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CONTENS / СОДЕРЖАНИЕ

RESEARCH ARTICLE

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ANALYTICAL FEATURES OF SYNTHETIC MDMB(N)-073F CANNABIMIMETICS AND ITS MARKERS IN BIOLOGICAL MATERIAL

S.S. Kataev[1], O.N. Dvorskaya[2], M.A. Gofenberg[4,5], A.V. Labutin[6], A.B. Melentyev[7]

[1] Perm Regional Bureau of Forensic-Medical Expertise, 61, Startsev Str, Perm, Russia, 614077
[2] Perm State Pharmaceutical Academy, 2, Polevaya Str., Perm, Russia, 614990
[3] Sverdlovsk Regional Narcological Hospital, l44 A, Khalturin Str., Yekaterinburg, Russia, 620030
[4] Yekaterinburg State Publicly Funded Health Facility "Regional Clinical Psychiatric Hospital", 8km, Sibirsky Trakt, Yekaterinburg, Russia, 620034
[5] Ural State Medical University, 3, Repin Str., Yekaterinburg, Russia, 620028
[6] Tomsk Regional Drug Abuse Clinic, 4, Lebedev Str., Tomsk, Russia, 634061
[7] Chelyabinsk Regional Bureau of Forensic-Medical Expertise, 4b, Varna Str., Chelyabinsk, Russia, 454076

E-mail: dvoksnik@gmail.com

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The aim of the research is to study both analytical features of synthetic MDMB(N)-073F cannabimimetics of indazole carboxamides group by gas chromatography methods combined with tandem mass spectrometry (GC-MS) and high performance liquid chromatography with high-resolution mass spectrometry (HPLC-HRMS) as well as characteristics of the major MDMB(N)-073F metabolite, its glucuronide and derivatives, using gas chromatography with mass-spectrometric (GC-MS) detection and high-performance liquid chromatography (HPLC) with MS/MS mass spectrometry (HPLC-MS/MS) in urine samples to be applied in expert practice, chemical-toxicological and forensic and chemical analyses.

Materials and methods. To carry out the study, the following materials were used: plant-based objects with narcotic drugs withdrawn from illegal trafficking and applied to them;. urine samples to be studied under chemical-toxicological and forensic and chemical analyses. For solid-phase epitaxy, SampliQ EVIDEX TFE cartridges – 200 mg – 3 ml (Agilent, USA) were used for sample preparation; β-glucuronidase, Type HP-2, From Helix Pomatia, 100000 UA/ml (Sigma-ALDRICH CHEMI, Germany) was used for enzymatic hydrolysis. GC-MS/MS analysis was made using Agilent 7890 gas chromatograph with a tandem quadrupolar mass-spectrometer Agilent 7000 (Agilent, США); GC-MS analysis was carrid out using gas chromatograph Agilent 7820 with mass-selective detector Agilent 5975 (Agilent, USA); HPLC-HRMS research was made on liquid chromatograph Agilent 1260 with tandem hybrid high-resolution quadrupole-time-of-flight detector Agilent 6540 (Agilent, США); liquid chromatograph Agilent 1260 with Agilent 6460 (Agilent, USA) with tandem mass-spectrometer were used for making HPLC-MS/MS research.

Results. The structure of MDMB(N)-073F compound has been confirmed and an exact mass of the protonated molecule corresponding to the chemical formula $C_{19}H_{27}FN_3O_3$ fixed by GC-MS/MS and HPLC-HRMS methods. Spectral characteristics of MDMB(N)-073F have been given. One of the branches in MDMB(N)-073F biotransformation in the human body found out by GC-MS and HPLC-MS/MS methods, is the ester decomposition with further conjugation of the resulting acid. The product interacting with glucuronic acid, is found to be the conjugate of major MDMB(N)-073F metabolite of the Ist phase in biotransformation. Metabolites appearing due to the ester decomposition and its conjugate with glucuronic acid, are recommended to be used as markers for synthetic MDMB(N)-073F cannabimimetics in the analysis by chromatographic methods; they can be used for regular screening of biological samples.

Conclusion. The research results presented here, are the following: the analytical features characteristic for synthetic MDMB(N)-073F cannabimimetics found out by gas chromatography methods combined with tandem mass spectrometry (GC-MS/MS) and liquid chromatography of hybrid high-resolution quadrupole-time-of-flight mass spectrometry (HPLC-HRMS), as well as characteristics of major MDMB(N)-073F metabolite, its glucuronide and derivatives with the use of gas chromatography with mass-spectrometric detection (GC-MS) and liquid chromatography combined with tandem mass spectrometry (HPLC-MS/MS) in urine samples to be applied in expert practice, chemical-toxicological, forensic and chemical analyses.

Keywords: MDMB(N)-073F, cannabimimetics, gas chromatography – mass spectrometry (GC-MS), high performance liquid chromatography (HPLC), high-resolution mass spectrometry (HRMS), hybrid high-resolution quadrupole-time-of-flight mass spectrometry

Abbreviations: MRM – multiple reaction monitoring, GC – gas chromatography, MS mass spectrometry, HPLC – high performance liquid chromatography, HRMS – high-resolution mass spectrometry, a.u. – antitoxic unit.

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АНАЛИТИЧЕСКИЕ ХАРАКТЕРИСТИКИ СИНТЕТИЧЕСКОГО КАННАБИМИМЕТИКА MDMB(N)-073F И ЕГО МАРКЕРОВ В БИОЛОГИЧЕСКОМ МАТЕРИАЛЕ

С.С. Катаев[1], О.Н. Дворская[2], М.А. Гофенберг[4,5], А.В. Лабутин[6], А.Б. Мелентьев[7]

[1] Государственное казенное учреждение здравоохранения особого типа Пермского края «Пермское краевое бюро судебно-медицинской экспертизы». 614077, Россия, г. Пермь, ул. Старцева, 61
[2] Федеральное государственное бюджетное образовательное учреждение высшего образования «Пермская государственная фармацевтическая академия» Министерства здравоохранения Российской Федерации. 614990, Россия, г. Пермь, ул. Полевая, 2
[3] Государственное автономное учреждение здравоохранения Свердловской области «Областная наркологическая больница». 620030, Россия, г. Екатеринбург, ул. Халтурина, 44А
[4] Государственное бюджетное учреждение здравоохранения Свердловской области «Свердловская областная клиническая психиатрическая больница». 620034, Россия, г. Екатеринбург, Сибирский тракт, 8 км
[5] Федеральное государственное бюджетное образовательное учреждение высшего образования «Уральский государственный медицинский университет» Министерства здравоохранения Российской Федерации. 620028, Россия, г. Екатеринбург, ул. Репина, 3
[6] Областное государственное бюджетное учреждение здравоохранения «Томский областной наркодиспансер». 634061, Россия, г. Томск, ул. Лебедева, 4
[7] Государственное бюджетное учреждение здравоохранения «Челябинское областное бюро судебно-медицинской экспертизы». 454080, Россия, Челябинская обл., г. Челябинск, ул. Варненская, 4Б

E-mail: dvoksnik@gmail.com

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Целью исследования является изучение аналитических характеристик синтетического каннабимиметика группы индазолкарбоксамидов MDMB(N)-073F методами газовой хроматографии с тандемной масс-спектрометрией (ГХ-МС/МС) и жидкостной хроматографии с гибридной квадруполь-времяпролетной масс-спектрометрией высокого разрешения (ВЭЖХ-МСВР), а также характеристик главного метаболита MDMB(N)-073F, его глюкуронида и дериватов с использованием газовой хроматографии с масс-спектрометрическим детектированием (ГХ-МС) и жидкостной хроматографии с тандемной масс-спектрометрией (ВЭЖХ-МС/МС) в моче для целей экспертной практики, химико-токсикологического и судебно-химического анализа.

Материалы и методы. Объекты растительного происхождения с нанесенными на них наркотическими средствами, изъятые в нелегальном обороте. Образцы мочи, поступившие на химико-токсикологическое и судебно-химическое исследование. Для пробоподготовки использовались патроны для ТФЭ *SampliQ EVIDEX* – 200 мг-3 мл (*Agilent*, США), для ферментативного гидролиза использовалась β-глюкуронидаза, *Type HP-2, From Helix Pomatia*, 100000 ЕД/мл (*Sigma-ALDRICH CHEMI*, Германия). ГХ-МС/МС анализ проводили на газовом хроматографе Agilent 7890 с тандемным квадрупольным масс-спектрометром Agilent 7000 (Agilent, США); ГХ-МС анализ выполнен на газовом хроматографе Agilent 7820 с масс-селективным детектором Agilent 5975 (Agilent, США); ВЭЖХ-МСВР исследование проводили на жидкостном хроматографе Agilent 1260 с тандемным гибридным квадруполь – времяпролетным детектором высокого разрешения Agilent 6540 (Agilent, США); ВЭЖХ-МС/МС исследование выполнено на жидкостном хроматографе Agilent 1260 с тандемным масс-спектрометром Agilent 6460 (Agilent, США).

Результаты. В результате исследования, проведенного методами ГХ-МС/МС и ВЭЖХ-МСВР, подтверждена структура соединения MDMB(N)-073F, определена точная масса протонированной молекулы, соответствующая брутто-формуле $C_{19}H_{27}FN_3O_3$. Приведены спектральные характеристики MDMB(N)-073F. Методами ГХ-МС и ВЭЖХ-МС/МС установлено, что одним из направлений биотрансформации MDMB(N)-073F в организме человека является гидролиз сложноэфирной связи с последующей конъюгацией образующейся кислоты. Установлено, что конъюгатом главного метаболита MDMB(N)-073F фазы I биотрансформации является продукт взаимодействия с глюкуроновой кислотой. Метаболиты, образующиеся в результате гидролиза сложноэфирной связи, и его конъюгат с глюкуроновой кислотой рекомендованы в качестве маркеров употребления синтетического каннабимиметика MDMB(N)-073F при анализе хроматографическими методами они могут быть использованы при систематическом аналитическом скрининге биологических образцов.

Заключение. Приведены аналитические характеристики синтетического каннабимиметика MDMB(N)-073F методами газовой хроматографии с тандемной масс-спектрометрией (ГХ-МС/МС) и жидкостной хроматографии гибридной квадруполь-времяпролетной масс-спектрометрией высокого разрешения (ВЭЖХ-МСВР), а также характеристики главного метаболита MDMB(N)-073F, его глюкуронида и дериватов с использованием газовой хроматографии с масс-спектрометрическим детектированием (ГХ-МС) и жидкостной хроматографии с тандемной масс-спектрометрией (ВЭЖХ-МС/МС) в моче для целей экспертной практики, химико-токсикологического и судебно-химического анализа.

Ключевые слова: MDMB(N)-073F, каннабимиметики, метаболизм, газовая хроматография – масс-спектрометрия, высокоэффективная жидкостная хроматография, тандемная масс-спектрометрия, гибридная квадруполь-времяпролетная масс-спектрометрия высокого разрешения

Список сокращений: ГХ-МС/МС – газовая хроматография с тандемной масс-спектрометрией, ВЭЖХ-МСВР – жидкостная хроматография с гибридной квадруполь-времяпролетной масс-спектрометрией высокого разрешения, ГХ-МС – газовая хроматография с масс-спектрометрическим детектированием

INTRODUCTION

The analysis of toxicants' properties and processes of metabolism in the human body, methods of their extraction and identification is the main part in both forensic and toxicological chemistry. This is particularly true for new hazardous psychoactive substances including synthetic cannabimimetics, regularly appearing in illegal drug market.

All the factors – various equipment in expert institutions, various approaches to analyzing synthetic cannabimimetics to be found in biological materials, lack of analytical standards – make it difficult to interpret and compare the results obtained from different sources.

Gas chromatography with mass-spectrometric (GC/MS) detection is widely applied in laboratory practice in the Russian Federation, with the application thereof being combined with labor-intensive and time-consuming sample preparation to make qualitative sampling. In this case metabolite conjugation, extraction and derivatization are to be made when testing urine for cannabimimetics metabolites [1, 2].

Liquid chromatography combined with with a tandem mass-spectrometry (HPLC-MS/MS) method is considered to be the best, as it makes it possible to simplify the sample preparation process to detect metabolites of synthetic cannabimimetics as no deconjugation and derivatization are required [3-5].

In this scientific work, the results of the research of analytical features of synthetic MDMB(N)-073F cannabimimetics found out by gas chromatography methods combined with MS/MS mass spectrometry (GC-MS/MS) and liquid chromatography of hybrid high-resolution quadrupole-time-of-flight mass spectrometry (HPLC-HRMS), as well as characteristics of major MDMB(N)-073F metabolite and glucuronide and derivatives thereof using gas chromatography with mass-spectrometric detection (GC-MS) and liquid chromatography with MS/MS mass spectrometry (HPLC-MS/MS) in the urine samples to be applied in expert practice, chemical-toxicological, forensic and chemical analyses have been presented here.

OBJECTS OF RESEARCH

To carry out the study, the following materials were used: plant-based objects with narcotic drugs withdrawn from illegal trafficking and applied to them;. urine samples to be studied under chemical-toxicological and forensic and chemical analyses.

MATERIALS AND METHODS

Synthetic cannabimimetics in plant-based objects were detected and identified by gas-liquid chromatography methods with tandem mass-spectrometry and high performance liquid chromatography with high resolution MS/MS quadrupole-time-of-flight detector.

Gas-liquid chromatography methods with tandem quadrupole mass-spectrometric detector and high performance liquid chromatography with tandem hybrid high-resolution quadrupole-time-of-flight mass spectrometry (HPLC-HRMS) were applied to detect cannabimimetics markers and metabolites in biological material.

Equipment

- Gas chromatograph Agilent 7890 (capillary column DB-5MS, similar to (5% phenyl)-methylpolysiloxane), ID = 0.25mm, length = 30m, thickness of stationary phase film = 0.25 μm) with tandem quadrupole mass-spectrometer MS/MS Agilent 7000 (Agilent, USA);
- Gas chromatograph Agilent 7820 (capillary column HP-5MS (5% phenyl)- methylpolysiloxane), ID = 0.25 mm, length = 30m, thickness of film=0.25 μm) with mass-selective detector Agilent 5975 (Agilent, USA);
- Liquid chromatograph Agilent 1260 (chromatographic column Zorbax Extend C-18 2.1*50 mm, sorbent grain diameter = 1.8 μm) with tandem hybrid high-resolution quadrupole-time-of-flight detector Agilent 6540 (resolution at least 40000) (Agilent, USA);
- Liquid chromatograph Agilent 1260 (chromatographic column 3*150 mm with reversed-phase sorbent Poroshell 120 EC-C18, grain size = 2.7 μm) with tandem mass-spectrometer Agilent 6460 (Agilent, USA);
- 12 position SPE Vacuum Manifold System (Supelco);
- low vacuum pump (KNF lab LABOPORT (France);
- thermal block PE-4030 (Ecros, Russia);
- single-channel vaporizer PE-2300 (“Ecros”, Russia);
- microshaker PE-2 (“Ecros”, Russia);
- microwave Supra MWS-1824SW (Russia);
- solid-phase extraction cartridges SampliQ EVIDEX (200 mg / 3 ml) (Agilent, USA);
- semi-automatic dispensers with 4–40, 40–200 μl and 0.2–1, 1–5 ml range.

Materials

Bis-trimethylsilyl-trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane; 2,2,3,3,3-pentafluoropropanol, 2,2,3,3,3-pentafluoropropionic anhydride, methyl iodide, β-glucuronidase, *Type HP-2, From Helix Pomatia,* 100,000 EU / ml *(Sigma-ALDRICH CHEMI,* Germany). The chemicals used in the study are of the “chemically pure” brand. The storage of the urine samples before the study was carried out at + 4°C.

Sample preparation

Plant-based objects preparation (for GC-MS/MS and HPLC-HRMS analyses)

A weighed quantity of 10 mg of the plant-based object was extracted with 10ml of ethanol for 5 minutes. The resulting extract was separated from the plant matrix by centrifugation, diluted with ethanol by 10 times and analyzed by GC-MS/MS and HPLC-HRMS methods.

ОРИГИНАЛЬНАЯ СТАТЬЯ
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Preparation of urine samples (applying enzymatic hydrolysis, for GC-MS analysis)

50 µl of an internal standard alcohol solution (0.2 mg/ml Hexenal, 250 µl of 1/15 M pH 6 phosphate buffer and 50 µl of β-glucuronidase) was added to 1 ml of the urine sample. Then the vial was corked up and exposed to 45°C for 2 hours. After cooling down, 2 ml 1/15 M phosphate buffer (pH 4,8) was added. The contents of the vials were centrifugated at 3000 rpm for 10 minutes, the centrifugate was separated from the residue.

For extraction, SPE cartriges SampliQ EVIDEX (200 mg/3 ml) with a mixed phase were used. Conditioning of a sorbent was conducted via succesive transfer of 2 ml of 95% ethanol and 2 ml of 1/15 M phosphate buffer (pH 4.8) through the cartridge. After that, the sample was downloaded at the speed of 1 ml/min. Flushing was conducted in a successive manner: first 1 ml of 1/15 M phosphate buffer (pH 4.8) and then 1 ml of 10% ethanol. Drying the cartridge was carried out in vacuum for 20 minutes. Eluate was derived via double transfer of 2 ml of n-hexane – ethylacetate (2:1) concoction through the cartridge. Eluate was vaporized in the nitrogen flow at 45°C.

Derivatization

Methylation

500 µl of anhydrous acetone, 40 µl of iodomethane and 20–25 mg of anhydrous potassium carbonate were added to the dry residue of eluate I. The vial was corked up and heated at 60°C in the thermal block for 60 minutes. Then the vial was cooled down, the fluid fraction of the reactive concoction was separated and transferred into a clean vial, then vaporized at 40 °C in the nitrogen flow. The dry residue was dissolved in 100 µl of anhydrous ethylacetate and 1 µl of this solution was put into the gas chromatograph evaporator.

Esterification with 2,2,3,3,3-pentafluoropropanol

20µl of 2,2,3,3,3-pentafluoropropanol and 60µl of 2,2,3,3,3-pentafluoropropionic anhydride (washing off the vial walls) were added to the dry residue of eluate, the vial was sealed and MW-irradiated in the 560W microwave oven for 5 minutes. After cooling down, the vial was opened and excess reagents were vaporized in the nitrogen flow (not above 40°C). The dry residue was dissolved in 100µl of anhydrous ethylacetate, and 1µl thereof was added to chromate-mass-spectrometer's evaporator.

Trimethylsilyl esters acquisition

100 µl of BSTFA containing 1% of trimethylchlorsilane was added to the dry residue of eluate, the vial was corked up, shaken with the microshaker and heated at 80°C in the thermal block for 60 minutes. The vial was cooled down and 2 µl was put into the chromato-mass-spectrometer's evaporator.

Preparation of urine samples (without hydrolysis, for HPLC analysis)

0.45ml of internal standard mixture in acetonitrile (with 0.03µm/ml concentration of ethylmorphine and cyclizine *ana*) was added to the urine sample (0.05ml) in Eppendorf tube. The tube was centrifuged at 10000 rpm for 15 minutes, the supernatant layer was transferred to the vial for autosampling, and 2ml of the resulting solution was added to the chromatograph.

Operation mode for GC-MS/MS analysis (gas chromatograph Agilent 7890 with MS/MS quadrupole mass-spectrometer Agilent 7000)

- Chromatograph vaporizer temperature – 280ºC;
- vaporizer operation mode: split/splitless (15:1 split ratio, with 1 min delay after the sample injection);
- detector interface temperature – 280°C;
- initial temperature of the column heating oven – 220°C;
- final temperature of the column heating oven – 300°C;
- variation of the column temperature – 20 degrees/min;
- exposure at the final temperature – 5 min;
- carrier gas – helium, column flow rate – 1 ml/min;
- injection volume – 1 ml;
- collision cell gas – nitrogen, 1.5ml/min;
- «cooling gas» – helium, flow rate – 2.25 ml/min;
- collision energy – 10–20eV.

Operation mode for HPLC-HRMS analysis (liquid chromatograph Agilent 1260 with high resolution MS/MS hybrid quadrupole-time-of-flight detector Agilent 6540)

- Gradient eluation with phase A (a 0.1% formic acid solution in deionized water) and phase B (acetonitrile) at the increase of the content of phase B from 1% to 100% for 10 minutes;
- volume of injected sample – 1 ml;
- flow rate – 0.3 ml/min;
- column temperature – 45°C;
- electrospray ionization in positive ion mode;
- drying gas temperature (nitrogen) – 350°C;
- drying gas flow rate (nitrogen) – 8 l/min;
- nebulizer gas pressure (nitrogen) – 20 psi;
- capillary voltage – 3500 V;
- fragmentor voltage – 100 V and 180 V;
- mass-spectrometer operation mode: Auto MS/MS;
- equipment calibration and accuracy correction of

mass measuring in the course of the analysis were made by standard calibrators recommended by the equipment manufacturers.

A part of the previously obtained alcoholic extract to be analyzed was diluted with water and studied under the above conditions.

Operation mode for GC-MS analysis (gas chromatograph Agilent 7820 with mass-selective detector Agilent 5975)

- Flow rate of the carrier gas (helium) through the column – 1.5 ml/min;
- working mode of vaporizer split/splitless (low-splitting – 15:1 with the impulse delay of 1 minute after the sample injection);
- the temperatures of the injection port and the line connecting to the mass spectrometer were 250°C and 280°C, respectively;
- the initial temperature of the column was 70°C for 2 minutes; then, the column was heated up to 280°C at the programming speed of 20 degrees/min. and kept at the final temperature for 8 minutes;
- the temperatures of the ion source and the quadrupole were at 230 and 150°C, respectively;
- voltage of the multiplier of the mass-spectrometric detector was set equal to that of the automatic routine adjustment of the detector.
- The registration of mass spectrum for methyl derivatives in the full ion scanning mode was in mass range of 42–450 a.u. The registration of mass spectrum for trimethylsilyl and pentafluoropropyl derivatives in the full ion scanning mode was in the mass range of 43–650 a.u.

The conjugation degree of the major MDMB(N)-073F metabolite of biotransformation phase I in the urine was determined by the ratio of the peak area of methyl ethers for the ion with m/z value 219 and the peak area of the ion m/z 235 for *N*-methylhexenal (internal standard) in eluate I of the urine with enzymatic hydrolysis and by similar procedure without hydrolysis.

Operation mode for HPLC-MS/MS analysis (liquid chromatograph Agilent 1260 with MS/MS mass-spectrometer Agilent 6460)

- Gradient eluation with phases A (a 10 mM solution of ammonium formate and 0.1% formic acid in deionized water) and B (0,01% formic acid in methanole);
- eluent flow rate was 0.6 ml/min;
- column temperature – 50ºC;
- gradient mode: 0 – 1.0 min – reaching 95% of phase A, by the 5th minute reducing to 50% of phase A, by the 15th – by 2%, by the 17th – by 2%, by the 17.1 – by 95% and the column regeneration within 3 minutes was 95% of phase A;
- injection volume – 2 mcl;
- electrospray ionization in positive ion mode;
- flow of desiccant gas (nitrogen) to the ion source 6 l/min;
- spray gas pressure (nitrogen) 40 psi;
- temperature of desiccant gas – 300°C;
- capillary voltage – 3500 V;
- fragmentor voltage – 125 V;
- mass-spectrometer operation modes: dynamic MRM and Product Ion Scan (mass range: 100–550 Da).

Processing of the chromatograms in order to identify the components of the samples was carried out using *MSD ChemStation E.02.01.1177* (*Agilent*), *MassHunter B.08.02 (Agilent)* and *AMDIS* (The Automatic Mass Spectral Deconvolution and Identification System, NIST) software.

RESULTS AND DISCUSSION

As has been previously shown on the basis of the relative metabolite content in the urine samples, the principal pathway for MDMB(N)-073F cannabimimetics metabolism is MDMB(N)-073F ester decomposition with further conjugation of the resulting product (Fig.1). This metabolite of biotransformation phase I has both the maximum signal intensity in chromatograms of MDMB(N)-073F users and a characteristic mass-spectrum thereby making it possible to be used as a marker for this cannabimimetics [6, 7]. It should be also considered, that in the urine, this metabolite of biotransformation phase I is significantly associated with the conjugated form (Table 1), so in case of applying GC-MS research methods, hydrolysis of conjugates is required.

Table 1 – Marker conjugation values in 10 urine samples of MDMB(N)-073F cannabimimetics users

Sample	561	663	717	721	722	224	705	752	754	756	Median, %
Marker conjugation, %	96	0	49	98	97	97	99	99	99	100	97.5

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2019-7-4-184-197

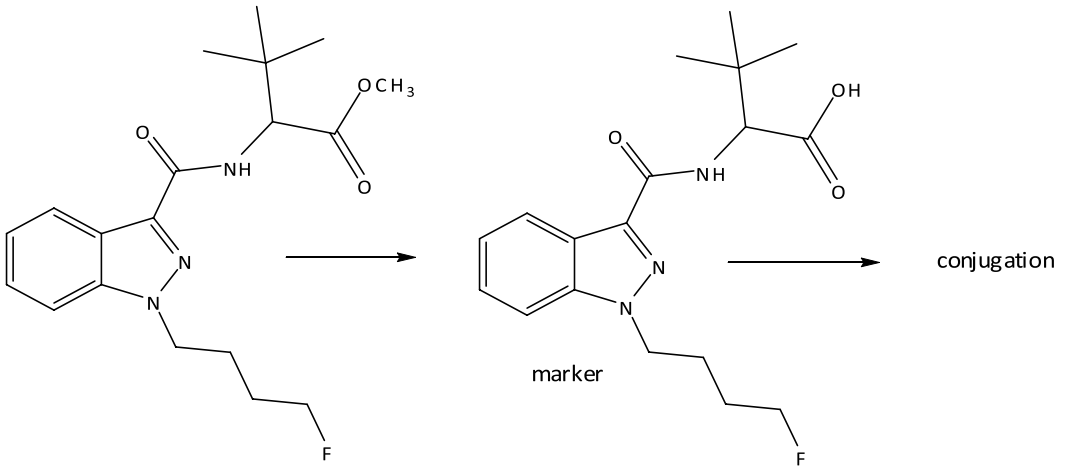

Figure 1 – Principal MDMB(N)-073F metabolic pathway

Obtaining–methyl, trimethylsilyl and 2,2,3,3,3-pentafluoropropyl esters is considered the most common variant of derivatization in cannabimimetics markers screening in the biological material based on gas chromatography with a quadrupole mass-spectrometric detector.

The formation of methyl ester MDMB(N)–073F marker corresponding to the original compound (Fig. 2), takes place during methylation, thereby simplifying the compound identification. Mass-spectra, retention indices and structures of trimethylsilyl and 2,2,3,3,3-pentafluoropropyl esters of MDMB(N)-073F marker, are given in Fig. 3 and 4, respectively.

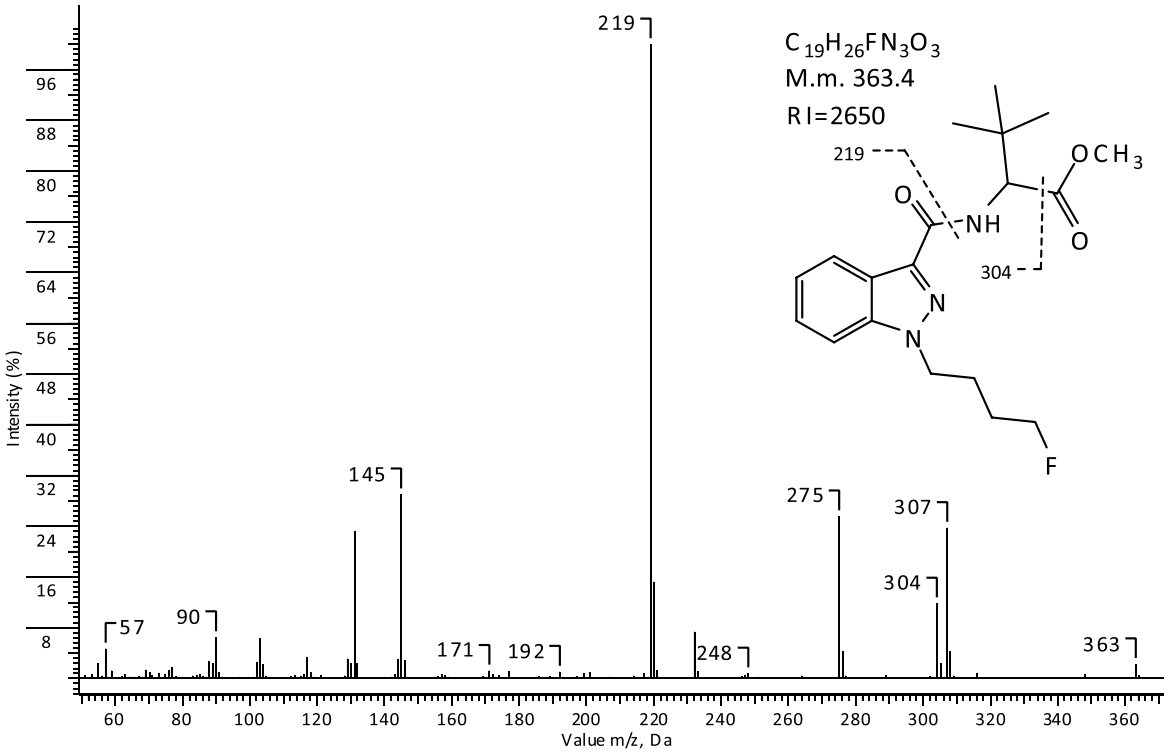

Figure 2 – Mass-spectrum, retention index and structure of methyl ester of MDMB(N -073F marker

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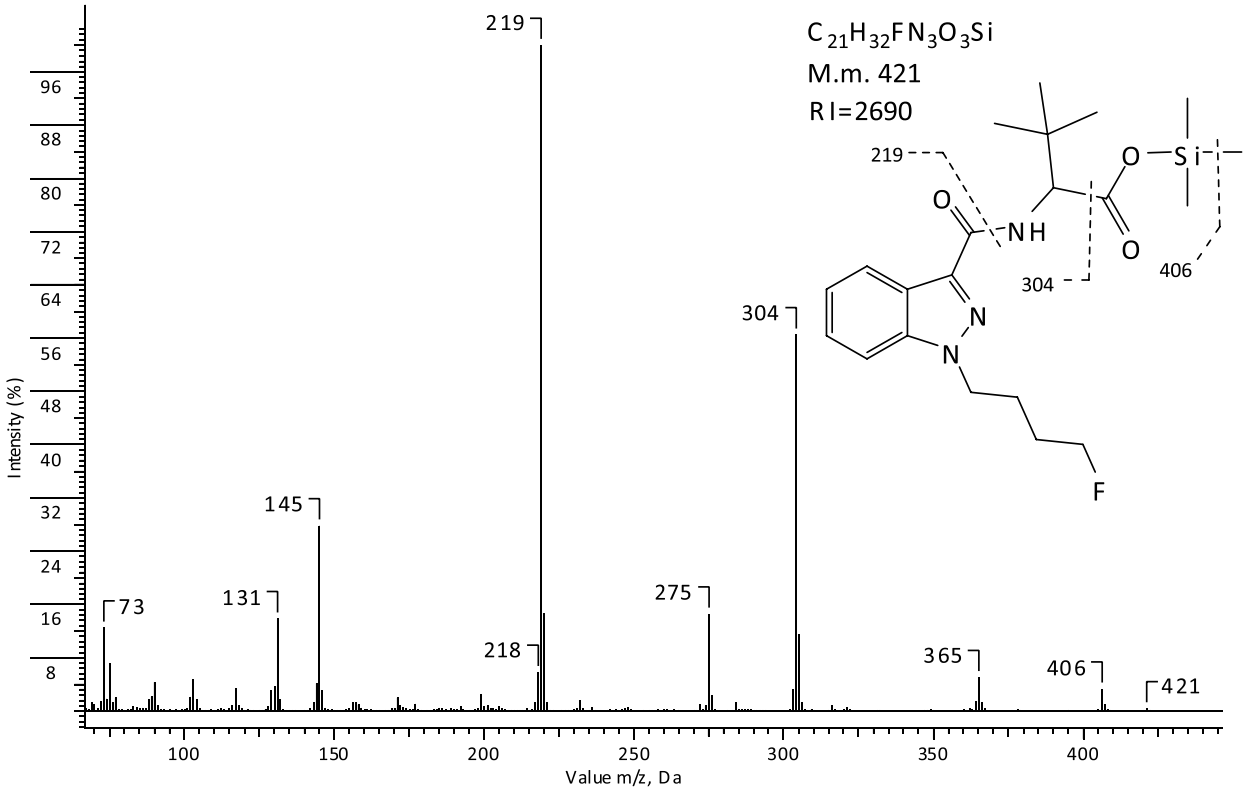

Figure 3 – Mass-spectrum, retention index and structure of trimethylsilyl ester of MDMB(N)-073F marker.

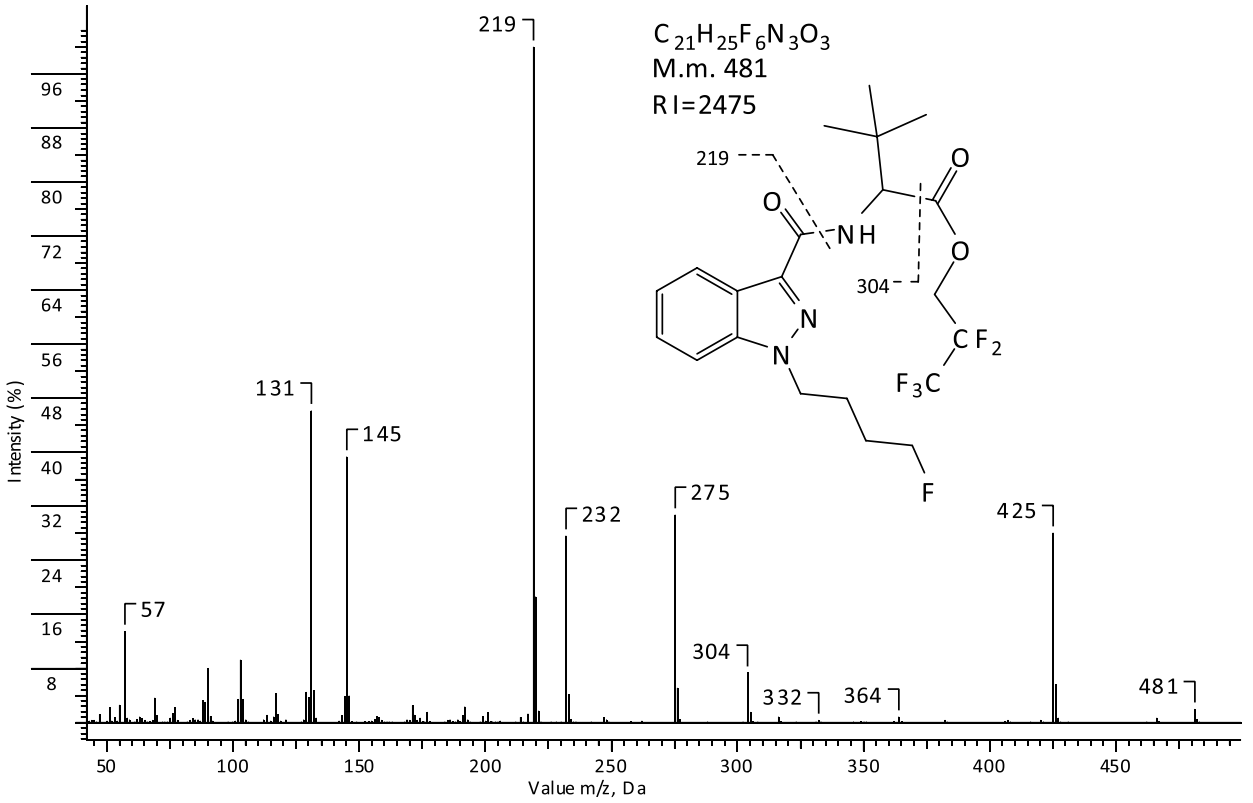

Figure 4 – Mass-spectrum, retention index and structure of 2,2,3,3,3-pentafluoropropyl ester of MDMB(N)-073F marker.

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2019-7-4-184-197

Taking into account the identical structures of MDMB (N)-073F and the methyl derivative of its main metabolite (Fig. 1), to study the properties of MDMB(N)-073F, the analysis of the original cannabimimetic MDMB (N) -073F was carried out on the basis of the methods of GC-MS / MS and HPLC-HRMS**.** When applying gas chromatography method with a tandem mass-spectrometric triple quadrupole detector, the fragmentation of basic ions being formed under the electron impact ionization from MDMB(N)-073F was analyzed (Fig. 5–10).

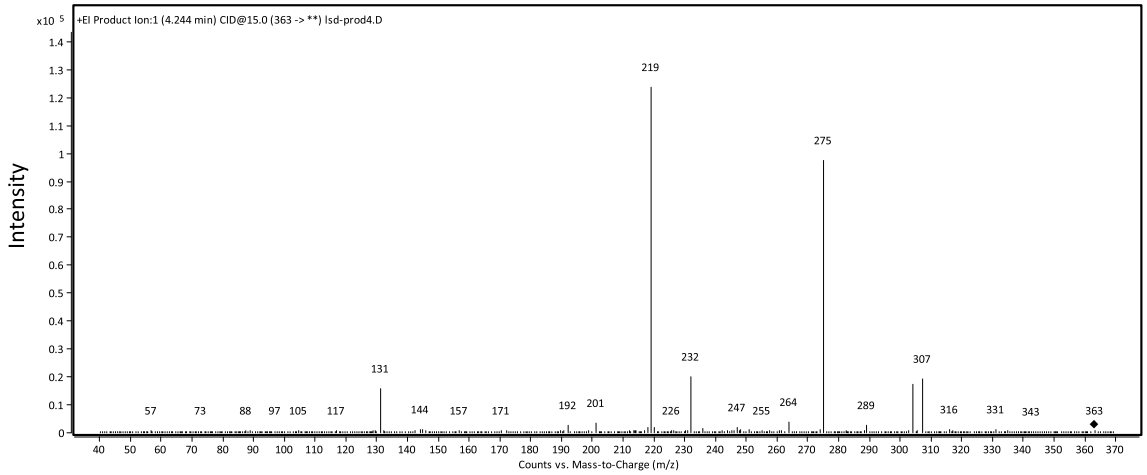

Figure 5 – MS/MS ion spectrum with m/z 363 (collision energy – 15 eV).

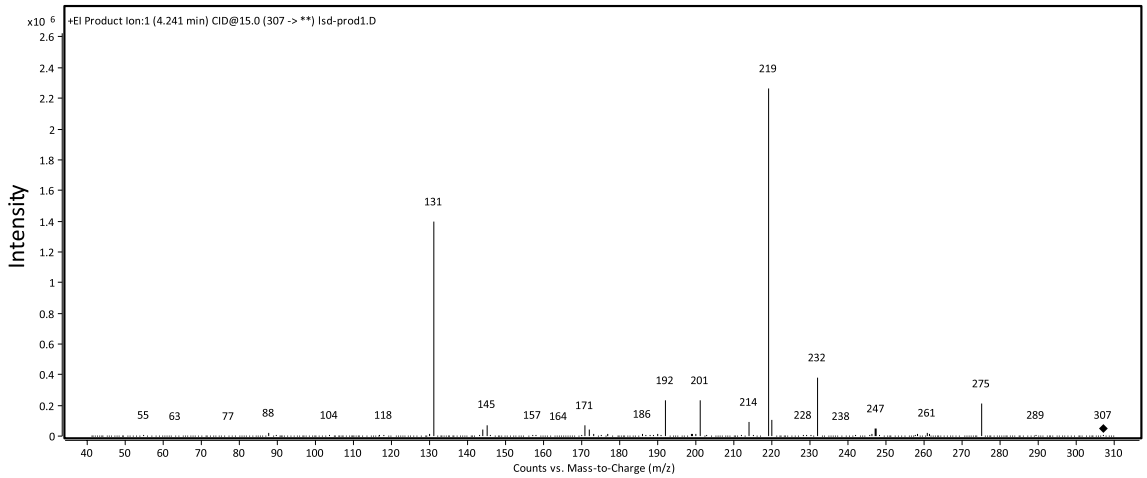

Figure 6 – MS/MS ion spectrum with m/z 307 (collision energy – 15 eV).

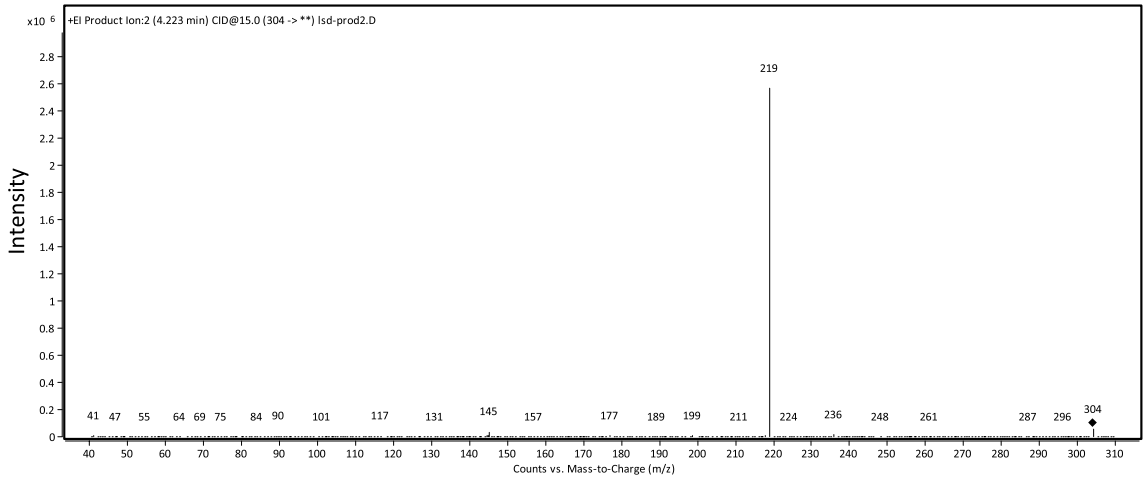

Figure 7 – MS/MS ion spectrum with m/z 304 (collision energy – 15 eV).

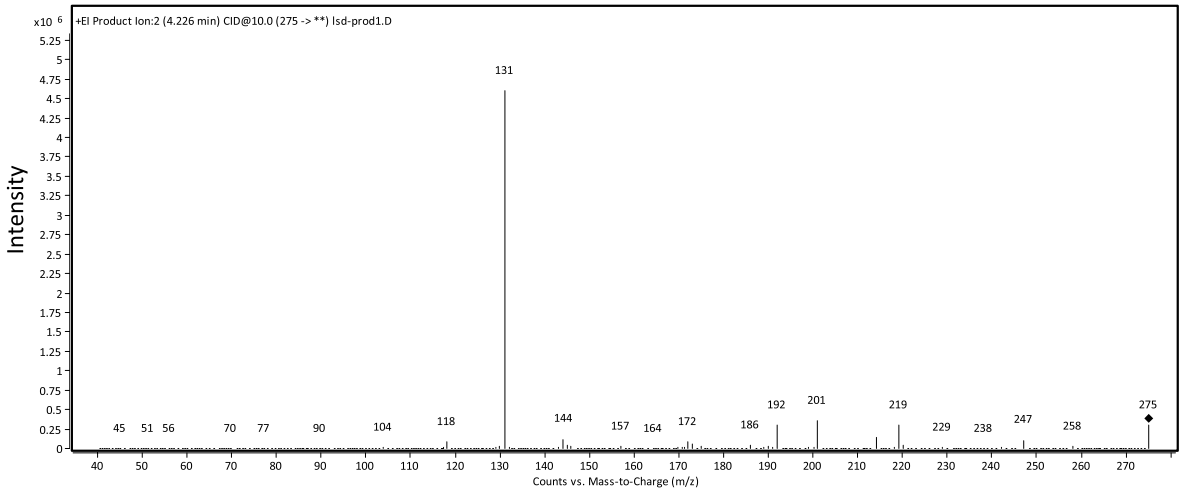

Figure 8 – MS/MS ion spectrum with m/z 275 (collision energy – 10 eV)

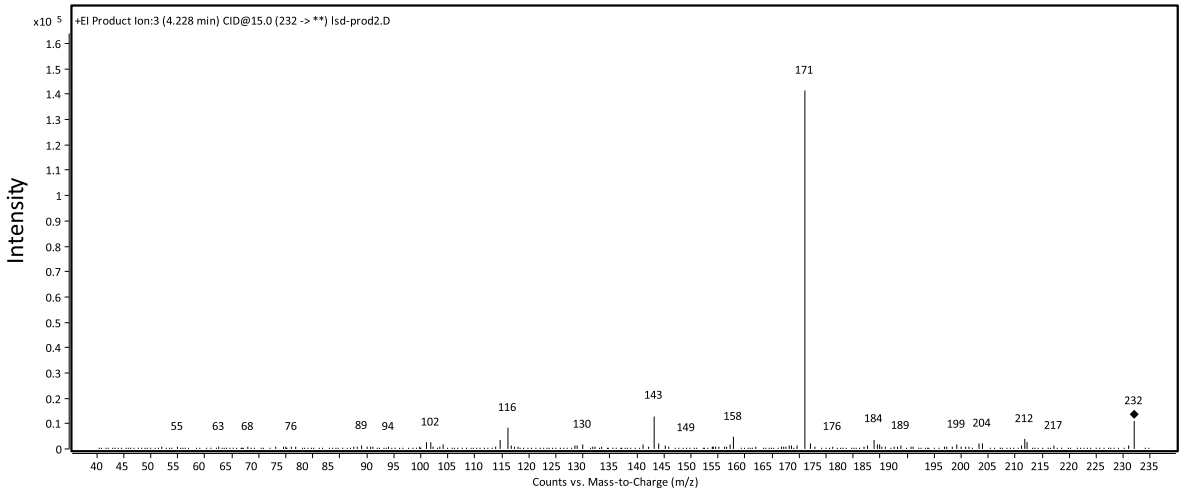

Figure 9 – MS/MS ion spectrum with m/z 232 (collision energy – 15 eV).

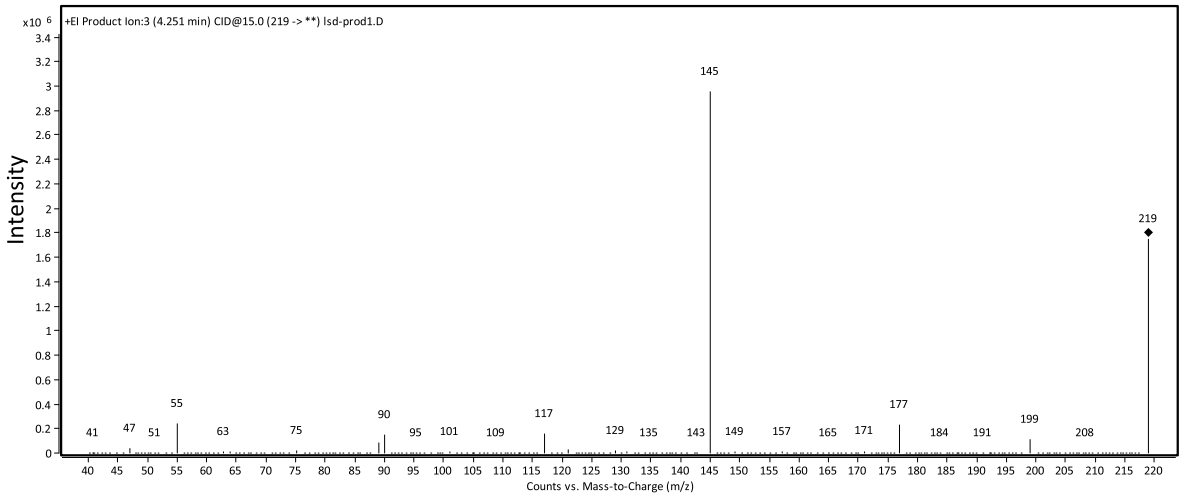

Figure 10 – MS/MS ion spectrum with m/z 219 (collision energy – 15 eV).

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2019-7-4-184-197

According to the mass spectra of individual ions presented in Fig. 5–10, all the ions are seen to be structurally bound together. So, ion with m/z value of 304 a.e.m., comprises ions with m/z values of 219 and 145 a.e.m., and ion with m/z value of 219 a.e.m. comprises ion 145 a.e.m., and ion with m/z value of 307 comprises ions with m/z values of 232, 275 and 131 a.e.m. The obtained results presented below, comply with the fragmentation structure impacted by electron impact (Fig. 11).

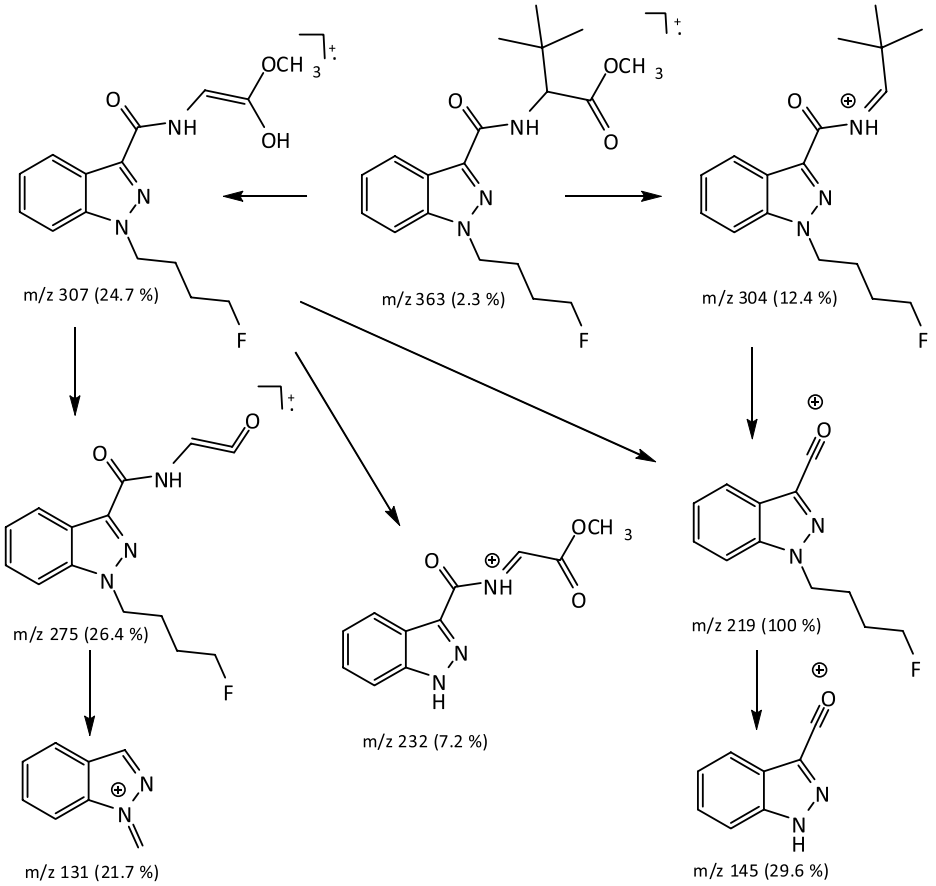

Figure 11 – Proposed structure of MDMB(N)-073F fragmentation.

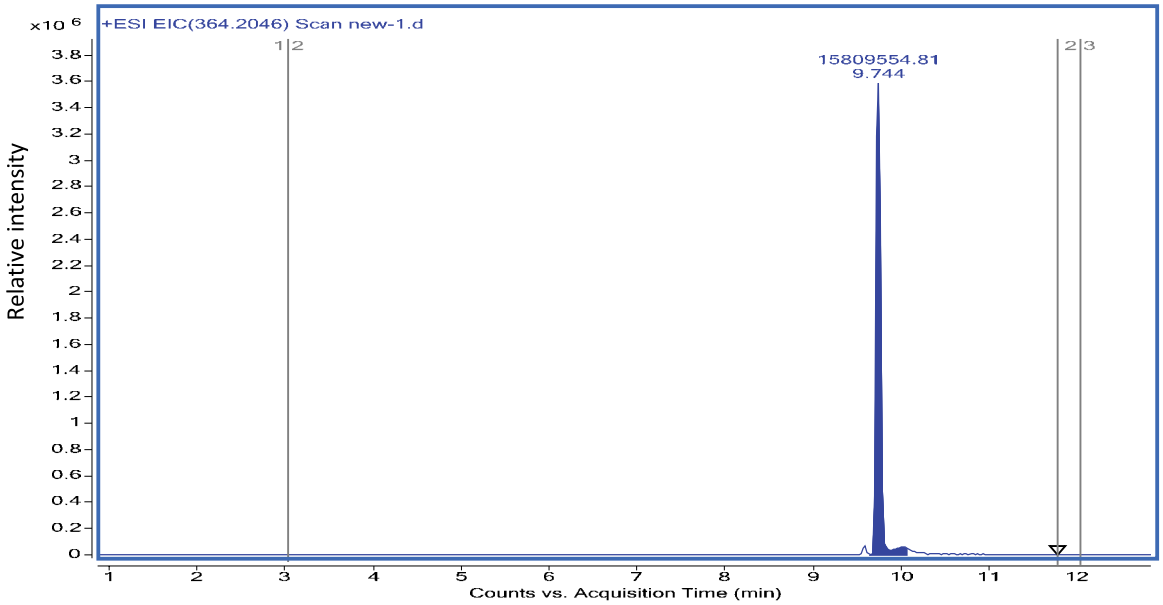

Figure 12 – Chromatogram of the plant-based MDMB(N)-073F object (HPLC-HRMS, m/z 364.203, image range ±5 mDa).

The data on the fragmentation of the main ions, obtained in the investigations of MDMB(N)-073F were found in the plant-based objects. The analysis was carried by HPLC-HRMS methods, taking into account the exact masses of the main ions. The chromatogram and spectrum of MDMB(N)-073F obtained in its analysis, are given in Fig.12 and 13, accordingly. Both theoretical and experimentally found exact masses of MDMB(N)-073F protonated molecule and fragment ions are presented in Table 2. The computed error is also given there.

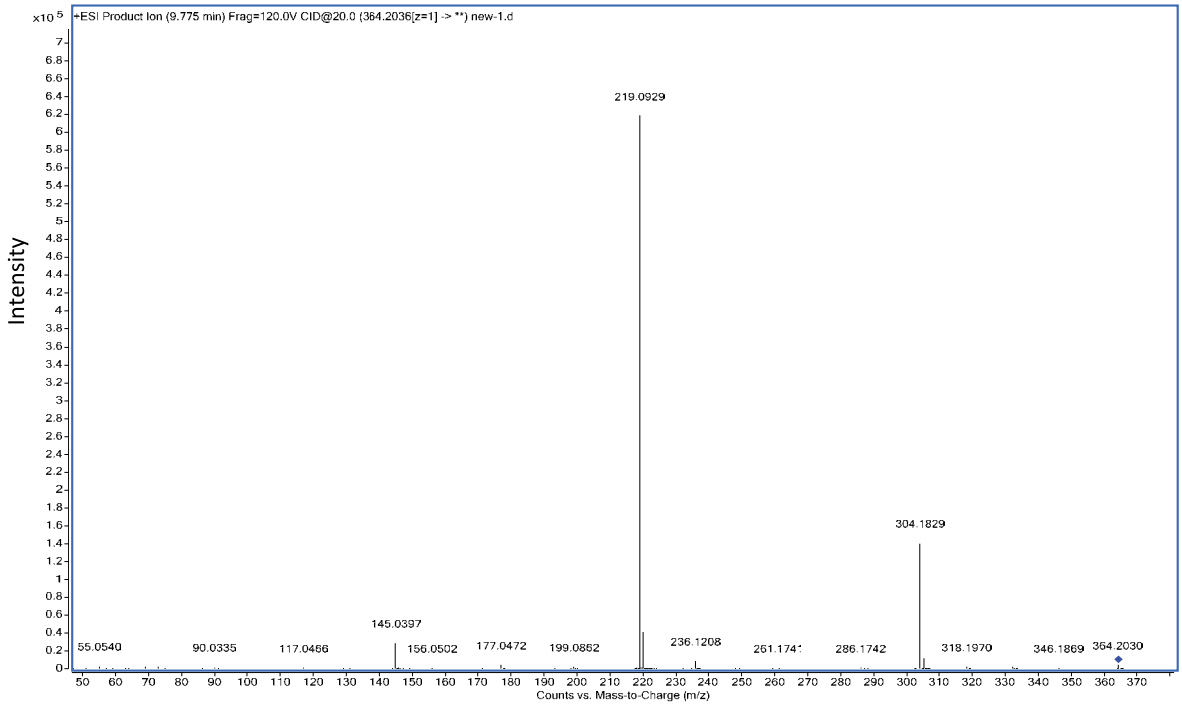

Figure 13 – MS/MS ion spectrum with m/z 364, 203.

Ions corresponding to protonated molecules of the original substance, are known to be within positive electrospray conditions. The ion with the chemical formula of $C_{19}H_{27}FN_3O_3$ and the exact mass of 364, 2031 Da is to be used in the combination with MDMB(N)-073F structure. The exact molecular mass of the ion measured in the experiment, is different from the mass calculated for 0.27 ppm, thereby proving the accuracy of the proposed chemical formula.

Table 2 – Determination of exact masses of protonated molecule and fragment ions of MDMB(N)-073F

Ion formula	Theoretical mass, Da	Measured mass, Da	Error, ppm
$C_{19}H_{27}FN_3O_3$	364.2031	364.2030	0.27
$C_{17}H_{23}FN_3O$	304.1852	304.1829	7.56
$C_{12}H_{15}FN_3O$	236.1193	236.1208	6.35
$C_{12}H_{12}FN_2O$	219.0928	219.0929	0.46
$C_8H_5N_2O$	145.0396	145.0397	0.69
C_4H_7	55.0542	55.0540	3.63

A study of the consumer's urine, containing MDMB (N)-073F, by HPLC-MS/MS methods and using multiple reaction registration (MRR) showed the following: apart from the marker, the conjugate with glucuronic acid are excreted together with the urine. The chromatograms are presented in Fig. 14–17.

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2019-7-4-184-197

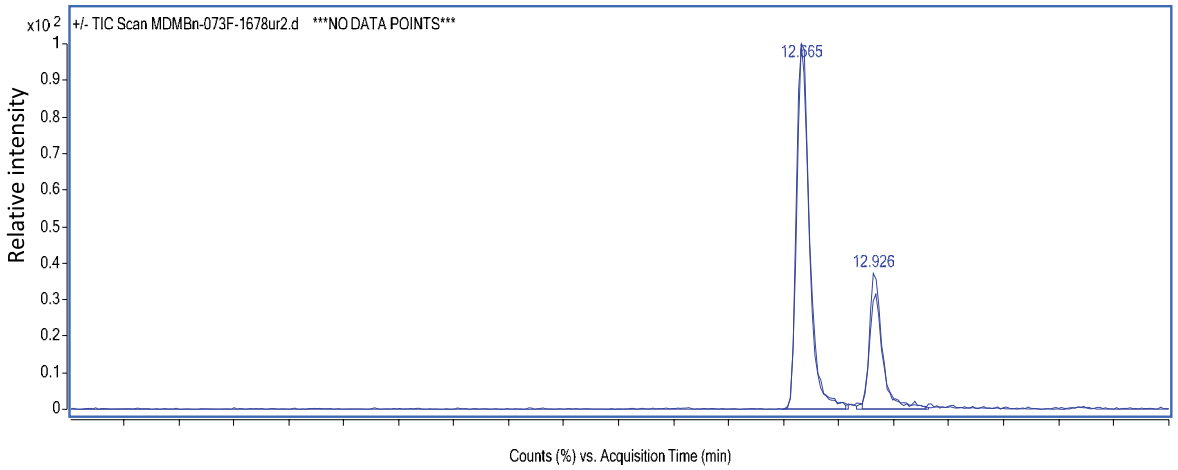

Figure 14 – Chromatogram of MDMB(N)-073F user's urine (HPLC-MS/MS, the total ion current for MRM transitions 350.1 > 145.0 and 350.1 > 219.0). Retention time for MDMB(N)-073F marker and its glucuronide is thereof 12.926 and 12.665 min., respectively

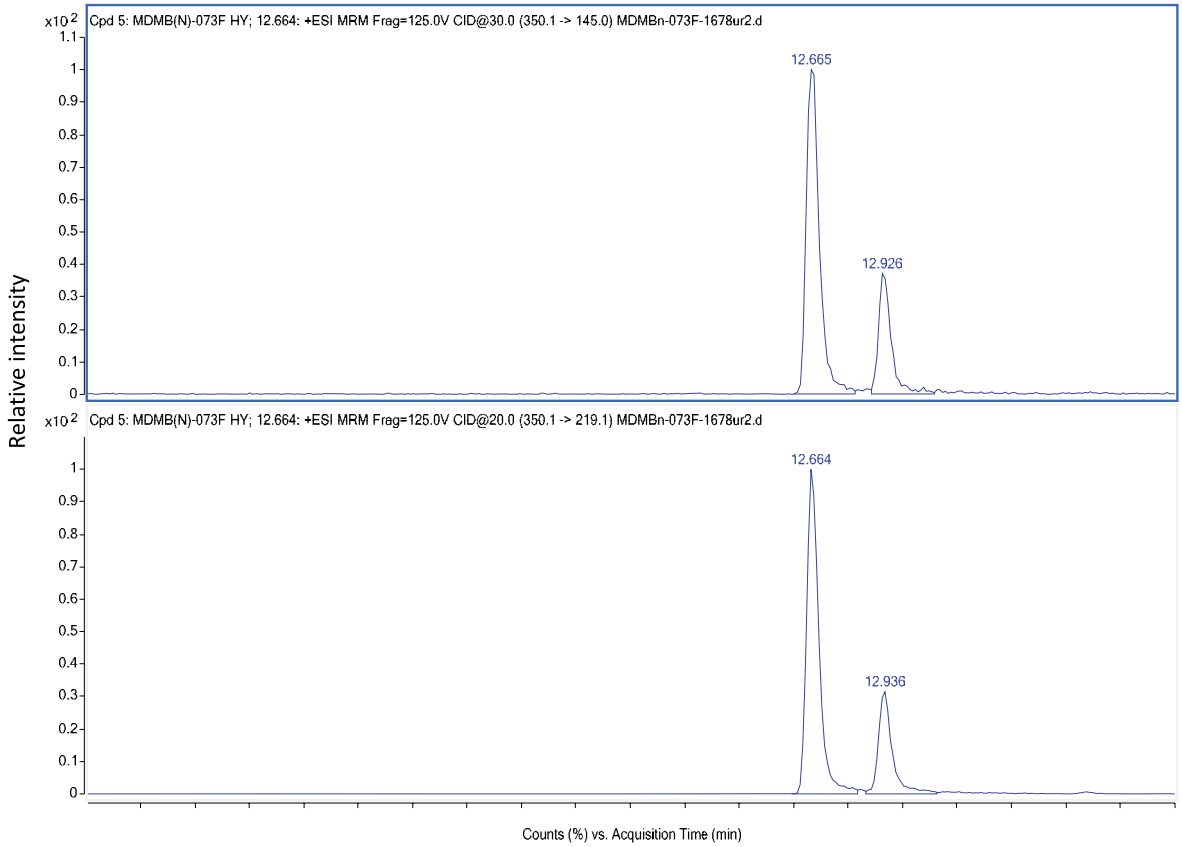

Figure 15 – Chromatogram of MDMB(N)-073F user's urine (HPLC-MS/MS; MRM transitions: above 350.1 > 145.0, below 350.1 > 219.0)

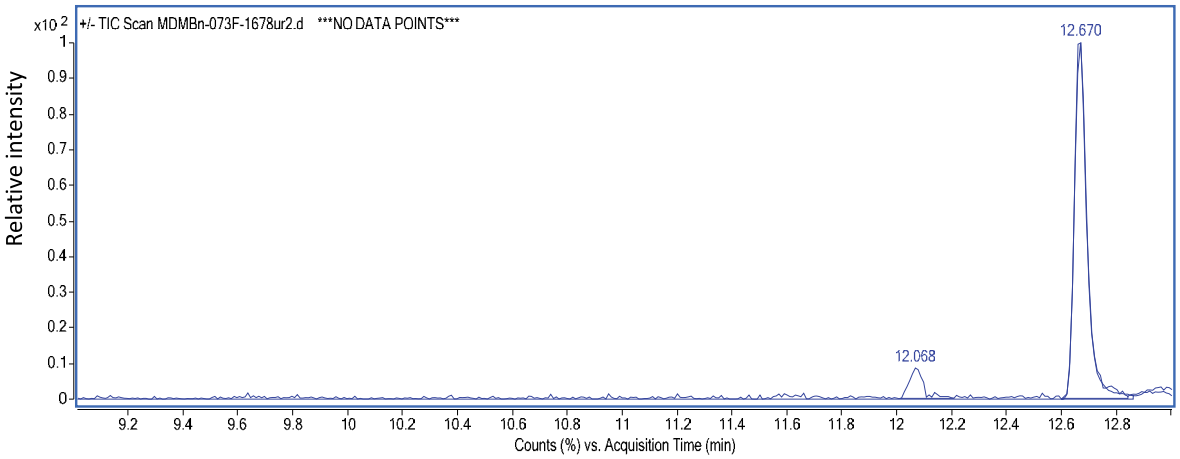

Figure 16 – Chromatogram of MDMB(N)-073F user's urine (HPLC-MS/MS, total ion current for MRM transitions 526.3 > 145.0 and 526.3 > 219.0). Retention time for glucuronide marker of MDMB(N)-073F– 12.670 min.

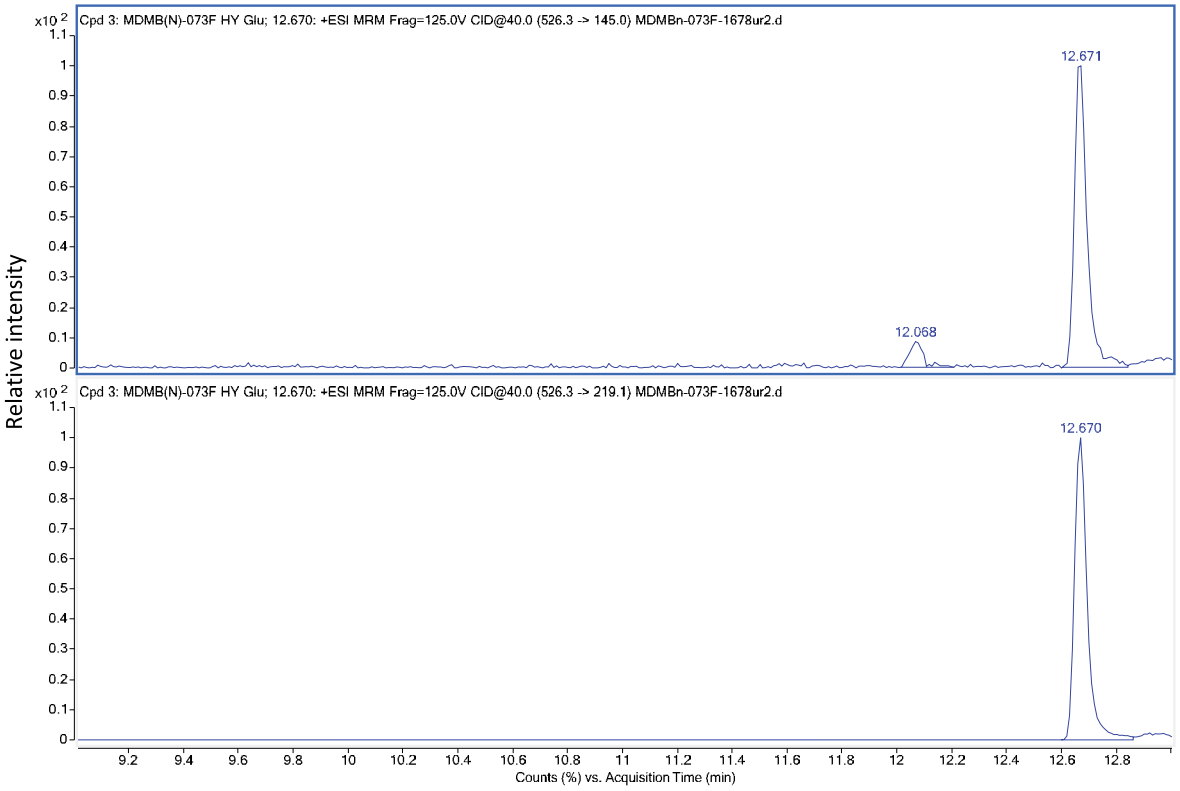

Figure 17 – Chromatogram of MDMB(N)-073F user's urine (HPLC-MS/MS; MRM transitions: above 526.3 > 145.0, below 526.3 > 219.0).

Mass-spectrum of ions – products of MDMB(N)-073F marker with m/z value of 350 for a protonated molecule – is similar to the spectrum of the unchanged compound (Fig. 13),; the ions with m/z values of 219 and 145 are presented therein (Figures 14, 15). The spectrum of ions – glucuronide products of MDMB(N)-073F marker (m/z 526 for the protonated molecule) – also contains these ions (Fig. 16, 17), thereby making it possible to use them in the registration of MRM transitions for both compounds.

Glucuronide of MDMB(N)-073F marker is a compound ester. This leads to its partial fragmentation in the ion source of liquid mass spectrometers with positive ionization [3]. Fragmentation of glucuronide in the source is basically due to the elimination of glucuronic acid residue. The resulting ion with m/z value of 350 corresponds to the protonated molecule of this MDMB(N)-073F marker itself. Glucuronide instability of MDMB(N)-073F marker makes it possible to use ion with m/z value of 350 as a precursor when finding both compounds (Fig. 14, 15).

ОРИГИНАЛЬНАЯ СТАТЬЯ
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The data presented herein connfirm that the major part of MDMB(N)-073F marker is found in the urine of its users as a conjugate with glucuronic acid.

CONCLUSIONS

The structure of MDMB(N)-073F compound has been confirmed by methods of gas chromatography with MS/MS mass-spectrometry and liquid chromatography with hybrid high-resolution quadrupole-time-of-flight mass spectrometry. Mass-spectral characteristics of MDMB(N)-073F have been given herein.

Ester decomposition with further conjugation of the resulting acid has been found one of MDMB(N)-073F biotransformation in the human body. The product interacting with glucuronic acid, is the conjugate of MDMB(N)-073F metabolite of phase I in biotransformation.

Metabolite formed as a result of ester decomposition and its conjugate with glucuronic acid, are recommended to be applied as markers for synthetic MDMB(N)-073F cannabimimetics in the analysis by chromatographic methods; they can be used in regular analytical screening of biological samples.

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AUTHOR CONTRIBUTIONS

All authors had equally contributed to the research work.

CONFLICTS OF INTEREST

The authors and peer reviewers of this paper report no conflicts of interest.

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AUTHORS

Sergey S. Kataev – Candidate of sciences (Chemistry), Chief of the forensic-chemistry division. Perm Regional Bureau of Forensic-Medical Expertise, Perm. ORCID 0000-0001-6742-2054. E-mail: forenschemist@yandex.ru

Oksana N. Dvorskaya – Candidate of sciences (Pharmacy), Assistant professor of the Chair of Toxicological Chemistry, Perm State Pharmaceutical Academy, Perm. ORCID 0000-0003-4774-8887. E-mail: dvoksnik@gmail.com

Maria A. Gofenberg – Head of Chemical-Toxicological Laboratory of the Regional Narcological Clinic; pharmacist-analyst at Chemical and Toxicology Laboratory of Sverdlovsk Regional Clinical Psychiatric Hospital; assistant of the Department of Pharmacy and Chemistry of Ural State Medical University, Yekaterinburg. ORCID: 0000-0003-2877-1301. E-mail: hoffenberg@yandex.ru

Andrei V. Labutin – expert chemist at Chemical and Toxicology Laboratory of Tomsk Regional Drug Abuse Clinic, Tomsk. ORCID 0000-0002-2719-1578. E-mail: lav877@rambler.ru

Aleksey B. Melentyev – Candidate of sciences (Chemistry), Chief of the forensic-chemistry division. Chelyabinsk Regional Bureau of Forensic-Medical Expertise, Chelyabinsk. ORCID 0000-0003-0470-1114. E-mail: melentjev-a@yandex.ru

RESEARCH ARTICLE

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EFFECT OF PUMPKIN (CUCURBITA PEPO L.) AND MARIGOLD (TAGETES PATULA L.) EXTRACTS ON HIPPOCAMPAL MITOCHONDRIA FUNCTIONAL ACTIVITY WITHIN CONDITIONS OF EXPERIMENTAL ACUTE BRAIN HYPOMETABOLISM

A.V. Voronkov, D.I. Pozdnyakov, S.L. Adzhiakhmetova, N.M. Chervonnaya, K.A. Miroshnichenko, A.V. Sosnovskaya, E.I. Chereshkova

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

E-mail: pozdniackow.dmitry@yandex.ru

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The aim of the study is to evaluate the effect of pumpkin *(Cucurbita pepo* L.*)* and marigold extracts *(Tagetes patula* L.*)* on the hippocampal mitochondria functional activity within the conditions of experimental acute brain hypometabolism.
Materials and methods. The work was performed on 50 male Wistar rats, which reproduced an acute brain hypometabolic state by administration of a 3M sodium azide solution in hippocampus (n = 40 and n = 10 – a group of sham-operated animals). The test extracts and the reference drug – EGb 761 – were prophylactically administered at the dose of 100 mg/kg *per os* for 10 days. 24 hours after the last administration, sodium azide was injected, the brain was taken, the hippocampus was isolated to obtain a supernatant and determine the parameters of mitochondrial respiration, the intensity of anaerobic processes, the concentration of the apoptosis-inducing factor, endonuclease G, and β-amyloid.
Results. The carried out study established that the prophylactic administration of pumpkin and marigold extracts contributed to the restoration of a mitochondrial function and a decrease in the intensity of anaerobic processes. In the group of the rats treated with pumpkin and marigold extracts, an increase of ATP concentration in the hippocampal supernatant by 65.7% ($p<0.002$) was observed; it was 66.2% ($p<0.002$) relative to the animals deprived of pharmacological support. ,When the rats were treated with pumpkin and marigold extracts, a decrease in the concentration of apoptosis-inducing factor (by 33% ($p<0.002$) and 38.3% ($p<0.002$), respectively) and endonuclease G (by 3.6 times ($p<0.002$) and 4.4 times ($p<0.002$), respectively) was also noted. The administration of pumpkin and marigold extracts reduced the amyloid β-peptide concentration in the rats' hippocampus by 54.4% ($p<0.0002$) and 54.4% ($p<0.0002$), respectively. The test-extracts had an equivalent therapeutic efficacy with the reference drug.
Conclusion On the basis of the obtained data, it is possible to suggest the prospect of a further study of pumpkin and marigold extracts as the drugs of a targeted correction of cerebral hypometabolism.
Keywords: plant extracts, hypometabolism, hippocampus, mitochondria

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ВЛИЯНИЕ ЭКСТРАКТА ТЫКВЫ ОБЫКНОВЕННОЙ (CUCURBITA PEPO L.) И ЭКСТРАКТА БАРХАТЦЕВ РАСПРОСТЕРТЫХ (TAGETES PATULA L.) НА ФУНКЦИОНАЛЬНУЮ АКТИВНОСТЬ МИТОХОНДРИЙ ГИППОКАМПА В УСЛОВИЯХ ЭКСПЕРИМЕНТАЛЬНОГО ОСТРОГО ГИПОМЕТАБОЛИЗМА ГОЛОВНОГО МОЗГА

А.В. Воронков, Д.И. Поздняков, С.Л. Аджиахметова, Н.М. Червонная, К.А. Мирошниченко, А.В. Сосновская, Е.И. Шерешкова

Пятигорский медико-фармацевтический институт – филиал ФГБОУ ВО «Волгоградский государственный медицинский университет» Минздрава России
Россия, 357532, г. Пятигорск, пр. Калинина, д. 11

E-mail: pozdniackow.dmitry@yandex.ru

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Цель исследования. Оценить влияние экстракта тыквы обыкновенной (*Cucurbita pepo* L.) и экстракта бархатцев распростертых (*Tagetes patula* L.) на функциональную активность митохондрий гиппокампа в условиях экспериментального острого гипометаболизма головного мозга.

Материалы и методы. Работа выполнена на 50 крысах-самцах линии *Wistar*, которым моделировали острое гипометаболическое состояние путем введения 3М раствора натрия азида в гиппокамп (n=40 и n=10 – группа ложнооперированных животных). Исследуемые экстракты и препарат сравнения – EGb 761 вводили в дозе 100 мг/кг *per os*, профилактически на протяжении 10 дней. Через 24 часа после ведения натрия азида осуществляли забор головного мозга, выделяли гиппокамп с получением супернатанта и определением в нем параметров митохондриального дыхания, интенсивности анаэробных процессов, концентрации апоптоз-индуцирующего фактора, эндонуклеазы G и β-амилоида.

Результаты исследования. В ходе исследования было установлено, что профилактическое введение изучаемых экстрактов тыквы и бархатцев способствовало восстановлению митохондриальной функции и снижению интенсивности анаэробных процессов. При этом в группах крыс, получавших экстракт тыквы и бархатцев, отмечено повышение концентрации АТФ в супернатанте гиппокампа относительно животных без фармакологической поддержки на 65,7% (p<0,002) и 66,2% (p<0,002); соответственно. Также при введении крысам экстрактов тыквы и бархатцев наблюдалось снижение концентрации апоптоз-индуцирующего фактора (на 33 % (p<0,002) и 38,3% (p<0,002) соответственно) и эндонуклеазы G (в 3,6 раза (p<0,002) и в 4,4 раза (p<0,002) соответственно). При применении исследуемых экстрактов тыквы и бархатцев отмечено снижение концентрации амилоидного β-пептида в гиппокампе крыс на 54,4% (p<0,0002) и 54,4% (p<0,0002) соответственно. При этом исследуемые экстракты обладали эквивалентной терапевтической эффективностью с препаратом сравнения.

Заключение. На основании полученных данных можно предположить перспективность дальнейшего изучения экстрактов тыквы и бархатцев как средств целенаправленной коррекции церебрального гипометаболизма.

Ключевые слова: растительные экстракты, гипометаболизм, гиппокамп, митохондрии

INTRODUCTION

Alzheimer's disease (AD) is a worsening neurodegenerative disease which accounts for 50–70% of dementia cases, comprising more than 12 million people [1]. Generally, clinical manifestations of AD are observed in the late stages of the disease and are associated with deposition of cytotoxic β-amyloid in the brain structures [2]. Amyloid β-peptide is formed as a result of proteolysis of transmembrane protein – amyloid precursor protein (APP) during catalysis of secretase enzymes. To date, it has been established that the accumulation of β-amyloid is one of the most reliable and early markers of irreversible neurodegeneration [3]. In the processes of neuronal degradation, various β-amyloid isoforms form two types of cytotoxic conglomerates: the aggregates not stabilized by metal ions (usually soluble in water and having little cytotoxic potential) and associates in which divalent metal ions are present (these conglomerates form covalently cross-linked oligomers, which are then deposited in the cytoplasm of neurons (mainly the hippocampus) in the form of amyloid plaques) [4]. The excess of amyloid β-peptide in brain cells leads to irreversible phosphorylation of tau protein, resulting in the increased production of 4-hydroxynonenal – membrane-toxic aldehyde, which, in its turn, initiates oxidative processes in neuronal membranes, accompanied by a deterioration of the ion transport ATPases, functioning as glucose and glutamate transporters [5]. As a result,

the described processes lead to the impairment in synaptic transmission and AD worsening.

The research group of *Del Prete D, et al., 2017* found out that the amyloid β-peptide precursor protein can be localized on mitochondrial membranes. In addition, a secretase complex, mainly represented by γ-secretase, is found on mitochondrial membranes, which, in turn, provides a rapid synthesis of β-amyloid from its precursor [6].

In a number works it is noted that mitochondrial β-amyloid can play a leading role in the pathological cascade, adducting to early neurons death within AD conditions. Thus, a study by *Lustbader J W, et al., 2004* showed that there is a direct relationship between a mitochondrial dysfunction and a hypometabolic brain state, on the one hand, and an increase of the β-amyloid cytotoxicity, on the other [7]. Further on, *Chen X. et al., 2006* found out that within AD conditions, brain hypometabolism arising with an increase of the amyloid β-peptide concentration is associated with a malfunction of electron transport reactions in the mitochondrial respiratory chain, activation of anaerobic oxidative processes, and intensification of the internal apoptosis pathway (the effector system – endonuclease G) [8]. However, it should be noted that activation of the mitochondrial γ-secretase complex is observed only with a decrease in the intensity of metabolic reactions — a hypometabolic state [9]. Thus, it can be assumed that a targeted pharmacological correction of cerebral hypometabolism may be a promising method for the early treatment of AD. In this regard, the **aim of this study** was to assess the effect of pumpkin *(Cucurbita pepo L.)* and marigold extracts *(Tagetes patula* L.*)* on the hippocampal mitochondria functional activity within experimental acute brain hypometabolism conditions.

MATERIALS AND METHODS

Experimental animals

In the experiment, 50 sexually mature male Wistar rats with a body weight of 220–240 grams) were used. The animals had been obtained from "Rappolovo", the Federal State Unitary Enterprise "Nursery of laboratory animals (Leningrad Region). During the experiment, the rats were kept under standard vivarium conditions at the air temperature of 22 ± 2°C, the relative humidity of 60 ± 5% and a natural circadian cycle change. The animals were kept in macrolon cells by 10 individuals, where a granular beech fraction was used as a nesting material. The rats received extruded food and water *ad libitum*. Manipulations with the experimental animals were performed in accordance with the generally accepted ethical standards adopted by the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (1986) and taking into account the International recommendations of the European Convention for the protection of vertebrate animals used in experimental studies (1997) [24, 25].

Test objects. Experiment design

In this work, a thick pumpkin extract *(Cucrbita pepo L.)* and a thick marigold extract *(Tagetes patula L.)* were used as test objects. Marigold inflorescences *(Tagetes patula L.)* of the «Carmen» variety were collected in the botanical garden of Pyatigorsk Medical and Pharmaceutical Institute – branch of Volgograd State Medical University. The pumpkin fruits *(Cucurbita pepo L.) of the* «Atlant srednepozdnij» variety were collected in the «Aleksandrovskij» district, Stavropol region. The raw materials samples were harvested in the period from June to September 2016–2018. Fresh pumpkin fruits and marigold inflorescences, dried in shadow, were used as raw materials.

EGb 761 (a standardized *Ginkgo biloba* extract manufactured by *Hunan Warrant Pharmaceuticals*, China) was a reference drug. The reference drug was administered *per os* at the dose of 100 mg/kg [10] before a surgery operation for 10 days. The test objects were administered similarly to the reference drug (prophylactically, at the dose of 100 mg/kg for 10 days). During the experiment, the following experimental groups of animals were formed: sham-operated rats (SO, n = 10); a negative control group of rats, deprived of pharmacological support (NC, n = 10); groups of animals treated with the reference drug and test–objects (n = 10 in each experimental group). The study design is shown in Fig. 1.

Experimental model of cerebral hypometabolism

Cerebral hypometabolism was modeled by an intrahippocampal injection of a 3M sodium azide solution, for which the animals had been anesthetized (chloral hydrate 350 mg/kg, intraperitoneally), the rats' heads were fixed, the pelage was removed and the skull was scalped. Then, in the left and right hemispheres, trepanation holes were drilled with a 1 mm diameter trepanation bur with the stereotactic coordinates P: –3.0; ML: ± 3.0, V: –3.0 (from Bregma), which corresponded to the dorsal part of the hippocampus [11]. Then, a 30G needle was slowly inserted into the trepanation hole to the depth of 1.5 mm; a 3 M sodium azide solution (pH = 7.4) was injected in 0.5 µl volume, alternating with the right and left hemispheres (totally, there were 4 injections made). The needle was removed after 30 seconds since the insertion. The wound was sutured, the seam treated with a 5% iodine solution. Biomaterial was taken 24 hours after the surgery [12].

ОРИГИНАЛЬНАЯ СТАТЬЯ
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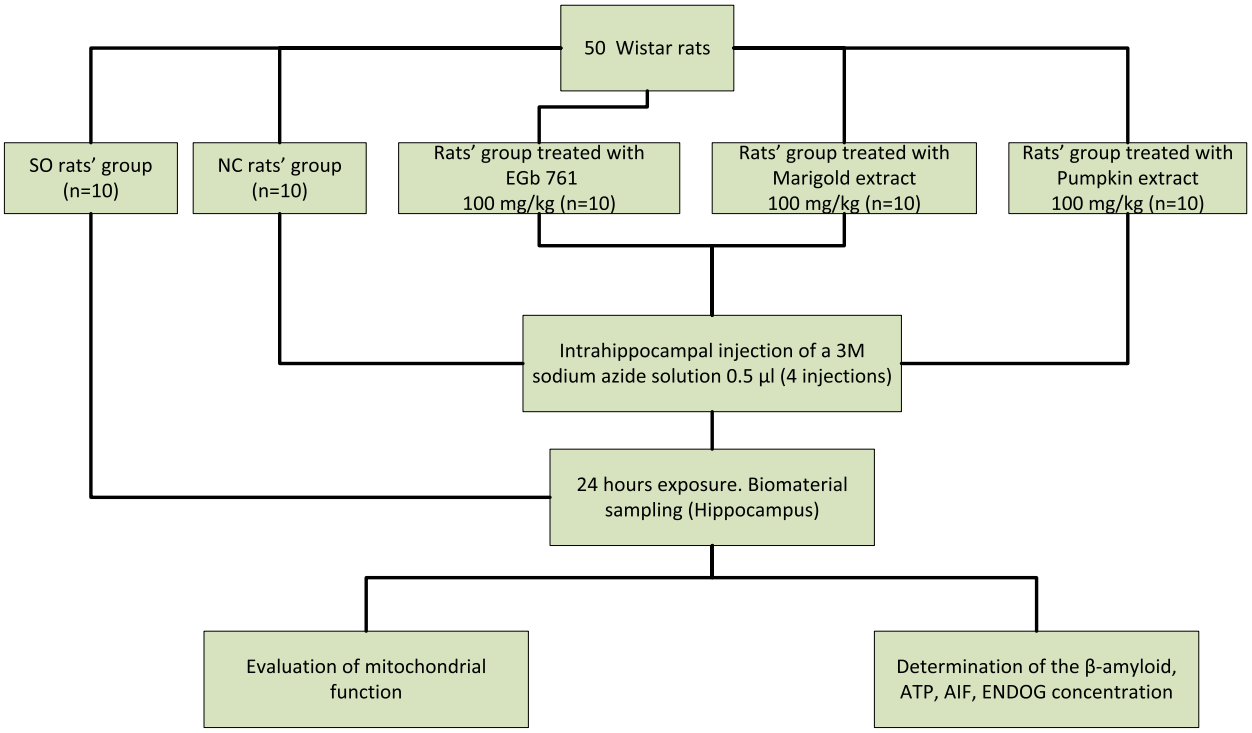

Figure 1 – Experiment design

Note: SO – a sham-operated rats group; NC – a negative control anmals group; ATP – adenosine triphosphate; AIF – apoptosis-inducing factor; ENDOG – endonuclease G.

Biomaterial sampling

In the work, the rat hippocampus was used as biomaterial. The hippocampus was removed according to the standard procedure. The isolated hippocampus was divided into 2 parts: the first was homogenized in the following medium: 1 mmol EDTA + 215 mmol mannitol + 75 mmol sucrose + 0.1% BSA solution + 20 mmol HEPES (pH 7.2), followed by a double centrifugation in modes of 1,400g → 3 min. at 4^0C (supernatant was removed) and 13000g → 10 min. The resulting secondary supernatant was removed for a respirometric analysis. The second part of the hippocampus was homogenized in PBS with pH 7.4 in a ratio of 1:7, centrifuged in the mode of 10000g → 5 min, and then the resulting supernatant was taken for ELISA.

Respirometric analysis

In the work, the previously described approach to assessing mitochondria respirometric functions had been used. The study was performed according to the SEAHORSE protocol on a laboratory AKPM-01L respirometer (Alfa-Bassens, Russian Federation). During the analysis, the change in oxygen consumption in the medium containing native mitochondria, was assessed against the background of the injection of mitochondrial respiration disconnectors: oligomycin 1 µg/ml; 4-(trifluoromethoxy) phenyl) hydrazono) malononitrile (FCCP – 1 µM); rotenone – 1 µM; sodium azide – 20 mmol. Glucose (15 mmol) was used as an oxidation substrate in the process of the study of the intensity of anaerobic processes. At the same time, the following parameters characterizing the mitochondria respiratory function were calculated: ATP-generating capacity; maximum respiratory rate, respiratory capacity. The glycolysis intensity, glycolytic capacity, and glycolytic reserve were also determined [13].

Enzyme-linked immunosorbent assay immunoassay

The standard ELISA reagents kits manufactured by *Cloud clone* (USA) was used. During the study, the changes in the following parameters were evaluated: β-amyloid concentration, apoptosis-inducing factor (AIF), endonuclease G (ENDOG) and ATP concentration. The analysis was carried out in accordance with the manufacturer's recommendations (a protocol analysis instruction was attached to each kit). The results measurement was carried out on a microplate reader *Infinite* F50 (Tecan, Austria).

Methods of statistical analysis

Statistical analysis of the obtained results was performed in the STATISTICA 6.0 software package (StatSoft, USA). The data were expressed as M ± SEM. The comparison of means was carried out by the one-way analysis of the variance method with the post-hoc Newman-Keuls test.

RESULTS

The influence of the test objects and the reference drug on the change of the β-amyloid concentration in the rats' hippocampus within the conditions of experimental acute brain hypometabolism

During this part of the work it was found out that the β-amyloid concentration (Fig. 2) in the SO group was 9.46 ± 0.09 pg/ml. At the same time, in the NC group of the animals within the conditions of the experimental cerebral hypometabolism, an increase of the amyloid β-peptide concentration in comparison with the SO group of the animals was by 9 times (p <0.0001).

The use of EGb 761 reduced the content of cytotoxic β-amyloid in the rats' hippocampus in relation to the NC group of the animals by 54.4% (p<0.0002). Against the background of the test pumpkin and marigold extracts administration, the β-amyloid concentration decreased relative to the NC group of the animals by 54.4% (p<0.0002) and by 49% (p<0.0002), respectively (Fig. 2).

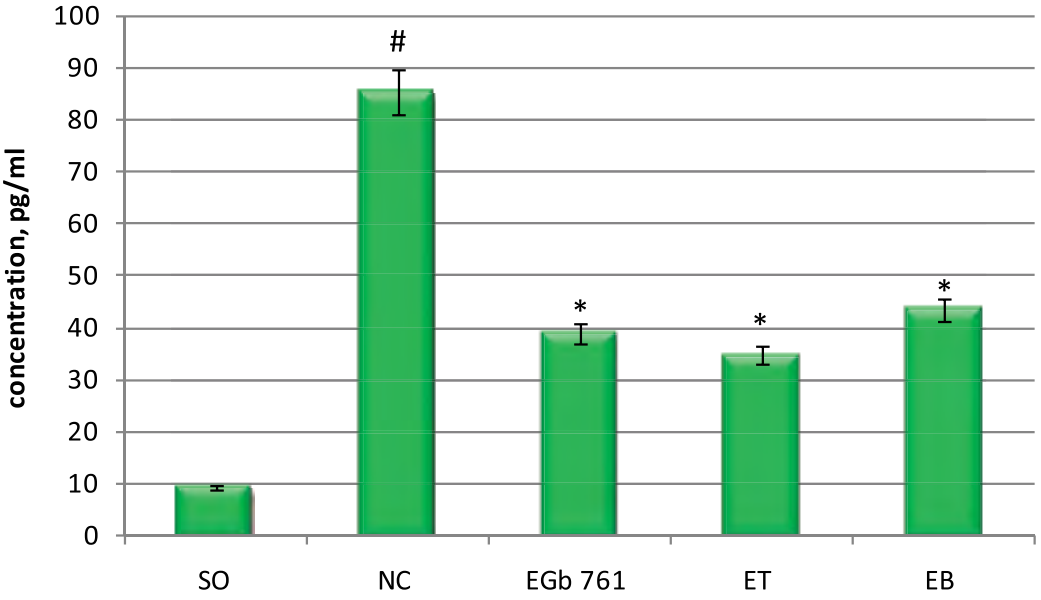

Figure 2 – Effect of the test objects and the reference drug on the change of the β-amyloid concentration in the rats' hippocampus within the conditions of the experimental acute brain hypometabolism

*Note. SO – a sham-operated group of rats; NC – a negative control group of rats; ET – a group of rats treated with the test pumpkin extract; EB – a group of rats treated with the test marigold extract; EGb 761 – a group of rats treated with EGb 761; # – statistically significant relative to the SO animals group (Newman-Keusle test, p <0.0001); * – statistically significant relative to the NC animals group (Newman-Keusle test, p <0.0002).*

The influence of the test objects and the reference drug on the change of the rats' hippocampal mitochondria respirometric function within the conditions of the experimental acute brain hypometabolism

When assessing the effect of the test extracts and EGb 761 on the change of the hippocampal mitochondria respirometric function, it was found out that in the NC group within the conditions of the experimental brain hypometabolism compared with the SO group of rats, a decrease in the ATP-generating capacity was by 10.8 times (p<0.0001), the maximum respiratory rate was by 19.4 times (p<0.0001) and the respiratory capacity was by 9.9 times (p<0.0001) (Fig. 3).

In the animals deprived of pharmacological support, in relation to the SO group of rats, the glycolysis intensity increased by 8.5 times (p <0.0001), and the glycolytic capacity and glycolytic reserve decreased by 5.8 times (p<0.0001) and 6.1 times (p <0.0001), respectively (Fig. 4). As a result, the ATP concentration in the hippocampal supernatant of the NC group decreased by 2.2 times (p<0.0001) relative to the SO group (Fig. 5).

ОРИГИНАЛЬНАЯ СТАТЬЯ
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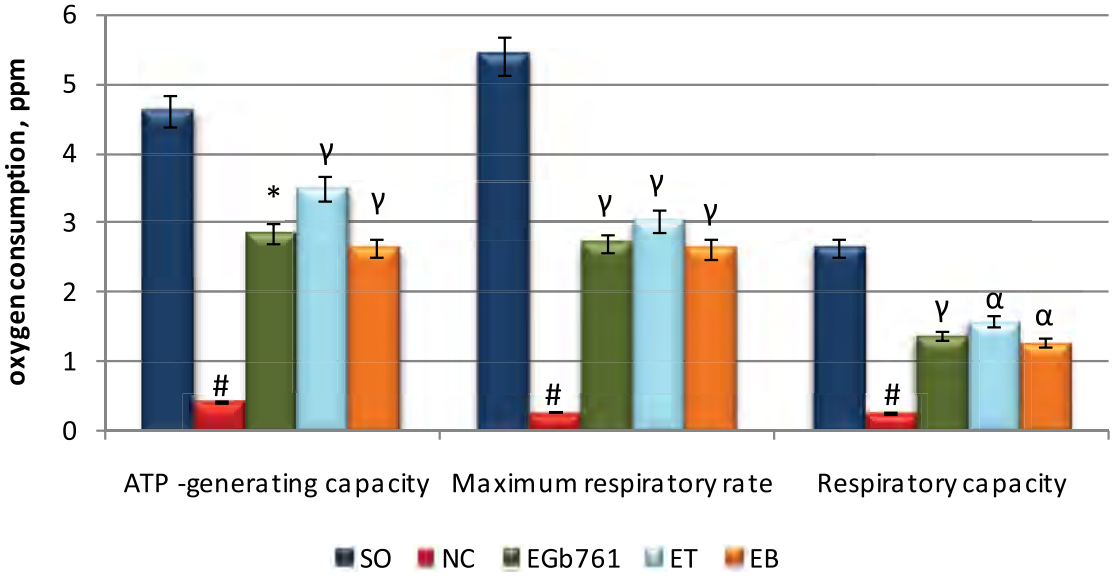

Figure 3 – Effect of the test objects and the reference drug on the change of the mitochondrial respiration in the rats' hippocampus within the conditions of the experimental acute brain hypometabolism

*Note. SO – a sham-operated group of rats; NC – a negative control group of rats; ET – a group of rats treated with the test pumpkin extract; EB – a group of rats treated with the test marigold extract; EGb 761 – a group of rats treated with EGb 761; # – statistically significant relative to the SO group of animals (Newman-Keusle test, p<0.0001)); statistically significant relative to the NC group of animals (Newman-Keusle test * – p <0.02; γ – p<0.001; α-p<0.004).*

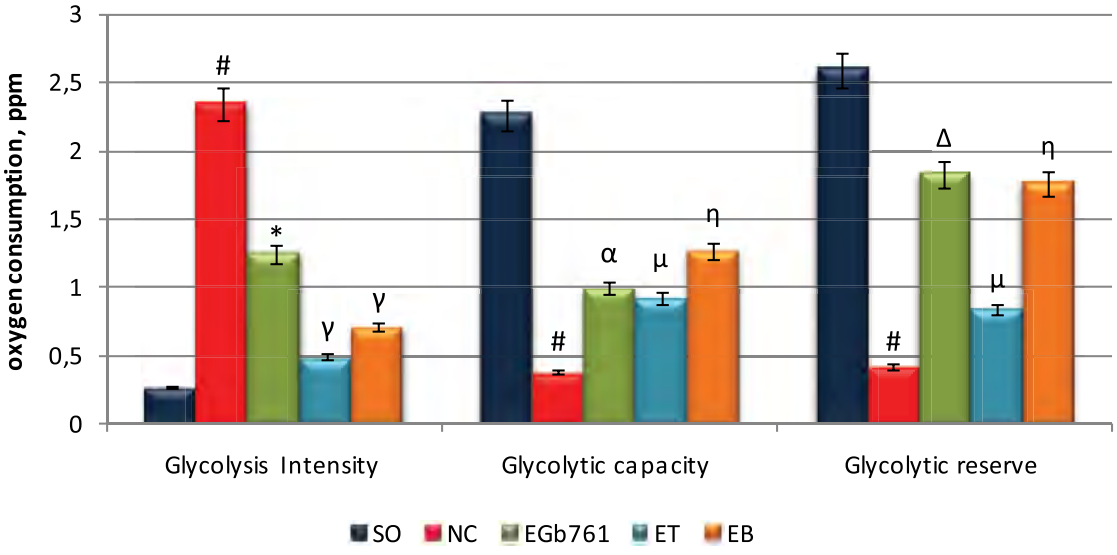

Figure 4 – The effect of the test objects and the reference drug on the change of the anaerobic processes activity in the rats' hippocampus within the conditions of the experimental acute brain hypometabolism

*Note. SO – a sham-operated group of rats; NC – a negative control group of rats; ET – a group of rats treated with the test pumpkin extract; EB – a group of rats treated with the test marigold extract; EGb 761 – a group of rats treated with EGb 761; # – statistically significant relative to the SO animals' group (Newman-Keusle test, p <0.0001); * – statistically significant relative to the NC group of animals (Newman-Keusle test, * – p <0.005; γ–p <0.002; α–p <0.007; Δ- p <0.03; μ–p <0.01; η–p <0.004)*

Against the background of EGb 761 administration in rats, an increase (relative to the NC group of animals) of ATP-generating capacity (Fig. 3) by 6.7 times (p<0.002), the maximum respiratory rate by 9.7 times (p<0.001) and respiratory capacity – by 5.1 times (p<0.001) was noted. In the rats treated with EGb 761, there was also an increase in glycolytic capacity and glycolytic reserve (Fig. 4) in comparison with the same parameters of the NC animals' group by 2.6 times (p<0.007) and by 4.3 times (p <0.03), respectively, while the intensity of glycolysis decreased by 1.9 times (p<0.005). In addition, the concentration of ATP in the animals treated with EGb 761 was 65.7% (p<0.002) higher than that in the rats without any pharmacological correction.

In the animals treated with the pumpkin extract, relative to the NC rats' group, an increase in ATP-generating capacity was by 8.2 times (p <0.001); the maximum level of respiration was by 10.9 times (p <0.001) and respiratory capacity was by 5.9 times (p <0.004) (Fig. 3). At the same time, the intensity of glycolysis in the animals that were administrated pumpkin extract, was 4.7 times (p <0.002) lower than that in the NC group of rats, while the glycolytic capacity and glycolytic reserve (Fig. 4) in the animals treated with the test pumpkin extract, increased in comparison with the rats deprived of pharmacological support by 2.4 times (p <0.01) and twice (p <0.004), respectively. The administration of the pumpkin extract in the animals contributed to an increase of ATP content (Fig. 5) in the hippocampus by 66.2% (p <0.002) in relation to the NC group of rats.

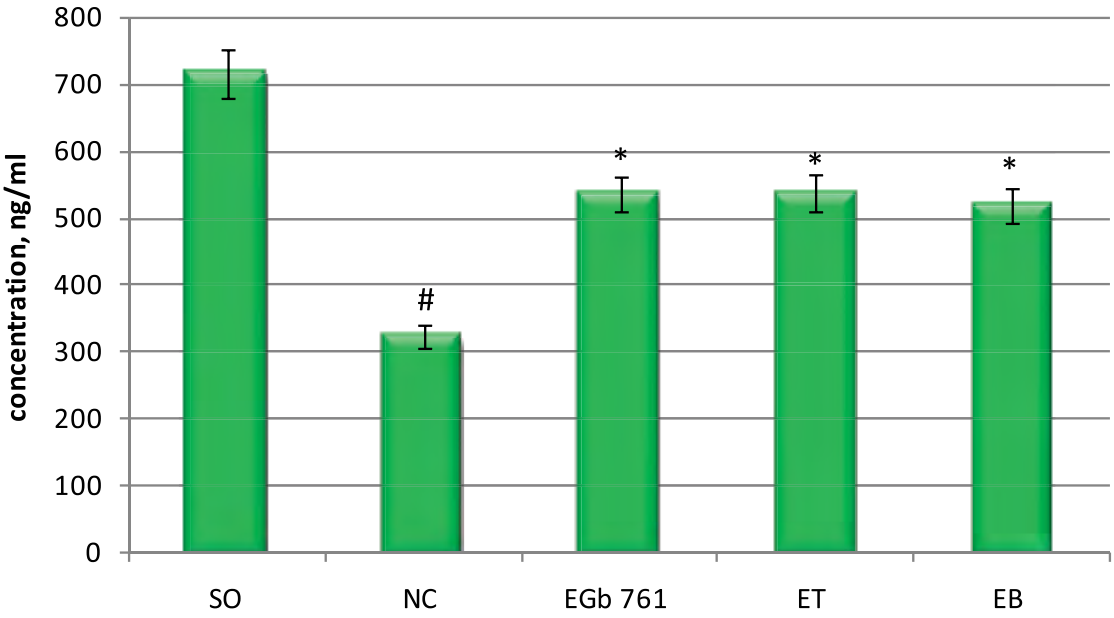

Figure – 5. The effect of the test-objects and the reference drug on the change of the ATP concentration in the rats' hippocampus within the conditions of the experimental acute brain hypometabolism

*Note. SO – a sham-operated group of rats; NC – a negative control group of rats; ET – a group of rats treated with the test pumpkin extract; EB – a group of rats treated with the test marigold extract; EGb 761 – a group of rats treated with EGb 761; # – statistically significant relative to the SO group (Newman-Keusle test, p<0.0001); * – statistically significant relative to the NC group (Newman-Keusle test, p<0.002).*

When the marigold extract was administrated in the animals, an increase (relative to the NC rats' group) of ATP-generating capacity, the maximum respiratory rate and respiratory capacity (Fig. 3) by 6.2 times (p<0.001); by 9.4 (p<0.001) and 4.7 times (p<0.004), respectively, were noted. In addition, the intensity of glycolysis in the animals treated with marigold extract was 3.3 times lower (p<0.002) than that in the NC group of rats, while the glycolytic capacity and glycolytic reserve (Fig. 4) increased in the animals treated with marigold extract compared with the rats deprived of pharmacological support by 3.3 (p<0.01) and 4.2 times (p<0.004), respectively. As a result, the ATP concentration in the hippocampus of the animals treated with the marigold extract was 60.7% (p<0.002) higher than that of the rats of the NC group (Fig. 5).

The influence of the test objects and the reference drug on the change of the internal apoptosis pathway activity within the conditions of experimental acute brain hypometabolism

In the course of this part of the study it was found out that the concentration of AIF and ENDOG in the animals of the NC group exceeded the similar values of the SO rats' group by 3 (p<0.0001) and 21.7 times (p<0.002), respectively (Table 1). At the same time, the animals treated with EGb 761 showed a decrease in AIF concentration by 36.8% (p<0.002) and ENDOG by 2.7 times

ОРИГИНАЛЬНАЯ СТАТЬЯ
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(p<0.0001) compared with the group of rats deprived of pharmacological support (Table 1).

Against the background of using the test pumpkin extract in the animals, the contents of AIF and ENDOG in the hippocampus decreased (relative to the NC group of rats) by 33% (p<0.002) and 3.6 times (p<0.002), respectively. A decrease in the concentration of proapoptotic markers of the internal apoptosis pathway was also observed while using the marigold extract. So, in the animals that were administrated the test marigold extract, the concentration of AIF and ENDOG was 38.3% (p<0.002) and 4.4 times (p<0.002), respectively, lower than the similar parameters of the NC rats' group (Table 1).

Table 1 – The influence of the test objects and the reference drug on the change of the internal apoptosis pathway activity within the conditions of experimental acute brain hypometabolism

Group	SO	NC	EGb 761	ET	EB
AIF, ng/ml	6.16±0.413	18.69±0.04#	11.81±0.043*	12.61±0.426*	11.54±0.446*
ENDOG, ng/ml	72.03±1.069	1564.42±63.59#	578.41±60.45*	433.09±42.41*	352.35±10.796*

*Note. AIF – apoptosis-inducing factor; ENDOG – endonuclease G; SO – a sham-operated group of rats; NC – a negative control group of rats; ET – a group of rats treated with the test pumpkin extract; EB – a group of rats treated with the test marigold extract; EGb 761 – a group of rats treated with EGb 761; # – statistically significant relative to the SO animals' group (Newman-Keusle test, p <0.0001); * – statistically significant relative to the NC group of animals (Newman-Keusle test, p<0.0002).*

DISCUSSION

Currently, it has been established that the cerebral hypometabolism is one of the leading factors for the AD development. An experimental study by *Riha et al., 2008* provides the data that hypometabolism of the posterior cingulate cortex contributes to the deposition of toxic amyloid β-peptide in this brain structure, resulting in characteristic clinical manifestations of AD in animals: decreased cognitive functions, activation of lipid peroxidation and decreased synaptic transmission [14]. This study was completed by *Scheltens et al., 2018*, who proved that in addition to impaired memory and associated brain functions, a decrease in metabolic processes contributes to the development of neurological symptoms, motor and sensory areflexion [15]. Brain hypometabolism is largely associated with impairment of the cellular bioenergetic processes, depending on the functional activity of mitochondria [16]. *In vitro* and *in vivo* approaches to the carried out studies have shown that a decrease in the mitochondrial function is observed before the development of deep structural changes in the brain, characteristic to Alzheimer's pathology, and include a decrease of mitochondrial respiration, expression and activity of metabolic enzymes, an increase oxidative stress and expression of a proapoptotic signal [17]. Hereby, the activation of the apoptotic cascade occurs mainly due to the increase of the intensity of internal apoptosis pathway reactions (depending on mitochondria) with an increased apoptosis-inducing factor releasing and endonuclease G activation [18]. The induction of apoptosis enhances the cytotoxicity of β-amyloid, which accelerates the death of neurons and, accordingly, worsens the course of disease [19]. Previously, it was established that about 90% AD patients have hippocampal hypometabolism, and the clinical symptoms of AD varied greatly: from minor neurological disorders to diffuse cognitive deficit, accompanied by motor fluctuations and loss of sensory perception [20]. In addition, people with cerebral hypometabolism are, as a rule, β-amyloid-positive patients whose prognosis is most unfavorable [21].

In this regard, it can be assumed that the correction of hippocampal hypometabolism can be a promising approach to preventive pharmacotherapy of AD, which was partially confirmed by *Villain et al., 2008* [22]. However, in spite of the probable expediency of early correction of metabolic disorders, provided they are diagnosed in a timely manner, currently existing AD treatment methods are aimed at reducing the cytotoxicity of β-amyloid, and the spectrum of compounds which can restore the metabolic activity of cells, is quite limited [23]. In addition, the developed drugs of targeted AD correction often have undesirable toxicological parameters. An example of this agent is semagacestat, a γ-secretase inhibitor, a potentially effective drug for the treatment of AD, which reduces the toxicity of β-amyloid, but has a high oncogenic potential, and therefore its further study has been stopped [24].

In this regard, a study to evaluate the effect of pumpkin *(Cucurbita pepo* L.*)* and marigold extracts *(Tagetes patula* L.*)* on the functional activity of hippocampal mitochondria within the conditions of the experimental acute brain hypometabolism was conducted. During the study, it was found out that the prophylactic administration of the tests pumpkin and marigold extracts contributed to the restoration of the mitochondrial function and a decrease of the anaerobic processes intensity equally with the reference drug – a standardized *Ginkgo biloba extract* (EGb 761).

Moreover, in the groups of the rats treated with marigold, pumpkin extracts and EGb 761, there was an increase of ATP concentration in the hippocampal supernatant compared to the same indicator in the group of the rats deprived of pharmacological support by 65.7% (p<0.002); 66.2% (p<0.002); and 60.7% (p<0.002), respectively. It may indicate normalization of bioenergetic

processes in the hippocampus within the conditions of acute cerebral hypometabolism when using the test extracts and the reference drug [25].

In addition, the restoration of the optimal ATP synthesis can prevent the destabilization of mitochondrial membranes, which prevents their decomposition and release of AIF, thereby inhibiting internal apoptotic cascade reactions [26]. That was also established during the study. So, when the animals were treated with pumpkin and marigold extracts, a decrease of the AIF concentration (by 33% ($p<0.002$) and 38.3% ($p<0.002$), respectively), and ENDOG (by 3.6 times ($p <0.002$) and by 4. 4 times ($p<0.002$), respectively), was noted.

Against the background of the test extracts and EGb 761, a decrease in the concentration of amyloid β-peptide in the rats' hippocampus by 54.4% ($p<0.0002$); by 54.4% ($p<0.0002$) and by 49% ($p<0.0002$), respectively, was noted. As a whole with a decrease of apoptosis intensity, it can prevent neuronal destruction [27].

CONCLUSION

On the basis of the obtained data, it can be assumed that the prophylactic administration of pumpkin extract and marigold extract helps to normalize bioenergetic processes, decrease the concentration of proapoptotic markers and β-amyloid in the rats' hippocampus under acute hypometabolism caused by intrahippocampal administration of a 3M sodium azide solution.

At the same time, the test extracts showed an equivalent therapeutic efficacy with the reference drug – a standardized extract of *Ginkgo biloba* (EGb 761), which makes these extracts promising objects for a further study in order to create drugs for targeted correction of AD in the early hypometabolic stage of the disease.

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AUTHOR CONTRIBUTIONS

All authors had equally contributed to the research work.

CONFLICTS OF INTEREST

The authors and peer reviewers of this paper report no conflicts of interest.

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AUTHORS

Andrey V. Voronkov – Doctor of Science (Med.), professor, head of the department of pharmacology with a course of clinical pharmacology. Pyatigorsk medical-pharmaceutical Institute. ORCID ID:0000-0001-6638-6223. E-mail: prohor77@mail.ru

Dmitry I. Pozdnyakov – Candidate of l Sciences (Pharm.), Senior Lecturer of the Department of Pharmacology with a course of clinical pharmacology. Pyatigorsk medical-pharmaceutical Institute. ORCID ID:0000-0003-0889-7855. E-mail: pozdniackow.dmitry@yandex.ru

Similya L. Adzhiakhmetova – PhD, lecturer of the Department of Organic Chemistry of the Pyatigorsk Medical Pharmaceutical. E-mail: similla503@mail.ru

Nadezhda M. Chervonnaya – PhD, lecturer of the Department of Organic Chemistry of the Pyatigorsk Medical Pharmaceutical Institute. E-mail: nadezhda.chervonnaya@yandex.ru

Kirill A. Miroshnichenko – PhD-student of the Department of Pharmacology with a course of clinical pharmacology. Pyatigorsk medical-pharmaceutical Institute. E-mail: K220436@yandex.ru

Anastasia V. Sosnovskaya – 5th year student of the Pharmaceutical department. Pyatigorsk medical-pharmaceutical Institute. ORCID ID: 0000-0002-5037-994X. E-mail: 88misi88@yandex.ru

Elizaveta I. Shereshkova – 5th year student of the Pharmaceutical department. Pyatigorsk medical-pharmaceutical Institute. ORCID ID: 0000-0002-7854-5401. E-mail: elizaveta.shereshkova@yandex.ru

RESEARCH ARTICLE

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INFLUENCE OF HEPARINOID FROM PAEONIA LACTIFLORA ON HEMOSTATIC SYSTEM WITHIN CONDITIONS OF PRETHROMBOSIS

M.G. Lyapina, M.S. Uspenkaya, E.S. Maistrenko, M.D. Kalugina

Lomonosov Moscow State University,
Block 12, 1, Leninskie Gory, Moscow, Russia, 119234

E-mail: lyapinal@mail.ru

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The search and development of direct and rapid anticoagulants used *per os,* is an urgent problem in physiological and medical science. A number of plants contain heparin-like components with a positive effect on the hemostatic system, both within normal and in some pathological conditions of the body.

The aim of the work was to study the complex effect of fibrin, a heparin-like substance (heparinoid) from the roots of *Paeonia lactiflora,* on fibrinolytic, anticoagulant systems of the body and polymerization processes, when it is administered *per os* in animals within normal conditions and when modeling the state of prethrombosis.

Materials and methods. To carry out the research, the roots of *Paeonia lactiflora* growing in the Botanical Garden of Moscow State University, and laboratory animals – male Wistar rats – were used. To study the antithrombotic effects of the extract from roots containing heparinoid, the state of prethrombosis was modeled in rats. The determined parameters of hemostasis were: anticoagulant activity according to the tests of activated partial thromboplastin time and thrombin time, fibrinolytic activity according to the test of total fibrinolytic activity, fibrin polymerization according to the test of fibrindepolymerization activity of blood plasma.

Results. With repeated (every 24 hours within 3 days) oral administration of the extract containing heparinoid, in animals within normal conditions and with prethrombosis, the following anticoagulant effects were established in the blood: an increase in anticoagulant, fibrindepolymerization and fibrinolytic plasma activity. Possible mechanisms of the activating effect of heparinoid on fibrinolysis and anticoagulant properties of plasma due to the excretion of tissue plasminogen activator into the bloodstream from the endothelium, thrombin inhibition, and fibrin polymerization are described. Moreover, the anticoagulant effect of the use of the extract from the peony roots was equivalent to that of the reference drug of low molecular weight heparin from Celsus (USA). For the first time, it was revealed that when modeling experimental prethrombosis, the administration of heparinoid in rats at the dose of 37.5 IU/kg ME/кг body weight restored impaired hemostasis, which requires a further study.

Conclusion. The ability of heparinoid from peony roots to normalize the functional state of the anticoagulant system during the development of prethrombosis in animals has been established. The restriction of fibrin polymerization during oral administration of heparinoid from peony in animals by increasing the enzymatic fibrinolytic and fibrindepolymerization activity of blood plasma was revealed. In the future, heparinoid can be used as an antithrombotic agent.

Keywords: *Paeonia lactiflora,* peony extract, hemostatic system, prethrombosis, anticoagulant, heparinoid

List of abbreviations: CLA – conjugated linoleic acid; TFA – total fibrinolytic activity; GAGs – glycosaminoglycans; LMWH – low molecular weight heparin; PTT – partial thromboplastin time; FDPA – fibrindepolymerization activity; TT – thrombin time; TFA – total fibrinolytic activity, ACS – anticoagulative system; TTPA – tissue-type plasminogen activator

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ВЛИЯНИЕ ГЕПАРИНОИДА ИЗ ПИОНА (PAEONIA LACTIFLORA) НА СИСТЕМУ ГЕМОСТАЗА В УСЛОВИЯХ ПРЕДТРОМБОЗА

М.Г. Ляпина, М.С. Успенская, Е.С. Майстренко, М.Д. Калугина

Московский государственный университет им.М.В. Ломоносова
119234, Россия, Москва, Ленинские горы, д. 1, стр. 12

E-mail: lyapinal@mail.ru

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Поиск и разработка антикоагулянтов прямого и быстрого действия, применяемых пероральным методом, является актуальной задачей физиологической и медицинской науки. Ряд растений содержит гепариноподобные компоненты с положительным влиянием на систему гемостаза как в норме, так и при некоторых патологических состояниях организма.

Цель исследования – изучение комплексного воздействия на фибринолитическую, антикоагулянтную системы организма и процессы полимеризации фибрина гепариноподобного вещества (гепариноида) из корней пиона молочноцветкового при его пероральном введении животным в норме и при моделировании состояния предтромбоза.

Материалы и методы. Корни пиона молочноцветкового, произрастающего в Ботаническом саду МГУ. Использовались лабораторные животные – крысы-самцы линии Wistar. Для изучения противотромботических эффектов экстракта из корней, содержащего гепариноид, моделировали у крыс состояние предтромбоза. Определяемыми параметрами гемостаза служили антикоагулянтная активность по тестам активированного частичного тромбопластинового времени и тромбинового времени, фибринолитическая активность по тесту суммарной фибринолитической активности, полимеризации фибрина по тесту фибриндеполимеризационной активности плазмы крови,

Результаты. При многократном (в течение 3-х сут. через каждые 24 ч.) пероральном введении животным в норме и при предтромбозе экстракта, содержащего гепариноид, в крови установлены противосвертывающие эффекты: повышение антикоагулянтной, фибриндеполимеризационной и фибринолитической активности плазмы. Описаны возможные механизмы активирующего действия гепариноида на фибринолиз и антикоагулянтные свойства плазмы вследствие экскреции в кровоток из эндотелия тканевого активатора плазминогена, ингибирования тромбина и полимеризации фибрина. При этом антикоагулянтный эффект от применения экстракта из корней пиона был равноценен таковому у препарата сравнения низкомолекулярного гепарина фирмы Celsus (США). Впервые выявлено, что при моделировании экспериментального предтромбоза введение крысам гепариноида в дозе 37,5 МЕ/кг массы тела восстанавливало нарушенную функцию гемостаза, что требует дальнейшего его изучения.

Заключение. Установлена способность гепариноида из корней пиона нормализовать функциональное состояние противосвертывающей системы при развитии предтромбоза у животных. Выявлено ограничение процесса полимеризации фибрина при пероральном введении животным гепариноида из пиона путем увеличения ферментативной фибринолитической и фибриндеполимеризационной активности плазмы крови. В перспективе гепариноид может применяться как антитромботический агент.

Ключевые слова: экстракт пиона, система гемостаза, предтромбоз, антикоагулянт, гепариноид

Список сокращений: АЧТВ – активированное частичное тромбопластиновое время; ГАГ – гликозаминогликаны; ГП – гепариноводобное вещество (гепариноид); МЕ – международная единица гепарина; НМГ – низкомоекулярный гепарин; ПСС – противосвертывающая система; СЛК – сулодексид; СФА – суммарная фибринолитическая активность; ТАП – тканевой активатор плазминогена; ТВ – тромбиновое время; ФДПА– фибриндеполимеризационная активность

INTRODUCTION

The search and development of direct and rapid anticoagulants used *per os*, is an urgent problem in physiological and medical science. Currently, new oral anticoagulants are widely used for the prevention of thrombosis and the intensive care without a laboratory control [1], which are inferior to low molecular weight heparins in terms of speed of action. Low molecular weight heparin (LMWH) preparations have universally recognized advantages, which include: 1) ease of administration – once a day; 2) no need for laboratory monitoring; 3) relative safety without adverse side effects in the form of bleeding [2]. Despite the creation of ever new drugs for anticoagulant therapy, LMWHs remain the preparations of choice for the prevention and long-term treatment of thrombosis. Traditionally, LMWH is considered a variety of interchangeable drugs of the same type. However, their relative antithrombin activity varies depending on the average molecular weight. It is known that LMWH drugs are used only subcutaneously or intravenously [3]. Of all the clinic drugs containing LMWH, only Sulodexide can be administered *per os*. It is a highly purified mixture of glycosaminoglycans consisting of LMWH (80%) and dermatan sulfate (20%). The low molecular weight of both fractions of conjugated linoleic acid (CLA) provides a high absorption of the drug when taken *per os* [4]. The pharmacological properties of CLA are significantly different from other glycosaminoglycans (GAGs) and are characterized by a long half-life and

a limited effect on the parameters of the hemostatic system. Due to the presence of two fractions of GAG, CLA potentiates the activity of antithrombin III and cofactor heparin II simultaneously. When taken orally, CLA additionally detects a fibrinolytic activity due to the release of tissue plasminogen activator from vascular endothelium [5]. The antithrombotic and antithrombin activity of CLA is of significant pharmacological interest and makes it possible to use it for the prevention and treatment of diseases complicated by thrombosis [6, 7]. However, this drug is obtained from tissues of the animal origin, namely, from the mucous membrane of the pig's small intestine. At the same time, the search for drugs of a similar action of a plant nature is of an undoubted interest. Many plants serve a source of medicinal raw materials for the isolation of anticoagulants, fibrinolytics and thrombolytics [8–11].

It was previously established that a number of plants contain the components that are an integral part of heparin and other GAGs [12, 13] with a positive effect on the hemostatic system both within normal and in some pathological conditions of the body [14]. An anticoagulant agent of the heparin nature with an inhibitory activity against factor Xa and thrombin was obtained from birch bark [15]. A heparin-like substance was found in the roots of herbaceous peonies. It exerts anticoagulant effects *in vitro* and when administered in animals intravenously [16].

The aim of the work was to establish a comprehensive effect on the fibrinolytic and anticoagulant systems of the body, as well as on the polymerization of fibrin of a heparin-like substance from the roots of *Paeonia lactiflora* administered in animals *per os* both within normal conditions and when modeling the state of prethrombosis.

MATERIALS AND METHODS

Animals

The experiments were performed on 66 laboratory animals – white male Wistar rats weighing 190–210 g. The animals were obtained from the nursery of the Stolbovaya station in the Moscow region. Before and during the experiments, the rats were on a normal laboratory diet and kept under standard conditions in the vivarium of the Faculty of Biology of Moscow State University with a free access to water and food and observing a 12-hour light regime of the day. All the animal experiments were carried out in accordance with the generally accepted ethical principles and standards recommended by the European Convention for the Protection of Vertebrate Animals (Strasbourg 06/15/2006), the Basel and Helsinki Declaration on the Humane Treatment of Animals.

Raw Materials

Paeonia lactiflora roots were obtained from environmentally friendly plants growing in the Botanical Garden of Moscow State University. (Moscow, Russia). This peony species was determined by the employees of the Botanical Garden of Moscow State University chaired by M.S. Uspenskaya. The raw materials were harvested in autumn (from late August to mid-October 2016) and stored at the temperature of + 3…5°C. For the experiments, the primary 5% extract was prepared from dry, clean roots, which were ground in a porcelain mortar to a powder state. The presence of a heparin-like substance was determined in the extract [16] by photoelectrocolorimetric method using Azur A (a dye for heparin) and protamine sulfate (a heparin inhibitor). The heparin-like substance in the amount of 0.5 mg, dissolved in 0.5 ml of physiological solution of sodium chloride, contained 37.5 IU of heparin.

Study Design

The heparin-like substance (heparinoid) in the indicated dose (0.5 mg) and volume (0.5 ml) was administered orally in experimental rats weighing 200 g every day.

The first series of experiments

The studies were conducted on healthy rats kept under standard vivarium conditions. The rats were divided into three groups: the 1st group received heparinoid *per os* once, the 2nd group received heparinoid for 3 days every 24 hours and the 3rd (control) one – a 0.85% sodium chloride solution.

The second series of experiments

The study was carried out under experimental prethrombosis. The state of prethrombosis in rats was modeled by creating depression of the anticoagulative system function by administering a 2.5% solution of chlorpromazine (0.06 ml/200 g of body weight) to turn off the autonomic nervous system, followed (after 30–40 minutes) by intravenous injection in rats of a 1% solution of coagulant – tissue (brain) thromboplastin in the volume of 0.6 ml/200 g of body weight.

Rats with experimental prethrombosis were divided into 3 groups: the 1st (experimental) group preliminarily received -heparinoid three times in the indicated dose and volume; the 2nd one (control) was given a 0.85% sodium chloride solution – at the same time and in the same volume; the 3rd (comparison) group received commercial low molecular weight heparin (LMWH) from Celsus (release date 2016 with antifactorial Xa activity) three-times —on the same time and in the same volume (37.5 IU of heparin per 200 g of a rat body weight). At the same time, in this series of experiments, 2 groups of healthy rats were used for comparison, one of which did not receive any drugs (Norm group), and the second one was treated with heparinoid only three times.

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2019-7-4-208-214

Research practice

The blood was taken from the jugular vein *(vena jugularis)* 20 hours after the last injection of drugs using 3.8% sodium citrate as a preservative in the ratio of 9:1. Then it was centrifuged at 3000g for 10–12 minutes to obtain platelet-poor blood plasma.

To characterize the parameters of the fibrinolytic anticoagulative blood unit, two types of fibrin plates were prepared: 1) unstabilized by factor XIIIa, characterized by the presence of hydrogen bonds in the soluble fibrin polymer; 2) stabilized by factor XIIIa, having strong covalent bonds in an insoluble fibrin polymer. The following biochemical parameters of hemostasis were determined in blood plasma: on the unstabilized fibrin plates there was a total fibrinolytic activity, including the activity of heparin complexes with blood plasma components and plasmin activity, as well as fibrindepolymerization activity, reflecting the processes of fibrin depolymerization; on the standard stabilized fibrin plates there was the activity of a tissue-type plasminogen activator and plasmin enzyme. The anticoagulant activity of plasma was determined by the partial thromboplastin time (PTT) test, which characterizes the internal mechanism of blood coagulation, and thrombin time (TT), which reflects the general path of blood coagulation. In addition, fibrinogen concentration was measured [17].

Statistical processing of results

All data were statistically processed using the non-parametric Wilcoxon test and Student t-test *(*Statistica 7.0 software package*).*

RESULTS AND DISCUSSION

In the first series of experiments with a single oral administration of heparinoid after 20 hours, a significant increase by 28% in the anticoagulant activity (according to the PTT test) was found stastically reliable; an increase by 37% in the total fibrinolytic activity (TFA) on unstabilized fibrin due to the total activity of heparin complexes and plasmin activity was established. As evidenced by an increase in the fibrindepolymerization (FDP) activity by 56% compared with the control, the fibrin polymerization process was significantly reduced. An additional confirmation of the inhibition of the fibrin polymerization process was a decrease in the concentration of fibrinogen by 33% compared with the control.

20 hours after a triple oral administration of heparinoid, an even sharper change in the studied parameters towards hypocoagulation was observed. Thus, anticoagulant activity increased by 50% (according to the PTT test) and by 23% (according to the TT test) compared to the control, and exceeded the same indices in the acute experiment by 22–13%, respectively. At the same time, TFA increased by 52%, and, according to the FDA test, the fibrin depolymerization increased by 49% compared with the control (Table 1).

Table 1 – Anticoagulant, fibrinolytic, fibrindepolymerization activities; fibrinogen concentration in 20 hours after once and triple oral administration of heparinoid from *Paeonia Lactiflora* roots in healthy animals (M ± m)

Blood values	Administrated *per os*			
	Heparinoid, administrated once (Group 1)	Heparinoid, administrated 3 times (Group 2)	0.85% sodium chloride solution, administrated once Control 1	0.85% sodium chloride solution, administrated 3 times Control 2
Anticoagulant activity – (%) by PTT test – by TT test	 39.7 ± 0.4** (128%) 29.0 ± 0.6 110%	 46.6 ± 2.7** (150%) 34.5± 1.4** (123%)	 30.6 ± 1.7 (100%) 28.0 ± 1.1 (100%)	 31.0 ± 0.7 (100%) 28.0 ± 1.1 (100%)
TFA, mm^2 (%)	55.3 ± 1.3** (137%)	61.6 ± 1.1** (152%)	40.5 ± 1.0 (100%)	40.3 ± 1.5 (100%)
FDPA, mm^2 (%)	36.0 ± 1.1** (156%)	38.9 ± 1.3** (149%)	23.0 ± 0.9 (100%)	26.0 ± 0.9 (100%)
TTPA, mm^2 (%)	40.1 ± 1.3** (413%)	42.3 ± 1.3** (436%)	9.5 ± 0.8 (100%)	9.7 ± 1.0 (100%)
Plasmin activity, mm^2 (%)	20.2 ± 1.5** (230%)	24.2 ± 1.1** (275%)	9.0 ± 0.6 (100%)	8.8 ± 0.8 (100%)
Fibrinogen concentration, mg (%)	187.0 ± 13.2* (67%)	210.0 ± 9.8 (70%)	305.0 ± 10.0 (100%)	308.0 ± 11.3 (100%)

*Note: statistical indicators are calculated relative to the corresponding control samples taken as 100%. *p < 0.05; **p < 0.01*

As Table 1 shows, the most significant changes after the exposure to heparinoid compared with the control, were observed in enzymatic plasma fibrinolysis of the rats. So, 20 hours after the oral administration of heparinoid once or three times, the activity of plasmin significantly increased by 130–175%, respectively. But especially significant differences from the control under these conditions were found in the activity of TTPA, which exceeded the control level with single and triple use of heparinoid by 313 and 336%, respectively.

The second series of experiments involved the creation of a model of rats with the state of prethrombosis against the background of depression of the ACS function. In pre-thrombosis (Control group), a sharp inhibition was compared with the Norm anticoagulant plasma activity group (by the PTT test) by 21.4%, TFA decrease – by 65%, FDPA – by 155%, TTP activity – by 115%, the plasmin level – by 69%. At the same time, in prethrombosis, the fibrinogen concentration was increased by 30% compared with the Norm group. In the experimental group of rats, in which prethrombosis was simulated 20 hours after the last triple use of heparinoid, 30 minutes after the administration of tissue thromboplastin in rats (Table 2), a protective antithrombotic effect was established.

Table 2 – Anticoagulant, fibrinolytic, fibrindepolymerization activities; fibrinogen concentration 30 minutes after modeling prethrombosis in rats against the background of triple oral administration of heparinoid from *Paeonia Lactiflora* (M ± m)

Blood values	Heparinoid, administrated3 (Group 1 – before prethrombosis)	30 minutes after modeling experimental prethrombosis against the background triple administration of:			Healthy rats (Norm group)
		heparinoid – Experiment	0.85%-sodium chloride – Control	LMWH (Celsus) – reference drug	
Anticoagulant activity – (%) by PTT test	39.7 ± 0.4** (163%)	30.0 ± 1.7 (123%)	24.3 ± 2.2 (100%)	28.9 ± 0.7 (122%)	31.0 ± 0.7 (121.4%)
TFA, mm^2 (%)	55.3 ± 1.3** (229%)	41.5 ± 1.0** (173%)	23.9 ± 2,1 (100%)	30.0 ± 1.1* (125%)	40.3 ± 1.5 (169%)
FDPA, mm^2 (%)	36.0 ± 1.1** (400%)	26.0 ± 0.9** (279%)	9.0± 1.5 (100%)	–	23.0 ± 0.9 (255%)
TTPA, mm^2 (%)	40.1 ± 1.3** (817%)	8.3 ± 0.8** (184%)	4.5 ± 0.1 (100%)	–	9.7 ± 1.0 (215%)
Plasmin activity, mm^2 (%)	20.2 ± 1.5** (348%)	9.0 ± 0.6** (161%)	5.5 ± 0.2 (100%)	–	8.8 ± 0.8 (160%)
Fibrinogen concentration, mg (%)	187.0 ± 13.2 ** (45%)	326,0 ± 12,0** (78%)	415.0 ± 15.3 (100%)	400.0 ± 12.0 (96%)	308.0 ± 11.3 (70%)

*Note: statistical indicators are calculated relative to the corresponding control samples taken as 100%. * p <0.05; ** p <0.01*

So, in experimental rats (Experience), the anticoagulant activity, according to the PTT test, increased by 23% compared with the control, and approached the same indicator in healthy rats (Norm group). Moreover, TFA increased by 73% compared with the Control and also almost corresponded to the normal level (Norm group). According to the FDPA test, fibrin depolymerization increased by 179% under the influence of heparinoid, while in the Norm group this value exceeded the control level by 155%. In the Experience, TTPA increased by 84% compared with the Control but, however, did not reach (by 31%) the level noted in the Norm group. Plasmin activity in the Norm and Experience groups exceeded the control level by 60–61%. The fibrinogen concentration after the exposure to heparinoid corresponded to the normal values (Table 2). In the reference group (commercial LMWH was administrated), an increase in anticoagulant and the total fibrinolytic activity was found to be 22 and 25%, respectively.

Analyzing the results obtained, it should be noted that the extract from the roots of *Paeonia lactiflora*, like SLK [5], has not only an anticoagulant effect, like heparin [18] and the reference drug (Table 2), but also a wide range of fibrinolytic effects when administered into the body *per os*. It enhances the activity of tissue plasminogen activator in the bloodstream more than 4 times, both with single and triple uses.

It indicates its high affinity for endothelial cells and indicates its ability to interact with endothelial receptors, expressing plasminogen activators into the bloodstream from the vessel wall, as was shown in other works, under the action of other plant anticoagulants [19]. It was also revealed that this process is associated with the increased plasmin activity under the influence of heparinoid. We are the first to have established the fact that plant-based heparinoid, when administered *per os*, interferes with the polymerization of fibrin, as a result of which its FDPA in blood plasma increases by more than 50–60%. Earlier [20], it was reported that thrombin interacts with fibrinogen under the influence of plant inhibitors, which was observed in our studies.

It is noteworthy that a protective antithrombotic effect, which was found when modeling the state of prethrombosis in animals, was revealed in heparinoid. The heparinoid studied by us when taken *per os*, like the SLK administered *per os* [6], has both antithrombin and an-

ОРИГИНАЛЬНАЯ СТАТЬЯ
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tithrombotic activity. Heparinoid, like SLK, is of pharmacological interest in terms of its use for the prevention of diseases complicated by thrombosis.

CONCLUSION

Based on the study of the effects of the plant heparinoid from *Paeonia lactiflora*, its anticoagulant effect is revealed even with a single administration *per os*. It is enhanced when administered every 24 hours at the daily dose of 37.5 IU (by heparin content) per 200 g of rat body weight for 3 days. Its protective and corrective role in restoring the impaired function of the anticoagulant system during the development of the state of prethrombosis in animals has been established. It was found out that heparinoid, when administered *per os*, reduces the plasma concentration of fibrinogen, limits the process of fibrin polymerization, increases the enzymatic plasma fibrinolytic activity by increasing the level of plasmin and the activity of tissue plasminogen activator, and enhances the total fibrinolytic activity, as well as the fibrin-depolymerization activity caused by the complex of heparin with proteins, peptides and blood amino acids in the blood. Plant heparinoid has a functional similarity with low molecular weight heparin by the mechanism of an antithrombotic action. Moreover, the anticoagulant effect caused by the use of the extract from the roots of *Paeonia lactiflora,* was not inferior to that of the drug for comparison of low molecular weight, heparin from Celsus (USA). The studied preparation from the roots of *Paeonia lactiflora* is a complex agent with anticoagulant, fibrinolytic, fibrindepolymerization properties that protects the body from the development of thrombotic conditions. It can ensure the lysis of fresh fibrin clots that have just appeared in the bloodstream. It can do that both due to its direct effect on polymerizable fibrin and through an indirect effect on plasma hemostasis. All of these effects of plant heparinoid prove the effectiveness and prospects of studying this agent in order to prevent and treat thromboembolic diseases and thrombotic complications.

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AUTHORS’ CONTRIBUTION

All the authors equally participated in physiological experiments and in determining the parameters of hemostasis. Lyapina M. G has made up the experimental strategy, Uspenskaya M. S. was responsible for the determination of the type of peony, harvesting and storage of raw materials; Maistrenko E. S. was responsible for the preparation of extract from peony, statistical processing of the results; Kalugina M. D. – for the literature search.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHORS

Lyapina Margarita Grigoryevna – Leading specialist at the laboratory of protective blood systems n. a. prof. B.A. Kudryashov, Department of Human and Animal Physiology, Faculty of Biology, Moscow State University. ORCID 0000-0001-6039-5161. E-mail: lyapinal@mail.ru.

Uspenskaya Marianna Sergeevna – Candidate of Sciences (Biology), associate Professor, senior researcher at the Botanical garden of the Faculty of Biology, Moscow State University. ORCID 0000-0002-5368-4026. E-mail: ms-uspenskaya@yandex.ru

Maistrenko Evgenia Semenovna – Candidate of Sciences (Biology), senior researcher at the laboratory of protective blood systems n. a. prof. B.A. Kudryashov, Department of Human and Animal Physiology, Faculty of Biology, Moscow State University. ORCID ID: 0000-0003-3227-1465. E-mail: ms-uspenskaya@yandex.ru

Kalugina Marina Dmitrievna – Researcher at the Laboratory of Protective Blood Systems n. a. prof. B.A. Kudryashov, Department of Human and Animal Physiology, Faculty of Biology, Moscow State University. Istina ResearchD (IRID) 8945687. E-mail: laboratory7@mail.ru

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APPLICATION OF DRUG-RELATED PROBLEMS APPROACH TO ANALYSIS OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS' SAFETY

A.V. Matveev[1,2], A.E. Krasheninnikov[1], E.A. Egorova[2], E.I. Konyaeva[2]

[1] National Pharmacovigilance Research Center,
2/2, Malaya Sukharevskaya sq., Moscow, Russia, 127051
[2] Medical Academy n. a. S.I. Georgievsky of Vernadskjy CFU,
5/7, Lenin Avenue, Simferopol, Republic of Crimea, Russia, 295051

E-mail: elena212007@rambler.ru

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Summary. A Drug-Related Problem is an event or circumstance involving drug therapy that actually or potentially interferes with desired health outcomes.

The aim of the study was the analysis of the adverse drug reactions (ADR) associated with prescription of the non-steroidal anti-inflammatory drugs (NSAIDs) using the DRP PCNE V5.01 qualification system.

Materials and methods. The objects of the study were 415 notification forms about adverse drug reactions of NSAIDs recorded in the regional database of spontaneous reports and called ARCADe (Adverse Reactions in Crimea, Autonomic Database) for the period from 1 January 2009 to 31 December of 2018. The study and analysis of the problems associated with drugs were carried out using the qualification system DRP PCNE V5.01 (Pharmaceutical Care Network Europe) 2006 in the modification of Prof. Zimenkovsky.

Results. Among other representatives of the NSAID group, Ibuprofen and Diclofenac became the "leaders" in the incidence of ADR. The frequency of ADR cases for Ibuprofen was 142 reports (34.22% of the total number of ADR for NSAIDs), and for Diclofenac it was 90 cases (21.69%). The calculation of DRP values for each of the presented cases made it possible to determine that in 81 (19.51%) and 91 (21.9%) cases, the DRP value was 6 and 7, respectively. DRP values in the range of 8–10 were found in 92 reports. The highest DRPs value was observed after the administration of Parecoxib (13 problems but only one case was found in the database), the DRPs value of Dexketoprofen was 12.5 (95% CI: 7–17) and the DRPs value of Diclofenac combinations was 10 DRPs; 95% CI: 5–17 DRP). The minimum DRPs values were associated with Naproxen, Rofecoxib, and Etoricoxib prescriptions.

Conclusion. Using the DRP system in the analysis of NSAIDs, ADRs allow to identify the medicines which have a high risk of causing safety problems, such as Parecoxib, Dexketoprofen and Diclofenac combinations. The prescription of these drugs should be carried out with special cautions and control to the indications and contraindications, the dose and duration of treatment, as well as to a possible interaction of them with concomitant drugs.

Keywords: drug-related problems (DRPs), non-steroidal anti-inflammatory drugs (NSAIDs), adverse drug reactions (ADRs)

List of abbreviations: DRP – drug-related problems; MP – medicinal products; ADRs – adverse drug reactions; NSAIDs – non-steroidal anti-inflammatory drugs; COX – cyclooxygenase; ATC-code – code of product according to Anatomical Therapeutic Chemical (ATC) classification system.

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RESEARCH ARTICLE

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ПРИМЕНЕНИЕ СИСТЕМЫ ПРОБЛЕМ, СВЯЗАННЫХ С ЛЕКАРСТВЕННЫМИ ПРЕПАРАТАМИ (DRUG-RELATED PROBLEMS), НА ПРИМЕРЕ ГРУППЫ НЕСТЕРОИДНЫХ ПРОТИВОВОСПАЛИТЕЛЬНЫХ СРЕДСТВ

А.В. Матвеев[1,2], А.Е. Крашенинников[1], Е.А. Егорова[2], Е.И.Коняева[2]

[1]Автономная некоммерческая организация «Национальный научный центр Фармаконадзора», 127051, Российская Федерация, Москва, ул. Малая Сухаревская площадь, д. 2, корп. 2

[2] Медицинская академия им. С.И. Георгиевского ФГАОУ ВО «КФУ им. В.И. Вернадского», 295051, Российская Федерация, г. Симферополь, бул. Ленина, 5/7

E-mail: elena212007@rambler.ru

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Проблемы, связанные с лекарственными препаратами (drug-related problems, DRP), – это события или изменения, связанные с фармакотерапией, которые реально или потенциально препятствуют достижению желаемых результатов в процессе лечения.

Цель – анализ случаев нежелательных реакций (НР) на препараты группы нестероидных противовоспалительных средств (НПВС) с помощью квалификационной системы DRP PCNE V5.01.

Материалы и методы. Объектами исследования стали 415 карт-извещений о нежелательных реакциях НПВС, зарегистрированные в региональной базе спонтанных сообщений ARCADe (Adverse Reactions in Crimea, Autonomic Database) за период 2009–2018 гг. Изучение и анализ проблем, связанных с лекарственными препаратами, проводился с использованием квалификационной системы DRP PCNE V5.01 (Pharmaceutical Care Network Europe) 2006 г. в модификации А.Б. Зименковского.

Результаты. «Лидерами» по частоте развития НР среди отдельных представителей группы НПВС стали препараты ибупрофен и диклофенак. Частота встречаемости случаев НР для ибупрофена составила 142 случая (34,22% от всего количества случаев НР на НПВС), а для диклофенака – 90 случаев (21,69%). Расчет значений DRP для каждого из представленных случаев НР позволил определить, что в 81 (19,51%) и 91 (21,9%) случае значение DRP было равно 6 и 7 соответственно. Значения DRP в пределах значений 8–10 встречались в 92 случаях. Наиболее высокие значения DRP наблюдались при назначении парекоксиба– 13 проблем (зарегистрирован 1 случай), дексketопрофена – 12,5 DRP (95% ДИ: 7–17) и комбинированных препаратов, содержащих диклофенак – 10 DRP (95% ДИ: 5–17 DRP). Минимальные значения DRP были выявлены при назначении напроксена, рофекоксиба и эторикоксиба.

Заключение. Использование системы проблем, связанных с лекарственными препаратами (DRP), для проведения анализа нежелательных реакций препаратов группы НПВС позволило выделить отдельных представителей группы, обладающих высоким риском развития неблагоприятных последствий при их применении в практической медицине. Среди таких препаратов стоит отметить парекоксиб, дексketопрофен и комбинированные препараты, содержащие диклофенак. Назначение представленных выше препаратов должно проводиться со строгим учетом показаний и противопоказаний к применению, дозы и курса лечения, а также вероятности возможного взаимодействия этих препаратов с сопутствующими лекарственными средствами.

Ключевые слова: проблемы, связанные с лекарственными препаратами, DRP, нестероидные противовоспалительные средства

Список сокращений: РГЗТ – реакция гиперчувствительности замедленного типа, РПГА – реакция прямой гемагглютинации, ФИ – фагоцитарный индекс, ФЧ – фагоцитарное число

INTRODUCTION

Drug-related problems are defined as “any circumstance related to the patient’s use of a drug that actually or potentially prevents the patient from gaining the intended benefit of the drug” [1, 2]. Such undesirable consequences due to the use of medicinal products (MPs) are common in medical institutions both at the stage of primary medical care (in hospitals) and at the stage of outpatient (ambulatory) treatment of patients. Most of these problems are revealed at the stage of prescribing, dosing or intake of drugs by patients. Potential causes of DRPs are: the simultaneous prescription of five or more drugs (polypharmacy), complex regimens for the use of medicinal products, as well as the use of new MPs in clinical practice, which is associated with a high risk of adverse drug reactions (ADRs) during their use [3, 4]. Another important reason for the development of DRPs in patients is the lack of clear recommendations from doctors about the use of concomitant medications or alternative methods of treatment [5].

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2019-7-4-215-223

The medical conditions resulting from DRPs, are serious issues for patients and society, as the suffering of patients and the costs of corrections caused by these problems are significant [6–8]. The economic consequences of DRPs in the United States of America (USA) are estimated at more than $ 150 billion annually [9]. Moreover, the estimate of the costs associated with medical errors when using drugs ambulatory varies from 30.1 to 136.8 billion US dollars [10, 11].

Among the main causes of DRPs, the following ones are distinguished: off-label prescription and use of drugs, discrepancies between the doctor-prescribed and real regimes of the use of MPs, irrational choice of drugs, poor patients' compliance to treatment, drug interactions, as well as interactions with the food components, adverse reactions, overdose, the use of subtherapeutic doses and the need for additional drug therapy [12–15]. According to the systematic review published by Krähenbühl Melcher et al., about 8% of hospitalized patients have adverse drug reactions and 5–10% of prescriptions in these patients are erroneous [16]. Forster A.J. and colleagues studied the frequency of ADRs and revealed that those reactions occurred in 15% of patients at the hospitalization stage, and in 12–17% of patients after their discharge from a medical institution [17–19].

The identification and study of drug-related problems, as well as the identification of the main groups and individual representatives of the drugs with a high risk of developing DRPs, are important stages in the treatment of patients. They can reduce morbidity, mortality and increase compliance of patients to treatment [20].

According to our search in Russian medical literature, the DRPs methodology is practically not used in domestic practice, and the number of such studies is occasional [21, 22].

The aim of the current study is to analyze the cases of ADRs caused by non-steroidal anti-inflammatory drugs (NSAIDs) using the DRP PCNE V5.01 qualification system.

MATERIALS AND METHODS

The objects of this study were the cases of ADRs development after the use of NSAIDs described in official notification forms and recorded in the regional database of spontaneous reports called "ARCADs (Adverse Reactions in Crimea, Autonomic Database)" for the period of January 2009 – December 2018. For the covered period, 415 forms containing information about the ADRs of varying severity in the patients treated in medical institutions, in outpatient settings and the cases of self-treatment were found.

The detection of ADRs was carried out by the codes of the Anatomical Therapeutic Chemical (ATC) classification of medicines of the World Health Organization [23], the data from the instructions for medical use (analogue of Short Medical Product Characteristics) found in the State Drug Registers of the Russian Federation and Ukraine (for the cases registered before the incorporation of the Republic of Crimea into the Russian Federation).

The study of the frequency and severity of DRPs and their further analysis were carried out using the DRP PCNE V5.01 (Pharmaceutical Care Network Europe) 2006 qualification system in the modification of Andriy B. Zimenkovsky [24]. This system is based on the coding in 4 main categories: P – problems, C – causes, I – interventions and O – consequences. According to the "P" code, DRPs are divided into the following groups: manifestations of ADRs (allergic/non-allergic), the rationality of the choice of a suspect and concomitant drugs, their dosage regimen and dosage form, the errors that occur during the drug intake, the possibility of drug interactions and some others problems. The causes of DRPs standardized by code "C" are classified as follows:

- ✓ DRP associated with the choice of the suspected drug, and its dose;
- ✓ DRP caused by the use of concomitant drugs and the possibility of drug interactions;
- ✓ patient-oriented psychological factors (i.e. a patient's inability to take a suspected and/or concomitant drug);
- ✓ DRPs resulting from the low awareness of doctors and patients about a rational use of drugs (lack of knowledge of the instructions for medical use and/or patients' treatment protocols);
- ✓ DRP related to pharmaceutical logistics (drug availability).

The interventions of a doctor or a clinical pharmacist, indicated by the code "I", are divided into 3 subgroups: the first one is the level of the specialist who prescribed the medicine, the second subgroup is the level of the patient and the third subgroup is the level of the medicine itself. Among such interventions, the cancellation of a suspected drug, the withdrawal of concomitant MPs, as well as the administration of a new drug to correct the consequences caused by ADRs have been distinguished. Among the outcomes of DRPs (code "O"), there are 2 main options: the drug-related problem is completely solved and the drug-related problem is not solved [24–27].

RESULTS AND DISCUSSION

To study the drug-related problems, in the regional database of spontaneous reports about adverse reac-

tions, an automatic search was performed and 415 notification forms corresponding to the M01 ATC classification code were selected. They amounted to 5.96% of the total number of ADRs for the period from 1 January 2009 till 31 December 2018 (the total number of records was 6960).

The first stage of the work was devoted to identifying the frequency of ADRs for each representative of the NSAIDs group. Ibuprofen and Diclofenac became the "leaders" by the incidence of reactions. The occurrence of cases for Ibuprofen was 142 cases (34.22% of the total number of records for NSAIDs), and for Diclofenac it was 90 cases (21.69%). Much less frequently, ADRs occurred when patients took Nimesulide (45 cases, 10.84%), Ketorolac (44 records, 10.6%) and Meloxicam (23 cases, 5.54%). The frequency of ADRs for other active substances from M01 group is presented in Table 1.

Table 1 – Frequency of adverse reactions associated with prescription of NSAIDs and registered in the Republic of Crimea for the period of 2009–2018

NSAIDs	ATC code	Quantity of reports (absolute)	Quantity of reports (% of total NSAIDs ADRs)
Ibuprofen	M01AE01	142	34.22
Diclofenac	M01AB05	90	21.69
Nimesulide	M01AX17	45	10.84
Ketorolac	M01AB15	44	10.60
Meloxicam	M01AC06	23	5.54
Dexketoprofen	M01AE17	14	3.37
Diclofenac combinations	M01AB55	14	3.37
Lornoxicam	M01AC05	9	2.17
Nimesulide combinations	M01AX67	5	1.20
Etoricoxib	M01AH05	5	1.20
Aceclofenac	M01AB16	5	1.20
Celecoxib	M01AH01	3	0.72
Rofecoxib	M01AH02	3	0.72
Naproxen	M01AE02	2	0.48
Parecoxib	M01AH04	2	0.48
Other NSAIDs combinations	M01BX	2	0.48
Ibuprofen combinations	M01AE51	1	0.24
Mefenamic acid	M01AG01	1	0.24
Glucosamine	M01AX05	1	0.24
Chondroitin sulfate	M01AX25	1	0.24
The combination of Glucosamine and Chondroitin sulfate	M09AX	1	0.24
Ketoprofen	M01AE03	1	0.24
Indomethacin	M01AB01	1	0.24

The calculation of DRPs values for each of the recorded cases made it possible to determine the following: in 81 (19.51%) and 91 (21.9%) cases, the DRPs value was 6 and 7, respectively. The DRPs values in the range of 8–10 were found in 92 cases (8 DRPs – 38 records (9.16%), 9 DRP – 20 records (4.8%) and 10 DRP – 34 reports (8.2%)). Special attention was paid to forms, in which the DRPs values amounted to more than 10 problems per case (124 reports, 29.9%).

Such high rates most likely indicate an irrational choice of the NSAIDs, incorrect dosing or possible drug interactions. The frequency of DRPs values for NSAIDs is shown in Fig. 1. The total number of DRPs for all the cases was 3785 (therefore, the average value was 9 DRPs / a patient).

At the next stage of the work, the DRPs values for each NSAID were calculated. Our analysis of the ADRs records revealed that the highest DRPs values were observed for Parecoxib (13 problems) though the only case for this substance was found in the database. The results also included Dexketoprofen with an average amount of 12.5 DRPs / a case (95% CI: 7–17) and Diclofenac combinations with 10 DRPs / a case (95% CI: 5–17 DRP).

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2019-7-4-215-223

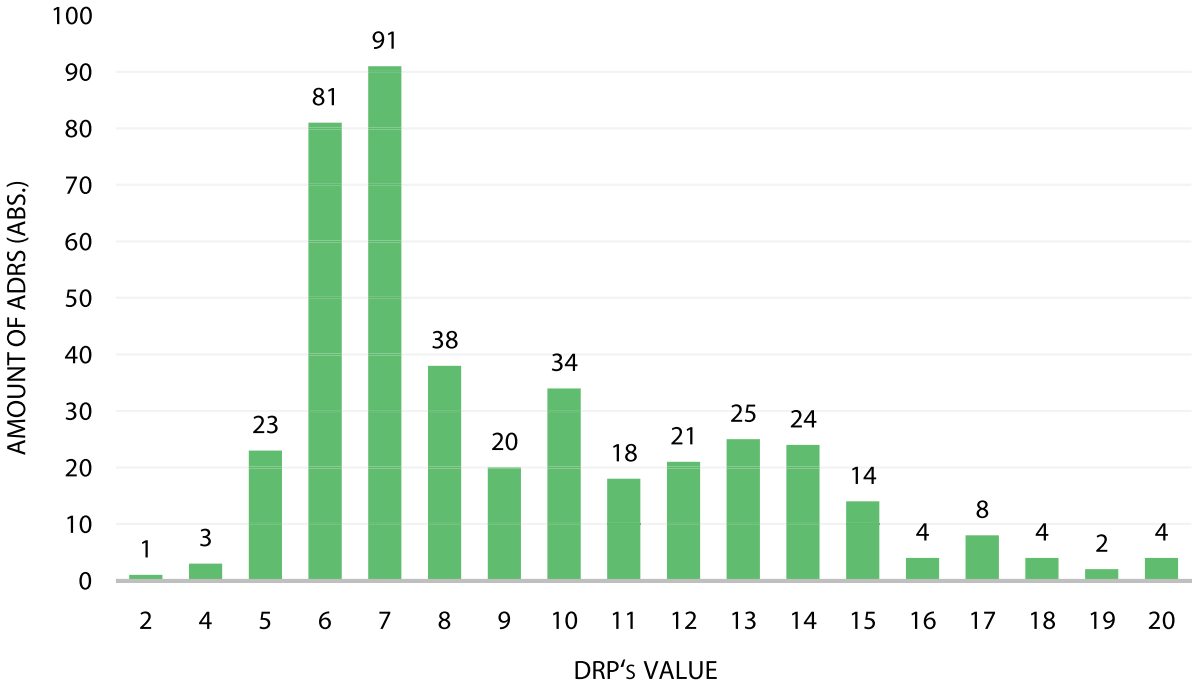

Figure 1 – Frequency of DRP's Values in NSAIDs ADRs records

The average values of DRPs / a case in the range of 9-9.5 were observed in the records for Ketorolac, Meloxicam and Aceclofenac. High maximum DRPs values for Ketorolac and Meloxicam (20 and 19 DRP, respectively) are noteworthy, as they indicate the irrational prescription of MPs and a high risk of serious ADRs developing during their use. The distribution of the NSAIDs according to the median, maximum and minimum DRPs values is presented in Table 2.

Table 2 – Median, maximum and minimum of NSAIDs-related problems

NSAID	Maximum	Minimum	Median	Range
Dexketoprofen	17	7	12.5	10
Diclofenac combinations	17	5	10	12
Ketorolac	20	5	9.5	15
Meloxicam	19	5	9	10
Aceclofenac	12	5	9	7
Diclofenac	19	6	8	13
Ibuprofen	16	4	7	12
Lornoxicam	10	6	7	4
Nimesulide	17	2	7	15
Celecoxib	13	6	7	7
Nimesulide combinations	14	4	7	10
Naproxen	7	5	6	2
Rofecoxib	8	6	6	2
Etoricoxib	11	6	6	5

The results presented above, indicate that Naproxen (a non-selective inhibitor of cyclooxygenase-2 (COX-2)), Rofecoxib and Etoricoxib (highly selective COX-2 inhibitors) have a better safety profile compared to other NSAIDs [28]. When administrated, the amount of this drug-related problems was lower than that of the other representatives of the group.

The fourth stage of the analysis was devoted to the study of NSAIDs' administration routes (local, rectal, intravenous, intramuscular and per os) and their association with DRPs values. The results of the analysis showed that most often ADRs occurred when NSAIDs were taken per os. The frequency of such cases was 270 (65%). Less often, adverse reactions were observed after intramuscular (125 cases, 30.1%), intravenous (9 cases, 2.2%), rectal (9 cases, 2.2%) and local (2 cases, 0.5%) administration routes (Fig. 2).

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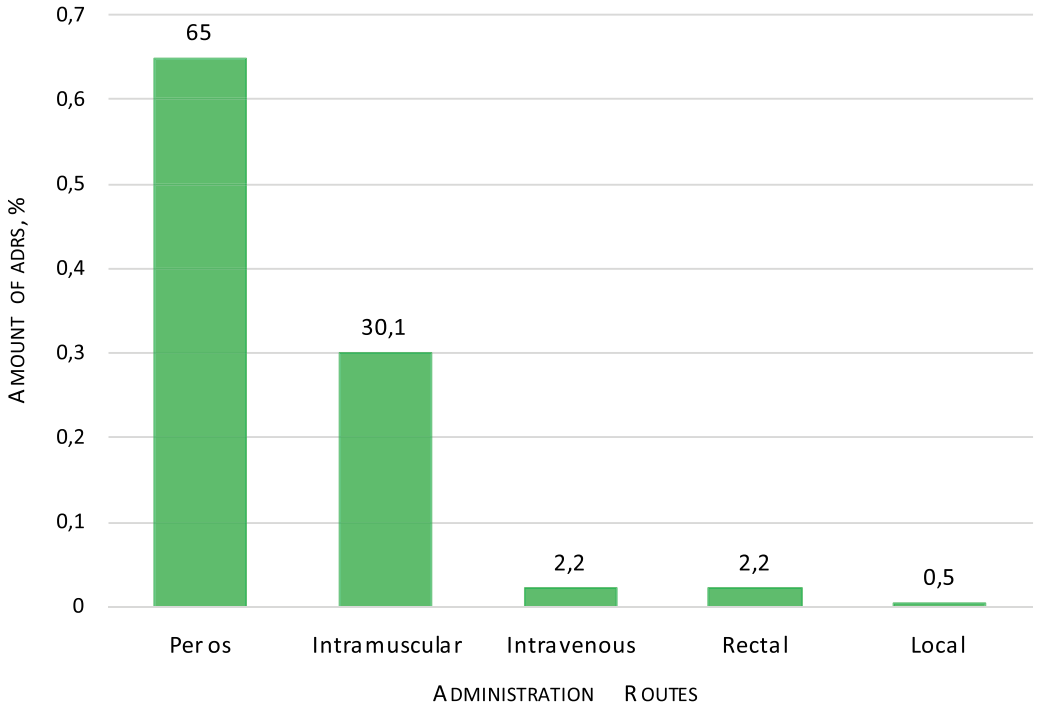

Figure 2 – Distribution of ADR according to administration routes

The analysis of DRPs values for each of the presented routes revealed that high DRPs values were observed in case of rectal (4 records with DRPs values of more than 11, which amounted to 44.4% of all the cases for this administration route) and intramuscular routes (50 cases with DRPs values of more than 11 cases, which amounted to 40% of all the cases with intramuscular administration). For oral route, high DRPs values (DRP ≥ 11) were observed in 64 records, which amounted to 23.7% of the total number of peroral intake of NSAIDs. The distribution of DRPs values for all the routes of administration is presented in Table 3.

Table 3 – Distribution of DRPs values according to administration routes

Route (total amount of records)	Range of DRPs values	Cases of ADRs (abs.)	Cases of ADR (% from total amount of cases per route)
Per os 270 records	0–5	23	8.5
	6–10	183	67.8
	11–15	55	20.4
	16–20	9	3.3
Intramuscular 125 records	0–5	3	2.4
	6–10	72	57.6
	11–15	37	29.6
	16–20	13	10.4
Intravenously 9 records	6–10	6	66.7
	11-15	3	33.3
Per rectum 9 records	0–5	1	11.1
	6–10	5	55.5
	11–15	4	44.4
Local 2 records	6–10	2	100

The obtained results indicate a higher risk of DRPs when parenteral routes of administration are used. In our opinion, it is primarily due to the peculiarities of the pharmacokinetics of NSAIDs after intramuscular and intravenous injections.

Special attention was paid to the analysis of ADRs

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2019-7-4-215-223

records with DRPs values equal to 18–20. The total number of such reports was 10: 5 were associated with Ketorolac use, 3 and 2 cases – with Meloxicam and Diclofenac, respectively.

Such high values of DRPs in 8 cases were were caused by an irrational combination of two or more NSAIDs in one patient. In 3 cases there was an excess of single and/or daily doses of the suspected drug and in 3 clinical situations, there was an excess of the course of treatment. In some cases, two or more reasons for such high rates of DRPs were found.

In our opinion, the range, i.e. the differences between the maximal and minimal DRPs values, is of special interest, as it can reflect the potential risk of a drug problem for each drug included in the analysis. As follows from the data in Table 2, the highest risk of problems determined by the range of the values is associated with Nimesulide, Ketorolac and Diclofenac prescriptions. Undoubtedly, the final and clear index should include not only the range of values but also the minimum value.

The analysis of violations of the dosing regimen (a low dose or an extended duration / an extended dose or a short duration) is also of a scientific interest. It is *a priori* a determining factor in the occurrence of DRPs. Information on a dosing regimen is presented in category "P" of the DRPs PCNE V5.01 qualification system. It made possible to obtain the data on the frequency of such mistakes for NSAIDs. The excess of the dose was found in 23 cases (5.53% of the total amount of NSAIDs ADRs), the doses below the minimum therapeutic ones were found in 16 cases (3.85%). The distribution of dosing violations is presented in Fig. 3.

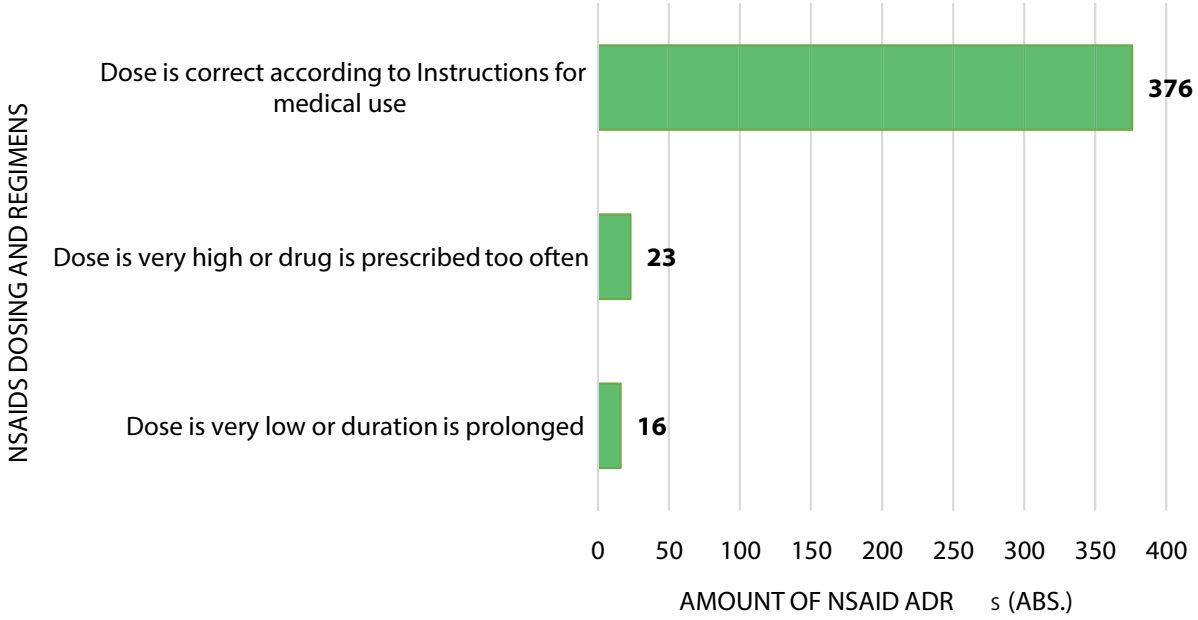

Figure 3 – Distribution of ADRs cases with violations of the dosage regimen

It is worth noting that the use of the DRPs system in the practice of doctors, clinical pharmacologists and pharmacists is an important and promising tool which makes it possible to improve the quality of pharmacotherapy and adherence to treatment. For drug safety professionals, the DRPs system makes it possible to identify priority areas in pharmacovigilance.

The results of the carried out analysis made it possible to identify NSAIDs, the prescription of which needs special attention due to the high risks of drug-related problems (Parecoxib, Dexketoprofen, Nimesulide, Diclofenac and Ketorolac).

In addition, the analysis of DRPs values using the DRP PCNE V5.01 qualification system made it possible to identify NSAIDs with a good safety profile. Among these drugs, it is worth to note Naproxen, Rofecoxib and Etoricoxib associated with a low amount of DRPs.

CONCLUSION

Using the drug-related problems (DRPs) system in the analysis of the safety of NSAIDs made it possible to identify the representatives of this group which may have an extra risk of ADRs in real medical practice. Among NSAIDs, it is worth to note Parecoxib, Dexketoprofen and Diclofenac combinations. The prescription of the mentioned products should be carried out with strict regard to the approved indications and contraindications for use, dose and duration of treatment, as well as taking into consideration all kinds of possible drug-drug interaction.

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AUTHOR CONTRIBUTIONS

All authors had equally contributed to the research work.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHORS

Matveyev Alexander Vasilyevich – PhD (Medicine), docent of the Department of Internal Medicine No.1 with the course of Clinical Pharmacology of Medical Academy n.a. S.I. Georgievsky of Vernadskjy CFU; executive director of ANO “National Pharmacovigilance Research Center”. ORCID 0000-0002-6636-3950. E-mail: avmcsmu@gmail.com

Krasheninnikov Anatoly Evgenievich – PhD (Pharmacy), CEO of ANO “National Pharmacovigilance Research Center”. ORCID 0000-0002-7791-6071. E-mail: anatoly.krasheninnikov@drugsafety.ru.

Egorova Elena Aleksandrovna – PhD (Pharmacy), assistant of the Department of Internal Medicine No.1 with the course of Clinical Pharmacology of Medical Academy n.a. S.I. Georgievsky of Vernadskjy CFU. ORCID 0000-0003-4012-2523. E-mail: elena212007@rambler.ru

Konyaeva Elena Ivanovna – PhD (Medicine), docent of the Department of Internal Medicine No.1 with the course of Clinical Pharmacology of Medical Academy n.a. S.I. Georgievsky of Vernadskjy CFU. E-mail: konyaeva.simferopol@gmail.com

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GLYPROLINES AS MODULATORS OF IMMUNOREACTIVITY WITHIN CONDITIONS OF "SOCIAL" STRESS

M.A. Samotrueva[1], A.L. Yasenyavskaya[1], O.A. Bashkina[1], N.F. Myasoedov[2], L.A. Andreeva[2]

[1] Astrakhan State Medical University,
121, Bakinskaya Str., Astrakhan, Russia, 414000
[2] Institute of Molecular Genetics of the Russian Academy of Sciences,
2, Academician Kurchatov Square, Moscow, Russia, 123182

E-mail: yasen_9@mail.ru

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The most important direction in the development of modern medical science is the study of protective, compensatory and pathological reactions of the organism that occur in response to various stress factors. The aim of the study is the subsequent development of methods for pharmacological correction of these reactions. The remedies for the correction of stress-induced immunity disorders are represented by the glyprolin group – the Selank drug and the Pro-Gly-Pro peptide compound – and are of particular interest.

The aim of the experiment was to study the immunomodulating effect of glyprolines on the basis of the "social stress" model.

Materials and methods. The experiment was performed on non-linear male rats aged 6-8 months. A model of a sensor contact was used as a model of the experimental "social stress". The animals were divided into groups (n = 10): a "control" group was represented by individuals with aggressive and submissive types of behavior, formed within the conditions of the experimental "social stress" for 20 days; and 2 experimental groups in which the animals were intraperitoneally administered Selank (100 μg/kg) and Pro-Gly-Pro (100 μg/kg) against the background of the experimental "social" stress once a day for 20 days. A functional activity of the immune system was studied on the basis of standard immunopharmacological tests: a delayed-type hypersensitivity test (DTH test), a direct agglutination test (DAT), a latex test for studying the Neutrophil phagocytic rate of peripheral blood, and the essessment of the leucogram.

Results. It has been established, that within the conditions of the "social" stress, the changes in the immune response are multidirectional. That fact confirms the theory of "the immune disbalance" caused by the action of stressors. As a result of studying the effect of glyprolines within the conditions of "the social stress", Selank and Pro-Gly-Pro proved to be effective immunocorrectors, restoring cellular and humoral immunogenesis reactions as well as the phagocytic activity of neutrophils and leucogram indices.

Conclusion. The carried out study expands understanding of the immunoreaction pathogenesis within the stress-induced conditions in order to further develop a pharmacological strategy for correcting the revealed disorders through the substances of the neuropeptide structure.

Keywords: glyprolines, Selank, Pro-Gly-Pro, social stress; delayed-type hypersensitivity (DTH) test; direct agglutination test (DAT); phagocytic index (PhI); phagocytic number (PhN), leucogram

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ГЛИПРОЛИНЫ КАК МОДУЛЯТОРЫ ИММУНОРЕАКТИВНОСТИ В УСЛОВИЯХ «СОЦИАЛЬНОГО» СТРЕССА

М.А. Самотруева[1], А.Л. Ясенявская[1], О.А. Башкина[1], Н.Ф. Мясоедов[2], Л.А. Андреева[2]

[1] Федеральное государственное бюджетное учреждение высшего образования «Астраханский государственный медицинский университет» Министерства здравоохранения Российской Федерации, Россия, 414000, г. Астрахань, ул. Бакинская, д. 121

[2] Федеральное государственное бюджетное учреждение науки Институт молекулярной генетики Российской академии наук, Россия, 123182, г. Москва, площадь академика И.В. Курчатова, д. 2

E-mail: yasen_9@mail.ru

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Важнейшим направлением развития современной медицинской науки является изучение защитных, компенсаторных и патологических реакций организма, возникающих в ответ на действие различных стрессогенных факторов с целью последующей разработки способов фармакологической коррекции. В качестве перспективных средств коррекции стресс-индуцированных нарушений иммунитета интерес представляют представители группы глипролинов: лекарственный препарат Селанк и пептидное соединение Pro-Gly-Pro.

Цель исследования – изучение иммуномодулирующего действия глипролинов на модели «социального» стресса.

Материалы и методы. Эксперимент выполнен на нелинейных крысах-самцах (6–8 мес.). В качестве экспериментального «социального» стресса использовали модель сенсорного контакта. Животные были разделены на группы (n=10): группа «контроль»; группа животных с агрессивным и субмиссивным типами поведения, сформированные в условиях экспериментального «социального» стресса в течение 20 дней и 2 опытные группы – животные, которым на фоне «социального» стресса внутрибрюшинно вводили Селанк (100 мкг/кг) и Pro-Gly-Pro (100 мкг/кг) 1 раз в сутки в условиях стрессорного воздействия в течение 20 дней. Функциональную активность иммунной системы изучали с помощью стандартных иммунофармакологических методов: реакции гиперчувствительности замедленного типа (РГЗТ), реакции прямой гемагглютинации (РПГА), латексного теста по изучению фагоцитарной активности нейтрофилов периферической крови и оценки лейкоцитарной формулы.

Результаты. Установлено, что в условиях «социального» стресса изменения иммунного реагирования имеют разнонаправленный характер, что подтверждает теорию «иммунного дисбаланса» при действии стрессирующих факторов. В результате изучения влияния глипролинов в условиях «социального» стресса было установлено, что Селанк и Pro-Gly-Pro проявили себя как эффективные иммунокорректоры, восстанавливая клеточную и гуморальную реакции иммуногенеза, а также фагоцитарную активность нейтрофилов и показатели лейкоцитарной формулы.

Заключение. Данное исследование расширяет представление об общих закономерностях иммунореагирования в условиях стресс-индуцированных состояний с целью последующей разработки фармакологической стратегии коррекции выявленных нарушений посредством веществ нейропептидной структуры.

Ключевые слова: глипролины, Селанк, Pro-Gly-Pro, «социальный» стресс, реакция гиперчувствительности замедленного типа (РГЗТ), реакция прямой гемагглютинации (РПГА), фагоцитарный индекс (ФИ), фагоцитарное число (ФЧ), лейкоцитарная формула

Список сокращений: РГЗТ – реакция гиперчувствительности замедленного типа, РПГА – реакция прямой гемагглютинации, ФИ – фагоцитарный индекс, ФЧ – фагоцитарное число

INTRODUCTION

The studies devoted to the the mechanisms of the dysfunctional changes in the immune system within the conditions of the “social” stress, as well as the development of methods for their correction, are the main directions of development in modern immunology and pharmacology. The action of stressors, regardless of their nature, leads to the “strain” of homeostasis of the organism on the whole and the immune system, in particular. That was confirmed by a lot of works on the study of Hans Selye’s general adaptation syndrome, which consider the involution processes of the thymic lymphatic apparatus as an obligatory part of the stress triad. Stress-induced immune disorders can develop at any stage of the stress response, being the cause of decline in adaptive skills of the body and the development of, for example, secondary immunodeficiency states, autoimmune and allergic processes [1–3].

The interest in research aimed at finding means for correcting changes in the immune response, morphofunctional disorders in the immunocompetent cells and organs against the background of the influence of vari-

ous stress factors, has recently increased significantly [4, 5]. The greatest achievement of molecular biology and medicine was the possibility of synthesizing bioregulators, in particular the ones of peptide nature, and the creation of new highly effective drugs on their basis. These drugs exhibit, among others, stress protective properties [6–8].

As a result of the research in recent years, a new class of regulatory peptides – glyprolines – has been isolated; most of them are promising as therapeutic agents [9]. It should be noted that at the moment, a representative of this group, Selank, synthesized at the Institute of Molecular Genetics of the Russian Academy of Sciences, is in active use in clinical medicine. This drug was created by attaching Pro-Gly-Pro tripeptide to the C-termini of the unstable regulatory peptide Taftsin which solved the problem of *in vivo* stabilization and supplemented it with the effects of Pro-Gly-Pro itself [10]. As well as the already registered drug Selank, Pro-Gly-Pro tripeptide itself is of considerable interest from the standpoint of long-term benefits of practical implementation in clinical pharmacology. This peptide is a structural fragment of Selank, and, in addition, it has a physiological activity of its own [9, 11]. The analysis of the experimental data confirms the uniqueness of the properties of the preparations with the glyproline structure which consists in the combination of psycho-, neuro- and immunotropic kinds of activity [9, 12, 13], and makes it promising to study various aspects of the pharmacological action of glyprolines in order to expand the possibilities of their practical application.

The aim of the research is to study immunomodulatory effects of glyprolines on the "social" stress model.

MATERIAL AND METHODS

Laboratory experiments

The study was performed on 70 non-linear male white rats, aged 6–8 months, obtained from the vivarium of the laboratory of physiology, morphology, genetics and biomedicine of Astrakhan State Medical University (Russia, Astrakhan). Throughout the experiment, the rats were kept in standard vivarium conditions of Astrakhan State Medical University. By the Order of the Ministry of Health of the Russian Federation No. 199n dated 04 January, 2016 "On Approval of the Laboratory Practice Rules" and the Protocol of the Ethical Committee of Federal State Budget Educational Institution of Higher Education "Astrakhan State Medical University" of the Ministry of Health of Russia No. 8 dated 24 November, 2015, all the animal manipulations were performed in accordance with the requirements of the Directive of the European Parliament and the Council of the European Union on the protection of the animals used for scientific purposes (2010/63/ EU), and the rules adopted by the International Convention for the Protection of Vertebrate Animals used for experimental and scientific purposes (Strasbourg, 1986).

Experimental model

The "social" stress was chosen as an experimental model. The main methodological technique of this experimental model is a permanent residence of partners against the conditions of sensor contacts, which form aggressive and submissive kinds of behavior in animals [14–17]. This model is widely used in studying various aspects of the influence of chronic aggression on brain neurochemistry, physiological functions, behavior and catastasis of animals. Therefore, and it is highly productive in terms of obtaining new and original data and their interpretation. These factors suggest similarity between the animals' state and the one observed in humans.

The males were placed in pairs in experimental cells, separated by a septum preventing them from a physical contact, but it had holes providing their sensor contacts. Every day, the septum was removed for 10 minutes. It overwhelmingly led to agonistic collisions (confrontations) [14].

The groups of the animals with alternative types of behavior were formed: an aggressive type (in case of repeated victories experience – a winner / an aggressor) and a submissive type (in case of defeats – a victim). In the experimental animals, the manifestation of aggression was expressed in the forms of upright and sideways offensive postures – "threat" or attack. The social passivity was manifested by various acts of individual behavior: locomotion, sniffing, autogrumming, movements in place, upright defensive postures and immobility.

Experimental groups

The laboratory animals with aggressive and submissive kinds of behavior were divided into groups of 10 individuals:

- group of intact males;
- group of the animals that were exposed to stress for 20 days (sensor contacts);
- group of the individuals which were treated with Selank intraperitoneally at the dose of 100 mcg/kg a day from the 1st day of exposure to stress (sensor contacts) for 20 days;
- group of the rats which were treated with Pro-Gly-Pro intraperitoneally at the dose of 100 mcg/kg a day from the 1st day of exposure to stress (sensor contacts) for 20 days.

Methods

The study of the functional activity of the immune system of the animals was carried out according to the "Guidelines for preclinical studies of drugs" on the basis of standard immunopharmacological methods [18]: a delayed-type hypersensitivity test (DTH), a direct agglutination test (DAT), a latex test for studying the phagocytic activity of peripheral blood neutrophils. In addition, the total number of leukocytes in the blood and leucogram were determined. It is important to note that in the formation of a specific immune response in the laboratory animals within the conditions of the experiment, a corpuscular T-depen-

ОРИГИНАЛЬНАЯ СТАТЬЯ
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dent antigen (sheep erythrocytes) was used as an antigenic stimulus in the formulation of DTH and DAT.

To determine the phagocytic activity of peripheral blood neutrophils, a latex test on the basis of heparinized blood of the animals was used. Melanomaldehyde latexes were used as a test object. The activity of neutrophils was determined by the following indices: a phagocytic index or percentage of phagocytosis (the number of neutrophils with latex/100); a phagocytic number (the number of latex particles / 100).

To determine the content of the blood leukocyte count of the test animals, the blood was taken during the removal of the animals from the experiment from large vessels of the cervical area. The calculation was carried out in Gorjaev's chamber. The percentage of individual forms of leukocytes was evaluated in blood smears stained by Romanovsky-Giemsa staining.

Statistical processing of results

The results of the experiment were statistically processed using the following programs: Microsoft Office Excel 2007 (Microsoft, USA), BIOSTAT 2008 Professional 5.1.3.1. To process the obtained results, a parametric method with the Student t-test (with Bonferroni correction) was used. Statistically significant differences were considered at $p<0.05$.

RESULTS

In the course of the experiments, it was established that long-term inter-male confrontations caused the suppression of DTH and DAT in the animals with both aggressive and submissive types of behavior compared to the control animals. The index of the delayed-type hypersensitivity test in the aggressors decreased by more than 45% ($p<0.01$), in the victims – by more than 30% ($p<0.05$). In relation to the humoral immunity in the animal aggressors, more pronounced changes in the indices were observed: a decrease in antibody titer in the aggressors – by more than 80% ($p<0.001$), in the victims – by more than 50% ($p<0.001$) compared with the control indices (Table 1).

Table 1 – Effect of glyprolines on the formation of DTH and DAT within the conditions of the "social" stress

Experimental groups (n = 10) / Indices (M ± m)	Index DTH, %	Titer of antibodies in DAT, log
Animals with an aggressive type of behavior		
Control	30.83 ± 3.52	224.77 ± 23.27
"Social" stress	16.57 ± 1.75**	40.46 ± 5.81***
"Social" stress" + Selank (100 mcg/kg a day)	30.38 ± 3.48##	210.56 ± 22.54###
"Social stress"+ Pro-Gly-Pro (100 mcg/kg a day)	29.40 ± 3.63##	253.21 ± 23.27###
Animals with a submissive type of behavior		
Control	30.83 ± 3.52	224.77 ± 23.27
"Social" stress	20.78 ± 2.54*	103.55 ± 11.64***
"Social" stress+ Selank (100 mcg /kg a day)	28.26 ± 2.66#	231.19 ± 34.91##
"Social" stress+ Pro-Gly-Pro (100 mcg/kg a day)	28.57 ± 2.55#	138.71 ± 12.84#

*Note: * – p<0.05; ** – p<0.01;*** – p<0.001 – relative to the control group; # – p<0.05; ## – p<0.01; ### – p<0.001 – relative to the "stress" group (Student's t-test with Bonferroni correction for multiple comparisons)*

As the results presented in Table 1 show, glyprolines contributed to the restoration of the indices of both immunity units. The index of DTH in aggressors increased in terms of the administration of Selank and Pro-Gly-Pro by an average of 80% ($p<0.01$), in victims – by an average of 30% ($p<0.05$). As for the formation of anti-erythrocyte antibodies in DAT, the indices of the hemagglutinin titer also remained higher than those of the animals of the "stress" group with an aggressive type of behavior by an average of more than 5 times ($p<0.001$). In the animals with a submissive type of behavior, when Selank was administered, the index was 2.2 times higher than that in the "stress" group and 1.3 times higher in the group after the administration of Pro-Gly-Pro ($p<0.05$, $p<0.01$) (Table 1).

When studying the phagocytic activity indices of peripheral blood neutrophils in the animals exposed to the "social" stress, an increase in the phagocytic index (PhI) and a phagocytic number (PhN) was found in the rats with both aggressive and submissive types of behavior. There was an increase in the phagocytic index by 18% in aggressors ($p>0.05$) and by almost 30% in victims ($p<0.05$), a phagocytic number was increased by 40% in aggressors and by 20% in victims ($p>0.05$). These factors indicate a hyperreactivity of a nonspecific element of the immune system (Table 2).

Table 2 – Effect of glyprolines on the phagocytic activity of neutrophils within the conditions of the "social" stress

Experimental groups (n = 10) / Indices (M ± m)	Phagocytic index	Phagocytic number, %
Animals with an aggressive type of behavior		
Control	17.7 ± 1.68	53.3 ± 3.66
"Social" stress,	21.0 ± 1.85	74.3 ± 7.37*
"Social" stress + Selank (100 mcg /kg a day)	16.3 ± 1.87	57.6 ± 4.23
"Social" stress + Pro-Gly-Pro (100 mcg/kg/a day)	15.9 ± 1.74#	58.7 ± 3.23
Animals with a submissive type of behavior		
Control	17.7 ± 1.68	53.3 ± 3.66
"Social" stress	22.9 ± 1.61*	63.7 ± 4.73
"Social" stress + Selank (100 mcg/kg a day)	18.4 ± 1.58	50.5 ± 4.65
"Social" stress+ Pro-Gly-Pro (100 mcg/kg a day)	20.2 ± 1.83	54.0 ± 3.72

*Note: * – p<0.05; ** – p<0.01;*** – p<0.001 – relative to the control group; # – p<0.05; ## – p<0.01; ### – p<0.001 – relative to the "stress" group (Student's t-test with Bonferroni correction for multiple comparisons)*

When assessing the phagocytosis indices in the group of the animals treated with glyprolines (Selank, Pro-Gly-Pro) against the background of the impact of the "social" stress, it has been found out that the administration of these compounds leads to the restoration of the parameters of nonspecific immunoreactivity. A decrease of phagocytic number was noted among the aggressors and victims by an average of 20% (p>0.05) compared with the stressed animals. The assessment of the phagocytic index in the aggressors showed its slight decrease; and in the victims, it remained at the level of the intact animals. At the same time, it should be mentioned that the most pronounced changes in the studied parameters were notified within the conditions of the administration of Pro-Gly-Pro tripeptide to the aggressors, and less pronounced ones were notified in the victims (Table 2).

An important stage of the work was to determine the total number of leukocytes, as well as to study the indices of the leucogram. Within the conditions of the "social" stress, a decrease in the total number of leukocytes (p<0.05) was observed in both, the aggressors (–29.1%) and the victims (–28.2%) relative to the control group indices. The stressed animals' leucogram showed a decrease in the percentage of eosinophils by 28.6% (p<0.05) in the aggressors and by more than 40% (p<0.01) in the victims. A statistically significant increase in segmented neutrophils by an average of 2 times (p<0.001), in band staple ones – by more than 50% (p<0.01) in the aggressors and by almost 2 times in the victims (p<0.01) should be also notified (Table 3).

It was established that in the "stress" group animals treated with glyprolins, the total number of leukocytes was higher relative to the control values. The administration of these drugs to the stressed animals contributed to an increase in the total number of leukocytes relative to the "stress" group: after the administration of Selank and Pro-Gly-Pro, in the aggressors' group the number of leukocytes was almost twice as high (p<0.001 and p<0.01 respectively); in the group treated with Selank it was more than 1.5 times as high as in the victims (p<0.01); and after the administration of Pro-Gly-Pro, only slight changes were noted (p>0.05).

Under the influence of Selank, the number of eosinophils in the aggressors decreased less than in the animals without any pharmacological support. The administration of Pro-Gly-Pro to the stressed animals contributed to the conservation and was higher relative to the stressed group by 30% (p<0.05). In the leucogram of the rats with a submissive type of behavior, the percentage of eosinophils increased by an average of 31% (p<0.05). In addition, in the individuals with an aggressive type of behavior, the administration of Selank against the background of stress led to the maintenance of the content of band neutrophils relative to the intact animals, and that was by 12% lower (p<0.05) relative to the stressed animals. Within the conditions of Selank and Pro-Gly-Pro administration, in the animals with a submissive type of behavior the number of band neutrophils was lower than the values of the stressed group by an average of 50% (p<0.01 and p<0.01, respectively). The percentage of the segmented forms of neutrophils decreased in all the groups by more than 40% relative to the stressed animals (Table 3).

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2019-7-4-224-230

Table 3 – The effect of glyprolines on the leucogram of the animals under the "social" stress

Experimental groups (n = 10) / Indices (M ± m)	Total number of leukocytes, x10^9/l	Eosinophils, %	Band neutrophils, %	Segmented neutrophils, %	Lymphocytes, %	Monocytes, %
Animals with an aggressive type of behavior						
Control	11.7 ± 0.93	2.8 ± 0.33	2.2 ± 0.23	12.7 ± 1.59	81.5 ± 5.95	0.83 ± 0.15
"Social" stress	8.3 ± 0.82*	2.0 ± 0.21*	3.4 ± 0.25**	26.7 ± 1.81***	67.1 ± 4.27	0.71 ± 0.10
"Social" stress+ Selank (100 mcg/kg a day)	15.7 ± 1.24###	2.4 ± 0.20	2.1 ± 0.36#	16.0 ± 2.10##	78.7 ± 4.87	0.86 ± 0.11
"Social" stress+ Pro-Gly-Pro (100 mcg/kg a day)	15.9 ± 1.54##	2.6 ± 0.21#	3.0 ± 0.60	16.1 ± 1.52##	77.0 ± 3.81	0.86 ± 0.11
Animals with a submissive type of behavior						
Control	11.7 ± 0.93	2.8 ± 0.33	2.2 ± 0.23	12.7 ± 1.59	81.5 ± 5.95	0.83 ± 0.15
"Social" stress	8.4 ± 0.77*	1.6 ± 0.11**	4.1 ± 0.40**	27.1 ± 2.11***	66.4 ± 4.77	0.71 ± 0.10
"Social" stress+ Selank (100 mcg/kg a day)	13.1 ± 0.58###	2.1 ± 0.22#	2.1 ± 0.37##	16.1 ± 2.57##	78.9 ± 4.87	0.86 ± 0.11
"Social" stress+ Pro-Gly-Pro (100 mcg /kg a day)	9.7 ± 0.69	2.1 ± 0.22#	2.0 ± 0.30##	15.0 ± 1.51###	79.9 ± 5.01	1.0 ± 0.10#

*Note: * – p<0.05; ** – p<0.01;*** – p<0.001 – relative to the control group; # – p<0.05; ## – p<0.01; ### – p<0.001 – relative to the "stress" group (Student's t-test with Bonferroni correction for multiple comparisons)*

DISCUSSION

The experiment has resulted in the following. It has been proved that under the influence of "the social stress", the changes in immunoreactivity are multidirectional, which indicates the formation of an immune imbalance, manifested by activation of some and suppression of other elements of the immune system. Thus, against the background of an increase in the phagocytic index and phagocytic number, stressing of animals with aggressive and submissive types of behavior was accompanied by the suppression of cellular and humoral immunity indices. In addition, some characteristic manifestations of stress-reactions on behalf of blood have been revealed: a decrease in the total number of leukocytes and eosinophils, as well as an increase in neutrophils.

As a result of studying the activity of glyprolines against the conditions of the "social" stress, it has been found out that the compounds used in the experimental groups proved to be effective immunocorrector, preserving immunogenesis (cellular and humoral) reactions and indices of phagocytic activity, as well as providing a protective effect on leukocyte blood lineage. The data obtained, indicate the presence of immunomodulatory properties in the studied compounds. It is important to note that the effect of glyprolins on the immune system used to be observed before [19–21], but in this aspect the effect of peptides is studied for the first time.

CONCLUSION

Thus, this study actualizes the search for new immunocorrectors among the representatives of a new class of regulatory peptides – glyprolins, most of which are promising as therapeutic agents.

The fundamental approach used in this work, emphasizes the importance of scientific research in the field of immunoreactivity against stress-induced conditions, in particular, the "social" stress. The aim of further studies is developing a pharmacological correction strategy using Selank and its fragments as representatives of glyprolines, characterized by a wide spectrum of action, as well as a high degree of safety.

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AUTHOR CONTRIBUTIONS

All authors had equally contributed to the research work.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHORS

Samotrueva Marina Aleksandrovna – Doctor of Sciences (Medicine), Professor, Head of the Department of Pharmacognosy, Pharmaceutical Technology and Biotechnology of Astrakhan State Medical University. ORCID: 0000-0001-5336-4455. E-mail**:** ms1506@mail.ru

Yasenyavskaya Anna Leonidovna – Candidate of Sciences (Medicine), Associate Professor of the Department of Pharmacognosy, Pharmaceutical Technology and Biotechnology of Astrakhan State Medical University. ORCID: 0000-0003-2998-2864. E-mail: yasen_9@mail.ru

Bashkina Ol'ga Aleksandrovna – Doctor of Sciences (Medicine), Professor, Head of the Department of Pediatrics Faculty of Astrakhan State Medical University. ORCID: 0000-0003-4168-4851. E-mail: bashkina1@mail.ru

Myasoedov Nikolai Fedorovich – Academician of the Russian Academy of Sciences, Professor, Institute of Molecular Genetics of the Russian Academy of Sciences. ORCID: 0000-0003-1294-102X. E-mail: nfm@img.ras.ru

Andreeva Liudmila Aleksandrovna – Sector Leader, Institute of Molecular Genetics of the Russian Academy of Sciences. ORCID: 0000-0002-3927-8590. E-mail: landr@img.ras.ru

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ОРИГИНАЛЬНАЯ СТАТЬЯ

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STUDY OF ANTISECRETORY ACTIVITY OF DINITRATE 2-PHENYL-9-DIETHYLAMINOETHYLIMIDAZO[1,2-A] BENZIMIDAZOLE BY METHOD OF CONTINUOUS PERFUSION OF RATS' STOMACHS

M.V. Chernikov, M.A. Oganova, A.S. Gerasimenko, E.A. Artemyev

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

E-mail: pharmax@list.ru

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Nowadays, effective pharmacotherapy of acid-dependent gastrointestinal diseases remains an urgent problem of modern gastroenterology. In this regard, the search for new drugs with a pronounced antisecretory activity still continues; their aim is to keep the control over the acid production safe and effective.

The aim of this study was an experimental study of the antisecretory activity of the substance and the finished dosage form (FDF) of dinitrate 2-phenyl-9-diethylaminoethylimidazo[1,2-a]benzimidazole.

Materials and Methods. The study of antisecretory activity was performed by method of a continuous perfusion of rats' stomachs. The studied substance was administered at the doses of 3, 10 and 30 mg/kg, and the FDF – at the doses of 13 and 26 mg/kg. The substance of Ranitidine (Sigma Aldrich, USA) was used as a reference object in the study of the antisecretory activity of the substance under study, and Ranitidine (Hemofarm A.D., Serbia) was used as a reference drug in the study of the FDF. In order to determine the stimulated secretion immediately before collecting the samples of the perfusate, histamine was administered subcutaneously at the dose of 5 mg/kg. The content of hydrochloric acid in the perfusate was determined by titration of a 0.01 M sodium hydroxide solution. The acidity value was determined in terms of the debit-hour of hydrochloric acid.

Results and discussion. The obtained experimental data showed that the studied substance at the dose of 30 mg/kg decreased the basal hydrochloric acid secretion by 54%, which significantly exceeded the antisecretory effect of Ranitidine by 1.8 times. The FDF at the dose of 26 mg/kg, statistically reliable relative to the control and the group treated with Ranitidine, decreased the basal secretion of gastric juice by 33%. The substance at the dose of 30 mg/kg reliably suppressed the stimulated secretion of hydrochloric acid by 80%, while Ranitidine did it by 56%. The FDF at the dose of 26 mg/kg decreased the histamine-stimulated secretion by 66%, and Ranitidine did it by 52%, which was statistically reliable.

Conclusions. The studied substance and its dosage form are more effective in suppressing basal activities and exceed the anisecretory activity of H_2-histamine antagonists of Ranitidine under the conditions of the secretion stimulated by histamine.

Keywords: dinitrate 2-phenyl-9-diethylaminoethylimidazo[1,2-a]benzimidazole, antisecretory effect, basal secretion, stimulated secretion, preclinical studies

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ИЗУЧЕНИЕ АНТИСЕКРЕТОРНОЙ АКТИВНОСТИ ДИНИТРАТА 2-ФЕНИЛ-9-ДИЭТИЛАМИНОЭТИЛИМИДАЗО[1,2-А] БЕНЗИМИДАЗОЛА МЕТОДОМ НЕПРЕРЫВНОЙ ПЕРФУЗИИ ЖЕЛУДКА КРЫС

М.В. Черников, М.А. Оганова, А.С. Герасименко, Е.А. Артемьев

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

E-mail: pharmax@list.ru

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Эффективная фармакотерапия кислотозависимых заболеваний ЖКТ на сегодняшний день остается актуальной проблемой современной гастроэнтерологии. В связи с этим, продолжается поиск новых лекарственных препаратов, обладающих выраженной антисекреторной активностью, с целью безопасного и эффективного контроля кислотопродукции.

Целью данного исследования – экспериментальное изучение антисекреторной активности субстанции и готовой лекарственной формы (ГЛФ) динитрата 2-фенил-9-диэтиламиноэтилимидазо[1,2-а]бензимидазола.

Материалы и методы. Исследование антисекреторной активности выполняли методом непрерывной перфузии желудка крыс. Изучаемая субстанция вводилась в дозах 3, 10 и 30 мг/кг, а ГЛФ в дозах 13 и 26 мг/кг. В качестве объекта сравнения при исследовании антисекреторной активности субстанции исследуемого вещества была использована субстанция ранитидина (Sigma Aldrich, США), а в качестве препарата сравнения при изучении ГЛФ – Ранитидин (Хемофарм А.Д., Сербия). С целью определения стимулированной секреции непосредственно перед началом сбора образцов перфузата подкожно вводился гистамин в дозе 5 мг/кг. Содержание соляной кислоты в перфузате определялось титрованием 0,01М раствором натрия гидроксида. Величину кислотности определяли в пересчете на дебит-час соляной кислоты.

Результаты. Полученные экспериментальные данные показали, что изучаемая субстанция в дозе 30 мг/кг снижала базальную секрецию соляной кислоты на 54%, что достоверно превышало антисекреторное действие ранитидина в 1,8 раз. ГЛФ в дозе 26 мг/кг, достоверно относительно контроля и группы, получавшей ранитидин, снижала базальную секрецию желудочного сока на 33%. Субстанция в дозе 30 мг/кг достоверно подавляла стимулированную секрецию соляной кислоты на 80%, в то время как ранитидин на 56%. ГЛФ в дозе 26 мг/кг снижала стимулированную гистамином секрецию на 66%, а ранитидин на 52%, что статистически достоверно.

Заключение. Изучаемые субстанция и ГЛФ более эффективно подавляют базальную и превосходят антисекреторную активность H_2-гистаминоблокатора ранитидина в условиях стимулированной гистамином секреции.

Ключевые слова: 2-фенил-9-диэтиламиноэтилимидазо[1,2-а]бензимидазол, антисекреторное действие, базальная секреция, стимулированная секреция, доклинические исследования

INTRODUCTION

Nowadays, the treatment of acid-dependent diseases, such as a gastroesophageal reflux disease (GERD), gastric and duodenal kinds of ulcer, functional dyspepsia, appears to be an urgent problem in modern clinical gastroenterology [1–4]. In the treatment of acid-dependent diseases, the main pathogenetic principle is the suppression of the secretion of hydrochloric acid by parietal cells of the gastric mucosa, which eliminates or attenuates the main clinical manifestations of the above mentioned diseases [5, 6].

The inhibition of HCl secretion also significantly eliminates the manifestations of gastrointestinal complications when non-steroidal anti-inflammatory drugs (NSAIDs), antiaggregants and anticoagulants are taken. In the cases of infection with Helicobacter pilory, the inhibition of HCl secretion increases the effectiveness of the eradication therapy by increasing the anti-Helicobacter activity of antibacterial drugs [7, 8].

Hydrogen-potassium adenosine triphosphatase blockers (proton pump inhibitors), histamine H2 receptor antagonists, selective M-cholinoblockers, antacids, and partly gastrin and cholecystokinin CCK-2 receptor blockers are used as antisecretory agents [2, 4, 5, 9].

Of course, the positive effects of the drugs of these groups are undeniable and all of them are widely used in the treatment of acid-dependent diseases, but they have certain disadvantages and side effects.

Before the introduction of proton pump inhibitors into the clinical practice, H2-histamine receptor antagonists were “the gold standard” of the antisecretory therapy [9]. However, along with the resistance to their

ОРИГИНАЛЬНАЯ СТАТЬЯ
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use in about 1/5 patients with acid-dependent diseases, there is a rapid development of tolerance in previously susceptible patients, withdrawal syndrome. In addition, the side effects characteristic of this group of drugs, such as decreased libido, bradycardia, hepatotoxicity and others, largely limit the intake and prescription of these preparations [10–14].

It has been proved that a long-term treatment with proton pump inhibitors can cause a number of undesirable effects, such as magnesium deficiency, hypergastrinemia and a risk of tumors, vitamin B12 deficiency, acute interstitial nephritis, osteoporosis and an increased risk of fractures, an intestinal bacterial overgrowth syndrome, a risk of cardiovascular accidents [15].

M-cholinoblockers, including selective ones, such as pyrenzepine, have a full range of side effects, characteristic of the "classic" non-selective drugs, although less pronounced, such as dry mucous membranes, tachycardia, accommodation disorders and photophobia, intestinal and bladder atony, dizziness, headaches [11, 16–18].

Under modern conditions, antacid agents can be considered only as additional means of auxiliary therapy of acid-dependent diseases [11].

In this regard, it remains relevant to search for new acid-dependent diseases treatment. They should be safer, more effective in suppressing acidity and, at the same time, available to the consumer.

Currently, the pharmaceutical market offers a wide range of drugs based on benzimidazole derivatives, such as anti-ulcer agents, inhibitors of hydrogen-potassium adenosine triphosphatase – omeprazole, lansoprazole; antihistamines – astemizole; antihypertensive agents – telmisartan, candesartan; antiviral agents – enviradin, maribavir; antiparasitic agents – albendazole, oxfendazole and many others[19–22]. This factor indicates a significant medical value of this group of chemicals and provides a high interest in them.

The aim of this study was the experimental study of the antisecretory activity of the substances and the finished dosage forms of dinitrate 2-phenyl-9-diethylaminoethylimidazo[1,2-a]benzimidazole by the method of a continuous perfusion of the rats' stomachs.

MATERIALS AND METHODS

Animals

The studies of the antisecretory activity were performed on outbred Wistar rats of both sexes (aged 10–12 weeks) weighing 180.0–250.0 grams. The variation in the initial mass of the animals in the group did not exceed 10% [23]. The animals were received from "Rappolovo", the Federal State Unitary Enterprise "Nursery of laboratory animals".

The conditions of keeping the animals met the requirements of the Decree of the Chief State Sanitary Doctor of the Russian Federation No.51 dated 29.08.2014 "On approval of SP 2.2.1.3218-14 "Sanitary and epidemiological requirements for the device, equipment and maintenance of experimental biological clinics (vivaria)".

During the experiment, the animals were kept under controlled conditions: the ambient temperature of 20-26°C and the relative humidity of 30–70%. Macrolon cells T-3 (for rats) equipped with steel grating covers were used to accommodate the animals. Sawdust was used as a nesting material. The animals were on a standard diet with a free access to feed (Complete feed recipe PK-120 for the maintenance of laboratory animals, GOST R 50258-92, the manufacturer of "Laboratory Feed"), and water. A nesting material, cages and accessories, drinking bowls changed weekly.

Manipulations with the experimental animals were performed in accordance with the generally accepted ethical standards adopted by the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (1986) and taking into account the International recommendations of the European Convention for the protection of vertebrate animals used in experimental studies (1997) [24, 25].

Investigated substances

The studied pharmaceutical substance of dinitrate 2-phenyl-9-diethylaminoethylimidazo[1,2-a]benzimidazole is a fine crystalline powder of a white or light gray color. It is moderately soluble in water. For the studies, purified water was used as a solvent of the substance during intragastric administration.

The finished dosage form of dinitrate 2-phenyl-9-diethylaminoethylimidazo[1,2-a]benzimidazole was presented as film-coated tablets: 60 mg, biconvex, light green, odour-free.

For the research, a weighed portion of the finished dosage form or reference preparations, previously ground into powder, was taken and dissolved in purified water. The resulting suspension was administered intragastrically using an atraumatic gastric catheter. The maximum volume for intragastric administration to rats did not exceed 3.0 ml for the animals weighing up to 200 g, 5.0 ml for the animals from 200 to 240 g, and 6.0 ml for the animals weighing more than 240 g.

Reference preparations

In the experiments on the study of basal and stimulated secretion of a pharmaceutical substance, Ranitidine (Sigma Aldrich, USA) was chosen as the reference preparation. In the study of the antisecretory action of the finished dosage form, Ranitidine was used (Hemofarm A.D., Serbia, series M703084).

Study design

In the first series of the experiments, the influence of the studied substance at the doses of 3 mg/kg, 10 mg/

kg and 30 mg/kg on the basal and histamine-stimulated secretion of hydrochloric acid was evaluated. The substance of Ranitidine at the doses of 3 mg/kg, 10 mg/kg and 30 mg/kg (Sigma Aldrich, USA) was used as the reference object. The data obtained were used to calculate the ED50 value for the substance of dinitrate 2-phenyl-9-diethylaminoethylimidazo[1,2-a]benzimidazole. Further on, in the study of the antisecretory activity, the doses of the finished dosage form (FDF) equal to the ED50 values were used, and the doses of the reference drug were calculated relative to the therapeutic dose for a person, taking into account the interspecific transfer coefficient; it amounted to 28 mg/kg. The control group of the animals received a solvent (purified water) in the equivolume amount of the administered drugs.

Before the experiment, the animals were subjected to a 24-hour food deprivation with a free access to water. After the anesthesia with chloral hydrate (350 mg/kg), a median laparotomy was performed. The stomach was removed to the surgical wound. A cannula was inserted through the discission in the proximal part of the duodenum up to the level of the pyloric sphincter. It was fixed with a ligature. The second cannula was inserted into the lumen of the stomach through the discission in the cardiac part and then fixed to the lower food sphincter. The stomach was perfused through the esophageal cannula with a 0.1 M phosphate buffer solution (pH 7.4, the temperature 37°C, 77.4 ml of 1M disodium hydrogen phosphate and 22.6 ml of 1M sodium dihydrogen phosphate per 100 ml of buffer) using a peristaltic pump at the constant speed of 0.5 ml/min. The perfusion samples were collected from the pyloric cannula.

In order to study basal secretion, 3 samples of the perfusate were collected at a 20-minute interval for 1 hour. The test substance was injected intragastrically through the gastric catheter 1 hour before the beginning of the sample collection. The finished dosage form (FDF) was administered intragastrically through the gastric catheter 2 hours before the beginning of the sample collection.

The acid content in the perfusate was determined by titration with a 0.01 M sodium hydroxide solution. The acidity value was determined in terms of the debit-hour of hydrochloric acid.

In order to determine the stimulated secretion, immediately before the collection of the perfusate samples, histamine dihydrochloride was administered subcutaneously at the dose of 5 mg/kg. The subcutaneous administration was carried out with sterile syringes. The test substance was administered 1 hour before the collection of perfusate samples intragastrically through the gastric catheter.

The acid content in the perfusate was determined by titration with a 0.01 M sodium hydroxide solution. The acidity value was determined in terms of the debit-hour of hydrochloric acid.

The calculation of the debit-hour of hydrochloric acid:

Dh = V1*E1*0,001+V2*E2*0,001+V3*E3*0,001,

where Dh is the debit-hour of hydrochloric acid, mmol; V is the volume of the portion of the gastric contents, ml; E is the concentration of free hydrochloric acid of the same portion in the titration units; 0.001 is the amount of hydrochloric acid in 1 ml of the gastric contents at the concentration of 1 mmol/l.

Determined indicators

To evaluate the antisecretory effect, the content of hydrochloric acid in the gastric juice was determined under the conditions of basal and histamine-stimulated secretion (total acidity), and the debit-hour of hydrochloric acid was calculated. According to the results of the study, the ED50 values were calculated for the studied substance, the finished dosage form and Ranitidine.

Statistical processing

The obtained experimental data were analyzed using the method of variation statistics. The summary tables show the group averages (M) and the standard error of the mean (m). The intergroup differences were analyzed using a nonparametric test – Mann-Whitney U-test. The differences were determined at a 0.05 significance level. For statistical processing of the results, the "StatPlus 2009" software package was used.

RESULTS

The analysis of the experimental data showed that the test substance at the dose of 10 mg/kg and 30 mg/kg statistically reliably decreased the basal hydrochloric acid secretion relative to the control by 35% and 54%, respectively. The administration of the substance at the dose of 3 mg/kg did not significantly affect the basal secretion of hydrochloric acid. Ranitidine, at the doses similar to the studied substance, also contributed to the suppression of the secretion statistically reliably by 22% and 29% relative to the control, respectively. Hereby, the substance of the test drug at the dose of 30 mg/kg decreased the basal secretion relative to Ranitidine significantly more effectively (Table 1).

ОРИГИНАЛЬНАЯ СТАТЬЯ
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Table 1 – Influence of the pharmaceutical substance of dinitrate 2-phenyl-9-diethylaminoethylimidazo[1,2-a]benzimidazole on basal gastric secretion

Substance	Dose	Debit-hour of hydrochloric acid mg-EQ/hour	Percentage of secretion suppression (%)
Control Purified water	–	0.27±0.010	–
Test substance	3 mg/kg	0.22±0.023	–18
Test substance	10 mg/kg	0.18±0.004*	–35
Test substance	30 mg/kg	0.13±0.009*#	–54
Ranitidine	3 mg/kg	0.26±0.017	–4
Ranitidine	10 mg/kg	0.21±0.014*	–22
Ranitidine	30 mg/kg	0.19±0.007*	–29

*Note: * – reliability relative to control P<0.05*
 # – reliability compared to the group treated with Ranitidine, P<0.05

The data reflecting the percentage of the decreased basal secretion relative to the control, made it possible for us to calculate the ED50 values for the test substance and Ranitidine (Fig. 1, 2). The calculated data were 26 mg/kg for the test substance, and 54.0 mg/kg for Ranitidine.

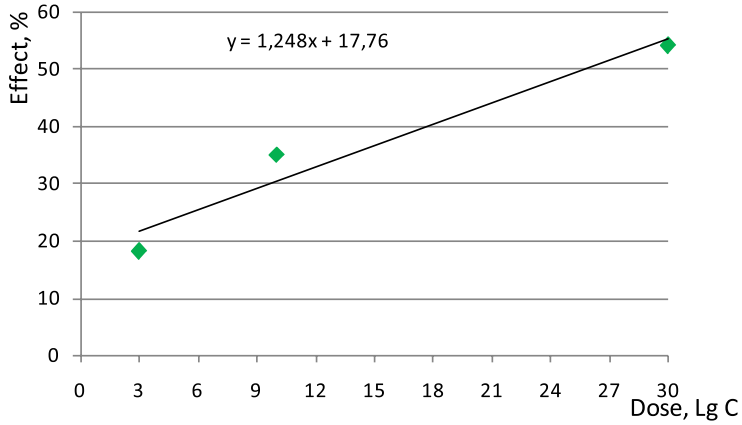

Figure 1 – Calculation of the ED50 value of basal secretion suppression of hydrochloric acid by the substance of dinitrate 2-phenyl-9-diethylaminoethylimidazo[1,2-a]benzimidazole

The finished dosage form at the dose of 26 mg/kg contributed to a decrease in the basal level of the secretion reliably relative to the control by 33%. The results obtained are statistically reliable relative to the control and the values of the group treated with Ranitidine at the dose of 28 mg/kg (Table 2).

Table 2 – Influence of the finished dosage form of dinitrate 2-phenyl-9-diethylaminoethylimidazo[1,2-a] benzimidazole on basal gastric secretion

Substance	Dose	Debit-hour of hydrochloric acid, mg-Eq/hour	Percentage of secretion suppression (%)
Control Purified water	–	0.259±0.005	-
Finished dosage form	26 mg/kg	0.172±0.004*#	–33
Ranitidine	28 mg/kg	0.207±0.007*	–20

*Note: * – statistical reliability relative to control P <0.05*
 # – statistical reliability relative to the group treated with Ranitidine P <0.05

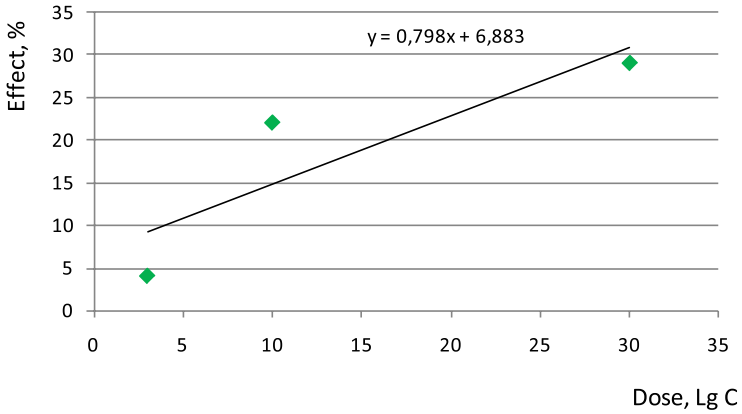

Figure 2 – Calculation of the ED50 value of basal secretion suppression of hydrochloric acid by the substance of Ranitidine

According to the results of the study stimulated by histamine secretion, it was established that the administration of histamine, increased the production of hydrochloric acid by 1.8 times relative to the basal level. The studied substance significantly inhibited the secretion of hydrochloric acid at all the studied doses. At the dose of 30 mg/kg, the secretion decreased by 80%, while Ranitidine inhibited the secretion by 56%, which is statistically reliable. The studied substance and Ranitidine at the dose of 10 mg/kg also contributed to a statistically reliable suppression of the acid production relative to the control, but no statistically reliable intergroup differences were observed. The administration of the dose of 3 mg/kg did not have the expected pharmacological effect either in the experimental group or in the reference group (Table 3).

Table 3 – Influence of the pharmaceutical substance of dinitrate 2-phenyl-9-diethylaminoethylimidazo[1,2-a]benzimidazole on histamine-stimulated gastric secretion

Substance	Dose	Debit-hour of hydrochloric acid mg-Eq/hour	Percentage of secretion suppression (%)
Control	–	0.51±0.022	–
Test substance	3 mg/kg	0.40±0.015*	–22
Test substance	10 mg/kg	0.23±0.011*	–56
Test substance	30 mg/kg	0.10±0.008*#	–80
Ranitidine	3 mg/kg	0.46±0.032	–10
Ranitidine	10 mg/kg	0.29±0.013*	–43
Ranitidine	30 mg/kg	0.22±0.013*	–56

*Note: * – statistical reliability relative to control P <0.05*
 # – statistical reliability relative to the group treated with Ranitidine P <0.05

The value of ED50 was calculated by the trend equation; for the substance under study it was 13.0 mg/kg (Fig. 3); for Ranitidine it was 23.6 mg/kg (Fig. 4).

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2019-7-4-231-240

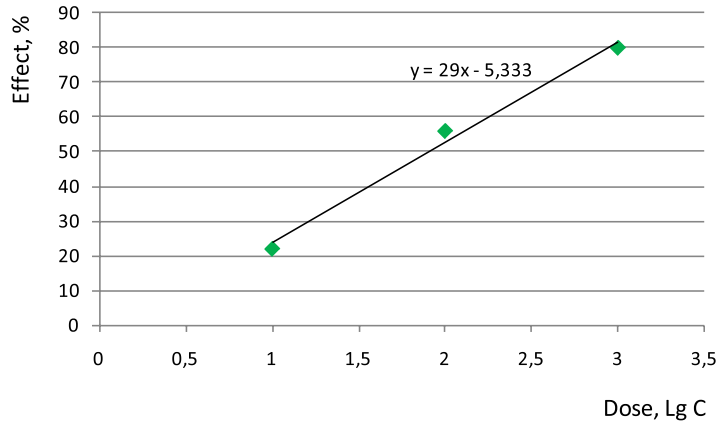

Figure 3 – Calculation of the ED50 value for the substance of dinitrate 2-phenyl-9-diethylaminoethylimidazo[1,2-a]benzimidazole under the conditions of histamine-stimulated secretion

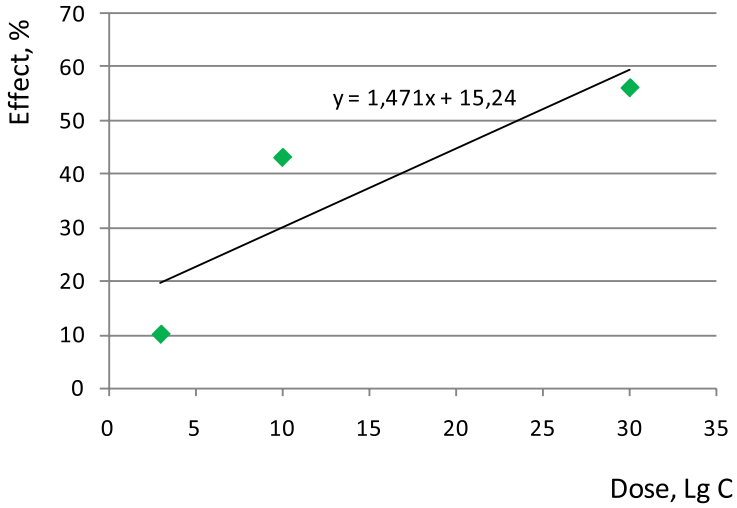

Figure 4 – Calculation of the ED50 value for the substance of Ranitidine under the conditions of histamine-stimulated secretion

According to the results of the study of histamine-stimulated secretion, it was established that the FDF of dinitrate 2-phenyl-9-diethylaminoethylimidazo [1,2-a]benzimidazole at the dose of 26 mg/kg statistically reliably inhibited hydrochloric acid secretion by 66%, while Ranitidine did it by 52% (Table 4).

Table 4 – Influence of the finished dosage form of dinitrate 2-phenyl-9-diethylaminoethylimidazo[1,2-a] benzimidazole on histamine-stimulated gastric secretion

Substance	Dose	Debit-hour of hydrochloric acid mg-Eq/hour	Percentage of secretion suppression (%)
Control	–	0.522±0.010	–
FDF	13 mg/kg	0.179±0.008*#	–66
Ranitidine	28 mg/kg	0.250±0.008*	–52

Note: * – statistical reliability relative to control P <0.05
– statistical reliability relative to the group treated with Ranitidine P <0.05

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AUTHOR CONTRIBUTIONS

All authors had equally contributed to the research work.

CONFLICTS OF INTEREST

The authors and peer reviewers of this paper report no conflicts of interest.

DISCUSSION

Benzimidazole contains nitrogen atoms of two types (pyrrole and pyridine) both acting as a donor and a proton acceptor, and existing in two tautomeric forms. Benzimidazole Bicycle can not only attach or give a proton, but also easily enter into various non-valent interactions (hydrogen bonds, van der Waals interactions, stacking interactions). These features of the structure of the benzimidazole molecule cause the possibility of its binding to a variety of therapeutic targets, providing a wide range of biological activity of benzimidazole derivatives.

The spectrum of benzimidazole derivatives' biological activity includes antiviral [26], antifungal [27], antimicrobial [28], anticancer [29], anthelmintic [30], analgesic and antipyretic [31], antidiabetic [32], antiprotozoal [33], antioxidant [33, 34], anticonvulsant [27], antipsychotic [35], antiulcer [36], anesthetic [37] and other types of activity.

A significant medical value of benzimidazole-containing drugs provides a high interest and an intensive development of this direction in pharmacology.

The analysis of the data obtained during the experiment revealed the capability of the studied substance of dinitrate 2-phenyl-9-diethylaminoethylimidazo[1,2-a] benzimidazole to suppress basal and histamine-stimulated secretion of hydrochloric acid in a model of a continuous gastric perfusion.

The results are consistent with the specific pharmacological activity of H2-histamine-blocking action of the studied compound identified in the course of a comprehensive study of the receptor effect on isolated atrial tissues.

However, taking into account the fact that the receptor effect did not exceed the Ranitidine indices, the results obtained in vivo demonstrating a more pronounced suppression of acid production, may be associated with the presence of additional mechanisms of influence on the secretion of hydrochloric acid, which creates the preconditions for further studies.

CONCLUSIONS

Thus, the substance of dinitrate 2-phenyl-9-diethylaminoethylimidazo[1,2-a]benzimidazole at the dose of 30 mg/kg and its finished dosage form at the doses equal to ED50, has an antisecretory activity, statistically reliably superior to the average Ranitidine H2-histamine-blocking agent twice on average, at the level of both – basal and histamine-stimulated – secretion. The finished dosage form in the administrated doses showed a generally comparable pharmacological effect compared with the data obtained in the study of the substance, as adjusted for the calculated nature of the ED50 values. The obtained results provide a sufficient justification for further study of the possibility of using the substance and the finished dosage form of dinitrate 2-phenyl-9-diethylaminoethylimidazo[1,2-a]benzimidazole as an effective pharmaco-therapeutic agent for acid-dependent diseases of the gastrointestinal tract.

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AUTHORS

Chernikov Maxim Valentinovich – PhD (Medicine), Head of the Department of Biology of Pyatigorsk Medical and Pharmaceutical Institute – branch of Volgograd State Medical University. ORCID 0000-0001-8340-1296. E-mail: pharmax@list.ru

Oganova Marina Albertovna – PhD (Pharmacy), Associate Professor of the Department of Biology of Pyatigorsk Medical and Pharmaceutical Institute – branch of Volgograd State Medical University. E-mail: marina-oganova81@mail.ru

Gerasimenko Anna Sergeevna – lecturer of the Department of pathology of Pyatigorsk Medical and Pharmaceutical Institute – branch of Volgograd State Medical University. E-mail: ger_ann5@ro.ru

Artemyev Evgeny Albertovich – post-graduate student of the Department of biology and physiology of Pyatigorsk Medical and Pharmaceutical Institute – branch of Volgograd State Medical University. E-mail: johni1001@rambler.ru


1943
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