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

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

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

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

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

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

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

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ОРИГИНАЛЬНАЯ СТАТЬЯ

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

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

INTRODUCTION

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

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

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

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

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

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

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

MATERIALS AND METHODS

Bioanalysis

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

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

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

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

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

Design of animal experiment

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

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2023-11-3-240-254

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

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

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

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

Synthesis of OXSA metabolites

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

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

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

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

Statistical processing

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

[2] Ibid.

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

[4] Russian State Pharmacopoeia. XV edition. GPhM.1.2.1.1.0008 Mass-spectrometry. Available from: https://pharmacopoeia.regmed.ru/pharmacopoeia/izdanie-15/1/1-2/1-2-1/1-2-1-1-metody-spektralnogo-analiza/mass-spektrometriya/. Russian

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

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

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

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

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

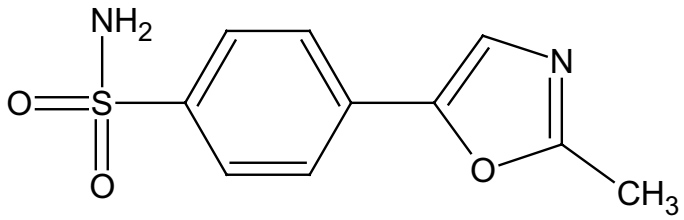

Figure 1 – Structure of 4-(2-methyl-1,3-oxazol-5-yl)-benzenesulfonamide

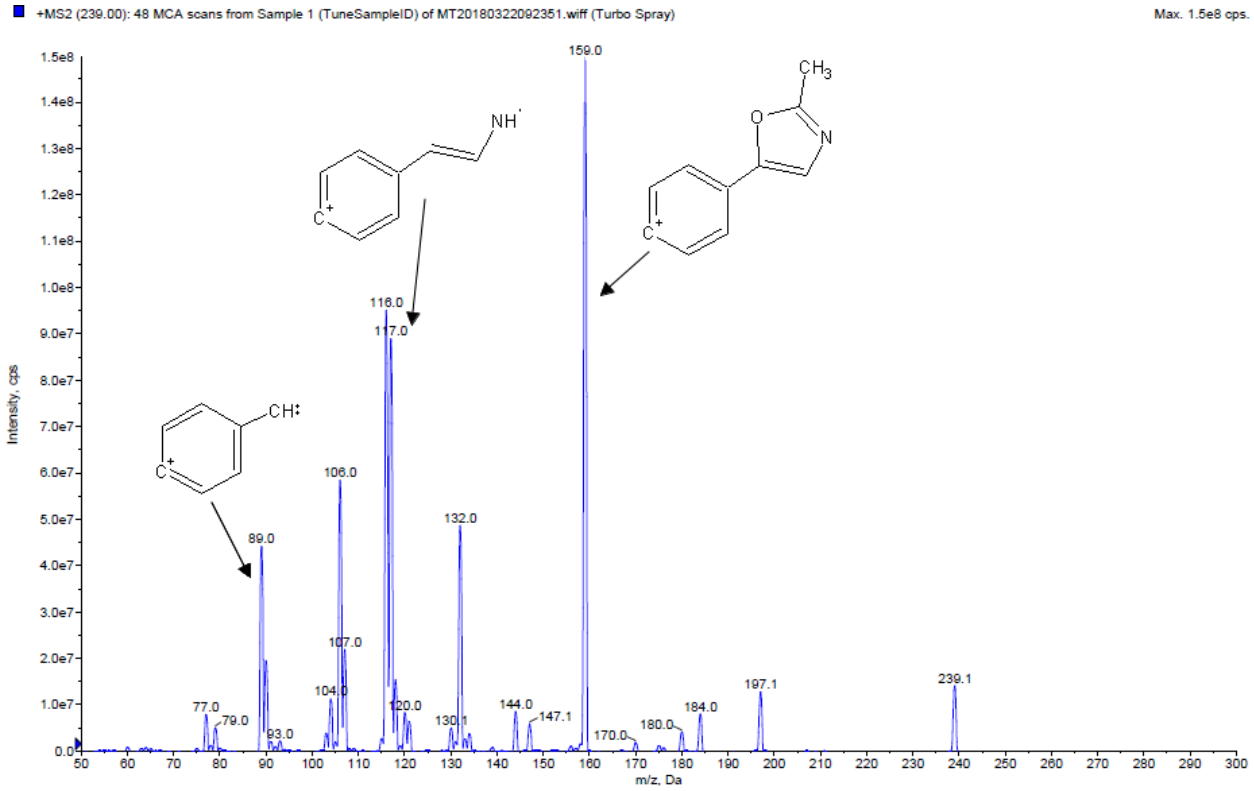

Figure 2 – Mass-spectrum of OXSA (MS2-mode; positive polarity; CE=40eV)

ОРИГИНАЛЬНАЯ СТАТЬЯ
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Table 3 – MRM-transitions for identification of possible metabolites

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

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

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

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

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

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

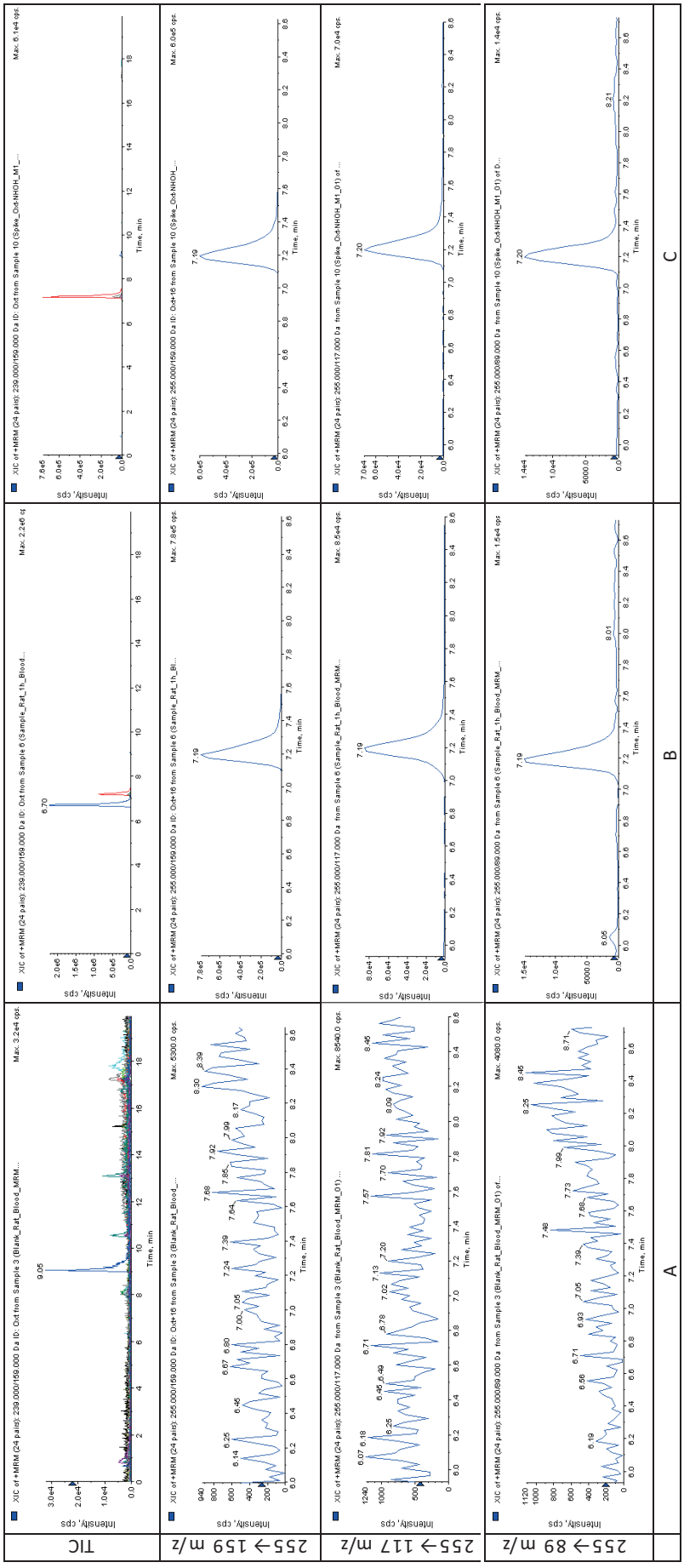


Figure 3 – Examples of blank sample chromatograms (A – rat's blood), rat's sample (B – rat's blood at 1 h point), blood sample spiked by N-hydroxy-4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonamide (C – analyte spiked blood sample of rat at concentration of 500 ng/ml)
Note: TIC – chromatograms of all MRM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

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2023-11-3-240-254

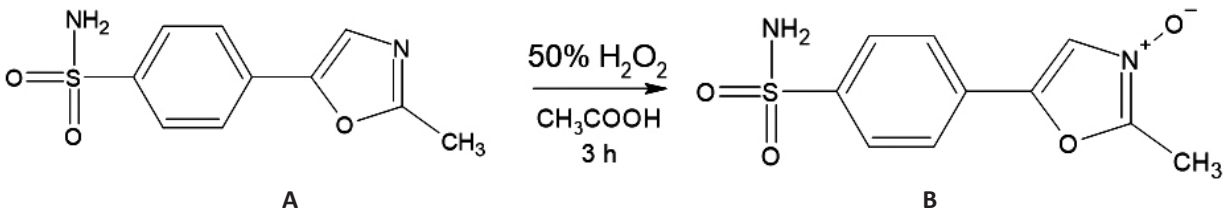
Table 5 – MRM- transitions for identification of possible conjugates of hydroxylated metabolite

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

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

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

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



A B

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

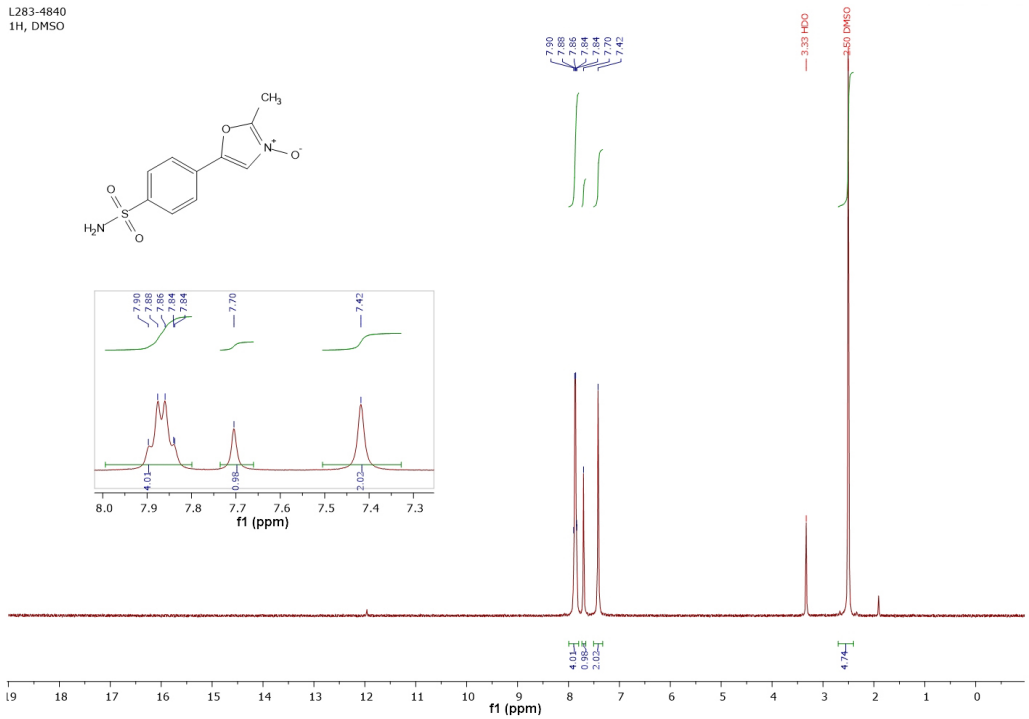


Figure 5 – ^{1}H-NMR-spectrum of N-oxide of 4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonamide in DMSO-D$_6$

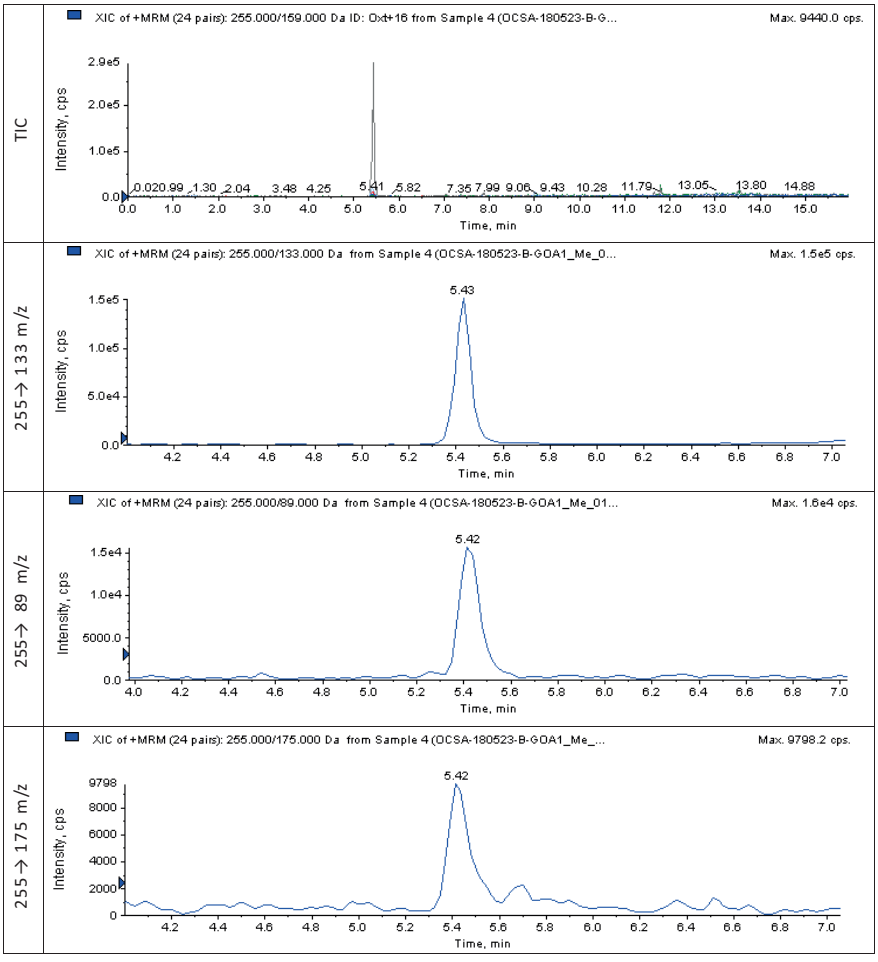
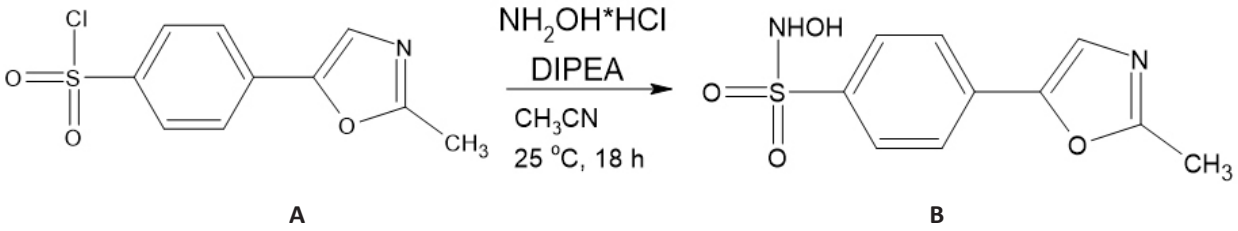


Figure 6 – Example of MRM-chromatogram of N-oxide OXSA in spiked blood sample in concentration of 500 ng/ml

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

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2023-11-3-240-254



A B

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

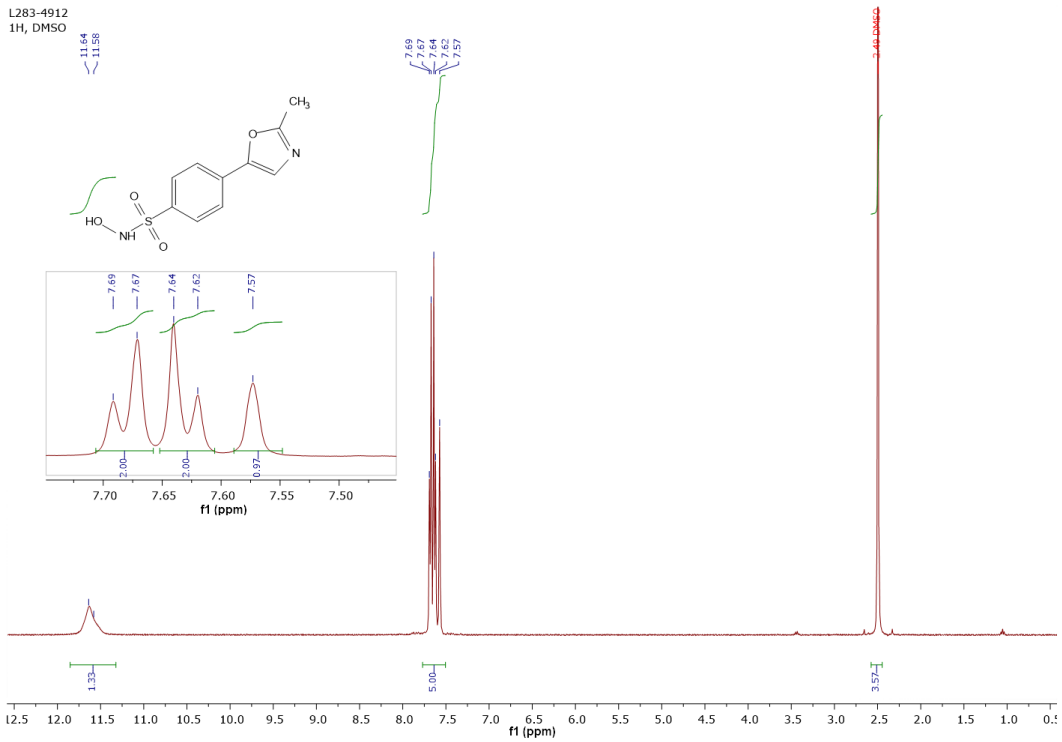


Figure 8 – ^{1}H-NMR-spectrum of N-hydroxy-4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonamide in DMSO-D_6

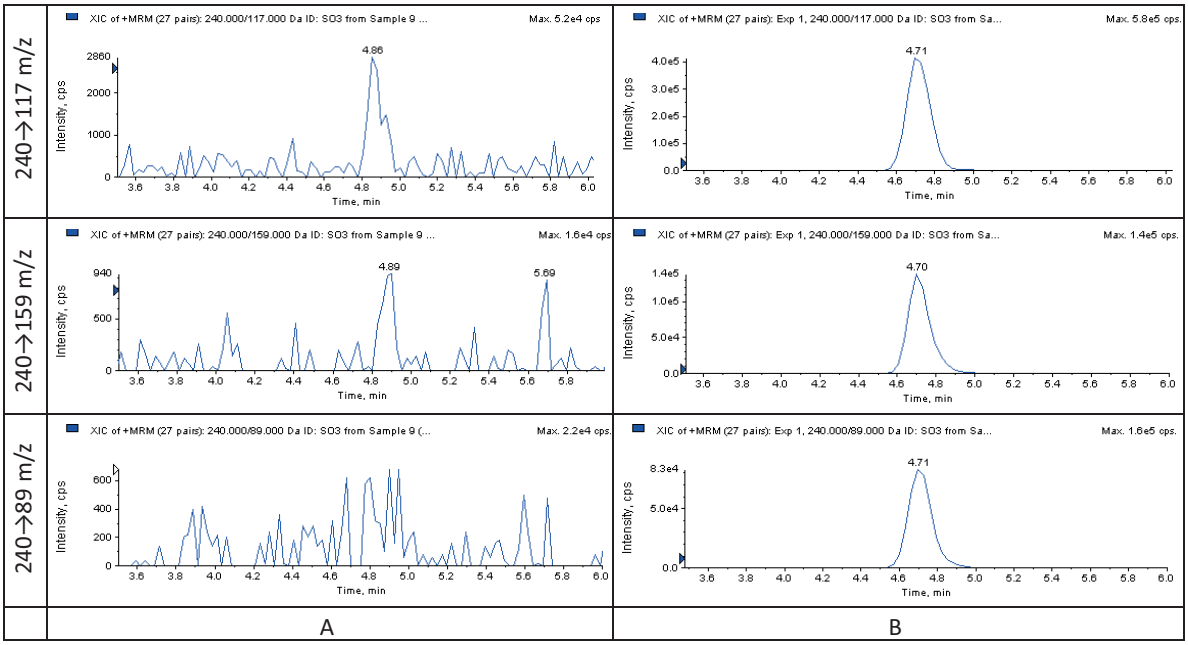


Figure 9 – MRM-chromatogram examples of plasma sample spiked by metabolite after preparation (A) and spiked metabolite sample after 48 h of storage at room temperature (B)

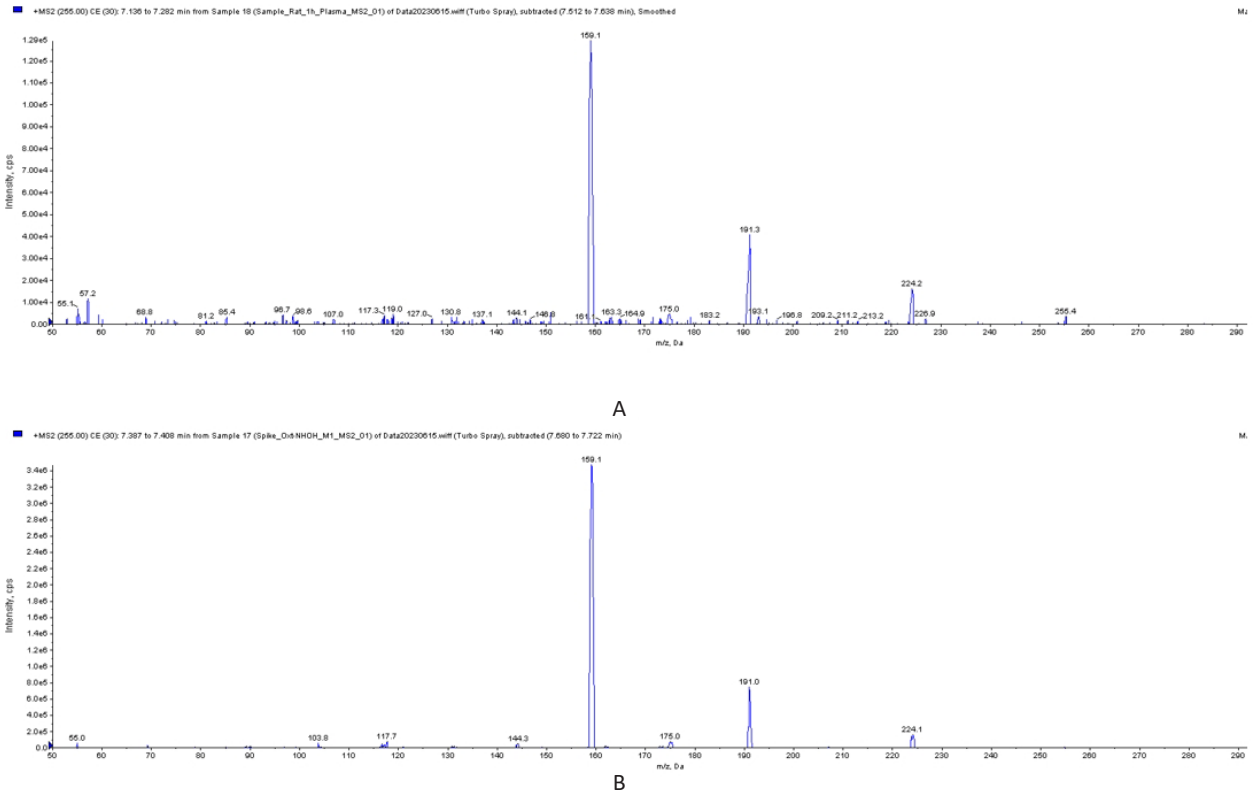


Figure 10 – Mass-spectra examples of N-hydroxy-4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonamide in rat plasma sample (1 h point) (A) and in spiked plasma sample at analyte concentration of 500 ng/ml (B)

RESULTS AND DISCUSSION

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

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

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

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

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

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

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2023-11-3-240-254

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

CONCLUSION

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

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

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

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