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Development of a method for quantitative determination of nitric oxide (NO) in rat tissues based on high-performance liquid chromatography and mass spectrometry

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A quantitative assessment of nitric oxide (NO) production in body tissues is an urgent problem in pharmacology and biochemistry. The study of physiological processes occurring with the participation of NO, as well as the metabolism and pharmacodynamics of pharmacological agents from the group of NO donors, requires the introduction of accurate and reproducible methods for the quantitative determination of this metabolite in biological media.

The aim of the study was to develop the HPLC-MS/MS methods for the quantitative determination of NO in various tissues of rats.

Materials and methods. The indirect NO quantification was based on estimation of the level of more stable metabolites: nitrites and nitrates extracted from rat tissues by homogenization with water. The reduction of nitrates to nitrites was carried out using nitrate reductase. The derivatization of nitrites was based on a reaction with Griess reagent. The resulting azo dye was determined by HPLC-MS/MS using an Agilent InfinityLab Poroshell 120 EC-C18 4.6×100 mm, 2.7 µm analytical column. The total chromatographic analysis time was 12 minutes, and the analyte retention time was 6.1 minutes. The analytical range of the method was 0.1–100.0 nmol (in terms of nitrite) per 1 ml of plasma or tissue homogenate.

Results. The developed a bioanalytical method was validated according to the following parameters: a selectivity, a matrix effect, a recovery degree, a sample transfer, an analytical range linearity, a lower limit of quantification (LLOQ), an intra- and inter-assay accuracy and precision, and a stability at all the stages of the analysis. To test the method, the NO content in the plasma, brain, heart, aorta and lungs of rats was determined.

Conclusion. The developed bioanalytical HPLC-MS/MS methods fully meets the validation requirements. The metrological characteristics of the technique make it possible to highly accurately estimate the NO production in various tissues of rats, which is undoubtedly relevant and in demand in the study of pathological processes as well as the mechanism of action of pharmacological agents from the group of NO donors.

Keywords: HPLC-MS/MS; chromatography; mass spectrometry; endothelial relaxing factor; nitric oxide

Abbreviations: NO – nitric oxide / endothelial relaxing factor; HPLC-MS/MS – high-performance liquid chromatography with mass spectrometric detection; IS – internal standard; AUC – area under the concentration-time curve; NADH – nicotinamide adenine dinucleotide; MRM – multiple reaction monitoring; LLOQ – lower limit of quantitation; EDTA – ethylenediaminetetraacetic acid; NMF – normalized matrix factor; CV – coefficient of variation; SD – standard deviation; DP – declustering potential; EP – entrance potential; CE – collision energy; CXP – collision cell exit potential.

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Разработка методики количественного определения оксида азота (NO) в тканях крыс с применением высокоэффективной жидкостной хроматографии и масс-спектрометрии

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Количественная оценка продукции оксида азота (NO) в тканях организма является актуальной задачей фармакологии и биохимии. Изучение физиологических процессов, протекающих с участием NO, а также метаболизма и фармакодинамики фармакологических средств из группы донаторов NO требует внедрения точных и воспроизводимых методик количественного определения данного метаболита в биологических средах.

Цель. Разработка ВЭЖХ-МС/МС методики количественного определения оксида азота в различных тканях крыс.

Материалы и методы. Непрямое количественное определение NO было основано на оценке уровня более стабильных метаболитов: нитритов и нитратов, которые извлекали из тканей крыс путем гомогенизации с водой. Восстановление нитратов до нитритов осуществляли с помощью нитрат редуктазы. В основе дериватизации нитритов использовали реакцию с реактивом Грисса. Полученный азокраситель определяли с помощью ВЭЖХ-МС/МС с использованием аналитической колонки Agilent InfinityLab Poroshell 120 EC-C18 4,6×100 мм, 2,7 мкм. Общее время хроматографического анализа составило 12 минут, время удерживания аналита составило 6,1 минут. Аналитический диапазон методики составил 0,1–100,0 нмоль (в пересчете на нитрит) на 1 мл плазмы или гомогената ткани.

Результаты. Разработанная биоаналитическая методика была валидирована по следующим параметрам: селективность, матричный эффект, степень извлечения, перенос пробы, линейность аналитического диапазона, нижний предел количественного определения (НПКО), внутри- и межсерийная точность и прецизионность, стабильность на всех этапах анализа. Для апробации методики было проведено определение содержания NO в плазме, головном мозге, сердце, аорте и легких крыс.

Заключение. Разработанная биоаналитическая ВЭЖХ-МС/МС-методика полностью соответствует валидационным требованиям. Метрологические характеристики методики позволяют с высокой точностью оценить продукцию NO в различных тканях крыс, что, несомненно, представляет актуальным и востребованным в исследовании патологических процессов, а также механизма действия фармакологических средств из группы донаторов NO.

Ключевые слова: ВЭЖХ-МС/МС; хроматография; масс-спектрометрия; эндотелиальный релаксирующий фактор; оксид азота

Список сокращений: NO – оксид азота / эндотелиальный релаксирующий фактор; ВЭЖХ-МС/МС – высокоэффективная жидкостная хроматография с масс-спектрометрическим детектированием; IS – внутренний стандарт; AUC – площадь под кривой хроматографического пика; NADH – никотинамидааденинадинуклеотид; MRM – мониторинг множественных реакций; НПКО – нижний предел количественного определения; ЭДТА – этилендиаминтетрауксусная кислота; NMF – нормированный матричный фактор; CV – коэффициент вариации; SD – стандартное отклонение; DP – декластеризующий потенциал; CEP – напряжение на входе в ячейку соударений; CE – энергия столкновений; CXP – выходной потенциал ячейки соударений.

INTRODUCTION

To date, there is a sufficient amount of data on the participation of nitric oxide (NO) in the regulation of a number of biological processes in the body [1, 2]. It is known that NO involved in the regulation of vascular tone and systemic hemodynamic reactions [3, 4], is an inhibitor of a smooth muscle cell proliferation [5, 6]; it plays an important role in the hemostasis system [7, 8] and protection of the mucous membrane of the gastrointestinal tract [9, 10]. In addition, NO has been proven to play a role in the development and progression of many neurological, psychiatric and neurodegenerative disorders [11–13]. A decrease in the NO production, which is one of the causes of an endothelial dysfunction, is one of the early signs of a coronary heart disease and atherothrombosis [14]. At

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the same time, the endothelial dysfunction is important in the development of pathological conditions such as hypercholesterolemia, type 2 diabetes mellitus, arterial hypertension, and a heart failure [15, 16].

The accumulation of data on the NO participation in numerous bioregulation pathways has made it possible not only to study the pathogenesis of many diseases, but also to expand the possibilities of using organic nitrates and other NO donors in the clinical practice. It is known that the kinetics of the NO release from pharmacologically active substances depends on many factors, among which the chemical structure, reactivity, and activity of certain enzymes play a key role [17].

The study of physiological processes occurring with the NO participation, as well as metabolism and pharmacodynamics of pharmacological agents from the group of the NO donors, requires the introduction of accurate and reproducible methods for the quantitative determination of this metabolite in biological media. The assessment of the NO production is mainly carried out by measuring the concentration of its more stable metabolites – nitrites, which, in turn, are determined colorimetrically after the interaction with the Griess reagent [18]. However, this widely used method for the indirect determination of NO has a number of significant drawbacks. First, the Griess reagent can interact chemically with various components of the biological matrix with the formation of colored products that change the optical density of the analyzed samples [19, 20].

Second, this method requires a careful removal of protein components, since their presence in samples ready for the photometric analysis also affects the value of the measured optical density [21]. Third, a photometric detection does not often provide the required level of sensitivity [22]. In addition, this method does not provide for the internal control throughout all stages of analysis (use of an internal standard – IS). The above disadvantages require the improvement of this method for a quantitative determination of the NO production in the direction of increasing the selectivity and sensitivity of the reaction product detection.

One of such solutions is the use of high-performance liquid chromatography in combination with a mass selective detection for this purpose.

The aim of the study was to develop the HPLC-MS/MS methods for the quantitative determination of NO in various tissues of rats.

MATERIALS AND METHODS

Animals

In the process of developing and validating the method, the biological materials obtained from 6 intact male Wistar rats weighing 200–220 g (kennel of SMK STEZAR LLC, Russia), were used. The animals were kept in the vivarium of Tver State Medical University in plastic cages with a mesh lid, equipped with a feeder and drinking bowl. Sterile wood shavings were used as bedding. The rat cages were kept under controlled environmental conditions (temperature of 20–26°C and the relative humidity of 30–70%). In the animal rooms, a 12-hour lighting cycle and 8–10-fold changes in the air volume per hour were maintained. The rats were fed with complete feed PK-120 (Provimi LLC, Russia), food and filtered tap water were given *ad libitum*. The cages were cleaned daily, and water bottles were replaced with new ones every day. Wet cleaning of rooms was carried out daily. The evening before the experiment, the animals were deprived of food.

The animals were removed from the experiment by decapitation using a guillotine (manufacturer – Open Science) under a light general anesthesia using a combination of zolazepam and tiletamine (Zoletil® 100 mg/ml, Virbac, France). The blood was collected into tubes containing EDTA, and plasma was obtained by centrifugation at 3000 g for 10 min. Then fragments of internal organs (brain, heart, aorta, lungs) were selected and then used to obtain homogenates.

Ethical approval

This study was approved by the Local Ethics Committee of Tver State Medical University (Minutes of meeting No. 4 dated 29 May 2019). All the experiments were performed in accordance with the Rules of Laboratory Practice approved by Order of the Ministry of Health of Russia No. 708n dated 23 August 2010 and Directive of the European Parliament and the Council of the European Union dated 2010/63/EU on the protection of vertebrate animals used for scientific purposes.

Description of experimental part

Since NO is a gaseous metabolite with a relatively short half-life in living body tissues, its indirect quantification was based on assessing the level of more stable metabolites – nitrites and nitrates (Fig. 1).

The derivatization of nitrites was based on the well-known reaction with the Griess reagent (Fig. 2). The reduction of nitrates to nitrites was carried out using nitrate reductase.

A sample preparation of rat tissues included several stages: homogenization with deionized water, nitrates reduction, derivatization of the resulting nitrites, and deproteinization. The preparation of homogenates was carried out as follows: a tissue fragment was placed in

a 2 ml Eppendorf test tube pre-tared on an analytical scales VL-124 ("Gosmetr", Russia). After determining the exact mass, water was added at the rate of 400 µl per 100 mg of the tissue using an automatic variable volume dispenser ("Eppendorf Research Plus", Germany). A quartz glass ball with a diameter of 5 mm was placed, and then homogenized in a vibrating mill with a reciprocating frequency of 50 Hz and an amplitude of 30 mm for 15 min. To increase the yield of nitrates and nitrites from the biomaterial, the tubes were additionally kept in an ultrasonic bath (Megeon, China) for 10 min. The separation of the liquid part of the homogenate was carried out by centrifugation at 16 000 g and the temperature of +4°C for 15 min. The supernatant was transferred into separate 0.5 ml Eppendorf tubes, immediately frozen and stored at –40°C until the analysis. At the initial stage, the sample preparation of the blood plasma included a 5-fold waterdilution.

Before the procedure of obtaining the nitrate reduction and derivatization of the nitrite, the necessary solutions and reagents were prepared. The phosphate buffer solution was prepared as follows: 3.75 g of monopotassium phosphate ("PanReac Applichem", Spain), 10 mg of ethylenediaminetetraacetate sodium dihydrate ("PanReac Applichem", Spain), 1.4 g of potassium hydroxide ("Millipore", Germany) were added to a 1000 ml volumetric flask and brought to the mark with deionized water. The resulting solution was stored in the refrigerator at +4°C for no longer than 6 months. The resulting reagent, 1 ml, was transferred into Eppendorf tubes, used immediately or frozen and stored at –20°C for 1 month. The NADH working solution was prepared by diluting 1 ml of the original solution with a phosphate buffer 10 times and used throughout the day. The nitrate reductase stock solution ("R&D Systems", USA) was prepared by reconstituting 1 U of the lyophilized substance in 1 ml of the phosphate buffer and stored on ice for no longer than 8 hours. Immediately before the use, the original solution was 5-fold diluted. The supernatant of tissue homogenates (or diluted plasma) obtained at the previous stage was placed in clean microcentrifuge tubes in the volume of 20 µl, then 10 µl of NADH working solution was added and the reconstituted nitrate reductase was mixed on a V1 plus vortex ("Biosan", Latvia) for 10 seconds, after which it was incubated in a solid-state thermostat TDB-120 ("Biosan", Latvia) at 37°C for 30 min.

The final stage of the sample preparation included the nitrite derivatization using the Griess reagent and subsequent deproteinization. For this purpose, after thermostatting, the samples were transferred to an ice bath, 20 µl of a cooled 1% solution of sulfanilamide ("Merck", Germany) in a 2 N aqueous solution of hydrochloric acid ("Merck", Germany) was added, and left for 5 minutes. Then 20 µl of a 0.1% solution of N-(1-naphthyl)ethylenediamine ("Merck", Germany) in a 2 N aqueous solution of hydrochloric acid was added to the tubes and incubated at room temperature for 15 minutes. Protein precipitation was carried out by adding 200 µl of methanol cooled to –20°C containing IS (4-[4-amino-1-naphthylazo] benzenesulfonamide, 1000 ng/ml) to the samples (Fig. 3). The samples were kept in a thermostated shaker TS-100 C ("Biosan", Latvia) at temperature of 4°C and an oscillation frequency of 1 400 rpm for 5 min, after which the supernatant was separated by centrifugation at the acceleration of 16 000 g and the temperature of 4°C for 15 min. The resulting supernatant was transferred into polyethylene inserts for chromatographic vials and used for the HPLC-MS/MS analysis.

A chromatographic separation was carried out using HPLC 1260 Infinity II ("Agilent Technologies", Germany) in a reverse phase mode using a Poroshell InfinityLab 120 EC-C18 4.6×100 mm, 2.7 µm analytical column ("Agilent Technologies", USA) in combination with precolumn Zorbax Eclipse Plus C18 4.6×12.5 mm, 5 µm ("Agilent Technologies", USA).

The detection of the analyte and IS during the chromatographic analysis was carried out using an AB Sciex QTrap 3200 MD tandem quadrupole mass spectrometer ("Sciex", Singapore), equipped with an ion source with a chemical ionization probe at atmospheric pressure. The selection of the optimal parameters for the mass spectrometric detection was carried out by a continuous injection of individual solutions (100.0 ng/ml) of the azo dyes into the ion source individual solutions (100.0 ng/ml) of azo the dyes (an analyte and an IS) in methanol with the addition of 0.1% formic acid using a syringe pump at the speed of 10 µl/min. At the first stage, the *m/z* of protonated molecules (the first order mass spectrum) was determined for the azo dyes, and the optimal values of the declustering potential (DP) and voltages at the entrance potential to the collision cell (EP) were selected. At the second stage, the mass spectra of product ions were determined for the identified precursor ions; 2 characteristic ions were selected, for which the optimal values of the collision energy (CE) and the collision cell exit potential (CXP) were selected. The obtained values were used for the detection of the azo dyes in the multiple reaction monitoring (MRM) mode and provided the best sensitivity.

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Statistical processing

The primary data processing of the chromatography-mass spectrometric analysis was carried out using built-in Software AB Sciex Analyst 1.3.6; Microsoft Office Excel 365 (Microsoft, USA) Software was used to calculate the values of validation parameters.

The method was validated according to the following parameters: selectivity, a matrix effect, a recovery degree, a sample transfer, a linearity of the analytical range, a lower limit of quantification (LLOQ), the intra- and inter-run accuracy and precision, a stability at all stages of the analysis.

RESULTS AND DISCUSSION

The first- and second-order mass spectra were obtained for the nitrite derivatization product (azo dye) and the IS (Fig. 4). For the analyte and the IS, two characteristic product ions were singled out; herewith, the detection conditions had been selected to ensure the maximum ion current (Table 1).

The chromatographic determination of azo dyes was carried out using a reverse phase column. The use of methanol as a component of the mobile phase with a higher elution force compared to acetonitrile resulted in narrower and higher peaks for both the analyte and the IS. In addition, the use of acetonitrile significantly increased the LLOQ of 4-[4-(2-aminoethylamino)-1-naphthylazo]benzenesulfonamide, which did not allow achieving the required level of sensitivity. Thus, elution was carried out with a mixture of deionized water (A) and methanol (B) and the addition of 0.1% formic acid in a gradient mode (Table 2). The retention times of 4-[4-(2-aminoethylamino)-1-naphthylazo] benzenesulfonamide and 4-[4-amino-1-naphthylazo] benzenesulfonamide (IS) were 6.1 and 6.5 min, respectively (Fig. 5).

To determine the metrological characteristics of the developed method, a series of standard samples with a nitrite content of: 0.1; 0.5; 1.0; 5.0; 10.0; 50.0; 100.0 nmol/ml in terms of homogenate. Due to the lack of a nitrite-free biological matrix, a proof of the feasibility of using deionized water as a substitute is required. To do this, an experiment with the addition of working solutions of nitrite to aliquots of rat tissue homogenates, followed by a sample preparation procedure, including the stages of nitrate reduction and derivatization of the resulting nitrites, was carried out [23]. The calibration curve obtained from the analysis of the samples prepared with deionized water was used to determine the nitrite content in standard samples prepared with homogenates. The difference between the nitrite concentration established by the calibration dependence and the concentration of the standard solutions with the additive without taking into account the endogenous level of the analyte, was determined. The standard solutions were prepared by adding 10 µl of a corresponding 10-fold working solution of nitrite to 90 µl of the deionized water (or tissue homogenate), after which the resulting samples were prepared according to the procedure described above. In addition, the slope coefficients of the calibration curves obtained from the analysis of a series of standard samples prepared in water and in the corresponding homogenates, were compared.

According to the results of the experiment, it was found out that the coefficient of variation of the concentration difference for homogenates of the brain tissue, myocardium, aorta, and rat blood plasma throughout the entire analytical range, were from 7.9 to 10.3%. Only for the lung homogenate, the relative standard deviation was close to 15%. The ratio of the slope coefficients of the calibration curves obtained from the analysis of the deionized water samples (0.0741) and tissue homogenates, ranged from 0.887 to 1.114 (Table 3). Thus, the use of the calibration standard samples prepared with deionized water had not affected the accuracy of determining the concentration of nitrites in biological objects.

The assessment of the matrix effect and the extraction degree was carried out based on the results of the samples analysis with the addition of 4-[4-(2-aminoethylamino)-1-naphthylazo] benzenesulfonamide (analyte) in the quantities equivalent to the conditional nitrite content in low (8.0 nmol/ml) and high (80.0 nmol/ml) concentrations from the analytical range of the technique. The matrix effect was calculated as the ratio of the chromatographic peak area of the analyte in the unextracted sample (*post-spike sample*) to the average signal value of the analyte in deionized water (*solvent-spike sample, n*=6). The normalized matrix factor (NMF) was taken to be the ratio of the analyte peak area value normalized to the internal standard (IS) in the *post-spike sample* to the average value of the ratio of the analyte peak area value normalized to IS in the *solvent-spike sample*.

The extraction degree was determined as the ratio of the chromatographic peaks areas in the extracted sample (*pre-spike sample*) and the unextracted sample (*post-spike sample*), expressed as a percentage. The matrix effect and recovery results are presented in Table 4.

The coefficient of variation values for NMF less

than 15% indicated[1] that the use of 4-[4-amino-1-naphthylazo]benzenesulfonamide as an IS can effectively compensate the influence of the biological matrix components on the signal intensity of the analyte. The average degree of the analyte recovery is in the range of 91.78–94.97% with a maximum coefficient of variation (CV) of 7.79%[2], which indicates an almost quantitative extraction of the nitrite derivatization product from the biological material.

The selectivity of the methods was assessed by comparing the ratios of the chromatographic peak areas of two azo dye product ions (analyte and IS) when analyzing the samples in the deionized water and tissue homogenates. It was found that for high and low nitrite concentrations the difference between these values did not exceed 5% for the analyte and 1% for IS[3].

In addition, a comparative characterization of the chromatograms of biological samples was carried out; this analysis was accomplished without a preliminary procedure of nitrite derivatization and samples to which 4-[4-(2-aminoethylamino)-1-naphthylazo]benzenesulfonamide (analyte) was added in the amount equivalent to the nitrite content at the LDL level. The results of assessing the selectivity of the methods are presented in Table 5.

The analyte response in the intact samples did not exceed 10.6% of the response in the LLOQ samples; for IS, the similar indicator is less than 0.22%, which proves a high selectivity of the developed methods[4].

Based on the results of the analysis of a standard samples series, a calibration graph was constructed. It reflects the ratio dependence of the peak area of the nitrite derivatization product to the peak area of the IS on the concentration of the analyte in the standard sample (Fig. 6). This dependence is presented in the form of a linear regression equation y=0.0735x+0.0123 (normalization 1/x2) with a correlation coefficient of 0.9959. The analytical range of the methods was from 0.1 to 100 nmol/ml of a conditional nitrite content.

Since there is no biological matrix free of NO metabolic products in nature, the LLOQ for nitrites was estimated based on the results of chromatographic analysis of analytical standards prepared on deionized water. The LLOQ was taken to be the minimum conventional nitrite content in 1 ml of tissue homogenate, which can be determined with a relative standard deviation and relative error values of no more than 20%[5], while the signal-to-noise ratio in the chromatogram should be 5:1[6]. The LLOQ value for nitrites was 0.1 nmol/ml. A fragment of a chromatogram of a standard sample with a nitrite content at the LLOQ level is shown in Fig. 7.

The transfer of substances during the analysis was assessed by comparing the chromatograms of blank samples analyzed after six injections of a sample with a nitrite concentration of 100 nmol/ml with chromatograms of LLOQ samples (0.1 nmol/ml). The analysis revealed that the ratio of peak areas in the blank samples to the peak areas in LLOQ samples was below the maximum permissible level (20% for the analyte and 5% for IS), indicating a little transfer of substances from higher to lower concentrations.

The accuracy and precision of the methods was calculated based on the results of the analysis of 5 control samples for each of the four levels of nitrite concentrations: LLOQ (0.1 nmol/ml), a low concentration (LC – 8.0 nmol/ml), a medium concentration (MC – 40.0 nmol/ml), a high concentration (HC – 80.0 nmol/ml) in three independent series. The accuracy was expressed as a percentage, as the ratio of the measured concentration in the control samples to the nominal nitrite content (E). The precision was determined by the coefficient of variation (CV) of the five-fold determination results of the nitrite concentration. The acceptance criterion was the value of the CV and a relative error for the LLOQ level – no more than 20%, for other concentrations – no more than 15%. The intra- and inter-assay accuracy and precision results are presented in Table 6.

The results of the stability assessment confirmed the inalterability of the initial and working nitrite solutions for 3 months. The solutions of sulfonamide and N-(1-naphthyl)ethylenediamine used for the nitrite derivatization remained reactive for 1 year when stored in a refrigerator. An aqueous solution of β-nicotinamide adenine dinucleotide (2 mg/ml) remained stable when frozen at –20°C for 1 month. The reactivity of the reconstituted nitrate reductase was not assessed after storage; a freshly prepared solution was used each time. The IS stock solution in methanol (1 mg/ml) remained stable for 1 year at –20°C.

[1] Requirements for the validation of bioanalytical test methods and analysis of biological samples under study (Decision of the Council of the Eurasian Economic Commission dated November 3, 2016 No. 85 (as amended on February 15, 2023) On approval of the Rules for conducting bioequivalence studies of medicinal products within the framework of the Eurasian Economic Union).
[2] Ibid.
[3] Ibid.
[4] Ibid.
[5] Ibid.
[6] Ibid.

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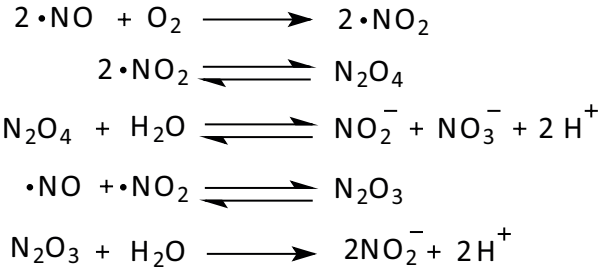

Figure 1 – Equations of chemical reactions for metabolites formation of endothelial relaxing factor (NO)

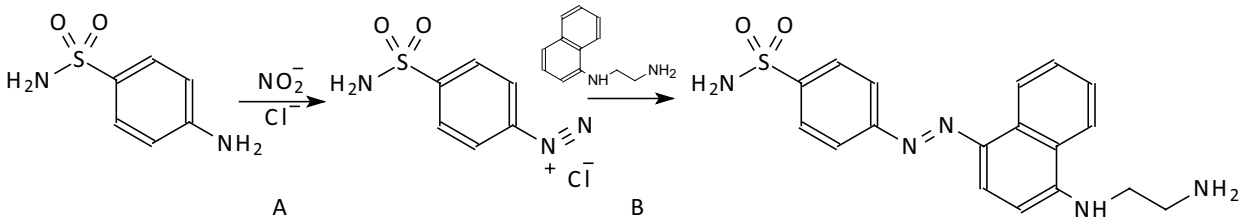

Figure 2 – Reaction of azo dye formation (A – diazotization of sulfonamide, B – azo coupling of diazonium ion with N-(1-naphthyl)ethylenediamine)

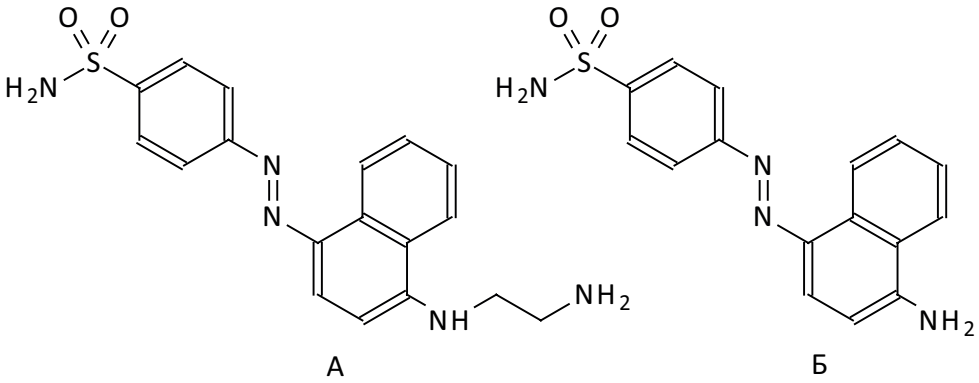

Figure 3 – Chemical structure of analyte (A – 4[4(2aminoethylamino)1naphthylazo]benzenesulfonamide) and internal standard (B – 4-[4-amino-1-naphthylazo]benzenesulfonamide)

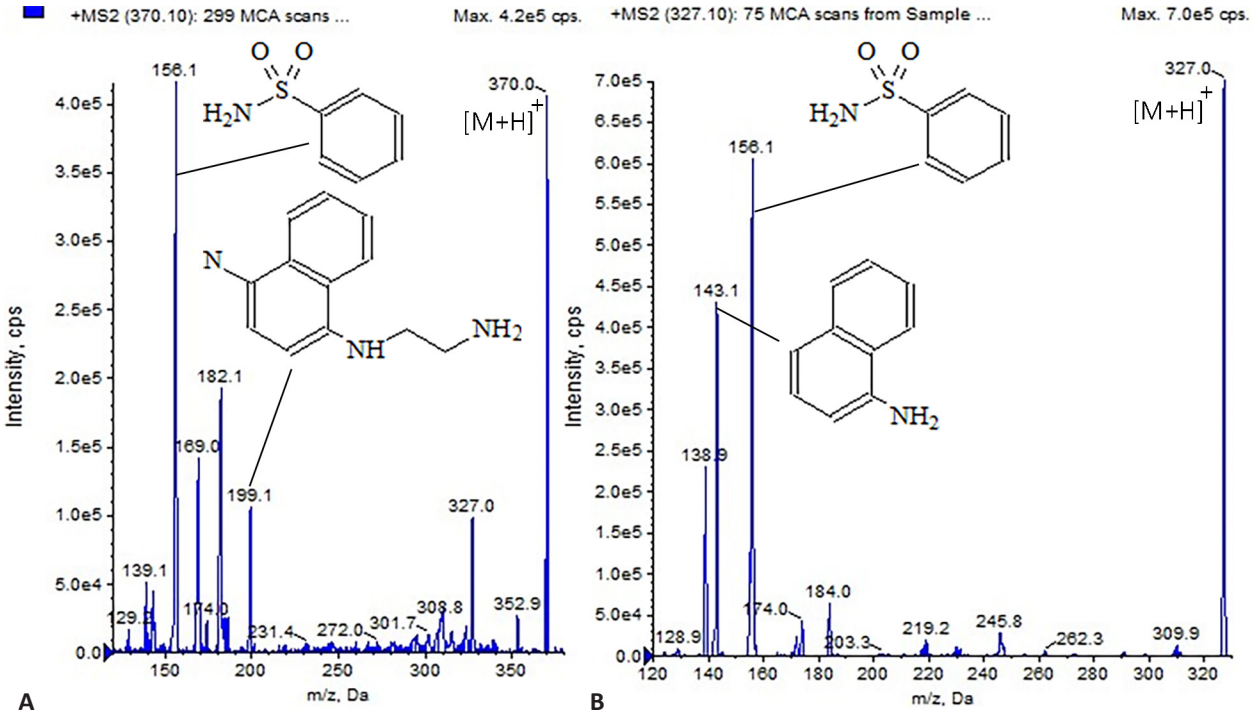

Figure 4 – Fragment ion mass spectra of analyte (A – 4-[4-(2-aminoethylamino)-1-naphthylazo] benzenesulfonamide) and internal standard (B – 4-[4-amino-1-naphthylamino]benzenesulfonamide)

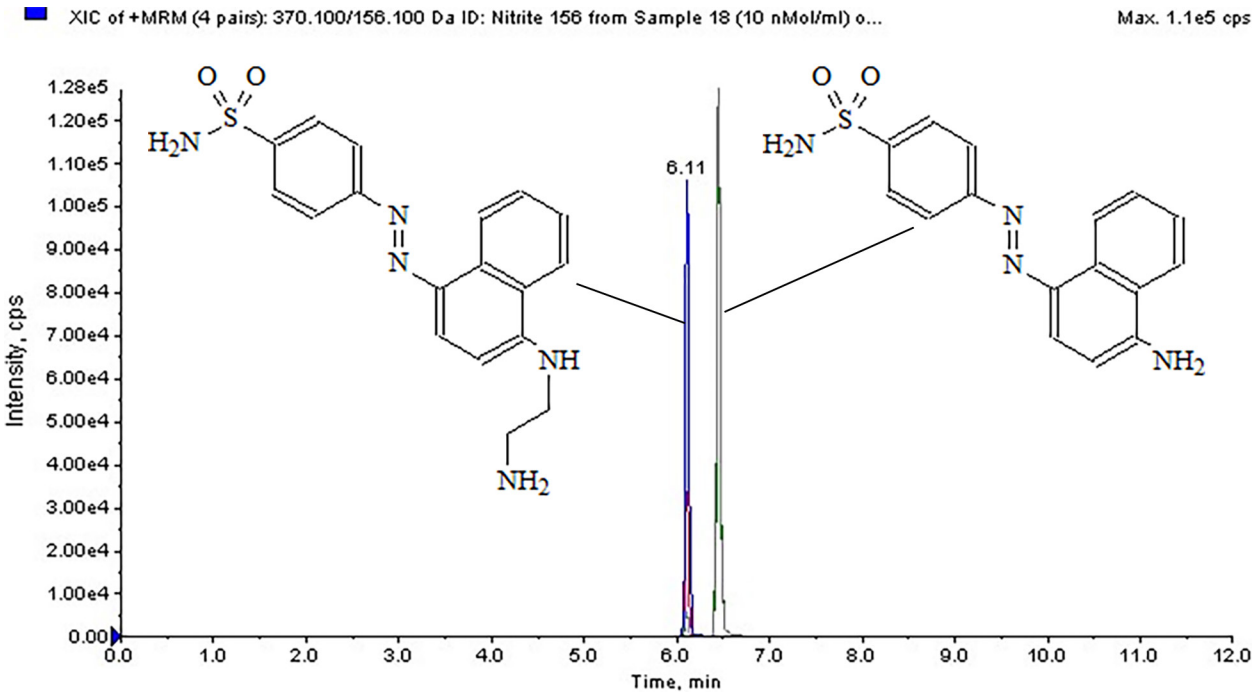

Figure 5 – Chromatogram of standard sample with nitrite concentration of 10.0 nmol/ml

Note: mobile phase – methanol and deionized water with the addition of 0.1% formic acid; elution mode – gradient; chromatographic column – InfinityLab Poroshell 120 EC-C18 4.6×100 mm, 2.7 µm; column temperature – 30°C; aliquot – 5 µl.

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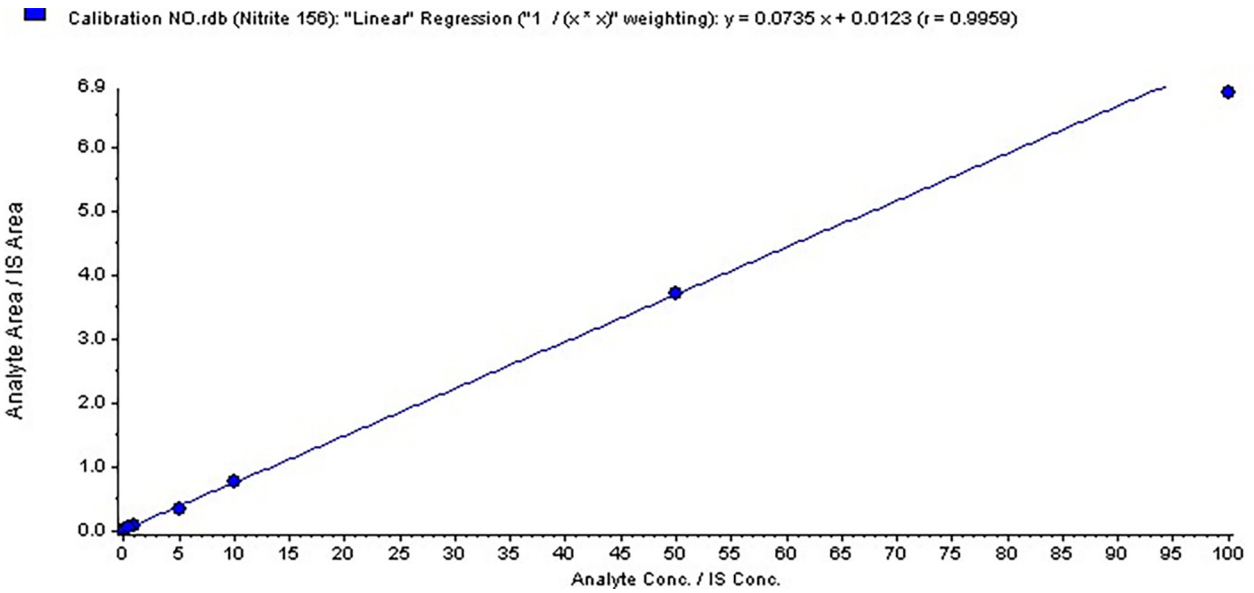

Figure 6 – Calibration curve for quantitative determination of nitrites in rat tissue homogenates
Note: horizontal axis – nitrite concentration in the standard sample, nmol/ml; the vertical axis is the ratio of the area of the chromatographic peak of the analyte to the area of the IS peak).

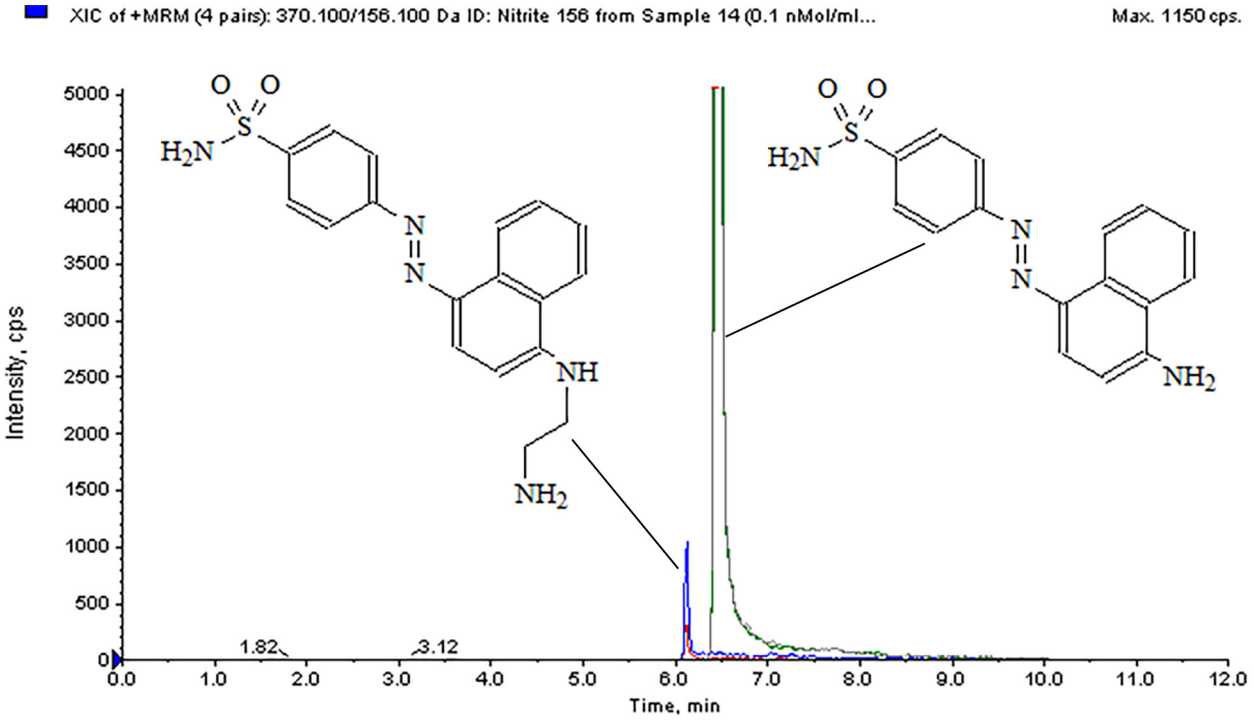

Figure 7 – Fragment of a standard sample chromatogram with a nitrite concentration at the LLOQ of 0.1 nmol/ml
Note: signal-to-noise ratio – 28:1; mobile phase – methanol and deionized water with the addition of 0.1% formic acid; elution mode – gradient; chromatographic column – InfinityLab Poroshell 120 EC-C18 4.6×100 mm, 2.7 µm; column temperature – 30°C; aliquot – 5 µl.

Table 1 – Parameters of mass spectrometric detection of nitrite derivatization product (azo dye) and internal standard

Ion source	Heated Nebulizer				
Ionization method	Atmospheric pressure chemical ionization (APCI)				
Ionization mode	Positive				
Ion source temperature, °C	400.0				
Ion source voltage, V	5500.0				
Gas curtain pressure, *psi*	20.0				
Ion source gas pressure, *psi*	30.0				
Input voltage, V	10				
Dwell time, msec	200				
Product ions	MRM, *m/z*	DP, V	EP, V	CE, eV	CXP, V
Analyte 4-[4-(2-aminoethylamino)-1-naphthylazo]benzenesulfonamide	370.1/156.1 370.1/199.1	67.0	21.0	37.0 28.0	2.5 3.3
Internal standard 4-[4-amino-1-naphthylamino]benzenesulfonamide	327.1/156.1 327.1/143.1	86.0	18.2	32.8 37.0	2.7 2.6

Note: DP – declustering potential; EP – entrance potential to the collision cell; CE – collision energy; CXP – collision cell exit potential.

Table 2 – Chromatographic parameters for detection of nitrite derivatization product

Chromatographic column	Agilent InfinityLab Poroshell 120 EC-C18 4.6×100 mm, 2.7 µm			
Eluent A	Deionized water+0,1% formic acid			
Eluent B	Methanol+0,1% formic acid			
Gradient program	Time, min	Flow rate, µl/min	% A	%B
	0.0	500	90	10
	4.0		0	100
	8.0		0	100
	8.01		90	10
	12.0		90	10
Column thermostat temperature, °C	30			
Sample volume, µl	5			
Total analysis time, min	12			
Injector flushing	Through rinse port, 3 seconds, 50% methanol aqueous solution			

Table 3 – Experiment results of studied homogenates with addition of standards

Nitrite concentration in water, nmol/ml	0.1	0.5	1.0	5.0	10.0	50.0	100.0	Average value, nmol/ml	SD, nmol/ml	CV, %
Blood plasme										
Nitrite concentration with additive, nmol/ml	6.3	6.0	6.8	11.8	16.8	56.4	106.0	–		
Concentration difference, nmol/ml	6.2	5.5	5.8	6.8	6.8	6.4	6.0	6.2	0.5	**7.9**
Calibration curve slope factor	0.0751		Ratio of slope coefficients of calibration curves						**1.014**	
Brain homogenate										
Nitrite concentration with additive, nmol/ml	25.2	24.3	24.9	29.8	32.6	71.3	118.4	–		
Concentration difference, nmol/ml	25.1	23.8	23.9	24.8	22. 6	21.3	18.4	22.8	2.4	**10.3**
Slope of linear regression equation	0.0657		Ratio of slope coefficients of calibration curves						**0.887**	
Heart homogenate										
Nitrite concentration with additive, nmol/ml	20.7	21.3	22.1	26.9	33.1	74.4	125.1	–		
Concentration difference, nmol/ml	20.6	20.8	21.1	21.9	23.1	24.4	25.1	22.4	1.8	**8.0**
Slope of linear regression equation	0.0825		Ratio of slope coefficients of calibration curves						**1.114**	
Aorta homogenate										
Nitrite concentration with additive, nmol/ml	18.6	18.8	19.4	26.4	28.1	69.4	116.2	–		
Concentration difference, nmol/ml	18.5	18.3	18.4	21.4	18.1	19.4	16.2	18.6	1.6	**8.4**
Slope of linear regression equation	0.0711		Ratio of slope coefficients of calibration curves						**0.959**	
Lungs homogenate										
Nitrite concentration with additive, nmol/ml	7.8	7.6	7.8	11.6	16.4	55.3	105.1	–		
Concentration difference, nmol/ml	7.7	7.1	6.8	6.6	6.4	5.3	5.1	6.4	0.9	**14.6**
Slope of linear regression equation	0.0695		Ratio of slope coefficients of calibration curves						**0.938**	

Note: SD – standard deviation; CV – coefficient of variation.

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Table 4 – Results of assessing matrix effect and recovery degree

Values	AUC of analyte *post-spike sample*	AUC IS *post-spike sample*	AUC of analyte/ AUC IS	AUC of analyte *solvent-spike sample*	AUC IS *solvent-spike sample*	AUC of analyte *pre-spike sample*	NMF	Extraction rate, %
Blood plasma (8,0 nmol/ml)								
Average	247 200	397 600	0.62	246 400	391 600	232 600	**0.99**	**94.48**
SD	16 783	8 561	0.04	13 446	5 941	3 781	0.07	7.36
CV, %	6.79	2.15	7.19	5.46	1.52	1.63	7.19	7.79
Brain homogenate (8,0 nmol/ml)								
Average	221 800	372 400	0.60	246 400	391 600	208 600	**0.95**	**94.02**
SD	9 338	8 324	0.03	13 446	5 941	12 915	0.06	3.50
CV, %	4.21	2.24	5.87	5.46	1.52	6.19	5.87	3.72
Heart homogenate (8,0 nmol/ml)								
Average	221 400	375 000	0.59	246 400	391 600	203 200	**0.94**	**91.78**
SD	24 130	18 439	0.06	13 446	5 941	22 286	0.10	1.18
CV, %	10.90	4.92	10.96	5.46	1.52	10.97	10.96	1.29
Aorta homogenate (8,0 nmol/ml)								
Average	229 200	364 400	0.63	246 400	391 600	215 400	**1.00**	**94.11**
SD	13 179	7 893	0.04	13 446	5 941	7 829	0.06	3.76
CV, %	5.75	2.17	6.41	5.46	1.52	3.63	6.41	3.99
Lungs homogenate (8,0 nmol/ml)								
Average	198 600	362 200	0.55	246 400	3 916 00	185 600	**0.87**	**93.48**
SD	10 064	13 311	0.03	13 446	5 941	9 710	0.05	2.58
CV, %	5.07	3.68	6.20	5.46	1.52	5.23	6.20	2.76
Blood plasma (80,0 nmol/ml)								
Average	2 232 000	395 400	5.64	2 268 000	398 800	2 104 000	**0.99**	**94.28**
SD	101 341	6 107	0.19	106 864	10 710	85 029	0.03	0.91
CV, %	4.54	1.54	3.36	4.71	2.69	4.04	3.36	0.96
Brain homogenate (80,0 nmol/ml)								
Average	2 122 000	385 200	5.51	2 268 000	398 800	2 014 000	**0.97**	**94.97**
SD	107 564	13 368	0.31	106 864	10 710	92 897	0.05	3.09
CV, %	5.07	3.47	5.57	4.71	2.69	4.61	5.57	3.25
Heart homogenate (80,0 nmol/ml)								
Average	2 214 000	375 000	2214000	2 268 000	398 800	2 084 000	**1.04**	**93.92**
SD	241 309	18 439	241309	106 864	10 710	290 740	0.11	4.96
CV, %	10.90	4.92	10.90	4.71	2.69	13.95	10.96	5.28
Aorta homogenate (80,0 nmol/ml)								
Average	2 136 000	393 800	5.42	2 268 000	398 800	1 996 000	**0.95**	**93.33**
SD	154 370	16 407	0.32	106 864	10 710	185 687	0.06	2.59
CV, %	7.23	4.17	5.93	4.71	2.69	9.30	5.93	2.78
Lungs homogenate (80,0 nmol/ml)								
Average	1 884 000	387 200	4.87	2 268 000	398 800	1 744 000	**0.86**	**92.47**
SD	59 414	17 922	0.27	106 864	10 710	132 778	0.05	4.16
CV, %	3.15	4.63	5.57	4.71	2.69	7.61	5.57	4.50

Note: AUC – area under the curve of chromatographic peak; IS – internal standard; NMF – normalized matrix factor; SD – standard deviation; CV – coefficient of variation.

Table 5 – Results of assessing methods selectivity

Biomaterial	AUC of analyte peak (average value)		Selectivity, %	AUC of IS peak (average value)		Selectivity, %
	Intact sample	LLOQ Sample		Intact sample	LLOQ Sample	
Blood plasma	204.00	2 105.00	9.72	361.50	374 333,33	0.10
Brain homogenate	188.83	2 333.00	8.19	420.17	372 166,67	0.11
Heart homogenate	188.17	2 308.33	8.32	820.83	365 166,67	0.22
Aorta homogenate	190.33	2 911.67	6.57	381.33	382 666,67	0.10
Lungs homogenate	221.00	2 085.00	10.58	545.33	391 500,00	0.14

Note: AUC – area under the curve of chromatographic peak; IS – internal standard; LLOQ – lower limit of quantitation.

Table 6 – Results of assessing precision and accuracy of the method

Sample	LLOQ 0.1 nmol/ml	LC 8.0 nmol/ml	MC 40.0 nmol/ml	HC 80.0 nmol/ml
Accuracy and precision, batch 1				
Measured concentration, nmol/ml	0.104±0.010	8.67±0.33	38.1±1.0	83.9±2.1
Accuracy, %	104.0	108.4	95.2	104.9
CV, %	9.90	3.84	2.52	2.48
Accuracy and precision, batch 2				
Measured concentration, nmol/ml	0.093±0.009	8.33±0.05	38.1±0.9	82.9±1.5
Accuracy, %	93.4	104.1	95.2	103.7
CV, %	9.41	0.61	2.31	1.84
Accuracy and precision, batch 3				
Measured concentration, nmol/ml	0.092±0.008	8.63±0.29	38.0±1.5	84.6±2.7
Accuracy, %	91.8	104.5	95.1	105.8
CV, %	9.13	3.42	3.84	3.22
Inter-batch accuracy and precision				
Measured concentration, nmol/ml	0.096±0.010	8.45±0.29	38.1±1.0	83.8±2.1
Accuracy, %	96.4	105.7	95.1	104.8
CV, %	10.57	3.38	2.75	2.54

Note: LLOQ – lower limit of quantitation; LC – low concentration; MC – medium concentration; HC – high concentration.

Table 7 – NO content in homogenates in terms of nitrite

Sample	Blood plasma	Brain	Heart	Aorta	Lungs
Content of nitric oxide, nmol/ml	31.8±3.0	122.4±29.5	108.9±14.5	87.9±17.4	35.2±9.4

The results of assessing the post-preparative stability confirmed the safety of the samples ready for a chromatographic analysis within 24 hours (the maximum time for the analysis of all samples in the chromatograph autosampler). The results of studying biological samples after 3 cycles of freezing and thawing showed high values of stability of the metabolic NO products. Thus, the areas of chromatographic peaks of the nitrite derivatization product before and after the procedure did not differ by more than 10%. Storing rat tissue homogenates for 3 months at –40°C did not result in a significant decrease in NO. Thus, the areas of chromatographic peaks of the same samples analyzed 1 day and 3 months after obtaining the biomaterial did not differ by more than 15%.

A 10-fold dilution factor was validated to confirm that the possibility of using this methods could be used to quantify NO metabolites well above the upper limit of quantitation (100 nmol/ml as nitrite). It was found that a 10-fold dilution with water of standard nitrite solutions (1000 and 5000 nmol/ml) prepared on the tissue homogenates does not reduce the precision and accuracy of the analysis.

The developed methods was used to analyze the content of NO metabolites in the tissues of intact Wistar rats (n=6), kept in the vivarium of Tver State Medical University and removed from the experiment in the morning by decapitation. The results of the NO determination are presented in Table 7.

The developed methods can be also used without performing the nitrate reduction stage. In this case, measuring the level of nitrites can play an important role, for example, when studying the mechanisms of cytoprotection under hypoxic conditions using a model of ischemia-reperfusion [24]. In addition, assessing nitrite levels can be useful in studying the replication processes of viruses, for example, SARS-CoV-2 [25]. The use of this methods in the conjunction with determining the level of cyclic guanosine monophosphate [26] in the tissues of the laboratory rats can serve as a useful tool in studying not only physiological and pathological processes occurring with the NO participation [28–31], but also the pharmacodynamics of drug candidates, which is an extremely important stage in the drug development [32–34].

Limitations of the study

According to the authors' data, the following factors may affect the reliability of the analysis results: a decrease in the activity of nitrate reductase and β-nicotinamide adenine dinucleotide, which is a consequence of an improper storage of lyophilisates or ready-made solutions; the presence of sulfonamide drugs in the biomaterial, as well as the intake of nitrates

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and nitrites into the body of laboratory animals in case of non-compliance with feeding requirements.

CONCLUSION

The developed chromatography-mass spectrometric methods for the NO determination in the rat tissues fully met the validation requirements. Compared to the common colorimetric method for assessing the NO production, this method is characterized by a high sensitivity and selectivity, as well as a low influence of interfering components of the biological matrix. The application of this methods makes it possible to accurately estimate the total content of NO metabolic products in the blood plasma, the tissues of the brain, heart, aorta and lungs of rats, which is in demand in the study of pathological processes, as well as the pharmacodynamics and effectiveness of pharmacological agents affecting the NO metabolism.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

All the authors made equivalent and equal contributions to the preparation of the publication. All the authors confirm that their authorship meets the international ICMJE criteria (all the authors have made significant contributions to the development of the concept, conduct of the study and preparation of the article, read and approved the final version before the publication. Nikita S. Popov – development, validation and testing of bioanalytical methods, preparation of the manuscript; Dmitry A. Gavrilenko – analysis of literary sources, conducting the biological part of the study, analysis of the results, preparation of the manuscript; Mikhail S. Baranov – development of the research concept, manuscript preparation; Vadim Yu. Balabanyan – purpose setting, study design development, manuscript preparation.

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