

Identification and synthesis of metabolites of the new antiglaucoma drug

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Abstract

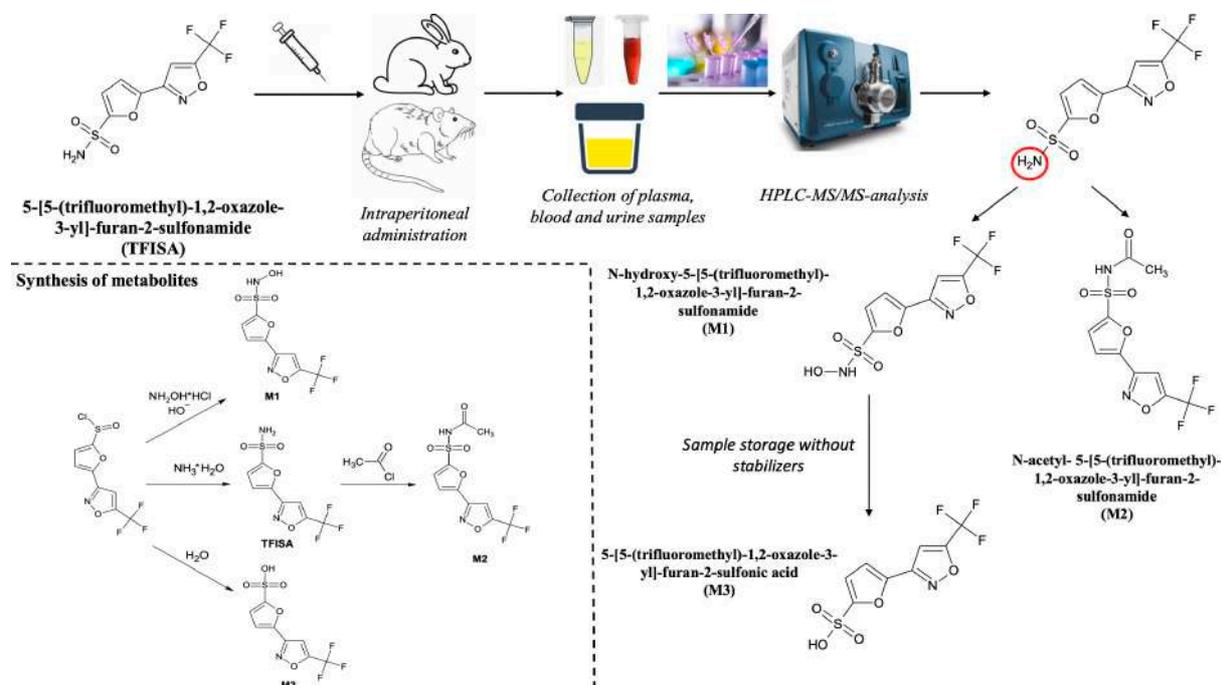
Introduction: The determination of biotransformation products is an essential part of the preclinical trial of original medicines. These studies have not been conducted before for the new drug 5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide. Identification and synthesis of metabolite substances are necessary for subsequent tests of bioavailability, linearity of pharmacokinetics, accumulation, distribution and excretion.

Materials and methods: The study was carried out on Wistar rats and rabbits of the Soviet Chinchilla breed. The substance of the drug was administered to animals intraperitoneally. The collection of animal blood samples was performed before administration and 1 h, 2 h, 4 h, 24 h after administration into K₃EDTA-tubes. A part of each sample was centrifuged to separate the plasma. Rat urine samples were taken before administration and at intervals of 0-2 h, 2-4 h, 4-6 h, 6-24 h after administration of the drug. The HPLC-MS/MS method was used to identify metabolites in biological fluids. The assumed biotransformation products were synthesized after preliminary analysis. The structure of the obtained substances was confirmed by NMR spectroscopy and high-resolution mass spectrometry. Then, a comparison was carried out with the compounds identified in biological fluids by retention time, ratios of chromatographic peak areas at the main MRM-transitions using HPLC-MS/MS.

Results and discussion: A metabolite formed by addition an oxygen atom to a drug molecule, as well as an acylated metabolite were detected during the analysis of plasma and blood samples. A compound with an increased molecular weight of 1 dalton compared to the drug substance was also present in a rat urine. N-hydroxy-5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide, 5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonic acid, which could potentially be obtained during biotransformation, as well as N-acetyl-5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide were synthesized. Repeated HPLC-MS/MS tests confirmed the correctness of the initial hypothesis. It was found that a sulfonic acid derivative is formed in urine as a result of decomposition of N-hydroxymetabolite during sample collection.

Conclusion: The studied drug is metabolized by formation of two metabolites: N-hydroxy-5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide and N-acetyl-5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide. N-hydroxymetabolite is able to decompose in biological fluids samples with formation of 5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonic acid.

Graphical abstract



Keywords

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

Introduction

Determination of pharmacokinetic parameters of active substance and its biotransformation products is required during preclinical study of each original drug. The qualitative composition of metabolites of a newly created pharmacologically active substance is unknown, but can be predicted “in-silico” (Mironov 2012; Khokhlov et al. 2019). The modeling of a process of microsomal oxidation and conjugation of xenobiotics is possible using microsomes, S9 fraction or hepatocytes (Khokhlov and Pyatigorskaya 2019; Alseekh et al. 2021). The most accessible method of experimental study of metabolism is the administration of a drug to laboratory animals. Thus, the biological samples, in which biotransformation products are determined, are collected from the subjects at certain time points (Reddy et al. 2021). HPLC-MS/MS is used as an analytical method for obtaining the metabolic profile of most low molecular compounds. The detected metabolites are synthesized after screening. The obtained substances are used as reference standards for further pharmacokinetic studies. Its pharmacological activity is also evaluated.

The 5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide (TFISA) (Fig. 1) is a new original molecule for treatment of open-angle glaucoma. Its mechanism of action is based on selective inhibition of the enzyme carbonic anhydrase II (iCA II). This drug exceeds previously developed *dorzolamide*, *brinzolamide*, as well as derivatives of *1,3-oxazole* of a new generation in its pharmacological activity (Khokhlov et al. 2023).

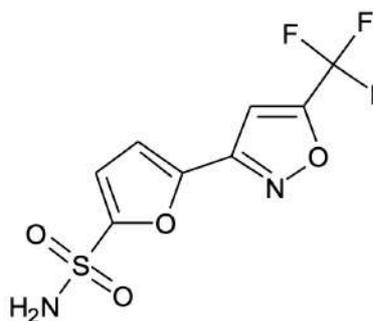


Figure 1. Structure of 5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide

The biotransformation process of TFISA has not been studied before. The first drug from the group of carbonic anhydrase inhibitors, acetazolamide, is eliminated unchanged (Begou et al. 2020). Dorzolamide and brinzolamide undergo dealkylation (Lo Faro et al. 2021; Dhandar et al. 2022). The N-hydroxylation of sulfonamide group is the main biotransformation pathway of 4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonamide (Khokhlov et al. 2023). Sulfanilamide antimicrobials, which are similar in structure to TFISA, are metabolized by N-acetylation and N-hydroxylation of the aromatic amino group. Substituents of sulfonamide and amino groups of these drugs can be methylated and hydroxylated (Vree et al. 1985; Ma et al. 2021).

Thus, the aim of the study is identification and synthesis of metabolites of 5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide.

Materials and Methods

Design of bioanalytical part

Analytical equipment

HPLC-MS/MS system consisting of a tandem mass spectrometric detector "AB Sciex QTRAP5500" (AB Sciex, Singapore) and a chromatograph "Agilent 1260 Infinity" (Agilent Technologies, Germany) was used for determination of metabolites in biological objects. The chromatograph consisted of the following units: binary pump G1312B, autosampler G1329B with thermostat G 1330 B, and a column thermostat G1316A. The device was controlled by the Analyst 1.6.2 software (AB Sciex, USA); the integration of the obtained chromatograms was carried out by the MultiQuant 3.0.5 software (AB Sciex, USA). The LightSight 2.3 software (AB Sciex, USA) was used for prediction of the structure of possible metabolites of the studied drug and creation of MRM-methods for its identification.

Sample preparation and analysis

Sample preparation was carried out by addition of methanol to the samples: 25 μL of blood, plasma or urine was mixed with 100 μL of the solvent using vortex shaker and centrifuged at 10000 rpm for 5 min. A supernatant was transferred to vials with micro-inserts.

Spiked by synthesized substance plasma, blood and urine were obtained by adding 2.5 μL of methanol metabolite solution at a concentration of 5 $\mu\text{g}/\text{mL}$. A separate series of spiked samples was prepared for each compound in 6 repetitions.

The Poroshell 120EC-C18 (50*3.0 mm, 2.7 microns) chromatographic column with a Zorbax Eclipse Plus C18 pre-column (12.5*2.1 mm, 5.0 microns) was used for chromatographic separation. It was thermostated at a temperature of 40° C. The mobile phase consisted of 0.1% aqueous solution of formic acid (Optima LC-MS-Grade, Thermo Fisher Scientific, USA) and methanol (Lichrosolv hypergrade for LC-MS, Merck KGaA, Germany). The parameters of gradient elution are shown in Table 1. The injected volume was 3 μL . Every standard and animal sample was analyzed once.

Mass spectrometric detection was carried out in negative polarity using electrospray ionization (ESI) and collision-activated dissociation (CAD). The temperature of the ion source was 700°C, and the voltage of the

electrospray was - 4500V.

Samples of plasma, blood or urine spiked by TFISA at a concentration of 1 ng/m were analyzed before beginning the analytical series for system suitability and sensitivity tests. The signal-to-noise ratio of the chromatographic peaks at the control MRM-transitions 281→136 m/z, 281→66 m/z, 281→189 m/z were at least 50:1, 30:1 and 10:1, respectively.

Table 1. The parameters of gradient elution of method of identification of metabolites of 5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide

<i>Mobile phase: 0,1% formic acid (water solution) – A, methanol – B</i>		
<i>Flow rate: 500 $\mu\text{L}/\text{min}$</i>		
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

Design of biotransformation part

Animals

The study of the metabolism of 5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide was performed on 6 Wistar rats weighing 288.67±14.15 g (Mean±SD) and 6 rabbits of the Soviet Chinchilla breed weighing 3.00±0.18 kg (Mean±SD). The test group consisted of 3 males and 3 females of each species. The animals were obtained from SMK Stezar LLC (Russian Federation). A catheter was previously inserted into the right jugular vein in rats. The sampling of rabbits was carried out from the ear vein.

The study was approved by the Ethics Committee of Yaroslavl State Pedagogical University (YSPU) named after K.D. Ushinsky (Minutes №2 of 10 October 2023).

Dosing of the drug and sample collection

The substance of the studied drug was administered intraperitoneally at a dosage of 10 mg/kg to rats and at a dosage of 0.8 mg/kg to rabbits. Blood samples were collected before administration of the drug and 1 hour, 2 hours, 4 hours, 24 hours after administration in a volume of 0.2 mL into K₃EDTA-test tubes. Then 50 μL of the sample was immediately processed and analyzed. The remaining part of blood was centrifuged for 10 min at 2500 rpm and a temperature of +4° C for plasma separation. Rat urine sampling was also performed. Urine was collected using a metabolic cage before the administration of the drug and at intervals from the moment of the administration to 2 h, from 2 h to 4 hours, from 4 hours to 6 hours, from 6 hours to 24 hours after the administration. Preparation of urine and plasma samples was also carried out immediately.

The animals were divided into two groups to conduct two stages of the experiment. It included 3 individuals of each species. The identification of metabolites in biological samples was performed at the first stage. The experiment was repeated on the second group of animals

after successful synthesis of the detected compounds. It was necessary for comparison of the structure of the analytes and confirmation of reproducibility. The analysis of fresh samples was also necessary due to instability of metabolite N-hydroxy-5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide in biological fluids. Therefore, a 10% ascorbic acid aqueous solution was added to urine and plasma samples at the rate of 5 μ L of solution per 25 μ L of biological fluid at the second stage.

Finally, 30 samples of rat plasma, blood and urine (including 6 control samples) and 30 samples of rabbit plasma and blood (including 6 control samples) were analyzed during the study. The total number of samples was 150 (120 test samples and 30 control samples). This number of observations is enough for reliable identification of TFISA metabolites.

Design of synthesis experiments

5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide and its metabolites was produced in M.V. Dorogov Pharmaceutical Technology Transfer Center of YSPU named after K.D. Ushinsky.

4,4,4-Trifluoro-1-(furan-2-yl)-butane-1,3-dione ($\geq 99\%$, Sigma Aldrich, USA), acetyl chloride (reagent grade ($\geq 98\%$), Sigma Aldrich, USA), chlorosulfonic acid ($\geq 99\%$, Sigma Aldrich, USA), thionyl chloride (reagent grade ($\geq 97\%$), Sigma Aldrich, USA), acetonitrile (chemically pure, OC Vecton, Russian Federation), hydroxylamine hydrochloride ($\geq 99\%$, OC Vecton, Russian Federation), sodium hydroxide (chemically pure, OC Vecton, Russian Federation), ammonia solution (chemically pure, OC Vecton, Russian Federation), acetone (chemically pure, OC Vecton, Russian Federation), trichlorometane (chemically pure, OC Vecton, Russian Federation) were used for synthesis without additional purification. High-resolution mass spectrometry and NMR spectroscopy were applied to confirm the structure of the compounds. Changes in melting/decomposition temperature were also measured. NMR spectra were recorded on a Varian UNITY Plus – 400 device (400 MHz) (Varian LLC, USA). Deuterated dimethyl sulfoxide was used to dissolve the samples. The signals of residual solvent protons in $^1\text{H-NMR}$ (δH 2.50 ppm) and carbons in $^{13}\text{C-NMR}$ (δC 39.5 ppm) were chosen as a reference for counting chemical shifts. The signal designation forms on NMR-spectra are: s – singlet, d – doublet, t – triplet, q – quartet, d,d – doublet of doublets, d,t – triplet of doublets, m – multiplet. The Bruker Daltonics micrOTOF-II mass spectrometer (Bruker Daltonics GmbH, USA) was used to determine the exact molecular weight of synthesized substances. The melting point was determined using a Büchi M-560 device (Büchi Labortechnik AG, Switzerland).

Criteria of comparison of analytical signals of metabolites and synthesized substances

The following parameters were used to compare the analytical signal of metabolites in animal samples with synthesized standard substance (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):

- MRM-transitions where chromatographic peaks were identified in comparison with the blank samples (qualitative parameter) – MRM-transitions of studied compounds should match);

- Retention times of chromatographic peaks (t_{R}) – the acceptable difference could be no more than 1% (% of match – 99-101%));
- The ratio of chromatographic peak areas at the main MRM-transitions of the metabolite – the acceptable difference could be no more than 20% (% of match – 80.0-120.%);
- Mass spectra of the molecular ion of the metabolite obtained using MS2-mode – matching could be no more than 85%.

Statistical analysis

The mean values of the quantitative parameters were compared during metabolite identification (formula 1):

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

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

The standard deviation (SD) is shown in the table as dispersion measure of obtained comparison data. Mean values of chromatographic peak area of M1 at each stability point (Mean $S_{\text{stab point}}$) were compared with the mean value of the chromatographic peak area of this analyte in fresh samples (Mean $S_{\text{fresh sample}}$) during stability study of N-hydroxy-5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide in urine (formula 2):

$$\% \text{ of initial concentration} = \frac{\text{Mean } S_{\text{stab. point}}}{\text{Mean } S_{\text{fresh sample}}} \times 100\% \quad (2)$$

Microsoft Excel 2016 (Microsoft Corporation, USA) was applied for statistical calculations.

Results and Discussion

During development of the method, it was found that the best signal intensity of 5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide was achieved using negative polarity due to the presence of an NH-acid center in sulfonamide group. The 3 main product ions – 66 m/z, 136 m/z, 189 m/z – are formed in the process of CAD-fragmentation of the molecular ion of this analyte (MS2 mode, Fig. 2). These ions were selected for prediction of MRM-transitions of metabolites. Possible hydroxylation, N-oxidation, methylation, acetylation, sulfonation, glucuronic conjugation, as well as elimination of the fluorine atom and formation of sulfonic acid (Khokhlov et al. 2023) were taken into account for creation of the method (Table 2).

Two metabolites were identified on chromatograms of plasma, blood and urine samples of laboratory animals. They were formed by the addition of an oxygen atom to the TFISA molecule ($\Delta m/z=16$) and its acetylation ($\Delta m/z=42$) (Table 3). The chromatographic peaks of the TFISA oxide derivative, which were absent in the blank samples, were detected at MRM-transitions 297 \rightarrow 136 m/z, 297 \rightarrow 66 m/z, 297 \rightarrow 205 m/z (Fig. 3). The increase in m/z of the product ion with a sulfonamide group 189 m/z by 16 Da, and no increase in m/z of fragments of furan 66 m/z and 3-(trifluoromethyl)-1,2-oxazole 136 m/z suggested that this metabolite is the N-hydroxy-5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide (Fig. 2).

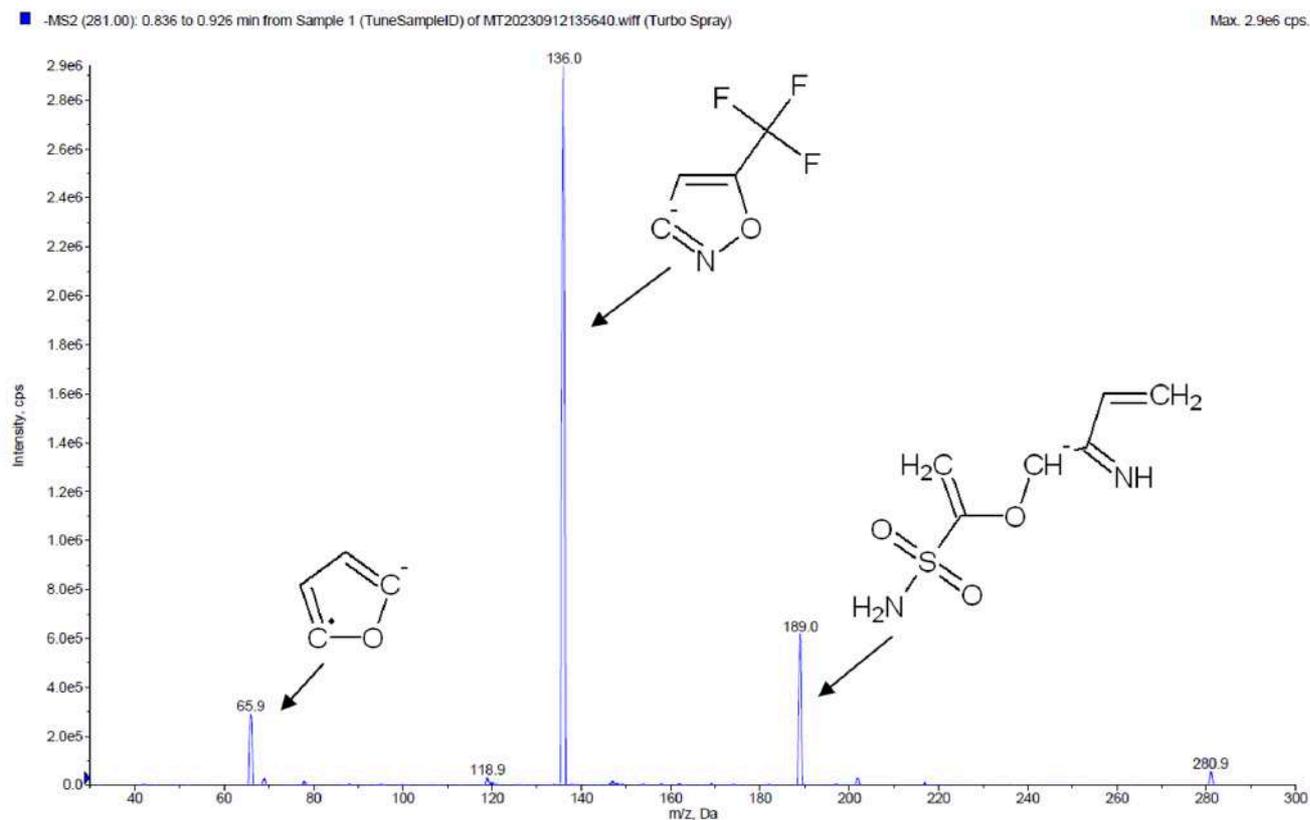


Figure 2. Mass spectrum of 5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide (MS2 mode; polarity negative; CE=30eV).

Table 2. MRM-transitions for identification of possible metabolites of 5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide

Modification	Difference of m/z	Predicted MRM-transitions	
		M+ Δ m/z \rightarrow D*	M+ Δ m/z \rightarrow D+ Δ m/z
Control (TFISA)	-	281 \rightarrow 136 m/z; 281 \rightarrow 66 m/z; 281 \rightarrow 189 m/z	-
Hydroxylation /N-oxidation	16	297 \rightarrow 136 m/z; 297 \rightarrow 66 m/z; 297 \rightarrow 189 m/z	297 \rightarrow 152 m/z; 297 \rightarrow 82 m/z; 297 \rightarrow 205 m/z
Methylation	14	295 \rightarrow 136 m/z; 295 \rightarrow 66 m/z; 295 \rightarrow 189 m/z	295 \rightarrow 150 m/z; 295 \rightarrow 80 m/z; 295 \rightarrow 203 m/z
Glucuronidation	176	457 \rightarrow 136 m/z; 457 \rightarrow 66 m/z; 457 \rightarrow 189 m/z	457 \rightarrow 312 m/z; 457 \rightarrow 365 m/z; 457 \rightarrow 242 m/z
Acetylation	42	323 \rightarrow 136 m/z; 323 \rightarrow 66 m/z; 323 \rightarrow 189 m/z	323 \rightarrow 178 m/z; 323 \rightarrow 108 m/z; 323 \rightarrow 231 m/z
Sulfonation	80	361 \rightarrow 136 m/z; 361 \rightarrow 66 m/z; 361 \rightarrow 189 m/z	361 \rightarrow 216 m/z; 361 \rightarrow 146 m/z; 361 \rightarrow 269 m/z
Defluorination	-18	263 \rightarrow 136 m/z; 263 \rightarrow 66 m/z; 263 \rightarrow 189 m/z	263 \rightarrow 118 m/z; 263 \rightarrow 48 m/z; 263 \rightarrow 171 m/z
Formation of sulfonic acid	1	282 \rightarrow 136 m/z; 282 \rightarrow 66 m/z; 282 \rightarrow 189 m/z	282 \rightarrow 137 m/z; 282 \rightarrow 190 m/z; 282 \rightarrow 67 m/z

The chromatographic peaks were also detected on rat urine chromatograms at MRM-transitions 282 \rightarrow 136 m/z and 282 \rightarrow 66 m/z on a retention time of 8.15 min. It indicated the presence of 5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonic acid (Fig. 4). The analytical signal of this analyte was absent in plasma and blood samples. There were no other modifications of TFISA identified in the studied samples (Fig. 3).

The N-hydroxy derivative (M1) and the derivative of sulfonic acid (M3) of TFISA can theoretically undergo to conjugation. Therefore, an additional MRM-method was created for testing this hypothesis (Table 4), and the prepared samples were analyzed again. However, new metabolites were not detected on the obtained chromatograms.

Table 3. The detected metabolites of 5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide

Modification	Objects in which the metabolite is identified	Retention time, min.	The main MRM-transitions	Predicted metabolite
Hydroxylation	Plasma, blood and urine of rat; Plasma and blood of rabbit	8.73	297→136 m/z; 297→66 m/z; 297→205 m/z*	 N-hydroxy-5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide (M1)
Acetylation	Plasma, blood and urine of rat; Plasma and blood of rabbit	8.92	323→136 m/z; 323→66 m/z	 N-acetyl-5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide (M2)
Formation of sulfonic acid derivate	Rat urine	8.15	282→136 m/z; 282→66 m/z	 5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonic acid (M3)

There were no chromatographic peaks at MRM-transitions 297→66 m/z and 297→205 m/z on the chromatograms of many urine samples due to low concentration of the analyte

Table 4. MRM-transitions for identification of possible conjugates of a hydroxylated metabolite and sulfonic acid derivate

Modification	$\Delta m/z$	Predicted MRM-transitions							
		N-hydroxymetabolite			Sulfonic acid derivate				
		M+16+ $\Delta m/z$ →D*	M+16+ $\Delta m/z$ →D*+16	M+16+ $\Delta m/z$ →D+ $\Delta m/z$	M+16+ $\Delta m/z$ →D+16+ $\Delta m/z$	M+1+ $\Delta m/z$ →D*	M+1+ $\Delta m/z$ →D+1	M+1+ $\Delta m/z$ →D+ $\Delta m/z$	M+1+ $\Delta m/z$ →D+1+ $\Delta m/z$
Glucuronidation	176	473→	473→	473→	473→	458→	458→	458→	458→
		136 m/z;	152 m/z;	312 m/z;	328 m/z;	136 m/z;	137 m/z;	312 m/z;	313 m/z;
		473→	473→	473→	473→	458→	458→	458→	458→
		66 m/z;	82 m/z;	242 m/z;	258 m/z;	66 m/z;	67 m/z;	365 m/z;	366 m/z;
		473→	473→	473→	473→	458→	458→	458→	458→
		189 m/z	205 m/z	365 m/z	381 m/z	189 m/z	190 m/z	242 m/z	243 m/z
Acetylation	42	339→	339→	339→	339→	324→	324→	324→	324→
		136 m/z;	152 m/z;	178 m/z;	194 m/z;	136 m/z;	137 m/z;	178 m/z;	179 m/z;
		339→	339→	339→	339→	324→	324→	324→	324→
		66 m/z;	82 m/z;	108 m/z;	124 m/z;	66 m/z;	67 m/z;	108 m/z;	109 m/z;
		339→	339→	339→	339→	324→	324→	324→	324→
		189 m/z	205 m/z	231 m/z	247 m/z	189 m/z	190 m/z	231 m/z	232 m/z
Sulfonation	80	377→	377→	377→	377→	362→	362→	362→	362→
		136 m/z;	152 m/z;	216 m/z;	232 m/z;	136 m/z;	137 m/z;	216 m/z;	217 m/z;
		377→	377→	377→	377→	362→	362→	362→	362→
		66 m/z;	82 m/z;	146 m/z;	162 m/z;	66 m/z;	67 m/z;	146 m/z;	147 m/z;
		377→	377→	377→	377→	362→	362→	362→	362→
		189 m/z	205 m/z	269 m/z	285 m/z	189 m/z	190 m/z	269 m/z	270 m/z
Methylation	14	311→	311→	311→	311→	296→	296→	296→	296→
		136 m/z;	152 m/z;	150 m/z;	166 m/z;	136 m/z;	137 m/z;	150 m/z;	151 m/z;
		311→	311→	311→	311→	296→	296→	296→	296→
		66 m/z;	82 m/z;	80 m/z;	96 m/z;	66 m/z;	67 m/z;	80 m/z;	81 m/z;
		311→	311→	311→	311→	296→	296→	296→	296→
		189 m/z	205 m/z	203 m/z	219 m/z	189 m/z	190 m/z	203 m/z	204 m/z

Note: *M – m/z value of the molecular ion TFISA 281 m/z; $\Delta m/z$ – changing of m/z due to presence of modification; D – m/z value of the product ions 136, 66, 189 m/z; 16 and 1 – increase in m/z due to hydroxylation/ formation of sulfonic acid

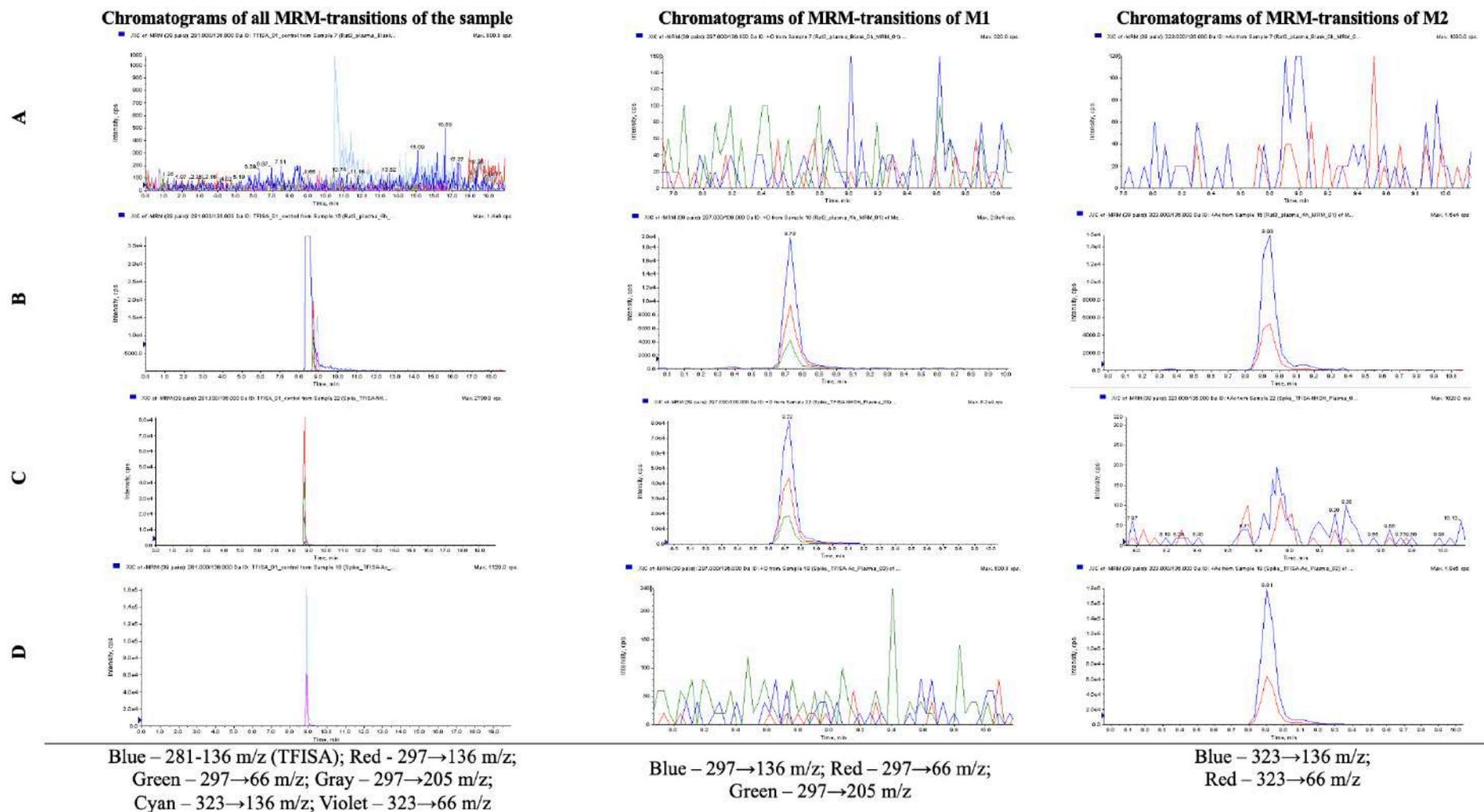


Figure 3. Examples of chromatograms of rat plasma samples before administration of drug (A), rat samples at point 4 h (B), plasma samples with the addition of N-hydroxy-5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide (C) and N-acetyl-5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide (D).

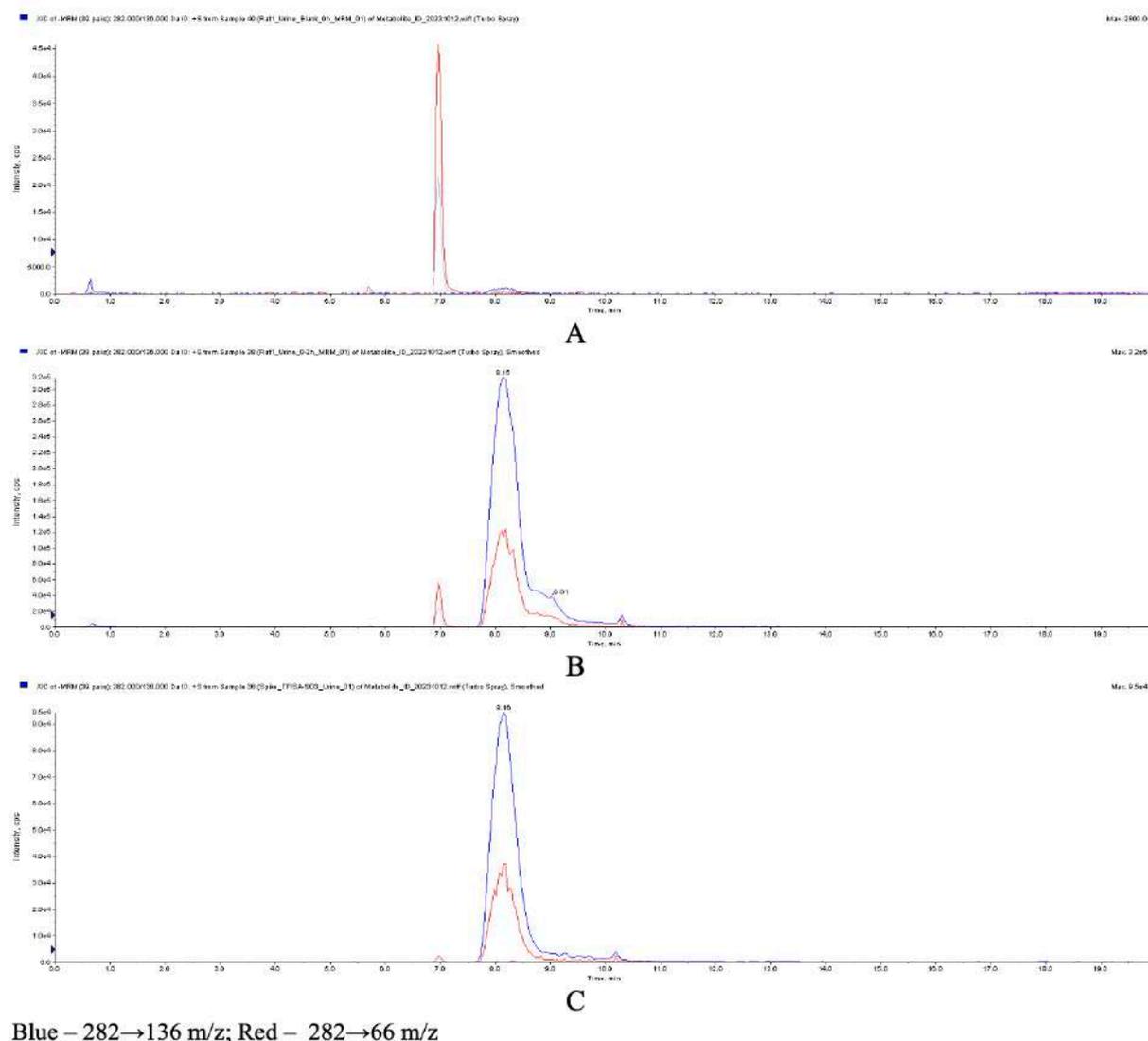


Figure 4. Examples of MRM-chromatograms of rat urine samples before administration of the drug (A) and urine samples in the range of 0-2 hours after administration of the drug (B), urine samples with the addition of 5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonic acid at concentration of 250 ng/ml (C).

The synthesis of the detected metabolites N-hydroxy-5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide (M1), N-acetyl-5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide (M2) and 5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonic acid (M3) was carried out after analyzing the samples of laboratory animals.

The initial product 5-[5-(trifluoromethyl)-1,2-oxazol-3-yl]-furan-2-sulfonyl chloride (Fig. 5-3), which was necessary for synthesis of M1 and M3 metabolites, as well as TFISA, was obtained by the method in (Sibinčić, Kalinin, Sharoyko et al. 2023). The synthesis of this compound was carried out using 4,4-trifluoro-1-(furan-2-yl)-butane-1,3-dione (Fig. 5-1), which was converted by condensation with hydroxylamine hydrochloride into a hydroxy derivative (Fig. 5-2), and then treated with a mixture of thionyl chloride and chlorosulfonic acid. The substance of M1 metabolite was obtained by reaction of the sulfonyl chloride (Fig. 5-3) with an aqueous solution of hydroxylamine in the presence of sodium hydroxide.

Hydroxylamine hydrochloride (5 g, 72 mmol) was dissolved of purified water (5 g, 278 mmol) for this purpose. The resulting solution was cooled to 0°C and 2.88 g of sodium hydroxide (72 mmol) was added. The precipitate was filtered out with formation a 50% aqueous solution of hydroxylamine. Next, 0.42 g of the resulting solution was added drop by drop to cooled at 0°C the acetonitrile solution of 5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonyl chloride (10 mL, concentration 76 mmol/L). The mixture was stirred at room temperature for 3 hours and when cooling had been stopped. The precipitate was filtered out and the filtrate was evaporated. Water was added to dry residue. The obtained solid was filtered, washed with acetonitrile and dried at 50°C. M1 (0.11 g, 0.37 mmol, yield 48%) was obtained as white crystalline substance with a melting point of (97-99)°C.

The structure confirmation results are:

- ¹H-NMR – spectroscopy (400 MHz, DMSO-d₆) δ, ppm: 10.24 (d, J=3.0 Hz, 1H), 9.90 – 9.85 (m, 1H),

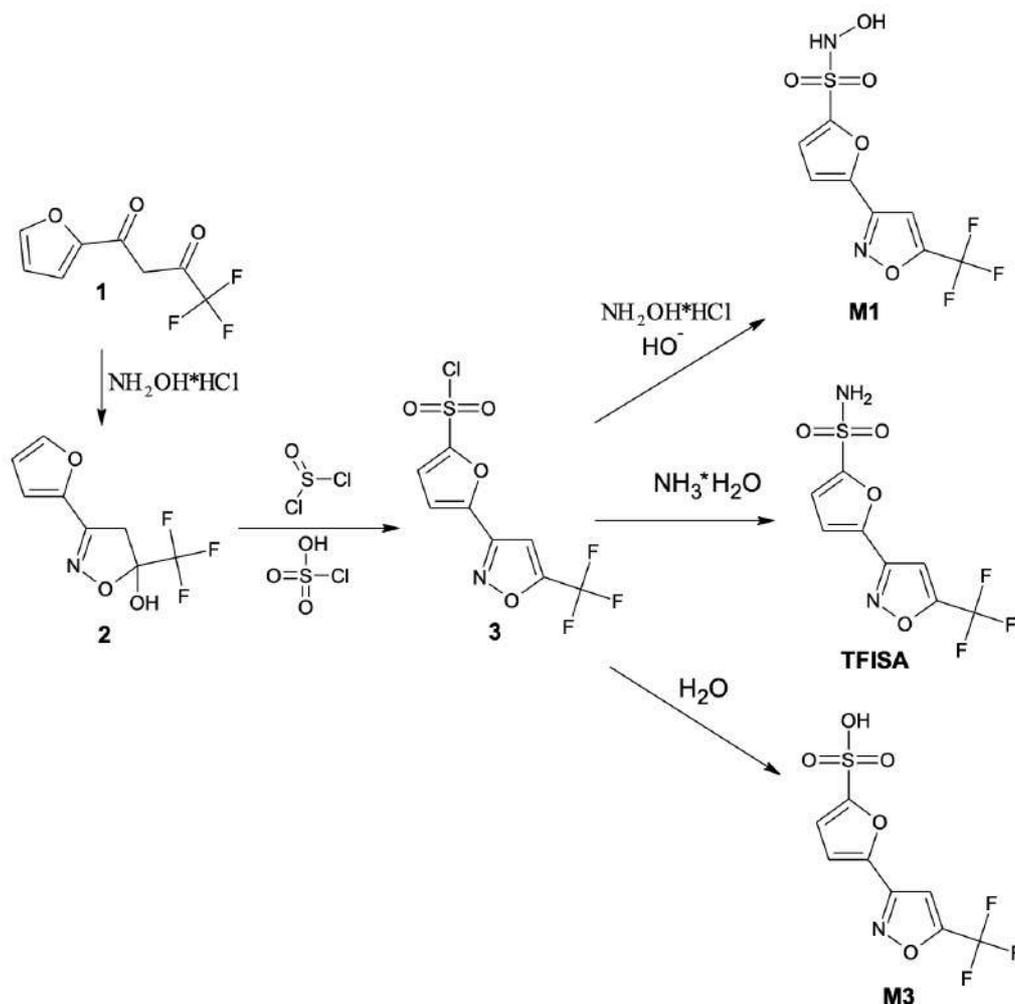


Figure 5. Synthesis scheme of 5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide (TFISA), N-hydroxy-5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide (M1), 5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonic acid (M3). **Note:** **1** – 4,4,4-trifluoro-1-(furan-2-yl)-butane-1,3-dione; **2** – 3-(furan-2-yl)-5-(trifluoromethyl)-4,5-dihydro-1,2-oxazol-5-ol; **3** – 5-[5-(trifluoromethyl)-1,2-oxazol-3-yl]-furan-2-sulfonyl chloride.

8.05 (s, 1H), 7.52 (d, $J=3.7$ Hz, 1H), 7.47 (d, $J=3.4$ Hz, 1H);

- ^{13}C - NMR – spectroscopy (100 MHz, DMSO-d_6) δ , ppm: 158.43, 156.70, 154.67, 148.69, 145.68, 120.91, 115.92, 106.45;

- Mass-spectrometry: the m/z value of the molecular ion $[\text{M} + \text{H}]^+$: 298,9944 m/z ; $\Delta m/z=-2,00$ ppm (calculated for the theoretical value $\text{C}_8\text{H}_6\text{F}_3\text{N}_2\text{O}_5\text{S}^+$: 298,9950 m/z).

The M3 metabolite was synthesized by hydrolysis of the sulfonyl chloride (Fig. 5-3) in water while heating. A 1.09 g of 5-[5-(Trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonyl chloride (3.61 mmol) was dissolved in 1 mL of acetone, and 3 mL of purified water was added. The mixture was stirred under reflux for 3 hours, and then cooled to room temperature and filtered. Next, extraction of impurities was carried out by 5 mL of chloroform. The water layer was separated and evaporated under vacuum, obtaining of 0.56 g (1.97 mol) of beige-colored substance with a melting point of 100-102 °C. The yield of M3 was 55%.

The structure confirmation results are:

- ^1H -NMR – spectroscopy (400 MHz, DMSO-d_6) δ , ppm: 7.91 (s, 1H), 7.22 (d, $J=5.3$ Hz, 1H), 6.64 (d, $J=3.3$ Hz, 1H), 5.50 (s, 1H);

- ^{13}C - NMR – spectroscopy (100 MHz, DMSO-d_6) δ , ppm: 160.55, 157.68 (d, $J=42.1$ Hz), 155.23, 141.16, 118.36 (d, $J=270.0$ Hz), 115.07, 110.58, 105.90; Mass-spectrometry: the m/z value of the molecular ion $[\text{M} + \text{H}]^+$: 283.9836 m/z ; $\Delta m/z=-1,76$ ppm (calculated for the theoretical value $\text{C}_8\text{H}_5\text{F}_3\text{NO}_5\text{S}^+$: 283.9841 m/z).

TFISA substance was reacted with an excess of acetyl chloride at room temperature for synthesis of M2 (Fig 6). The target product was obtained selectively without impurity of the bisacylated molecule of TFISA.

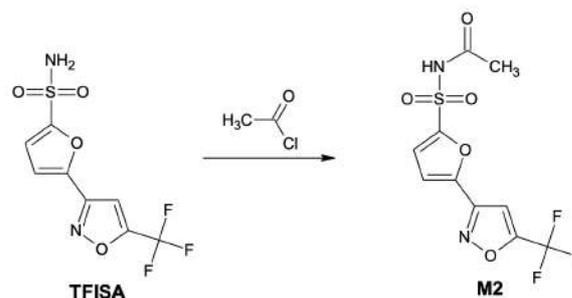


Figure 6. Synthesis scheme of N-acetyl-5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide (M2). **Note:** TFISA - 5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide.

A 3.32 g of acetyl chloride (42.2 mmol) was cooled to 0°C and 0.1 g (0.35 mmol) of TFISA was added to it by small portions. Next, cooling was stopped and the reaction mixture was stirred at room temperature for 10 hours. The resulting solution was decomposed with ice, the precipitate was filtered and washed by water and dried at 50°C. The white crystalline product M2 (0.09 g, 0.28 mmol, yield 82%) with a melting point of 158-159°C was obtained.

The structure confirmation results are:

- ¹H-NMR – spectroscopy (400 MHz, DMSO-D₆) δ, ppm: 12.60 (s, 1H), 8.05 (s, 1H), 7.56 (d, J=3.7 Hz, 1H), 7.51 (dd, J=3.6, 1.0 Hz, 1H), 2.01 (s, 3H);
- ¹³C- NMR – spectroscopy (100 MHz, DMSO-D₆) δ, ppm: 169.64, 158.43, 158.02, 154.58, 149.51, 145.68, 120.72, 115.83, 106.53, 24.10;
- Mass-spectrometry: the m/z value of the molecular ion [M + H]⁺: 325.0101 m/z; Δm/z=-1,54 ppm (calculated for the theoretical value C₁₀H₈F₃N₂O₅S⁺: 325.0106 m/z).

Plasma, blood and urine samples with addition of M1 and M2 at a concentration of 250 ng/mL, as well as rat urine samples with addition of M3 at a concentration of 250 ng/mL were prepared to confirm the structure of the metabolites. It was found after analyzing the spiked samples that retention times and main MRM-transitions of the synthesized substances coincide with these parameters of metabolites in animal samples (Figs 3C, D, Fig. 4C). Analytical signal of N-hydroxy-5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide (M1) was not detected on chromatograms after repeated analysis of processed samples of rat plasma, rat urine and rabbit plasma for comparison with standard samples, but the chromatographic peaks were identified on the MRM-transitions 282→136 m/z and 282→66 m/z of sulfonic acid derivate M3 at t_R=8.2 min. Animal blood samples contained trace amounts of N-hydroxymetabolite M1. This indicated the decomposition of M1 with the formation of M3. It could be the reason for the presence of this compound in fresh urine samples. Animal blood samples contained both M1 and M3. Probably, the degradation of M1 occurs due to oxidation, and antioxidant system of red blood cells in the blood slows down this process (Khokhlov et al. 2023) (Fig. 7).

Therefore, the stability of N-hydroxy-5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide in urine at room temperature was studied on samples spiked by TFISA, M1 and M2 at concentration of 250 ng/mL. Aliquots of the samples were analyzed immediately after preparation, and then 1 hour, 2 hours, 4 hours, 8 hours, 24 hours after preparation.

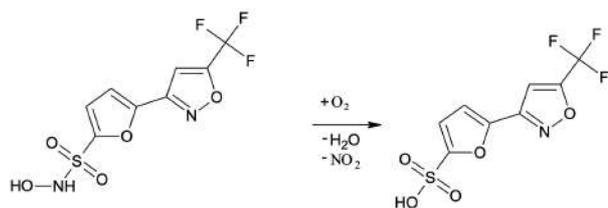


Figure 7. Reaction of decomposition of N-hydroxy-5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide in biological fluids.

Rapid decomposition of M1 was observed during the experiment. Thus, the content of M1 in the urine samples decreased by more than 50% from the initial level after 1 hour of storage, and signal of the analyte was below detection limit of the method after 8 hours of storage (Fig. 8). Thus, the chemical degradation of N-hydroxy-5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide during urine sampling may cause the presence of 5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonic acid.

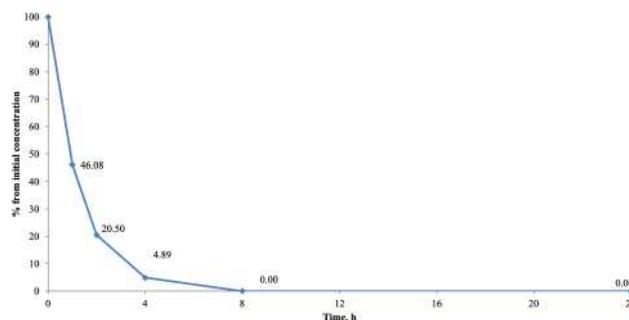


Figure 8. Results of stability study of N-hydroxy-5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide in urine. **Note:** An aliquots from 3 spiked samples were analyzed at each time point.

The drug was administered to second group of the animals for carrying out a confirmatory experiment on the next stage of the study. At the same time, a 10% aqueous solution of ascorbic acid was added to the plasma and urine samples in a volume ratio of 1:5 (Khokhlov et al. 2023). This stabilizer simultaneously slowed down oxidation of M1 and lowered pH for prevention of formation of sulfonic acid.

Plasma, blood and urine samples at concentration of 1 ng/mL spiked by 5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonic acid were additionally analyzed for system suitability test: signal-to-noise ratio of chromatographic peaks at MRM-transitions 282→136 m/z and 282→66 m/z was more than 10:1. The results of comparing the tested animal samples and model mixtures of synthesized compounds are shown in Table 5. Retention times and ratios of chromatographic peak area at the main MRM-transitions of M1 and M2 in rat and rabbit plasma and blood samples matched the parameters of standard substances. Thus, the percentage of coincidence of t_R of M1 and M2 was within the range of 99.96 – 100.20% and 99.93-100.14%, respectively; the percentage of coincidence of peak area ratios of M1 and M2 was within the range of 96.82-100.98% and 94.91-106.04%, respectively. Its values did not exceed the required ranges of 99.0-101.0% (t_R) and 80.0-120.0% (peak area ratio), respectively.

Presence of M1, M2 and M3 was confirmed in urine. Retention times of M1, M2, M3 in test and spiked samples matched by 99.97, 99.98 and 99.72%, respectively. Coincidence of ratios of chromatographic peak area at the main MRM-transitions of M1, M2, M3 was 102.81%, 97.88%, 100.01%, respectively. But M1 was present in this object in trace amounts. It reduced the number of observations. The percentage of matching of the MS2-mass spectra of 297 m/z, 323 m/z and 282 m/z molecular ions of metabolites exceeded 89% (Fig. 9). Plasma and blood samples at 2 h points and urine samples at 6-24 h intervals were used for spectral evaluation, because the concentration of M1, M2 and M3 was maximum in them. Thus, the chemical structure of the identified biotransformation products was confirmed.

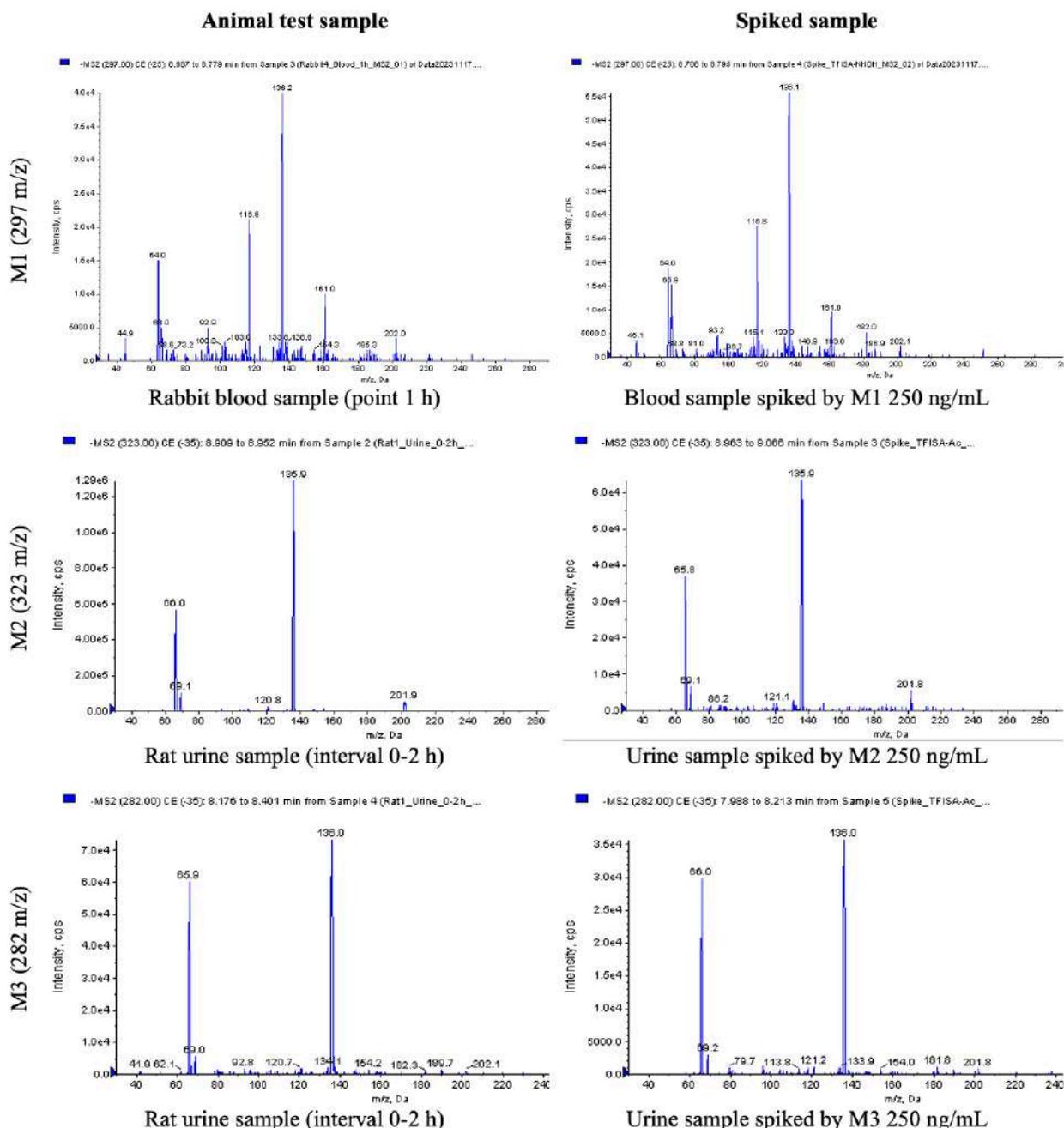


Figure 9. Examples of MS2-mass spectra of 5-[3-(trifluoromethyl)-1,2-oxazole-5-yl]-furan-2-sulfonamide metabolites in test samples and samples spiked by standard substances.

The sulfonic acid derivative M3 was not detected in freshly collected rat and rabbit plasma and blood (Table 5), while the chromatographic peaks area at MRM-transitions $282 \rightarrow 136$ m/z and $282 \rightarrow 66$ m/z in rat urine samples exceeded the area of chromatographic peaks in a spiked by M3 samples at concentration of 250 ng/mL (Fig. 4). This allows us to conclude that this compound is formed due to decomposition of N-hydroxy-5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide during sampling. Thus, M3 is not a product of TFISA biotransformation. Besides, examples of enzymatic deamination of the sulfonamide group during

metabolism have not been previously published (Begou et al. 2020; Lo Faro et al. 2021; Dhandar et al. 2022). Hydroxylation of this functional group during microsomal oxidation is typical for similar structure of 4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonamide (Khokhlov et al. 2023). N-acetylation is one of the main mechanisms of biotransformation of sulfonamide antimicrobials, but the substituent is attached to the aromatic amino group in these drugs (Vree et al. 1985; Ma et al. 2021). Examples of this modification of the sulfonamide group have not been published before.

Table 5. Results of confirmation of the structure of metabolites of 5-[5-(trifluoromethyl)-1,2-oxazole-5-yl]-furan-3-sulfonamide

Parameter	N-hydroxy-5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide			N-acetyl-5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide			5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonic acid		
	t _R , min.	Ratio of peak area 297→136 m/z / 297→66 m/z	% of MS2-mass spectrum matches (Min.-max., %)	t _R , min.	Ratio of peak area 297→136 m/z / 297→66 m/z	% of MS2-mass spectrum matches (Min.-max., %)	t _R , min.	Ratio of peak area 297→136 m/z / 297→66 m/z	% of MS2-mass spectrum matches (Min.-max., %)
Rat plasma samples	Test samples (n=12)	8.74±0.01	1.839±0.140	8.94±0.01	2.459±0.211	-	-	-	-
	Spiked samples (n=6)	8.72±0.00	1.821±0.038	92-96%	8.93±0.02	2.534±0.216	93-97%	-	-
	% of matches*	100.20	100.98	100.08	97.03	-	-	-	-
Rat blood samples	Test samples (n=12)	8.73±0.01	1.855±0.130	8.92±0.01	2.559±0.223	-	-	-	-
	Spiked samples (n=6)	8.73±0.00	1.839±0.085	91-94%	8.93±0.01	2.413±0.091	92-98%	-	-
	% of matches	99.96	100.84	99.93	106.04	-	-	-	-
Rat urine samples	Test samples (n=12)	8.73±0.01	1.926±0.224 (n=6)*	The comparison was not carried out due to low concentrations	8.93±0.01	2.473±0.059	8.13±0.03	2.856±0.163	-
	Spiked samples (n=6)	8.73±0.00	1.873±0.087		8.94±0.01	2.527±0.060	93-98%	8.16±0.02	2.856±0.030
	% of matches	99.97	102.81		99.98	97.88	99.72	100.01	91-93%
Rabbit plasma samples	Test samples (n=12)	8.73±0.01	1.771±0.184	8.92±0.01	2.597±0.136	-	-	-	-
	Spiked samples (n=6)	8.72±0.01	1.827±0.018	93-97%	8.91±0.01	2.646±0.092	89-92%	-	-
	% of matches	100.13	96.82	100.14	98.17	-	-	-	-
Rabbit blood samples	Test samples (n=12)	8.72±0.01	1.739±0.098	8.91±0.01	2.484±0.246	-	-	-	-
	Spiked samples (n=6)	8.72±0.01	1.78±0.04	94-97%	8.91±0.01	2.617±0.062	90-93%	-	-
	% of matches	100.01	97.44	99.93	94.91	-	-	-	-

Note: Plasma and blood samples at the points of 1h, 2h, 4h, 24h, urine samples at intervals of 0-2 h, 2-4 h, 4-6 h, 6-24 h were used as test samples; Mean ± SD is given in each cell of the table; * The number of observations is lower due to the low concentration of the analyte and the absence of a chromatographic peak of the analyte at the MRM-transition 297→66 m/z.

Conclusion

Thus, the studied drug undergoes biotransformation by N-hydroxylation and N-acetylation of the sulfonamide group. The structure of N-hydroxy-5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide and N-acetyl-5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide was confirmed by using ¹H- and ¹³C-NMR spectroscopy and high-resolution mass spectrometry. The content of these compounds in plasma, blood and urine was proved by comparing the retention time, the ratio of the areas of chromatographic peaks at the main MRM transitions on the chromatograms, MS2-mass spectra of the tested animal samples and samples spiked by synthesized substances. The pharmacokinetic properties of the identified metabolites will be studied during the study of bioavailability, distribution, elimination and accumulation of 5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonamide. Its pharmacological activity will also be evaluated. The formation of 5-[5-(trifluoromethyl)-1,2-oxazole-3-yl]-furan-2-sulfonic acid occurs during urine sampling, as

well as during storage of plasma and blood samples without the addition of a stabilizer. The synthesized substance of this compound is necessary for further study of the excretion of drugs with urine and feces.

Conflict of interest

The authors declare the absence of a conflict of interests.

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Data availability

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

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