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Research Article

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

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Abstract

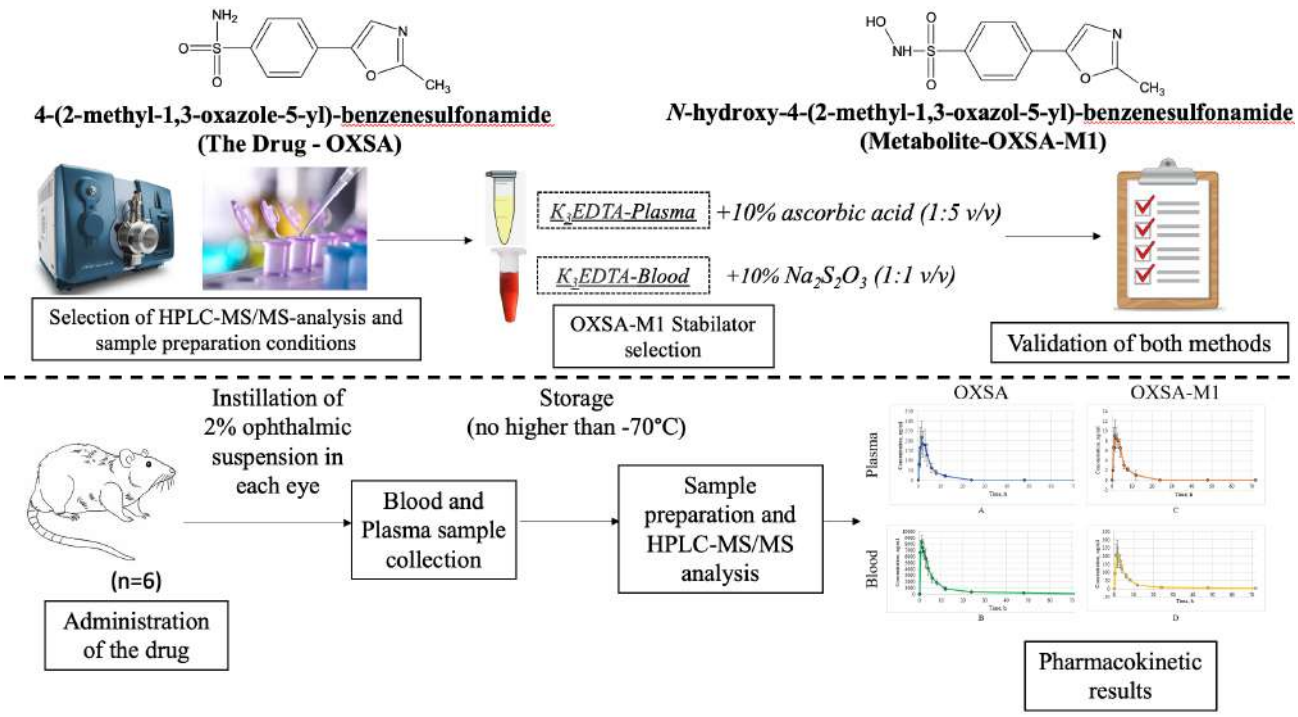
Introduction: Development of new bioanalytical methods is required for studying the systemic exposure of new selective inhibitor of carbonic anhydrase II, 4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonamide, and its N-hydroxymetabolite in plasma and in whole blood. The results of the experiment with a single administration of an ophthalmic suspension of the drug are necessary to optimize the subsequent design of a full pharmacokinetic study.

Materials and Methods: HPLC-MS/MS method was used to measure a concentration of analytes in plasma and whole blood. Chromatographic separation was performed on the Poroshell 120EC-C18 column (50*3.0 mm, 2.7 μm). Pharmacokinetics was studied on 6 Wistar rats weighing 287.50±18.64 g (Mean±SD). Each animal was instilled with 40 μL of the ophthalmic suspension in concentration of 2% in each eye. Blood samples were collected before administration of the drug and 30 min, 1 h, 1 h 30 min, 2 h, 3 h, 4 h, 6 h, 8 h, 12 h, 24 h, 48 h, and 72 h after administration. Non-compartment approach was used for the evaluation of pharmacokinetic parameters.

Results and Discussion: The protein precipitation was chosen for a sample preparation of biological fluids. A solution of ascorbic acid in concentration of 10% was added to plasma, and a solution of sodium thiosulfate in concentration of 10% was added to blood to prevent the degradation of N-hydroxymetabolite of the drug. The analytical range of determination of 4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonamide and its N-hydroxyderivative in blood was 50-10000 ng/mL and 5-1000 ng/mL, respectively, in plasma – 10-2000 ng/mL and 1-200 ng/mL, respectively. The maximum plasma concentration of the studied drug was 264.32±68.47 ng/mL (Mean±SD) 1.92±0.92 h (Mean±SD) after administration, and its metabolite was 10.43±1.79 ng/mL 2.17±1.13 h after administration. The maximum concentration of the drug in blood reached 8705.23±1301.84 ng/mL (Mean±SD) 1.17±0.52 h (Mean±SD) after administration, and the maximum concentration of N-hydroxymetabolite reached 230.00±69.54 ng/mL (Mean±SD) 1.33±0.41 h (Mean± SD) after administration.

Conclusion: The developed methods have been fully validated according to the requirements of Russian and internatonal guidelines and have been successfully used for pharmacokinetic research. It was found that a content of 4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonamide and its main metabolite in whole blood is significantly higher than in plasma.

Graphical Abstract



Keywords

HPLC-MS/MS, stabilization, pharmacokinetics, selective carbonic anhydrase II inhibitor

Introduction

Glaucoma is one of the main causes of irreversible blindness. Increased intraocular pressure (IOP) is the important symptom and the initial element in the pathogenesis of the disease. One of the ways of decreasing IOP in glaucoma is reduction of the secretion of intraocular fluid by inhibiting carbonic anhydrase II of a ciliary body of the eye (Strakhov et al. 2023). The new selective carbonic anhydrase II inhibitor (iCAII) 4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonamide (OXSA) (Fig. 1A) demonstrates pharmacological activity to this isoform in picomolar concentrations. The drug was developed in the form of a ophthalmic suspension in concentration of 2% (Ferraroni et al. 2017). OXSA undergoes biotransformation with formation the single metabolite of N-hydroxy-4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonamide (OXSA-M1) (Fig. 1B).

The pharmacokinetic study is a mandatory part of the preclinical investigation of any drug including ophthalmic drug forms. The initial stage of the study is researching the systemic exposure of the drug and its metabolites in plasma or serum with a single administration (Mironov 2012). The obtained results may be used for determination of the time intervals of excreta collection in the elimination study, correction of time points of organ and tissue collection in the distribution study, as well as calculation of the analytical range of bioanalytical methods for quantification of the analytes in these biological objects. Previously developed drugs of the group iCAII such as dorzolamide (Lo Faro et al. 2021; Kintz et al. 2022) and brinzolamide (Foivas et al. 2016; Madrewar et al. 2022) are able to accumulate in red blood cells. Therefore, additional calculation of pharmacokinetic parameters of OXSA and OXSA-M1 in blood is necessary for full evaluation of systemic exposure of the substances. Bioanalytical quantification methods of the drug and its metabolite in biological fluids have not been developed. The most rapid and selective method for bioassay is HPLC-MS/MS (Khokhlov et al. 2018).

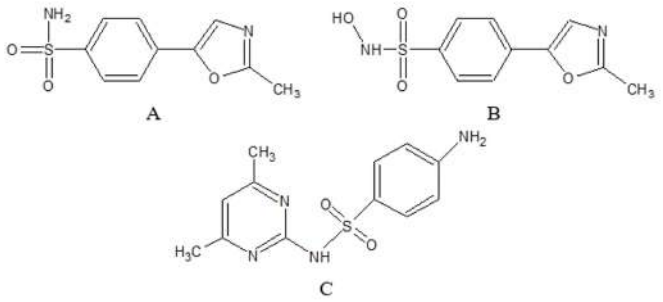


Figure 1. Structures of 4-(2-methyl-1,3-oxazol-5-yl)-benzenesulfonamide (**A**), its metabolite *N*-hydroxy-4-(2-methyl-1,3-oxazol-5-yl)-benzenesulfonamide (**B**) and sulfamethazine (**C**)

Materials and Methods

Design of bioanalytical part

Studied compounds

Substances 4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonamide and N-hydroxy-4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonamide produced by M.V. Dorogov Pharmaceutical Technology Transfer Center of Yaroslavl State Pedagogical University (YSPU) named after K.D. Ushinsky were used as standards.

Reagents

Sulfamethazine (Sulf) (CAS 57-68-1) was applied as an internal standard (Sigma-Aldrich (S6256-25G)) (Fig. 1C). Methanol for LC-MS (CAS 67-56-1; Merck LLC, LiChrosolv hypergrade for LC-MS 1060352500) and formic acid for LC-MS (Thermo Fisher Scientific LLC, Optima LC-MS-Grade A117) were used for mobile phase preparation. Ascorbic acid (CAS 50-81-7, chemically pure grade), sodium sulfite (CAS 7757-83-7, pure for analysis grade), sodium thiosulfate pentahydrate (CAS 10102-17-7, pure for analysis grade), sodium metabisulfite (CAS 7681-57-4, pure for analysis grade), ammonium acetate (CAS 631-61-8, HPLC-Grade) were investigated as stabilizers for OXSA-M1.

Analytical equipment

The development and validation of the methods, as well as the analysis of the rat samples were carried out on a HPLC-MS/MS system, including a liquid chromatograph "Agilent 1260 Infinity" (Germany) (pump G1312B, autosampler G1329B with thermostat G1330B, column thermostat G1316A) and a tandem mass spectrometric detector AB Sciex QTRAP5500 (Singapore) (device control – software "Analyst 1.6.2" (USA), chromatogram processing – software MultiQuant 3.0.5"(USA)).

Validation parameters

Validation of the methods was carried out according to the requirements of Russian (On Approval of the Rules for Conducting Bioequivalence Studies on Medicines in the Eurasian Economic Union. Decision of the Council of the Eurasian Economic Commission № 85, 2016; Mironov A.N. (ed), 2014) and international (ICH Guideline M10 on Bioanalytical Method Validation and Study Sample Analysis 2022) guidelines. The concentration levels of calibration (K1-K8) and control samples (LLOQ, LQC, MQC, HQC), as well as samples for evaluating the effect of dilution are presented in Table 1.

Table 1. The concentration of calibration samples and QC samples

Designation	Concentration, ng/ml			
	Plasma		Blood	
	OXSA	OXSA-M1	OXSA	OXSA-M1
K1 (LLOQ)	10	1	50	5
K2	50	5	250	25
K3	100	10	500	50
K4	250	25	1000	100
K5	500	50	2500	250
K6	1000	100	5000	500
K7	1500	150	7500	750
K8	2000	200	10000	1000
LQC	30	3	150	15
MQC	750	75	3500	350
HQC	1600	160	8000	800
Dil	3200	320	16000	1600

Note: OXSA – 4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonamide; OXSA-M1 – N-hydroxy-4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonamide.

Design of pharmacokinetic part

Animals

The pharmacokinetics of an ophthalmic suspension of 4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonamide in a concentration of 2% was studied on 6 Wistar rats weighing 287.50±18.64 g (Mean±SD) obtained from SMK Stezar LLC (Russian Federation). The study group included 3 males and 3 females. The animals were previously catheterized into the right jugular vein. The study was approved by the Ethics Committee of YSPU named after K.D. Ushinsky (Minutes №1 of 10 September 2023).

Dosing of the drug and sample collection

The administration was carried out by instillation of 40 µL of ophthalmic suspension of the drug in a concentration of 2% into each eye. Blood samples collection was performed before the administration of OXSA (blank sample) and 30 min, 1 h, 1 h 30 min, 2 h, 3 h, 4 h, 6 h, 8 h, 12 h, 24 h, 48 h, 72 h after administration in an amount of 0,2 mL. K_3EDTA was used as an anticoagulant. Each sample was divided into 2 parts: 30 µl of sodium thiosulfate solution 10% was added to 30 µl of whole blood and frozen to a temperature no higher than -70°C. The remaining part was centrifuged for 10 min at 2500 rpm. The plasma was separated,

stabilized by an ascorbic acid solution 10% and frozen.

Statistical analysis

Non-compartment approach was used for the evaluation of pharmacokinetic parameters of OXSA and OXSA-M1. The following pharmacokinetic constants were calculated using R software v. 3.3.2 with package Bear v. 2.7.7: maximum drug concentration in plasma and blood (C_{max}), time-to-peak concentration in plasma and blood (T_{max}), area under the pharmacokinetic «concentration – time» curve from zero to the last blood sampling procedure (AUC_{0-t}), area under the pharmacokinetic curve from time zero to infinity ($AUC_{0-\infty}$), terminal elimination rate constant (K_{el}), half-life elimination of the drug ($T_{1/2}$), and average drug retention time in blood and plasma (mean resident time (MRT)). Descriptive statistics (mean, standard deviation (SD), coefficient of variation (CV), standard error of the mean (SEM)) were calculated using Statsoft Statistica 10.0.1011 software. Pharmacokinetic curves were plotted using Microsoft Excel 2016.

Results and Discussion

The parameters of chromatography and mass spectrometric detection were selected at the initial stage of development of the bioanalytical methods. Chromatographic separation was carried out on a Poroshell 12EC-C18 column (50*3.0 mm, 2.7 µm) with a Zorbax Eclipse Plus C18 pre-column (12.5*2.1 mm, 5.0 µm) at a thermostat temperature of 40°C. The gradient elution parameters are presented in Table 2. Mass spectrometric detection was performed in MRM mode with positive polarity (Table 3).

Table 2. The parameters of gradient elution of OXSA and its metabolite

Mobile phase: 0,1% formic acid aqueous solution – A, methanol – B			
Time, min	**Flow rate, µL/min**	**A, %**	**B, %**
0.00	500	75	25
0.50	500	75	25
2.00	500	20	80
4.00	500	20	80
4.10	500	75	25
7.00	500	75	25

Protein precipitation was used for sample preparation: 125 µL of sulfamethazine solution in methanol (IS solution) was added to 25 µL of plasma, and 500 µL of IS solution was added to 25 µL of blood. Mixtures were vortexed and centrifuged for 5 min at 10000 rpm. The supernatant was injected in HPLC-MS/MS-system. It was found during the method development that N-hydroxy-4-(2-methyl-1,3-oxazol-5-yl)-benzenesulfonamide in samples of biological objects underwent degradation with a formation of 4-(2-methyl-1,3-oxazole-5-yl)-benzenesulfonic acid (Fig. 2). Thus, OXSA-M1 is practically absent in plasma samples after 24 hours of storage at room temperature and 3 freeze/thaw cycles (Fig. 3). Its concentration in blood during storage also decreases below the minimum permissible 85% of the nominal value (Fig.4). Besides, OXSA-M1 is more stable in whole blood than in thawed hemolyzed blood.

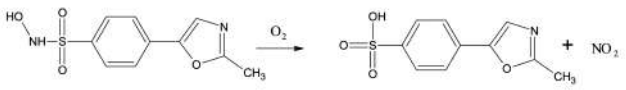


Figure 2. Reaction of oxidative degradation of N-hydroxy-4-(2-methyl-1,3-oxazol-5-yl)-enzene-1-sulfonamide in plasma and blood.

A stabilizer solution was selected by studying short-term stability for 24 hours at room temperature (STS), stability after 3 freeze/thaw cycles (FTS), and stability of the analyte in prepared samples in an autosampler (ASS) to prevent the decomposition of N-hydroxymetabolite of OXSA in plasma (Khokhlov et al. 2018). Initially, an aqueous solution of ammonium acetate 250 mM with pH=3.8 was added to the plasma sample with HQC concentration level (according to levels in Table 1) in a volume ratio of 1:5 and 1:2 (stabilizer: plasma). Acidification of the sample made it possible to stabilize only the prepared samples in the autosampler and also to slow down the degradation of OXSA-M1 in STS and FTS tests (Fig. 3). Preliminary tests of STS and FTS using whole blood and thawed hemolyzed blood showed that the studied metabolite was less exposed to degradation compared to plasma (Fig. 4). This may indicate that the formation of 4-(2-methyl-1,3-oxazol-5-yl)-benzenesulfonic acid occurs due to the oxidation of the N-hydroxysulfonamide group. Therefore the effect of antioxidant solutions on the decomposition process of OXSA-M1 was subsequently studied.

Table 3. The parameters of mass spectrometry detection of OXSA and its metabolite OXSA-M1

№	Analyte	ESI Voltage, V	SRM-transition		DP	EP	CE	CXP
			Q1	Q3				
1	OXSA	5500	239	159	150	10	40	10
2	OXSA (Control)	5500	239	117	150	10	40	10
3	OXSA-M1	5500	255	159	120	10	40	10
4	OXSA-M1 (Control)	5500	255	117	120	10	40	10
5	Sulf (IS)	5500	279	124	120	10	60	10

Nos 1,3 were used for quantification, Nos 2,4 were used for proof of correctness of identification.

Note: OXSA – 4-(2-methyl-1,3-oxazol-5-yl)-benzene-1-sulfonamide; OXSA-M1 – N-hydroxy-4-(2-methyl-1,3-oxazol-5-yl)-benzene-1-sulfonamide; Sulf – sulfamethazine; ESI – electrospray ionization; Q1 – m/z of parent ion; Q3- m/z of product ion; DP – declustering potential; EP-entrance potential; CE – collision energy; CXP – collision cell exit potential.

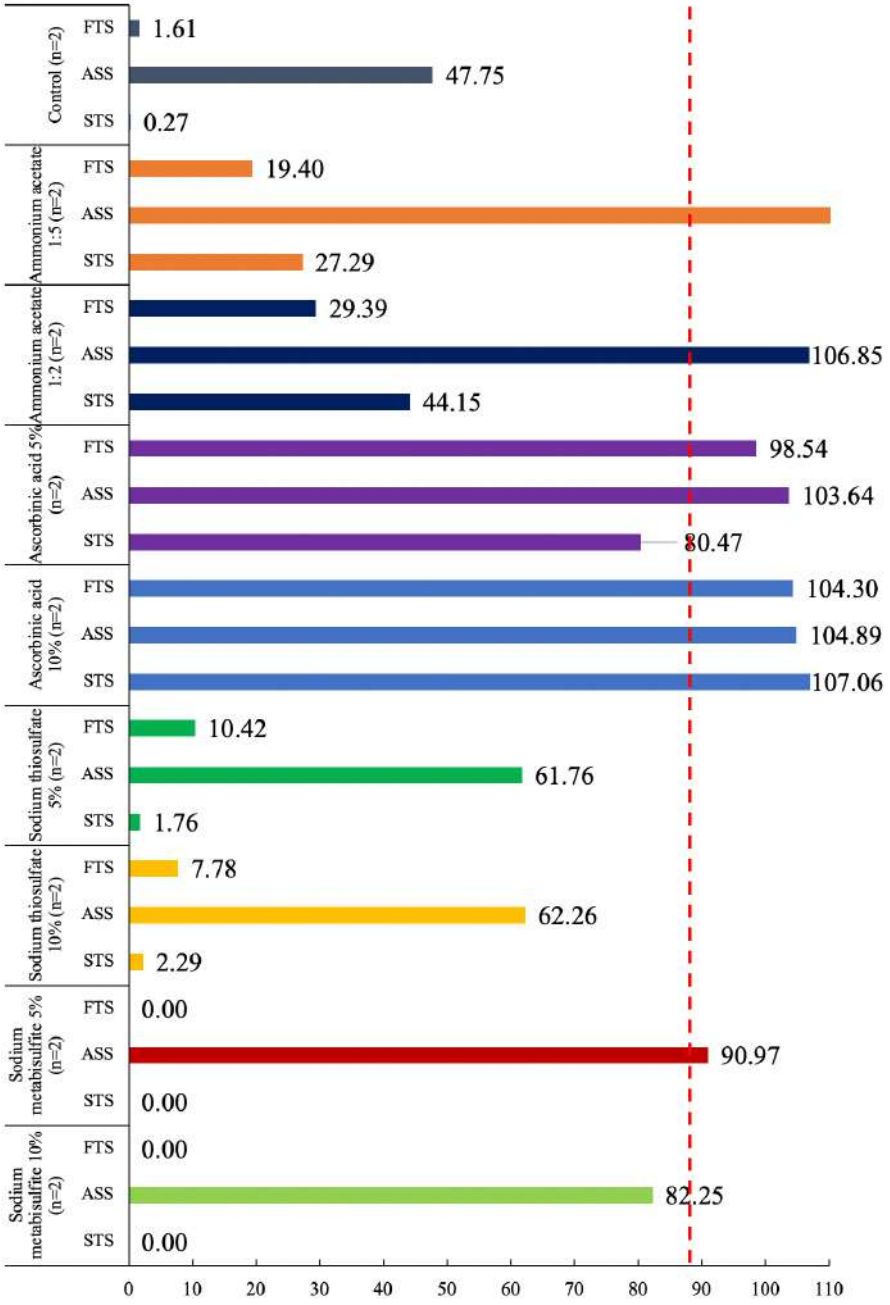


Figure 3. The results of preliminary stability investigation of N-hydroxy-4-(2-methyl-1,3-oxazol-5-yl)-benzene-1-sulfonamide in plasma. ***Note:*** STS – short-term stability; FTS – stability after 3 freeze/thaw cycles; ASS – autosampler stability.

Solutions of ascorbic acid, sodium sulfite, sodium thiosulfate, sodium metabisulfite in concentrations of 5 and 10% were added to plasma at the rate of 20 µL of solution per 100 µL of plasma (1:5, v/v) for the investigation. There were 2 replicates of each kind of stabilized samples. The results of preliminary FTS, STS, ASS tests were within the acceptable range of 85-115% of the nominal value only after using the solution of ascorbic acid at a concentration of 10% (Fig. 3). Therefore, this stabilizer was chosen for subsequent studies. The chromatographic peak OXSA-M1 was absent in samples with the addition of sodium sulfite solution. Therefore these results are not presented in Figure 3.

The antioxidant solutions listed above and 250mM ammonium acetate solution were also added to hemolyzed thawed blood to stabilize OXSA-M1 at the rate of 50 µL of solution per 100 µL of blood (1:2, v/v). STS test was performed using an ice bath. Blood samples, which were stabilized by solutions of ascorbic acid 10%, sodium sulfite and sodium metabisulfite, ammonium acetate, thickened during storage. It did not allow the sample preparation. Therefore, these results are not presented in Figure 4. FTS and STS test results were close to the acceptable concentration range after usage of 10% sodium thiosulfate solution. Subsequent tests were carried out with the addition of $Na_2S_2O_3$ solutions to blood at concentrations of 10% and 20% in a volumetric ratio of 1:1. Also 25 µL of a 5% formic acid solution was added to the prepared methanol deproteinizates after mixing to prevent the formation of sulfonic acid. The obtained results met the established requirements after applying these stabilizers, as well as acidification of the prepared samples (Fig. 4). A 10% $Na_2S_2O_3$ solution in the ratio of 1:1 (v/v) was selected for subsequent tests,

because the signal-to-noise ratio of the chromatographic peaks of OXSA and OXSA-M1 was approximately 2 times higher than after usage of a 20% $Na_2S_2O_3$ solution.

Thus, the preparation of blood samples was carried out as follows: 500 µL of IS solution was added to 25 µL of stabilized blood. The mixture was vortexed and stabilized by 25 µL of a 5% formic acid aqueous solution, re-vortexed and centrifuged for 5 min at 10000 rpm. The supernatant was separated and analyzed. The method of plasma sample preparation of ascorbic acid stabilized plasma samples did not differ from the initial method.

The full validation of the methods was carried out after choosing the optimal conditions of sample storage. The analytical range of quantification of OXSA in blood was 50-10000 ng/mL, and in plasma – 10-2000 ng/mL. Concentrations of OXSA-M1 were measured in the range of 5-1000 ng/mL in blood and 1-200 ng/mL in plasma. The dependence of the ”analyte/internal standard” peak area ratio from the concentration of the studied compounds in blood and plasma was linear. There was no indicated chromatographic peaks in regions of the analytes and internal standard retention time on main and control MRM-traces of chromatograms of blank samples of the studied biological fluids (Figs 5-6).

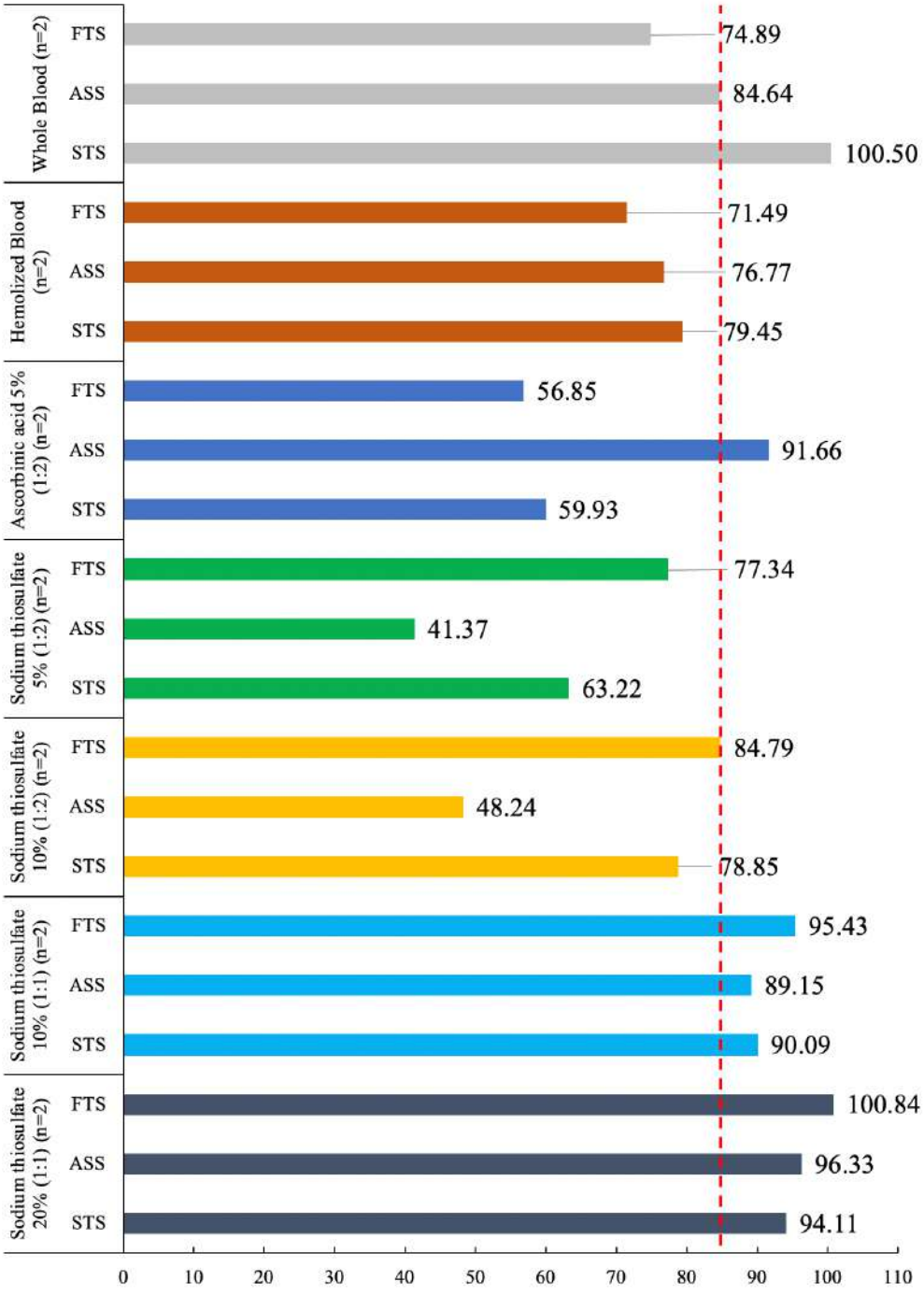


Figure 4. The results of preliminary stability investigation of N-hydroxy-4-(2-methyl-1,3-oxazol-5-yl)-benzene-1-sulfonamide in blood. ***Note:*** STS – short-term stability; FTS – stability after 3 freeze/thaw cycles; ASS – autosampler stability.

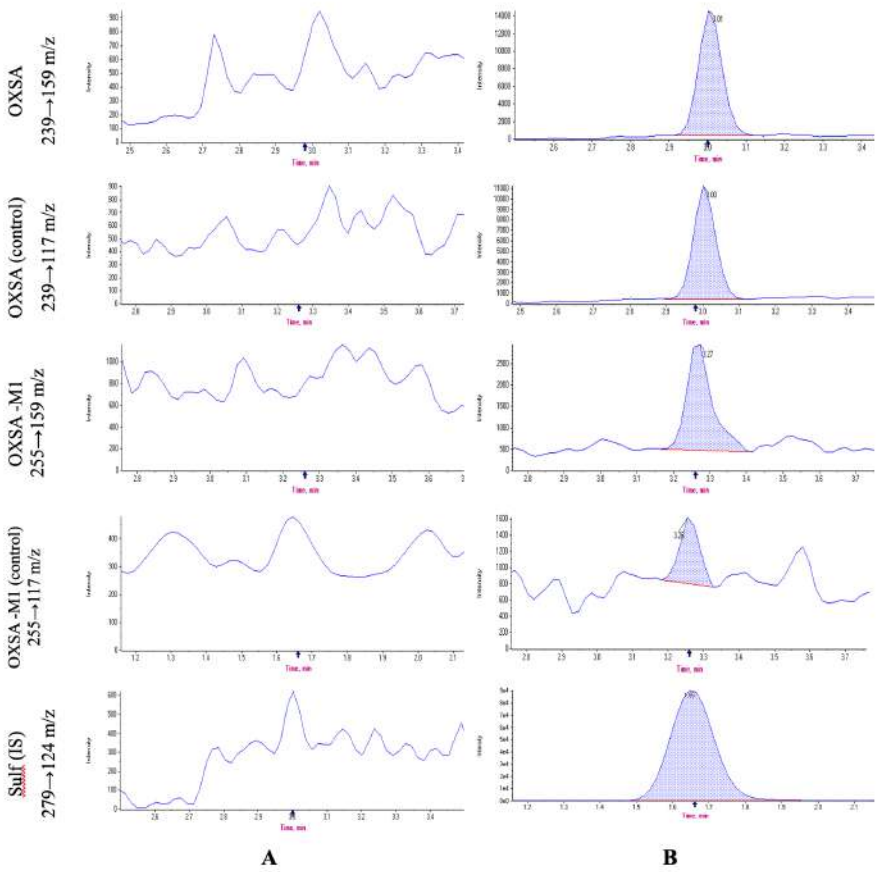


Figure 5. Examples of chromatograms of blank plasma sample (**A**), LLOQ plasma sample (**B**)

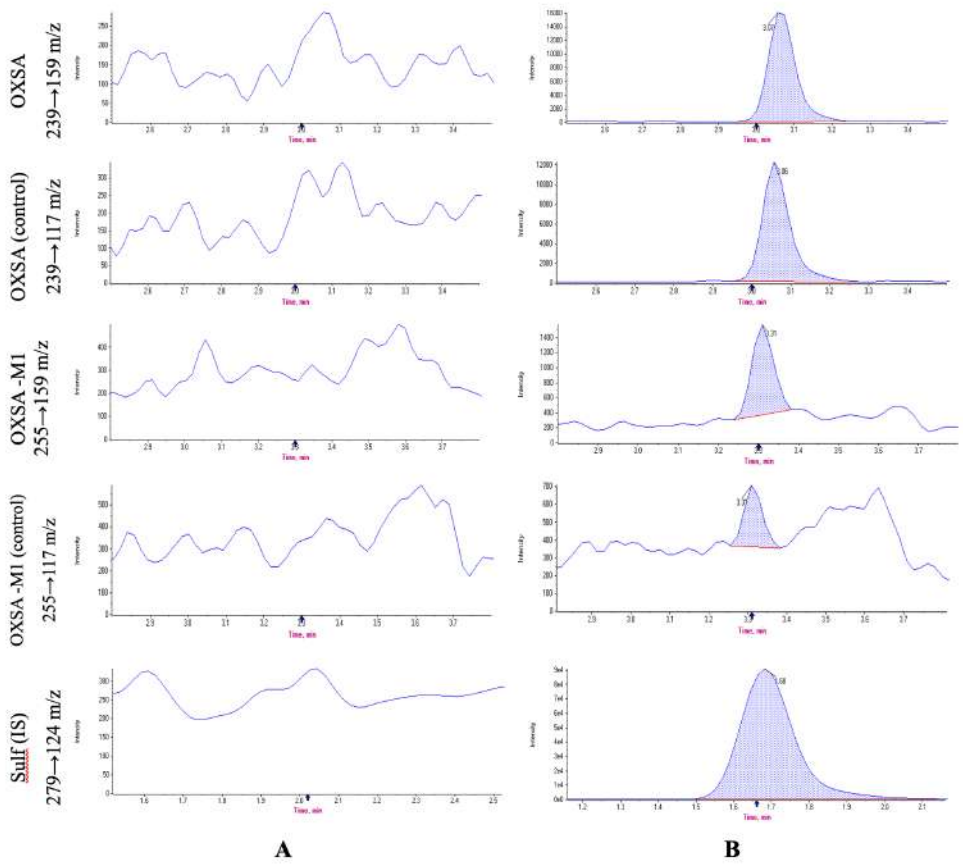


Figure 6. Examples of chromatograms of blank blood sample (**A**), LLOQ blood sample (**B**)

The average value of the relative error in the study of within-run and between-run accuracy of the developed methods, as well as reinjection reproducibility was in the range of 80.0-120.0% for the LLOQ concentration level, and in the range of 85.0-115.0% for the LQC, MQC, HQC concentration levels (Table 4). The coefficient of variation of the concentrations measured in these tests did not exceed 20% at the LLOQ concentration level and 15% at the LQC, MQC, HQC concentration levels. Twofold dilution of blood and plasma samples with Dil concentration level (according to levels in Table 1) by a blank matrix did not affect the accuracy of the determination of analytes in these objects. The matrix effect was studied in two ways on samples with LQC and HQC levels (according to levels in Table 1) prepared using plasma and blood obtained from 6 different rats: by evaluation of accuracy and precision of the measured concentrations (Table 4, Batch 1) (ICH Guideline M10 on Bioanalytical Method Validation and Study Sample Analysis, 2022), as well as by calculation the CV of the normalized matrix factor (NMF) (On Approval of the Rules for Conducting Bioequivalence Studies on Medicines in the Eurasian Economic Union, Decision of the Council of the Eurasian Economic Commission № 85. 2016; Mironov 2014). The results of both tests of the developed methods met the established requirements. There was no carry-over of analytes from the previous sample in both matrices.

Table 4. The results of validation of developed methods

Parameter		Plasma				Blood			
		OXSA		OXSA-M1		OXSA		OXSA-M1	
Selectivity		Interference in the area of analyte retention times in blank samples did not exceed 20% of the LLOQ level and in the area of internal standard retention times did not exceed 5% of the peak area*							
LLOQ		10 ng/mL		1 ng/mL		50 ng/mL		5 ng/mL	
Calibration range (linear dependence)		10-2000 ng/mL		1-200 ng/mL		50-10000 ng/mL		5-1000 ng/mL	
Accuracy and precision		Acc., %	CV, %	Acc., %	CV, %	Acc., %	CV, %	Acc., %	CV, %
Batch 1* (n=6**)	LLOQ	85.82	6.54	92.52	8.21	96.90	13.79	115.67	6.78
	LQC	90.13	8.85	93.56	11.76	105.97	6.07	103.67	7.23
	MQC	94.64	5.15	91.00	7.14	99.87	4.23	90.45	6.94
	HQC	90.57	7.08	90.0	8.44	89.94	4.32	92.37	5.38
Batch 2 (n=6**)	LLOQ	95.65	8.96	110.21	9.96	98.90	11.95	97.33	16.11
	LQC	98.36	6.61	109.71	6.64	101.76	7.10	109.44	12.08
	MQC	98.65	5.01	98.87	5.40	96.23	7.52	97.76	5.55
	HQC	93.54	6.18	95.45	4.12	94.62	7.13	87.47	4.70
Batch 3 (n=6**)	LLOQ	106.56	7.62	104.78	13.13	103.73	13.28	114.33	7.12
	LQC	98.88	6.74	96.58	7.32	87.77	7.60	104.78	3.72
	MQC	92.31	4.60	91.67	5.24	101.72	2.65	102.26	4.88
	HQC	95.74	1.82	95.09	2.08	107.24	14.07	100.52	4.30
Inter-batch accuracy and precision (n=18**)	LLOQ	95.06	10.39	102.92	12.74	99.84	12.59	109.11	12.42
	LQC	95.94	8.66	100.37	10.80	98.50	10.39	105.96	8.39
	MQC	94.92	5.32	93.84	6.83	99.27	5.36	96.82	7.49
	HQC	93.25	5.92	93.51	5.78	97.27	12.22	93.45	7.44
Reinjection reproducibility	LLOQ	87.46	14.58	96.74	11.89	100.23	9.97	102.33	9.20
	LQC	92.22	9.44	95.45	11.04	102.69	5.28	100.44	9.53
	MQC	98.61	6.76	99.83	5.09	99.68	7.91	93.86	4.19
	HQC	93.51	6.87	96.00	8.21	105.82	7.60	86.39	4.09
Dilution integrity (n=6)	2-fold	98.25	9.18	98.68	8.22	107.19	3.17	105.60	2.69
Matrix effect (CV NMF, %)	LQC	7.37		4.90		5.84		4.54	
	HQC	7.49		8.28		3.19		4.05	

* The selectivity evaluation was performed at Batch 1; Matrix effect evaluation accordance to ICH M10 was performed at Batch 1; **number of samples at each concentration level;

***Note*:** OXSA – 4-(2-methyl-1,3-oxazol-5-yl)-benzene-1-sulfonamide; OXSA-M1 - N-hydroxy-4-(2-methyl-1,3-oxazol-5-yl)-benzene-1-sulfonamide; Acc. – relative accuracy; LLOQ – lower limit of quantification; LQC – low concentration quality control samples; MQC – middle concentration quality control samples; HQC – high concentration quality control samples; CV NMF – coefficient of variation of the normalized matrix factor.

The performed validation tests of short-term stability, stability after 3 freeze/thaw cycles, stability in prepared samples in the autosampler, long-term stability in the freezer at a temperature no higher than -70˚C (LTS) meet the acceptance criteria (Table 5). Thus, the selected antioxidant solutions prevent the decomposition of OXSA-M1 during the storage of plasma and blood samples. Blood and plasma samples obtained during the pharmacokinetic study were analyzed at the next stage using the developed methods. The obtained values of the pharmacokinetic parameters of the studied substances are presented in Table 6, and the pharmacokinetic profiles are shown in Figure 7.

Table 5. The results of evaluation of stability of OXSA and OXSA-M1 in plasma and blood

Parameter			Plasma		Blood	
			OXSA	OXSA-M1	OXSA	OXSA-M1
% from initial concentration	STS (24 h, room temperature- for plasma; ice bath – for blood) (n=6*)	LQC	95.71	100.04	106.44	92.56
		HQC	99.01	97.60	98.74	87.93
	FTS (n=6*) (3 cycles)	LQC	96.18	100.16	104.94	93.56
		HQC	94.76	93.02	103.75	90.68
	ASS (48 h at +4°C) (n=6*)	LQC	95.71	87.25	107.28	100.22
		HQC	99.01	100.36	98.68	89.74
	LTS (28 days at temp. no higher than -70˚C) (n=6*)	LQC	98.70	101.56	107.84	93.89
		HQC	97.71	95.18	103.22	88.64

* number of samples at each concentration level

Note: OXSA – 4-(2-methyl-1,3-oxazol-5-yl)-benzene-1-sulfonamide; OXSA-M1 - N-hydroxy-4-(2-methyl-1,3-oxazol-5-yl)-benzene-1-sulfonamide; LQC – low concentration quality control samples; HQC – high concentration quality control samples; STS – short-term stability; FTS –stability after 3 freeze/thaw cycles; ASS – autosampler stability; LTS – long-term stability.

Table 6. The pharmacokinetic parameters of 4-(2-methyl-1,3-oxazol-5-yl)-benzenesulfonamide and its N-hydroxymetabolite in plasma and blood

Parameter			C_{max}, ng/ml	Tmax, h	AUC_{0-t}, ng*h/mL	$AUC_{0-\infty}$, ng*h/mL	T1/2, h	K_{el}, h^{-1}	MRT, h
Plasma	OXSA (n=6)	Mean	264.32	1.92	982.06	1113.38	4.25	0.1760	4.09
		SD	68.47	0.92	281.55	287.32	1.35	0.0502	0.41
		CV	25.90	47.87	28.67	25.81	31.84	28.49	10.07
		SEM	27.95	0.37	114.94	117.30	0.55	0.0205	0.17
	OXSA-M1 (n=6)	Mean	10.43	2.17	45.07	54.26	3.79	0.1942	4.17
		SD	1.79	1.13	8.95	10.79	1.12	0.0468	0.54
		CV	17.16	41.83	19.86	19.89	29.52	24.11	12.96
		SEM	0.73	0.34	3.65	4.41	0.46	0.0191	0.22
Blood	OXSA (n=6)	Mean	8705.23	1.17	57243.17	62829.17	26.02	0.0342	13.11
		SD	1301.84	0.52	15124.99	17844.11	10.85	0.0238	2.45
		CV	14.95	44.26	26.42	28.40	41.68	69.67	18.71
		SEM	531.47	0.21	6174.75	7284.83	4.43	0.0097	1.00
	OXSA-M1 (n=6)	Mean	230.00	1.33	1439.43	1577.36	16.66	0.0787	10.18
		SD	69.54	0.41	396.97	444.47	13.07	0.0763	3.88
		CV	30.23	30.62	27.58	28.18	78.47	96.85	38.07
		SEM	28.39	0.17	162.06	181.45	5.34	0.0311	1.58

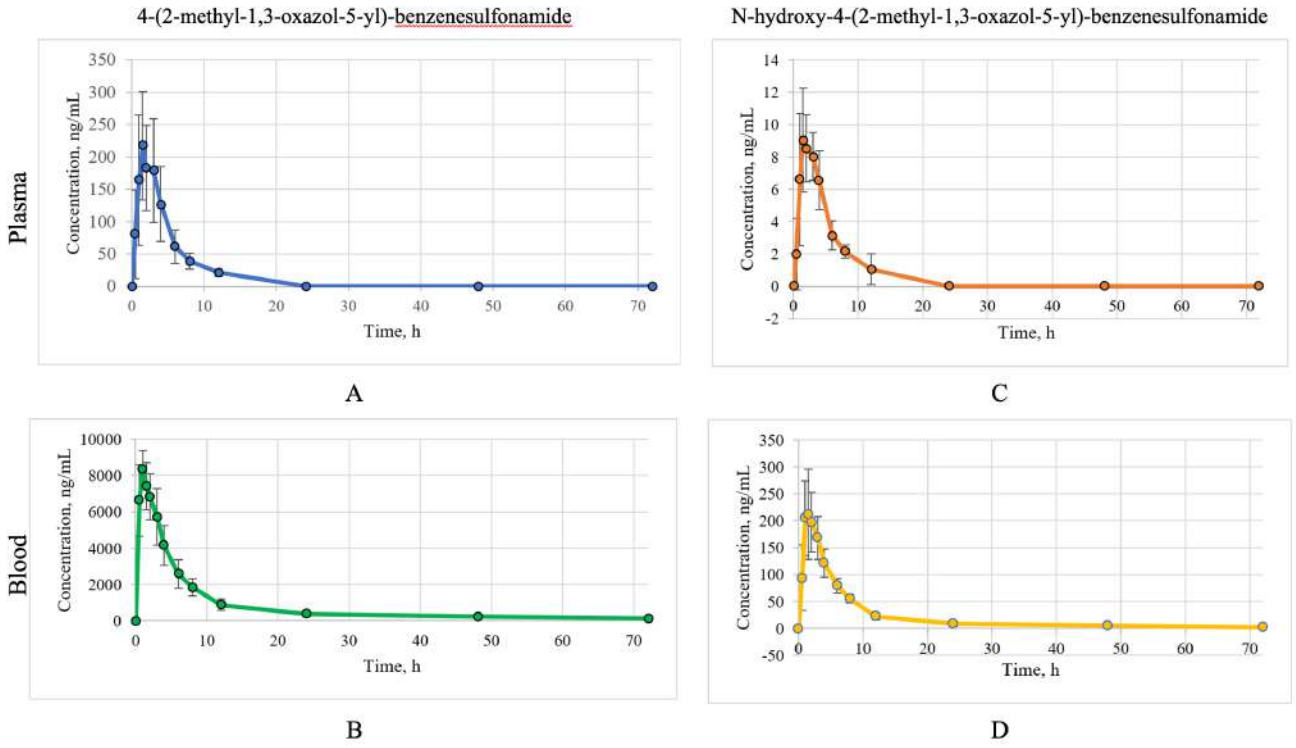


Figure 7. Averaged Pharmacokinetic profiles of 4-(2-methyl-1,3-oxazol-5-yl)-benzenesulfonamide in plasma (A) and in blood (B) and Averaged Pharmacokinetic profiles of N-hydroxy-4-(2-methyl-1,3-oxazol-5-yl)-benzenesulfonamide in plasma (C) and in blood (D) (error intervals: ±SD)

OXSA and its metabolite have monoexponential pharmacokinetic profiles in plasma. The concentration of OXSA increases to a maximum value of 264.32±68.47 ng/mL (Mean±SD) 1.92±0.92 h (Mean±SD) after administration. C_{max} of OXSA-M1 is 10.43±1.79 ng/mL (Mean±SD) which reaches 2.17±1.13 h (Mean±SD) after instillation. It is significantly lower than C_{max} of OXSA. The concentration of analytes in plasma begins to decrease rapidly after 4-hour point (Fig. 7 A,C) and its analytical signal on chromatograms becomes below the lower limit of quantification of the method after 12-hour point (LLOQ – Table 1). The plasma half-life of OXSA is 4.25±1.35 h (Mean±SD) and of OXSA-M1 – 3.79±1.12 h (Mean±SD).

The content of these substances in blood is significantly higher than in plasma. Thus, C_{max} of OXSA in blood is 8705.23±1301.84 ng/mL (Mean±SD), and C_{max} of its metabolite is 230.00±69.54 ng/mL (Mean±SD). The time-to-peak concentration of OXSA in blood comes in 1.17±0.52 hours after administration the drug and in 1.33 ± 0.41 hours for OXSA-M1. Amount of analytes in blood also rapidly decreases after 4-hour point (Fig. 7 B,D). However, the half-life of OXSA and OXSA-M1 in blood is longer than in plasma – 26.02±10.85 hours (Mean±SD) and 16.66±13.07 hours (Mean±SD), respectively. It is due to the deposition of these compounds in red blood cells.

The systemic exposure of the single metabolite OXSA-M1 in both biological fluids is significantly lower compared to OXSA. Probably, most part of active substance is eliminated unchanged. There was no additional increase in a concentration of analytes at the late points of 12-72 h of the pharmacokinetic curve. It indicates the absence of enterohepatic recirculation of these substances. Therefore, there is no necessity to study the bile excretion of OXSA and OXSA-M1.

Thus, the selected analytical ranges of methods are sufficient for subsequent tests with multiple ocular administration of the drug, as well as checking the hypothesis of linearity of pharmacokinetics. Twofold dilution of blood samples may be required with an increase in the volume of the installed drops. It will increase the upper limit of the quantitative determination of OXSA to 20000 ng/mL.

The high value of the upper limit of the quantification (ULOQ) of methods for OXSA of 10000 ng/mL should be chosen in the subsequent study of the distribution of the drug and its metabolite in tissues and urinary excretion, taking into account their high concentrations in blood. The ULOQ level can be reduced to 500 ng/mL, and the lower limit of quantitative determination can be reduced to 0.5 ng/mL for OXSA-M1 in order to detect trace concentrations of this analyte in samples 24, 48 and 72 hours after administration.

Conclusion

The developed methods for the quantitative determination of 4-(2-methyl-1,3-oxazol-5-yl)-benzenesulfonamide and N-hydroxy-4-(2-methyl-1,3-oxazol-5-yl)-benzenesulfonamide in plasma and blood samples have been fully validated in parameters of selectivity, calibration curve and range, within-run and between-run accuracy and precision, dilution integrity, carry-over, reinjection reproducibility, and stability. Stabilization of plasma and blood samples with 10% ascorbic acid solution and 10% sodium thiosulfate solution, respectively, guarantee the trueness of measurement of concentration of OXSA-M1. The methods have been successfully applied in the pharmacokinetic study with a single administration of ophthalmic suspension of OXSA. It was found that the analytes accumulate in red blood cells. Thus, the concentration of the drug in blood reaches high values of

8705.23±1301.84 ng/mL (Mean±SD). The selected sample collection period of up to 72 hours after administration is sufficient for a complete description of the pharmacokinetic properties of the studied substances in plasma and blood.

Conflict of Interests

The authors declare the absence of a conflict of interests.

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References

- Ferraroni M, Lucarini L, Masini E, Korsakov M, Scozzafava A, Supuran CT, Krasavin M (2017) 1,3-Oxazole-based selective picomolar inhibitors of cytosolic human carbonic anhydrase II alleviate ocular hypertension in rabbits: Potency is supported by X-ray crystallography of two leads. Bioorganic & Medicinal Chemistry 25 (17): 4560–4565. https://doi.prg/10.1016/j.bmc.2017.06.054 [PubMed]
- Foivas A, Malenović A, Kostić N, Božić M, Knežević M, Loukas YL, Dotsikas Y (2016) Quantitation of brinzolamide in dried blood spots by a novel LC-QTOF-MS/MS method. Journal of Pharmaceutical and Biomedical Analysis 119(5): 84–90. https://doi.org/10.1016/j.jpba.2015.11.043 [PubMed]
- ICH Guideline M10 on Bioanalytical Method Validation and Study Sample Analysis (2022) https://www.ema.europa.eu/en/documents/scientific-guideline/ich-guideline-m10-bioanalytical-method-validation-step-5_en.pdf. (access date: 15 May 2023)
- Khokhlov AL, Yaichkov II, Dzhurko YuA, Shitov LN, Shitova AM (2018) Methodical approaches to bioassay of substances containing unstable functional groups. Research Results in Pharmacology 4(1): 33–42. https://doi.org/10.3897/rrpharmacology.4.25253
- Kintz P, Gheddar L, Raul J-S (2022) Adverse analytical finding due to red blood cells transfusion: A rare case involving the diuretic dorzolamide. Drug Testing and Analysis 14(10): 1785–1790. https://doi.org/10.1002/dta.3342 [PubMed]
- 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]
- Madrewar BA, Deshpande A, Bhattacharya S (2022) Mini-Review on bioanalytical estimation of brinzolamide. Current Pharmaceutical Analysis 18(3): 265–272. https://doi.org/10.2174/1573412917666210812103414
- Mironov A N (ed.) (2012) Guidelines for Conducting Preclinical Studies of Medicines. Volume 1. Polygraph Plus, Moscow, 944 pp.
- Mironov AN (2014) Guidance on Inspection of Medicines. Volume 1. Polygraph Plus, Moscow, 328 pp. [in Russian]
- On Approval of the Rules for Conducting Bioequivalence Studies on Medicines in the Eurasian Economic Union. Decision of the Council of the Eurasian Economic Commission №85 of November 3, 2016 (2016) http://docs.cntd.ru/document/456026107 (access date: 15 May 2023).
- Strakhov VV, Korsakov MK, Fedorov VN, Vdovichenko VP, Shetnev AA, Popova AA, Volkhin NN (2023) Carbonic anhydrase inhibitors for the treatment of glaucoma. Medical Ethics 1: 44–50. https://doi.org/10.24075/medet.2023.001

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