Research Results in Pharmacology

Research Results in Pharmacology 10(4): 15–27
UDC: 615.015; 543.51+543.54
DOI 10.18413/rrpharmacology.10.498

Research Article

Identification and synthesis of metabolites of 4-(5-methyl-1,3,4-oxadiazole-2-yl)-benzenesulfonamide

Alexander L. Khokhlov[1], Ilya I. Yaichkov[1,2], Anton A. Shetnev[2], Valeria A. Panova[2], Yulia A. Efimova[2,3], Sergey A. Ivanovskiy[2], Mikhail K. Korsakov[2], Nikita N. Vol'khin[2], Sergey S. Petukhov[1,2]

1 Yaroslavl State Medical University, 5 Revolutsionnaya St., Yaroslavl 150000 Russia
2 Yaroslavl State Pedagogical University named after K.D. Ushinsky, 108/1 Respublikanskaya St., Yaroslavl 150000 Russia
3 Kosygin State University of Russia, 33 Sadovnicheskaya St, Bldg 1, Moscow 115035 Russia

Corresponding author: : Ilya I. Yaichkov (i.yaichkov@yspu.org)

Academic editor: Tatyana Avtina ♦ **Received** 16 July 2024 ♦ **Accepted** 31 October 2024 ♦ **Published** 21 November 2024

Citation: Khokhlov AL, Yaichkov II, Shetnev AA, Panova VA, Efimova YA, Ivanovskiy SA, Korsakov MK, Vol'khin NN, Petukhov SS (2024) Identification and synthesis of metabolites of 4-(5-methyl-1,3,4-oxadiazole-2-yl)-benzenesulfonamide. Research Results in Pharmacology 10(4): 15–27. https://doi.org/10.18413/rrpharmacology.10.498

Abstract

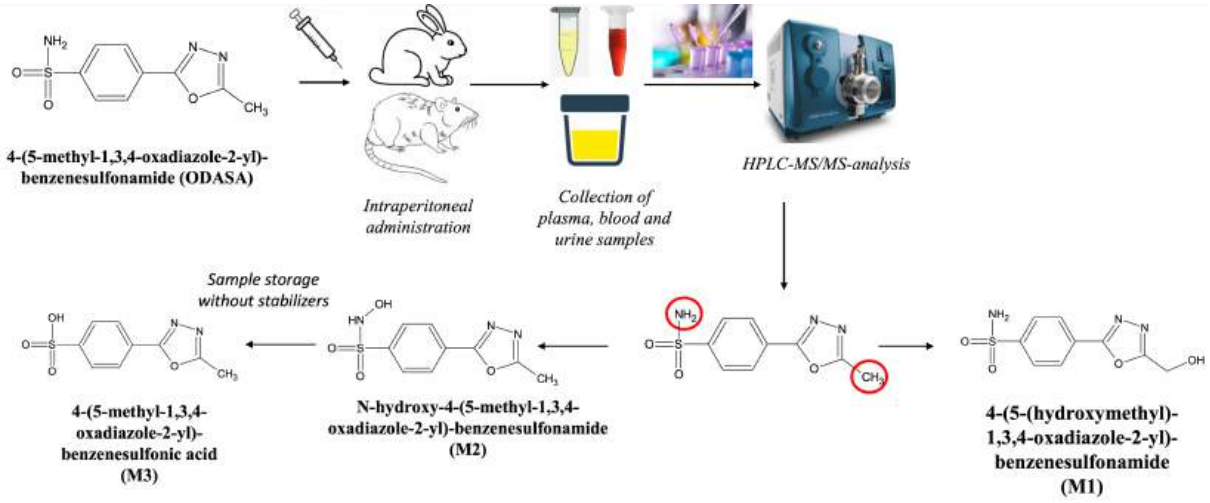
Introduction: 4-(5-methyl-1,3,4-oxadiazole-2-yl)-benzenesulfonamide is a new selective type II carbonic anhydrase inhibitor with local action through instillation into the eyes. For a complete pharmacokinetic study of this drug, it is necessary to detect and synthesize its metabolites for their systemic exposure evaluation.

Materials and Methods: The investigation was performed on 6 Wistar rats and 6 Soviet Chinchilla rabbits. The drug in the form of a 1% suspension was administered by intraperitoneal injection. Blood was sampled in a volume of 0.2 mL at the following time points: before administration and 1 h, 2 h, 4 h, 24 h after administration. Then 150 µL of each sample was centrifuged to produce plasma. Urine was simultaneously sampled in rats using metabolic cells: before administration and at intervals of 0-2 h, 2-4 h, 4-6 h, 6-24 h after administration of the drug. The identification of metabolites in these objects was performed using HPLC-MS/MS. Then the detected biotransformation products were synthesized. The structure of the obtained substances was confirmed by NMR spectroscopy and high-resolution mass spectrometry. At the final stage, animal biological fluids and model samples with the addition of the synthesized compounds were analyzed using HPLC-MS/MS to establish the structure of metabolites.

Results and Discussion: N-hydroxy-4-(5-methyl-1,3,4-oxadiazole-2-yl)-benzenesulfonamide, 4-(5-(hydroxymethyl)-1,3,4-oxadiazole-2-yl)-benzenesulfonamide and 4-(5-methyl-1,3,4-oxadiazole-2-yl)-benzenesulfonic acid were identified and synthesized. The complete coincidence of the structure of metabolites and the synthesized substances was established as a result of their comparison in retention time, the ratio of the areas of chromatographic peaks at the main MRM transitions, as well as mass spectra.

Conclusion: N-hydroxy-4-(5-methyl-1,3,4-oxadiazole-2-yl)-benzenesulfonamide and 4-(5-(hydroxymethyl)-1,3,4-oxadiazole-2-yl)-benzenesulfonamide are products of biotransformation of the studied drug. It was found that 4-(5-methyl-1,3,4-oxadiazole-2-yl)-benzenesulfonic acid is formed by decomposition of N-hydroxymetabolite in urine samples during the collection process.

Graphical abstract



Keywords

biotransformation, selective carbonic anhydrase II inhibitor, HPLC-MS/MS, N-hydroxysulfonamide

Introduction

Glaucoma can lead to decreasing or complete loss of vision. The number of cases of primary disability from this disease is about 0.35 per 1000 population. At the same time, 81% of disabled people had primary open-angle glaucoma (POAG) (Simakova et al. 2023). Selective carbonic anhydrase inhibitors of type II (CA_{II}) belong to the groups of drugs for the conservative treatment of POAG. The therapeutic effect is achieved by a decrease in the secretion of intraocular fluid into the anterior chamber of the eye (Kurysheva 2020). 4-(5-methyl-1,3,4-oxadiazole-2-yl)-benzenesulfonamide (ODASA) (Fig. 1) is one of the new highly active molecules capable of suppressing the activity of CA_{II} (Khokhlov et al. 2023). This compound is at the stage of preclinical trials.

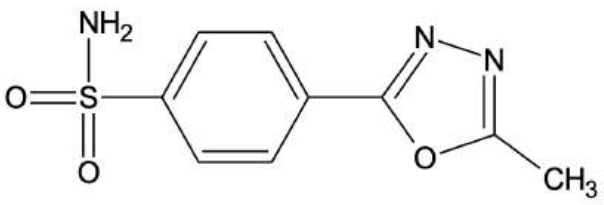


Figure 1. The structure of 4-(5-methyl-1,3,4-oxadiazole-2-yl)-benzenesulfonamide.

Before the pharmacokinetic study, it is necessary to establish the metabolite profile of ODASA, which was previously unknown. Synthesis of biotransformation product substances is also required for further quantitative determination of this compound in biological objects. The selective CA_{II} inhibitors dorzolamide and brinzolamide, which are widely used in POAG therapy, undergo N- and O-dealkylation in hepatocytes (Lo Faro et al. 2021; Dhandar et al. 2022). N-hydroxysulfonamides formation is characteristic for 4-(5-methyl-1,3-oxazole-2-yl)benzenesulfonamide (OXSA) (Khokhlov AL et al. 2023) and 5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide (TFISA) (Khokhlov et al. 2024), which are structurally similar to ODASA. These metabolites are difficult to detect in biological media due to their high reactivity and rapid decomposition. Therefore, additional measures to the sample stabilization after collection are necessary for the study. The sulfonamide group TFISA is also exposed to acetylation (Khokhlov et al. 2024).

The determination of metabolites in samples of animal biological fluids after administration of the drug is the most accessible and easiest way for study biotransformation. At the same time, it does not require biochemical reactions using microsomes (Trawinski et al. 2022; Alsibaee et al. 2023), S9 fraction (Peeters et al. 2020), hepatocyte cell cultures (Boyce et al. 2023; Chen et al. 2023), various oxidation coenzymes and coenzymes of the second phase (Peeters et al. 2020; Trawinski et al. 2022; Alsibaee et al. 2023). HPLC-MS/MS is most commonly used to identify derivatives of the investigated substances (Reddy et al. 2021).

The aim of the study is to identify the metabolites of 4-(5-methyl-1,3,4-oxadiazole-2-yl)-benzenesulfonamide in plasma, whole blood and urine and their synthesis.

Materials and Methods

Analytical equipment and reagents

The study was carried out using HPLC-MS/MS method. The tandem mass spectrometer detector AB Sciex QTRAP5500 (AB Sciex, Singapore) with chromatograph Agilent 1260 Infinity (Agilent Technologies, Germany).

The following special analytical software was applied:

- Analyst 1.6.2 (AB Sciex, USA) – for HPLC-MS/MS system control;
- MultiQuant 3.0.5 (AB Sciex, USA) – for MRM-chromatograms integration;
- LightSight 2.3 (AB Sciex, USA) – for creation of MRM-transitions of putative metabolites.

Methanol (Lichrosolv Hypergrade for LC-MS, Merck KGaA, Germany), formic acid (Optima LC-MS-Grade, Thermo Fisher Scientific, USA), aqueous ammonia (especially pure, Base №1 of Chemical Reagents JSC, Russian Federation) were used for mobile phase preparation. Methanol (especially pure, Chimmed, Russian Federation), dimethylsulfoxide (chemical pure, Ekos-1 JSC, Russian Federation), and ascorbic acid (chemical pure, Lenreactiv, Russian Federation) were applied for sample preparation.

Animals

The study was carried out on 6 rat and 6 rabbits from SMK Stezar LLC (Russian Federation):

- Rats: Wistar line, 238±19 g (M±SD), 3 males and 3 females;
- Rabbits: Soviet Chinchilla, 2.47± 0.22 kg (M±SD), 3 males and 3 females.
- Rats were catheterized into the right jugular vein. Rabbit sampling was performed from the ear vein without catheterization.

The study was approved by the Ethics Committee of Yaroslavl State Pedagogical University named after K.D. Ushinsky (Minutes №2 of 06 March 2024).

Drug dosing and sample collection

The drug substance was administered at a dosage of 10 mg/kg to rats and at a dosage of 0.8 mg/kg to rabbits using intraperitoneal injection. The sampling was performed at following time points:

- Blood sampling: before administration (0 h) and 1 h, 2 h, 4 h, 24 h after administration;
- Urine sampling (only for rats): before administration (0 h) and at intervals 0 h – 2 h, 2 h – 4 h, 4 h – 6 h, 6 h – 24 h after the administration.

Blood was collected in a volume of 200 µL into test tubes with mixture of sodium fluoride and potassium oxalate. Every sample was divided into 2 parts: 50 µL – for metabolite identification in whole blood; 150 µL – for obtaining plasma by centrifugation at 2500 rpm for 10 min. The 5% ascorbic acid aqueous solution was added to plasma at volume ratio of 1:2. Rat urine was also stabilized by this solution (1:5, v/v) after collection using a metabolic cage.

The study consisted of 2 stages. The animals were divided into 2 equal groups of 3 rats and rabbits. The aim of the initial part was identification of all drug metabolites. The next experiment was carried out for comparing analytical signals of the detected and synthesized compounds and confirming coincidence of their structure. Necessity of this stage was based on instability of N-hydroxy-4-(5-methyl-1,3,4-oxadiazol-2-yl)-benzenesulfonamide in biological fluids during storage.

Sample preparation and analysis

Methanol was added to biological fluid samples in volume ratio of 1:5. Then, mixture was shaken using vortex and centrifugated at 10000 rpm for 5 min. The supernatant was injected to HPLC-MS/MS-system in a volume of 5 µL. Solutions of ODASA metabolites in dimethylsulfoxide were added to blank matrix in a volume ratio of 1:19 for confirmation and stability tests.

Chromatographic separation was performed in gradient mode using Zorbax Eclipse Plus C18 column (150*3.0 mm, 3.5 µm) with similar precolumn (12.5*2.1 mm, 5.0 µm) (Table 1). Mobile phase consisted of 25mM ammonium formate aqueous solution and methanol. Flow rate was 0.5 mL/min.

Table 1. The parameters of gradient elution of the identification method

Mobile phase: 25 mM ammonium formate aqueous solution – A, methanol – B		
Time, min.	**A, %**	**B, %**
0.00	90	10
0.50	90	10
15.00	10	90
20.00	10	90
20.10	90	10
25.00	90	10

Mass spectrometric detection was performed using electrospray ionization (ESI) (negative polarity). The ion source temperature was 650 °C, ESI voltage was –4500V.

A system suitability test was carried out before each analytical batch: blood, plasma or urine sample spiked by ODASA. The concentration of the studied substance was 1 ng/mL. The signal-to-noise ratio of ODASA chromatographic peaks at main MRM-transitions 238→117 m/z, 238→131 m/z, 238→181 m/z was at least 10:1.

Design of synthesis experiments

The original product for synthesis of ODASA and its metabolites was 4-sulfamoyl benzoic acid hydrazide (Dayang Chem (Hangzhou) Co., Ltd, China). Reagents 2-chloroacetyl chloride (98%, Acros Organics, USA) and diisopropylethylamine (≥98%, Sigma-Aldrich, Germany) were obtained from foreign manufactures. Reagents N,N-dimethylacetamide (chemical pure, Ekos-1,), acetonitrile (chemical pure, Vecton), calcium chloride anhydrous (pure, Vecton), ethyl acetate (chemical pure, Vecton), hydrochloric acid (chemical pure, Vecton), hydroxylamine hydrochloride (≥99%, Vecton), methanol (chemical pure, Vecton), methylene chloride (chemical pure, Vecton), phosphorus oxychloride (Grade A, Ekotec), potassium acetate anhydrous (pure for analysis, Vecton), potassium carbonate (pure for analysis, Vecton),

sodium sulfate anhydrous (chemical pure, Vecton) were produced in the Russian Federation. Synthesis of ODASA and its metabolites was carried out at M.V. Dorogov Pharmaceutical Technology Transfer Center of Yaroslavl State Pedagogical University (YSPU) named after K.D. Ushinsky using the following methods:

- *Method of synthesis of 4-(2-acetylhydrazine-1-carbonyl)-benzenesulfonamide:* 1 mL of N,N-dimethylacetamide, 0.5 g of the initial 4-sulfamoyl benzoic acid hydrazide and 0.42 g of potassium carbonate are loaded into the flask. The reaction mass was mixed for 5 min at room temperature: suspension was observed without coloration. Then the flask was placed in an ice bath and cooled for 5 min. The calculated amount of acetyl chloride was added drop by drop for 3-5 min, and the flask was immediately closed with a tube with calcium chloride. The reaction mass was mixed at room temperature for 5 h. Next, the solvent was evaporated at 80° C, and 15 mL of purified water was added to the sticky viscous residue. The reaction mass was acidified by hydrochloric acid to neutralize potassium carbonate. The resulting precipitate was filtered out and washed with 0.5-1 mL of water. The resulting white powder is dried in air at room temperature. Yield of 4-(2-acetylhydrazine-1-carbonyl)-benzenesulfonamide was 68%.
- *Method of synthesis of 4-(5-methyl-1,3,4-oxadiazol-2-yl)benzenesulfonyl chloride:* 10 equivalents of phosphorus oxychloride were added to the flask with 4-(2-acetylhydrazine-1-carbonyl)-benzenesulfonamide and the reaction mass was heated at 105-110° C for 12 h. The excess of phosphorus oxychloride was distilled under vacuum after the end of the reaction, and the oily residue was poured onto ice. The obtained suspension was mixed for 2 h. The precipitate was filtered and washed with cold water. Yield of the light yellow powder was 50-53%.
- *Method of synthesis of 4-(5-methyl-1,3,4-oxadiazol-2-yl)-benzenesulfonamide (ODASA):* 2 equivalents of phosphorus oxychloride were added in a flask to 4-(2-acetylhydrazine-1-carbonyl)-benzenesulfonamide and the reaction mass was heated at 80° C for 4-5 h. The excess of phosphorus oxychloride was distilled under vacuum after the end of the reaction, and the oily residue was poured onto ice. The obtained suspension was neutralized by ammonia and stirred for 2 h. The precipitate was filtered and washed with cold water. Yield of white-beige powder of ODASA was 66%.
- *Method of synthesis of N-hydroxy-4-(5-methyl-1,3,4-oxadiazol-2-yl)-benzenesulfonamide (M2):* 1.5 equivalents of hydroxylamine hydrochloride were added to acetonitrile solution of 4-(5-methyl-1,3,4-oxadiazol-2-yl)-benzenesulfonyl chloride and 3 equivalents of diisopropylethylamine were added drop by drop during cooling. The reaction mass was stirred for 3 h and diluted with water. The obtained precipitate was filtered and washed with ice water. Yield of white-beige powder of M2 was 35%.
- *Method of synthesis of 4-(5-methyl-1,3,4-oxadiazole-2-yl)-benzenesulfonic acid (M3):* 4-(5-methyl-1,3,4-oxadiazol-2-yl)-benzenesulfonyl chloride was heated by boiling in water for about 5 h until the suspension dissolved and then the hot mixture was filtered. Next, the reaction mass was cooled and filtered and the sediment of sulfonic acid M3 was precipitated. Yield of white powder was 33%.
- *Method of synthesis of 4-(2-(2-chloroacetyl)hydrazine-1-carbonyl)-benzenesulfonamide:* The initial 4-sulfamoyl benzoic acid hydrazide was suspended in acetonitrile (10 mL per 1 g) and 2 equivalents of anhydrous potassium carbonate were added. Next, 2-chloroacetyl chloride (1.05 equivalents) was added to the reaction mass at room temperature. The mixture was stirred for 16 h. The solvent was distilled in vacuum. The residue was suspended in water, filtered, rinsed with water and dried in air for 12 h. Yield of white powder was 80%.
- *Method of synthesis of 4-(5-(chloromethyl)-1,3,4-oxadiazol-2-yl)-benzenesulfonamide:* 6 equivalents of phosphorus oxychloride was added to 4-(2-(2-chloroacetyl)hydrazine-1-carbonyl)-benzenesulfonamide in a flask and the reaction mass was heated at 80° C for 12 h. The excess of phosphorus oxychloride was distilled under vacuum after the end of the reaction, and the oily residue was poured onto ice. The resulting suspension was mixed for 2 h. The precipitate was filtered and washed with cold water. Yield of beige powder was 60%.
- *Method of synthesis of 5-(4-sulfamoylphenyl)-1,3,4-oxadiazol-2-yl)methyl acetate:* 4-(5-(chloromethyl)-1,3,4-oxadiazol-2-yl)-benzenesulfonamide was suspended in acetonitrile and 1.9 equivalents of anhydrous potassium acetate were added. The reaction mass was stirred at 60° C for 4 h; acetonitrile was evaporated in vacuum after the end of the reaction. The product was isolated by flash chromatography using methylene chloride. Yield of beige powder was 40%.
- *Method of synthesis of 4-(5-(hydroxymethyl)-1,3,4-oxadiazol-2-yl)-benzenesulfonamide (M1):* 250 mg of 5-(4-sulfamoylphenyl)-1,3,4-oxadiazol-2-yl)-methyl acetate was dissolved in 10 mL of methanol in a flask; 2 equivalents of sodium carbonate and 2 mL of water were added. The reaction mass was stirred at room temperature for 12 h. The solvent was evaporated and residue was dissolved by a fresh portion of water. Insoluble impurities were filtered out, and the aqueous filtrate was acidified by hydrochloric acid. Then extraction by ethyl acetate was performed. The organic layer was washed with brine, dried over sodium sulfate and evaporated under reduced pressure to afford the title compound as an off-white solid. Yield of light beige powder was 60%.

Confirmation of the structure of synthesized intermediates and metabolite substances was performed using ^{1}H-NMR- and ^{13}C-NMR-spectroscopy (Varian UNITY Plus – 400 (400 MHz), Varian LLC, USA) and high resolution mass spectrometry (micrOTOF-II, Bruker Daltonics GmbH, USA). Melting and decomposition temperature was additionally measured (Buchi M-560 melting point instrument, Büchi Labortechnik AG, Switzerland).

Statistical analysis and criteria of comparison of analytical signals of metabolites and synthesized substances

The comparison of analytical signals of metabolites and their synthesized substances was performed on retention times of chromatographic peaks (t_R), ratio of chromatographic peak areas at the main MRM-transitions, MS2-mass spectra (Khokhlov et al. 2023; Khokhlov et al. 2024; GPA.1.2.1.2.0001.15 The

Chromatography; GPA. 1.2.1.1.0008.15 The mass-spectrometry; The State Pharmacopoeia of Russian Federation. XV edition 2023).

The confirming calculation was carried out using formula 1:

$$\%\,of\ match = \frac{Mean\ of\ parameter\ of\ test\ animal\ sample}{Mean\ of\ parameter\ of\ spiked\ standard\ sample} \times 100\,\% \quad (1)$$

Note: Parameter – retention times of chromatographic peaks; ratio of chromatographic peak areas at the main MRM-transitions.

N-hydroxy-4-(5-methyl-1,3,4-oxadiazol-2-yl)-benzenesulfonamide stability test was carried out by comparison of mean value of chromatographic peak area of M2 after 24 h storage of urine sample at room temperature (Mean S_{24h}) and mean value of chromatographic peak area of this analyte in fresh urine samples (Mean $S_{fresh\ sample}$), formula 2:

$$\%\,of\ initial\ concentration = \frac{Mean\ S24_h}{Mean\ Sfresh} \times 100\,\% \quad (2)$$

Microsoft Excel 2016 (Microsoft Corporation, USA) was used for statistical calculations. Mean values (M) and standard deviation as dispersion measure (SD) are shown in tables.

Results and Discussion

The best sensitivity of 4-(5-methyl-1,3,4-oxadiazole-2-yl)-benzenesulfonamide detection was achieved in negative mode. Three main daughter ions 117, 131 and 181 m/z were selected for creating an MRM-screening method (Fig. 2). The m/z change in case of presence of modification was added to these ions, as well as to ODASA molecular ion 238 m/z. Hydroxylation, N-oxidation, methylation, acetylation, sulfonation, glucuronic conjugation was prognosed (Table 2). The formation of sulfonic acid due to potential decomposition of N-hydroxysulfonamide had also been checked (Khokhlov et al. 2023; Khokhlov et al. 2024).

Two metabolites were found in plasma and blood samples with changing m/z for 16 Da compared to ODASA molecule. The first metabolite with chromatographic peaks at MRM- transitions 254→117 m/z, 254→131 m/z and 254→181 m/z (t_R=6.7 min) had no increase in m/z of daughter ions (Table 3, Fig. 3). Most probably, this compound was formed by C-hydroxylation of the methyl group, since it was absent in the selected fragments of 117 m/z, 131 m/z, 181 m/z. The second metabolite, which was found at MRM- transitions 254→133 m/z, 254→147 m/z at 8.7 min, is N-hydroxysulfonamide (Table 3). A sulfonic acid derivative was also identified in rat urine samples, which could have been formed as a result of decomposition of N-hydroxy-4-(5-methyl-1,3,4-oxadiazol-2-yl)benzenesulfonamide (Khokhlov et al. 2023; Khokhlov et al. 2024) (Fig. 4).

An additional MRM-method was used to check the hypothesis of conjugation of hydroxylated metabolites, as well as 4-(5-methyl-1,3,4-oxadiazole-2-yl)-benzenesulfonic acid. The values of m/z after acetylation (+42 m/z), methylation (+14 m/z), conjugation with glucuronic (+176 m/z) and sulfuric (+80 m/z) acids were added to molecular ions 254 m/z and 239 m/z, as well as fragment ions 117 m/z, 131 m/z,181 m/z,133 m/z,147 m/z, 197 m/z, 159 m/z, 175 m/z (hydroxylated metabolites), 117 m/z, 131 m/z, 181 m/z, 118 m/z, 132 m/z, 182 m/z (sulfonic acid derivative) for creating the method. MRM-transitions with unchanged daughter ions were also made. New metabolites were not found as a result of the repeated analysis of samples of the first stage.

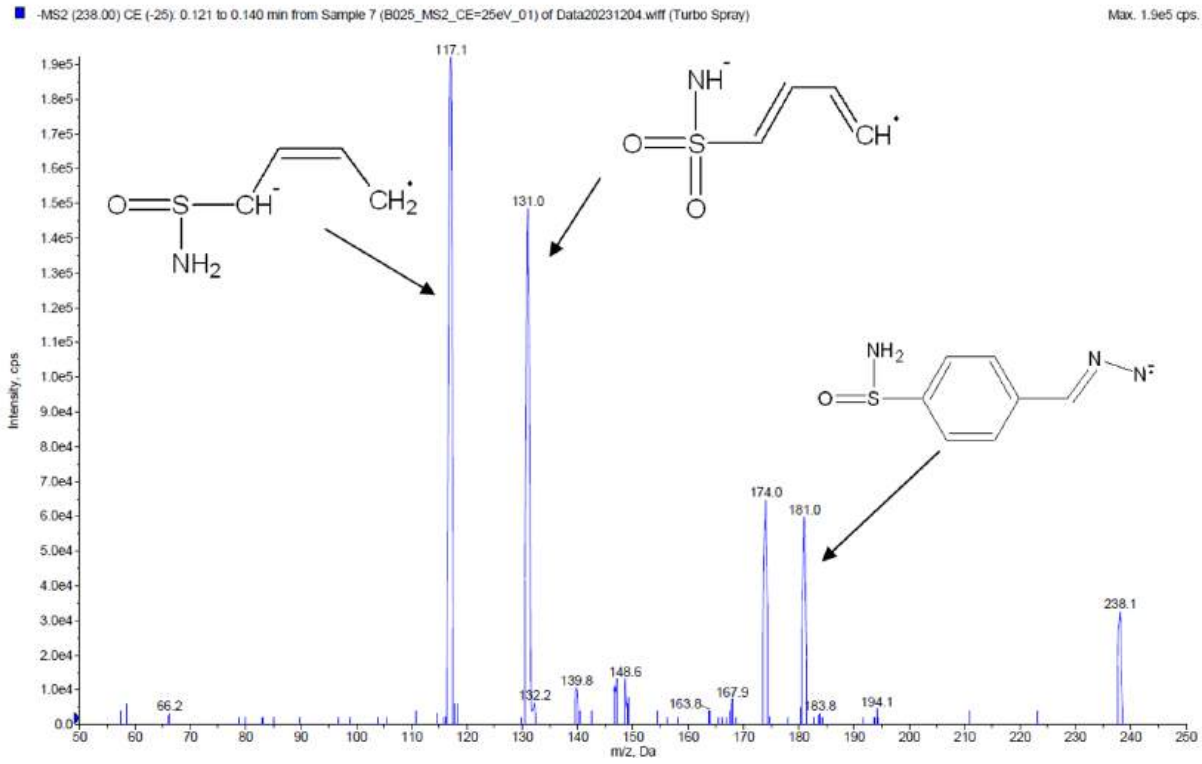


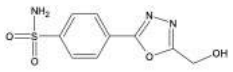
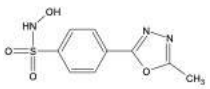
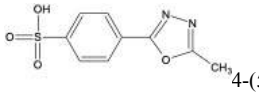
Figure 2. Mass spectrum of 4-(5-methyl-1,3,4-oxadiazole-2-yl)-benzenesulfonamide (MS2 mode; polarity negative; CE = -25eV).

Table 2. Predicted MRM-transitions for identification of possible metabolites of 4-(5-methyl-1,3,4-oxadiazole-2-yl)-benzenesulfonamide

Modification	Difference of m/z	Predicted MRM-transitions		
		Molecular ion M+Δm/z	Daughter ions	
			Non-modified ions M+Δm/z →m	Modified ions M+Δm/z →m+Δm/z
Control (ODASA)	-	238 m/z	117 m/z; 131 m/z; 181 m/z	-
Hydroxylation /N-oxidation	+ 16	254 m/z	117 m/z; 131 m/z; 181 m/z	133 m/z; 147 m/z; 197 m/z; 159 m/z*; 175 m/z*
Dihydroxylation/ Hydroxylation +N-oxidation	+ 32	270 m/z	117 m/z; 131 m/z; 181 m/z	+ 1 Oxygen: 133 m/z; 147 m/z; 197 m/z; 159 m/z*; 175* m/z + 2 Oxygen: 149 m/z; 163 m/z; 213 m/z; 191 m/z
Methylation	+ 14	252 m/z	117 m/z; 131 m/z; 181 m/z	145 m/z; 195 m/z
Glucuronidation	+ 176	414 m/z	117m/z; 133 m/z; 181 m/z	293 m/z; 307 m/z; 357 m/z
Acetylation	+ 42	280 m/z	117 m/z; 131 m/z; 181 m/z	159 m/z; 173 m/z; 223 m/z
Sulfonation	+ 80	318 m/z	117 m/z; 131 m/z; 181 m/z	197 m/z; 211 m/z; 261 m/z
Formation of sulfonic acid	+ 1	239 m/z	117 m/z; 131 m/z; 181 m/z	118 m/z; 132 m/z; 182m/z

Note: M – m/z value of the molecular ion of ODASA (238 m/z); m – m/z value of the product ion of ODASA (117 m/z, 131 m/z, 181 m/z); Δm/z – difference of m/z if modification is present; MRM-transitions 254→159 m/z, 254→175 m/z, 270→159 m/z, 270→175 m/z, 270→191 m/z were added after synthesis of the substance.

Table 3. Detected metabolites of 4-(5-methyl-1,3,4-oxadiazole-2-yl)-benzenesulfonamide

Modification	Objects in which the metabolite was detected	Retention time, min.	The main MRM-transitions	The presumed metabolite
C-hydroxylation	Plasma, rat and rabbit blood, rat urine	6.7 min	254→117 m/z, 254→131 m/z и 254→181 m/z	 4-[5-(hydroxymethyl)-1,3,4-oxadiazol-2-yl]-benzenesulfonamide (M1)
N-hydroxylation	Plasma, rat and rabbit blood, rat urine	8.7 min	254→133 m/z, 254→147 m/z, 254→159* m/z 254→175* m/z	 N-hydroxy-4-(5-methyl-1,3,4-oxadiazol-2-yl)-benzenesulfonamide (M2)
Formation of sulfonic acid	Rat urine	6.3 min	239→182 m/z, 239→118 m/z	 4-(5-methyl-1,3,4-oxadiazol-2-yl)-benzenesulfonic acid (M3)

Note: More intensive MRM-transitions 254→159 m/z and 254→175 m/z were detected after synthesis of substance of M2.

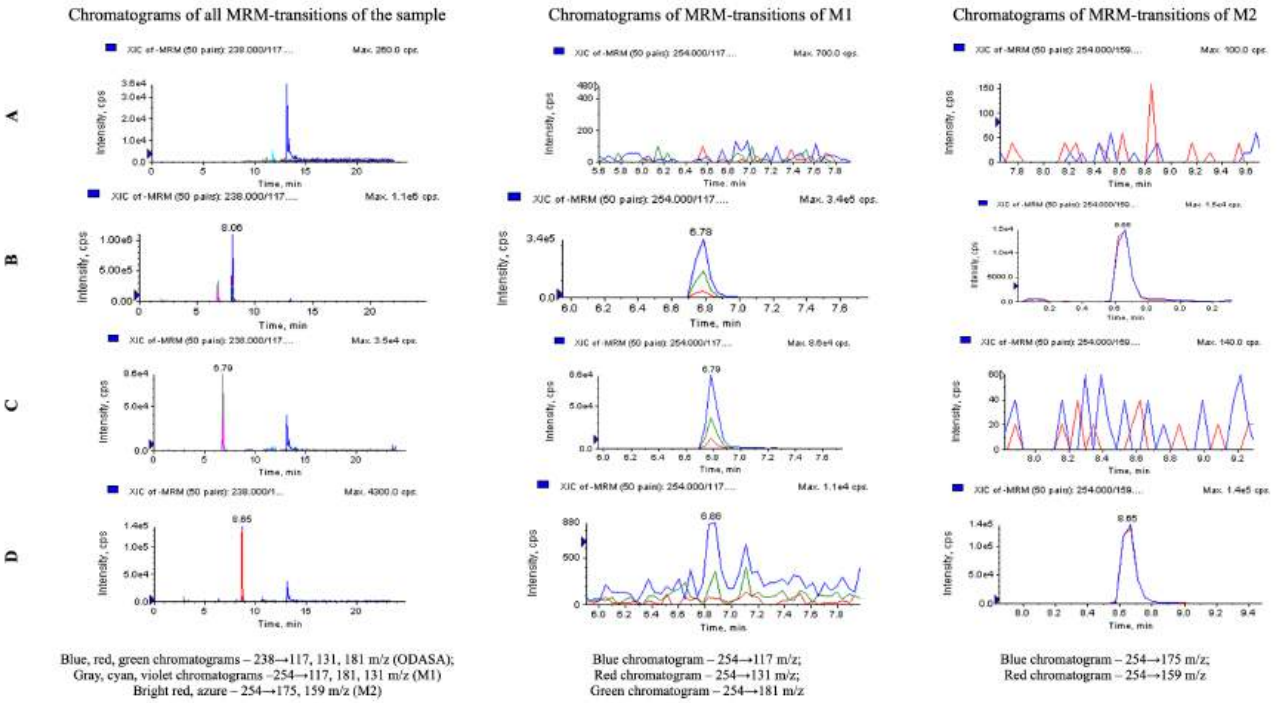


Figure 3. Examples of chromatograms of rabbit plasma samples before injection of drug (**A**), rabbit samples at point 2 h (**B**), blood samples with the addition of 4-[5-(hydroxymethyl)-1,3,4-oxadiazol-2-yl]-benzenesulfonamide (**C**) and N-hydroxy-4-(5-methyl-1,3,4-oxadiazol-2-yl)-benzenesulfonamide (**D**).

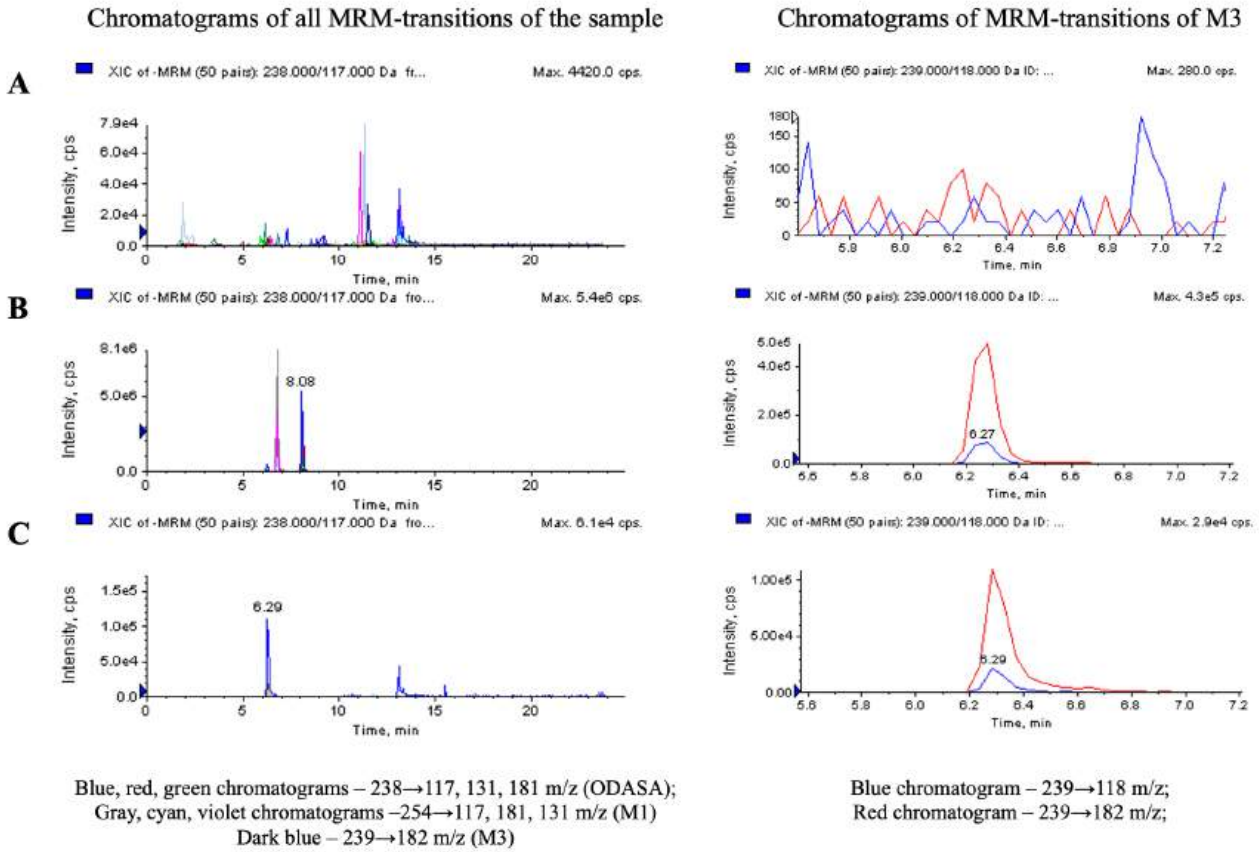


Figure 4. Examples of chromatograms of rat urine sample before injection of drug (**A**), rat urine sample at interval 2-4 h (**B**), urine sample with addition of 4-(5-methyl-1,3,4-oxadiazol-2-yl)-benzenesulfonic acid (**C**).

The synthesis of detected metabolites 4-(5-(hydroxymethyl)-1,3,4-oxadiazole-2-yl)-benzenesulfonamide (M1), N-hydroxy-4-(5-methyl-1,3,4-oxadiazole-2-yl)-benzenesulfonamide (M2), 4-(5-methyl-1,3,4-oxadiazole-2-yl)-benzenesulfonic acid (M3) was performed at the next stage of the study.

Scheme of the synthesis of M2 and M3 and drug candidate ODASA using commercially available 4-sulfamoyl benzoic acid hydrazide is shown in Fig. 5. Reagent 1 was converted by acylation with acetyl chloride in pyridine media into a diacyl derivative 2 (Fig. 5). It was subjected to cyclization under the action of phosphorus oxychloride at the same time. The sulfonamide fragment was hydrolyzed with regeneration of sulfochloride group and formation of intermediate 3 (Fig. 5). It was reacted with hydroxylamine hydrochloride in the presence of potassium carbonate for production of metabolite M2. Boiling of compound 3 (Fig. 5) in water resulted to moderate yield of target sulfonic acid M3.

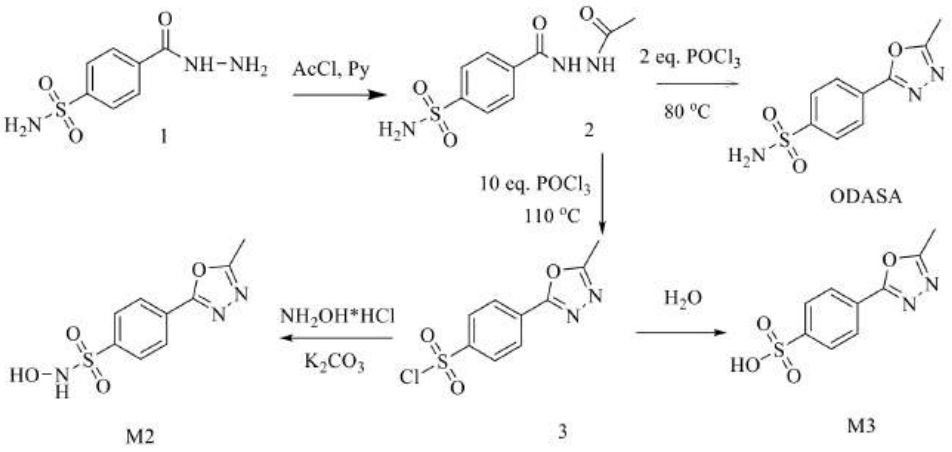


Figure 5. Synthesis scheme of 4-(5-methyl-1,3,4-oxadiazole-2-yl)-benzenesulfonamide (ODASA), N-hydroxy-4-(5-methyl-1,3,4-oxadiazole-2-yl)-benzenesulfonamide (M2) and 4-(5-methyl-1,3,4-oxadiazole-2-yl)-benzenesulfonic acid (M3). ***Note:*** 1 – hydrazide of 4-sulfamoyl benzoic acid; 2 – 4-(2-acetylhydrazine-1-carbonyl)-benzenesulfonamide; 3 – 4-(5-methyl-1,3,4-oxadiazol-2-yl)benzenesulfonyl chloride; AcCl – acetyl chloride; Py – Pyridine.

The hydroxymethyl derivative M1 was synthesized in accordance with the developed scheme (Fig. 6). The initial hydrazide 1 (Fig. 6) was acylated with chloroacetyl chloride in acetonitrile media in presence of diisopropylethylamine (DIPEA) as a base. The cyclization of intermediate 4) was carried out by action of phosphorus oxychloride for obtaining a chloromethyl derivative 5. It was determined that obtaining the target metabolite M1 from compound 5 using alkaline hydrolysis does not lead to preparative yields of the target product. The synthesis of M1 from a chlorine derivative through the formation of an acetate intermediate 6 (Fig. 6) gave reliable results and acceptable 35% total yield of the product.

Structure of all synthesized metabolites and intermediates was confirmed by NMR-spectroscopy and high-resolution mass-spectrometry (Table 4).

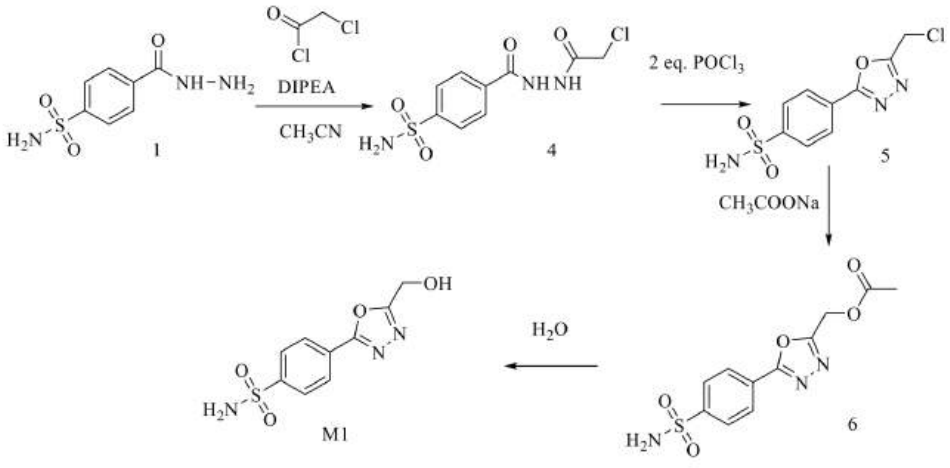


Figure 6. Synthesis scheme of 4-(5-(hydroxymethyl)-1,3,4-oxadiazole-2-yl)-benzenesulfonamide (M1). ***Note:*** 1 – hydrazide of 4-sulfamoyl benzoic acid; 4- 4-(2-(2-chloroacetyl)hydrazine-1-carbonyl)-benzenesulfonamide; 5-4-(5-(chloromethyl)-1,3,4-oxadiazol-2-yl)-benzenesulfonamide; 6-(5-(4-sulfamoylphenyl)-1,3,4-oxadiazol-2-yl)-methyl acetate **;** DIPEA – diisopropylethylamine.

Table 4. The results of structure confirmation of synthesized compounds

Compound	^{1}H-NMR-spectroscopy	^{13}C-NMR-spectroscopy	High resolution mass spectrometry	Melting point
4-(2-acetylhydrazine-1-carbonyl)-benzenesulfonamide (Fig. 5-2)	10.48 (s, 1H), 9.96 (s, 1H), 8.00 (d, J = 8.4 Hz, 2H), 7.92 (d, J = 8.4 Hz, 2H), 7.52 (s, 2H), 1.93 (s, 3H)	169.13, 165.18, 147.40, 136.08, 128.80, 126.43, 21.27	258.0539 m/z [M+H$^+$], Δm/z=-3.87 ppm	224-227°C
4-(5-methyl-1,3,4-oxadiazol-2-yl)-benzenesulfonyl chloride (Fig. 5-3)	8.27 (d, J = 8.1 Hz, 2H), 8.16 (d, J = 8.1 Hz, 2H), 2.67 (s, 3H)	165.19, 163.21, 146.37, 128.2, 128.0, 127.47, 11.41	258.9932 m/z [M+H$^+$], Δm/z=-4.63 ppm	175-180°C
N-hydroxy-4-(5-methyl-1,3,4-oxadiazol-2-yl)-benzenesulfonamide (M2)	9.78 (s, 1H), 9.75 (s, 1H), 8.21 (d, J = 8.2 Hz, 2H), 8.03 (d, J = 8.2 Hz, 2H), 2.62 (s, 3H)	165.40, 163.67, 140.53, 129.87, 128.15, 127.53, 11.39	256.0382 m/z [M+H$^+$], Δm/z=-3.91 ppm	220-225°C
4-(5-methyl-1,3,4-oxadiazole-2-yl)-benzenesulfonic acid (M3)	8.22 (d, J = 8.3 Hz, 2H), 8.05 (d, J = 8.3 Hz, 2H), 3.13 (s, CH_3)	164.57, 163.67, 147.54, 127.91, 127.28, 126.40, 33.66	241.0275 m/z [M+H$^+$], Δm/z=-3.31 ppm	>250°C
4-(2-(2-chloroacetyl)hydrazine-1-carbonyl)-benzenesulfonamide (Fig. 6-4)	10.74 (s, 1H), 10.47 (s, 1H), 8.02 (d, J = 8.3 Hz, 2H), 7.94 (d, J = 8.3 Hz, 2H), 7.53 (s, 2H), 4.21 (s, 2H, CH_2)	166.00, 165.10, 147.55, 135.80, 128.86, 126.49, 41.59	292.0148 m/z [M+H$^+$], Δm/z=-3.77 ppm	203-205°C
4-(5-(chloromethyl)-1,3,4-oxadiazol-2-yl)-benzenesulfonamide (Fig. 6-5)	8.21 (d, J = 7.4 Hz, 2H), 8.04 (d, J = 7.4 Hz, 2H), 7.61 (s, 2H), 5.16 (s, 2H)	164.78, 164.06, 147.77, 128.10, 127.48, 126.32, 33.87	274.0040 m/z [M+H$^+$], Δm/z=-4.74 ppm	206-208°C
(5-(4-sulfamoylphenyl)-1,3,4-oxadiazol-2-yl)-methyl acetate (Fig. 6-6)	8.20 (d, J = 8.2 Hz, 2H), 8.03 (d, J = 8.4 Hz, 2H), 7.61 (s, 2H), 5.41 (s, 2H), 2.14 (d, J = 0.7 Hz, 3H)	170.35, 164.43, 163.34, 147.67, 128.06, 127.45, 126.42, 55.87, 20.98	298.0490 m/z [M+H$^+$], Δm/z=-2.68 ppm	130°C (decomposition)
4-(5-(hydroxymethyl)-1,3,4-oxadiazol-2-yl)-benzenesulfonamide (M1)	8.19 (d, *J* = 8.3 Hz, 2H), 8.03 (d, *J* = 8.3 Hz, 2H), 7.58 (s, 2H), 4.74 (s, 2H)	167.33, 164.00, 147.46, 127.88, 127.46, 126.78, 54.39	256.0384 m/z [M+H$^+$], Δm/z=-3.12 ppm	190°C (decomposition)

Note: Deuterated chloroform was solvent for NMR-analysis of 4-(5-methyl-1,3,4-oxadiazol-2-yl)-benzenesulfonyl chloride;deuterated dimethyl sulfoxide was solvent for NMR-analysis of other substances; NMR signal designation: s – singlet, d – doublet, t – triplet, q – quartet, d.d – doublet of doublets, d.t – triplet of doublets, m – multiplet; br s -broad singlet.

The confirmation stage of the study was carried out using samples of biological fluids with the addition of synthesized substances. The most intense MRM-transitions 254→117 m/z and 254→131 m/z for M1, and 239→182 m/z and 239→118 m/z for M3 were selected to verify the structure. MRM-transitions of M2 254→159 m/z and 254→175 m/z were chosen after synthesis and obtaining MS2-mass spectrum of the substance. The retention time of M1 coincided with that of the standard sample by 99.90-100.09%, t_R M2 – coincided by 99.96-100.10%. Thus, the difference of t_R did not exceed the permissible 1% (Table 5). The percentage of matching the chromatographic peak area ratio at the main MRM-transitions of metabolites and their synthesized analogues was also in the established range of 80.0-120.0% (Khokhlov et al. 2023; Khokhlov et al. 2024 GPA.1.2.1.2.0001.15 The Chromatography; GPA. 1.2.1.1.0008.15 The mass-spectrometry. The State Pharmacopoeia of Russian Federation. XV edition 2023): 96.00-103.35% for M1 and 95.87-105.89% for M2 (Table 5). The MS2 mass spectra of the studied compounds were also compared (point 2h after administration): the mass spectra coincided by at least 93% for M1, by at least 88% for M2 (Fig. 7, Table 5). M3 and other new metabolites were not detected in plasma and blood samples of laboratory animals.

The presence of M2 decomposition product 4-(5-methyl-1,3,4-oxadiazol-2-yl)-benzenesulfonic acid was confirmed in rat urine. The coincidence of the retention time of M3 in test and spiked samples was 100.07%; the coincidence of the peak area ratio was 100.02%, and the coincidence of MS2 mass spectra was 93-96% (interval 2-4 hours) (Table 2).

The fact of M2 degradation was proven by its stability study. Rat urine samples (n=6) spiked by this analyte at a concentration of 100 ng/mL without the addition of ascorbic acid solution were analyzed immediately after preparation and after 24 h of storage at room temperature for this purpose. As a result, M2 was not detected in the stored samples (0% of the initial concentration), and the analytical signal M3, on the contrary, appeared. It indicates the complete decomposition of N-hydroxy-4-(5-methyl-1,3,4-oxadiazol-2-yl)-benzenesulfonamide.

Thus, 4-(5-methyl-1,3,4-oxadiazole-2-yl)-benzenesulfonamide undergoes biotransformation by hydroxylation of the sulfonamide group and methyl radical.

The formation of N-hydroxysulfonamides was also observed at OXSA (Khokhlov et al. 2023) and TFISA (Khokhlov et al. 2024) molecules. However, the methyl group of the isooxazole cycle of OXSA was not subject to any modification (Khokhlov et al. 2023). Modification of the substituent in 1,2-oxazol heterocycle in TFISA molecule is impossible due to its trifluorination (Khokhlov et al. 2024). Also, hydroxylation of aliphatic alkyl radicals was not identified for dorzolamide (Lo Faro et al. 2021), brinzolamide (Dhandar et al. 2022),

Table 5. Results of confirmation of the structure of metabolites of 4-(5-methyl-1,3,4-oxadiazol-2-yl)-benzenesulfonamide

Parameter		4-[5-(hydroxymethyl)-1,3,4-oxadiazol-2-yl]-benzenesulfonamide (M1)			N-hydroxy-4-(5-methyl-1,3,4-oxadiazol-2-yl)-benzenesulfonamide (M2)			4-(5-methyl-1,3,4-oxadiazol-2-yl)-benzenesulfonic acid (M3)		
		t_R, min.	Ratio of peak area 254→117 m/z 254→131 m/z	% of MS2-mass spectrum matches (Min.-max., %)	t_R, min.	Ratio of peak area 254→159 m/z 254→175 m/z	% of MS2-mass spectrum matches (Min.-max., %)	t_R, min.	Ratio of peak area 239→118 m/z/ 239→182 m/z	% of MS2-mass spectrum matches (Min.-max., %)
Rat plasma samples	Test samples (n=12)	6.90± 0.01	2.436±0.147		8.74± 0.01	1.040±0.121		N/A	-	
	Spiked samples (n=6)	6.9± 0.01	2.537±0.076	93-98	8.74± 0.01	1.038±0.052	89-92	N/A	-	-
	% of match*	99.90	96.00		99.96	100.24		N/A	-	
Rat blood samples	Test samples (n=12)	6.74± 0.01	2.479±0.500		8.65± 0.01	1.011±0.530		N/A	-	
	Spiked samples (n=6)	6.74± 0.01	2.398±0.103	94-99	8.65± 0.01	1.026±0.031	90-94	N/A	-	-
	% of match	100.04	103.35		100.05	98.60		N/A	-	
Rat urine samples	Test samples (n=12)	6.87± 0.02	1.971±0.139		8.66± 0.01	1.086±0.052		6.28± 0.01	5.018±0.166	
	Spiked samples (n=6)	6.87± 0.02	1.966±0.070	94-98	8.65± 0.01	1.025±0.039	89-91	6.28± 0.02	5.016±0.126	93-96
	% of match	99.93	100.24		100.10	105.89		100.07	100.02	
Rabbit plasma samples	Test samples (n=12)	6.90± 0.01	2.353±0.142		8.75± 0.01	0.983±0.105		N/A	-	
	Spiked samples (n=6)	6.91± 0.01	2.36±0.136	93-97	8.74± 0.01	1.026±0.087	89-93	N/A	-	-
	% of match	99.93	99.72		100.08	95.87		N/A	-	
Rabbit blood samples	Test samples (n=12)	6.74± 0.01	2.384±0.175		8.64± 0.01	1.004±0.099		N/A	-	
	Spiked samples (n=6)	6.74± 0.01	2.336±0.121	95-97	8.64± 0.01	1.042±0.066	91-94	N/A	-	-
	% of match	100.09	102.03		100.01	96.34		N/A	-	

Note: Mean ± SD is given in each cell of the table N/A – compound was not detected.

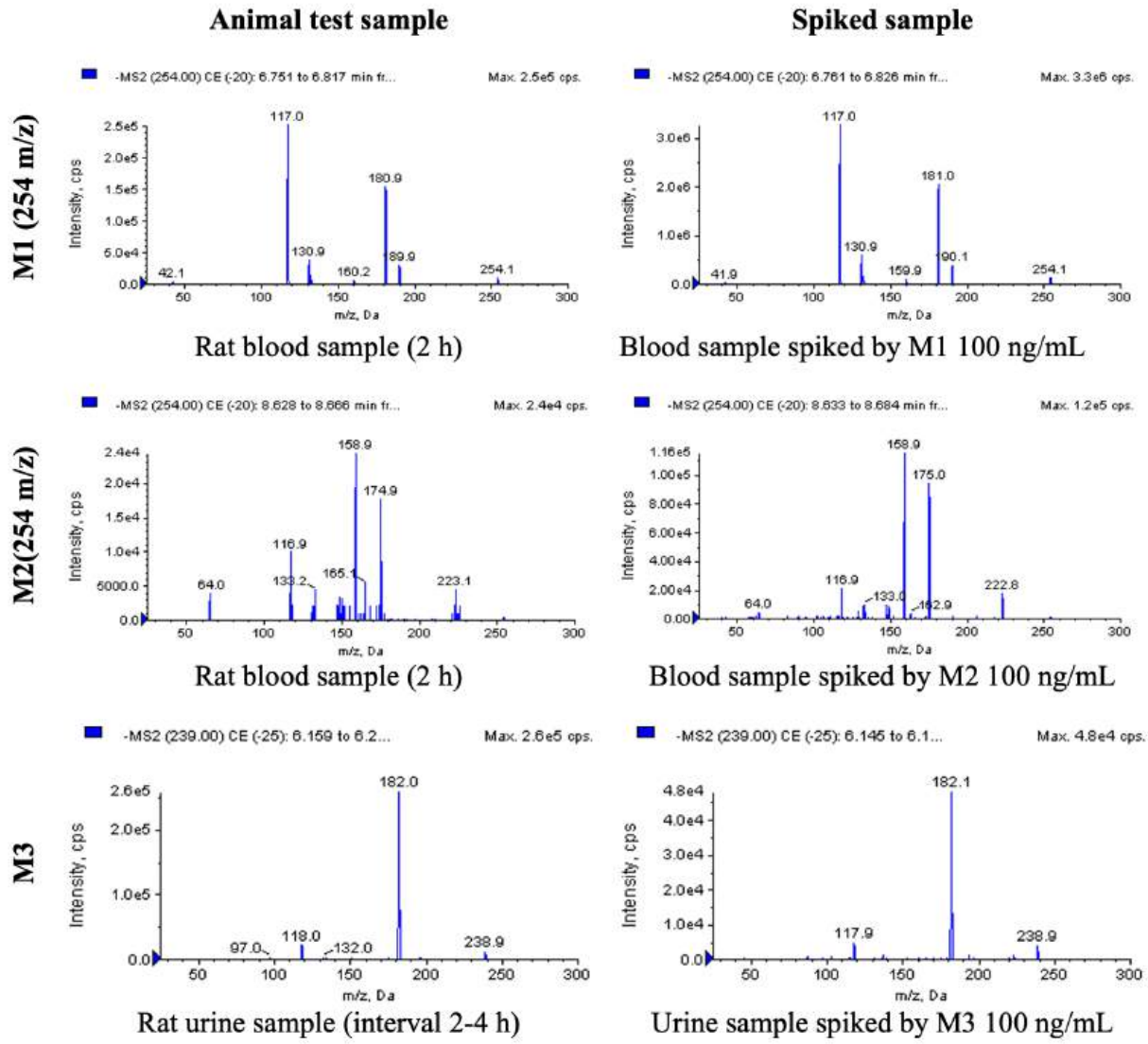


Figure 7. Examples of MS2-mass spectra of metabolites of 4-(5-methyl-1,3,4-oxadiazole-2-yl)-benzenesulfonamide in test and spiked samples.

and acetazolamide (Begou et al. 2020). ODASA does not form an N-acetyl derivative during metabolism, unlike TFISA (Khokhlov et al. 2024). The 4-(5-hydroxymethyl-1,3,4-oxadiazole-2-yl)-benzenesulfonamide (M1) is the most promising in pharmacological activity evaluation. Its sulfonamide group, which is necessary for binding to the active center of CA_{II}, does not undergo any modification.

Conclusion

Two main metabolites, 4-(5-hydroxymethyl-1,3,4-oxadiazole-2-yl)-benzenesulfonamide and N-hydroxy-4-(5-methyl-1,3,4-oxadiazole-2-yl)-benzenesulfonamide, are formed during the biotransformation of 4-(5-methyl-1,3,4-oxadiazole-2-yl)benzenesulfonamide. The N-hydroxy derivative is unstable in biological fluids after selection and decomposes with formation 4-(5-methyl-1,3,4-oxadiazole-2-yl)-benzenesulfonic acid. The structure of all detected compounds was confirmed using the HPLC-MS/MS method by comparison with their synthesized chemicals. The obtained substances will be certified as standard in accordance with the requirements of the State Pharmacopoeia of the Russian Federation XV and used during a complete pharmacokinetic study. The ability of these metabolites to inhibit carbonic anhydrase of various types will be investigated.

Conflict of interest

The authors declare the absence of a conflict of interests.

Funding

The study was carried out with the financial support of the Russian Scientific Fund (project № 22-13-20085).

Data availability

All of the data that support the findings of this study are available in the main text.

References

- Alsibaee AM, Aljohar HI, Attwa MW, Abdelhameed AS, Kadi AA (2023) Investigation of fenebrutinib metabolism and bioactivation using MS3 methodology in ion trap LC/MS. Molecules 28(10): 4225. https://doi.org/10.3390/molecules28104225 [PubMed] [PMC]
- Begou O, Drabert K, Theodoridis G, Tsikas D (2020) GC-NICI-MS analysis of acetazolamide and other sulfonamide (R-SO_2-NH_2) drugs as pentafluorobenzyl derivatives [R-SO_2-N($PFB)_2$] and quantification of pharmacological acetazolamide in human urine. Journal of Pharmaceutical Analysis 10(1): 49–59. https://doi.org/10.1016/j.jpha.2019.11.006 [PubMed] [PMC]
- Boyce M, Favela KA, Bonzo JA, Chao A, Lizarraga LE, Moody LR, Owens EO, Patlewicz G, Shah I, Sobus JR, Thomas RS, Williams AJ, Yau A, Wambaugh JF (2023) Identifying xenobiotic metabolites with in silico prediction tools and LCMS suspect screening analysis. Frontiers in Toxicology 5: 1051483. https://doi.org/10.3389/ftox.2023.1051483 [PubMed] [PMC]
- Chen W-H, Chiu C-H, Farn S-S, Cheng K-H, Huang Y-R, Lee S-Y, Fang Y-C, Lin Y-H, Chang K-W (2023) Identification of the hepatic metabolites of flumazenil and their kinetic application in neuroimaging. Pharmaceuticals 16(5): 764. https://doi.org/10.3390/ph1605076 [PubMed] [PMC]
- Dhandar AG, Chaudhari SR, Ganorkar SB, Patil AS, Surana SJ (2022) Mini-review on bioanalytical estimation of brinzolamide. Current Pharmaceutical Analysis 18(3): 265–272. https://doi.org/10.2174/1573412917666210812103414
- GPA. 1.2.1.1.0008.15 The Mass-spectrometry» (2023) The State Pharmacopoeia of Russian Federation. XV edition https://pharmacopoeia.regmed.ru/pharmacopoeia/izdanie-15/
- GPA.1.2.1.2.0001.15 The Chromatography (2023) The State Pharmacopoeia of Russian Federation. XV edition https://pharmacopoeia.regmed.ru/pharmacopoeia/izdanie-15/
- Khokhlov AL, Shetnev AA, Korsakov MK, Fedorov VN, Tyushina AN, Volkhin NN, Vdovichenko VP (2023) Pharmacological properties of sulfonamide derivatives – new inhibitors of carbonic anhydrase. Bulletin of Experimental Biology and Medicine 175(2): 166–170. https://doi.org/10.47056/0365-9615-2023-175-2-166-170 [PubMed]
- Khokhlov AL, Yaichkov II, Korsakov MK, Shetnev AA, Volkhin NN, Petukhov SS (2023) Development of quantification methods of a new selective carbonic anhydrase II inhibitor in plasma and blood and study of the pharmacokinetics of its ophthalmic suspension in rats. Research Results in Pharmacology 9(4): 53–64. https://doi.org/10.18413/rrpharmacology.9.10056
- Khokhlov AL, Yaichkov II, Shetnev AA, Ivanovsky SA, Korsakov MK, Alexeev MA, Gasilina OA, Volkhin NN, Petukhov SS (2024) Identification and synthesis of metabolites of the new antiglaucoma drug. Research Results in Pharmacology 10(1): 53–66. https://doi.org/10.18413/rrpharmacology.10.431
- Kurysheva NI (2020) Carbonic anhydrase inhibitors in the treatment of glaucoma. Review. Part II. Ophthalmology in Russia [Oftal'mologiya] 17(4): 676–682. https://doi.org/10.18008/1816-5095-2020-4-676-682 [in Russian]
- Lo Faro AF, Tini A, Gottardi M, Pirani F, Sirignano A, Giorgetti R, Busardò FP (2021) Development and validation of a fast ultra-high-performance liquid chromatography tandem mass spectrometry method for determining carbonic anhydrase inhibitors and their metabolites in urine and hair. Drug Testing and Analysis 13(8): 1552–1560. https://doi.org/10.1002/dta.3055 [PubMed] [PMC]
- Peeters L, Vervliet P, Foubert K, Hermans N, Pieters L, Covaci A (2020) A comparative study on the in vitro biotransformation of medicagenic acid using human liver microsomes and S9 fractions. Chemico-Biological Interactions 328: 109192. https://doi.org/10.1016/j.cbi.2020.109192 [PubMed]
- Reddy GN, Laltanpuii C, Sonti R (2021) Review on in vivo profiling of drug metabolites with LC-MS/MS in the past decade. Bioanalysis 13(22): 1697–1722. https://doi.org/10.4155/bio-2021-0144 [PubMed]
- Simakova IL, Grigoryan LA, Gorbacheva KS (2023) Modern possibilities of functional glaucoma screening (Part 1). National Journal Glaucoma 22(4): 99–111. https://doi.org/10.53432/2078-4104-2023-22-4-99-111 [in Russian]
- Trawinski J, Wronski M, Gawlik M, Skibinski R (2022) Identification of the new metabolite of nebivolol using liquid chromatography coupled with high-resolution mass spectrometry and chemometrics. Molecules 27(3): 763. https://doi.org/10.3390/molecules27030763 [PubMed] [PMC]

Author contributions

- **Alexander L. Khokhlov,** Doctor Habil. of Medical Sciences, Professor, Member of The Russian Academy of Sciences, Head of the Department of Pharmacology and Clinical Pharmacology, rector of Yaroslavl State Medical University; e-mail: al460935@yandex.ru, **ORCID ID**: https://orcid.org/0000-0002-0032-0341. The author's contribution: formulation and development of the aim and objectives; analysis and interpretation of the obtained data; critical review of the draft copy and provision of valuable comments.

- **Ilya I. Yaichkov**, Candidate of Pharmaceutical Sciences, research fellow of the Department of Analytical Development and Quality Control of M.V. Dorogov Pharmaceutical Technology Transfer Center of Yaroslavl State Pedagogical University named after K.D. Ushinsky; research fellow of the Institute of Pharmacy of Yaroslavl State Medical University; e-mail: i.yaichkov@yspu.org, **ORCID ID**: https://orcid.org/0000-0002-0066-7388. The author's contribution: concept development; development of design of biotransformation study; development of bioanalytical methods; analysis of samples; analysis and interpretation of the obtained data; writing the bioanalytical part and editing the manuscript.

- **Anton A. Shetnev,** Candidate of Chemical Sciences, Head of the Department of Pharmaceutical Development of M.V. Dorogov Pharmaceutical Technology Transfer Center of Yaroslavl State Pedagogical University named after K.D. Ushinsky; e-mail: a.shetnev@list.ru, **ORCID ID:** https://orcid.org/0000-0002-4389-461X. The author's contribution: formulation and development of the aim and objectives; development of synthesis technology of the drug and its metabolite; writing the synthesis part and interpretation of the obtained data.

- **Valeria A. Panova,** junior research fellow of the Department of Pharmaceutical Development of M.V. Dorogov Pharmaceutical Technology Transfer Center of Yaroslavl State Pedagogical University named after K.D. Ushinsky; e-mail: v.panova@yspu.org, **ORCID ID:** https://orcid.org/0000-0002-4775-5326. The author's contribution: synthesis of the drug and its metabolites.

- **Julia A. Efimova**, engineer of the Department of Pharmaceutical Development of M.V. Dorogov Pharmaceutical Technology Transfer Center of Yaroslavl State Pedagogical University named after K.D. Ushinsky, post-graduate student of Department of Organic Chemistry of A.N. Kosygin State University of Russia; e-mail: yua.efimova@yspu.org, **ORCID ID:** https://orcid.org/0000-0003-3027-0223. The author's contribution: synthesis of the drug and its metabolites.

- **Sergey A. Ivanovskiy,** Candidate of Chemical Sciences, Head of the Department of Analytical Development and Quality Control of M.V. Dorogov Pharmaceutical Technology Transfer Center of Yaroslavl State Pedagogical University named after K.D. Ushinsky; e-mail: main_engine@mail.ru, **ORCID ID:** https://orcid.org/0000-0002-1421-9236. The author's contribution: quality control and characterization of structure of the drug and its metabolites.

- **Mikhail K. Korsakov,** Doctor Habil. of Chemical Sciences, Professor of the Department of Chemistry, Theory and Methods of Teaching Chemistry, Head of The Center of Transfer of Pharmaceutical Technology named after M.V. Dorogov of Yaroslavl State Pedagogical University named after K.D. Ushinsky; e-mail: m.korsakov@yspu.org, **ORCID ID:** https://orcid.org/0000-0003-0913-2571. The author's contribution: formulation and development of the aim and objectives; analysis and interpretation of the obtained data; critical review of the draft copy and provision of valuable comments.

- **Nikita N**. **Volkhin,** junior research fellow of the Department of Pharmacological Studies of M.V. Dorogov Pharmaceutical Technology Transfer Center of Yaroslavl State Pedagogical University named after K.D. Ushinsky; e-mail: nnvolkhin@ysmu.ru, **ORCID ID:** https://orcid.org/0000-0002-4275-9037. The author's contribution: blood and plasma sample collection; analysis and interpretation of the data obtained.

- **Sergey S**. **Petukhov,** engineer of the Department of Pharmacological Studies of M.V. Dorogov Pharmaceutical Technology Transfer Center of Yaroslavl State Pedagogical University named after K.D. Ushinsky; junior research fellow of the Institute of Pharmacy of Yaroslavl State Medical University; e-mail: sspp465@mail.ru, **ORCID ID:** https://orcid.org/0009-0007-8435-7689. The author's contribution: blood and plasma sample collection; analysis and interpretation of the obtained data.