant Cell. 2007 Sep;19(9):2952–66. DOI: 10.1105/tpc.106.049981
14. Patent 2238554 Russian Federation, MCI G01N 33/15 No. 27/26. Sposob opredeleniya summarnoj antioksidantnoj aktivnosti biologicheski aktivnyh veshchestv [Method for determining the total antioxidant activity of biologically active substances] Pakhomov VP [et al.] (RF). No. 2003123072/15; application 25.07.03; publ. 20.10.04, Byul15:3. Russian
15. Yashin AYa, Yashin YaI. [A device for determining the antioxidant activity of herbal medicinal extracts and drinks]. J Int Inform Sys Resonant Technolog. 2004;(34): 10–14. Russian
16. Yashin AYa. [Injection-flow system with an amperometric detector for the selective determination of antioxidants in food products and drinks]. Rossijskij Khimicheskij Zhurnal (Zhurnal Rossijskogo Khimicheskogo Obshchestva Im. D.I. Mendeleeva). 2008; (2): 130–5. Russian
17. Adzhiakhmetova SL, Andreeva OA, Oganesyan ET. [Antioxidant activity of extracts from the leaves, the fruit and stems gooseberry rejected (*Grossularia reclinata* (L) Mill]. Fundamental research. 2013; 10(6): 1297–301. Russian. Available from: http://www.rae.ru/fs/pdf/2013/10-6/32535.
18. Melyantseva LP, Kreines VM, Melnikova VM, Gladshtein AI. [Influence of phosphatidylcholinesterol liposomes on the growth of some bacterial cultures]. J Microbiolog Epidemiolog & Immunobiolog. 1994; (2):14–7. Russian
19. Kulichenko EO, Andreeva OA, Sergeeva EO, Sigareva SS, Terekhov AYu, Oganesyan ET, Sidorskaya SYu. Pharmacological activity of extracts from plants of *Cosmos bipinnatus* Cav. species. Pharmacy & Pharmacology. 2022;10(1):82–92. DOI: 10.19163/2307-9266-2022-10-1-82-92
20. Bzhikhatlova MA, Andreeva OA, Oganesyan ET, Voronkov AV, Geraschenko AD. [Phenolic compounds of campsis radicans leaves]. Modern problems of science and education. 2015; 2 (Part 2). Russian. Available from: http://www.science-education.ru/ru/article/view?id=22562
21. Kodonidi IP, Sochnev VS, Terekhov AY, Sergeeva EO, Ryabukhin IY. [Synthesis and study of anti-inflammatory activity 2-vinyl derivatives of 4-(2,6-dimethyl-4-oxo-5-phenyl-4h-pyrimidin-1-yl)-benzsulfonamide]. Modern problems of science and education. 2021; 4: 101. DOI: 10.17513/ spno.31053. 21
22. Kobin AA, Dorkina EG. [Determination of antioxidant activity of dry extract of forsythia drooping on the model

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2022-10-4-343-353

of $Fe^{2+}$ escortinbrisbane lipid peroxidation]. Modern Science and Innovations. 2015;(1):124–6. Russian
23. Aguilar Diaz De Leon J, Borges CR. Evaluation of Oxidative Stress in Biological Samples Using the Thiobarbituric Acid Reactive Substances Assay. J Vis Exp. 2020 May 12;(159). DOI: 10.3791/61122
24. Potapova AA, Dorkina EG, Sergeeva EO, Kobin AA, Sajaia LA. [The study membrane stabilizing and antioxidant effects of baicalin]. Modern Science and Innovations. 2016;(1):148–52. Russian
25. Denisenko TA, Vishnikin AB, Tsiganok LP. Spectrophotometric determination of phenolic compounds sum in plants using aluminum chloride, 18-molybdodiphosphate and folin-ciocalteu reagents. Analytics and control. 2015; 19(4): 373–80. Russian. DOI: 10.15826/analitika.2015.19.4.012
26. Adzhiahmetova SL, Chervonnaja NM, Pozdnjakov DI, Oganesjan ET. [The content of phenols (including flavonoids) and antioxidants in the leaves of *Viscum album* L. and *Pyrus communis* L.] Problems of Biological, Medical and Pharmaceutical Chemistry. 2021. 24 (2): 15–22. DOI: 10.29296/25877313-2021-02-03
27. Makarova NV, Valiulina DF, Azarov OI, Kuznetsov AA. Omparative studies of the content of phenolic compounds, flavonoids and antioxidant activity of apples of different varieties. Chemistry of Plant Raw Material. 2018; 2: 115–22. DOI: 10.14258/jcprm.2018022205
28. Pascual J, Rahikainen M, Angeleri M, Alegre S, Gossens R, Shapiguzov A, Heinonen A, Trotta A, Durian G, Winter Z, Sinkkonen J, Kangasjärvi J, Whelan J, Kangasjärvi S. ACONITASE 3 is part of theANAC017 transcription factor-dependent mitochondrial dysfunction response. Plant Physiol. 2021 Aug 3;186(4):1859–77. DOI: 10.1093/plphys/kiab225
29. Ternette N, Yang M, Laroyia M, Kitagawa M, O'Flaherty L, Wolhulter K, Igarashi K, Saito K, Kato K, Fischer R, Berquand A, Kessler BM, Lappin T, Frizzell N, Soga T, Adam J, Pollard PJ. Inhibition of mitochondrial aconitase by succination in fumarate hydratase deficiency. Cell Rep. 2013 Mar 28;3(3):689–700. DOI: 10.1016/j.celrep.2013.02.013
30. Shepherd D, Garland PB. The kinetic properties of citrate synthase from rat liver mitochondria. Biochem J. 1969 Sep;114(3):597–610. DOI: 10.1042/bj1140597
31. Agati G, Brunetti C, Fini A, Gori A, Guidi L, Landi M, Sebastiani F, Tattini M. Are Flavonoids Effective Antioxidants in Plants? Twenty Years of Our Investigation. Antioxidants (Basel). 2020 Nov 9;9(11):1098. DOI: 10.3390/antiox9111098
32. Nabavi SM, Šamec D, Tomczyk M, Milella L, Russo D, Habtemariam S, Suntar I, Rastrelli L, Daglia M, Xiao J, Giampieri F, Battino M, Sobarzo-Sanchez E, Nabavi SF, Yousefi B, Jeandet P, Xu S, Shirooie S. Flavonoid biosynthetic pathways in plants: Versatile targets for metabolic engineering. Biotechnol Adv. 2020 Jan-Feb;38:107316. DOI: 10.1016/j.biotechadv.2018.11.005
33. De Bellis L, Luvisi A, Alpi A. Aconitase: To Be or not to Be Inside Plant Glyoxysomes, That Is the Question. Biology (Basel). 2020 Jul 12;9(7):162. DOI: 10.3390/biology9070162
34. Kumari A, Pathak PK, Bulle M, Igamberdiev AU, Gupta KJ. Alternative oxidase is an important player in the regulation of nitric oxide levels under normoxic and hypoxic conditions in plants. J Exp Bot. 2019 Aug 29;70(17): 4345–54. DOI: 10.1093/jxb/erz160
35. Zhao H, Chen G, Sang L, Deng Y, Gao L, Yu Y, Liu J. Mitochondrial citrate synthase plays important roles in anthocyanin synthesis in petunia. Plant Sci. 2021 Apr;305:110835. DOI: 10.1016/j.plantsci.2021.110835
36. Jardim-Messeder D, Caverzan A, Rauber R, de Souza Ferreira E, Margis-Pinheiro M, Galina A. Succinate dehydrogenase (mitochondrial complex II) is a source of reactive oxygen species in plants and regulates development and stress responses. New Phytol. 2015 Nov;208(3):776–89. DOI: 10.1111/nph.13515
37. Liu T, Hu X, Zhang J, Zhang J, Du Q, Li J. $H_2O_2$ mediates ALA-induced glutathione and ascorbate accumulation in the perception and resistance to oxidative stress in Solanum lycopersicum at low temperatures. BMC Plant Biol. 2018; 18: 34. DOI: 10.1186/s12870-018-1254-0

## AUTHORS

**Similla L. Adzhiakhmetova** – Candidate of Sciences (Pharmacy), Associate Professor of the Department of Organic Chemistry, Pyatigorsk Medical and Pharmaceutical Institute – branch of Volgograd State Medical University. ORCID ID: 0000-0001-6924-8563. E-mail: simillа503@mail.ru

**Dmitry I. Pozdnyakov** – Candidate of Sciences (Pharmacy), Associate Professor of the Department of Pharmacology with a Course of Clinical Pharmacology, Pyatigorsk Medical and Pharmaceutical Institute – branch of Volgograd State Medical University. ORCID ID: 0000-0002-5595-8182. E-mail: pozdniackow.dmitry@yandex.ru

**Nadezhda M. Chervonnaya** – Candidate of Sciences (Pharmacy), Senior Lecturer, Department of Organic Chemistry, Pyatigorsk Medical and Pharmaceutical Institute – branch of Volgograd State Medical University. ORCID ID: 0000-0001-6924-8563. E-mail: nadezhda.chervonnaya@yandex.ru

**Evgenia O. Kulichenko** – postgraduate student of the Department of Organic Chemistry, Senior Lecturer of the Department of Microbiology and Immunology with a Course of Biological Chemistry, Pyatigorsk Medical and Pharmaceutical Institute – branch of Volgograd State Medical University. ORCID ID: 0000-0002-0727-6689. E-mail: evgenia.kuli4encko@yandex.ru

**Eduard T. Oganesyan** – Doctor of Sciences (Pharmacy), Professor, Head of the Department of Organic Chemistry, Pyatigorsk Medical and Pharmaceutical Institute – branch of Volgograd State Medical University. ORCID ID: 0000-0002-2756-9382. E-mail: edwardov@mail.ru

*RESEARCH ARTICLE*

*ISSN 2307-9266 e-ISSN 2413-2241*

Scientific and Practical Journal
PHARMACY & PHARMACOLOGY



# SAFETY STUDY OF ROMIPLOSTIM BIOSIMILAR

**A.N. Afanasyeva[1], V.B. Saparova[1,2], D.D. Karal-ogly[3], E.I. Mukhametzyanova[3], D.V. Kurkin[4], A.V. Kalatanova[1], I.E. Makarenko[1,2], A.L. Khokhlov[5], I.A. Lugovik[1]**

[1] Closed Joint-Stock Company "Farm-Holding",
Bld. A, 34, Svyaz Str., Strelna Vil., St. Petersburg, Russia, 198515
[2] A.I. Yevdokimov Moscow State University of Medicine and Dentistry,
Bld. 1, 20, Delegatskaya Str., Moscow, Russia, 127473
[3] Scientific Research Institute of Medical Primatology,
Bld. 177, Mir Str., Vesyoloye Vil., Sochi, Russia, 354376
[4] Volgograd State Medical University,
1, Pavshikh Bortsov Sq., Volgograd, Russia, 400131
[5] Yaroslavl State Medical University,
5, Revolutsionnaya Str., Yaroslavl, Russia, 150000

E-mail: Alina.Afanaseva@geropharm.com

Received 04 July 2022 After peer review 20 Aug 2022 Accepted 26 Aug 2022

Idiopathic thrombocytopenic purpura is a chronic autoimmune hematological disease caused by an increased destruction of platelets and associated thrombocytopenia, for the treatment of which the imported drug romiplostim is used. Creation of the drug biosimilar provides a reduction in the cost of therapy and an access for the treatment to more patients.

**The aim** of the study was to compare the safety indicators of the reference drug and its biosimilar *in vivo* and in *vitro.*

**Materials and methods.** In the *in vitro* study, a model of "complement-dependent cytotoxicity" induced by the complement was formed on the 32D hTPOR clone 63-cell line, followed by a cell viability measurement with the CellTitter Glo® kit. *An in vivo* part of the study was carried out on Javanese macaque monkeys *(Macaca fascicularis).* During the experiment, the clinical condition, mortality, appetite of the animals, their body weight, body temperature, respiratory rate were assessed, the clinical parameters of blood and urine of the animals were also monitored, and the hemostasis indicators were additionally measured.

**Results.** In the *in vitro* experiment, the original drug romiplostim and its biosimilar GP40141 were compared in terms of EC50 values. The indicatirs did not show complement-dependent cytotoxicity. According to the *in vivo* results, no deviations were recorded in the clinical status of the animals and their feed intake, and no lethality was fixed out in the groups either. For all the parameters studied (body weight and temperature, respiratory rate, clinical urinalysis, clinical and biochemical blood tests, coagulation hemostasis), GP40141 and romiplostim, when administered at the doses equivalent to 10 toxic doses (TDs), had comparable effects.

**Conclusion.** In the comparison of safety performance both *in vitro* and *in vivo*, the original drug romiplostim and its biosimilar GP40141 showed similar results.

**Keywords:** romiplostim; biosimilar GP40141; Nplate®; complement-dependent cytotoxicity; drug safety studies; *in vivo; in vitro;* idiopathic thrombocytopenic purpura; toxicological profile; thrombopoietin receptor; TPO-R

**Abbreviations:** ITP – idiopathic thrombocytopenic purpura; TPO – tropmbopoetin; TPO-R – tropmbopoetin receptor; CDC – complement-dependent cytotoxicity; CS – complement system; CP – classical pathway; BA – biological activity; RS – reference standard; ICS – internal control sample; TO – test object; $EC_{50}$ – half maximal effective concentration; $IC_{50}$ – half-maximal inhibitory concentration; MABs – monoclonal antibodies; MP – medicinal preparation; RR – respiratory rate; Density – specific density; pH – pH value; TP – Total protein; GLU – glucose; BIL – bilirubin (in urine); UBG – urobilinogen; KET – ketone bodies; RBC – red blood cell count; MCV – mean cell volume; Hgb – hemoglobin; MCH – mean cell hemoglobin; MCHC – mean corpuscular hemoglobin concentration; HCT – hematocrit; PLT CNT – platelet count; PCT – plateletcrit; MPV – mean platelet volume; PDW – platelet distribution width; WBCs – white blood cells/leucocytes; MON – monocytes; LYM – lymphocytes; NEU – neutrophils; EOS – eosinophils; BA – basophils; APTT – activated partial thromboplastin time; PT – prothrombin time; AP – alkaline phosphatase; ALT – alanine transaminase; AspAT – aspartate aminotransferase; LDH – lactate dehydrogenase; TB – total bilirubin (blood); UREA – urea; CRE – creatinine; CHOL – cholesterine; TG – triglycerides; alb – albumine; GLB – globulin; ALB/GLB – albumin/globulin ratio; SM – statistical mean; SD – standard deviation; t-test – Student's t-test; Me – median; Q1 – quartile 1; Q3 – quartile 3; U-test – Mann-Whitney U-test.

**For citation:** A.N. Afanasyeva, V.B. Saparova, D.D. Karal-ogly, E.I. Mukhametzyanova, D.V. Kurkin, A.V. Kalatanova, I.E. Makarenko, A.L. Khokhlov, I.A. Lugovik. Safety study of romiplostim biosimilar. *Pharmacy & Pharmacology.* 2022;10(4):354-370. **DOI:** 10.19163/2307-9266-2022-10-4-354-370



**Для цитирования:** А.Н. Афанасьева, В.Б. Сапарова, Д.Д. Карал-оглы, Е.И. Мухаметзянова, Д.В. Куркин, А.В. Калатанова, И.Е. Макаренко, А.Л. Хохлов, И.А. Луговик. Исследование безопасности биоаналога ромиплостима. *Фармация и фармакология.* 2022;10(4):354-370. **DOI:** 10.19163/2307-9266-2022-10-4-354-370

Научно-практический журнал
ФАРМАЦИЯ И ФАРМАКОЛОГИЯ

*ОРИГИНАЛЬНАЯ СТАТЬЯ*

*DOI: 10.19163/2307-9266-2022-10-4-354-370*

# ИССЛЕДОВАНИЕ БЕЗОПАСНОСТИ БИОАНАЛОГА РОМИПЛОСТИМА

**А.Н. Афанасьева[1], В.Б. Сапарова[1,2], Д.Д. Карал-оглы[3], Е.И. Мухаметзянова[3], Д.В. Куркин[4], А.В. Калатанова[1], И.Е. Макаренко[1,2], А.Л. Хохлов[5], И.А. Луговик[1]**

[1] Закрытое акционерное общество «Фарм-Холдинг»,
198515, Россия, г. Санкт-Петербург, пос. Стрельна, ул. Связи, д. 34-А
[2] Федеральное государственное бюджетное образовательное учреждение высшего образования «Московский государственный медико-стоматологический университет имени А.И. Евдокимова» Министерства здравоохранения Российской Федерации,
127473, Россия, г. Москва, ул. Делегатская, д. 20/1
[3] Федеральное государственное бюджетное научное учреждение «Научно-исследовательский институт медицинской приматологии»,
354376, Россия, г. Сочи, с. Весёлое, ул. Мира, стр. 177
[4] Федеральное государственное бюджетное образовательное учреждение высшего образования «Волгоградский государственный медицинский университет» Министерства здравоохранения Российской Федерации,
400131, Россия, г. Волгоград, пл. Павших Борцов, д. 1
[5] Федеральное государственное бюджетное образовательное учреждение высшего образования «Ярославский государственный медицинский университет» Министерства здравоохранения Российской Федерации,
150000, Россия, Ярославская область, г. Ярославль, ул. Революционная, д. 5

E-mail: Alina.Afanaseva@geropharm.com

Получена 04.07.2022 После рецензирования 20.08.2022 Принята к печати 26.08.2022

Идиопатическая тромбоцитопеническая пурпура – это хроническое аутоиммунное гематологическое заболевание, обусловленное повышенным разрушением тромбоцитов и ассоциированной тромбоцитопенией, для лечения которого применяется импортный препарат ромиплостим. Создание биоаналога данного препарата может поспособствовать снижению стоимости терапии и доступности большему числу пациентов к лечению.

**Цель.** Сравнение показателей безопасности референтного препарата и его биоаналога в условиях *in vivo* и *in vitro*.

**Материалы и методы.** В *in vitro* исследовании сформирована модель «комплемент-зависимой цитотоксичности», индуцированного комплементом, на клеточной линии 32D hTPOR clone 63, с последующим измерением жизнеспособности клеток набором CellTitter Glo®. *In vivo* часть исследования была проведена на макаках яванских (*Macaca fascicularis*). В ходе эксперимента оценивали клиническое состояние, смертность, аппетит животных, массу тела, температуру тела, частоту дыхательных движений, также смотрели клинические показатели крови и мочи животных, дополнительно измерялись показатели гемостаза.

**Результаты.** В эксперименте *in vitro* оригинальный препарат ромиплостим и его биоаналог GP40141 сравнивали по значениям $EC_{50}$, которые не показали комплемент-зависимой цитотоксичности. По результатам *in vivo* не было зафиксировано отклонений в клиническом статусе животных и потреблении ими корма, летальности в группах не зафиксировано. По всем исследуемым показателям (масса и температура тела, частота дыхательных движений, клинический анализ мочи, клинический и биохимический анализы крови, коагуляционный гемостаз) GP40141 и ромиплостим, при введении их в дозах эквивалентных 10 ТД, оказывали сопоставимые эффекты.

**Заключение.** При сравнении показателей безопасности *in vitro* и *in vivo* оригинальный препарат ромиплостим и его биоаналог GP40141 показали аналогичные результаты.

**Ключевые слова:** ромиплостим; биоаналог GP40141; Энплейт®; комплемент-зависимая цитотоксичность; исследования безопасности препаратов; *in vivo*; *in vitro*; идиопатическая тромбоцитопеническая пурпура; токсикологический профиль; тромбопоэтиновый рецептор; TPO-R

**Список сокращений:** ИТП – идиопатическая тромбоцитопеническая пурпура; ТРО – тропмбопоэтин; TPO-R – тромбопоэтиновый рецептор; КЗЦ – комплемент-зависимая цитотоксичность; СК – система комплемента; КП – классический путь; БА – биологическая активность; СО – стандартный образец; ВКО – внутренний контрольный образец; ОИ – объект испытания; $EC_{50}$ – полумаксимальная эффективная концентрация; $IC_{50}$ – концентрация полумаксимального ингибирования; МАТ – моноклональные антитела; ЛП – лекарственный препарат; ЧДД – частота дыхательных движений; плотность – удельный вес; pH – водородный показатель; TP – общий белок; GLU – глюкоза; BIL – билирубин (в моче); UBG – уробилиноген; KET – кетоновые тела; RBC – количество эритроцитов; MCV – средний объем эритроцита; Hgb – гемоглобин; MCH – среднее содержание гемоглобина в эритроците; MCHC – средняя концентрация гемоглобина в эритроците; Hct – гематокрит; Plt – количество тромбоцитов; PCT– тромбокрит; MPV – средний объем тромбоцитов; PDW – относительная ширина распределения тромбоцитов по объему; WBC – лейкоциты; MON – моноциты; LYM – лимфоциты; NE – нейтрофилы; EO – эозинофилы; BA – базофилы;

АЧТВ – активированное частичное тромбопластиновое время; ПВ – протромбиновое время; ALP – щелочная фосфатаза; ALT – аланинаминотрансфераза; AST – аспартатаминотрансфераза; LDH – лактатдегидрогеназа; TB – общий билирубин (крови); UREA – мочевина; CRE – креатинин; CHOL – холестерин; TG – триглицериды; ALB – альбумин; GLB – глобулин; ALB/GLB – альбумин/глобулиновое соотношение; Mean – среднее статистическое; SD – стандартное отклонение; T-тест – t-критерий Стьюдента; Me – медианна; Q1 – 1 квартиль; Q3 – 3 квартиль; U-критерий – U-критерий Манна-Уитни.

## INTRODUCTION

Idiopathic thrombocytopenic purpura (ITP) is a severe disease characterized by thrombocytopenia (<100×$10^9$/l) and requires thrombopoiesis stimulants to maintain blood clotting [1–3]. Currently, only 2 drugs belonging to this group are registered in the Russian Federation (RF) – Nplate® (Nplate, AMGEN EUROPE, B.V.)[1] and Revolade® (Revolade, NOVARTIS PHARMA, AG)[2]. The active substance of Nplate®, romiplostim, has been repeatedly tested for effectiveness and has shown its therapeutic activity in the treatment of idiopathic thrombocytopenic purpura. This made it possible for patients to receive modern therapy that improves the quality of life [1, 4–6]. However, this drug is produced outside the Russian Federation, which increases its cost for the consumer, and when creating a biosimilar, newer technologies are used, which ultimately affects the cost of their production [7]. As thrombopoiesis stimulants are required on an ongoing basis in ITP [3, 8], their availability and affordability are vital for patients. The creation of a romiplostim biosimilar will increase the availability of treatment in the Russian Federation.

Romiplostim (Fig. 1) is a peptid antibody containing four TPO-R (thrombopoetin receptor, MPL) binding domains with a high affinity for TPO-R and one IgG1 Fc-carrier domain, which has no sequence homology with endogenous thrombopoietin (TPO) [9–11]. Romiplostim binds to and activates TPO-R on megakaryocyte precursors in the bone marrow. It binds in the same way as endogenous TPO and can displace TPO from binding to the receptor. Like TPO, romiplostim activates many of the same pathways resulting in a sustained increase in platelet counts [9, 12–15]. Much less is known about the effects mediated by the Fc area of the romiplostim molecule. For example, complement-dependent cytotoxicity (CDC) may be one of the possible options, since this mechanism is implemented in monoclonal antibodies by binding the Fc fragment to activate the complement system [16–18].

The complement system (CS) is known to be an integral part of both the innate and adaptive immune systems. This complex consists of a group of plasma proteins that interact in a cascade manner [19]. CS can be activated in three different ways, one of which is the classical pathway (CP) usually activated by antibodies. It mediates specific immune responses and functions as a part of the adaptive immunity. CP is triggered when the C1q complement molecule binds to the Fc antigen elements. Binding to the antibody causes conformational changes in the C1q molecule, leading to the activation of two C1r proteases and a further degradation of the two C1s molecules (another serine protease). The C1 complex now binds to C2 and C4 and cleaves them. The C4b product binds covalently to the cell surface and forms C4bC2 complexes. Activated C1s further cleave C4bC2 and generate C4bC2a, which is a C3/C5 CP convertase. Then, if the complement activation is not limited, it proceeds to the formation of a membrane attack complex (MAC) and the lysis of the target cell [19]. It has been generally accepted that monoclonal antibodies (MABs), can mediate the effects that cannot be fully elucidated by *in vitro* studies. These factors make bioequivalence studies on animals reasonable[3].

**THE AIM** of the study was to compare the safety indicators of the reference drug romiplostim and its biosimilar *in vivo* and in *vitro.*

## MATERIALS AND METHODS

### *In vitro* determination of complement-dependent cytotoxicity

Complement-dependent cytotoxicity (CDC) for romiplostim was evaluated in comparison with the indicative CDC method for rituximab. This drug is a chimeric MAB that specifically binds to the CD20 antigen on the surface of normal and malignant B-lymphocytes and initiates immunological reactions that mediate the B-cell lysis. One of the working mechanisms of the drug goes through the CDC [20]. In the CDC test for romiplostim, a mouse lymphoblast cell line *(Mus musculus)* with a stable expression of the human TPO

[1] Russian State Register of Medicinal Products. Nplate®. Available from: https://grls.rosminzdrav.ru/Grls_View_v2.aspx?routingGuid=e7a25c3e-1caa-44c0-81fe-5967882a071a.

[2] Russian State Register of Medicinal Products. Revolade® Available from: https://grls.rosminzdrav.ru/Grls_View_v2.aspx?routingGuid=e7a25c3e-1caa-44c0-81fe-5967882a071a.

[3] Decision of Council of the Eurasian Economic Commission of November 3, 2016 No. 89 "About approval of Rules of carrying out researches of biological medicines of the Eurasian Economic Union". Available from: http://www.consultant.ru/document/cons_doc_LAW_207925/.

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2022-10-4-354-370

receptor 32D hTPOR clone 63 (Selvita Group) was used as a test system. The effect of complement-dependent cytotoxicity was compared with the original drug romiplostim (AMGEN, the Netherlands) and its biosimilar GP40141 (OOO GEROPHARM, Russia). The complement concentration was selected on the basis of the toxicity absence of the complement itself; and a dilution by a factor of 14 was chosen.

On the first day of the experiment, the cells were seeded and the test objects (TOs) were introduced with the rabbit blood serum complement (Cedarlane, CL3441-S50). The concentrations used for the assessment of a specific biological activity (BA) on the 32D-hTPOR clone cell line, were the following: 63:10; 3.3; 1.1; 0.4; 0.1; 0.04; 0.005; 0.002; 0.001; 0.0002 ng/mL). These concentrations were added to the RPMI Basal assay medium, consisting of RPMI 1640 (Biolot, 1.3.4.), 1 mM HEPES (Biolot, 1.2.6.1.), 10 mM sodium pyruvate (Biolot, 1.4.004.), up to 1, 5 g/l sodium bicarbonate (Sigma-Aldrich, S5761), up to 4.5 g/l anhydrous glucose (PanReac AppliChem, 141341), penicillin-streptomycin (Biolot, 1.3.18.), to which a 10% fetal bovine serum FBS (Capricorn, FBS-11A) was added. After the test object (TO) preparation, the rabbit serum complement was diluded by a factor of 14 and the line was seeded into the wells of a 96-well white plate (Corning, 3917) at the concentration of 5000 cells/well, the cell counts were performed using a Countess II FL cell counter (ThermoFisher Scientific, USA). The test objects with complements and without complements were added to the cells in different wells. They were incubated with 5% carbon dioxide for 24±4 hours at 37°C. Then the plate was cooled down to room temperature for 1 h, and the CellTitter-Glo reagent (Promega, G7571) was added to each well with the culture liquid in the equivalent volume ratio. The cells were lysed mechanically on an orbital shaker for also 2 min at room temperature. Then the plates were incubated for 10 min to stabilize the luminescent signal, after which the luminescence was recorded on a CLARIOstar multimodal microplate reader (BMG Labtech, Germany). The settings on the instrument included Presets Ultra Glo, the integration time was 0.4 sec per 1 well.

In the CDC test for rituximab, the human B-lymphoblast cell line WIL2-S was used as a test system. A rituximab reference standard (RS) (Mabxience, Spain) and a rituximab internal control sample (ICR) were used as TOs. On the first day of the experiment, similarly to the CSC test for romiplostim, the cells were seeded and the TO with the complement of the rabbit blood serum was added. For rituximab CSC, the complement concentration was selected at the ratio of 1:7, where there was no toxicity of the complement itself. For RI, concentrations of 5000, 2000, 800, 320, 128, 51, 20, 8, 3, 1.3, and 0.5 ng/ml were selected; their dilution was carried out in a medium similar to the rituximab CDC for the analysis. After preparing the TO, the rabbit blood serum complement was prepared and the line was seeded into the wells of a 96-well black plate (Corning, 3603, USA) at the concentration of 10,000 cells/well. The plate was incubated at 37°C and 5% carbon dioxide for 2 hours, and then the alamarBlue™ Cell Viability Reagent (Thermo Fisher Scientific, DAL1100, USA) was added and incubated again for up to 24 hours. On the expiry of time, fluorescence was recorded on a CLARIOstar multimodal microplate reader. The data analysis for the two tests was carried out in the integrated MARS Data Analysis software, and the statistical analysis – in GraphPad Prism 9.3.1.471, where the data were normalized as a percentage relative to the zero control, then a four-parameter dose-response curve was built up to calculate the $EC_{50}$ value (a half maximum effective concentration).

### *In vivo* safety study

The study was carried out on the basis of Scientific Research Institute of Medical Primatology (Russia, Sochi) in the strict accordance with Rus-LASA standards[4,5] [20]. The study was approved by the independent local ethics committee (Protocol statement No. 61 dated February 9, 2021).

Clinically healthy Javanese macaque monkeys *(Macaca fascicularis)* were used as a test system. *Macaca fascicularis* are phylogenetically quite close to humans and, at the same time, in the framework of preclinical safety studies of the original drug, they did not show any formation of neutralizing antibodies to romiplostim, unlike rats, mice and rhesus monkeys in the studies of a similar duration. That makes it possible to predict the risks associated with primary pharmacodynamics in humans, to the greatest extent[6]. All these factors made it possible to consider these animals as the most relevant ones for a comparative safety study.

In accordance with the decision of the Economic

[4] GOST 33218-2014 «Guide for the Care and Management of Laboratory Animals. Rules of Care and Management of Nonhuman Primates», 2014.

[5] GOST standard 33215-2014 «Guide for the Care and Use of Laboratory Animals. Rules of Fitting of Facilities and of Organization of Procedures», 2014.

[6] CHMP assessment report for Nplate®. Procedure No. EMEA/H/C/942. EMEA/654269/2008, 2022. Available from: https://www.ema.europa.eu/en/documents/assessment-report/nplate-epar-public-assessment-report_en.pdf.

Commission for Europe Council 89 (ECE Council 89), when forming experimental groups, it is necessary to adhere to a flexible approach. It is permissible to use animals of only one sex and only one dose of drugs, which makes it possible to fully follow the Reduce principle (the reduction in the number of the animals used) from the fundamental principles of humanity 3R[7]. However, the use of such methods to reduce the number of the animals requires justification. Animals of the same sex were used because the toxicological profile of the original drug romiplostim did not differ between males and females in numerous toxicological experiments, indicating the absence of specific toxic effects associated with sex[8]. It follows that the introduction of animals of two sexes will not lead to additional information about the safety of the substance and is contrary to the bioethical Reduce principle. The next step to reduce the number of animals was the abolition of the control group. According to the Decision of EU Directive 89, it is permissible to conduct a study with a modified design (using only one dose of a biosimilar medicinal product (MP) and the original (reference) MP. In order to compare the dynamics of animal parameters, all main laboratory and functional parameters had been taken before dosing, so that the initial data of the animals acted as some control for subsequent measurement points.

In this regard, 12 mature males weighing 2.65–3.95 kg were selected for the experiment. The animals were divided into groups according to the level of platelets, which had been measured 7 days before the administration of the drugs, after which, to eliminate the preferences of the researcher, the experimental groups were composed by randomization of the animals from the formed groups. The animals were divided into 2 groups, 6 males in each. Group 1 received the biosimilar GP40141, group 2 received the original drug romiplostim (Table 1).

When selecting doses in safety studies, a critical point is the choice of dosage and frequency of administration of the test object and reference standard. On the one hand, they should allow the potential adverse effects to be fully assessed and thereby protect participants in clinical trials. On the other hand, they should not be redundant allowing unnecessary suffering of animals in the framework of the research[9,10,11]. In this regard, as a reasonable compromise, the dosage of 100 mcg/kg was chosen, as it allows a significantly (10 times) higher therapeutic dose for clinical use[12], and had been applied in earlier safety studies of the original drug romiplostim[13]. This dose has been directly extrapolated from the clinical animal practice and no dose conversion factor has been applied.

The drugs were administered repeatedly, over 28 days, with a frequency of once/72 hours, since this frequency of administration repeats the design of the study of the original drug romiplostim and allows achieving a stable systemic exposure in animal plasma[14]. The contents of the vial with test objects and the reference standard were a powder diluted in 0.72 ml of sterile water for injections to obtain a concentration of 250 μg/0.5 ml (500 μg/ml) immediately after the preparation. The drugs were administered to a specific animal at the same place throughout the study (28 days).

The animals were kept and fed in accordance with Directive 2010/63/EU of the European Parliament and the European Council dated 22 September 2010 on the protection of animals used for scientific purposes. Throughout the experiment, the animals were kept in individual cages, which indicated the animal's inventory number, group, and gender. The cells were equipped with a central water supply. The ambient temperature was 21–28°C, the relative humidity was 40–70%, daylight hours were natural (Sochi). The diet of the animals consisted of granulated feed, eggs, dried and fresh fruits and bread. Feeding was carried out in three stages: 8–9 o'clock – granulated feed mixture, 11–12 o'clock – juicy feeds, rice porridge and biscuits, 14–15 o'clock – granulated feed mixture. The water was provided *ad libitum* and met the requirements of SanPiN 2.1.4.1074-01, GN 2.1.5.1315-03, GN 2.1.5.2280-07[15,16,17].

[7] Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Available from: https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ%3AL%3A2010%3A276%3A0033%3A0079%3Aen%3APDF/

[8] Decision of Council of the Eurasian Economic Commission of November 3, 2016 No. 89 "About approval of Rules of carrying out researches of biological medicines of the Eurasian Economic Union".

[9] Ibid.

[10] Product monograph including patient medication information Nplate® romiplostim for injection. Available from: https://www.amgen.ca/-/media/Themes/CorporateAffairs/amgen-ca/amgen-ca/documents/products/en/nplate_pm.pdf.

[11] CHMP assessment report for Nplate®. Procedure No. EMEA/H/C/942. EMEA/654269/2008, 2022.

[12] Decision of Council of the Eurasian Economic Commission of November 3, 2016 No. 89 "About approval of Rules of carrying out researches of biological medicines of the Eurasian Economic Union".

[13] Ibid.

[14] Ibid.

[15] SanPin 2.1.4.1074-01. Drinking water Hygienic requirements for water quality of centralized drinking water supply systems. Quality control.

[16] GN 2.1.5.1315-03 Maximum allowable concentrations (MACs) of chemicals in the water of water objects used for drinking and domestic recreation purposes. Available from: https://files.stroyinf.ru/Data2/1/4294815/4294815336.pdf

[17] GN 2.1.5.2280-07 "Maximum permissible concentration (MPC) of chemical substances in water of water bodies of household, drinking and cultural and household water use. Available from: http://pravo.gov.ru.

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2022-10-4-354-370

Mortality, appearance (skin, hair, eyes, nose, respiration, stool, mucous membranes, posture, behavior and coordination) and feed intake were assessed daily during the study. Weight, body temperature, respiratory rate (RR) were assessed on days 0, 15 and 29 along with urine and blood sampling of the animals.

Urine samples were taken from the cell tray into test tubes. The following indicators were analyzed: color, transparency, specific gravity (density), pH, protein (UP), glucose (GLU), bilirubin (BIL), ketone bodies (KET), erythrocytes (ERY), leukocytes (LEY), urobilinogen (UBG). The analysis was carried out on a DocUReader 2 PRO analyzer using test strips from the same company.

Blood sampling for clinical analyses was carried out in Microvette Sarstedt capillary tubes, 200 µl, with K3-EDTA. The following indicators were determined in the blood samples of the experimental animals: hemoglobin (Hgb), erythrocyte count (RBC), leukocyte count (WBC), leukocyte formula (LYM), monocytes (MON), neutrophils (NE), eosinophils (EO), basophils (BA), platelet count (Plt), hematocrit (Hct), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), thrombocrit (PCT), mean platelet volume (MPV), relative platelet distribution width (PDW). The analysis was carried out on an automatic hematological analyzer MEK 7300K (EKO-MED-S M LLC, Russia). The reagents for the hematological analyzer from Nihon Kohden (Firenze S.r.l., South Italy) were used for the analyses. The number of cells was counted by the volume resistance method; the hemoglobin concentration was determined by a spectrophotometric method; hematocrit, thrombocrit, platelet and erythrocyte distribution widths were calculated by histograms.

To determine biochemical parameters, 18 hours before blood sampling, the animals were deprived of food, while maintaining a free access to the water. Blood sampling was carried out in test tubes to obtain serum with a clot activator and gel VACUETTE (5 or 8 ml) from the left inguinal vein. The serum was obtained from the blood by the usual method: incubation at room temperature for 30 min, centrifugation at 3500 rpm. The following parameters were evaluated: alanine aminotransferase (ALT), aspartate aminotransferase (AST), de Ritis ratio, total protein (TP), albumin (ALB), globulin (GLB), albumin/globulin ratio (ALB/GLB), alkaline phosphatase (ALP), total bilirubin (TB), total cholesterol (CHOL), creatinine (CRE), glucose (GLU), lactate dehydrogenase (LDH), triglycerides (TGs), sodium (Na+), potassium (K+), urea (UREA). Biochemical blood analysis was carried out on a BioLit-8020 biochemical analyzer (URIT Medical Electronic Group Co., Ltd, China). The reagents produced by DAC, Moldova were used for the analysis. The measurement method used was colorimetry (kinetics, the end point).

For the coagulometric analysis, the blood was taken into S-Monovette® vacuum tubes (Germany) with a piston of 1.4 ml. To study the effect on the blood coagulation system, activated partial thromboplastin time (APTT), prothrombin time (PT), and fibrinogen were analyzed. The analysis for the coagulation hemostasis was carried out on a coagulometer TS 4000 Plus (Russia). The PV-clotting method is based on determining the clotting time of citrated blood plasma under the action of a mixture of thromboplastin and calcium ions. The method for determining APTT is the following: a reagent, which is an aqueous solution of ellagic acid in combination with soy phospholipids, and calcium chloride are sequentially added to the studied blood plasma. In the process of measuring the APTT, the time from the moment of adding calcium ions to the moment of clot formation is recorded. Fibrinogen was determined by the Clauss method.

### Statistical processing of analysis results

The *in vivo* statistical data processing part was carried out using software packages R version 3.5.2. For all the data, descriptive statistics was applied: the data were checked for compliance with the normal distribution law using the Shapiro-Wilk`s W test. In case of a normal distribution, the mean value and standard deviation were calculated, which, together with the value of n (number of observations), are presented in the final tables. If the data did not comply with the normal distribution law, the median and quartile range were calculated. When identifying intergroup differences in the data with signs of the normal distribution, the Student's t-test was used, and in the data with signs of abnormal distribution, it was the Mann-Whitney U-test.

In the applicable cases, in intergroup and intragroup comparisons, the indicators obtained for the animals at different times of the experiment, were compared. To assess the intragroup dynamics in the data with signs of normal distribution, the Student's t-test was used, and in the data with signs of abnormal distribution, it was Friedman's test. Fisher's exact test was used to analyze the manifestation frequencies of some parameters (the level of erythrocytes in the urine).

The comparison results were considered statistically significant at $p \leq 0.05$. The data graphical presentation was carried out in GraphPad Prism 9.3.1.471.

**Table 1 – Scheme of experimental animals distribution by groups**

| Group No. | Animals | | Test object/ reference standard | Route of administration | Dose, µg/kg | Duration of administration |
|---|---|---|---|---|---|---|
| | Gender | Number in the group | | | | |
| 1 | ♂ | 6 | GP40141 | Subcutaneously | 100 | 28 days, once/72 hours |
| 2 | ♂ | 6 | Romiplostim | | 100 | |

Note: ♂ – male.

**Table 2 – Indicators of CDC relative activity for romiplostim**

| Sample | $EC_{50}$, ng/ml | $R^2$ | Relative activity, % |
|---|---|---|---|
| GP40141 (ng/ml) + Complement (1:14) | 0.128 | 0.995 | 72% |
| GP40141 (ng/ml) | 0.093 | 0.989 | n/a |
| Romiplostim (ng/ml) + Complement (1:14) | 0.084 | 0.994 | 87% |
| Romiplostim (ng/ml) | 0.073 | 0.992 | n/a |
| Sample | $IC_{50}$, ng/ml | $R^2$ | Relative activity, % |
| Rituximab RM-RX-03 + complement (1:7) | 125.6 | 0.951 | n/a |
| Rituximab RM-RX-02 + complement (1:7) | 121.7 | 0.948 | n/a |

Note: n/a – not applicable, $R^2$ – coefficient of determination, $EC_{50}$ – half-maximal effective concentration, $IC_{50}$ – half-maximal inhibition concentration. $EC_{50}$ and $IC_{50}$ were calculated using 11 concentration points in 3 replicates.

**Table 3 – Dynamics of changes in experimental animals' average weight (g) by groups, n=6**

| Group | Parameter | Measurement day | | |
|---|---|---|---|---|
| | | 0 | 15 | 29 |
| GP40141 (Group 1) | Mean±SD | 3 483±397.1 | 3 525±248,5 | 3 600±249.0 |
| | T-test, p Background/Day | n/a | 0.83 | 0.56 |
| Romiplostim (Group 2) | Mean±SD | 3 150±405.0 | 3 116.7±469.8 | 3 158±453.2 |
| | T-test, p Background/Day | n/a | 0.90 | 0.97 |
| | T-test, p, Group 1/Group 2 | 0.07 | 0.06 | 0.05 |

Note: Mean – statistical mean, SD – standard deviation, T-test – Student's t-test.

**Table 4 – Dynamics changes in the average body temperature by groups of experimental animals, n=6**

| Group | Parameters | Measurement day | | |
|---|---|---|---|---|
| | | 0 | 15 | 29 |
| GP40141 (Group 1) | Mean±SD | 38.68±0.24 | 38.80±0.18 | 38.63±0.26 |
| | T-test, p Background/Day | n/a | 0.36 | 0.74 |
| Romiplostim (Group 2) | Mean±SD | 38.68±0.35 | 38.60±0.27 | 38.50±0.36 |
| | T-test, p Background/Day | n/a | 0.79 | 0.39 |
| | T-test, p, Group 1/Group 2 | 1.0 | 0.24 | 0.48 |

Note: Mean – statistical mean, SD – standard deviation, T-test – Student's t-test.

**Table 5 – Dynamics of changes in average RR/min of experimental animals by groups, n=6**

| Group | Parametrs | Measurement day | | |
|---|---|---|---|---|
| | | 0 | 15 | 29 |
| GP40141 (Group 1) | Mean±SD | 39.00±4.10 | 34.83±4.83 | 32.17±2.48 |
| | T-test, p Background/Day | n/a | 0.14 | 0.006 |
| Romiplostim (Group 2) | Mean±SD | 34.17±2.71 | 33.50±3.02 | 32.83±2.14 |
| | T-test, p Background/Day | n/a | 0.70 | 0.37 |
| | T-test, p, Group 1/Group 2 | 0.04 | 0.58 | 0.63 |

Note: Mean – statistical mean, SD – standard deviation, T-test – Student's t-test.

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2022-10-4-354-370

**Table 6 – Indicators dynamics of clinical urine analysis, n=6**

| Group | Date | Parameter | Indicators | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | eDensity, g/ml | pH | UP, mg/dl | GGLU, ml/dl | LEY, cells/µl | BIL, ml/dl | UBG, mg/dl | KET, mg/dl |
| GP40141 (Group 1) | Background | Me ($Q_1$; $Q_3$) | 1 (1;1) | 8.0 (7.0; 8.6) | 0 (0;0) | 0 (0;0) | 0 (0;0) | 0 (0;0) | 0 (0;0) | 0 (0;0) |
| | 15 days | Me ($Q_1$; $Q_3$) | 1 (1;1) | 9.0 (8.9; 9.0) | 0 (0;0) | 0 (0;0) | 0 (0;0) | 0 (0;0) | 0 (0;0) | 0 (0;0) |
| | 29 days | Me ($Q_1$; $Q_3$) | 1 (1;1) | 8,25 (7.5; 8.5) | 0 (0;0) | 0 (0;0) | 0 (0;0) | 0 (0;0) | 0 (0;0) | 0 (0;0) |
| | | Friedman test, p | >0.05 | >0.05 | >0.05 | >0.05 | >0,05 | >0.05 | >0.05 | >0.05 |
| Romiplostim (Group 2) | Background | Me ($Q_1$; $Q_3$) | 1 (1;1) | 8.0 (7.4; 9.0) | 0 (0;0) | 0 (0;0) | 0 (0;0) | 0 (0;0) | 0 (0;0) | 0 (0;0) |
| | 15 days | Me ($Q_1$; $Q_3$) | 1 (1;1) | 8.5 (8.4; 9.0) | 0 (0;0) | 0 (0;0) | 0 (0;0) | 0 (0;0) | 0 (0;0) | 0 (0;0) |
| | | U-criterion, p Group 1/ Group 2 | >0.05 | >0.05 | >0.05 | >0.05 | >0,05 | >0.05 | >0.05 | >0.05 |
| | 29 days | Me ($Q_1$; $Q_3$) | 1 (1;1) | 8.25 (6.9; 8.6) | 0 (0;0) | 0 (0;0) | 0 (0;0) | 0 (0;0) | 0 (0;0) | 0 (0;0) |
| | | Friedman test, p | >0.05 | >0.05 | >0.05 | >0.05 | >0.05 | >0.05 | >0.05 | >0.05 |
| | | U-criterion, p Group 1/ Group 2 | >0.05 | >0.05 | >0.05 | >0.05 | >0.05 | >0.05 | >0.05 | >0.05 |

Note: Me – median; Q1 – 1 quartile; Q3 test – 3 quartile; U-test – Mann-Whitney U-test; TP – total protein; GLU – glucose; LEY – leukocytes; BIL – bilirubin; UBG – urobilinogen; KET – ketone bodies.

**Table 7 – Frequency of erythrocytes manifestation in urine (5–10 cells/µl), according to results of clinical urine analysis, n=6**

| Measurement day | GP40141 (Group 1) | Romiplostim (Group 2) | Fisher's exact test (intragroup analysis), p |
|---|---|---|---|
| Background | 1/6 | 1/6 | >0.05 |
| Day 15 | 1 | 1/6 | >0.05 |
| Day 29 | 1 | 1 | >0.05 |
| Fisher's exact test (intragroup analysis), background/day 15, p | >0.05 | >0.05 | – |
| Fisher's exact test (intragroup analysis), background/day 29, p | >0.05 | >0.05 | – |

**Table 8 – Intralaboratory norms for indicators of clinical urine analysis**

| Indicator, unit of measurement | Value range |
|---|---|
| Specific gravity (density), g/ml | 1.0–1.030 |
| Hydrogen index (pH) | 5–9 |
| Total protein (TP), mg/dl | 0–15 |
| Glucose (GLU), ml/dl | 0–40 |
| Erythrocytes (ERY), cells/µl | 0–10 |
| Leukocytes (LEY), cells/µl | 0–20 |
| Bilirubin (BIL), ml/dl | 0–0.5 |
| Urobilinogen (UBG), mg/dl | 0–1.8 |
| Ketone bodies (KET), mg/dl | 0–50 |

**Table 9 – Hematological parameters of experimental animals groups, n=6**

| Group | Date | Parameter | Parameter | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | RBC, $10^{12}$/l | MCV, fl | Hgb, g/l | MCH, pg | MCHC, g/l | Hct, % | Plt×$10^9$/l | PCT, % | MPV, fl | PDW, % |
| GP40141 (Group 1) | Back-ground | Mean±SD | 6.6±0.56 | 63.7±4.55 | 126±4.4 | 19.3±1.70 | 303±6.9 | 41.5±1.24 | 309±68.0 | 0.2±0.04 | 8.0±0.75 | 16.5±0.36 |
| | 15 days | Mean±SD | 6.1±0.62 | 63.8±4.86 | 115±2.1 | 18.9±1.73 | 297±7.7 | 38.7±1.57 | 757±183.2 | 0.5±0.09 | 7.0±0.66 | 17.8±0.74 |
| | | T-test, p Back-ground/ 15 days | 0.24 | 0.97 | 0.0003 | 0.71 | 0.21 | 0.006 | 0.0002 | <0.0001 | 0.03 | 0.003 |
| | 29 days | Mean±SD | 5.8±0.28 | 62.7±4.83 | 109±7.0 | 19.0±1.70 | 303±7.1 | 36.2±2.06 | 717±153.6 | 0.5±0.07 | 6.4±0.66 | 17.3±0.72 |
| | | T-test, p Back-ground/ 29 days | 0.01 | 0.74 | 0.0007 | 0.73 | 0.84 | 0.0003 | 0.0001 | 0.0001 | 0.002 | 0.04 |
| | | T-test, p 15/29 days | 0.25 | 0.77 | 0.10 | 0.97 | 0.29 | 0.04 | 0.68 | 0.16 | 0.13 | 0.25 |
| Romiplostim (Group 2) | Back-ground | Mean±SD | 6.6±0.53 | 63.2±2.45 | 123±4.4 | 18.8±1.02 | 298±6.7 | 41.4±2.24 | 308±99.5 | 0.2±0.06 | 7.7±0.75 | 16.9±0.93 |
| | 15 days | Mean±SD | 6.4±0.57 | 63.0±2.93 | 120±7.6 | 18.7±1.22 | 297±8.5 | 40.4±2.91 | 818±249.5 | 0.6±0.16 | 7.0±0.39 | 17.8±0.85 |
| | | T-test, p Background/ 15 days | 0.65 | 0.93 | 0.35 | 0.90 | 0.82 | 0.51 | 0.0009 | 0.0006 | 0.09 | 0.11 |
| | | T-test, p, Group 1/ Group 2 | 0.41 | 0.82 | 0.18 | 0.79 | 0.86 | 0.24 | 0.64 | 0.54 | 0.96 | 0.94 |
| | 29 days | Mean±SD | 5.6±0.48 | 62.3±2.71 | 105±5.3 | 18.7±1.24 | 300±10.5 | 35.0±2.62 | 661±194.3 | 0.5±0.12 | 7.0±0.42 | 17.5±0.98 |
| | | T-test, p Background/ 29 days | 0.009 | 0.56 | <0.0001 | 0.92 | 0.66 | 0.001 | 0.003 | 0.002 | 0.08 | 0.34 |
| | | T-test, p 15/29 days | 0.03 | 0.66 | 0.003 | 0.98 | 0.56 | 0.007 | 0.25 | 0.19 | 0.89 | 0.52 |
| | | T-test, p, Group 1/ Group 2 | 0.50 | 0.84 | 0.22 | 0.78 | 0.66 | 0.39 | 0.59 | 0.91 | 0.08 | 0.69 |

Note: Mean – statistical mean; SD – standard deviation; T-test – Student's t-test; RBC – erythrocyte count; MCV – mean cell volume; Hgb – hemoglobin; MCH – mean cell hemoglobin; MCHC – mean cell hemoglobin concentration; Hct – hematocrit; Plt – platelet count; PCT – thrombocrit; MPV – mean platelet volume; PDW – relative platelet distribution width.

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2022-10-4-354-370

**Table 10 – Experimental animals' leukocyte formula by groups, n=6**

| Group | Date | Parameter | WBC, $\times 10^9$/l | MON, % | LYM, % | NE, % | EO, % | BA, % |
|---|---|---|---|---|---|---|---|---|
| GP40141 )Group 1) | Back ground | Mean±SD | 9.5±2.91 | 4.2±1.13 | 34.4±6.42 | 59.0±6.89 | 1.9±1.38 | 0.6±0.41 |
| | 15 days | Mean±SD | 11.0±2.53 | 4.4±0.83 | 33.7±6.00 | 59.0±6.66 | 2.4±1.41 | 0.6±0.27 |
| | | T-test, p Background/15 days | 0.34 | 0.82 | 0.85 | 0.98 | 0.59 | 1.00 |
| | 29 days | Mean±SD | 11.8±2.65 | 4.7±1.84 | 30.1±5.87 | 63.0±4.33 | 1.4±0.67 | 0.8±0.32 |
| | | T-test, p Background/29 days | 0.17 | 0.57 | 0.25 | 0.25 | 0.47 | 0.41 |
| | | T-test, p 15/29 days | 0.60 | 0.65 | 0.32 | 0.25 | 0.18 | 0.31 |
| Romiplostim (Group 2) | Back-ground | Mean±SD | 9.2±1.73 | 4.2±1.06 | 35.4±8.41 | 58.0±7.60 | 1.6±1.28 | 0.8±0.27 |
| | 15 days | Mean±SD | 11.3±2.25 | 3.9±0.99 | 32.1±10.08 | 61.9±11.38 | 1.5±0.96 | 0.6±0.37 |
| | | T-test, p Background /15 days | 0.10 | 0.62 | 0.55 | 0.50 | 0.88 | 0.39 |
| | | T-test, p, Group 1/Group 2 | 0.87 | 0.43 | 0.75 | 0.61 | 0.26 | 0.93 |
| | 29 days | Mean±SD | 14.2±5.53 | 5.2±1.47 | 24.5±14.08 | 67.4±15.58 | 2.0±2.87 | 0.9±0.29 |
| | | T-test, p Background /29 days | 0.06 | 0.21 | 0.13 | 0.21 | 0.75 | 0.43 |
| | | T-test, p 15/29 days | 0.26 | 0.11 | 0.31 | 0.50 | 0.68 | 0.15 |
| | | T-test, p, Group 1/Group 2 | 0.37 | 0.64 | 0.39 | 0.52 | 0.63 | 0.58 |

Note: Mean – statistical mean; SD – standard deviation; T-test – Student's t-test; WBC – leukocytes; MON – monocytes; LYM – lymphocytes; NE – neutrophils; EO – eosinophils; BA – basophils.

**Table 11 – Biochemical parameters of animal blood (part 1), n=6**

| Group | Date | Parameter | Indicators | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | GLU Mmol/l | ALP U/l | ALT U/l | AST U/l | dRR | LDH U/l | TB Mmol/l | UREA Mmol/l |
| GP40141 (Group 1) | Background | Mean±SD | 4.0±0.96 | 1 064±322.3 | 26.7±5.99 | 37.0±8.85 | 0.8±0.29 | 579±197.3 | 4.7±1.30 | 6.1±0.37 |
| | 15 days | Mean±SD | 4.3±0.55 | 1 238±141.3 | 31.2±4.49 | 45.7±12.31 | 0.7±0.16 | 651±165.2 | 7.5±2.99 | 6.2±0.37 |
| | | T-test, p Background/15 days | 0.54 | 0.053 | 0.17 | 0.19 | 0.90 | 0.51 | 0.06 | 0.60 |
| | 29 days | Mean±SD | 4.5±1.64 | 1 142±365.3 | 28.5±5.82 | 41.2±16.74 | 0.8±0.19 | 543±314.0 | 4.6±1.93 | 6.2±0.57 |
| | | T-test, p Background/29 days | 0.48 | 0.71 | 0.60 | 0.60 | 1.00 | 0.82 | 0.96 | 0.68 |
| | | T-test, p, 15/29 days | 0.70 | 0.15 | 0.40 | 0.61 | 0.87 | 0.47 | 0.07 | 1.00 |
| Romiplostim (Group 2) | Background | Mean±SD | 5.7±1.29 | 1 294±245.9 | 29.2±9.93 | 39.0±17.33 | 0.8±0.15 | 693±96.4 | 4.9±1.11 | 8.8±1.13 |
| | 15 days | Mean±SD | 4.0±1.39 | 1 363±133.1 | 31.3±7.94 | 36.0±10.26 | 0.9±0.12 | 675±83.7 | 6.9±1.31 | 8.8±0.97 |
| | | T-test, p Background/15 days | 0.06 | 0.56 | 0.69 | 0.72 | 0.31 | 0.73 | 0.02 | 0.98 |
| | | T-test, p, Group 1/ Group 2 | 0.67 | 0.75 | 0.97 | 0.17 | 0.14 | 0.76 | 0.64 | 0.0001 |
| | 29 days | Mean±SD | 4.7±1.24 | 1 322±159.0 | 35.5±12.60 | 37.5±15.14 | 1.0±0.23 | 699±141.5 | 5.1±1.35 | 9.0±1.16 |
| | | T-test, p Background/ 15 days | 0.22 | 0.82 | 0.36 | 0.88 | 0.08 | 0.93 | 0.73 | 0.77 |
| | | T-test, p 15/29 days | 0.37 | 0.64 | 0.51 | 0.84 | 0.23 | 0.72 | 0.04 | 0.77 |
| | | T-test, p, Group 1/ Group 2 | 0.85 | 0.29 | 0.24 | 0.70 | 0.06 | 0.29 | 0.61 | 0.0003 |

Note: Mean – statistical mean; SD – standard deviation; T-test – Student's t-test; GLU – glucose; ALP – alkaline phosphatase; ALT – alanine aminotransferase; AST – aspartate aminotransferase; LDH – lactate dehydrogenase; TP – total protein; UREA – urea; dRR - de Ritis ratio.

**Table 12 – Biochemical parameters of animal blood (part 2), n=6**

| Group | Date | Parameter | Indicators | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | CRE Mmol/l | CHOL Mmol/l | TG Mmol/l | TP g/l | ALB g/l | GLB g/l | ALB/ GLB | $K^+$ Mmol/l | $Na^+$ Mmol/l |
| GP40141 (Group 1) | Back-ground | Mean±SD | 144±25.2 | 3.5±1.18 | 0.4±0.09 | 71.7±15.00 | 41.7±9.14 | 30.0±7.4 | 1.4±0.29 | 4.2±0.90 | 118±2.4 |
| | 15 days | Mean±SD | 164±18.9 | 3.9±0.81 | 0.5±0.23 | 79.2±11.58 | 49.2±3.87 | 30.0±12.8 | 2.1±1.41 | 5.2±0.86 | 115±2.7 |
| | | T-test, p Background/ 15 days | 0.16 | 0.47 | 0.18 | 0.36 | 0.09 | 1.00 | 0.26 | 0.07 | 0.07 |
| | 29 days | Mean±SD | 138±28.6 | 5.1±0.71 | 0.5±0.26 | 70.8±10.63 | 40.3±6.80 | 30.5±3.94 | 1.32±0.09 | 4.23±1.04 | 116±10.3 |
| | | T-test, p Background/ 29 days | 0.71 | 0.34 | 0.25 | 0.91 | 0.78 | 0.89 | 0.41 | 0.89 | 0.63 |
| | | T-тест, p 15/29 days | 0.10 | 0.35 | 0.95 | 0.22 | 0.02 | 0.93 | 0.19 | 0.12 | 0.85 |
| Romiplostim (Group 2) | Back-ground | Mean±SD | 143±9.5 | 4.1±0.84 | 0.3±0.18 | 83.0±4.29 | 45.2±1.72 | 37.8±3.92 | 1.2±0.13 | 4.7±0.50 | 144±9.6 |
| | 15 days | Mean±SD | 148±16.6 | 4.6±1.02 | 0.5±0.14 | 84.5±5.28 | 47.3±3.67 | 37.2±5.42 | 1.3±0.29 | 5.2±0.71 | 150±5.1 |
| | | T-test, p Background/ 15 days | 0.54 | 0.42 | 0.11 | 0.60 | 0.22 | 0.81 | 0.45 | 0.15 | 0.22 |
| | | T-test, p, Group 1/ Group 2 | 0.14 | 0.23 | 0.92 | 0.33 | 0.42 | 0.23 | 0.19 | 0.91 | <0.0001 |
| | 29 days | Mean±SD | 140±9.3 | 3.9±0.38 | 0.4±0.32 | 84.5±5.54 | 46.5±3.83 | 38.0±3.95 | 1.2±0.17 | 4.9±0.55 | 148±4.1 |
| | | T-test, p Background/ 29 days | 0.59 | 0.61 | 0.70 | 0.61 | 0.46 | 0.94 | 0.73 | 0.40 | 0.39 |
| | | T-test, p 15/29 days | 0.33 | 0.17 | 0.49 | 1.00 | 0.71 | 0.77 | 0.62 | 0.46 | 0.47 |
| | | T-test, p, Group 1/ Group 2 | 0.93 | 0.35 | 0.55 | 0.02 | 0.08 | 0.01 | 0.31 | 0.17 | <0.0001 |

Note: Mean – statistical mean; SD – standard deviation; T-test – Student's t-test; CRE – creatinine; CHOL – cholesterol; TG – triglycerides; TB – total bilirubin; ALB – albumin; GLB – globulin; ALB/GLB – albumin/globulin ratio.

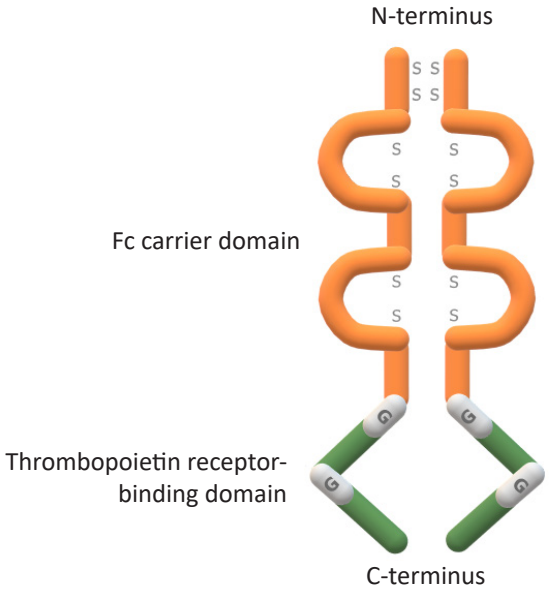



**Figure 1 – Chemical structure of romiplostim**

Note: romiplostim is a recombinant protein consisting of an Fc receptor domain at the N-terminus fused to a thrombopoietin receptor-binding domain at the C-terminus.

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2022-10-4-354-370

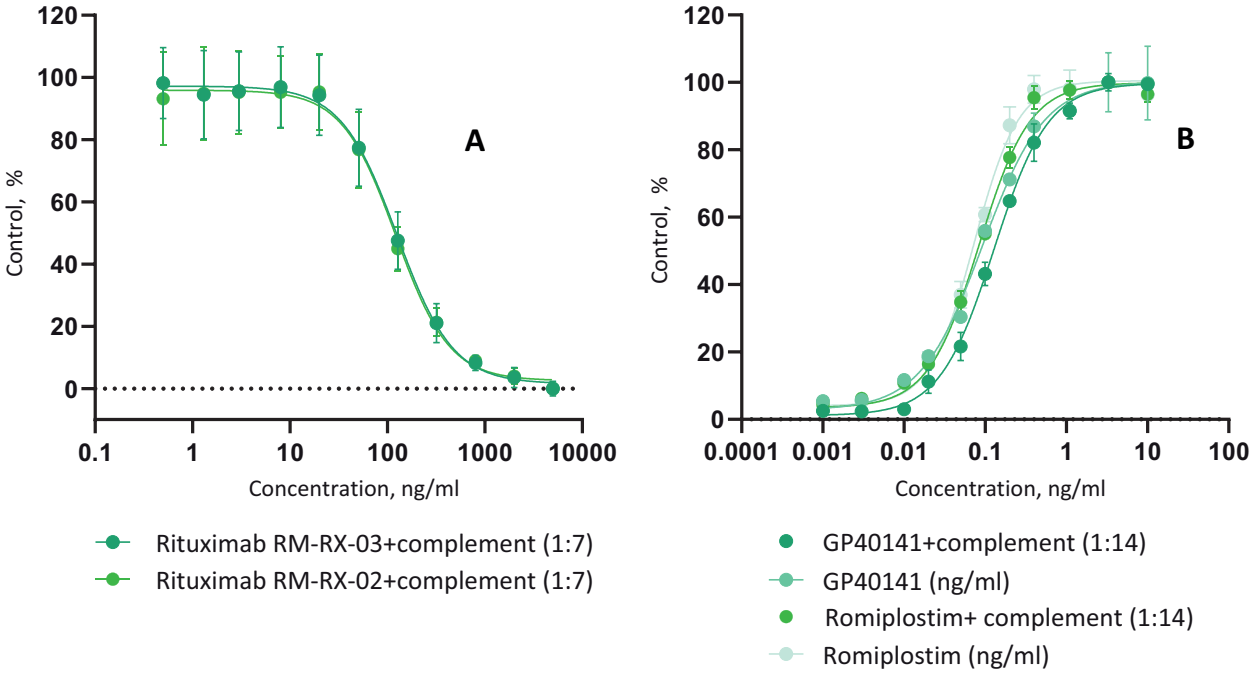



**Figure 2 – Graphs of complement-dependent cytotoxicity.**
Note: A – rituximab CDC, B – romiplostim CDC.

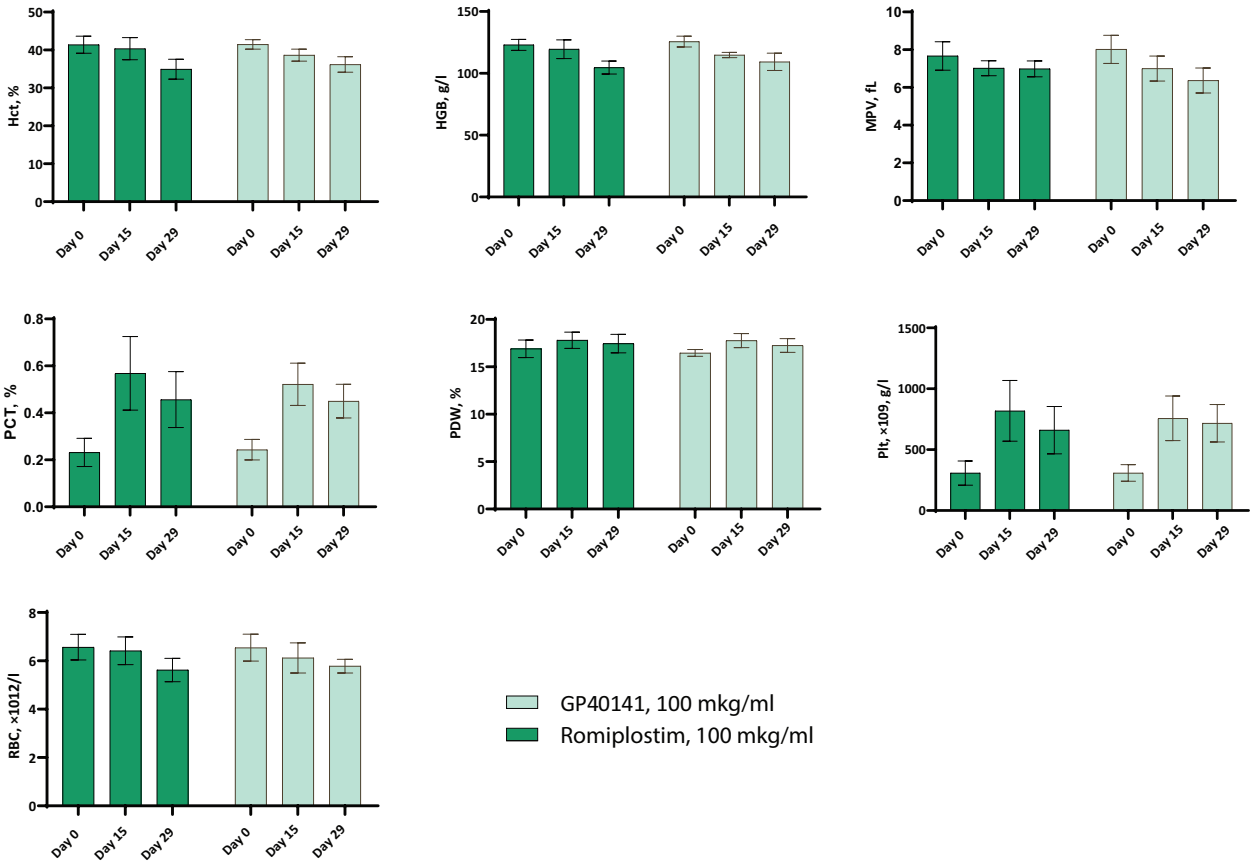



**Figure 3 – Indicators of hematological analysis**

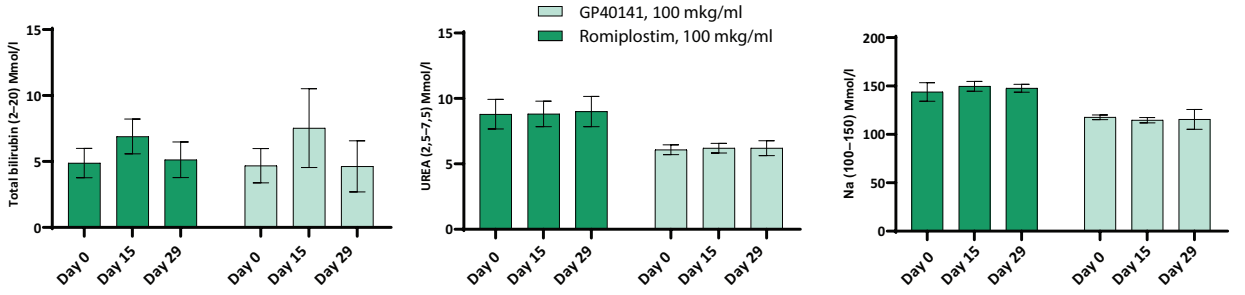



**Figure 4 – Indicators of biochemical blood test**

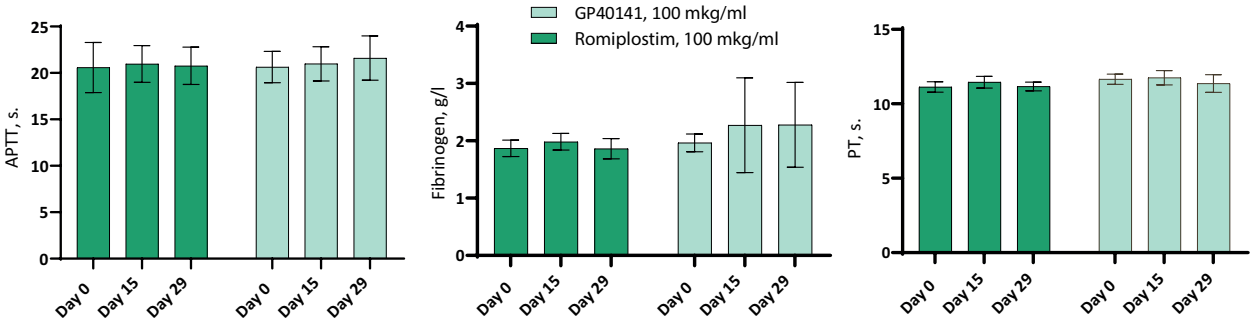



**Figure 5 – Indicators of coagulation hemostasis**

## RESULTS

### *In vitro* complement dependent cytotoxicity

Series 1107017A of the innovator romiplostim and its biosimilar GP40141 were analyzed to evaluate CDC; the data are presented in Fig. 2 and Table 2. The rituximab CDC test was considered as a reference example of complement-dependent cytotoxicity (Fig. 2A). For the CDC romiplostim test, the EC50 was calculated relative to the control without the addition of the TO and the complement. For this, first the values were normalized in the GraphPad Prism 9 Program, and then a logarithmic response curve was built up. It has a symmetrical sigmoidal shape and is also called a four-parameter dose-response curve. Rituximab CDC was calculated in a similar way.

The main action mechanism of rituximab is realized through complement-dependent cytotoxicity [21-24]. Fig. 2A shows the dose-dependence of this effect: as the concentration of rituximab increases, the viable cell signal decreases. In contrast to rituximab for romiplostim (Fig. 2B), on the contrary, with an increase in the concentration of the drug, the signal of viable cells increases, which is a consequence of its direct biological action – binding to thrombopoietin receptors and inducing cell proliferation. In order to distinguish the proliferation from CDC, the samples with and without complement were additionally considered: there were no differences and there was no clear decrease in the signal for the samples in the presence of the complement.

For the obtained $EC_{50}$ values, the relative activity was calculated by comparing the values for the drug without complement versus the drug with complement in percentage. For the original drug, romiplostim, and GP40141, similar values of 87% and 72% were obtained; they can be considered insignificant due to the absence of a decrease in the cell viability signal when the drug is stimulated together with the complement.

### *In vivo* safety study

The comparative safety results of the original drug romiplostim and its biosimilar GP40141 on 12 male *Macaca fascicularis* are presented in Tables 3–5 and Fig. 3–5.

### Influence on animals' general condition

Throughout the entire period of the experiment, there were no deviations in the animals' clinical status of feed intake in any of the experimental groups, and there was no mortality observed.

### Influence on body weight dynamics

The experimental animals' body weight data and their statistical processing are presented in Table 3.

According to the results of statistical processing, no significant differences were found between the experimental animals' average weight before the start

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2022-10-4-354-370

of the experiment with subsequent days of observation within the groups (days 15 and 29). Intergroup differences on the 29th day are due to the fact that one of the males showed a pronounced increase in body weight (11%), which led to a decrease in SD and the emergence of statistically significant differences between the groups in the absence of the difference in the dynamics of body weight gain. These differences not related to the effect of drugs, but due to the individual growth dynamics of this animal.

### Influence on body temperature

The values of body temperature and the results of statistical processing are shown in Table 4.

At the studied time points, the experimental animals' body temperature remained within the normal range and did not change significantly relative to the background values.

### Influence on respiratory rate

The respiratory rate was measured by counting the respiratory movements of the animals before the administration of the drug, as well as on the 15th and 29th days. The results of statistical processing are shown in Table 5.

From the results of statistical processing, it follows that in *Macaca fascicularis* of the both groups, the background values of the respiratory rate were higher than on the following days: the dynamics of changes was the same. A significant difference in the first group relative to the initial values in 29 days after the introduction of the test object is due to the high background values in some (2 out of 6) animals. A significant difference in the background values between the *Macaca fascicularis* groups is also a consequence of a large respiratory rates variability in *Macaca fascicularis* of the 1st group. Given this, it can be said that the drugs had a comparable effect on the animals' respiratory rates.

### Influence on indicators of clinical urine analysis

The analysis was carried out in dynamics – before the introduction of the objects, on the 15th and 29th days. All the animals had yellow and clear urine throughout the experiment. A summary table with the main results of the clinical urine analysis is presented in Table 6.

Table 7 separately presents the results of erythrocytes indicators in the animals' urine. This is due to the fact that in some animals during the experiment, the number of erythrocytes equal to 5–10 cells/µl was recorded. Fisher's exact test was used for comparison. According to the results of the comparison, it was found out that there were no differences in the dynamics of groups or between the groups at similar time points.

Throughout the experiment, the urine indicators did not go beyond the limits of intralaboratory norms, either (Table 8). All these make it possible to conclude that the studied preparations did not affect the animals' urine.

### Influence on parameters of clinical blood analysis

The analysis was carried out in dynamics – before the introduction of the objects, on the 15th and 29th days. Summary tables with the results of the clinical analysis are presented in Tables 9 and 10. The data of the clinical blood test indicators, for which the intergroup differences were observed, are presented in Fig. 3.

There were no differences between the groups in any of the clinical blood tests. However, when analyzing the differences in the dynamics within the groups, an increase in the number of platelets (Plts), thrombocrit (PCT), as well as a decrease in the number of erythrocytes (RBCs), hemoglobin (Hgb) and hematocrit (Hct) was observed in both groups. In the first group, on the 15th and 29th days, these indicators differed significantly from the background ($p < 0.05$), in the second group, they differed on the 29th day relative to the background and the 15th day ($p < 0.05$). There was also an increase in the platelet distribution index (PDW) in group 1 and a similar trend in group 2, as well as a decrease in the mean platelet volume (MPV) within the groups on days 15 and 29 relative to the baseline values (Fig. 6). Since these indicators had similar dynamics (relative to the baseline values) in the groups treated with GP40141 and romiplostim, with a high degree of probability, it can be argued that they had comparable effects on hematological parameters. Similar changes were notified in the studies of the original drug and associated with its primary pharmacodynamics[18,19].

### Influence on the parameters of a biochemical blood test

The analysis was carried out in dynamics – before the introduction of the objects, on the 15th and 29th days. The data of biochemical blood indicators analysis are presented in Tables 11 and 12, and the indicators for which intergroup differences were observed are presented in Fig. 4.

The evaluation of intragroup dynamics showed only an increase in the level of bilirubin on the 15th

[18] CHMP assessment report for Nplate®. Procedure No. EMEA/H/C/942. EMEA/654269/2008.
[19] Product monograph including patient medication information Nplate® romiplostim for injection.

day, compared with baseline values in both groups ($p < 0.05$). When evaluating the intergroup dynamics, it was notified that throughout the experiment, groups 1 and 2 did not differ from each other in any indicator, except the content of urea and sodium in the blood ($p < 0.05$).

### Influence on blood coagulation system

In dynamics – on days 0, 15 and 29 – the blood was taken from the experimental animals to assess the parameters of coagulation hemostasis. The data of the obtained results are presented in Fig. 5.

The administration of the original drug romiplostim and GP40141 did not lead to changes in the parameters of coagulation hemostasis, no significant differences were found out either between the groups of the monkeys, or between the background values and the results on days 15 and 29 after the introduction of the objects.

## DISCUSSION

Complement-dependent cytotoxicity for the original drug romiplostim, as well as for its analogue GP40141, was not observed for the 32D hTPOR clone 63 lines. The main mechanism of the romiplostim action is associated with the activation of signaling pathways that promote cells viability, their growth, megakaryocyte endomitosis, and maturation of megakaryocytes and, what is important, a platelet production [9]. The Fc–area of the romiplostim molecule does not specifically bind to complement proteins and therefore does not elicit an immune response.

According to the results of *in vivo* studies, the original drug romiplostim and its biosimilar GP40141 can be considered comparable in terms of the safety profile. During the study, no deviations in the clinical status of the animals were recorded, and neither mortality in the groups was notified. Body weight and respiratory rate had similar dynamics throughout the experiment. There were no differences in the indicators of coagulation hemostasis and clinical urinalysis either; the dynamics according to these indicators was similar. The values of most of the clinical blood analysis indicators remained stable compared to the baseline values. The exception was an increase in the number of platelets and thrombocrit, as well as a decrease in the level of erythrocytes, hemoglobin and hematocrit, the intergroup differences were not observed in any of the indicators. On the 15$^{th}$ day, the analysis of blood biochemical parameters revealed only an increase in the level of bilirubin, compared with the initial values in both groups, and when assessing the intergroup dynamics, it was notified that throughout the experiment, groups 1 and 2 did not differ from each other in any indicator, except urea and sodium levels in the blood. Considering that the differences were observed at each measurement point (before the administration, on days 15 and 29), while the dynamics of the indicators did not differ between the groups, we it can be said that these differences are not due to the action of the drugs, but reflect the initial difference in the groups. All these indicate that the study drugs had a comparable effect on the blood biochemical parameters.

## CONCLUSION

The comparison of safety profiles for the original drug romiplostim and its biosimilar GP40141 both *in vitro* and *in vivo* showed similar results. For the *in vitro* CDC test, when the complement was added, the drugs showed a proliferative activity, and there was no cells death. Based on the data obtained as a result of the *in vivo* study, it can be concluded that GP40141 (TO) and romiplostim (RF) were satisfactorily tolerated by the animals, there were no deviations in food intake, no deviations in the clinical status and deaths were recorded. The introduction of the test object and the standard object did not lead to a significant change in the weight and body temperature of the experimental animals compared to the initial values. There were no differences in urine and hemostasis parameters throughout the study either. The revealed changes in hematological parameters were unidirectional in both groups and were associated with the primary pharmacodynamics of GP40141 (TO) and romiplostim (RF). The changes in biochemical blood parameters were also unidirectional in both groups. According to the results of *in vivo* studies, it can be concluded that the toxicological profile for the drugs is similar and they are comparable in terms of the safety profile.

## FUNDING

The work was carried out with the financial support of LLC GEROPHARM group. The sponsor had no influence on the choice of materials for the publication, analysis and interpretation of the data.

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2022-10-4-354-370

## CONFLICT OF INTERESTS

The *in vitro* and *in vivo* study was organized by Pharm-Holding, which is a part of the LLC GEROPHARM group of companies. The *in vitro* study was carried out at Pharm-Holding division under a financing agreement with LLC GEROPHARM. The *in vivo* study was carried out at the Scientific Research Institute of Medical Primatology under the financing agreement of the LLC GEROPHARM. The manufacturer of GP40141, a biosimilar of romiplostim, is the LLC GEROPHARM.

## AUTHORS' CONTRIBUTION

ANA – writing and editing the text, analyzing and interpreting the results of the *in vitro* study, conducting the *in vitro* study; VBS – development of the *in vitro* study design, analysis and interpretation of the *in vitro* study results, text editing; JJKO – conducting the *in vivo* study, editing the text; EIM – conducting the *in vivo* study; DVK – interpretation of the results of the study *in vitro* and *in vivo*, approval of the text; AVK – development of the *in vivo* study design, interpretation of the *in vivo* results; IEM – development of the study design, analysis critical revision of the content of the article, approval of the final version for publication; ALK – interpretation of the results of the *in vitro* and *in vivo* studies, approval of the text; IAL – development of the *in vivo* study design, writing and editing the *in vivo* text.

## REFERENCES

1. Kuter DJ, Newland A, Chong BH, Rodeghiero F, Romero MT, Pabinger I, Chen Y, Wang K, Mehta B, Eisen M. Romiplostim in adult patients with newly diagnosed or persistent immune thrombocytopenia (ITP) for up to 1 year and in those with chronic ITP for more than 1 year: a subgroup analysis of integrated data from completed romiplostim studies. Br J Haematol. 2019 May;185(3):503–13. DOI: 10.1111/bjh.15803
2. Birocchi S, Podda GM, Manzoni M, Casazza G, Cattaneo M. Thrombopoietin receptor agonists for the treatment of primary immune thrombocytopenia: a meta-analysis and systematic review. Platelets. 2021 Feb 17;32(2):216–26. DOI: 10.1080/09537104.2020.1745168
3. Pietras NM, Pearson-Shaver AL. Immune Thrombocytopenic Purpura. 2022 May 10. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2022 Jan.
4. Newland A, Godeau B, Priego V, Viallard JF, López Fernández MF, Orejudos A, Eisen M. Remission and platelet responses with romiplostim in primary immune thrombocytopenia: final results from a phase 2 study. Br J Haematol. 2016 Jan;172(2):262–73. DOI: 10.1111/bjh.13827
5. Pasquet M, Aladjidi N, Guiton C, Courcoux MF, Munzer M, Auvrignon A, Lutz P, Ducassou S, Leroy G, Munzer C, Leverger G; Centre de Référence National des Cytopénies Auto-immunes de l'Enfant (CEREVANCE). Romiplostim in children with chronic immune thrombocytopenia (ITP): the French experience. Br J Haematol. 2014 Jan;164(2):266–71. DOI: 10.1111/bjh.12609
6. Vishnu P, Aboulafia DM. Long-term safety and efficacy of romiplostim for treatment of immune thrombocytopenia. J Blood Med. 2016 May 25;7:99–106. DOI: 10.2147/JBM.S80646
7. Wolff-Holz E, Tiitso K, Vleminckx C, Weise M. Evolution of the EU Biosimilar Framework: Past and Future. BioDrugs. 2019 Dec;33(6):621–34. DOI: 10.1007/s40259-019-00377-y
8. Suntsova EV, Chikvina II, Sadovskaya MN, Kotskaya NN, Hachatryan LA, Baydildina DD, Kalinina II, Petrova UN, Pshonkin AV, Lutfullin IY, Lemazina EN, Osipova IV, Galeeva AV, Naumova OS, Fisyn IV, Bikova GV, Kazaryan GR, Maschan AA, Novichkova GA. Use of romiplostim for newly diagnosed immune thrombocytopenia in children. Pediatric Hematology/Oncology and Immunopathology. 2020;19(1):18–26. DOI: 10.24287/1726-1708-2020-19-1-18-26 Russian
9. Bussel JB, Soff G, Balduzzi A, Cooper N, Lawrence T, Semple JW. A Review of Romiplostim Mechanism of Action and Clinical Applicability. Drug Des Devel Ther. 2021 May 26;15:2243–68. DOI: 10.2147/DDDT.S299591
10. Yang AS. Development of romiplostim: a novel engineered peptibody. Semin Hematol. 2015 Jan;52(1):12-5. DOI: 10.1053/j.seminhematol.2014.10.007
11. Kuter DJ. Romiplostim. Cancer Treat Res. 2011;157:267–88. DOI: 10.1007/978-1-4419-7073-2_16
12. Bussel JB, Kuter DJ, George JN, McMillan R, Aledort LM, Conklin GT, Lichtin AE, Lyons RM, Nieva J, Wasser JS, Wiznitzer I, Kelly R, Chen CF, Nichol JL. AMG 531, a thrombopoiesis-stimulating protein, for chronic ITP. N Engl J Med. 2006 Oct 19;355(16):1672–81. DOI: 10.1056/NEJMoa054626. Erratum in: N Engl J Med. 2006 Nov 9;355(19):2054.
13. Kuter DJ, Bussel JB, Lyons RM, Pullarkat V, Gernsheimer TB, Senecal FM, Aledort LM, George JN, Kessler CM, Sanz MA, Liebman HA, Slovick FT, de Wolf JT, Bourgeois E, Guthrie TH Jr, Newland A, Wasser JS, Hamburg SI, Grande C, Lefrère F, Lichtin AE, Tarantino MD, Terebelo HR, Viallard JF, Cuevas FJ, Go RS, Henry DH, Redner RL, Rice L, Schipperus MR, Guo DM, Nichol JL. Efficacy of romiplostim in patients with chronic immune thrombocytopenic purpura: a double-blind randomised controlled trial. Lancet. 2008 Feb 2;371(9610):395–403. DOI: 10.1016/S0140-6736(08)60203-2
14. Kuter DJ, Rummel M, Boccia R, Macik BG, Pabinger I, Selleslag D, Rodeghiero F, Chong BH, Wang X, Berger DP.

Romiplostim or standard of care in patients with immune thrombocytopenia. N Engl J Med. 2010 Nov 11;363(20):1889–99. DOI: 10.1056/NEJMoa1002625

15. Shirasugi Y, Ando K, Miyazaki K, Tomiyama Y, Okamoto S, Kurokawa M, Kirito K, Yonemura Y, Mori S, Usuki K, Iwato K, Hashino S, Wei H, Lizambri R. Romiplostim for the treatment of chronic immune thrombocytopenia in adult Japanese patients: a double-blind, randomized Phase III clinical trial. Int J Hematol. 2011 Jul;94(1):71–80. DOI: 10.1007/s12185-011-0886-8
16. Beck A, Reichert JM. Therapeutic Fc-fusion proteins and peptides as successful alternatives to antibodies. MAbs. 2011 Sep-Oct;3(5):415-6. DOI: 10.4161/mabs.3.5.17334
17. Czajkowsky DM, Hu J, Shao Z, Pleass RJ. Fc-fusion proteins: new developments and future perspectives. EMBO Mol Med. 2012 Oct;4(10):1015–28. DOI: 10.1002/emmm.201201379
18. Lee CH, Delidakis G. Engineering IgG1 Fc Domains That Activate the Complement System. Methods Mol Biol. 2022;2421:187–200. DOI: 10.1007/978-1-0716-1944-5_13.
19. Sommarhem A. Resistance mechanisms of non-Hodgkin lymphomas against Rituximab treatment. Helsinki: University of Helsinki, 2007.
20. Amoroso A, Hafsi S, Militello L, Russo AE, Soua Z, Mazzarino MC, Stivala F, Libra M. Understanding rituximab function and resistance: implications for tailored therapy. Front Biosci (Landmark Ed). 2011 Jan 1;16(2):770–82. DOI: 10.2741/3719
21. Cerny T, Borisch B, Introna M, Johnson P, Rose AL. Mechanism of action of rituximab. Anticancer Drugs. 2002 Nov;13 Suppl 2:S3-10. DOI: 10.1097/00001813-200211002-00002
22. Pescovitz MD. Rituximab, an anti-cd20 monoclonal antibody: history and mechanism of action. Am J Transplant. 2006 May;6(5Pt1):859–66. DOI: 10.1111/j.1600-6143.2006.01288.x
23. Winkler MT, Bushey RT, Gottlin EB, Campa MJ, Guadalupe ES, Volkheimer AD, Weinberg JB, Patz EF Jr. Enhanced CDC of B cell chronic lymphocytic leukemia cells mediated by rituximab combined with a novel anti-complement factor H antibody. PLoS One. 2017 Jun 28;12(6):e0179841. DOI: 10.1371/journal.pone.0179841
24. Pawluczkowycz AW, Beurskens FJ, Beum PV, Lindorfer MA, van de Winkel JG, Parren PW, Taylor RP. Binding of submaximal C1q promotes complement-dependent cytotoxicity (CDC) of B cells opsonized with anti-CD20 mAbs ofatumumab (OFA) or rituximab (RTX): considerably higher levels of CDC are induced by OFA than by RTX. J Immunol. 2009 Jul 1;183(1):749–58. DOI: 10.4049/jimmunol.0900632

## AUTHORS

**Alina N. Afanasyeva** – Junior Researcher, CJSC Pharm-Holding. ORCID ID: 0000-0002-1443-4294. E-mail: Alina.Afanaseva@geropharm.com

**Valeria B. Saparova** – Head of the Laboratory of Pharmacology, CJSC Pharm-Holding; researcher, A.I. Yevdokimov Moscow State University of Medicine and Dentistry. ORCID ID: 0000-0002-8445-1129. E-mail: Valeriya.Saparova@geropharm.com

**Jina J. Karal-Ogly** – Candidate of Sciences (Biology), Deputy Director for Science, Scientific Research Institute of Medical Primatology. ORCID ID: 0000-0003-3606-1668. E-mail: karal_5@mail.ru

**Evgenia I. Mukhametzyanova** – Researcher, Scientific Research Institute of Medical Primatology. ORCID ID: 0000-0002-1238-7594. E-mail: evg.m89@mail.ru

**Denis V. Kurkin** – Doctor of Sciences (Pharmacy), Associate Professor, Professor of the Department of Clinical Pharmacology and Intensive Care, Volgograd State Medical University, First Deputy Director of the Scientific Center for Innovative Medicines, Volgograd State Medical University. ORCID ID: 0000-0002-1116-3425. E-mail: strannik986@mail.ru

**Anna V. Kalatanova** – Researcher, CJSC Pharm-Holding. ORCID ID: 0000-0003-2623-6014. E-mail: Anna.Kalatanova@geropharm.com

**Igor E. Makarenko** – Candidate of Sciences (Medicine), Head of the Medical Department, CJSC Pharm-Holding; researcher, A.I. Yevdokimov Moscow State University of Medicine and Dentistry. ORCID ID: 0000-0003-2308-0608. E-mail: Igor.Makarenko@geropharm.com

**Alexander L. Khokhlov** – Academician of the Russian Academy of Sciences, Professor, Doctor of Sciences (Medicine), Head of the Department of Clinical Pharmacology and Ethics of the Use of Medicines at UNESCO; Rector, Yaroslavl State Medical University. ORCID ID: 0000-0002-0032-0341. E-mail: al460935@yandex.ru

**Ilya A. Lugovik** – Researcher, CJSC Pharm-Holding. ORCID ID: 0000-0003-3840-5049. E-mail: Ilia.Lugovik@geropharm.com

Научно-практический журнал
ФАРМАЦИЯ И ФАРМАКОЛОГИЯ

*ОРИГИНАЛЬНАЯ СТАТЬЯ*

*DOI: 10.19163/2307-9266-2022-10-4-371-386*



# MODERN DIRECTED ANTIVIRAL COVID-19 THERAPY: RESULTS OF MULTICENTER CLINICAL EFFECTIVENESS AND SAFETY STUDY OF FIXED NIRMATRELVIR+RITONAVIR COMBINATION

**L.A. Balykova[1], N.M. Selezneva[1], E.I. Gorshenina[1], O.I. Shepeleva[1], N.V. Kirichenko[2], E.N. Simakina[3], K.B. Kolontarev[4,5], D.Yu. Pushkar[4,5], D.N. Zemskov[1], K.Ya. Zaslavskaya[1], S.M. Noskov[6,7], A.V. Taganov[8], P.A. Bely[4]**

[1] National Research Ogarev Mordovia State University,
68, Bol'shevistskaya Str., Saransk, Republic of Mordovia, Russia, 430005
[2] Ivanovo Clinical Hospital named after the Kuvaevs,
Bld. 2, 52, Ermak Str., Ivanovo, Russia, 153025
[3] Smolensk clinical hospital No. 1,
40, Frunze Str., Smolensk, Russia, 214006
[4] Evdokimov Moscow State Medical and Dental University,
Bld. 1, 20, Delegatskaya Str., Moscow, Russia, 127473
[5] Spasokukotsky City Clinical Hospital,
21, Vuchetich Str., Moscow, Russia, 127206
[6] Yaroslavl State Medical University,
5, Revolutionary Str., Yaroslavl, Russia, 150000
[7] Clinical Hospital No. 3,
61, Mayakovsky Str., Yaroslavl, Russia, 150007
[8] Peoples' Friendship University of Russia,
6, Miklukho-Maklay Str., Moscow, Russia, 117198

E-mail: matis87177@yandex.ru

Received 03 Jule 2022 After peer review 15 Aug 2022 Accepted 30 Aug 2022

The article presents the data from an open, two-stage, multicenter study on the efficacy and safety evaluation of a combined drug (a fixed combination of nirmatrelvir 300 mg and ritonavir 100 mg) in the complex therapy in COVID-19 patients.

**The aim** of the study was to assess the safety, tolerability and pharmacokinetic parameters of the fixed combination of nirmatrelvir 300 mg and ritonavir 100 mg in healthy volunteers, the efficacy and safety assessment of the drug in the combination therapy compared with the standard therapy in COVID-19 patients.

**Material and methods.** An open two-stage multicenter clinical study to assess the main pharmacokinetic parameters, safety, and efficacy against COVID-19 of the drug nirmatrelvir 300 mg and ritonavir 100 mg combination (Skyvira® PROMOMED RUS LLC, Russia) in the adult population, included 2 stages. At stage 1, safety, tolerability and pharmacokinetic parameters were evaluated in healthy volunteers (over 18 years of age) in order to confirm their comparability with the literature data known for a set of active substances. Phase 2 assessed efficacy and safety in COVID-19 patients. As a part of the second stage, the study involved 264 patients (men and women aged 18 to 80 years), who had been divided into two groups. The first group patients (n=132) received the study drugs (nirmatrelvir 300 mg and ritonavir 100 mg) – 1 tablet twice a day with an interval of 12±2 hours for 5 days in combination with pathogenetic and symptomatic therapy. The second group patients (n=132) received standard therapy in accordance with the approved Temporary Guidelines for the Prevention and Treatment of Novel Coronavirus Infection (Version 15 dated February 22, 2022).

**Results.** During the study, none of the patients from the (nirmatrelvir + ritonavir) group experienced a transition of the COVID-19 course to a heavier severity level, in contrast to the patients in the standard therapy group. The study participants included patients with comorbidities (68% of the general population), with risk factors for COVID-19 progression to a heavier severity level and the risk of hospitalization (75% of the general population). There were no cases of COVID-19 progression

**For citation:** L.A. Balykova, N.M. Selezneva, E.I. Gorshenina, O.I. Shepeleva, N.V. Kirichenko, E.N. Simakina, K.B. Kolontarev, D.Yu. Pushkar, D.N. Zemskov, K.Ya. Zaslavskaya, S.M. Noskov, A.V. Taganov, P.A. Bely. modern directed antiviral COVID-19 therapy: results of multicenter clinical effectiveness and safety study of fixed nirmatrelvir+ritonavir combination. *Pharmacy & Pharmacology.* 2022;10(4):371-386. **DOI:** 10.19163/2307-9266-2022-10-4-371-386



**Для цитирования:** Л.А. Балыкова, Н.М. Селезнева, Е.И. Горшенина, О.И. Шепелева, Н.В. Кириченко, Е.Н. Симакина, К.Б. Колонтарев, Д.Ю. Пушкарь, Д.Н. Земсков, К.Я. Заславская, С.М. Носков, А.В. Таганов, П.А. Белый. Современная направленная противовирусная терапия COVID-19: результаты многоцентрового клинического исследования эффективности и безопасности фиксированной комбинации, содержащей нирматрелвир и ритонавир. *Фармация и фармакология.* 2022;10(4):371-386. **DOI:** 10.19163/2307-9266-2022-10-4-371-386

to a heavier severity level in the study drug group. By the 6th day, in the nirmatrelvir + ritonavir group, the proportion of the patients who had achieved a complete recovery was twice more and amounted to 35.61% (p=0.0001), and the proportion of the patients with a negative RNA analysis to SARS-CoV-2 was 20% higher than in the comparison group, and amounted to 82.58% (p=0.0001). The fixed nirmatrelvir + ritonavir combination therapy has a favorable safety profile comparable to the standard therapy. The identified adverse reactions were transient in nature and did not require discontinuation of therapy or changes in the treatment regimen.

**Conclusion.** The fixed nirmatrelvir + ritonavir combination has a favorable safety profile in COVID-19 patients, comparable to the standard therapy. The data obtained demonstrate a clinical and pharmacoeconomic feasibility of including the fixed (nirmatrelvir + ritonavir) combination in the COVID-19 treatment regimen.

**Keywords:** coronavirus; COVID-19; nirmatrelvir; ritonavir; Skyvira®; adverse events

**Abbreviations:** RDS – respiratory distress syndrome; WHO – World Health Organization; FDA – Food and Drug Administration (USA); SBP – systolic blood pressure; EMA – European Medicines Agency; AE – adverse event; DBP – diastolic blood pressure; HR – heart rate; RR – respiratory rate; BMI – body mass index; SAE – serious adverse events; IG – Interim Guidelines "Prevention, diagnosis and treatment of a new coronavirus infection"; Cmbs – comorbidities; NAAT – nucleic acid amplification test; NSAIDs – non-steroidal anti-inflammatory drugs; ALT – alanine aminotransferase; AST – aspartate aminotransferase; ULN – upper limit of normal; MedDRA (Medical Dictionary for Regulatory Activities) – medical dictionary of terms of international medical terminology; EPIC-HR (Evaluation of Protease Inhibition for Covid-19 in High-Risk Patients) – assessment of protease inhibition in COVID-19 in patients at high risk; CI – confidence interval; SARS-CoV-2 – severe acute respiratory syndrome coronavirus; COVID-19 – CoronaVIrus Disease 2019; CTs – clinical trials; IWRS – Interactive web randomization system.

# СОВРЕМЕННАЯ НАПРАВЛЕННАЯ ПРОТИВОВИРУСНАЯ ТЕРАПИЯ COVID-19: РЕЗУЛЬТАТЫ МНОГОЦЕНТРОВОГО КЛИНИЧЕСКОГО ИССЛЕДОВАНИЯ ЭФФЕКТИВНОСТИ И БЕЗОПАСНОСТИ ФИКСИРОВАННОЙ КОМБИНАЦИИ, СОДЕРЖАЩЕЙ НИРМАТРЕЛВИР И РИТОНАВИР

**Л.А. Балыкова[1], Н.М. Селезнева[1], Е.И. Горшенина[1], О.И. Шепелева[1], Н.В. Кириченко[2], Е.Н. Симакина[3], К.Б. Колонтарев[4,5], Д.Ю. Пушкарь[4,5], Д.Н. Земсков[1], К.Я. Заславская[1], С.М. Носков[6,7], А.В. Таганов[8], П.А. Белый[4]**

[1] Федеральное государственное бюджетное образовательное учреждение высшего образования «Национальный исследовательский Мордовский государственный университет им. Н.П. Огарёва», 430005, Россия, г. Саранск, ул. Большевистская, д. 68

[2] Областное бюджетное учреждение здравоохранения «Ивановская клиническая больница имени Куваевых», 153025, Россия, г. Иваново, ул. Ермака, д. 52/2

[3] Областное государственное бюджетное учреждение здравоохранения «Клиническая больница № 1», 214006, Россия, Смоленск, ул. Фрунзе, д. 40

[4] Федеральное государственное бюджетное образовательное учреждение высшего образования «Московский государственный медико-стоматологический университет имени А.И. Евдокимова» Министерства здравоохранения Российской Федерации, 127473, Россия, г. Москва, ул. Делегатская, д. 20, стр. 1

[5] Городская клиническая больница имени С.И. Спасокукоцкого Департамента здравоохранения города Москвы, 127206, Россия, г. Москва, ул. Вучетича, д. 21

[6] Федеральное государственное бюджетное образовательное учреждение высшего образования «Ярославский государственный медицинский университет» Министерства здравоохранения Российской Федерации, 150000, Россия, г. Ярославль, ул. Революционная, д. 5

[7] Государственное бюджетное учреждение здравоохранения Ярославской области «Клиническая больница № 3», 150007, Россия, г. Ярославль, ул. Маяковского, д. 61

[8] Федеральное государственное автономное образовательное учреждение высшего образования «Российский университет дружбы народов», 117198, Россия, г. Москва, ул. Миклухо-Маклая, д. 6

E-mail: matis87177@yandex.ru

Получена 03.07.2022 После рецензирования 15.08.2022 Принята к печати 30.08.2022

Научно-практический журнал
ФАРМАЦИЯ И ФАРМАКОЛОГИЯ

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2022-10-4-371-386

В статье представлены данные открытого двухэтапного многоцентрового исследования по оценке эффективности и безопасности комбинированного препарата (фиксированная комбинация нирматрелвир 300 мг и ритонавир 100 мг) в комплексной терапии у пациентов с COVID-19.

**Цель.** Оценить безопасность, переносимость и фармакокинетические параметры фиксированной комбинации нирматрелвир 300 мг и ритонавир 100 мг у здоровых добровольцев, эффективность и безопасность применения препарата в комплексной терапии в сравнении со стандартной терапией у пациентов с COVID-19.

**Материал и методы.** Открытое двухэтапное многоцентровое клиническое исследование по оценке основных фармакокинетических параметров, безопасности, а также эффективности в отношении COVID-19 лекарственного препарата нирматрелвир 300 мг и ритонавир 100 мг (Скайвира® ООО «ПРОМОМЕД РУС», Россия) у взрослой популяции включало 2 этапа. На I этапе оценивалась безопасность, переносимость и фармакокинетические параметры у здоровых добровольцев (старше 18 лет) с целью подтверждения их сопоставимости с литературными данными, известными для набора действующих веществ. На II этапе оценивалась эффективность и безопасность у пациентов с COVID-19. В рамках II этапа в исследовании участвовало 264 пациента (мужчины и женщины в возрасте от 18 до 80 лет), которые были распределены на две группы. Пациенты первой группы (n=132) получали исследуемый препарат (нирматрелвир 300 мг и ритонавир 100 мг) по 1 таблетке 2 раза в день с интервалом 12±2 ч в течение 5 дней в комплексе с патогенетической и симптоматической терапией. Пациенты второй группы (n=132) получали стандартную терапию в соответствии с утвержденными Временными методическими рекомендациями по профилактике и лечению новой коронавирусной инфекции, утвержденными Министерством здравоохранения Российской Федерации (Версия 15 от 22.02.2022).

**Результаты.** За время исследования ни у одного пациента из группы (нирматрелвир + ритонавир) не наблюдалось перехода течения COVID-19 в более тяжелую степень в отличие от пациентов группы стандартной терапии. Среди участников исследования были пациенты с сопутствующими заболеваниями (68% от общей популяции), с факторами риска прогрессирования COVID-19 до тяжелого течения и риска госпитализации (75% от общей популяции). В группе исследуемого препарата не было ни одного случая перехода COVID-19 в более тяжелую степень течения. К 6-му дню в группе (нирматрелвир + ритонавир) доля пациентов, достигших полного выздоровления, была больше в 2 раза и составляла 35,61% (p=0,0001), а доля пациентов с отрицательным анализом РНК к SARS-CoV-2 была на 20% выше, чем у группы сравнения и составила 82,58% (p=0,0001). Терапия фиксированной комбинацией (нирматрелвир + ритонавир) характеризуется благоприятным профилем безопасности, сопоставимым со стандартной терапией. Выявленные нежелательные реакции носили транзиторный характер и не требовали отмены терапии или изменения схемы лечения.

**Заключение.** Фиксированная комбинация (нирматрелвир + ритонавир) характеризуется благоприятным профилем безопасности у пациентов с COVID-19, сопоставимым со стандартной терапией. Полученные данные свидетельствуют о клинической и фармакоэкономической целесообразности включения фиксированной комбинации нирматрелвира с ритонавиром в схему лечения COVID-19.

**Ключевые слова:** коронавирус; COVID-19; нирматрелвир; ритонавир; Скайвира®; нежелательные явления

**Список сокращений:** РДС – респираторный дистресс-синдром; ВОЗ – Всемирная организация здравоохранения; FDA – Управление по санитарному надзору за качеством пищевых продуктов и медикаментов (США); САД – систолическое артериальное давление; EMA – Европейское агентство лекарственных средств; НЯ – нежелательное явление; ДАД – диастолическое артериальное давление; ЧСС – частота сердечных сокращений; ЧДД – частота дыхательных движений; ИМТ – индекс массы тела; СНЯ – серьёзные нежелательные явления; ВМР – Временные методические рекомендации «Профилактика, диагностика и лечение новой коронавирусной инфекции»; СЗ – сопутствующие заболевания; МАНК – метод амплификации нуклеиновых кислот; НПВП – нестероидные противовоспалительные препараты; АЛТ – аланинаминотрансферазы; АСТ – аспартатаминотрансфераза; ВГН – верхняя граница нормы; MedDRA – медицинский словарь терминов международной медицинской терминологии; EPIC-HR – оценка ингибирования протеазы при COVID-19 у пациентов с высоким риском; ДИ – доверительный интервал; SARS-CoV-2 – коронавирус, возбудитель COVID-19; COVID-19 – коронавирусная инфекция; КИ – клинические исследования; IWRS – модуль рандомизации пациентов.

## INTRODUCTION

COVID-19 can follow several scenarios – from asymptomatic carrier state to pneumonia of varying severity with the development of acute respiratory distress syndrome (ARDS). Despite the alleged “weakening” of SARS-CoV-2 new variants, the medical community has come to the consolidated opinion that without targeted antiviral therapy and vaccination, the COVID-19 pandemic may “approach” the scale of the 1894 plague (12 million deaths) and the flu pandemic A(H1N1) 1918 (50 million deaths) [1].

To date, the spread of the coronavirus disease (COVID-19) is still unstoppable (the number of confirmed cases and deaths continues to rise). The World Health Organization (WHO) reported the detection of cases in 216 different countries, which dictates the need to find new treatment strategies that can withstand the pandemic [2, 3].

The unprecedented global spread of SARS-CoV-2 has created serious challenges for the healthcare system. The global research community calls for the development of effective treatment protocols with the inclusion of new drugs with a high efficacy and safety profile that can significantly affect the containment and elimination of the COVID-19 pandemic [3, 4].

Currently, along with a standard supportive care, therapeutic approaches for the treatment of COVID-19 include the use of drugs that interfere with the life cycle of SARS-CoV-2 and block viral replication. At the moment, according to the medical community, the most promising is the combination of nirmatrelvir and ritonavir. It has been established that nirmatrelvir (PF-07321332) stops the spread of COVID-19 in animal models, and ritonavir slows down its metabolism and helps maintain therapeutic plasma concentrations. Despite frequent mutations in the SARS-CoV-2 genome, nirmatrelvir exhibits an effective antiviral effect against recent mutations and variants of coronavirus [5].

All coronaviruses, including SARS-CoV-2, encode two proteases required for the processing of the PP1A and PP1AB polyproteins. The main protease 3CL (chemotrypsin-like) gives rise to the formation of NSP11/16 proteins. The 3CL protease was chosen as one of the possible therapeutic targets for the development of antiviral drugs against SARS-CoV-2 due to its highly conserved sequence and structure. Nirmatrelvir (PF-00835231) has a high inhibitory activity against this particular protease. The antiviral activity of the drug was detected during the SARS-CoV-1 epidemic [6, 7].

Subsequently, a high antiviral activity of the nirmatrelvir and ritonavir combination against SARS-CoV-2 has been demonstrated [8]. Pharmacokinetic studies have shown off a significant increase in the systemic exposure of nirmatrelvir when co-administered with the CYP3A4 inhibitor ritonavir, consistent with the predominant role of CYP3A4 in the metabolism of nirmatrelvir [9, 10].

Further, in a number of clinical studies, the effectiveness of the nirmatrelvir + ritonavir combination was confirmed. Nirmatrelvir + ritonavir showed a greater reduction in the risk of hospitalization and death than molnupiravir compared with placebo. The both drugs were prescribed in the first five days after the onset of symptoms [11, 12].

In the EPIC-HR study, the use of the nirmatrelvir + ritonavir combination in the patients with a coronavirus infection resulted in an 88% reduction in hospitalization or mortality among unvaccinated outpatients with early COVID-19. The overall risk of hospitalization was 45% lower among the patients treated with nirmatrelvir + ritonavir [13]. An interim analysis demonstrated an 89% reduction in the risk of hospitalization or deaths from any cause associated with COVID-19 compared with placebo in the patients starting treatment within three days of the symptom onset (the primary endpoint) [14].

In a double-blind (randomized) study, unvaccinated, non-hospitalized adults at high risk of progression to the severe disease of COVID-19 were given nirmatrelvir (300 mg) + ritonavir (100 mg) every 12 hours for 5 days [15–16]. The primary aim of the study was to evaluate the efficacy of nirmatrelvir + ritonavir by comparing the percentage of patients with hospitalization or deaths from any cause related to COVID-19 within 28 days in the two groups.

A total of 2246 patients were randomized; 1120 patients received nirmatrelvir+ritonavir (a nirmatrelvir group) and 1126 patients received placebo (a placebo group). The incidence of hospitalizations or deaths associated with COVID-19 by day 28 in the nirmatrelvir group was 6.32% lower than in the placebo group (95% confidence interval (CI), –9.04 to –3.59; p <0.001, the reduction in the relative risk of hospitalization was 89.1%). All 13 deaths occurred in the placebo group.

The frequency of adverse events (AEs) was similar in the two groups (any AE: nirmatrelvir + ritonavir group/ placebo group – 22.6%/23.9%; serious AEs – 1.6%/6.6%; AEs leading to the drug withdrawal –2.1%/4.2%). Dysgeusia (5.6% vs 0.3%) and diarrhea (3.1% vs 1.6%) were more common in the groups treated with nirmatrelvir + ritonavir than with placebo. The treatment with nirmatrelvir in combination with ritonavir resulted in an 89% reduction in the risk of progression to severe COVID-19 compared with placebo and was characterized by a favorable safety profile [15–18].

In the study by Najjar-Debbiny R. et al. (2022), it was also proved that in the “Era of Omicrons”, nirmatrelvir in combination with ritonavir was very effective in reducing the risk of severe COVID-19 and/or mortality [19].

It is important to notify that the main target (3CL) is practically not amenable to mutations and modifications, and therefore nirmatrelvir in combination with ritonavir will be effective regardless of SARS-CoV-2 strains, which was also proven in a number of studies [20, 21].

The combination (nirmatrelvir + ritonavir) received a conditional approval in the United Kingdom in December 2021 for the treatment of adults at a high risk of progressing to severe COVID-19 patients who do not require supplemental oxygen. In January 2022, the nirmatrelvir + ritonavir combination was approved in the European Union to be used in adults and children over 12 years of age for the same indication and also has been approved for an emergency use in the USA [22, 23].

Due to the rapid spread of the Omicron SARS-CoV-2 virus variant worldwide, the Food and Drug

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2022-10-4-371-386

Administration (FDA, USA) has issued an emergency use authorization of the nirmatrelvir + ritonavir combination for the outpatient treatment of mild to moderate COVID-19 patients who are susceptible to risk of progression [15, 24–25]. Nirmatrelvir is included in the WHO guidelines for the COVID-19 treatment [26].

**THE AIM** of the study was to assess the safety, tolerability and pharmacokinetic parameters of the fixed combination of nirmatrelvir and ritonavir in healthy volunteers, the efficacy and safety assessment of the drug in the combination therapy compared with the standard therapy in COVID-19 patients.

## MATERIAL AND METHODS

An open two-stage multicenter study has been conducted to assess the main pharmacokinetic parameters and safety, as well as to evaluate the effectiveness of the drug Skyvira® (LLC PROMOMED RUS, Russia) against COVID-19 in the adult population.

This study was conducted with the aim of registering the drug in the Russian Federation, and included an assessment of the main pharmacokinetic parameters, safety, and efficacy against COVID-19[1].

The present study included 2 stages. At Stage 1, the safety, tolerability and pharmacokinetic parameters of the drug were evaluated in healthy volunteers (n=16), at Stage 2, the efficacy and safety of the drug were evaluated in COVID-19 patients.

The study was conducted in accordance with the principles of good clinical practice from February 17, 2021 to June 1, 2022 in 8 cities of the Russian Federation (Moscow, Saransk, Kirov, Ivanovo, Smolensk, St. Petersburg, Ryazan, Yaroslavl), on the basis of 12 medical institutions involved in the treatment of patients with a novel coronavirus infection.

**Stage 1, research centers of clinical trials:**

1. "Clinical Hospital No. 3" (Yaroslavl);
2. Analytical laboratory: LLC Center for Pharmaceutical Analytics (Moscow).

**Stage 2, research centers of clinical trials:**

1. Clinical Hospital No. 3 (Yaroslavl);
2. Spasokukotsky City Clinical Hospital, Moscow City Health Department (Moscow);
3. National Research Ogarev Mordovia State University, (Saransk);
4. Clinical Hospital No. 1 (Smolensk);
5. Ivanovo Clinical Hospital n. a. after the Kuvaevs (Ivanovo);
6. Ryazan Pavlov State Medical University (Ryazan);
7. Kirov State Medical University (Kirov);
8. Smolensk State Medical University (Smolensk);
9. LLC "Aurora MedFort" (St. Petersburg);
10. OrKli Hospital Limited Liability Company (St. Petersburg);
11. LLC "Uromed" (Smolensk).

### Research objectives

Stage 1 of the study included a non-randomized cohort design. All volunteers were screened and then hospitalized per cohort (the volunteers of cohort 2 were invited to hospitalization after the completion of participation in the study of cohort 1volunteers, subject to approval of a further study by the Expert Council for Drug Safety).

At this stage of the study, after the use of a fixed combination of nirmatrelvir + ritonavir, the following factors were evaluated in healthy volunteers: drug tolerability, basic vital signs, physical examination data, ECG, laboratory parameters (a clinical blood test, a biochemical blood test, a clinical urinalysis), the frequency and the AE severity. The pharmacokinetic parameters of the active substances (nirmatrelvir + ritonavir) after single and multiple uses of the drug were also studied.

### Inclusion criteria for Stage I:

1. Possession of a signed and dated informed consent form, as well as the ability and willingness to comply with all the requirements of the study protocol;
2. Men between 18 and 45 years old (inclusive) at the time of signing the informed consent form;
3. A verified diagnosis of "healthy" according to the standard clinical, laboratory and instrumental methods of examination;
4. Hemodynamic parameters: systolic blood pressure (SBP) 100–130 Mmhg, diastolic blood pressure (DBP) 60–85 Mmhg, heart rate (HR) 60–90 beats per minute, respiratory rate (RR) 16-20 breaths per minute;
5. Negative test results for human immunodeficiency virus (HIV), syphilis, hepatitis B (HbsAg), and hepatitis C (HCV);

[1] Open two-stage multicenter study has been conducted to assess the main pharmacokinetic parameters and safety, as well as to evaluate the effectiveness of the drug Skyvira® (LLC PROMOMED RUS, Russia) against COVID-19 in the adult population film-coated tablets (LLC PROMOMED RUS, Russia). Research sponsor LLC PROMOMED RUS, Russia. Study protocol: No. NR-012022. Phase of clinical development: I–III phase. Stage I – open incompare cohort. Stage II – open, randomized multicenter comparative. Dates of the study: 2021 Feb 17 – 2022 Jan 06. Contract research organization: SOLYUR-PHARM LLC, office 2, room 1, floor 3, Bldg 2, 4, Ivana Franko Str., Moscow, Russia, 121108.

ISSN 2307-9266 e-ISSN 2413-2241
Scientific and Practical Journal
PHARMACY & PHARMACOLOGY

6. Negative test results for the use of alcohol, narcotic drugs and psychotropic substances.

7. Body mass index (BMI): 18.5–30 kg/m$^2$;

8. Willingness to abstain from alcohol within 72 hours prior to screening and during the study period;

9. A volunteer's consent to use reliable methods of contraception during the study period, within 3 weeks after its completion.

**Criteria for non-inclusion, Stage 1:**

1. Aggravated allergological anamnesis, drug intolerance. Hypersensitivity to the components of the study drug;

2. Lactase deficiency, lactose intolerance, glucose-galactose malabsorption;

3. Impossibility to install a venous catheter for blood sampling;

4. Difficulties in swallowing tablets;

5. Donation or loss of blood (≥450 ml of blood or plasma) less than 3 months prior to screening;

6. Acute and chronic diseases of the cardiovascular, bronchopulmonary, endocrine and nervous systems, including organic diseases of the central nervous system (CNS), as well as diseases of the gastrointestinal tract (GIT), liver, kidneys, blood;

7. Any deviations from normal values during laboratory and/or instrumental examinations.

8. Surgical interventions on the gastrointestinal tract (with the exception of appendectomy) in anamnesis;

9. Acute infectious diseases or symptoms of SARS less than 4 weeks before the screening visit;

10. Detection of SARS-CoV-2 RNA or SARS-CoV-2 antigen within 6 months prior to the screening;

11. Presence of at least one of the epidemic signs:

• return from an overseas travel 14 days prior to the screening and no test results for SARS-CoV-2 RNA or SARS-CoV-2 antigen;

• close contact with a person under observation for COVID-19 who subsequently became ill, in the past 14 days prior to the screening;

• close contact with a laboratory confirmed case of COVID-19 in the past 14 days prior to the screening;

• professional contacts with individuals who have a suspected or confirmed case of COVID-19 in the last 14 days prior to the screening.

12. Regular intake of medications, including herbal and homeopathic preparations, vitamins and/or dietary supplements (BASs) less than 4 weeks before the screening visit;

13. Taking medications that have a pronounced effect on hemodynamics and/or liver function (barbiturates, omeprazole, cimetidine, etc.) less than 2 months before the screening visit;

14. Special diet (e.g., vegetarian, salt-restricted) less than 2 months prior to the screening visit;

15. Special lifestyle (night work, an extreme physical activity) less than 2 months prior to the

16. Alcohol ingestion in anamnesis: more than 10 units of alcohol per week (1 unit of alcohol is equivalent to 500 ml of beer, 200 ml of wine or 50 ml of hard liquor), or anamnestic data on alcoholism, drug addiction, drug abuse;

17. Smoking more than 10 cigarettes per day at the time of the screening visit;

18. Performing piercing procedures, tattooing/ tattooing less than 1 month before screening procedures and throughout the study;

19. Mental, physical and other reasons that do not allow the volunteer to adequately assess their behavior and correctly fulfill the conditions of the Research Protocol;

20. Participation in another clinical trial less than 3 months before the screening visit;

21. Other reasons that do not allow the volunteer, in the opinion of the research physician, to take part in this study.

**Duration of Stage I**

The total duration of a volunteer's participation in the study was no more than 11 days, herewith, the screening duration was no more than 7 days, the duration of a hospital stay was no more than 3.5 days, the duration of the study drug was no more than 3 days.

**Assessed safety indicators for Stage I:**

1. The frequency and severity of AEs registered on the basis of complaints, changes in the well-being of volunteers according to abnormal results of laboratory tests, physical examinations, assessment of vital signs, ECG;

2. Number of cases of participants' early termination in the study due to the development of AEs and/or SAEs, including those related to the investigated drug;

3. Assessment of the overall tolerability of the investigated drug on the Likert scale by the investigator.

**Method of application in Stage I**

It was supposed to include 2 cohorts of 8 healthy volunteers each: Cohort 1 – 300 mg + 100 mg once; Cohort 2 – 300 mg + 100 mg every 12 hours, 5 doses in total.

The study began with the use of the drug by Cohort 1 volunteers. Taking the drug by the volunteers Cohort 2

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2022-10-4-371-386

was started only after assessing the safety of the drug by the volunteers of Cohort 1.

The decision on the possibility of switching to the multiple use of the drug within Cohort 2 was made by the Expert Council for Drug Safety based on the assessment of AE/SAE. The transition to Cohort 2 could be stopped if at least one of the following stopping criteria was found in Cohort 1 of the volunteers. These criteria are as follows: the development of a serious adverse reaction with a possible, probable or definite connection with the study drug in ≥1 healthy volunteers; the development of a severe adverse reaction with a possible, probable or definite connection with the use of the study drug in ≥2 healthy volunteers, regardless of whether they belong to the same class of organ systems or not.

### Randomization of Stage II

Randomization was performed using the IWRS (Interactive web randomization system) built into a patient's electronic individual registration card. At Stage 2 of the study, 264 patients who had finished the study all over in accordance with the approved protocol were randomized.

In the group of the drugs combination nirmatrelvir + ritonavir (group 1), 84 female patients (63.64%) and 48 male patients (36.36%) were randomized, in the standard therapy group – 82 female patients (62.12%) and 50 male patients (37.88%). Subgroup 1–1 (n=33) was without any presence of a risk factor for the development of a severe COVID-19 course. Subgroup 1–2 (n=99) was characterized by the presence of at least one risk factor for the development of a severe COVID-19 course.

The mean age of patients in the fixed combination (nirmatrelvir + ritonavir) group was 46.61±15.75 years (from 19 to 79 years of age), the mean body weight was 80.69±14.31 kg (from 49.0 to 116 .0 kg), the height was 170.44±7.78 cm (from 152 to 190 cm), BMI – 27.81± 4.89 kg/m$^2$ (from 18.37 to 40.90 kg/m$^2$).

The mean age of patients in the standard therapy group was 46.62±15.97 years (from 18 to 77 years old), the mean body weight was 79.32±14.57 kg (from 50.0 to 122.0 kg), the mean height – 170.01±7.74 cm (from 148 to 193 cm), the mean BMI – 27.45±4.81 kg/m$^2$ (from 17.71 to 43.74 kg/m$^2$).

The first group (n=132) received 1 tablet of the study drug (nirmatrelvir + ritonavir) twice a day with an interval of 12±2 hours for 5 days in combination with pathogenetic and symptomatic therapy, presented in the Interim Guidelines "Prevention, diagnosis and treatment of novel coronavirus infection" (IG)[2], valid at the time of the study.

The second group (n=132) received standard therapy in accordance with the IGs in force at the time of the study[3].

Each group included 2 subgroups depending on the presence/absence of at least one risk factor for the development of severe COVID-19 in the ratio of 3:1.

### Inclusion criteria for Stage 2:

1. Availability of an informed consent form signed and dated by the patient;

2. Men and women aged from 18 to 80 years old inclusive at the time of signing the informed consent form;

3. A confirmed case of COVID-19 at the screening time: a positive laboratory test for the presence of SARS-CoV-2 RNA using nucleic acid amplification methods (NAAT) or SARS-CoV-2 antigen using an immunochromatographic analysis[4];

4. A mild or moderate infection caused by SARS-CoV-2;

5. At the time of screening and randomization, there is at least one of the following symptoms characteristic of COVID-19: nasal congestion or a running nose, a sore throat, shortness of breath on exertion, cough, fatigue, muscle or body pain, headache pain, chills, fever (body temperature >38°C), nausea, vomiting, diarrhea, loss of smell (anosmia), loss of taste sensation (ageusia).

6. The onset of the disease (appearance of the first symptom) no more than 5 days before randomization;

7. A patient is willing and able to take oral medications;

8. A patient's consent to use reliable methods of contraception throughout the study and for 3 weeks after the end of the study.

The study could also include the women who are unable to bear children (anamnesis: hysterectomy, tubal ligation, infertility, menopause for more than 10 years), as well as men with infertility or anamnesis of vasectomy.

[2] Interim guidelines "Prevention, diagnosis and treatment of a new coronavirus infection" (COVID-19), version 16 (2022 Aug 18), approved by the Ministry of Health of Russian Federation.
[3] Interim guidelines "Prevention, diagnosis and treatment of a new coronavirus infection" (COVID-19), version 15 (2022 Feb 22), approved by the Ministry of Health of Russian Federation.
[4] SARS-CoV-2 test results obtained up to 5 days prior to randomization could be considered if supporting documentation was available.

**Criteria for non-inclusion, Stage 2:**

1. Hypersensitivity to the components of the study drug;

2. Lactase deficiency, lactose intolerance, glucose-galactose malabsorption;

3. Use of direct-acting antivirals within 10 days prior to the screening;

4. At the screening time, the use of the drugs, clearance of which is highly dependent on the CYP3A isoenzyme, or which are strong inducers of CYP3A (for more information, see the section "Prohibited Therapy");

5. The need for the use of drugs from the list of prohibited therapies;

6. The need for oxygen therapy at the time of screening;

7. The need for hospitalization at the time of screening, or expecting the need for hospitalization caused by COVID-19 within 48 hours of randomization, other than the need for hospitalization in an observatory for social reasons (e.g., living in a hostel, cohabiting with people, including risk factors for severe COVID-19, who, after a contact with a patient, have a negative result in SARS-CoV-2 RNA/antigen, etc.);

8. Availability of criteria for severe and extremely severe course of the disease at the time of screening;

9. Vaccination less than 4 weeks prior to the screening;

10. Presence of a probable or confirmed case of moderate COVID-19 within 6 months prior to the screening;

11. Presence of a probable or confirmed case of severe and extremely severe COVID-19 in anamnesis;

12. Patients with the established moderate to severe renal insufficiency (the estimated glomerular filtration rate (GFR) <60 ml/min/1.73m$^2$ according to the CKD-EPI formula) or receiving renal replacement therapy at the screening time;

13. A severe liver failure (class C in Child-Pugh's classification) at the moment of screening or anamnesis (within 6 months prior to the screening) of alanine aminotransferase (ALT) and/or aspartate aminotransferase (AST) ≥2.5 upper limit of normal (ULN) ), and/or total bilirubin ≥2 ULN (≥3 ULN in Gilbert's syndrome);

14. A positive result of the analysis for the presence of HIV, syphilis, hepatitis B and/or C at the screening;

15. Alcohol, pharmacological and/or drug dependence in anamnesis and/or at the screening time;

16. Schizophrenia, schizoaffective disorder, a bipolar disorder or other psychiatric pathology in anamnesis or suspicion of their presence at the screening time;

17. Any anamnestic data that, in the opinion of the investigator, may complicate the interpretation of the results of the study or create any kind of an additional risk for a patient as a result of his participation in the study;

18. Unwillingness or inability of a patient to comply with the procedures of the Protocol (according to the investigator);

19. Pregnant or lactating women or women planning a pregnancy;

20. Participation in another clinical study within 3 months prior to the enrollment in the study;

21. Other conditions that prevent the inclusion of the patient in the study.

**Duration of Stage 2**

The total duration of a patient's participation in the study was no more than 31 days (Table 1).

**Criteria for safety assessment, Stage II:**

1. Total number of AEs stratified by severity and frequency.

2. Frequency of adverse events.

3. Frequency of SAEs, including those associated with study drug/standard therapy.

4. Proportion of patients with at least one AE.

5. Percentage of patients interrupting the treatment due to AE/SAE.

**Criteria for evaluating effectiveness at Stage 2:**

Primary efficacy criterion:

Incidence of patients with COVID-19 progressing to a more severe Interim Guidelines from baseline at visit 4 (Day 16).

Secondary efficacy criteria:

1. Dynamics of clinical status on a categorical ordinal scale of clinical improvement;

2. Frequency of worsening clinical status on a categorical ordinal scale of clinical improvement by ≥1 category at visits 2, 3 and 4;

3. Frequency of patients with category 0 on the categorical ordinal scale of clinical improvement at visits 2, 3 and 4;

4. Rate of SARS-CoV-2 RNA negative patients at visits 2, 3 and 4;

5 Symptom scores for Visits 2–6 on the COVID-19 Major Symptom Rating Scale.

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2022-10-4-371-386

### Statistical processing of research results

For the statistical analysis, software with validated algorithms for performing statistical analyzes and proper documentation StatSoft Statistica 10.0., IBM SPSS Statistics 22 (current version, GPL-2/GPL-3 license) was used.

Descriptive statistics is presented for all indicators of efficacy, safety, tolerability, and pharmacokinetic parameters collected during the study.

Continuous (quantitative) data are presented using a number of observations, arithmetic mean, 95% confidence interval (CI) for mean, standard deviation, median, interquartile range ($25^{th}$ and $75^{th}$ centiles), minimum and maximum (if not specified otherwise).

Ordinal, categorial, and qualitative data are presented as absolute frequencies (a number of observations), relative frequencies (percentage), and 95% CI (unless noted otherwise).

Checking for the normality of the distribution was carried out by one of the generally accepted methods (the Shapiro-Wilk test, the Kolmogorov-Smirnov test). In case of a non-Gaussian distribution, non-parametric estimation methods were used to compare indicators.

To assess the parameters represented by ordinal values, non-parametric methods of the analysis were used. The Mann-Whitney test was used to compare indicators between the groups; to assess the dynamics of an indicator within each group, the Friedman criterion for several dependent variables was used; to compare between the start and the end points within each group, the Wilcoxon test for two dependent variables was applied. If all the expected values in the cells of the contingency table for this analysis were 5 or more, the Fisher's exact test or the chi-square test $\chi^2$ could be also used for the analysis,

For the comparison between the groups of continuous quantitative indicators, the Student's t-test or Mann-Whitney test was used (depending on the conclusion about the nature of the distribution). Between the start and the end points of the assessment within each group, a paired t-test or the Wilcoxon test for two dependent variables (depending on the accepted conclusion about the nature of the distribution) were used.

In the case of estimating the time to the event (time-to-event), taking into account censored observations, the Kaplan-Meier method and the construction of survival tables could be used as descriptive methods of the analysis; the Cox-Mentel or Log-rank test.

The differences were considered statistically significant at $p < 0.05$.

Significance levels and confidence intervals were calculated as two-tailed, statistical significance of the differences was by default two-tailed and referred to a significance level of 0.05 (unless indicated otherwise).

For Stages I and II of the study, demographic data (age, sex), baseline data are presented as absolute frequencies (numbers of observations), relative frequencies (percentage) or using the arithmetic mean, 95% CI for the mean, standard (root mean square) deviation, median, interquartile range ($25^{th}$ and $75^{th}$ centiles), minimum and maximum depending on the type of the variable.

At Stage 2, to test the hypothesis about the homogeneity of the study groups, the null hypotheses (about the absence of differences between groups) using the Student's t-test (for interval indicators with a normal distribution in the study population) were tested. The Mann-Whitney test (for ordinal indicators or for interval indicators with a distribution other than normal) or the Fisher's exact test and the $\chi^2$ test (for qualitative traits) were also used.

In the case of finding statistically significant differences between the groups, the magnitude of the differences between the study groups was assessed using CI.

## RESULTS AND DISCUSSION

### Results of study, Stage 1

As a part of the first stage of the investigation, the safety of the studied combination was initially confirmed and its pharmacokinetic profile was studied (Fig. 1, 2).

During the first phase of the study, the comparability of the main pharmacokinetic parameters of the studied fixed combination with the literature data [8, 9, 16] for both nirmatrelvir and ritonavir was shown, both with a single and multiple administration.

### Nirmatrelvir

Cohort 1 (a single administration): the median time to reach the maximum concentration ($C_{max}$ = 1988.92±1109.65 ng/ml) of nirmatrelvir was $T_{max}$ = 1.75 hours. Nirmatrelvir was eliminated from blood plasma with a mean of $T_{1/2}$ = 5.80 ± 1.96 hours. The $AUC0_{-\infty}$ and $AUC_{0-t}$ values were 15354.88±6258.84 ng×h/ml and 13105.22±5656.34 ng×h/ml, respectively. The total clearance (Cl) was 23.18 ± 11.21 l/h. The volume of the drug distribution corresponded to the value of $V_d$ = 189.08±98.03 liters. The value of the mean retention time of the drug substance in the blood plasma was MRT = 8.82±3.00 hours.

ISSN 2307-9266 e-ISSN 2413-2241

Scientific and Practical Journal
PHARMACY & PHARMACOLOGY

**Table 1 – Schedule of patients' visits at Stage 2 of the study**

| Visit No. | Interval (hours/days) | Study status |
|---|---|---|
| 0 | No more than 48 hours | Screening |
| 1 | 1 day* | Randomization |
| 2 | 6–7 day | – |
| 3 | 11–12 days | – |
| 4 | 14–15 days** | – |
| 5 | 21±1 days** | – |
| 6 | 28±1 days** | Completion |

Note: * – visit 1 may have coincided with visit 0. If visit 1 and visit 0 were the same, then physical examinations, vital signs, registration of concomitant therapy, pulse oximetry with $SpO_2$ measurement, symptom scores using the COVID-19 Symptom Scale were not repeated, inclusion and non-inclusion criteria were assessed immediately before randomization, and exclusion criteria were assessed after drug administration. ** – the visit could be carried out both in person and by a phone call. In case of a phone call visit, and in the presence of a positive SARS-CoV-2 RNA test at the previous visit, medical personnel were sent to collect a swab from the nasopharynx and/or oropharynx for SARS-CoV-2 RNA analysis.

**Table 2 – Risk factors in patients in study drug and standard therapy group**

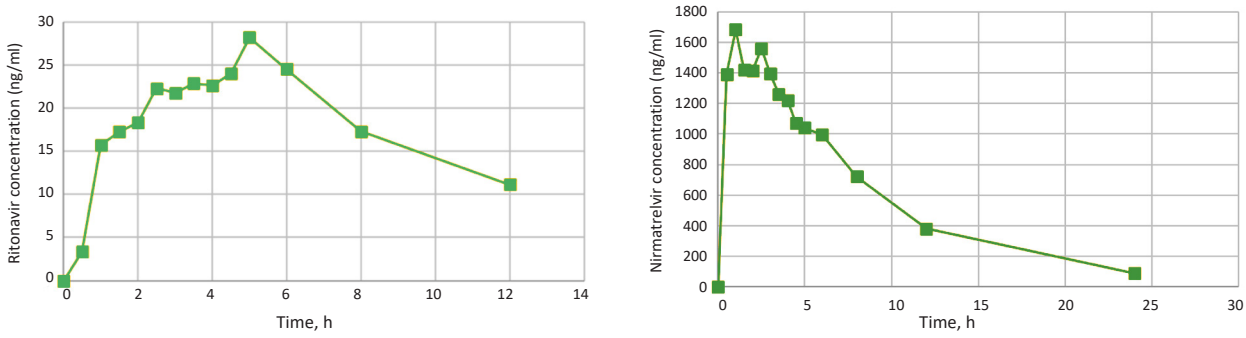
| Risk factors | Main group (n=132) | Comparison group (n=132) |
|---|---|---|
| BMI 30 or more kg/m$^2$ | 36.36% | 37.12% |
| Heart diseases | 31.06% | 30.30% |
| Age over 60 years | 22.73% | 21.97% |
| Chronic kidney diseases | 3.03% | 6.82% |
| Chronic lung diseases | 2.27% | 6.82% |
| Diabetes mellitus | 0.76% | 1.52% |
| Active malignancy | 0.76% | – |

**Table 3 – Analysis of adverse reactions frequency according to WHO classification associated with taking nirmatrelvir + ritonavir combination**

| System organ class and preferred MedDRA term | Number of events (abs/%) | | p-value (Fisher's criterion) |
|---|---|---|---|
| | Nirmatrelvir + ritonavir (n=132) | Standard therapy (n=132) | |
| Gastrointestinal disorders | | | |
| Diarrhea | 1 (0.8%) (infrequent) | 1 (0.8%) (infrequent) | 1.0000 |
| Dry mouth | 1 (0.8%) (infrequent) | Not observed | 1.0000 |
| Nausea | Not observed | 2 (1.5%) (frequent) | 0.4981 |
| Laboratory and instrumental data | | | |
| Increased ALT levels | 3 (2.3%) (frequent) | 2 (1.5%) (frequent) | 1.0000 |
| Increased AST levels | 3 (2.3%) (frequent) | 2 (1.5%) (frequent) | 1.0000 |
| Skin and subcutaneous tissue disorders | | | |
| Erythema | Not observed | 1 (0.8%) (infrequent) | 1.0000 |
| Nervous System Disorders | | | |
| Dysgeusia | 3 (2.3%) (frequent) | Not observed | 0.2472 |

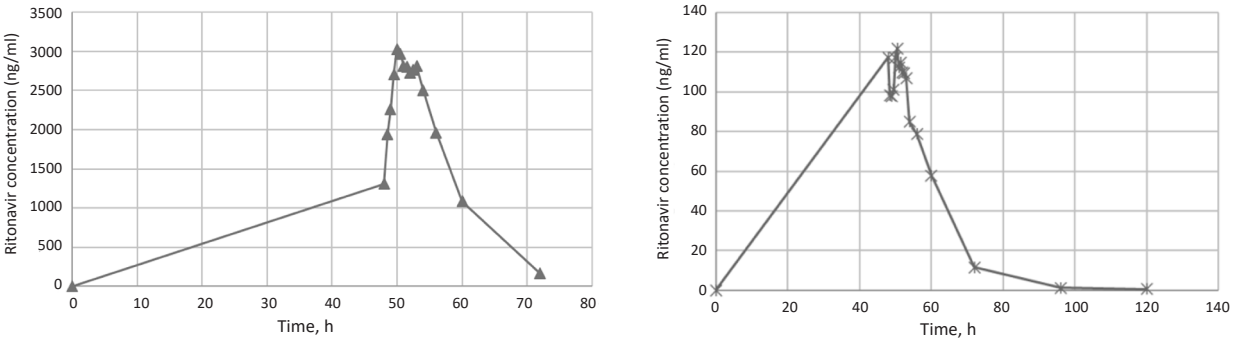
Note: The frequency of adverse reactions occurrence is defined as follows: very often (≥1/10), often (≥1/100, but <1/10), infrequently (≥1/1000, but <1/100), rarely (≥ 1/10,000 but <1/1000), very rarely (<1/10,000), not known (cannot be established based on available data). ALT – alanine aminotransferase, AST – aspartate aminotransferase.

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2022-10-4-371-386




a) b)

**Figure 1 – Pharmacokinetic profile of average concentration values of nirmatrelvir a) and ritonavir b) over time (in a linear transformation) after a single dose of the study drug**




a) b)

**Figure 2 – Pharmacokinetic profile of average concentration values of nirmatrelvir a) and ritonavir b) over time (in a linear transformation) after multiple doses of the study drug**

Cohort 2 (a repeated administration): the value of the maximum nirmatrelvir concentration in the volunteers' blood plasma was $C_{max,\ ma}$ = 3442.52±1078.88 ng/ml. The value of the minimum nirmatrelvir concentration in the blood plasma of volunteers was $C_{min,\ ma}$ = 246.30± 174.85 ng/ml. The $AUC_{48-\tau}$ value was 33334.03± 9770.52 ng×h/ml.

### Ritonavir

Cohort 1 (a single administration): the value of the median time to reach the maximum concentration ($C_{max}$ = 25.74±20.98 ng/ml) of ritonavir was $T_{max}$ = 5 hours. Ritonavir was eliminated from the blood plasma with a mean of $T_{1/2}$ = 8.13 ± 3.08 hours. $AUC_{0-\infty}$ and $AUC_{0-t}$ values were 652.80 ± 264.85 ng×h/ml and 183,89± 206,42 ng×h/ml, respectively. The total clearance (Cl) was 173.84±78.32 l/h. The volume of the drug distribution corresponds to the value of $V_d$ = 1991.30± 1049.61 liters. The value of the mean retention time of the drug substance in the blood plasma was MRT = 13.39±4.12 hours.

Cohort 1 (a repeated administration): the value of the maximum ritonavir concentration in the volunteers' blood plasma was $C_{max,\ ma}$ = 148.15±92.80 ng/ml. The value of the minimum ritonavir concentration in the volunteers' blood plasma was $C_{min,\ ma}$ = 16.78±9.48 ng/ml. The $AUC_{48-\tau}$ value was 1542.89±1207.18 ng×h/ml.

Since the results of the clinical trials (CTs) Stage 1 showed a favorable safety profile and a good tolerability in single and multiple oral administrations of the combination in the form of film-coated tablets, nirmatrelvir 300 mg and ritonavir 100 mg, during the Stage 1 study, no criteria for stopping the CTs were identified, and comparability of the obtained pharmacokinetic data for nirmatrelvir and ritonavir with the available literature data was demonstrated. The Ethics committee of the Ministry of Health[5] of Russian Federation considered it possible to proceed to the second stage of the study. It should be notified that the data obtained confirm the possibility and expediency of combining the active substances in the form of the fixed combination, which reduces polypharmacy and increases the convenience of use for patients.

### Results of study, Stage 2

182 (68.94%) of 264 patients had MedDRA-classified comorbidities (Cmbs).

[5] Study protocol: No. NR-012022. Contract research organization: SOLYUR-PHARM LLC, office 2, room 1, floor 3, Bldg 2, 4, Ivana Franko Str., Moscow, Russia, 121108.

The most common Cmbs were obesity (36.32%) and hypertension (32.48%), kidney diseases (15%), other Cmbs (11%), respiratory diseases (7%), etc. The groups were comparable in terms of the Cmbs presence. In the study drug group, it is interesting to notify the presence of patients with oncological diseases and anamnestic data on atrial fibrillation, i.e., the patients for whom a high safety profile of the drug is especially important.

A total of 264 patients including 198 patients with risk factors (99 (75%) patients in each group) were observed in the study (Stage 2). Of these, with the risk factor of "the age over 60 years" there were 30 patients (22.73%) in the study drug group and 29 patients (21.97%) in the comparison drug group; with the risk factor of "obesity (BMI 30 or more kg/m$^2$)" – 48 patients (36.36%) in the study drug group and 49 patients (37.12%) in the comparison drug group; with the risk factor of "diabetes mellitus" – 1 patient (0.76%) in the study drug group and 2 patients (1.52%) in the comparison drug group; with a risk factor of "a heart disease" – 41 patients (31.06%) in the study drug group and 40 patients (30.30%) in the comparison drug group; with the risk factor of "chronic lung diseases" – 3 patients (2.27%) in the study drug group and 9 patients (6.82%) in the comparison drug group; with the risk factor of "chronic kidney diseases" – 4 patients (3.03%) in the study drug group and 9 patients (6.82%) in the comparison drug group; with the risk factor of "active malignant neoplasm" – 1 patient (0.76%) in the study drug group. Information on the frequency of risk factors occurrence in the patients in the study drug group and a standard therapy group is presented in Table 2.

The intergroup analysis showed that the study groups were comparable in terms of demographic and clinical characteristics.

During the study, none of the patients in the nirmatrelvir + ritonavir group experienced a transition of the COVID-19 course to a heavier severity level, более тяжелую степень тяжести in contrast to the patients in the standard therapy group.

In the drug group (nirmatrelvir + ritonavir), the proportion of patients with the transition of the course of COVID-19 to a heavier severity level, более тяжелую степень тяжести compared to the initial state by day 16, was 0.00% (0/132), 95% CI [0.0000; 0.0352], in the standard therapy group – 6.06% (8/132), 95% CI [0.0285; 0.1198]. The difference in proportions between the drug groups (nirmatrelvir + ritonavir) and the standard therapy group was 0.0606 (6.06%), 95% CI [0.0129; 0.1198].

Hypothesis testing for the final statistical analysis was carried out at a one-sided significance level of 0.0275. As a result of a comparative analysis of the patients' frequency with the transition of the course of COVID-19 to a heavier severity level compared to the initial state by day 16, statistically significant differences were revealed between the drug group (nirmatrelvir + ritonavir) and the standard therapy group (p = 0.0035, i.e. p <0.0275).

Thus, the hypothesis of the therapy superiority with nirmatrelvir + ritonavir over the standard therapy can be considered proven. It has been also concluded that fixed combination therapy reduces the risk of deterioration in the patients' clinical condition and improves the prognosis of the disease course.

Due to the fact that among the study participants there were patients with Cmbs (68% of the general population), with risk factors for the progression of COVID-19 to a heavier severity level and the risk of hospitalization (75% of the total population), we can conclude that the therapy was highly effective (nirmatrelvir + ritonavir), regardless of the risk factors presence for an aggravated course of the disease.

Thus, the therapy under consideration is reasonable, clinically and pharmacoeconomically effective.

The dynamics of the mean value (Mean ± SD) of the clinical status on the categorical ordinal scale of the clinical improvement in the study drug group (nirmatrelvir and ritonavir) on the 6th day of the observation was 1.30±1.01 points compared to the standard therapy 1.76±0.79 (p=0.0001). This demonstrates the advantage of the fixed combination over the standard regimen in terms of reducing the severity of COVID-19 symptoms and improving the clinical condition of the patients and accelerating their recovery.

The frequency of patients with category 0 on the categorical ordinal scale of clinical improvement by the 6th day of observation in the drug group (nirmatrelvir and ritonavir) was 2 times more and amounted to 35.61% (47/132), compared to the standard therapy group – 14.39% (19/132) (p=0.0001). The data obtained indicate a high efficiency of the fixed combination (nirmatrelvir + ritonavir) and its significant advantages over the comparison group in terms of the improvement rate in the clinical status of the patients and the reduction of their recovery time.

The frequency of patients with a negative SARS-CoV-2 RNA analysis by the 6th day in the study drug group, was 20% higher than in the control group (82.58% of patients (109/132) and 61.36% of patients (81/132), respectively), which indicates the effective antiviral effect of the fixed combination (nirmatrelvir + ritonavir), characterized by a reduction in the elimination of the SARS-CoV-2 virus compared to the standard therapy. That leads to a faster disappearance of the infectious

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2022-10-4-371-386

disease symptoms and reduces the risk of developing COVID-19 complications.

As a result of a comparative analysis of the patients' frequency with varying severity symptoms by day 6, statistically significant differences were found between the drug group (nirmatrelvir and ritonavir) and the standard therapy group in terms of symptoms: nasal congestion or a running nose (p = 0.0027), a sore throat (p=0.0016), cough (p=0.0424), fatigue (p=0.0003), as well as the presence of cough symptoms at visit 4 (p=0.0016), indicating the effectiveness of the fixed combination in COVID-19 patients.

The assessment of the total score on the scale of the main COVID-19 symptoms shows that the average value of the total score for all the symptoms in the group taking the combination of nirmatrelvir + ritonavir (Mean ± SD) by day 6 (p <0.0001), was 1.39 ± 1.45 points (at screening – 5.43±2.16), in the standard therapy group – 2.21±1.77 points (at screening – 5.80 ± 2.46). I.e., in the study drug group, positive dynamics for all the symptoms occurred 60% faster than in the comparison group, and the changes were more pronounced. Thus, the use of the considered combination provides an improvement in the general condition and relief of the main symptoms of the disease.

Additionally, according to the WHO classification, an analysis of the adverse reactions frequency including AEs with a certain, probable and possible connection with the drugs nirmatrelvir + ritonavir (Table 3), was made.

The results of the comparative analysis also showed that taking the study drug contributed to a more effective decrease in the body temperature, which led to a decrease in the need for taking non-steroidal anti-inflammatory drugs (NSAIDs) and an increase in the therapy safety in general.

The incidence of patients in the study drug group with reported AEs was 7.58% (10/132). In total, 14 AEs were notified in 10 patients of the nirmatrelvir + ritonavir group. Among the reported AEs, 92.86% (13/14) were of a mild severity, 7.14% (1/14) were of a moderate severity. According to the study physicians, a causal relationship with the study drug therapy was assessed as "possible" in 71.43% (10/14) of cases, as "not related" in 14.29% (2/14) of cases, as "probable" in 7.14% (1/14) of cases, as "doubtful" in 7.14% (1/14) of cases. The analysis of the AEs outcomes frequency in the patients showed that in the group of patients who had received the study drug, "recovery without consequences" was notified in 100% (14/14) of cases.

The frequency of patients in the standard therapy group with reported cases of AEs was 6.06% (8/132). A total of 10 AEs were notified in 8 patients of the standard therapy group. Among the registered AEs, 90.00% (9/10) were of a mild severity, 10.00% (1/10) were of a moderate severity.

According to the investigators, a causal relationship with standard therapy was assessed as "possible" in 70.00% (7/10) of cases, as "not related" in 10.00% (1/10) of cases, as "probable" in 10.00% (1/10) of cases, as "doubtful" in 10.00% (1/10) of cases.

As a result of the AEs comparative analysis in terms of their presence, severity, causal relationship with the therapy and outcomes, no statistically significant differences were found between the observation groups. It was shown that taking the study drug does not adversely affect the ECG and other vital functions (BP, heart rate, etc.). No serious AEs, including deaths, were reported during the study.

In the nirmatrelvir + ritonavir group, the following adverse events classified by frequency, were identified: increased ALT levels – 3 (2.3%), increased AST levels – 3 (2.3%), dysgeusia – 3 (2.3%), diarrhea – 1 (0.8%), dry mouth – 1 (0.8%). In the standard therapy group, the following adverse events were identified: nausea 2 (1.5%), increased ALT levels – 3 (2.3%), increased AST levels – 3 (2.3%), diarrhea 1 (0.8%), erythema – 1 (0.8%).

The carried out analysis of adverse events in the therapy groups showed no statistically significant differences between the drug group (nirmatrelvir + ritonavir) and the standard therapy group.

## CONCLUSION

Thus, it can be argued that therapy with a fixed combination of nirmatrelvir + ritonavir is characterized by a favorable safety profile comparable to standard therapy. The identified adverse events were transient in nature and did not require discontinuation of therapy or changes in the treatment regimen.

The unique technology developed in the Russian Federation, which made it possible to combine both active substances into one fixed dosage form, reduces a number of tablets used, by 6 times compared to the American analogue, which reduces polypharmacy and increases adherence and safety of therapy in general [27].

As a result of the clinical study "Open-label two-stage multicenter study on evaluation of main pharmacokinetic parameters, safety, and efficacy against COVID-19 of drug Skyvira® in adult population", the advantage of therapy with a fixed nirmatrelvir (300 mg) + ritonavir (100 mg) combination was demonstrated over the standard therapy in COVID-19 patients, as well as the comparability of the pharmacokinetic profile of the drug with the available data on the components.

The advantage of Skyvira® therapy over standard

therapy in COVID-19 patients has been demonstrated in terms of reducing the risk of a heavier severity level of the disease and hospitalization, the rate of the virus elimination, the dynamics of reducing the symptoms severity of the infectious disease, improving the general condition of patients and their clinical status, reducing the risks of complications development in the course of COVID-19 in both patients without and those with risk factors for the progression of COVID-19 to a heavier severity level.

The results of the study demonstrated a favorable efficacy and safety profile of Skyvir (fixed combination: nirmatrelvir 300 mg + ritonavir 100 mg) when used in COVID-19 patients. The data obtained indicate a clinical and pharmacoeconomic feasibility of the therapy.

## FUNDING

The clinical study was carried out with the support of LLC PROMOMED RUS. The sponsor had no influence on the choice of material for publication, analysis and interpretation of the data.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## AUTHORS' CONTRIBUTION

LAB – development and implementation of research design, text writing and editing; NMS – study design implementation, data processing; EIG – study design implementation, data processing, OISh – study design implementation, data processing; NVK – study design implementation, processing of the obtained data; ENS – study design implementation, processing of the obtained data; KBK – study design development and implementation, research design implementation, text editing; DYuP – study design development and implementation, results analysis, text editing; DNZ – literary sources analysis, results analysis; KYaZ – research design development, text editing, literary sources analysis; SMN – study design implementation, processing of the obtained data; AVT – research data processing, results analysis; PAB – research design development, text editing.

## REFERENCES

1. To KK, Sridhar S, Chiu KH, Hung DL, Li X, Hung IF, Tam AR, Chung TW, Chan JF, Zhang AJ, Cheng VC, Yuen KY. Lessons learned 1 year after SARS-CoV-2 emergence leading to COVID-19 pandemic. Emerg Microbes Infect. 2021 Dec;10(1):507–35. DOI: 10.1080/22221751.2021.1898291
2. Tsang HF, Chan LWC, Cho WCS, Yu ACS, Yim AKY, Chan AKC, Ng LPW, Wong YKE, Pei XM, Li MJW, Wong SC. An update on COVID-19 pandemic: the epidemiology, pathogenesis, prevention and treatment strategies. Expert Rev Anti Infect Ther. 2021 Jul;19(7):877–88. DOI: 10.1080/14787210.2021.1863146
3. Sreepadmanabh M, Sahu AK, Chande A. COVID-19: Advances in diagnostic tools, treatment strategies, and vaccine development. J Biosci. 2020;45(1):148. DOI: 10.1007/s12038-020-00114-6
4. Wen W, Chen C, Tang J, Wang C, Zhou M, Cheng Y, Zhou X, Wu Q, Zhang X, Feng Z, Wang M, Mao Q. Efficacy and safety of three new oral antiviral treatment (molnupiravir, fluvoxamine and Paxlovid) for COVID-19:a meta-analysis. Ann Med. 2022 Dec;54(1):516–23. DOI: 10.1080/07853890.2022.2034936
5. Hung YP, Lee JC, Chiu CW, Lee CC, Tsai PJ, Hsu IL, Ko WC. Oral Nirmatrelvir/Ritonavir Therapy for COVID-19: The Dawn in the Dark? Antibiotics (Basel). 2022 Feb 9;11(2):220. DOI: 10.3390/antibiotics11020220
6. Anka AU, Tahir MI, Abubakar SD, Alsabbagh M, Zian Z, Hamedifar H, Sabzevari A, Azizi G. Coronavirus disease 2019 (COVID-19): An overview of the immunopathology, serological diagnosis and management. Scand J Immunol. 2021 Apr;93(4):e12998. DOI: 10.1111/sji.12998
7. Vangeel L, Chiu W, De Jonghe S, Maes P, Slechten B, Raymenants J, André E, Leyssen P, Neyts J, Jochmans D. Remdesivir, Molnupiravir and Nirmatrelvir remain active against SARS-CoV-2 Omicron and other variants of concern. Antiviral Res. 2022 Feb;198:105252. DOI: 10.1016/j.antiviral.2022.105252
8. Reina J, Iglesias C. Nirmatrelvir más ritonavir (Paxlovid) una potente combinación inhibidora de la proteasa 3CLpro del SARS-CoV-2 [Nirmatrelvir plus ritonavir (Paxlovid) a potent SARS-CoV-2 3CLpro protease inhibitor combination]. Rev Esp Quimioter. 2022 Jun;35(3):236–40. Spanish. DOI: 10.37201/req/002.2022
9. Paxlovid for treatment of COVID-19. Med Lett Drugs Ther. 2022 Jan 24;64(1642):9–10.
10. Eng H, Dantonio AL, Kadar EP, Obach RS, Di L, Lin J, Patel NC, Boras B, Walker GS, Novak JJ, Kimoto E, Singh RSP, Kalgutkar AS. Disposition of Nirmatrelvir, an Orally Bioavailable Inhibitor of SARS-CoV-2 3C-Like Protease, across Animals and Humans. Drug Metab Dispos. 2022 May;50(5):576–90. DOI: 10.1124/dmd.121.000801
11. Buxeraud J, Faure S, Fougere É. Le nirmatrelvir-ritonavir (Paxlovid®), un traitement contre la Covid-19 [Nirmatrelvir/ritonavir (Paxlovid®), a treatment for Covid-19]. Actual Pharm. 2022 Jun;61(617):10–2. French. DOI: 10.1016/j.actpha.2022.05.002
12. Saravolatz LD, Depcinski S, Sharma M. Molnupiravir and Nirmatrelvir-Ritonavir: Oral COVID Antiviral Drugs. Clin Infect Dis. 2022 Mar 4:ciac180. DOI: 10.1093/cid/ciac180
13. Wong CKH, Au ICH, Lau KTK, Lau EHY, Cowling BJ, Leung GM. Real-world effectiveness of molnupiravir and nirmatrelvir plus ritonavir against mortality, hospitalisation, and in-hospital outcomes among community-dwelling, ambulatory patients with confirmed SARS-CoV-2 infection during the omicron wave in Hong Kong: an observational study. Lancet. 2022 Oct 8;400(10359):1213–22. DOI: 10.1016/S0140-6736(22)01586-0
14. Drożdżal S, Rosik J, Lechowicz K, Machaj F, Szostak B,

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2022-10-4-371-386

Przybyciński J, Lorzadeh S, Kotfis K, Ghavami S, Łos MJ. An update on drugs with therapeutic potential for SARS-CoV-2 (COVID-19) treatment. Drug Resist Updat. 2021 Dec;59:100794. DOI: 10.1016/j.drup.2021.100794

15. Rubin EJ, Baden LR. The Potential of Intentional Drug Development. N Engl J Med. 2022 Apr 14;386(15):1463–4. DOI: 10.1056/NEJMe2202160
16. Hammond J, Leister-Tebbe H, Gardner A, Abreu P, Bao W, Wisemandle W, Baniecki M, Hendrick VM, Damle B, Simón-Campos A, Pypstra R, Rusnak JM; EPIC-HR Investigators. Oral Nirmatrelvir for High-Risk, Nonhospitalized Adults with Covid-19. N Engl J Med. 2022 Apr 14;386(15):1397–408. DOI: 10.1056/NEJMoa2118542
17. Vassilopoulos A, Mylonakis E. In patients with COVID-19 at risk for severe disease, nirmatrelvir + ritonavir reduced hospitalization or death. Ann Intern Med. 2022 Jun;175(6):JC63. DOI: 10.7326/J22-0038
18. Sacks HS. In nonhospitalized, unvaccinated adults with COVID-19, molnupiravir reduced hospitalization or death at 29 d. Ann Intern Med. 2022 Apr;175(4):JC40. DOI: 10.7326/J22-0017
19. Najjar-Debbiny R, Gronich N, Weber G, Khoury J, Amar M, Stein N, Goldstein LH, Saliba W. Effectiveness of Paxlovid in Reducing Severe COVID-19 and Mortality in High Risk Patients. Clin Infect Dis. 2022 Jun 2:ciac443. DOI: 10.1093/cid/ciac443
20. Lee JT, Yang Q, Gribenko A, Perrin BS Jr, Zhu Y, Cardin R, Liberator PA, Anderson AS, Hao L. Genetic Surveillance of SARS-CoV-2 Mpro Reveals High Sequence and Structural Conservation Prior to the Introduction of Protease Inhibitor Paxlovid. mBio. 2022 Aug 30;13(4):e0086922. DOI: 10.1128/mbio.00869-22
21. Greasley SE, Noell S, Plotnikova O, Ferre R, Liu W, Bolanos B, Fennell K, Nicki J, Craig T, Zhu Y, Stewart AE, Steppan CM. Structural basis for the in vitro efficacy of nirmatrelvir against SARS-CoV-2 variants. J Biol Chem. 2022 Jun;298(6):101972. DOI: 10.1016/j.jbc.2022.101972
22. Lamb YN. Nirmatrelvir Plus Ritonavir: First Approval. Drugs. 2022 Apr;82(5):585-591. DOI: 10.1007/s40265-022-01692-5. Erratum in: Drugs. 2022 Jun;82(9):1025.
23. Fishbane S, Hirsch JS, Nair V. Special Considerations for Paxlovid Treatment Among Transplant Recipients With SARS-CoV-2 Infection. Am J Kidney Dis. 2022 Apr;79(4):480–2. DOI: 10.1053/j.ajkd.2022.01.001
24. Arbel R, Wolff Sagy Y, Hoshen M, Battat E, Lavie G, Sergienko R, Friger M, Waxman JG, Dagan N, Balicer R, Ben-Shlomo Y, Peretz A, Yaron S, Serby D, Hammerman A, Netzer D. Nirmatrelvir Use and Severe Covid-19 Outcomes during the Omicron Surge. N Engl J Med. 2022 Aug 24. DOI: 10.1056/NEJMoa2204919
25. Marzi M, Vakil MK, Bahmanyar M, Zarenezhad E. Paxlovid: Mechanism of Action, Synthesis, and In Silico Study. Biomed Res Int. 2022 Jul 7;2022:7341493. DOI: 10.1155/2022/7341493
26. Agarwal A, Rochwerg B, Lamontagne F, Siemieniuk RA, Agoritsas T, Askie L, Lytvyn L, Leo YS, Macdonald H, Zeng L, Amin W, Barragan FAJ, Bausch FJ, Burhan E, Calfee CS, Cecconi M, Chanda D, Dat VQ, De Sutter A, Du B, Freedman S, Geduld H, Gee P, Gotte M, Harley N, Hashimi M, Hunt B, Jehan F, Kabra SK, Kanda S, Kim YJ, Kissoon N, Krishna S, Kuppalli K, Kwizera A, Lado Castro-Rial M, Lisboa T, Lodha R, Mahaka I, Manai H, Mino G, Nsutebu E, Preller J, Pshenichnaya N, Qadir N, Relan P, Sabzwari S, Sarin R, Shankar-Hari M, Sharland M, Shen Y, Ranganathan SS, Souza JP, Stegemann M, Swanstrom R, Ugarte S, Uyeki T, Venkatapuram S, Vuyiseka D, Wijewickrama A, Tran L, Zeraatkar D, Bartoszko JJ, Ge L, Brignardello-Petersen R, Owen A, Guyatt G, Diaz J, Kawano-Dourado L, Jacobs M, Vandvik PO. A living WHO guideline on drugs for covid-19. BMJ. 2020 Sep 4;370:m3379. doi: 10.1136/bmj.m3379. Update in: BMJ. 2020 Nov 19;371:m4475. Update in: BMJ. 2021 Mar 31;372:n860. Update in: BMJ. 2021 Jul 6;374:n1703. Update in: BMJ. 2021 Sep 23;374:n2219. Erratum in: BMJ. 2022 Apr 25;377:o1045.
27. Chulanov VP, Pushkar DYu, Gorelov AV, Balykova LA, Lioznov DA, Dmitriev AS, Kolontarev KB, Malyavin AG, Budnevsky AV, Esaulenko EV, Pshenichnaya NYu, Omarova HG, Taganov AV, Petrov VA. [Expert opinion. New possibilities of etiotropic therapy for coronavirus infection]. Infectious diseases. 2022;20(2):120–2. DOI: 10.20953/1729-9225-2022-2-120-122. Russian

## AUTHORS

**Larisa A. Balykova** – Doctor of Sciences (Medicine), Professor, Corresponding Member of the Russian Academy of Sciences, Head of the Department of Pediatrics, Director of National Research Ogarev Mordovia State University. ORCID ID: 0000-0002-2290-0013. E-mail: larisabalykova@yandex.ru

**Natalya M. Selezneva** – Doctor of Sciences (Medicine), Associate Professor of the Department of Hospital Therapy, National Research Ogarev Mordovia State University. ORCID ID: 0000-0002-3004-2063. E-mail: nata_rm@mail.ru

**Elena I. Gorshenina** – Doctor of Sciences (Medicine), Associate Professor, Department of Hospital Therapy, Medical Institute of National Research Ogarev Mordovia State University. ORCID ID 0000-0001-9342-0909. E-mail: lena.medfak@yandex.ru

**Olga I. Shepeleva** – Doctor of Sciences (Medicine), Associate Professor, Department of Hospital Therapy, Medical Institute, National Research Ogarev Mordovia State University. ORCID ID: 0000-0001-8307-2787. E-mail: shepeleva-oi@rambler.ru

**Natalya V. Kirichenko –** pulmonologist, Deputy Chief Physician for medical affairs, Ivanovo Clinical Hospital n. a. the Kuvaevs, Ivanovo, Russia. ORCID ID: 0000-0002-4272-9540. E-mail: igb2@ivreg.ru

**Elena N. Simakina** – infectious disease specialist, Head of the Infectious Diseases Department of Smolensk clinical hospital No. 1, Smolensk, Russia ORCID ID: 0000-0002-5709-8913. E-mail: e.simakina@mail.ru

**Konstantin B. Kolontarev** – Doctor of Sciences (Medicine), Professor of the Department of Urology, Evdokimov Moscow State Medical and Dental University; urologist, Spasokukotsky City Clinical Hospital, Department of Health of the city of Moscow. ORCID ID:

0000-0003-4511-5998. E-mail: kbk@mail.ru

**Dmitry Yu. Pushkar** – Doctor of Sciences (Medicine), Professor, Head of the Department of Urology, Evdokimov Moscow State Medical and Dental University; urologist, Spasokukotsky City Clinical Hospital, Department of Health of the city of Moscow; academician of the Russian Academy of Sciences. ORCID ID: 0000-0002-6096-5723. E-mail: pushkardm@mail.ru

**Dmitry N. Zemskov** – assistant of the Department of Biological and Pharmaceutical Chemistry with a Course of Organization and Management of Pharmacy, Medical Institute of National Research Ogarev Mordovia State University. ORCID ID: 0000-0002-0181-4327. E-mail: dizem1978@gmail.com

**Kira Ya. Zaslavskaya** – assistant of the Department of Biological and Pharmaceutical Chemistry with a Course of Organization and Management of Pharmacy, Medical Institute of National Research Ogarev Mordovia State University. ORCID ID: 0000-0002-7348-9412. E-mail: kiryonok@yandex.ru

**Sergey M. Noskov** – Doctor of Sciences (Medicine), Head of the Department of Hospital Therapy, Yaroslavl State Medical University; general practitioner, Clinical Hospital No. 3, Yaroslavl, Russia. ORCID ID: 0000-0003-3456-9409. E-mail: Noskov03@gmail.com

**Aleksey V. Taganov** – Doctor of Sciences (Medicine), Professor of the Department of Dermatovenereology with a Course of Cosmetology of the Faculty of Continuous Medical Education, Peoples' Friendship University of Russia. ORCID ID: 0000-0001-5056-374X. E-mail: matis87177@yandex.ru

**Petr A. Bely** – Candidate of Sciences (Medicine), Senior Laboratory Assistant, Department of Propaedeutics of Internal Diseases and Gastroenterology, Evdokimov Moscow State Medical and Dental University. ORCID ID: 0000-0001-5998-4874. E-mail: pbely@ncpharm.ru

Научно-практический журнал
ФАРМАЦИЯ И ФАРМАКОЛОГИЯ

*ОРИГИНАЛЬНАЯ СТАТЬЯ*

*DOI: 10.19163/2307-9266-2022-10-4-387-399*



# SYNERGIC EFFECT OF PREPARATION WITH COORDINATION COMPLEX "TRIMETHYDRAZINIUM PROPIONATE+ETHYMTH METHYLHYDROXYPIRIDINE SUCCINATE" ON ENERGY METABOLISM AND CELL RESPIRATION

**M.V. Zhuravleva[1,2], M.V. Granovskaya[3], K.Ya. Zaslavskaya[4], Yu.G. Kazaishvili[5], V.S. Shcherbakova[5], A.A. Andreev-Andrievsky[6], D.I. Pozdnyakov[7], M.Yu. Vysokikh[8]**

[1] Scientific Center for Expertise of Medicinal Products,
Bldg. 2, 8, Petrovsky Blvd., Moscow, Russia, 127051
[2] Sechenov First Moscow State Medical University (Sechenov University),
Bld. 2, 8, Trubetskaya Str., Moscow, Russia, 119991
[3] Institute of Systems Biology of University College Dublin,
4, Dublin, Belfield, Ireland, D04 V1W8
[4] National Research Ogarev Mordovia State University,
68, Bolshevistskaya Str., Saransk, Republic of Mordovia, Russia, 430005
[5] Tver State Medical University,
4, Sovetskaya Str., Tver, Russia, 170100
[6] Institute for Biomedical Problems of the Russian Academy of Sciences,
76A, Khoroshevskoe Hwy, Moscow, Russia, 123007
[7] Pyatigorsk Medical and Pharmaceutical Institute – branch of Volgograd State Medical University,
11, Kalinin Ave., Pyatigorsk, Russia, 357532
[8] A.N. Belozersky Institute of Physico-Chemical Biology of Moscow State University named after M.V. Lomonosov,
Bldg. 40, 1, Leninskie Gory, Moscow, Russia, 119234

E-mail: kiryonok@yandex.ru

Received 08 Jule 2022 After peer review 12 Aug 2022 Accepted 22 Aug 2022

The article presents the results of an *in vitro* study of the synergetic effect evaluation of the combined preparation based on coordination complex ethylmethylhydroxypyridine succinate and trimethylhydrazinium propionate on energy metabolism and cell respiration.

**The aim** of the study was to evaluate the mitochondria-directed action of the metabolic and antioxidant preparation based on succinic acid coordination complex with trimethylhydrazinium in relation to optimizing the energy metabolism in the cells under the oxidative stress conditions, as well as against the background of ischemic processes.

**Materials and methods.** The study of the hydroxysuccinate complex effect of the drug Brainmax® components was carried out on isolated mouse liver mitochondria. In the course of the study, the potential of mitochondria, the generation rate of hydrogen peroxide during the respiration, the respiration rate were evaluated in the following positions: a) unstimulated by malate and pyruvate, b) stimulated by malate and pyruvate (complex I substrates), by succinate (complex II substrates), c) against the background of the initial section of the electron transport chain blockade by rotenone, d) in phosphorylation blockade by oligomycin, e) against the background of the FCCP-induced uncoupling, and f) in cyanide-blocked complex IV (cytochrome C oxidase).

**Results.** It has been shown that the succinic acid coordination complex with trimethylhydrazinium, which is the active principle of the Brainmax® drug, significantly reduced the transmembrane potential of mitochondria ($IC_{50}$=197±5 μM), compared with the widely used preparations of ethylmethylhydroxypyridine succinate and trimethylhydrazinium propionate, which facilitates the transfer of the produced ATP into the cell and preserves a vital activity of mitochondria even under stress. In the study of

**For citation:** M.V. Zhuravleva, M.V. Granovskaya, K.Ya. Zaslavskaya, Yu.G. Kazaishvili, V.S. Shcherbakova, A.A. Andreev-Andrievsky, D.I. Pozdnyakov, M.Yu. Vysokikh. Synergic effect of preparation with coordination complex "trimethydrazinium propionate+ethymth methylhydroxypiridine succinate" on energy metabolism and cell respiration. *Pharmacy & Pharmacology.* 2022;10(4):387-399. **DOI:** 10.19163/2307-9266-2022-10-4-387-399



**Для цитирования:** М.В. Журавлева, М.В. Грановская, К.Я. Заславская, Ю.Г. Казаишвили, В.С. Щербакова, А.А. Андреев-Андриевский, Д.И. Поздняков, М.Ю. Высоких. Синергическое действие препарата с координационным комплексом триметилгидразиния пропионата и этилметилгидроксипиридина сукцината на энергетический обмен и дыхание клетки. *Фармация и фармакология.* 2022;10(4):387-399. **DOI:** 10.19163/2307-9266-2022-10-4-387-399

*RESEARCH ARTICLE*

*ISSN 2307-9266 e-ISSN 2413-2241*

Scientific and Practical Journal
PHARMACY & PHARMACOLOGY

the mitochondrial respiration stimulated by the substrates of complex I (NADP-coenzyme Q-oxidoreductase), pyruvate and malate, the studied drug led to a more pronounced increase in the oxygen consumption with $IC_{50}$=75±6 μM. When evaluating the effect of the complex on the production of ATP by mitochondria, the most pronounced effect was observed with the addition of studied complex, which indicated to the uncoupling of respiration and oxidative phosphorylation at the given concentrations of the studied compounds. When assessing the effect of the complex on the production of hydrogen peroxide by isolated mitochondria, a significant decrease in the peroxide production was shown in the samples containing the complex of trimethylhydrazinium propionate and EMHPS.

**Conclusion.** Based on totality of the results obtained, it can be assumed that a favorable conformation of the pharmacophore groups of ethylmethylhydroxypyridine succinate and trimethylhydrozinium propionate coordination complex included in the composition of Brainmax® leads to a synergetic interaction and more pronounced pharmacological effects on target cells. This complex provides stabilization of a mitochondrial function, intensification of the adenosine triphosphate energy production and the optimization of energy processes in the cell, reduces the severity of the oxidative stress and eliminates undesirable effects of an ischemic-hypoxic tissue damage.

**Keywords:** ethylmethylhydroxypyridine succinate; trimethylhydrazinium propionate; succinic acid coordination complex with trimethylhydrazinium; hydroxysuccinate complex with trimethylhydrazinium; mitochondria; breath; oxidative stress; peroxide production.

**Abbreviations:** EMHPS – ethylmethylhydroxypyridine succinate; TMHP – trimethylhydrozinium propionate; FCCP – carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; ATP – adenosine triphosphate; ATPase – adenosine triphosphatase; ROS – reactive oxygen species; DAMPs – damage associated molecular patterns; ADP – adenosine diphosphate; NADP – nicotinamide adenine dinucleotide phosphate; MUPs – mitochondrial uncoupling proteins.

# СИНЕРГИЧЕСКОЕ ДЕЙСТВИЕ ПРЕПАРАТА С КООРДИНАЦИОННЫМ КОМПЛЕКСОМ ТРИМЕТИЛГИДРАЗИНИЯ ПРОПИОНАТА И ЭТИЛМЕТИЛГИДРОКСИПИРИДИНА СУКЦИНАТА НА ЭНЕРГЕТИЧЕСКИЙ ОБМЕН И ДЫХАНИЕ КЛЕТКИ

**М.В. Журавлева[1,2], М.В. Грановская[3], К.Я. Заславская[4], Ю.Г. Казаишвили[5], В.С. Щербакова[5], А.А. Андреев-Андриевский[6], Д.И. Поздняков[7], М.Ю. Высоких[8]**

[1] Федеральное государственное бюджетное учреждение
«Научный центр экспертизы средств медицинского применения»
Министерства здравоохранения Российской Федерации,
127051, Россия, г. Москва, Петровский бульвар, д. 8, стр. 2
[2] Федеральное государственное автономное образовательное учреждение высшего образования
Первый Московский государственный медицинский университет имени И.М. Сеченова
Министерства здравоохранения Российской Федерации (Сеченовский Университет),
119991, Россия, г. Москва, ул. Трубецкая, д. 8, стр. 2
[3] Институт системной биологии, Университетский колледж Дублина,
D04 V1W8, Ирландия, Белфилд, Дублин, 4
[4] Федеральное государственное бюджетное образовательное учреждение высшего образования
«Национальный исследовательский Мордовский государственный университет им. Н.П. Огарёва»,
430005, Россия, Республика Мордовия, г. Саранск, ул. Большевистская, д. 68
[5] Федеральное государственное бюджетное образовательное учреждение высшего образования
«Тверской государственный медицинский университет Минздрава России,
170100, Россия, г. Тверь, ул. Советская, д. 4
[6] Федеральное государственное бюджетное учреждение науки
Государственный научный центр Российской Федерации Институт медико-биологических проблем
Российской академии наук,
123007, Россия, г. Москва, Хорошевское шоссе, д. 76А
[7] Пятигорский медико-фармацевтический институт – филиал федерального государственного
бюджетного образовательного учреждения высшего образования
«Волгоградский государственный медицинский университет»
Министерства здравоохранения Российской Федерации,
357532, Россия, г. Пятигорск, пр-т Калинина, д. 11
[8] Государственное учреждение «Научно-исследовательский институт физико-химической биологии
имени А.Н. Белозерского» Московского государственного университета имени М.В. Ломоносова,
119992, Россия, г. Москва, Ленинские горы, д. 1, стр. 40

E-mail: kiryonok@yandex.ru

Получена 08.07.2022 После рецензирования 12.08.2022 Принята к печати 22.08.2022

Научно-практический журнал
ФАРМАЦИЯ И ФАРМАКОЛОГИЯ

*ОРИГИНАЛЬНАЯ СТАТЬЯ*

*DOI: 10.19163/2307-9266-2022-10-4-387-399*

В статье представлены результаты *in vitro* исследования оценки синергического действия препарата лекарственного препарата на основе координационного комплекса этилметилгидроксипиридина сукцината и триметилгидразиния пропионата на энергетический обмен и дыхание клетки.

**Целью** данного исследования являлась оценка митохондриально-направленного действия метаболического и антиоксидантного лекарственного средства на основе янтарно-кислого координационного комплекса с триметилгидразинием в отношении оптимизации энергообмена в клетках в условиях оксидативного стресса, а также на фоне ишемических процессов.

**Материалы и методы.** Исследование действия гидросукцинатного комплекса компонентов препарата Брейнмакс® проводили на изолированных митохондриях печени мыши. В процессе исследования оценивали потенциал митохондрий, скорость генерации в ходе дыхания перекиси водорода, а также скорость дыхания: а) нестимулированного малатом и пируватом, б) стимулированного малатом и пируватом (субстраты комплекса I), сукцинатом (субстрат комплекса II), в) на фоне блокады начального участка электрон-транспортной цепи ротеноном, г) при блокаде фосфорилирования олигомицином, д) на фоне вызванного FCCP разобщения и е) при заблокированном цианидом комплексе IV (цитохром С оксидазе).

**Результаты.** Было показано, что янтарно-кислый координационный комплекс с триметилгидразинием, являющийся действующим началом лекарственного препарата Брейнмакс®, значимо снижал трансмембранный потенциал митохондрий ($IC_{50}$=197±5 μM), по сравнению с широко применяемыми препаратами этилметилгидроксипиридина сукцинатом и мельдонием, что облегчает перенос продуцируемых АТФ в клетку и сохраняет жизнедеятельность митохондрий даже в условиях стресса. При исследовании дыхания митохондрий, стимулированном субстратами комплекса I (НАДФ-коэнзимQ-оксидоредуктазы), пирувата и малата, изучаемый препарат приводил к более выраженному росту потребления кислорода с $IC_{50}$=75±6 μM. При оценке влияния комплекса на продукцию митохондриями АТФ, наиболее выраженное действие наблюдалось при добавлении изучаемого комплекса, что свидетельствовало о разобщении дыхания и окислительного фосфорилирования при данных концентрациях исследуемых соединений. При оценке влияния комплекса на продукцию изолированными митохондриями перекиси водорода, было показано значимое снижение продукции перекиси в пробах, содержащих комплекс триметилгидразиния пропионата и ЭМГПС.

**Заключение.** По совокупности полученных результатов можно предполагать, что выгодная конформация фармакофорных групп координационного комплекса этилметилгидроксипиридина сукцината и триметилгидрозиния пропионата в составе лекарственного препарата Брейнмакс® приводит к синергетическому взаимодействию и более выраженному фармакологическому воздействию на клетки-мишени. Данный комплекс обеспечивает стабилизацию митохондриальной функции, интенсификацию выработки энергии аденозинтрифосфата и оптимизацию энергетических процессов в клетке, снижает выраженность оксидативного стресса и устраняет нежелательные эффекты ишемически-гипоксического повреждения тканей.

**Ключевые слова:** этилметилгидроксипиридина сукцинат; триметилгидразиния пропионат; янтарно-кислый координационный комплекс с триметилгидразинием; гидроксисукцинатный комплекс с триметилгидразинием; митохондрии; дыхание; оксидативный стресс; продукция перекиси

**Список сокращений:** ЭМГПС – этилметилгидроксипиридинна сукцинат; ТМГП – триметилгидразиния пропионат; FCCP – карбонилцианид-n-трифторметокси-фенилгидразон; АТФ – аденозинтрифосфат; АТФ-азы – аденозинтрифосфатаза; АФК – активные формы кислорода; DAMPs – молекулярные паттерны клеточного повреждения; АДФ – аденозиндифосфат; НАДФ – никотинамидадениндинуклеотидфосфа́т; МРБ – митохондриальные разобщающие белки.

## INTRODUCTION

Pathological conditions associated with dyscirculatory disorders and tissue ischemia are the most common causes of death and a primary disability in the population. According to WHO Health Estimates[1], a coronary heart disease (terminal myocardial infarction) and an ischemic stroke occupy dominant positions in the list of leading non-communicable diseases with a high risk of death.

The pathogenesis of any ischemic tissue damage is based on an imbalance between the cells metabolic activity, expressed in the consumption of oxygen and substrates of biological oxidation, and an adequate delivery of essential nutrients [1].

Modern studies show: the key pathogenetic aspect that determines the severity of this imbalance is a violation of the cell mitochondria functional activity. Mitochondria are two-membrane organelles that perform many functions in cells. First of all, mitochondria are assigned the role of “energy stations” that provide an optimal pool of intracellular energy [2].

Mitochondria also regulate oxidation-reduction processes and apoptosis reactions. In this regard, disruption of a mitochondrial activity can lead to a deficiency of macroergic compounds, an increase in the generation of reactive oxygen species (ROS), and a premature cell death *via* the programmed pathway [3]. The main trigger initiating these processes is the lack of oxygen and oxidation substrates [4].

Vascular occlusion and subsequent hypoxia causes a number of severe biochemical and metabolic disorders that mediate a failure of the mitochondria functional activity. Cell metabolism switches from mitochondrial oxidative phosphorylation to anaerobic glycolysis, which leads to the intracellular accumulation of lactate and protons, lowering the pH with further activation adenosine triphosphatases (ATPases), primarily the $Na^+/H^+$ exchanger, but due to the rapid depletion of the energy resources in the form of ATP, there is an overload

[1] World Health Organization. The top 10 causes of death. Available from: https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death

of the cell with sodium ions and, as a result, calcium (under the conditions of sodium overload, $Na^+/Ca^{2+}$ ATPase is activated) [5].

A high intracellular calcium content in an ischemic cell disrupts the buffer capacity of mitochondria, and therefore the entry of calcium ions into the mitochondrial matrix is activated. [6]. Calcium ions entering the mitochondria cause the respiratory chain dysfunction, contributing to the hyperproduction of reactive oxygen species and activation of the cell death mechanism (necrosis, apoptosis). As a result of the calcium ions influx, mitochondria also "swell" (the phenomenon of mitochondrial blebbing) and are destroyed, releasing compounds into the cytosol increasing the degree of cellular damage [7].

First, these substances present damage associated molecular patterns (DAMPs) which activate the AGE/RAGE pathway, enhancing immunological reactivity in the ischemic focus [8].

Taking into account the peculiarities of the pathogenetic pathways of the ischemic cell damage described above and the central role of mitochondria in these processes, it is not surprising that the "energy stations" of cells have become the main target for the motivated cytoprotection. In order to correct mitochondrial dysfunction in ischemia, a number of chemically modified substances with a benz-γ-pyrone scaffold, ubiquinone, and a triphenylphosphonium linker [9], as well as the substances of a protein nature, are currently used. An example of them are peptides of the *Szeto-Schiller* group (SS-31) [9, 10]. However, a number of studies show that native, unmodified molecules can act as means of correcting a mitochondrial dysfunction. For example, succinates [11] or agents that bypass metabolic processes (trimethylhydrazinium propionate, trimetazidine) can prevent an irreversible damage to cell mitochondria [12, 13].

In the Russian Federation in 2022, a new original drug complex from the group of neuroprotectors and antioxidants, Brainmax®, was registered. This is an original fixed combination of ethylmethylhydroxypyridine succinate (EMHPS) and trimethylhydrazinium propionate (TMHP) in the form of capsules or a solution for intravenous and intramuscular injections. Trimethylhydrazinium propionate usually exists as a zwitterion (dihydrate) that has a positive charge on the hydrazine fragment and a negative charge on the carboxylate group [14]. It is described in the literature that salts of some polybasic acids (acid salts of fumaric and maleic acids, dihydrogen phosphate, acid salt of oxalic acid, mono- or disubstituted salt of mucic acid, salts of pamoic and orotic acids) in combination with trimethylhydrazinium propionate demonstrated special pharmacokinetic and pharmacodynamic properties [15]. A specific feature of the drug under consideration is the formation of a hydrosuccinate complex with trimethylhydrazinium during the preparation of the finished dosage form. The components of the complex are interconnected by hydrogen bonds and an electrostatic intermolecular interaction, which provides an advantageous conformation of pharmacophore fragments for better binding to receptors and a more pronounced effect. At the same time, it is important that the components of the complex have different action points of application, as a result of which, a synergistic effect can develop when they are used combined.

**THE AIM** of the study was to evaluate the mitochondria-directed action of the complex ethylmethylhydroxypyridine succinate and trimethylhydrazinium propionate included in composition of Brainmax®.

## MATERIALS AND METHODS

### Animals

The study included 50 CBA´B6 male mice aged 4-5 months, obtained from the Center for Genetic Resources of Laboratory Animals (Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences). The studies met the requirements of the Law of the Russian Federation "On the Protection of Animals from Cruelty" dated June 24, 1998, the rules of laboratory practice for preclinical studies in the Russian Federation (GOST 3 51000.3-96 and GOST R 53434-2009), and the directives of the European Community (86/ 609 EU), the rules of the International Recommendations of the European Convention for the Protection of Vertebrate Animals used in experimental studies (1997) and the Rules of Laboratory Practice adopted in the Russian Federation (Order of the Ministry of Health of the Russian Federation No. 708 dated 29.08.2010). The study protocol has undergone expert appraisal by the bioethics commission of the Scientific Research Institute of Mitoengineering of Moscow State University (Conclusion No. 171 dated 13 Jan 2022).

### Study design

The study was carried out on isolated mouse liver mitochondria. The potential of mitochondria, the generation rate of hydrogen peroxide, the respiration rate were evaluated in the following positions: a) unstimulated by malate and pyruvate, b) stimulated by malate and pyruvate (complex I substrates), by succinate (complex II substrates), c) against the background of the initial section of the electron transport chain blockade by rotenone, d) in phosphorylation blockade by oligomycin, e) against the background of the FCCP-induced uncoupling, and f) in cyanide-blocked complex IV (cytochrome C oxidase).

For each of the indicators in the three experiments, the following were recorded: 1) a reaction to meldonium, 2) a reaction to EMHPS, and 3) a reaction to the succinic acid coordination complex of trimethylhydrazinium propionate and EMHPS. For each of the experiments, 7 independent repetitions of the experiment were performed.

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2022-10-4-387-399

### Isolation of liver mitochondria

To obtain the liver, mice were euthanized by a cervical dislocation, after which the abdominal cavity of the animals was immediately opened and the liver was excised. The liver was placed in an ice-cold phosphate-buffered saline (pH=7.0) and kept on ice until mitochondria were isolated, but not longer than 3 min.

After homogenization in approximately 20 volumes of an isotonic solution, the suspension of liver tissue was transferred into test tubes and the remnants of intact tissues were precipitated by centrifugation at 1000 g and 4°C for 10 min. The supernatant was collected, avoiding the milky suspension being pipetted, on the surface and centrifuged at 14 000 g and 4°C for 10 min. The resulting dark precipitate of mitochondria was washed free from the upper light loose layer, collecting the latter with a pipette and washing the mitochondria with a buffer of the following composition: 250 mM sucrose, 20 mM Hepes/NaOH, pH 7.5, 0.5 mM EGTA, 0.1% BSA. The pellet was suspended in 0.5 ml of this buffer and carefully homogenized with 5–10 passes of a Teflon pestle in a 1 ml glass homogenizer (G-Biosciences, USA). The homogenate was quantitatively transferred into a new tube and mitochondria were precipitated by centrifugation at 12 000 g and 4°C for 10 min. The pellet was suspended on ice in 70–100 µl of the isolation buffer with a plastic pestle until a homogeneous suspension was obtained.

### Measurement of mitochondrial respiratory function

An aliquot of the obtained mitochondria preparation in the amount of 50 µg for the protein determined by the method with bicinchoninic acid (Pierce, USA) was used to determine the rate of respiration, the intensity of the oxidative phosphorylation, the degree of conjugation, and the calcium load of mitochondria. To determine the rate of respiration, the method of a direct registration of oxygen uptake using a highly sensitive oxigraph (Hansatech, England) was used. To do this, an aliquot of mitochondria was placed in a glass cuvette filled with 0.5 ml of a buffer containing: 120 mM sucrose, 75 mM KCl, 2 mM $MgCl_2$, 5 mM $KH_2PO_4$, 20 mM HEPES, pH=7.5 (titration with NaOH). The measurement of the oxygen consumption was carried out in a closed system at 37°C and constant stirring on a magnetic stirrer at the speed of 500 rpm.

The obtained values (a change in $O_2$ over time, $dO_2/dt$) were normalized to the protein content. Mitochondria were energized using substrates I and II of the respiratory chain complexes. After the registration of the respiration rate activated by adding 4 mM pyruvate to the system in the presence of 10 mM malate, the effect of the rotenone (2 µM) blocking complex I, was studied. To record the respiration rate of mitochondria with activated complex II, after the addition of rotenone, 1 mM potassium succinate was added to the measurement system.

The study of the conjugation degree of the obtained preparations was carried out in the presence of adenosine diphosphate (ADP) after the respiration stimulation by introducing 0.1 mM ADP into the system of 1 µg of oligomycin and the inhibition of the stimulated respiration was recorded. The ratio of the stimulated and unstimulated respiration rates was used as a characteristic value (respiratory control coefficient), which makes it possible to assess the quality of the mitochondria obtained preparation and their state in the tissues under study. The maximum rate of the uncoupled respiration was determined in the presence of 20–50 nM protonophore FCCP. When analyzing the respiration rates, the value corresponding to the oxygen consumption rate in the presence of 0.5 mM KCN was subtracted from all values.

To do this, 25 µl of mitochondrial protein was added to the microcuvette with a volume of 250 µl, the kinetics of changes in the 555/523 nm ratio were recorded in a two-wave mode on an Aminco DW2000 spectrophotometer (Olis Inc., USA) before and after the addition of the respiratory substrates and specific inhibitors of the electron transfer in the respiratory mitochondria chain such as rotenone, antimycin, malonate and myxothiazol. Dissipation of the transmembrane potential was achieved using FCCP.

### Measurement of calcium capacity

The calcium capacity of mitochondria was determined by titration while measuring the light scattering at 575 nm, spectrophotometrically in Cary Varian 300 (Agilent, США and in the medium of 250 mM sucrose, 2 mM $MgCl_2$, 5 mM $KH_2PO_4$, 20 mM HEPES, pH=7.4 (NaOH titration). At the same time, both the total amount of calcium, which induces a drop in absorption, corresponding to the maximum swelling of mitochondria in the iso-osmotic system (calcium capacity), and the kinetics of swelling, which characterizes the ability of mitochondria to transport calcium, were studied.

### Assessment of ATP synthesis

The level of adenosine triphosphate (ATP) synthesis was determined by the ATP-dependent luminescence of mitochondrial suspension in various states, when various respiratory chain complexes were energized by substrates. Since the level of the ATP production by mitochondria during aerobic oxidation of the substrates is determined by the activity of the ATP synthetase sensitive to oligomycin; the use of this inhibitor makes it possible to calculate the total maximum amount of the ATP synthesized in mitochondria, thus characterizing the differences in the ability of mitochondria to maintain energy metabolism.

*ISSN 2307-9266 e-ISSN 2413-2241*

Scientific and Practical Journal
PHARMACY & PHARMACOLOGY

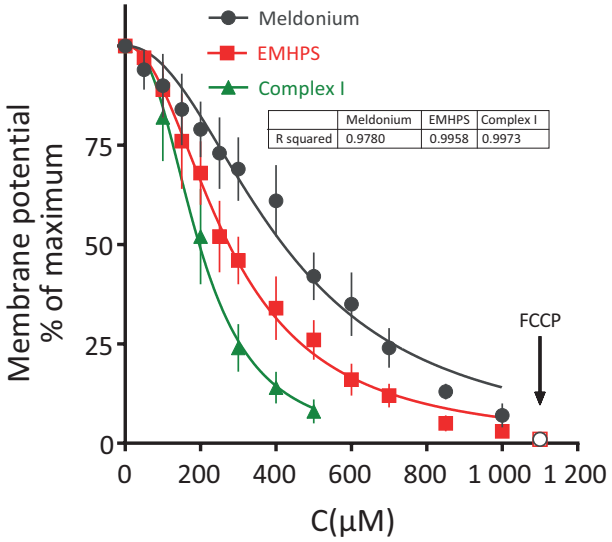



**Figure 1 – Dependence of mitochondria membrane potential on a meldonium dose, EMHPS or a complex of trimethylhydrazinium propionate and EMHPS (Complex I)**

Note: experimental data (M±SEM) are shown by dots, logistic regression is shown by lines; EMHPS – ethylmethylhydroxypyridine succinate.

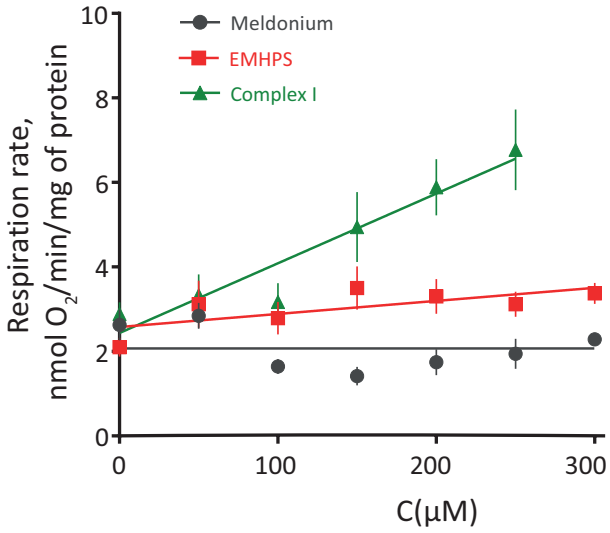



**Figure 2 – Basal respiration rate (without addition of exogenous substrates) of mouse liver mitochondria in presence of meldonium, EMHPS, or complex of components (Complex I)**

Note: experimental data (M±SEM) are shown by dots, logistic regression is shown by lines; EMHPS – ethylmethylhydroxypyridine succinate.

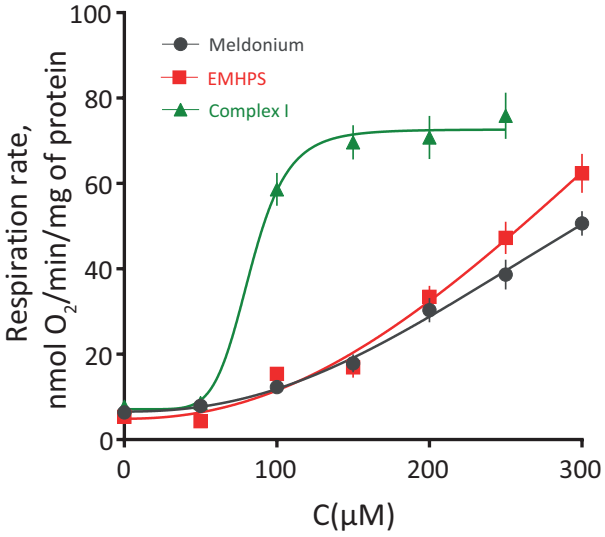



**Figure 3 – Respiration rate of mouse liver mitochondria stimulated with 5 mM pyruvate and 1 mM malate in presence of meldonium, EMHPS or a complex of components (Complex I)**

Note: experimental data (M±SEM) are shown by dots, logistic regression is shown by lines; EMHPS – ethylmethylhydroxypyridine succinate.

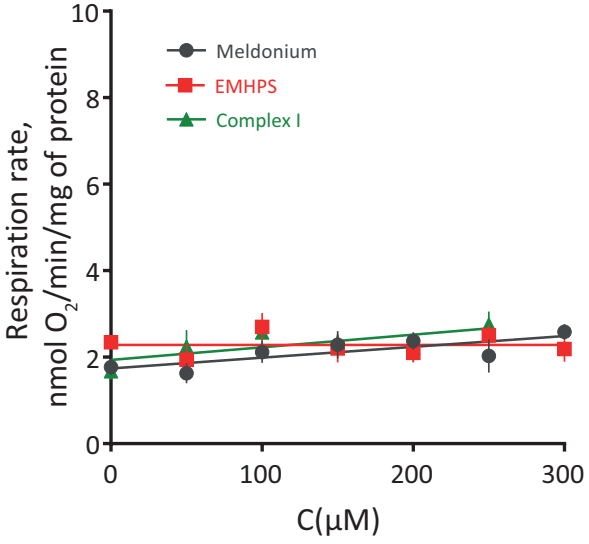



**Figure 4 – Respiratory rate of mouse liver mitochondria stimulated with 5 mM pyruvate and 1 mM malate in presence of meldonium, EMHPS or complex components (Complex I) and I inhibitor rotenone (2 μM)**

Note: experimental data (M±SEM) are shown by dots, logistic regression is shown by lines; EMHPS – ethylmethylhydroxypyridine succinate.

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2022-10-4-387-399

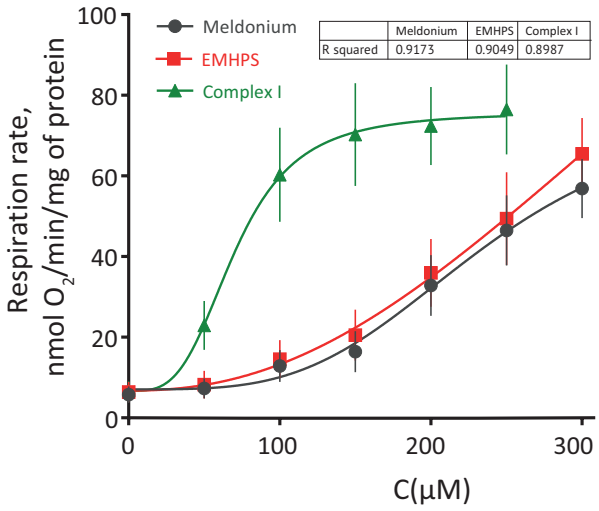



**Figure 5 – Respiratory rate of mouse liver mitochondria stimulated with 1 mM succinate in presence of meldonium, EMHPS, or studied complex (Complex I) and complex I inhibitor rotenone (2 μM)**

Note: experimental data (M±SEM) are shown by dots, logistic regression is shown by lines; EMHPS – ethylmethylhydroxypyridine succinate.

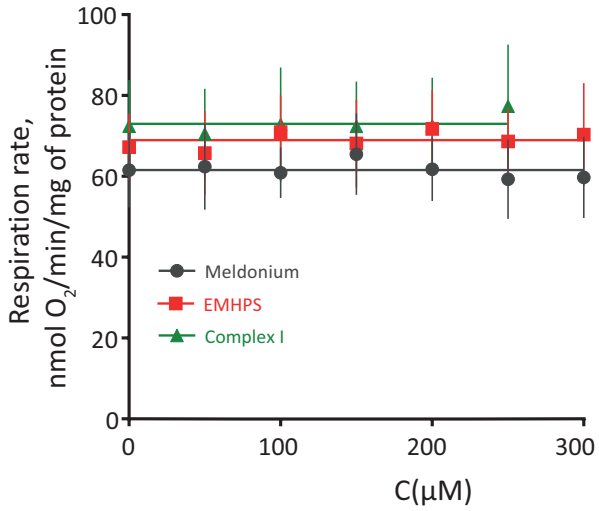



**Figure 6 – Respiration rate of mouse liver mitochondria stimulated by 1 mM ADP in presence of meldonium, EMHPS, or trimethylhydrazinium complex (Complex I)**

Note: experimental data (M±SEM) are shown by dots, logistic regression is shown by lines; EMHPS – ethylmethylhydroxypyridine succinate.

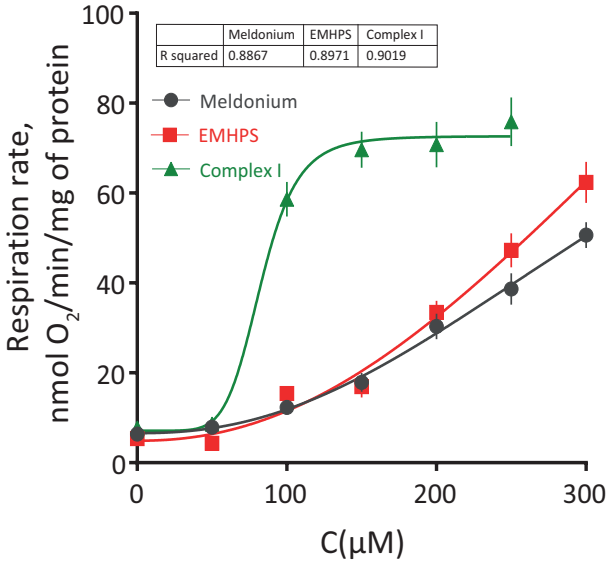



**Figure 7 – Respiratory rate of mouse liver mitochondria stimulated with 1 mM ADP in presence of meldonium, EMHPS, or complex under consideration (Complex I) and ATP synthase blocker oligomycin (1 μM)**

Note: experimental data (M±SEM) are shown by dots, logistic regression is shown by lines; EMHPS – ethylmethylhydroxypyridine succinate.

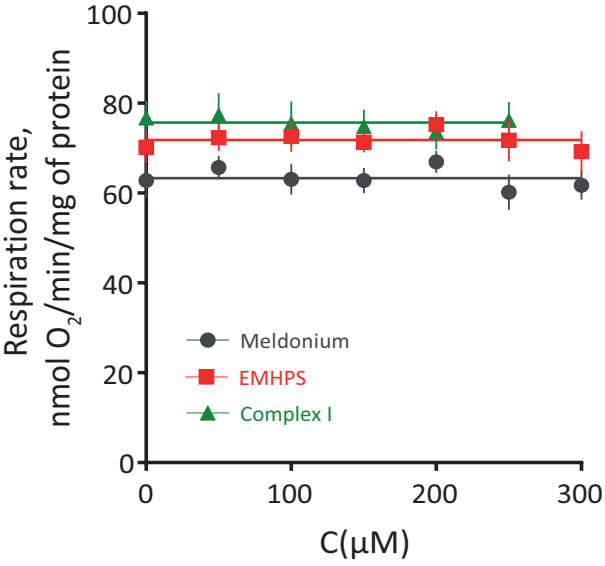



**Figure 8 – Respiration rate of mouse liver mitochondria stimulated with 1 mM ADP in presence of meldonium, EMHPS or complex under consideration (Complex I) and FCCP protonophore (1 μM)**

Note: experimental data (M±SEM) are shown by dots, logistic regression is shown by lines; EMHPS – ethylmethylhydroxypyridine succinate.

*ISSN 2307-9266 e-ISSN 2413-2241*

Scientific and Practical Journal
PHARMACY & PHARMACOLOGY

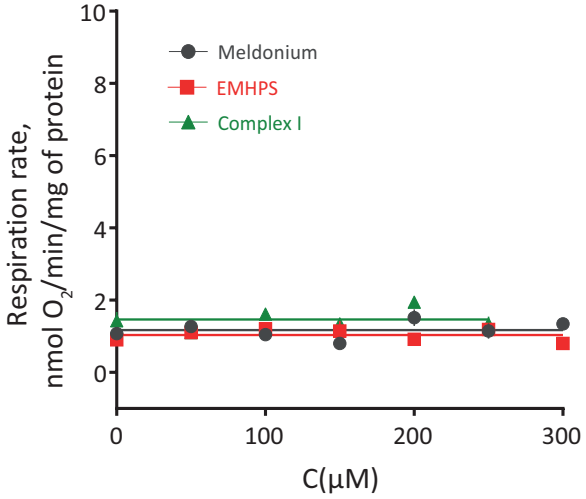



**Figure 9 – Respiration rate of mouse liver mitochondria stimulated by 1 mM ADP in presence of meldonium, EMHPS or studied complex (Complex I) and cyanide (1 μM)**

Note: experimental data (M±SEM) are shown by dots, logistic regression is shown by lines; EMHPS – ethylmethylhydroxypyridine succinate.

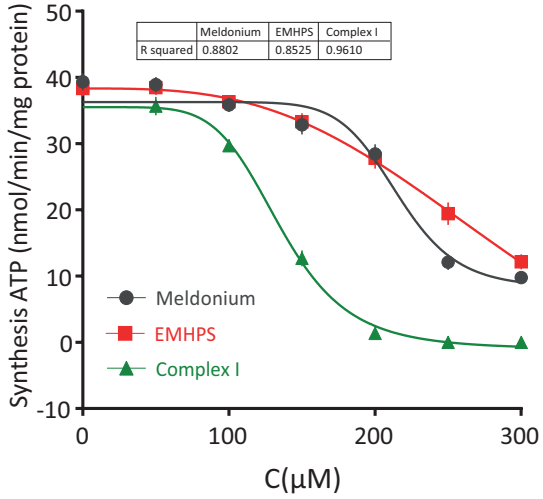



**Figure 10 – The ATP synthesis rate by mouse liver mitochondria in presence of meldonium, EMHPS or studied complex (Complex I)**

Note: experimental data (M±SEM) are shown by dots, logistic regression is shown by lines; EMHPS – ethylmethylhydroxypyridine succinate.

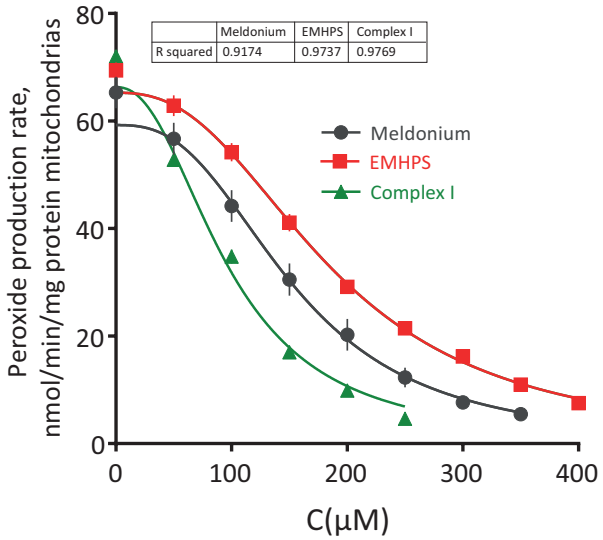



**Figure 11 – $H_2O_2$ production rate of mouse liver mitochondria in presence of meldonium, EMHPS or complex EMHPS with trimethylhydrazinium (Complex I)**

Note: experimental data (M±SEM) are shown by dots, logistic regression is shown by lines; EMHPS – ethylmethylhydroxypyridine succinate.

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2022-10-4-387-399

### Measurement of hydrogen peroxide generation

The generation of hydrogen peroxide by mitochondria was assessed using horseradish peroxidase (Thermo Scientific, USA) and its fluorogenic substrate Amplex Red reagent (Thermo Scientific, USA), on a Cary Eclipse fluorescent spectrophotometer (Agilent, USA) in the presence of a catalase inhibitor with a specific inhibitor of 3-amino-1,2,4-triazole.

### Statistical data analysis

For the primary analysis, the data were tabulated and descriptive statistics were calculated: mean (M), standard deviation (SD), a standard error of the mean (SEM). The data obtained submitted the Gaussian distribution law, on the basis of which parametric methods of statistic processing were chosen. Statistical analysis of the data was performed using non-linear regression methods, the Student's test for the comparison and a one-way variance analysis (for the comparison of several samples). The differences were considered significant at $p < 0.05$. The data were analyzed using Microsoft Excel 2016 (Microsoft, USA) and Prism 5.0 (Graphpad, USA) software.

## RESULTS

### Measurement of the transmembrane potential of mitochondria

When evaluating the effect of the studied drugs on the indicators of the mitochondria transmembrane potential, it was shown that when the increasing drugs concentrations were added, it decreased. The least pronounced effect was exerted meldonium, for which the value of the semi-effective concentration ($IC_{50}$) was 419±17 μM. EMHPS had a significantly ($F(1.22) = 82.90$; $p < 0.0001$) more pronounced effect on the potential ($IC_{50}$=275±6 μM).

The complex of trimethylhydrazinium propionate and EMHPS components reduced the potential of mitochondria with IC50=197±5 μM, which is significantly less compared to only meldonium ($F(1.15) = 166.5$; $p < 0.0001$) or only with EMHPS ($F(1.22) = 107.5$; $p < 0.0001$). When the uncoupler FCCP was added, the mitochondrial potential decreased to zero, regardless of the presence of meldonium, EMHPS, or the studied complex in the incubation mixture (Fig. 1).

### Mitochondrial respiration

In the absence of the exogenous respiration, the background respiration of mitochondria did not change with the addition of increasing concentrations of meldonium. EMHPS also insignificantly increased the rate of mitochondrial basal respiration from 2.2±0.2 to 3.4±0.25 nmol $O_2$/min/mg protein ($F(1.47) = 4.34$; $p = 0.0426$), and when the complex of the components was added, the rate of basal respiration increased from 2.9±0.3 to 6.8±1.0 nmol $O_2$/min/mg of protein ($F(1.40) = 28.95$; $p < 0.0001$), which may indicate the uncoupling of mitochondrial respiration by the action of the TMHP and EMHPS complex (Fig. 2). This effect makes it possible for protons to translocate into the intermembrane space *via* specific respiratory complexes of the electron transport chain and return to the mitochondrial matrix independently of ATP synthase. The established proton "leakage" is an important mechanism for the distribution of energy in the cell and accounts for up to 25% of the basal metabolism. Uncoupling of mitochondria against the background of the action of the components of the complex under study can be considered as a cytoprotective strategy mediated by mitochondrial uncoupling proteins (MUPs) under the conditions of the oxidative stress in any ischemic injury, including aging processes, as well as diabetes and resistance to anticancer drugs.

Then, the mitochondrial respiration stimulated by the substrates of complex I (NADP coenzyme Q oxidoreductases), pyruvate, and malate, was studied (Fig. 3). An increase in the respiration rate stimulated by the substrates of complex I, was found out with an increase in the concentration of the succinic acid complex of TMHP and EMHPS in the cell. Semi-effective concentrations of meldonium and EMHPS were 273±67 and 350±204 μM, respectively, and did not differ significantly ($F(1.90) = 0.21$; $p = 0.6470$). The addition of the studied complex led to a more pronounced increase in the oxygen consumption with $IC_{50}$=75±6 μM, which is significantly less compared to meldonium ($F(1.83) = 34.37$; $p < 0.0001$) and EMHPS ($F(1.83) = 30.17$; $p < 0.0001$).

After the addition of the complex inhibitor rotenone (2 μM), the rate of pyruvate-malate-stimulated mitochondrial respiration decreased almost to zero and did not change depending on the concentration of meldonium, EMHPS, or studied complex (Fig. 4).

Succinate is a complex II substrate of the electron transport chain; thus, in the presence of rotenone, the respiration stimulated by succinate makes it possible to assess the state of complexes II, III, and IV of the mitochondrial electron transport chain. As shown in Fig. 5, the studied complex effect on the rate of the oxygen consumption during the respiration supported by the complex II substrate of the electron transport chain (ETC) was similar. A semi-effective concentration for these two substances was 237±81 μM and 453±1059 μM and did not differ significantly ($F(1.90) = 0.20$; $p = 0.6596$). An equimolar mixture of the components in the complex stimulated the respiration with $IC_{50}$ = 141±27 μM. The differences with meldonium ($F(1.83) = 3.55$; $p = 0.0632$) and EMHPS ($F(1.83) = 3.90$; $p = 0.0516$) approached the statistical significance level.

To assess the mitochondrial conjugation, the respiration rate was measured in the presence of excess ADP and phosphate (Fig. 6), and then in the presence of 1 μM oligomycin, the ATP synthase inhibitor (Fig. 7). It was found out that in the presence of excess ADP, meldonium

and EMHPS did not affect the respiration rate, either alone or in the complex. Against the background of ATP synthase blockade by oligomycin meldonium, EMHPS and succinic acid complex with trimethylhydrazinium increased the respiratory rate from $IC_{50}$=380±699, 536±1578 and 165±40 μM. The significant differences in $IC_{50}$ did not reach the statistical significance.

The rate of the FCCP-uncoupled respiration did not change with the addition of meldonium, EMHPS, or the complex under consideration (Fig. 8).

Finally, the respiration rate did not change during the inhibition of cytochrome C oxidase by cyanide after the addition of meldonium, EMHPS, or component complex (Fig. 9).

Thus, the data on the effect of the studied complex on the mitochondrial respiration indicate the restoration of the oxygen exchange in the cells to ensure normal life and modulation of cellular metabolism under conditions of cardiovascular risks.

### Assessment of ATP synthesis

According to the results of the experiment, it was found out that meldonium, EPGMS and succinic acid coordination complex with trimethylhydrazinium have a pronounced effect on the ATP production by mitochondria (Fig. 10). Thus, the least pronounced effect on the rate of the ATP production was exerted by EMHPS, for which the concentration of the half-maximal inhibition was 321±168 μM. A half-maximal decrease in the ATP production with the addition of meldonium was observed at the substance concentration of 216±6 μM. The most pronounced decrease in the ATP production was observed with the addition of the TMHP coordination complex and EMHPS with $IC_{50}$=136±4 μM.

### Peroxide production rate

The peroxide production rate was evaluated fluorometrically. As shown in Fig. 11, TMHP, EMHPS and their complex reduced the hydrogen peroxide production by isolated mitochondria. EMHPS had the least effect on the peroxide generation ($EC_{50}$=186±6 μM), which is significantly less than that of TMHP ($IC_{50}$=153±11 μM, $F(1.113) = 16.36$, $p < 0.0001$). The greatest suppression of the peroxide production was observed when complex of meldonium and EMHPS were added to the isolated mouse liver mitochondria ($IC_{50}$=96±10 μM), which was significantly less than for meldonium alone ($F(1.92) = 68.94$, $p < 0.0001$) or for EMHPS alone ($F(1.99) = 310.2$, $p < 0.0001$). These results indicate a pronounced decrease in the production of reactive oxygen species and the antioxidant effect of the complex under consideration, which determines its protective effect on cells under conditions of ischemia and hypoxia.

## DISCUSSION

Means of metabolic therapy are increasingly used in practical medicine. The representatives of this pharmacotherapeutic group are trimethylhydrazinium propionate and ethylmethylhydroxypyridine succinate, widely known in the domestic pharmaceutical market. Meldonium is the tool that makes it possible you to "shunt" bioenergetic processes, switching the cell to a more energetically favorable mode of functioning. As a rule, this is reflected in a decrease in the intensity of fatty acid β-oxidation reactions and the predominance of carbohydrate metabolism reactions in the energy production. It is important that meldonium has a selective effect on the ischemic tissue, with virtually no effect on intact tissue areas. That makes it possible to avoid the "steal" effect [17].

The action of ethylmethylhydroxypyridine succinate is primarily aimed at suppressing the processes of lipid peroxidation and reducing the total ROS pool in the cell, as well as stimulating the energy production. The use of ethylmethylhydroxypyridine succinate limits the production of reactive oxygen and nitrogen species, eliminates negative endothelial effects in the form of an increase in the activity of inducible nitric oxide synthase, and increases the activity of endogenous antioxidant defense enzymes (superoxide dismutase, catalase). The presence of a succinate fragment in the structure of the molecule allows this compound to act not only as an antioxidant, but also as a direct substrate of mitochondrial complex II, which, given a high bioavailability, can contribute to the high metabolic activity. The variability of the targeted effects of complex TMHP and EMHPS may underlie their synergy with respect to the energy-producing function of cells. Currently, the domestic pharmaceutical market presents a neuro- and cytoprotector based on the coordination complex of trimethylhydrazinium propionate and ethylmethylhydroxypyridine succinate – Brainmax® – which has antiamnestic, antihypoxic, antioxidant and antiischemic effects [18].

In this regard, an investigation on the study of the mitochondria-directed action of the complex under consideration was conducted. As a result, it was shown that in the culture of mitochondria, compounds with *pK* of about 4 (trimethylhydrazinium propionate and ethylmethylhydroxypyridine succinate) act as moderate uncouplers of a mitochondrial respiration. This fact can be associated with the presence of a positively charged atom of tertiary nitrogen (trimethylhydrazinium) and heterocyclic nitrogen (ethylmethylhydroxypyridine succinate) in the structure of these compounds. In this connection, a formation of ion pairs between these compounds under physiological conditions and the corresponding pH value can be assumed. It is of interest to note that nitrogen with a positive charge closed by methyl groups can play the role of a penetrating cation and, upon the formation of an ion pair, increase the efficiency of the counterion delivery (in this case, succinate), including with subsequent proton dissociation. Thus, the coordination complex

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2022-10-4-387-399

under consideration can be a donor of additional protons (protonophores), which are so necessary for the respiratory chain.

The use of respiration uncouplers (the complex under study), which moderately increase the proton conductivity of mitochondria, can eliminate the negative effects caused by an increase in the ROS generation by mitochondria [19].

This assumption is confirmed, first, by the activation of the endogenous substrates (pyruvate and malate) utilization, but to a much greater extent, by the combined action of the studied substances in the concentration range of the tens of nmol order on the parameters of energized mitochondria. The obtained data on the dissipation of the membrane potential correlate well with the data on the decrease in the peroxide generation during a reverse transfer, while the kinetics of the peroxide generation suppression overtakes the kinetics of the ATP synthesis suppression, which makes it possible to attribute the observed phenomenon to the so-called soft depolarization, when the potential is below the threshold value for the formation of peroxides, but ATP synthesis is still possible.

In order for protonophores not to have a toxic effect and not to show their activity in cases where the cell needs ATP synthesis, it is necessary for their activity to depend on the functional state of mitochondria, e.g., on the potential on its inner membrane. In the state of hyperpolarization, the protonophore should remove only the excess potential, but not reduce it excessively, which will inevitably lead to the inhibition of the respiration process. An ideal protonophore should not inhibit the mitochondrial respiration even at relatively high concentrations. Previous attempts were made to synthesize substances with the properties of “soft” mitochondrial uncouplers, but they failed [20].

Thus, the considered hydrosuccinate complex probably acts as such a “soft” uncoupler, that it reduces the intensity of the ROS formation and optimizes ATP synthesis. As a result of the work, a high effect of the complex on the respiratory function of mitochondria has been shown. This study showed that a component complex of metabolic and antioxidant actions increases the basal level of the mitochondrial respiration, which may be relevant for increasing the initially normal respirometric function of mitochondria, for example, in the prevention of hypoxic conditions in the absence of pathology. An increase in the intensity of the stimulated respiration is also an interesting aspect of the metabolic action of the complex.

It was shown that the studied complex increased the intensity of the substrate respiration, and pronounced changes were obtained throughout the mitochondrial respiratory chain, which is an important therapeutic advantage in conditions of oxidation substrates deficiency – in ischemic-hypoxic damages.

The universal metabolic pattern of ischemia is the accumulation of the succinate precursor, cyclic citric acid, which is responsible for the mitochondrial production of reactive oxygen species. Excess succinate is re-oxidized by succinate dehydrogenase, which leads to a rapid accumulation of reactive oxygen species. Transferring the cell into an anaerobic cycle, the trimethylhydrazine component of the studied complex reduces the availability of molecular oxygen species for succinate oxidation, thus interrupting the pathological cascade of the formation of destructive free radicals and exerting a pronounced antihypoxic effect [21].

In addition, taking into account the metabolic profile of the trimethylhydrazinium propionate action, i.e., the restriction of oxygen-demanding processes of fatty acid oxidation with the cell transfer to the intensive carbohydrate metabolism and the shunting effect of ethylmethylhydroxypyridine succinate, an increase in the resistance of cells to oxygen deficiency at different conjugated metabolic levels can be assumed. Thus, by increasing the transport of carbohydrates into the cell and limiting glycolysis mediated by the inhibition of phosphofructokinase, the trimethylhydrazinium fragment can increase the efficiency of the influence degree of ethylmethylhydroxypyridine succinate on electron transport processes, thereby modulating the optimal production of ATP under conditions of ischemia, which is sufficient to maintain the cell normal functioning [22].

Under the prevailing conditions of tissue ischemia, modulation of ATP synthesis may be important for the cell survival. It is known that under conditions of an ischemic stroke, a decrease in the concentration of the intracellular ATP pool to a critical level mediates the activation of caspase-dependent apoptosis reactions, leading to the cell death and increased neuroinflammation reactions [23, 24]. During the manifestation of Alzheimer’s disease, one of the most common neurodegenerative diseases, an increase in the formation of ATP due to the activation of substrate-dependent respiration, i.e., the switching of bioenergetic processes from one used substrate to another, can also prevent spontaneous self-aggregation of the tau protein, thereby suppressing the main pathogenetic cascade of Alzheimer’s disease (in this case, ATP acts as a natural hydrotrope that stabilizes protein molecules) [25].

A significant increase in the succinate-dependent respiration under the influence of the complex (trimethylhydrazinium propionate + ethylmethylhydroxypyridine succinate) under conditions of the activity blockade of the mitochondrial complex I by rotenone, associated with the ROS-inhibiting activity, is likely to achieve certain therapeutic benefits in Parkinson’s disease. It has been established that one of the pathogenetic triggers of this disease is the mitochondrial complex I dysfunction, followed by an increase in electron leakage from the mitochondrial respiratory chain and the development of the oxidative

damage to substantia nigra neurons [26]. Under these conditions, the use of the considered components complex will make possible achieving the effect of "metabolic bypass" of complex I, which will reduce the retrograde current and electron leakage, reducing the formation of ROS.

## CONCLUSION

Based on the totality of the results obtained, it can be assumed that the drug Brainmax® leads to the stabilization of mitochondrial function, rationalization of cell function under stress, normalization of energy metabolism in the cell even under conditions of hypoxia, and the elimination of undesirable effects of the ischemic-hypoxic tissue damage. Moreover, for complex of active components with a synergistic interaction, these effects are more pronounced than when used separately. The spectrum of biochemical reactions occurring in the cell under the action of a succinic acid complex with trimethylhydrazinium and the corresponding pharmacological effects may be the subject of further, more detailed studies on the corresponding experimental models of pathological processes.

## FUNDING

The study was conducted with the support of PROMOMED RUS LLC. The sponsor had no influence on the choice of material for publication, analysis and interpretation of the data.

## CONFLICT OF INTERESTS

The authors declare no conflict of interest.

## AUTHORS' CONTRIBUTION

MVZ – analysis of results, text editing; MVG – interpretation of results;
KYaZ – research concept development, text editing; YuGK – data statistical processing, text writing;
VSSh – text writing, references searching; AAAA – organization and conduct of study, results interpretation;
DIP – data analysis, text writing; MYuV – development of design and writing of the research program.

## REFERENCES

1. Mui D, Zhang Y. Mitochondrial scenario: roles of mitochondrial dynamics in acute myocardial ischemia/reperfusion injury. J Recept Signal Transduct Res. 2021 Feb;41(1):1–5. DOI: 10.1080/10799893.2020.1784938.
2. Johnson J, Mercado-Ayon E, Mercado-Ayon Y, Dong YN, Halawani S, Ngaba L, Lynch DR. Mitochondrial dysfunction in the development and progression of neurodegenerative diseases. Arch Biochem Biophys. 2021 May 15;702:108698. DOI: 10.1016/j.abb.2020.108698
3. Voronkov AV, Pozdnyakov DI, Nigaryan SA, Khouri EI, Miroshnichenko KA, Sosnovskaya AV, Olokhova EA. Evaluation of the mitochondria respirometric function in the conditions of pathologies of various geneses. Pharmacy & Pharmacology. 2019;7(1):20–31. DOI: 10.19163/2307-9266-2019-7-1-20-31
4. Hernandez-Resendiz S, Prunier F, Girao H, Dorn G, Hausenloy DJ; EU-CARDIOPROTECTION COST Action (CA16225). Targeting mitochondrial fusion and fission proteins for cardioprotection. J Cell Mol Med. 2020 Jun;24(12):6571–85. DOI: 10.1111/jcmm.15384
5. Dia M, Gomez L, Thibault H, Tessier N, Leon C, Chouabe C, Ducreux S, Gallo-Bona N, Tubbs E, Bendridi N, Chanon S, Leray A, Belmudes L, Couté Y, Kurdi M, Ovize M, Rieusset J, Paillard M. Reduced reticulum-mitochondria $Ca^{2+}$ transfer is an early and reversible trigger of mitochondrial dysfunctions in diabetic cardiomyopathy. Basic Res Cardiol. 2020 Nov 30;115(6):74. DOI: 10.1007/s00395-020-00835-7
6. Wang P, Xu S, Xu J, Xin Y, Lu Y, Zhang H, Zhou B, Xu H, Sheu SS, Tian R, Wang W. Elevated MCU Expression by CaMKIIδB Limits Pathological Cardiac Remodeling. Circulation. 2022 Apr 5;145(14):1067–83. DOI: 10.1161/CIRCULATIONAHA.121.055841
7. Wolf P, Schoeniger A, Edlich F. Pro-apoptotic complexes of BAX and BAK on the outer mitochondrial membrane. Biochim Biophys Acta Mol Cell Res. 2022 Oct;1869(10):119317. DOI: 10.1016/j.bbamcr.2022.119317
8. Marchi S, Guilbaud E, Tait SWG, Yamazaki T, Galluzzi L. Mitochondrial control of inflammation. Nat Rev Immunol. 2022 Jul 25:1–15. DOI: 10.1038/s41577-022-00760-x
9. Kafkova A, Trnka J. Mitochondria-targeted compounds in the treatment of cancer. Neoplasma. 2020 May;67(3):450–60. DOI: 10.4149/neo_2020_190725N671
10. Zhu Y, Luo M, Bai X, Li J, Nie P, Li B, Luo P. SS–31, a Mitochondria-Targeting Peptide, Ameliorates Kidney Disease. Oxid Med Cell Longev. 2022 Jun 6;2022:1295509. DOI: 10.1155/2022/1295509
11. Chouchani ET, Pell VR, Gaude E, Aksentijević D, Sundier SY, Robb EL, Logan A, Nadtochiy SM, Ord ENJ, Smith AC, Eyassu F, Shirley R, Hu CH, Dare AJ, James AM, Rogatti S, Hartley RC, Eaton S, Costa ASH, Brookes PS, Davidson SM, Duchen MR, Saeb-Parsy K, Shattock MJ, Robinson AJ, Work LM, Frezza C, Krieg T, Murphy MP. Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. Nature. 2014 Nov 20;515(7527):431–5. DOI: 10.1038/nature13909
12. Wang D, Liu F, Yang W, Sun Y, Wang X, Sui X, Yang J, Wang Q, Song W, Zhang M, Xiao Z, Wang T, Wang Y, Luo Y. Meldonium Ameliorates Hypoxia-Induced Lung Injury and Oxidative Stress by Regulating Platelet-Type Phosphofructokinase-Mediated Glycolysis. Front Pharmacol. 2022 Apr 5;13:863451. DOI: 10.3389/fphar.2022.863451
13. Dedkova EN, Seidlmayer LK, Blatter LA. Mitochondria-mediated cardioprotection by trimetazidine in rabbit heart failure. J Mol Cell Cardiol. 2013 Jun;59:41–54. DOI: 10.1016/j.yjmcc.2013.01.016. Epub 2013 Feb 4
14. Balykova LA, Ivyanskiy SA, Samoshkina ES, Chiginyova KN, Varlashina KA, Pleshkov SA. [The banned drugs in sport and cardiovascular system]. Consilium Medicum, Pediatrics. 2017;4:78–83. Russian
15. Görgens C, Guddat S, Dib J, Geyer H, Schänzer W,

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2022-10-4-387-399

Thevis M. Mildronate (Meldonium) in professional sports – monitoring doping control urine samples using hydrophilic interaction liquid chromatography – high resolution/high accuracy mass spectrometry. Drug Test Anal. 2015 Nov-Dec;7(11–12):973–9. DOI: 10.1002/dta.1788

16. Wikstrom MK. Proton pump coupled to cytochrome c oxidase in mitochondria. Nature. 1977 Mar 17;266(5599):271–3. DOI: 10.1038/266271a0
17. Statsenko ME, Shilina NN, Turkina SV. [Use of meldonium in the combination treatment of patients with heart failure in the early post-infarction period]. Terapevticheskii Arkhiv. 2014;86(4):30-35. Russian
18. Tanashyan MM, Raskurazhev AA, Zaslavskaya KI, Kuznetsova PI, Merkulova IY. New opportunities for neuroprotective therapy of patients in the acute and early recovery period of ischemic stroke. Terapevticheskii arkhiv. 2022;94(6):748–55. DOI: 10.26442/00403660.2022.06.201743
19. Russian Federation Patent No. 2527519. [Soft cationic mitochondrial uncouplers]. Authors: Skulachev VP, Skulachev MV, Zinovkin RA, Severin FF, Antonenko YuN, Zorov DB, Plotnikov EYu, Isaev NK, Silachev DN, Knorre DA. Patent owner LLC Mitotech. 2011. Publication No. WO/2011/162633. Russian
20. Blaikie FH, Brown SE, Samuelsson LM, Brand MD, Smith RA, Murphy MP. Targeting dinitrophenol to mitochondria: limitations to the development of a self-limiting mitochondrial protonophore. Biosci Rep. 2006 Jun;26(3):231–43. DOI: 10.1007/s10540-006-9018-8
21. Jespersen NR, Yokota T, Støttrup NB, Bergdahl A, Paelestik KB, Povlsen JA, Dela F, Bøtker HE. Pre-ischaemic mitochondrial substrate constraint by inhibition of malate-aspartate shuttle preserves mitochondrial function after ischaemia-reperfusion. J Physiol. 2017 Jun 15;595(12):3765–80. DOI: 10.1113/JP273408
22. Wang D, Liu F, Yang W, Sun Y, Wang X, Sui X, Yang J, Wang Q, Song W, Zhang M, Xiao Z, Wang T, Wang Y, Luo Y. Meldonium Ameliorates Hypoxia-Induced Lung Injury and Oxidative Stress by Regulating Platelet-Type Phosphofructokinase-Mediated Glycolysis. Frontiers in pharmacology. 2022;13:863451. DOI: 10.3389/fphar.2022.863451
23. Voronkov AV, Pozdnyakov DI, Nigaryan SA. Cerebroprotective effect of some phenolic acids under conditions of experimental brain ischemia. Pharmacy & Pharmacology. 2019;7(6):332–9. DOI: 10.19163/2307-9266-2019-7-6-332-338
24. Sun Y, Zhang Y, Cheng B, Dong Y, Pan C, Li T, Xie Z. Glucose may attenuate isoflurane-induced caspase-3 activation in H4 human neuroglioma cells. Anesth Analg. 2014 Dec;119(6):1373–80. DOI: 10.1213/ANE.0000000000000383
25. Sarkar S, Mondal J. Mechanistic Insights on ATP's Role as a Hydrotrope. J Phys Chem B. 2021 Jul 22;125(28):7717–31. DOI: 10.1021/acs.jpcb.1c03964
26. Franco-Iborra S, Vila M, Perier C. The Parkinson Disease Mitochondrial Hypothesis: Where Are We at? Neuroscientist. 2016 Jun;22(3):266–77. DOI: 10.1177/1073858415574600

## AUTHORS

**Marina V. Zhuravleva** – Doctor of Sciences (Medicine), Professor, Professor of the Department of Clinical Pharmacology and Propaedeutics of Internal Diseases, Sechenov First Moscow State Medical University (Sechenov University); Deputy Head of the Scientific Department of Clinical Pharmacology, Scientific Center for Expertise of Medicinal Products. ORCID ID: 0000-0002-9198-8661. E-mail: mvzhuravleva@mai.ru

**Marina V. Granovskaya** – Associate Professor, Institute of Systems Biology of University College Dublin, Dublin, Ireland. ORCID ID: 0000-0002-6867-5376. E-mail: mgranovsk@gmail.com

**Kira Ya. Zaslavskaya** – Assistant of the Department of Biological and Pharmaceutical Chemistry with the course of organization and management of pharmacy, National Research Ogarev Mordovia State University. ORCID ID: 0000-0002-7348-9412. E-mail: kiryonok@yandex.ru

**Yuri G. Kazaishvili** – Candidate of Sciences (Biology), Assistant Professor, Department of Pharmacology, Tver State Medical University. ORCID ID: 0000-0003-0826-4177. E-mail: ykaza@bk.ru

**Victoria S. Scherbakova** – Candidate of Sciences (Biology), Assistant Professor, Department of Pharmacology, Tver State Medical University. ORCID ID: 0000-0002-7251-8744. E-mail: Victoria_kaptar@mail.ru

**Alexander A. Andreev-Andrievskiy** – Head of Animal Research, Institute for Biomedical Problems of the Russian Academy of Sciences. ORCID ID: 0000-0002-1173-8153. E-mail: aaa@mitotech.ru

**Dmitry I. Pozdnyakov** – Candidate of Sciences (Pharmacy), Associate Professor of the Department of Pharmacology with a Course of Clinical Pharmacology, Pyatigorsk Medical and Pharmaceutical Institute – branch of Volgograd State Medical University. ORCID ID: 0000-0002-5595-8182. E-mail: pozdniackow.dmitry@yandex.ru

**Mikhail Yu. Vyssokikh** – Candidate of Sciences (Biology), Head of the Laboratory of Molecular Mechanisms of Aging, A.N. Belozersky Institute of Physico-Chemical Biology of Moscow State University named after M.V. Lomonosov. ORCID ID: 0000-0002-4047-6201. E-mail: mikhail.vyssokikh@gmail.com

*EDITORIAL*
*ISSN 2307-9266 e-ISSN 2413-2241*
*DOI: 10.19163/2307-9266-2022-10-4-400*

Scientific and Practical Journal
PHARMACY & PHARMACOLOGY

# CORRIGENDUM: "EVALUATION OF WEIGHT REDUCTION EFFICACY AND SAFETY OF SIBUTRAMIN-CONTAINING DRUGS IN PATIENTS WITH ALIMENTARY OBESITY"

DOI: 10.19163/2307-9266-2022-10-3-289-304
Correction on: https://www.pharmpharm.ru/jour/article/view/1121

**T.Yu. Demidova[1], M.Ya. Izmailova[1], S.E. Ushakova[2], K.Ya. Zaslavskaya[3], A.A. Odegova[4], V.V. Popova[5], M.E. Nevretdinova[6], A.F. Verbovoy[7], P.A. Bely[8]**

[1] Pirogov Russian National Research Medical University,
1, Ostrovityanov Str., Moscow, Russia, 117997
[2] Ivanovo Clinical Hospital named after Kuvaev,
Bldg. 2, 52, Ermak Str., Ivanovo, Russia, 153025
[3] National Research Ogarev Mordovia State University,
Bldg. A, 26, Ulyanov Str., Saransk, Republic of Mordovia, Russia, 430005
[4] Kirov State Medical University,
112, K. Marx Str., Kirov, Russia, 610027
[5] Saint-Petersburg State Pediatric Medical University,
2, Litovskaya Str., Saint-Petersburg, Russia, 194100
[6] Limited Liability Company "The Practice of Health",
1, Skobelevskaya Str., Moscow, Russia, 117624
[7] Samara State Medical University,
89, Chapaevskaya Str., Samara, Russia, 443099
[8] A.I. Yevdokimov Moscow State University of Medicine and Dentistry,
Bldg. 1, 20, Delegatskaya Str., Moscow, Russia, 127473

E-mail: t.y.demidova@gmail.com

**Keywords:** obesity; pharmacotherapy; sibutramine; metformin; Reduxin®; Reduxin® Forte

In the published article "Evaluation of weight reduction efficacy and safety of sibutramin-containing drugs in patients with alimentary obesity", there was an error in the author list. Due to a technical error on author's part and without any malicious intent, the authors name A.F. Verbovoy, who was not directly involved in writing the article and not made a great contribution to the work were added. The corrected author list appears below.

**T.Yu. Demidova, M.Ya. Izmailova, S.E. Ushakova, K.Ya. Zaslavskaya, A.A. Odegova, V.V. Popova, M.E. Nevretdinova, P.A. Bely**

The error does not change the essence of the data presented in the article, does not violate their perception by readers or interpretation.

Authors confirm the accuracy of the information provided in this letter about the composition of the team of authors, and that the contribution of each author complies with the recommendations of the ICMJE. The original article has been updated.