












# Identification and synthesis of metabolites of the new 4,5-dihydroisoxazol-5-carboxamide derivate

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## Abstract

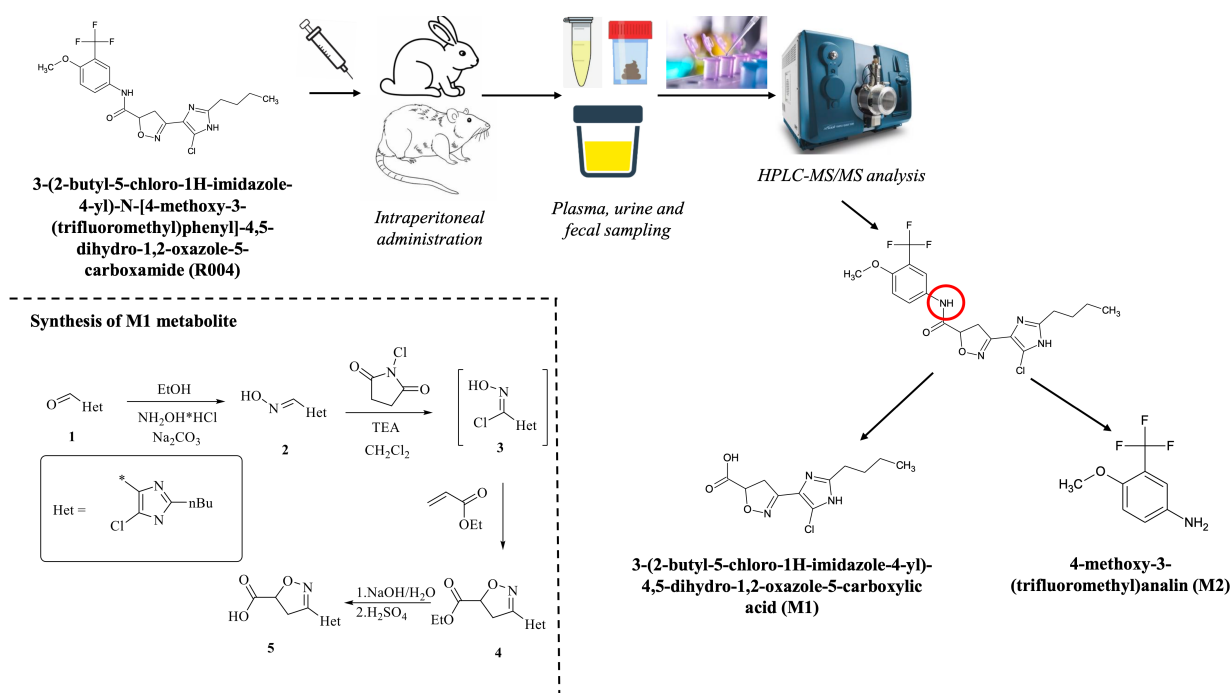
**Introduction:** 3-(2-butyl-5-chloro-1H-imidazole-4-yl)-N-[4-methoxy-3-(trifluoromethyl)phenyl]-4,5-dihydro-1,2-oxazole-5-carboxamide is new antirheumatic drug. It is necessary to identify and synthesize the biotransformation products for its complete pharmacokinetic study.

**Material and Methods:** A biotransformation study was carried out by intraperitoneal administration of the drug to Wistar rats and Soviet Chinchilla breed rabbits. Animal blood sampling was performed before the injection and 0.5 h, 1 h, 2 h, 4 h, 24 h after the injection of the investigated compound. The samples were immediately centrifuged for plasma separation. Urine was simultaneously collected from rats before the administration and at intervals of 0-2 h, 2-4 h, 4-6 h, 6-24 h after administration, faeces – before administration and at intervals of 0-12 h and 12-24 h after administration. The samples were analyzed by HPLC-MS/MS after immediate preparation by adding acetonitrile.

**Results and Discussion:** 3-(2-butyl-5-chloro-1H-imidazole-4-yl)-4,5-dihydro-1,2-oxazole-5-carboxylic acid and 4-methoxy-3-(trifluoromethyl)aniline – hydrolysis products of the active substance were found during the analysis of plasma, urine and fecal samples. The 4,5-dihydro-1,2-oxazole-5-carboxylic acid derivative has been synthesized. The second metabolite is a raw material for production of active pharmaceutical substance. During comparative tests, no significant difference between the retention times, ratio areas of chromatographic peaks at the main MRM-transitions and mass spectra of these metabolites on chromatograms of standard and animal samples was found, which indicates the correct identification of biotransformation products.

**Conclusion:** The studied drug undergoes biotransformation by hydrolysis to form two main metabolites: 3-(2-butyl-5-chloro-1H-imidazole-4-yl)-4,5-dihydro-1,2-oxazole-5-carboxylic acid and 4-methoxy-3-(trifluoromethyl)aniline. The structure of the metabolites was confirmed by comparison with the synthesized standard samples using HPLC-MS/MS.

## Graphical abstract



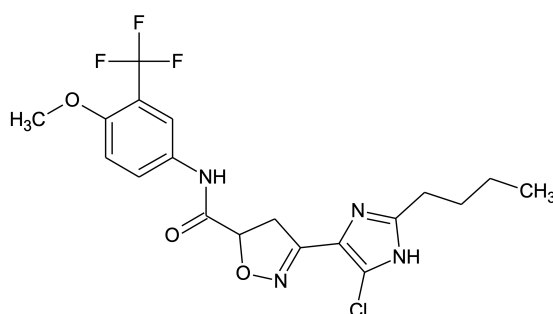
## Keywords

4,5-dihydroisoxazol-5-carboxamide derivative, biotransformation, PAR-2 inhibitor, HPLC-MS/MS, hydrolysis

## Introduction

Rheumatoid arthritis (RA) is characterized by chronic autoimmune process with joint destruction and systemic damage of internal organs. This disease is widespread: up to 1.0% of the population suffers from it. The morbidity of RA increases with age (Scherer et al. 2020). The inhibition of PAR-2 receptors of cell membranes of the immune system is one of the promising targets for the treatment of RA. Both experimental peptide molecules

and low molecular weight compounds are used for realization of this action mechanism (Romanycheva et al. 2023). 3-(2-butyl-5-chloro-1H-imidazole-4-yl)-N-[4-methoxy-3-(trifluoromethyl)phenyl]-4,5-dihydro-1,2-oxazole-5-carboxamide (R004) (Fig.1) is a new selective inhibitor of PAR-2 receptors. This drug has proven its pharmacological activity on a formaldehyde edema model in rats (Korsakov et al. 2023). Thus, R004 surpassed diclofenac sodium in its anti-inflammatory effect.



**Figure 1.** The structure of 3-(2-butyl-5-chloro-1H-imidazole-4-yl)-N-[4-methoxy-3-(trifluoromethyl)phenyl]-4,5-dihydro-1,2-oxazole-5-carboxamide.

Bioanalytical studies of this molecule have not been conducted before. Therefore, the main metabolites must be identified and synthesized before starting the full pharmacokinetic investigation. The biochemical reactions using microsomes, S9 fractions or hepatocytes, and the administration of drugs to laboratory animals are used for this purpose. The detection of biotransformation products is carried out in samples obtained within these experiments (Khokhlov and Pyatigorskaya 2019). The main analytical method for achieving these aims is high-performance liquid chromatography with tandem mass spectrometric detection (HPLC-MS/MS). In this case, it is possible to study the structure of metabolites by measuring the exact values of the mass-to-charge ratio of molecular and daughter ions, using multi-stage fragmentation of analytes ( $MS^n$ ) using ion traps, according to predicted MRM-transitions using fragmentation data of the drug molecular ion (Reddy et al. 2021). The metabolism study of this compound is complicated by the presence of an amide bond in the structure, which may be undergoes to hydrolysis (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 No 85, 2016; Mironov (Ed.) 2014). Therefore, it is necessary to demonstrate the stability of R004 in biological samples in order to distinguish decomposition and biotransformation products.

Thus, **the aim of the study** is identification and synthesis of biotransformation products of 3-(2-butyl-5-chloro-1H-imidazole-4-yl)-N-[4-methoxy-3-(trifluoromethyl)phenyl]-4,5-dihydro-1,2-oxazole-5-carboxamide.

## Materials and Methods

### Design of synthesis experiments

Synthesis of 3-(2-butyl-5-chloro-1H-imidazole-4-yl)-N-[4-methoxy-3-(trifluoromethyl)phenyl]-4,5-dihydro-1,2-oxazole-5-carboxamide (99.2%) and its main metabolite was performed in M.V. Dorogov Pharmaceutical Technology Transfer Center of Yaroslavl State Pedagogical University (YSPU) named after K.D. Ushinsky. Substance of 4-methoxy-3-(trifluoromethyl)anilin (CAS 393-15-7) was produced by Alfa Aesar (USA).

Organic reagents were used without further purification: 2-Butyl-5-chloro-1H-imidazole-4-carbaldehyde (CAS 83857-96-9, 95%, A Chemtek Inc., USA), N-Chlorosuccinimide (CAS 128-09-6, 98%, Sigma-Aldrich, USA), acrylic acid ethyl ester (CAS 140-88-5, 99%, Sigma-Aldrich, USA), triethylamine (CAS 121-44-8,  $\geq 99\%$ , Sigma-Aldrich, USA), ethanol (96%, Ferain, Russian Federation), and methylene chloride (chemical pure, Vecton, Russian Federation). Inorganic substances were also applied without additional purification: hydroxylamine hydrochloride ( $\geq 99\%$ , OC «Vecton», Russian Federation), sodium hydroxide (chemical pure, OC «Vecton», Russian Federation), sodium carbonate (chemical pure, Vecton, Russian Federation), sodium sulfate anhydrous (chemical pure, Vecton, Russian Federation), phosphorus oxychloride ( $\geq 99\%$ , Exotec, Russian Federation).

The following analytical methods were applied for confirmation of the structure of synthesized compounds:

- $^1H$ -NMR and  $^{13}C$ -NMR- spectroscopy (“Varian UNITY Plus – 400” (400 MHz) NMR-spectrometer, Varian LLC, USA);
- High resolution mass spectrometry (Bruker Daltonics micrOTOF-II mass spectrometer, Bruker Daltonics GmbH, USA);
- Measurement of melting/decomposition temperature (Buchi M-560 melting point instrument, Büchi Labortechnik AG, Switzerland).

### Analytical equipment and reagents

The investigation was performed using HPLC-MS/MS system (Analyst 1.6.2 software (AB Sciex, USA)) including an Agilent 1260 Infinity chromatograph (Agilent Technologies, Germany) and an AB Sciex QTRAP5500 tandem mass spectrometric detector (AB Sciex, Singapore). MultiQuant 3.0.5 software was applied for chromatograms integration. Predicted MRM-transitions of putative metabolites was created by LightSight 2.3 software (AB Sciex, USA).

The following reagents were used for analysis:

- Formic acid – Optima LC-MS-Grade, Thermo Fisher Scientific, USA;
- Acetonitril – Optima LC-MS-Grade, Fisher Chemicals, Belgium;
- Dimethylsulfoxide – chemical pure, Ekos-1 JSC, Russian Federation;
- Ammonium acetate – HPLC-Grade, High Purity Laboratory Chemicals Pvt. Ltd, India;
- Glacial acetic acid – chemical pure, JSC Base №1 of Chemical Reagents, Russian Federation;
- Aqueous ammonia – especially pure, JSC Base №1 of Chemical Reagents, Russian Federation.

### Sample preparation and analysis

An aliquot of 100  $\mu$ L of acetonitrile was added to 20  $\mu$ L of stabilized plasma and urine samples. The mixture was centrifugated at 10000 rpm and  $+4^\circ C$  for 5 min after vortexing. Supernatant in volume 1  $\mu$ L was injected to the chromatography system. Homogenates with acetonitrile in a ratio of 1:1 (weight/volume) were prepared from feces. They were centrifugated at 10000 rpm and  $+4^\circ C$  for 5 min, and 200  $\mu$ L of acetonitrile was added to 50  $\mu$ L of the supernatant. The mixture was also centrifugated and analyzed by HPLC-MS/MS.

The reversed phase gradient chromatography using Poroshell 120EC-C18 (50\*3.0 mm, 2.7 microns) chromatographic column with Zorbax Eclipse Plus C18 pre-column (12.5\*2.1 mm, 5.0 microns) was applied for sample analysis (Table 1). HPLC-MS-grade formic and acetonitril was used for mobile phase preparation.

**Table 1.** The parameters of gradient elution of method of identification metabolites of 3-(2-butyl-5-chloro-1H-imidazole-4-yl)-N-[4-methoxy-3-(trifluoromethyl)phenyl]-4,5-dihydro-1,2-oxazole-5-carboxamide

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

Mobile phase: 0,1% aqueous solution of formic acid – A, acetonitril – B. Flow rate: 500  $\mu$ L/min. Column temperature:  $40^\circ C$

Mass spectrometric detection was performed in MRM-mode with positive polarity. Eluate was ionized by electrospray under voltage 5500V and source temperature 650° C.

Sensitivity check of the HPLC-MS/MS-system was carried out by analysis spiked plasma, urine and feces samples at concentration of R004 of 1 ng/mL before beginning the batch. The signal-to-noise ratio of the R004 chromatographic peaks at MRM-transitions 445→198 m/z, 445→226 m/z, 445→281 m/z, 445→387 m/z was at least 10:1.

The concentration of the R004 in the samples for stability tests was 500 ng/mL (500 ng/g for feces). It was achieved by adding dimethylsulfoxide standard analyte solutions to blank matrix in a volume ratio of 1:19. Solutions of CH<sub>3</sub>COONH<sub>4</sub> with pH = 4.0; 5.0; 6.0; 8.0 were used in stability study. The pH value of 250 mM ammonium acetate solutions was adjusted by adding glacial acetic or aqueous ammonia.

### Animals

The 6 Wistar rats weighing 243.67±22.73 g (M±SD) and 6 Soviet Chinchilla rabbits weighing 2.59± 0.23 kg (M±SD) from SMK Stezar LLC Nursery (Russian Federation) was used for biotransformation study of R004. Three males and three females of each species were included in the experimental group. Catheterization of rat into the right jugular vein was performed before drug administration. The rabbit blood samples were collected from the ear vein.

The study was approved by the Ethics Committee of YSPU named after K.D. Ushinsky (Minutes №1 of 29 January 2024).

### Dosing of the drug and sample collection

The substance of R004 was injected intraperitoneally. Drug suspension was obtained using solution consisting of 1% tween-80 and 0.9% sodium chloride. The dosage was equal for rats and rabbits – 50 mg/kg. Blood was collected in volume of 0.2 mL in capillary K<sub>3</sub>EDTA-tube. There were following time points for both species: before administration and 0.5 h, 1 h, 2 h, 4 h, 24 h. The sample was centrifuged for 10 min at 2500 rpm and a temperature of +4°C. Obtained plasma was stabilized by addition of ammonium acetate solution 250 mM (pH=4.0) in volume ratio 1:5 (stabilizer: plasma). It was necessary to prevent *in-vitro* hydrolysis of R004.

Rat excreta sampling was performed using metabolic cells. Urine was collected before administration and at intervals of 0-2 h, 2-4 h, 4-6 h, 6-24 h after administration of the drug; feces were collected before administration and at intervals of 0-12 h and 12-24 h after administration of the drug. Feces were immediately homogenized by acetonitrile without any stabilizers. Ammonium acetate solution 250 mM (pH=4.0) was added to urine samples in volume ratio 1:5 (stabilizer: urine)

There were two groups, which included 3 individuals of each species. The first group was used for the preliminary detection of biotransformation products. The experiment on the second group was conducted for confirmation of the coincidence of the structure of the found metabolites with those of the synthesized substances. Analysis of fresh samples of second stage was also necessary due to hydrolytic instability of R004.

In total, the following samples were analyzed: 36 rat plasma samples, 30 rat urine samples, 18 rat feces

samples, and 36 rat rabbit samples. Every group of samples included 6 control samples. The total number of observations was 120 (including 30 control observations).

### Statistical analysis

All statistical calculations were carried out using Microsoft Excel 2016 (Microsoft Corporation, USA). The matching of quantitative parameters of metabolite and synthesized substances was carried out by comparison of their mean values (formula 1):

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

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

Dispersion measure of the data shown in the table 6 is standard deviation (SD).

Calculations during stability study of R004 in plasma, urine and feces was performed by external standard method (formula 2): the mean value of chromatographic peak area of R004 (MRM: 445→226 m/z) at test sample (Mean S<sub>test sample</sub>) was compared with the mean value of chromatographic peak area of R004 in fresh samples (Mean S<sub>fresh sample</sub>):

$$\% \text{ of initial concentration} = \frac{\text{Mean S}_{\text{test sample}}}{\text{Mean S}_{\text{fresh sample}}} \times 100\% \quad (2)$$

### Criteria of comparison of analytical signals of metabolites and synthesized substances

Comparison of metabolites in animal and standard spiked samples was performed by the following parameters (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):

- Coincidence of presence of chromatographic peaks on main MRM-transitions. In this case, chromatographic peaks in the blank sample should be absent (qualitative parameter);
- Deviation of retention times of chromatographic peak ( $t_R$ ) of standard should be no more than ±1% from  $t_R$  of the metabolite (quantitative parameter – % of match – 99-101%);
- Deviation of ratio of chromatographic peak areas at the main MRM-transitions of standard should be no more than 20% from the area ratio of the metabolite (quantitative parameter – % of match – 80-120%);
- Matching of MS<sub>2</sub>-mass spectra of metabolite and standard should be no less than 85%.

The matching of quantitative parameters of metabolite and synthesized substances was carried out by comparison of their mean values (formula 3):

$$\% \text{ of match} = \frac{\text{Mean of parameter} \times \text{test animal sample}}{\text{Mean of parameter} \times \text{spiked standard sample}} \times 100\% \quad (3)$$

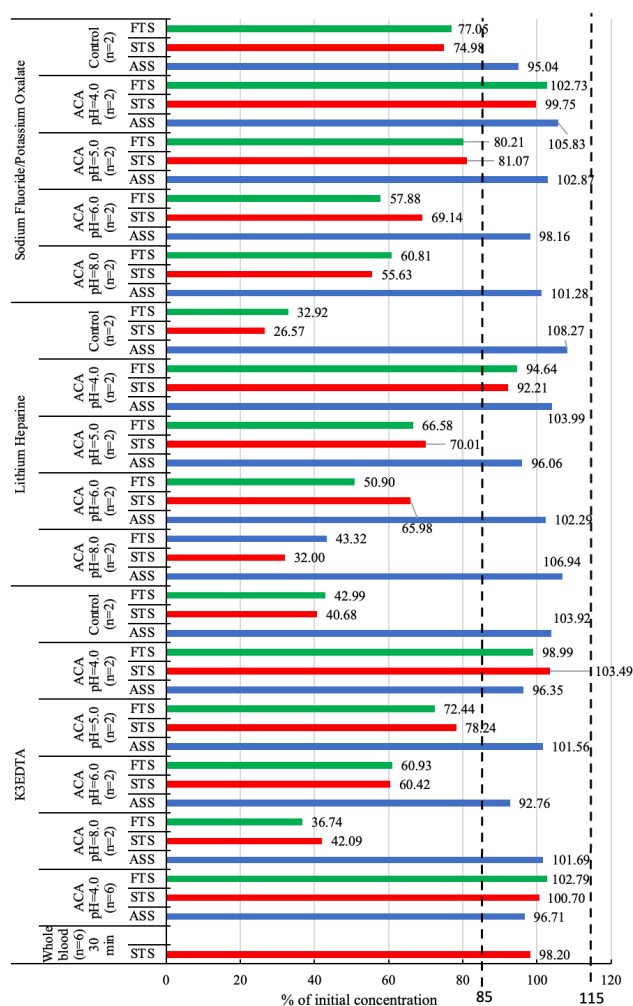
**Note:** parameter - retention times of chromatographic peaks; ratio of chromatographic peak areas at the main MRM-transitions.

Calculations during stability study of R004 in plasma, urine and feces was performed by external standard method (formula 4): the mean value of chromatographic peak area of R004 (MRM: 445→226 m/z) at test sample (Mean S<sub>test sample</sub>) was compared with the mean value chromatographic peak area of R004 in fresh samples (Mean S<sub>fresh sample</sub>):

$$\% \text{ of initial concentration} = \frac{\text{Mean S}_{\text{test sample}}}{\text{Mean S}_{\text{fresh sample}}} \times 100\% \quad (4)$$

## Results and Discussion

The selection of sampling and storage conditions was carried out due to the instability of the R004 molecule to hydrolysis in order to distinguish biotransformation products from the *in-vitro* decomposition products. Tests were performed on the short-term stability (STS) of R004 at room temperature, stability during 3 freeze/thaw cycles (FTS), and stability of prepared samples in an autosampler (ASS) in each studied biological object (Khokhlov and Pyatigorskaya 2019). The usage of  $K_3EDTA$ , lithium heparin and a mixture of sodium fluoride and potassium oxalate in plasma studies did not completely prevent the hydrolysis of the analyte. Application of combination of sodium fluoride and potassium oxalate with high antiesterase activity (Khokhlov and Pyatigorskaya 2019) provided the best results of STS (74.98±8.02% of the initial value) and FTS (77.05±5.45% of the initial value) (Fig. 2). However, these concentration values were not within the required range of 85-115% of the initial value. The addition of ammonium acetate solution with pH=4.0 in a volume ratio of 1:5 was allowed to stop hydrolysis using all anticoagulants.  $K_3EDTA$  was selected for further research, because 0.2 mL capillary tubes with this compound are the most commercially available. The short-term stability of R004 in whole blood was also

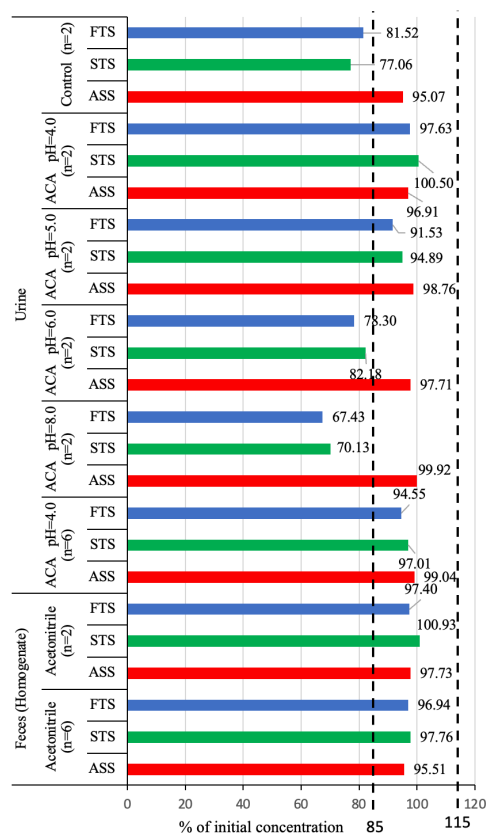


**Figure 2.** The results of stability study of R004 in plasma and blood samples. **Note:** ACA – ammonium acetate solution; STS – short-term stability; FTS – stability during 3 freeze/thaw cycles; ASS – stability of prepared samples in an autosampler; Results – mean values of percent

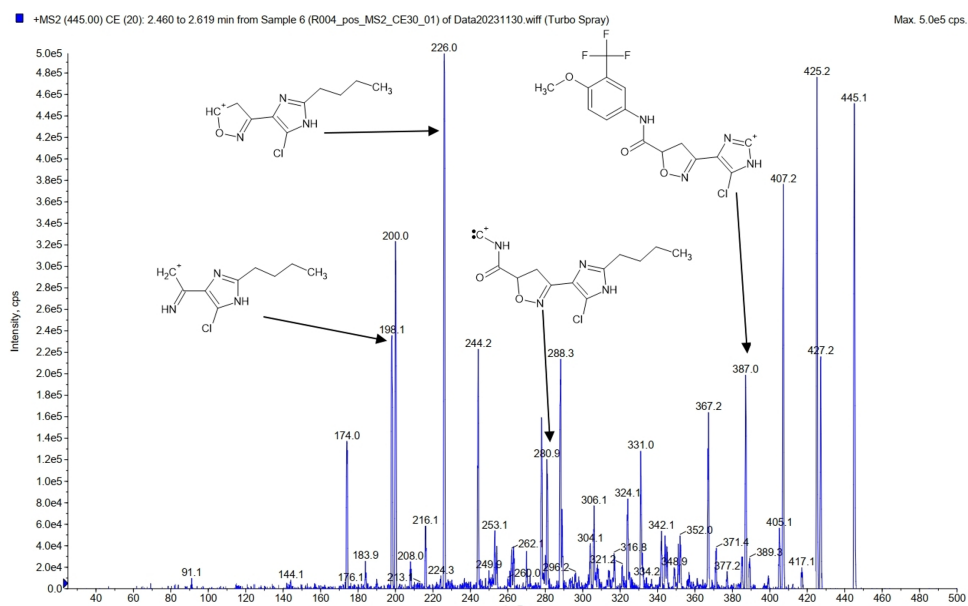
studied. The analyte content remained within the required range for at least 30 minutes (Fig. 2). This time is sufficient for plasma separation.

R004 was more stable in urine samples: after 24 hours of storage at room temperature, 77.06±3.95% of the initial concentration remained in the samples, and after 3 cycles of freezing/thawing – 81.52±1.01% of the initial concentration (Fig. 3). The addition of acidified solutions of ammonium acetate with pH=4.0 and pH=5.0 completely stopped hydrolysis (Fig. 3). The solution with pH=4.0 was also chosen to stabilize the samples, as well as for plasma. Decomposition of R004 in acetonitrile homogenates of feces was not observed: the results of the STS and FTS tests were within the permissible range of 85-115% of the initial value. Therefore, auxiliary measures for its storage were not required. All prepared samples with acetonitrile were also stable in the autosampler: the addition of the organic solvent completely stopped the hydrolysis of R004.

The identification of metabolites was carried out after confirmation of the absence of decomposition of R004 in the collected samples. Product ions 198 m/z, 281 m/z, 226 m/z, 387 m/z, which reflect the main fragments of the R004 structure, were selected to create the screening method (Fig. 4). Changing m/z of these ions makes it possible to accurately determine the localization of the modification. The following biotransformation ways were predicted: hydroxylation and dihydroxylation, N-oxidation, demethylation, demethylation with hydroxylation, and dehydration of 4,5-dihydro-1,2-oxazole cycle and hydrolysis (Table 2).



**Figure 3.** The results of stability study of R004 in urine and feces samples. **Note:** ACA – ammonium acetate solution; STS – short-term stability; FTS – stability during 3 freeze/thaw cycles; ASS – stability of prepared samples in an autosampler. Results – mean values of percent of initial content of R004 of 2 screening analyses (n=2) or 6 confirmatory analyses (n=6).



**Figure 4.** Mass spectrum of 3-(2-butyl-5-chloro-1H-imidazole-4-yl)-N-[4-methoxy-3-(trifluoromethyl)phenyl]-4,5-dihydro-1,2-oxazole-5-carboxamide (MS2 mode; positive polarity; CE = 20eV).

**Table 2.** Predicted MRM-transitions of metabolites of 3-(2-butyl-5-chloro-1H-imidazole-4-yl)-N-[4-methoxy-3-(trifluoromethyl)phenyl]-4,5-dihydro-1,2-oxazole-5-carboxamide

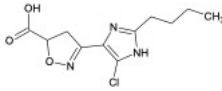
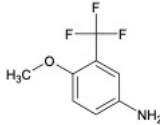
Modification	Difference of m/z	Predicted MRM-transitions		
		Molecular ion $M+\Delta m/z \rightarrow m$	Product ions	
			Non-modified ions $M+\Delta m/z \rightarrow m$	Modified ions $M+\Delta m/z \rightarrow m+\Delta m/z$
Control (R004)	-	445 m/z	198 m/z, 281 m/z, 226 m/z, 387 m/z	-
Hydroxylation /N-oxidation	16	461 m/z	198 m/z, 281 m/z, 226 m/z, 387 m/z	214 m/z, 297 m/z, 242 m/z, 403 m/z
Dihydroxylation/ Hydroxylation +N-oxidation	32	477 m/z	198 m/z, 281 m/z, 226 m/z, 387 m/z	+1 oxygen: 214 m/z, 297 m/z, 242 m/z, 403 m/z +2 oxygens: 230 m/z, 313 m/z, 258 m/z, 419 m/z
Methylation	14	459 m/z	198 m/z, 281 m/z, 226 m/z, 387 m/z	212 m/z, 295 m/z, 240 m/z, 401 m/z
Demethylation	-14	431 m/z	198 m/z, 281 m/z, 226 m/z, 387 m/z	184 m/z, 267 m/z, 373 m/z, 212 m/z Only demethylation: 184 m/z, 267 m/z, 373 m/z, 212 m/z;
Demethylation + Hydroxylation /N-oxidation	2	447 m/z	198 m/z, 281 m/z, 226 m/z, 387 m/z	Only hydroxylation /N-oxidation: 214 m/z, 297 m/z, 242 m/z, 403 m/z; Both modifications: 200 m/z, 283 m/z, 228 m/z, 389 m/z
Dehydration and aromatization of 4,5-dihydro-1,2-oxazole cycle	-2	443 m/z	198 m/z, 281 m/z, 226 m/z, 387 m/z	196 m/z, 279 m/z, 224 m/z, 385 m/z
Formation of 3-(2-butyl-5-chloro-1H-imidazole-4-yl)-4,5-dihydro-1,2-oxazole-5-carboxylic acid (Hydrolysis)	-	272 m/z	-	200 m/z, 157 m/z, 144 m/z
Formation of 4-methoxy-3-(trifluoromethyl)aniline (Hydrolysis)	-	192 m/z	-	108 m/z, 123 m/z, 161 m/z

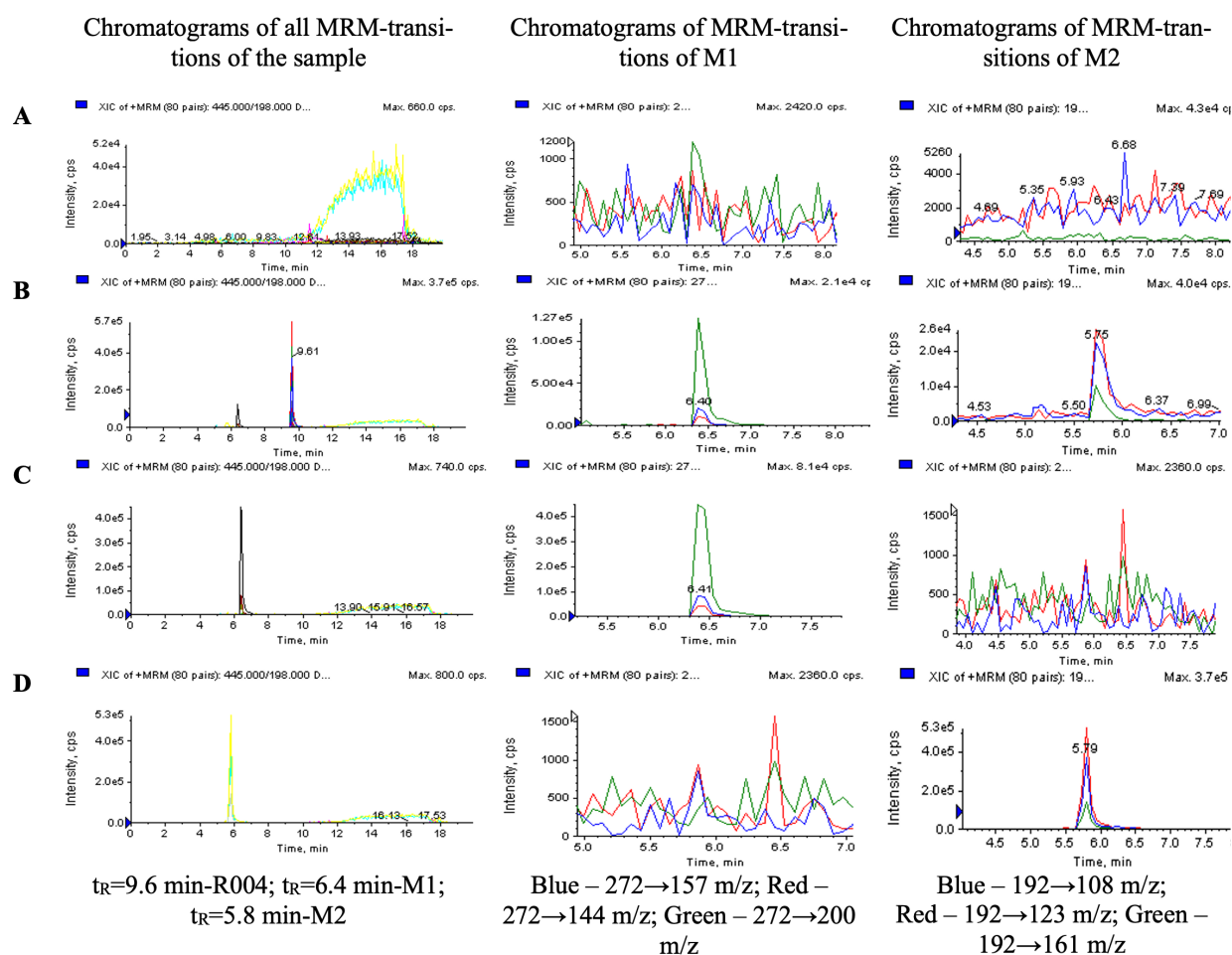
**Note:** M- m/z value of the molecular ion of R004 (445 m/z); m- m/z value of the product ion of R004 (198 m/z, 281 m/z, 226 m/z, 387 m/z);  $\Delta m/z$  - difference of m/z if modification is present

The hydrolysis products of R004 were found as a result of the analysis of the samples of the first stage: 3-(2-butyl-5-chloro-1H-imidazole-4-yl)-4,5-dihydro-1,2-oxazole-5-carboxylic acid and 4-methoxy-3-(trifluoromethyl)aniline (Table 3). The stabilization measures taken allow asserting that these compounds were formed during biotransformation and were not products of *in-vitro* decomposition. Both the active substance and its metabolites were identified in plasma

samples of rats and rabbits (Fig. 5). M1 and trace amounts of M2 were identified in urine (Fig. 6). Therefore, fecal samples were analyzed to determine the pathway of excretion or possible products of M2 biotransformation. M2, unchanged R004 as well as M1, were found in rat feces (Fig. 6). At the same time, the analytical signal M2 in fecal samples was much higher than in urine samples, and the analytical signal M1 was much lower than in urine samples (Fig. 6).

**Table 3.** Detected metabolites of 3-(2-butyl-5-chloro-1H-imidazole-4-yl)-N-[4-methoxy-3-(trifluoromethyl)phenyl]-4,5-dihydro-1,2-oxazole-5-carboxamide

Metabolite	Modification	The main MRM-transitions	Retention time, min.	Structure
3-(2-butyl-5-chloro-1H-imidazole-4-yl)-4,5-dihydro-1,2-oxazole-5-carboxylic acid	Hydrolysis	272→200 m/z, 272→157 m/z, 272→144 m/z	6.4	
4-methoxy-3-(trifluoromethyl)aniline	Hydrolysis	192→108 m/z, 192→123 m/z, 192→161 m/z	5.8	



**Figure 5.** Examples of chromatograms of rabbit plasma samples before administration of drug (A), rabbit samples at point 6 h (B), plasma samples with the addition of 3-(2-butyl-5-chloro-1H-imidazole-4-yl)-4,5-dihydro-1,2-oxazole-5-carboxylic acid (C) and 4-methoxy-3-(trifluoromethyl)aniline (D).

An additional MRM method was created to identify possible biotransformation products of M1 and M2 (Table 4). Glucuronidation, sulfonation, methylation, addition of hydroxyl group were predicted for metabolites. Acetylation and demethylation of M2 were additionally studied. Formation of benzoquinonimine derivate after demethylation as in paracetamol biotransformation (Athersuch et al. 2018)

was also checked. New metabolites have not been detected.

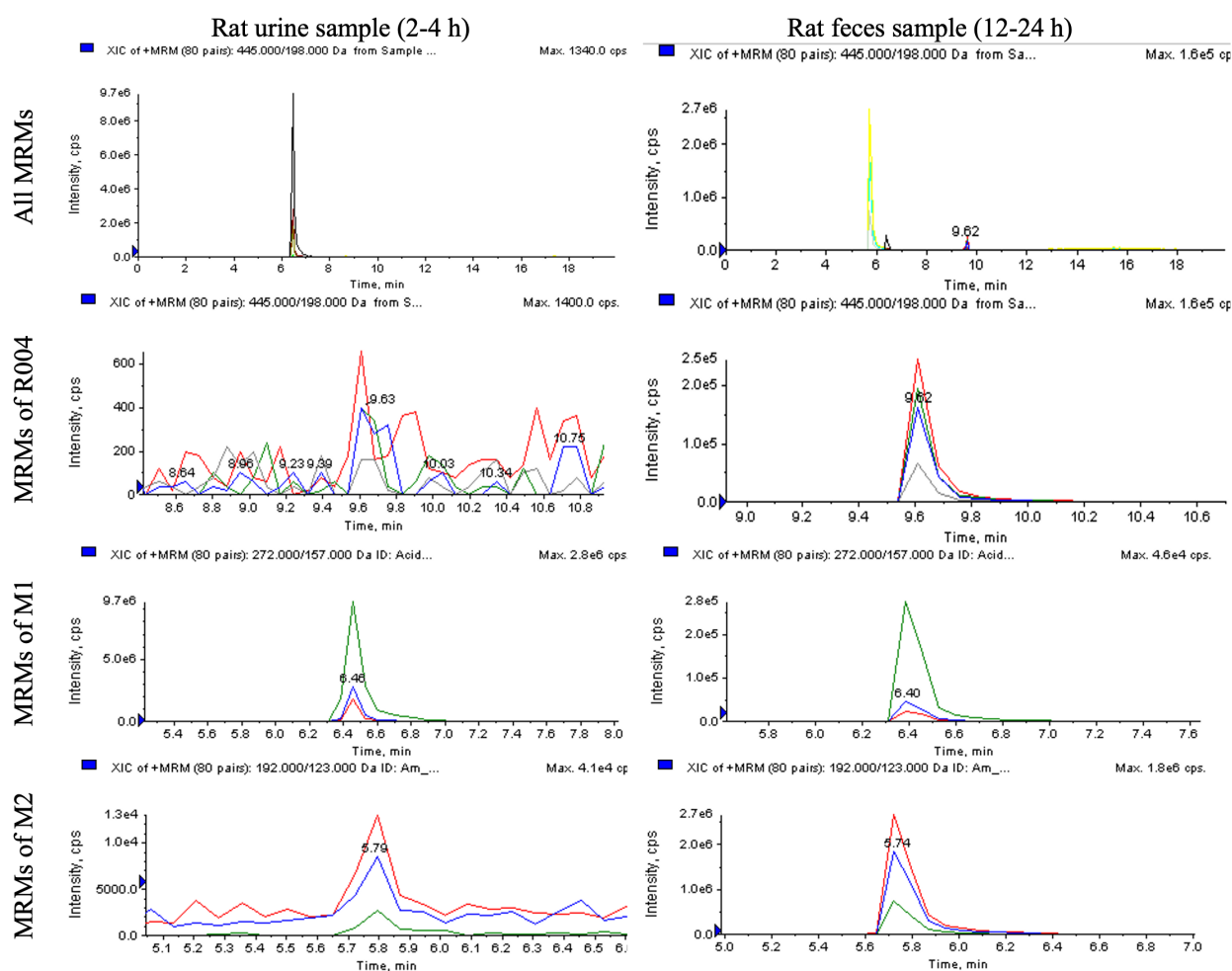
The 4-methoxy-3-(trifluoromethyl)aniline is a commercially available reagent and its production was not required. The 3-(2-butyl-5-chloro-1H-imidazole-4-yl)-4,5-dihydro-1,2-oxazole-5-carboxylic acid was synthesized on reaction scheme (Panova et al. 2023) (Fig. 6) with technological and reagent modifications to increase the yield.

**Table 4.** Predicted MRM-transitions of metabolites 3-(2-butyl-5-chloro-1H-imidazole-4-yl)-4,5-dihydro-1,2-oxazole-5-carboxylic acid and 4-methoxy-3-(trifluoromethyl)aniline

Metabolite	Modification	Difference of m/z	Predicted MRM-transitions		
			Molecular ion $M+\Delta m/z \rightarrow m$	Product ions	
				Non-modified ions $M+\Delta m/z \rightarrow m$	Modified ions $M+\Delta m/z \rightarrow m+\Delta m/z$
M1	Control (M1)	-	272 m/z	200 m/z, 157 m/z, 144 m/z	-
	Hydroxylation /N-oxidation	16	288 m/z	200 m/z, 157 m/z, 144 m/z m/z	216 m/z, 173 m/z , 160 m/z
	Dihydroxylation/ Hydroxylation +N-oxidation	32	304 m/z	200 m/z, 157 m/z, 144 m/z	+1 oxygen: 216 m/z, 173 m/z, 160 m/z; +2 oxygens: 232 m/z, 189 m/z, 176 m/z
	Dehydration and aromatization of 4,5-dihydro-1,2-oxazole cycle	-2	270 m/z	200 m/z, 157 m/z, 144 m/z	198 m/z, 155 m/z, 142 m/z
	Glucuronidation	176	448 m/z	200 m/z, 157 m/z, 144 m/z	376 m/z, 333 m/z, 320 m/z
	Sulfonation	80	352 m/z	200 m/z, 157 m/z, 144 m/z	280 m/z, 237 m/z, 224 m/z
	Methylation	14	286 m/z	200 m/z, 157 m/z, 144 m/z	214 m/z, 171 m/z, 158 m/z
M2	Control (M2)	-	192 m/z	108 m/z, 123 m/z, 161 m/z	-
	Hydroxylation /N-oxidation	16	208 m/z	108 m/z, 123 m/z, 161 m/z	124 m/z, 139 m/z, 177 m/z
	Methylation	14	206 m/z	108 m/z, 123 m/z, 161 m/z	122 m/z, 137 m/z, 175 m/z
	Acetylation	42	234 m/z	108 m/z, 123 m/z, 161 m/z	150 m/z, 165 m/z, 203 m/z
	Glucuronidation	176	368 m/z	108 m/z, 123 m/z, 161 m/z	284 m/z, 299 m/z, 337 m/z
	Sulfonation	80	272 m/z	108 m/z, 123 m/z, 161 m/z	188 m/z, 203 m/z, 241 m/z
	Demethylation	-14	178 m/z	108 m/z, 123 m/z, 161 m/z	94 m/z, 109 m/z, 147 m/z
	Demethylation and formation of benzoquinonimine	-16	176 m/z	108 m/z, 123 m/z, 161 m/z	Only demethylation: 94 m/z, 109 m/z, 147 m/z; Both modifications: 92 m/z, 107 m/z, 145 m/z

**Note:** M – m/z value of the molecular ion of M1 or M2 (272 or 192 m/z); m – m/z value of the product ion of M1 or M2;  $\Delta m/z$  – difference of m/z if modification is present





**Figure 6.** Examples of chromatograms of rat urine and feces samples. *Note:*  $t_R=9.6$  min-R004;  $t_R=6.4$  min-M1;  $t_R=5.8$  min-M2.

2-Butyl-5-chloro-1H-imidazole-4-carbaldehyde (Fig. 7-1) (20.53 g (0.11 mol)) was dissolved in 100 mL of ethyl alcohol, and a mixture of 30.21 g (0.43 mol) of hydroxylamine hydrochloride was added to the resulting solution and 34.50 g (0.32 mol) sodium carbonate in 100 mL of distilled water. The reaction mixture was stirred while boiling for 16 hours. After the time had elapsed, the ethyl alcohol was evaporated on a rotary evaporator, the precipitate that formed was filtered off, washed with 20 mL of distilled water twice, and dried at 50 °C for 24 hours. 8.71 g of (2-butyl-5-chloro-1H-imidazol-4-yl)methyl oxime was obtained (Fig. 7-2) in the form of a white powder. The product yield was 75%.

Preparation of 3-(2-butyl-5-chloro-1H-imidazol-4-yl)-4,5-dihydro-5-isoxazolecarboxylic acid ethyl ester (Fig. 7-4) was performed further using approach Basappa et al. (2003). To a suspension of 9.41 g (0.071 mol) of N-chlorosuccinimide in 100 mL of methylene chloride, 0.064 mol of oxime (Fig. 7-2) was added in one portion, after which the reaction mass was stirred at room temperature for 10 hours. After this, 12.87 g (0.128 mol) acrylic acid ethyl ester was added to the homogeneous mixture and 7.77 g (0.077 mol) triethylamine, maintaining a temperature not higher than 25°C. The resulting mixture was stirred at room temperature for 90 minutes. The organic solution was washed with 30 mL of distilled water four times, dried

with 11.00g of anhydrous sodium sulfate, passed through a layer of silica gel and evaporated in vacuum on a rotary evaporator. 17.45 g of 3-(2-butyl-5-chloro-1H-imidazol-4-yl)-4,5-dihydro-5-isoxazolecarboxylic acid ethyl ester (Fig. 7-4) was obtained in the form of a light yellow powder. The product yield was 91%.

Preparation of 3-(2-butyl-5-chloro-1H-imidazol-4-yl)-4,5-dihydro-5-isoxazolecarboxylic acid (M1) was performed on next stage. Ester (Fig. 7-4) (13.94 g (0.04 mol)) was dissolved in 150 mL of 80% ethyl alcohol, and 7.14 g (0.127 mol) of sodium hydroxide was added to the resulting mass. The reaction mixture was stirred at 50°C for 12 h, then cooled to 0°C. The reaction mass was acidified with concentrated sulfuric acid to pH=3.0 and left overnight to cool. The precipitate that formed was filtered off, washed with 20 mL of chilled water, and dried at 50°C for 24 hours. We obtained 9.35 g of 3-(2-butyl-5-chloro-1H-imidazol-4-yl)-4,5-dihydro-5-isoxazolecarboxylic acid (Fig. 7-5) in the form of a beige powder. The product yield was 74%. The results of structure confirmation of all synthesized compounds are shown in Table 5.

The second stage of the study was carried out after the successful synthesis of M1 and confirmation of the structure of M2. Samples of each biological object with the addition of metabolites were prepared for comparison

**Table 5.** The results of the confirmation of the structure of 3-(2-butyl-5-chloro-1H-imidazole-4-yl)-4,5-dihydro-1,2-oxazole-5-carboxylic acid with its intermediates and 4-methoxy-3-(trifluoromethyl)aniline

Compound	<sup>1</sup> H-NMR-spectroscopy	<sup>13</sup> C-NMR-spectroscopy	High resolution mass spectrometry	Melting point
<i>E,Z</i> mixture <i>N</i> -[(2-butyl-5-chloro-1 <i>H</i> -imidazol-4-yl)methylidene]hydroxylamine	11.73...12.55 (Br.s., 1H, NH), 7.85 (s, 1H, CH), 7.24 (s, 1H, OH), 2.66 (t, <i>J</i> = 7.4 Hz, 1H, CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> CH <sub>3</sub> ), 2.57 (t, <i>J</i> = 7.4 Hz, 2H, CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> CH <sub>3</sub> ), 1.52...1.65 (m, 2H, CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> CH <sub>3</sub> ), 1.20...1.36 (m, 2H, CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> CH <sub>3</sub> ), 0.87 (t, <i>J</i> = 7.4 Hz, 3H, CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> CH <sub>3</sub> )	<sup>13</sup> C NMR (101 MHz, DMSO) δ 150.40, 137.20, 133.46, 131.00, 129.13, 119.25, 117.49, 30.71, 30.44, 28.04, 27.84, 22.32, 22.25, 14.21.	202.0739 m/z [M+H <sup>+</sup> ], Δ <i>m/z</i> =-3.96 ppm (Theoretical <i>m/z</i> of C <sub>8</sub> H <sub>13</sub> ClN <sub>3</sub> O <sup>+</sup> = 202.0747)	143–145 °C
3-(2-butyl-5-chloro-1 <i>H</i> -imidazol-4-yl)-4,5-dihydro-5-isoxazolecarboxylic acid ethyl ester	12.67...12.89 (br.s., 1H, NH), 5.22 (dd, <i>J</i> <sub>1</sub> = 6.4 Hz, <i>J</i> <sub>2</sub> = 11.3 Hz, 1H, CH <sub>isoxazoline</sub> ), 3.69...3.79 (m, 1H, CH <sub>isoxazoline</sub> ), 3.55...3.65 (m, 1H, CH <sub>isoxazoline</sub> ), 2.59 (t, <i>J</i> = 7.3 Hz, 2H, CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> CH <sub>3</sub> ), 1.55...1.65 (m, 2H, CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> CH <sub>3</sub> ), 1.18...1.33 (m, 5H, CH <sub>3</sub> + CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> CH <sub>3</sub> ), 0.87 (t, <i>J</i> = 7.3 Hz, 3H, CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> CH <sub>3</sub> )	170.2, 151.3, 147.4, 129.9, 115.3, 78.0, 61.7, 56.8, 30.3, 27.9, 22.2, 18.6, 14.1.	300.1107 m/z [M+H <sup>+</sup> ], Δ <i>m/z</i> =-2.67 ppm (Theoretical <i>m/z</i> of C <sub>11</sub> H <sub>15</sub> ClN <sub>3</sub> O <sup>+</sup> = 300.1115)	89–92 °C
3-(2-butyl-5-chloro-1 <i>H</i> -imidazole-4-yl)-4,5-dihydro-1,2-oxazole-5-carboxylic acid	13.11...13.29 (br s, 1H, OH), 12.75...12.85 (br s, 1H, NH), 5.13 (d.d, <i>J</i> <sub>1</sub> = 6.4 Hz, <i>J</i> <sub>2</sub> = 11.5 Hz, 1H, CH <sub>isoxazoline</sub> ), 3.65...3.75 (m, 1H, CH <sub>isoxazoline</sub> ), 3.51...3.60 (m, 1H, CH <sub>isoxazoline</sub> ), 2.59 (t, <i>J</i> = 7.5 Hz, 2H, CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> CH <sub>3</sub> ), 1.55...1.65 (m, 2H, CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> CH <sub>3</sub> ), 1.22...1.33 (m, 2H, CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> CH <sub>3</sub> ), 0.87 (t, <i>J</i> = 7.3 Hz, 3H, CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> CH <sub>3</sub> )	169.04, 154.83, 151.48, 148.82, 148.56, 129.36, 127.42, 125.56, 125.42, 124.71, 122.00, 119.82, 119.32, 115.24, 112.76, 79.25, 79.15, 56.39, 40.09, 30.19, 28.60, 28.24, 22.42, 13.85.	272.0795 m/z [M+H <sup>+</sup> ], Δ <i>m/z</i> =-2.57 ppm (Theoretical <i>m/z</i> of C <sub>11</sub> H <sub>15</sub> ClN <sub>3</sub> O <sub>3</sub> <sup>+</sup> = 272.0802)	110–112°C
4-methoxy-3-(trifluoromethyl)aniline	6.87 (d, <i>J</i> = 2.74 Hz, 1H), 6.80 (m, 1H), 6.76 (dd, <i>J</i> = 2.74, 8.79 Hz, 1H), 3.78 (s, 3H), 3.53 (br s, 2H)	149.9, 139.3, 125.1, 121.1, 119.0, 118.8, 113.7, 113.6, 113.5, 56.2.	192.0625 m/z [M+H <sup>+</sup> ], Δ <i>m/z</i> =-5.21 ppm (Theoretical <i>m/z</i> of C <sub>8</sub> H <sub>9</sub> F <sub>3</sub> NO <sup>+</sup> = 192.0636)	

**Note:** Deuterated dimethyl sulfoxide was solvent for NMR; 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

with animal samples. The results are presented in Table 6. The retention time of M1 in the test and spiked samples coincided at 99.79-100.18%, and *t<sub>R</sub>* M2 coincided by 99.91-100.14%. Thus, the differences of this parameter did not exceed the maximum acceptable interval of 1%. The discrepancy in the area ratios of M1 chromatographic peaks at MRM-transitions with the most intense signal 272→200 *m/z* and 272→157 *m/z* was 99.64-101.41%. It was within the valid limit of ±20%. The percentage of coincidence of this parameter in M2 was within the range of 94.67-100.25%, which also meets the established requirements. The MS2-mass spectra of molecular ions 272 *m/z* (M1) and 192 *m/z* (M2) were compared to additional confirmation of the structure of the metabolites. The determination was carried out at points with the most intense analytical signal of the studied compounds: 4 h and 6 h – for rat and rabbit plasma, in the intervals of 2-4 h and 4-6 h – for rat urine, and in the interval of 12-24 h – for rat feces. Thus, the mass spectra of M1 matched by at least 90%, and the mass spectra of M2 – by 87% (Fig. 8). The low percentage of coincidence of mass spectra of M1 in fecal samples and mass spectra of M2 in urine samples was due to their low contents in these objects.

Thus, 3-(2-butyl-5-chloro-1*H*-imidazole-4-yl)-*N*-[4-methoxy-3-(trifluoromethyl)phenyl]-4,5-dihydro-1,2-oxazole-5-carboxamide undergoes biotransformation by hydrolysis. The structure of its metabolites has been

proven by comparison with synthetic substances. It was also found that the molecule of R004 is not stable in plasma and urine after collection. Therefore, acidification of samples is necessary for conduction of bioanalytical studies of this drug, as well as for study of statins (Khokhlov and Pyatigorskaya 2019).

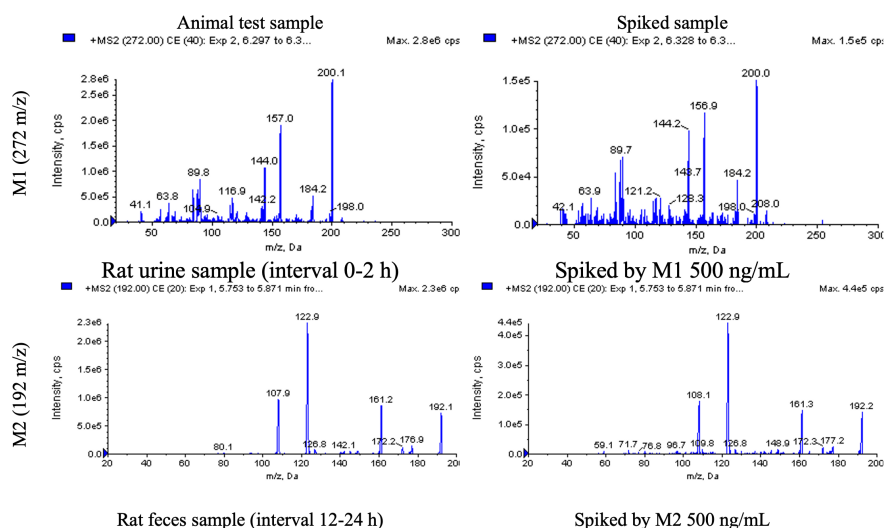
## Conclusion

The amide bond of the molecule 3-(2-butyl-5-chloro-1*H*-imidazole-4-yl)-*N*-[4-methoxy-3-(trifluoromethyl)phenyl]-4,5-dihydro-1,2-oxazole-5-carboxamide undergoes enzymatic hydrolysis. Metabolites of 3-(2-butyl-5-chloro-1*H*-imidazole-4-yl)-4,5-dihydro-1,2-oxazole-5-carboxylic acid and 4-methoxy-3-(trifluoromethyl)aniline have been identified in rat and rabbit plasma, as well as rat excretions. The measures for sample stabilization guarantee the trueness of the obtained results. The structure of the synthesized substances M1 and M2 has been confirmed by mass spectrometry and NMR-spectroscopy. In the future, these compounds will be used as standards for development of bioanalytical methods and analysis of laboratory animal samples. It is also reasonable to evaluate the anti-rheumatoid and anti-inflammatory activity of 3-(2-butyl-5-chloro-1*H*-imidazole-4-yl)-4,5-dihydro-1,2-oxazole-5-carboxylic acid.

**Table 6.** Results of confirmation of the structure of metabolites of 3-(2-butyl-5-chloro-1H-imidazole-4-yl)-N-[4-methoxy-3-(trifluoromethyl)phenyl]-4,5-dihydro-1,2-oxazole-5-carboxamide

Parameter	3-(2-butyl-5-chloro-1H-imidazole-4-yl)-4,5-dihydro-1,2-oxazole-5-carboxylic acid			4-methoxy-3-(trifluoromethyl)aniline		
	tr, min.	Ratio of peak area 272→200 m/z / 272→157 m/z	% of MS2- mass spectrum matches (Min.-max., %)	tr, min.	Ratio of peak area 192→123 m/z / 192→108 m/z	% of MS2- mass spectrum matches (Min.-max., %)
Test samples (n=15)	6.43±0.02	5.629±0.099		5.73±0.01	0.719±0.036	
Rat plasma samples						
Spiked samples (n=6)	6.44±0.02	5.650±0.074	92-95%	5.73±0.01	0.724±0.013	90-96%
% of match	99.79	99.64		99.91	99.41	
Test samples (n=12)	6.47±0.02	5.326±0.207		5.76±0.01	0.695±0.054	
Rat urine samples						
Spiked samples (n=6)	6.46±0.02	5.365±0.176	95-98%	5.75±0.01	0.734±0.021	87-92%
% of match	100.08	99.27		100.14	94.67	
Test samples (n=3)	6.46±0.01 (12-24 h, n=3)	5.437±0.199 (12-24 h, n=3)		5.75±0.01 (n=6)	0.727±0.024 (n=6)	
Rat feces samples						
Spiked samples (n=6)	6.45±0.01	5.361±0.159	90-98%	5.75±0.01	0.726±0.012	94-98%
% of match	100.18	101.41		99.97	100.25	
Test samples (n=15)	6.44±0.02	5.622±0.119		5.73±0.01	0.720±0.031	
Rabbit plasm samples						
Spiked samples (n=6)	6.44±0.02	5.565±0.118	94-99%	5.73±0.01	0.736±0.014	91-94%
% of match	99.96	101.03		100.08	97.83	

**Note:** Plasma samples at the points of 0.5h, 1h, 2h, 4h, 24h, urine samples at intervals of 0-2 h, 2-4 h, 4-6 h, 6-24 h, feces samples at intervals of 0-12 h, 12-24 h were used as test samples; Mean ± SD is given in each cell of the table; the number of M1 observations in fecal samples was 3, since this metabolite was not detected within the range of 0-12 hours.

**Figure 8.** MS2-spectra of 3-(2-butyl-5-chloro-1H-imidazole-4-yl)-4,5-dihydro-1,2-oxazole-5-carboxylic acid and 4-methoxy-3-(trifluoromethyl)aniline at animal and spiked samples.

### 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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