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Оригинальная статья / Research article

Simultaneous Determination of Nirmatrelvir and Ritonavir in Human Plasma by HPLC-MS/MS

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Abstract

Introduction. SARS-CoV-2 (severe acute respiratory syndrome-related coronavirus 2) is expected to remain a persistent global threat. Therefore, development of coronavirus disease 2019 (COVID-19) drugs is the most urgent global issue. Nirmatrelvir and ritonavir combination is an oral antiviral drug combination with activity against SARS-CoV-2. Nirmatrelvir and ritonavir combination is highly efficacious in reducing the risk of COVID-19. The study describes development and validation of high-performance liquid chromatography – tandem mass spectrometry (HPLC-MS/MS) method for the simultaneous determination of nirmatrelvir and ritonavir in human blood plasma. The method could be applied in pharmacokinetic study of nirmatrelvir and ritonavir.

Aim. The aim of this study is to develop and validate a HPLC-MS/MS bioanalytical method for the determination of nirmatrelvir and ritonavir in human plasma.

Materials and methods. The determination of nirmatrelvir and ritonavir in human plasma by HPLC-MS/MS. The samples were processed by acetonitrile protein precipitation. Internal standard: promethazine. Mobile phase: 0.1% formic acid solution in water (Eluent A), 0.1 % formic acid in acetonitrile (Eluent B). Column: Phenomenex Luna C18 50 × 2.0 mm, 5 µm. Analytical range: 50–10.000 ng/mL for nirmatrelvir, 5–1000 ng/mL for ritonavir in human plasma. Ionization source and ionization: electrospray ionization, positive. Detection conditions: 499.90 → 110.10 m/z, 499.90 → 319.20 m/z (nirmatrelvir), 720.90 → 426.00 m/z, 720.90 → 296.20 m/z, 720.90 → 268.10 m/z, 720.90 → 197.10 m/z, 720.90 → 139.90 m/z (ritonavir), 285.15 → 198.05 m/z (promethazine).

Results and discussion. This method was validated for selectivity, matrix effect, calibration curve, accuracy, precision, spike recovery, the lower limit of quantification, carry-over effect and stability.

Conclusion. The HPLC-MS/MS method for quantitative determination of nirmatrelvir and ritonavir in human plasma was developed and validated. The analytical range was 50–10,000 ng/mL for nirmatrelvir, 5–1000 ng/mL for ritonavir in human plasma. This method was applied to investigate the pharmacokinetics of nirmatrelvir and ritonavir.

Keywords: nirmatrelvir, ritonavir, COVID-19, plasma, HPLC-MS/MS, validation, pharmacokinetics

Conflict of interest. The authors declare that they have no obvious and potential conflicts of interest related to the publication of this article.

Contribution of the authors. Timofey N. Komarov, Olga A. Archakova, Dana S. Shchelgacheva, Polina K. Karnakova developed and validated the analytical method. Natalia S. Bagaeva carried out the statistical analysis. Igor E. Shohin, Kira Ya. Zaslavskaya and Petr A. Bely conceived the study and were in charge of direction and planning. All authors discussed the results.

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Совместное определение нирматрелвира и ритонавира в плазме крови человека методом ВЭЖХ-МС/МС

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Резюме
Введение. В настоящее время предполагается, что вирус SARS-CoV-2 (severe acute respiratory syndrome-related coronavirus 2), вызывающий новую коронавирусную инфекцию COVID-19 (Coronavirus Disease 2019) останется постоянной глобальной угрозой. В связи с этим, вопрос разработки лекарственных препаратов для лечения COVID-19 остается актуальным. Комбинация нирматрелвира с ритонавиром обладает прямым противовирусным действием: при ее применении удается повысить эффективность терапии и снизить риск осложнений COVID-19. Разработка и валидация методики совместного определения нирматрелвира и ритонавира в плазме крови человека является необходимой задачей для проведения аналитической части клинического исследования с целью дальнейшего изучения фармакокинетических параметров.
Цель. Целью исследования является разработка и валидация методики совместного определения нирматрелвира и ритонавира в плазме крови человека методом высокоэффективной жидкостной хроматографии с тандемным масс-селективным детектированием (ВЭЖХ-МС/МС) для дальнейшего изучения фармакокинетики.
Материалы и методы. Определение нирматрелвира и ритонавира в плазме крови человека проводили методом ВЭЖХ-МС/МС. В качестве пробоподготовки был использован способ осаждения белков ацетонитрилом. Внутренний стандарт: прометазин. Подвижная фаза: 0,1%-й раствор муравьиной кислоты в воде (элюент А), 0,1%-й муравьиной кислоты в ацетонитриле (элюент В). Колонка: Phenomenex Luna C18 50 × 2,0 мм, 5 мкм. Аналитический диапазон методики: 50–10,000 нг/мл для нирматрелвира, 5–1000 нг/мл для ритонавира в плазме крови. Источник ионизации и ионизация: электроспрей, положительная. Условия детектирования: 499,90 → 110,10 m/z, 499,90 → 319,20 m/z (нирматрелвир), 720,90 → 426,00 m/z, 720,90 → 296,20 m/z, 720,90 → 268,10 m/z, 720,90 → 197,10 m/z, 720,90 → 139,90 m/z (ритонавир), 285,15 → 198,05 m/z (прометазин).
Результаты и обсуждение. Разработанная методика была валидирована по следующим валидационным параметрам: селективность, эффект матрицы, калибровочная кривая, точность, прецизионность, степень извлечения, нижний предел количественного определения перенос пробы, стабильность.
Заключение. Разработана и валидирована методика совместного определения нирматрелвира и ритонавира в плазме крови человека методом ВЭЖХ-МС/МС. Подтвержденный аналитический диапазон методики составил 50–10,000 нг/мл для нирматрелвира, 5–1000 нг/мл для ритонавира в плазме крови. Полученный аналитический диапазон позволяет применять разработанную методику для проведения фармакокинетических исследований комбинированных препаратов нирматрелвира и ритонавира.

Ключевые слова: нирматрелвир, ритонавир, COVID-19, плазма, ВЭЖХ-МС/МС, валидация, фармакокинетика

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Вклад авторов. Т. Н. Комаров, О. А. Арчакова, Д. С. Щелгачева, П. К. Карнакова участвовали в разработке и валидации биоаналитической методики. Н. С. Багаева проводила статистическую обработку данных. И. Е. Шохин, К. Я. Заславская и П. А. Белый отвечали за организационную часть исследования. Все вышеуказанные авторы участвовали в обсуждении полученных результатов в форме научной дискуссии.

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INTRODUCTION

The COVID-19 pandemic (Coronavirus Disease 2019) caused by the SARS-CoV-2 (Severe acute respiratory syndrome-related coronavirus 2) virus has significantly changed the lives of the world's population. Morbidity and mortality rates around the world have reached extremely high values [1].

According to the statistics, as of January 2023, more than 663 million cases of the SARS-CoV-2 virus infection were recorded worldwide, of which about 6.7 million were fatal. To date, more than 21 million cases of this disease have been recorded in Russia, the number of deaths is more than 394 thousand[1]. More than a million people who have recovered from COVID-19 suffer from the long-term consequences of this disease, which are not yet fully understood [2]. COVID-19 is also expected to remain a constant global threat [3]. For this reason, development of drugs for the treatment of COVID-19 remains challenging.

Currently, the temporary guidelines of the Ministry of Health of the Russian Federation for the prevention, diagnosis and treatment of the novel coronavirus infection (version № 17 of 14.12.2022) highlight several drugs that can be used in the treatment of COVID-19, including the combination of nirmatrelvir with ritonavir. This combination is approved by the EMA and FDA for emergency use in patients with COVID-19; in Russia, it is used to treat mild to moderate COVID-19 in adults, including patients with an increased risk of progression to a more severe form and who do not require additional oxygen therapy[2].

[1] WHO Coronavirus (COVID-19) Dashboard. Available at:https://covid19.who.int/ Accessed: 19.01.2023.

[2] Temporary guidelines "Prevention, diagnosis and treatment of the novel coronavirus infection (COVID-19). Version 17 (14.12.2022)" (approved by the Ministry of Health of Russia). Available at: http://www.consultant.ru/document/cons_doc_LAW_347896/ Accessed at 01/19/2023.

Nirmatrelvir ((1R,2S,5S)-N-[(1S)-1-cyano-2-[(3S)-2-oxopyrrolidin-3-yl]ethyl]-3-[(2S)-3,3-dimethyl-2-[(2,2,2-trifluoroacetyl)amino]butanoyl]-6,6-dimethyl-3-azabicyclo[3.1.0]hexane-2-carboxamide), NIRM [Nirmatrelvir] is a drug with antiviral activity. The mechanism of action of nirmatrelvir is based on the inhibition of the protease of the SARS-CoV-2 3CL virus. Inhibition of this protease prevents the replication of the SARS-CoV-2 virus [4].

Ritonavir ([(1,3-thiazol-5-yl)methyl]{N-[(2S,3S,5S)-3-hydroxy-5-[(2S)-3-methyl-2-[(methyl{[2-(propan-2-yl)-1,3-thiazol-4-yl]methyl}carbamoyl)amino]butanamido]-1,6-diphenylhexan-2-yl]carbamate), RIT [Ritonavir, RIT]) is a protease inhibitor of human immunodeficiency virus type 1, human immunodeficiency virus type 2 and cytochrome P450 (CYP3A4) [5].

The combination of nirmatrelvir with ritonavir belongs to drugs of direct antiviral action. When administering this combination, it is possible to increase treatment efficacy and reduce the risk of complications of COVID-19 [6]. In this combination, ritonavir acts as a pharmacokinetic booster and slows down the metabolism of nirmatrelvir. Owing to this, nirmatrelvir is active for a longer time, higher concentrations of the drug remain in the body, elimination slows down. With oral administration of nirmatrelvir in combination with ritonavir, it is possible to achieve high bioavailability, which is a significant advantage, since good oral availability does not require parenteral administration and allows the use of the drug not only in hospitalized patients, but also among outpatients [7].

The development and validation of the method for the co-determination of both nirmatrelvir and ritonavir in human blood plasma is a procedure necessary for the analytical stage of the study in order to investigate further the pharmacokinetic parameters following a single and multiple oral administration of this combination by healthy volunteers.

Currently, a number of pharmacokinetic studies have been published on the quantification of nirmatrelvir and ritonavir in human body fluids. The methods of high performance liquid chromatography – tandem mass spectrometry (HPLC-MS/MS) and ultra-performance liquid chromatography – tandem mass spectrometry (UPLC-MS/MS) are used for the determination of the substances. In the methods under review, ionization is carried out by electrospray in a positive mode. As a sample preparation, a method for protein precipitation with methanol (MeOH) or acetonitrile (ACN) is used, as well as a method for protein precipitation with acetonitrile followed by dilution (Table 1).

After analysis of the literature data on the development of methods for the co-determination of nirmatrelvir and ritonavir, it was concluded that there were no published methods at the same time with a simple method of sample preparation and sufficient sensitivity. In this regard, it was decided to develop and validate independently a method for the co-determination of nirmatrelvir and ritonavir in human plasma by HPLC-MS/MS. As a sample preparation, a method of precipitation of proteins with acetonitrile was used.

Table 1. Bioanalytical methods of nirmatrelvir and ritonavir quantitative determination

Analytical method	Object	Sample preparation	Analytical range, ng/mL		Reference
UPLC-MS/MS	Human plasma	Protein precipitation	RIT	10–1000	[8]
			NIRM	40–4000	
HPLC-MS/MS	Human plasma	Protein precipitation by methanol	RIT	2–2000	[9]
			NIRM	10–10,000	
HPLC-MS/MS	Human plasma	Protein precipitation by acetonitrile, followed by dilution	RIT	5–5000	[6]
			NIRM	10–10,000	
HPLC-MS/MS	Human plasma, serum	Protein precipitation by acetonitrile	RIT	10.87–3516	[10]
			NIRM	14.69–4753	
HPLC-MS/MS	Human plasma	Protein precipitation	RIT	10–1000	[11]
			NIRM	50–5000	
HPLC-MS/MS	Human plasma	Protein precipitation by acetonitrile	RIT	10–50,000	[12]
			NIRM	10–50,000	

MATERIALS AND METHODS

Equipment

Chromatographic separation and detection were carried out on a Nexera XR high-performance liquid chromatograph equipped with a gradient pump, a column and sample thermostat, a degasser, an autosampler, a rack changer, a high-pressure flow switching valve and a tandem mass spectrometric detector LCMS-8040 with a triple quadrupole. Primary data were processed with LabSolutions software (Ver. 5.91) (Shimadzu Corporation, Japan).

Reagents and solutions

During the study, the following chemical reagents were used: acetonitrile (class UHPLC Supergradient, ACS, PanReac & AppliChem, Germany, Spain); acetonitrile (chemically pure, TH CHIMMED, LLC, Russia); methanol (chemically pure, TH CHIMMED, LLC, Russia); formic acid (98 % pure, PanReac, Spain); demineralized water (I class of purity).

For the preparation of stock and working solutions, reference samples of nirmatrelvir (Ningbo Mindo Pharmaceutical Co., Ltd, India, assay 99.50 %), ritonavir (Mylan Laboratories Limited, India, assay 100.40 %) and promethazine hydrochloride (USP reference standard, France, assay 99.90 %) were used.

Stock standard solutions (SSS) of nirmatrelvir and ritonavir were prepared by dissolving weighed amounts of the substances in methanol. SSS of the internal standard (IS) of promethazine was prepared by dissolving a sample of the substance of promethazine hydrochloride in acetonitrile. The concentration of nirmatrelvir in SSS was 400,000 ng/mL, ritonavir – 50,000 ng/mL, promethazine – 60,000 ng/mL. Mixed working standard solutions (WSS) of nirmatrelvir and ritonavir were prepared from stock standard solutions, by diluting them with methanol to obtain plasma concentrations corresponding to calibration levels 1–8 and quality control (QC) levels: the lower limit of quantification (LLOQ), low [L (low)], medium [M1 and M2 (middle 1, middle 2)] and high levels [H (high)] (Table 2). Working standard solutions was prepared by diluting SSS with acetonitrile until plasma conconcentrations of 1 ng/mL.

Table 2. Concentrations at calibration levels and quality control samples

Level	Analyte concentration, ng/mL		IS concentration, ng/mL
	NIRM	RIT	PROM
1	50	5	1
2	250	25	1
3	500	50	1
4	1000	100	1
5	2500	250	1
6	5000	500	1
7	7500	750	1
8	10,000	1000	1
LLOQ	50	5	1
L	150	15	1
M1	2000	200	1
M2	4000	450	1
H	8000	800	1

Samples of blank plasma, stock standard solutions and working standard solutions were stored in the freezer at a temperature of –42.5 ± 7.5 °C.

Sample preparation

A sample was aliquoted into the Eppendorf centrifuge microtube, working standard solution of promethazine IS was added, then the proteins were precipitated using acetonitrile (1 : 3). Next, the sample was mixed on a vortex and centrifuged. After centrifugation, the supernatant was transferred to vials and placed in an autosampler. The sample preparation scheme is shown in Figure 1.

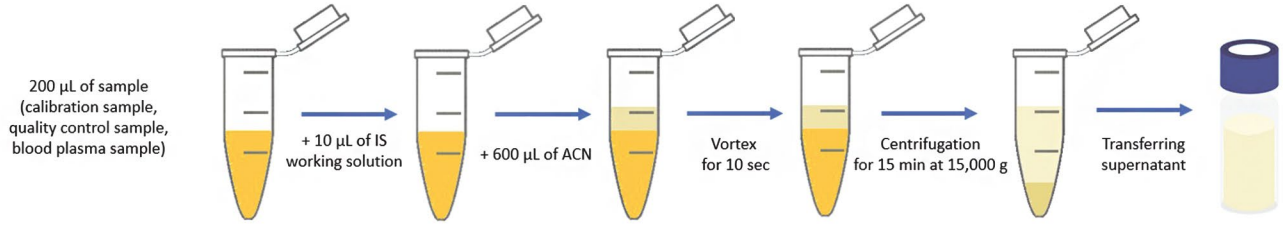


Figure 1. Sample preparation

Conditions of chromatographic separation and detection

- Column: Phenomenex Luna C18 50 × 2.0 mm, 5 µm.
- Precolumn: Phenomenex SecurityGuard™ Cartridges C18 4 × 3.0 mm, 5 µm.
- Thermostat temperature: 40 °C.
- Mobile phase: eluent A: 0.1 % solution of formic acid in water (v/v); eluent B: 0.1 % solution of formic acid in acetonitrile (v/v).
- Flow rate of the mobile phase: 1.0 mL/min.
- Gradient by composition of the mobile phase is presented in Table 3.

Table 3. Gradient elution

Time, min	Eluent A, %	Eluent B, %
0.00	95.00	5.00
0.50	95.00	5.00
1.50	0.00	100.00
2.50	0.00	100.00
2.60	95.00	5.00
4.00	95.00	5.00

- Sample volume: 1 µL.
- Run time of the mass spectrometric detector: 0.00–4.00 min.
- Ionization source parameters (electric spray): spray gas 3 L/min, drying gas 20 L/min, heating unit 400 °C, desolvation line 200 °C.
- Capillary voltage, ionization mode and detection conditions are presented in Table 4.

Table 4. Ion-source parameters and detection conditions

Name	Capillary voltage	Ion mode	Detection conditions
NIRM	4.5	+	499.90 → 110.10; 499.90 → 319.20
RIT	4.5	+	720.90 → 426.00; 720.90 → 296.20; 720.90 → 268.10; 720.90 → 197.10; 720.90 → 139.90
PROM	4.5	+	285.15 → 198.05

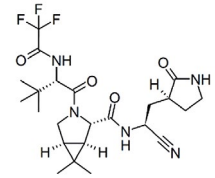
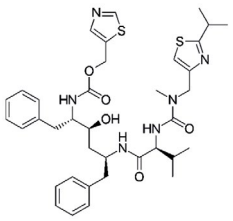
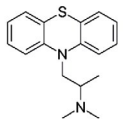
RESULTS AND DISCUSSION

Method development

Promethazine ((RS)-N,N,α-trimethyl-10H-phenothiazine-10-ethanamine) was selected as the internal standard (IS) for nirmatrelvir and ritonavir.

To obtain the necessary chromatographic parameters, a substance similar to nirmatrelvir and ritonavir in structure and physicochemical properties was selected (Table 5).

Table 5. Structure and physicochemical properties of the analytes and IS

Name	Physicochemical properties		Molecular structure
NIRM[1]	pKa	7.1	
	logP	2.12	
	Molecular weight	499.535	
RIT[2]	pKa	13.68	
	logP	4.24	
	Molecular weight	720.944	
PROM[3]	pKa	9.05	
	logP	4.29	
	Molecular weight	284.419	

Note. [1] Nirmatrelvir. Drugbank. Available at: https://go.drugbank.com/drugs/DB16691. Accessed: 19.01.2023.

[2] Ritonavir. Drugbank. Available at: https://go.drugbank.com/drugs/DB00503. Accessed: 19.01.2023.

[3] Promethazine. Drugbank. Available at: https://go.drugbank.com/drugs/DB01069. Accessed: 19.01.2023.

The conditions of mass spectrometric detection were used, allowing to obtain peaks of the analytes and IS with the highest intensity. During the development of the method, fragments obtained at different collision energies were analyzed, and the most intense transitions were selected (see Table 4). The assumed fragmentation ways of nirmatrelvir and ritonavir are presented in Figures 2, 3.

Acetonitrile was selected as a precipitant, since when it was used, the most complete precipitation of blood plasma proteins occurred, and it was also possible to achieve the optimal form of chromatographic peaks. At the same time, the method of sample preparation is the

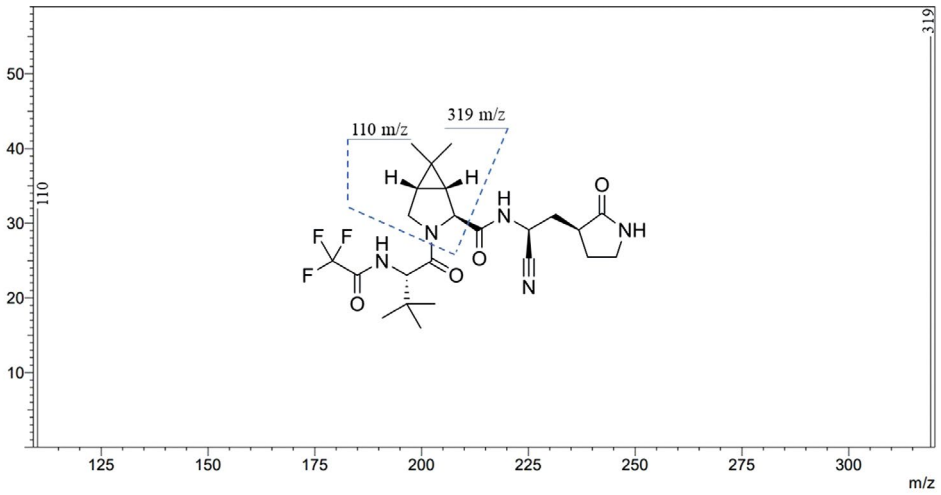


Figure 2. **MS/MS fragmentation of nirmatrelvir**

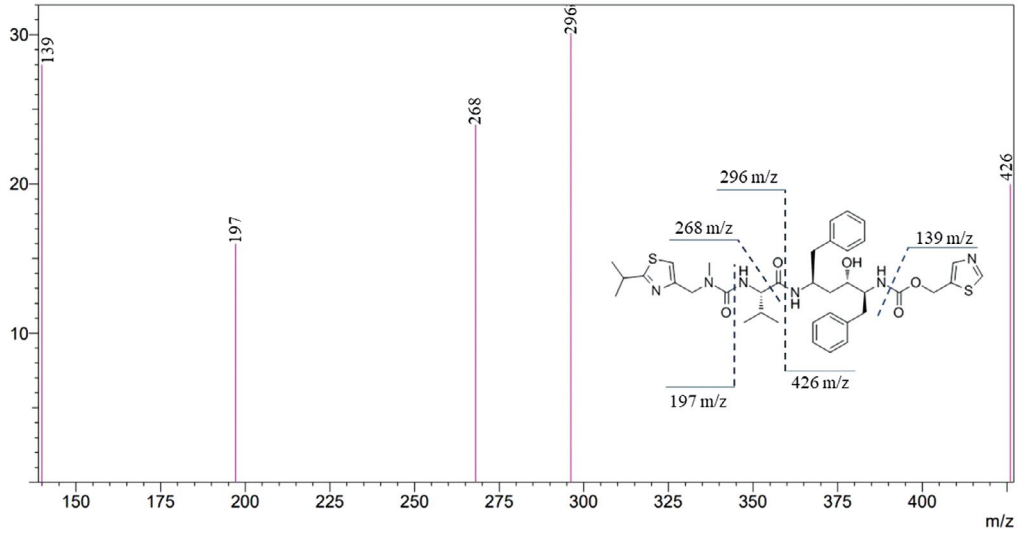


Figure 3. **MS/MS fragmentation of ritonavir**

least time-consuming, since it does not require additional manipulations and separate conditions for the analysis and does not affect the stability of samples when they are stored in an autosampler, in contrast to liquid-liquid extraction, protein precipitation with trifluoroacetic acid or methanol.

During the method development, based on the physicochemical properties of nirmatrelvir and ritonavir, Phenomenex Luna C18 column, 50 × 2.0 mm, 5 µm was selected for chromatographic separation of this combination of substances.

Method validation

The developed bioanalytical method was validated out in accordance with the Rules for conducting bioequivalence studies in the Eurasian Economic Union[1] based on the EMA[2] and FDA[3] guidelines. The method was fully validated for the following parameters: selectivity, calibration curve, accuracy and precision, lower limit of quantification (LLOQ), recovery, matrix effect, carryover, stability (short-term stability of the analyte in

[1] Rules for conducting bioequivalence studies in the Eurasian Economic Union (approved by decision of the Council of the Eurasian Economic Commission № 85 of 03.11.2016). Available at: https://docs.cntd.ru/document/456026107/ Accessed at 19.01.2023.

[2] European Medicines Agency. Available at: https://www.ema.europa.eu/en/bioanalytical-method-validation/ Accessed at: 19.01.2023.

[3] Food and Drug Administration. Available at: https://www.fda.gov/regulatory-information/search-fda-guidancedocuments/bioanalytical-method-validation-guidanceindustry/ Accessed: 19.01.2023.

the matrix ("bench-top" and "post-preparative"); freeze and thaw stability; SSS and WSS stability; long-term stability of the analyte in the matrix). The main validation characteristics with acceptance criteria are presented in Table 6.

Table 6. Validation characteristics

Validation characteristics	Acceptance criteria
Selectivity	Blank samples: the analyte ≤20 % LLOQ, the IS response ≤5 %
Calibration curve	$R \geq 0{,}99$; $-15\ \% \leq E \leq 15\ \%$; except $-20\ \% \leq E \leq 20\ \%$ at LLOQ
Accuracy (inter-day, intra-day)	$-15\ \% \leq E \leq 15\ \%$; except $-20\ \% \leq E \leq 20\ \%$ at LLOQ
Precision (inter-day, intra-day)	RSD ≤ 15 %, except RSD ≤ 20 % at LLOQ
LLOQ	RSD ≤ 20 %, $-20\ \% \leq E, \% \leq 20\ \%$
Recovery	RSD ≤ 15 %;
Matrix effect	IS-normalized matrix factors of the analytes: RSD ≤ 15 %
Stability	$-15\ \% \leq E, \% \leq 15\ \%$
Carryover	Blank samples: the analyte ≤20 % LLOQ, the IS response ≤5 %

Note. RSD, % – relative standard deviation. *E*, % – relative error.

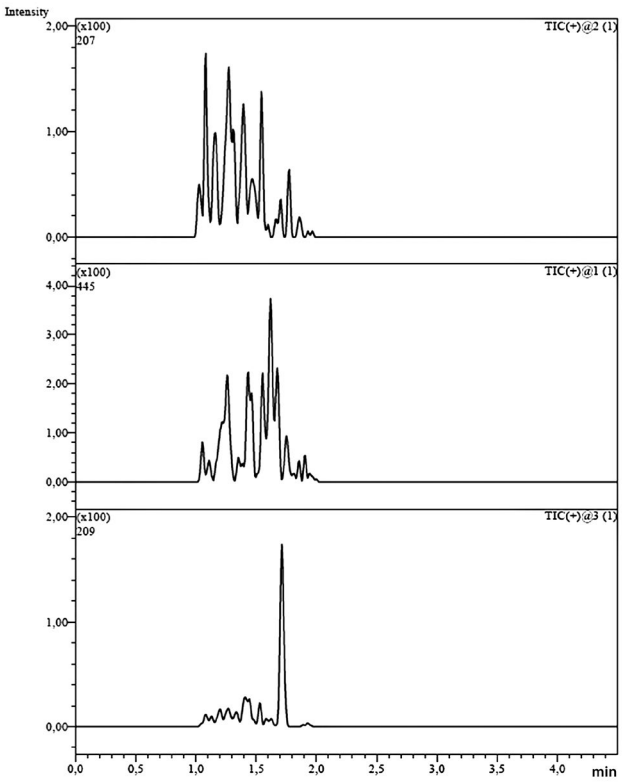


Figure 4. Chromatogram of blank human plasma sample

Selectivity

Six different samples of blank plasma, two different samples of hemolyzed blank plasma and hyperlipidemic blank plasma not containing any analytes, as well as samples with the addition of working standard solutions up to concentrations corresponding to the LLOQ level were analyzed (see Table 2). A chromatogram of a sample of blank human plasma is shown in Figure 4.

Calibration curve

Eight samples of blank blood plasma were analyzed with the addition of working standard solutions of promethazine IS to obtain a concentration of 1 ng/mL in a sample and mixed WSS of nirmatrelvir and ritonavir up to concentrations of nirmatrelvir in the range of 50–10,000 ng/mL and ritonavir in the range of 5–1000 ng/mL. Based on the values obtained, calibration curves were plotted in the coordinates of the ratio of the peak area of nirmatrelvir to the peak area of promethazine from the ratio of the concentration of nirmatrelvir to the concentration of promethazine in blood plasma, as well as calibration curves in the coordinates of the ratio of the peak area of ritonavir to the peak area of promethazine from the ratio of the concentration of ritonavir to the concentration of promethazine in the blood plasma.

The calibration curves were linear. The equations of calibration curves and correlation coefficients (R) for calibration plots in validation cycles № 1–4 are given in Table 7.

Accuracy and precision

QC samples corresponding to LLOQ, L, M1, M2 and H levels were analyzed (see Table 2). The analysis was carried out in four sequences of five sample injections for each concentration level of nirmatrelvir and ritonavir. The study was carried out within cycles and between cycles for 1–4 sequences. To calculate the relative standard deviation (RSD, %) and relative error (E, %) at the level between cycles, data obtained during 1–3 sequences (between cycles, $n = 15$), as well as during 1–4 sequences (between cycles, $n = 20$) were used. The data are presented in Table 8.

Lower limit of quantification

The lower limit of quantification of nirmatrelvir and ritonavir in human plasma was 50 ng/mL for nirmatrelvir and 5 ng/mL for ritonavir. A chromatogram of blood plasma containing nirmatrelvir and ritonavir at the LLOQ level is presented in Figure 5.

Table 7. **Calibration equation and correlation coefficients**

№	NIRM		RIT	
	Calibration equation	R	Calibration equation	R
1	$y = 0.00477758 \cdot x + 0.00871658$	0.9974542	$y = 0.0228336 \cdot x + 0.0752374$	0.9972169
2	$y = 0.00502899 \cdot x + 0.0104544$	0.9985304	$y = 0.0456889 \cdot x + 0.225216$	0.9958858
3	$y = 0.00424893 \cdot x + 0.00205931$	0.9984737	$y = 0.0320798 \cdot x + 0.103687$	0.9991287
4	$y = 0.00341493 \cdot x + 0.0257467$	0.9988095	$y = 0.0114315 \cdot x + 0.0427539$	0.9977584

Table 8. **Accuracy and precision of nirmatrelvir and ritonavir determination**

Injected (μg/mL)	RSD, %						*E*, %					
	Inter-day				Intra-day		Inter-day				Intra-day	
	n = 5 (№ 1)	*n* = 5 (№ 2)	*n* = 5 (№ 3)	*n* = 5 (№ 4)	*n* = 15	*n* = 20	*n* = 5 (№ 1)	*n* = 5 (№ 2)	*n* = 5 (№ 3)	*n* = 5 (№ 4)	*n* = 15	*n* = 20
	NIRM											
50	3.54	8.39	4.31	6.17	6.00	6.17	–4.71	–10.91	–7.69	–9.22	14.46	–9.22
150	4.75	2.95	2.54	6.61	6.88	6.61	–4.60	–9.37	4.32	–4.40	6.58	–4.40
2000	3.46	2.06	2.08	8.33	5.51	8.33	–4.75	–2.82	–13.07	–3.13	10.69	–3.13
4000	3.98	2.80	1.82	7.96	8.74	7.96	1.61	–14.57	1.92	–2.81	7.67	–2.81
8000	2.50	2.27	2.76	7.22	7.46	7.22	–1.49	–14.51	–0.18	–3.67	6.35	–3.67
	RIT											
5	4.51	11.96	8.49	13.44	–7.77	13.44	13.84	–6.28	–13.36	–3.70	–1.93	–3.70
15	3.83	7.40	2.33	7.47	–3.22	7.47	8.68	–2.44	0.43	–0.08	2.22	–0.08
200	1.04	5.09	1.61	10.38	–6.88	10.38	12.76	2.71	–11.74	3.96	1.24	3.96
450	3.58	4.24	3.00	7.07	–3.68	7.07	14.23	–1.68	0.99	3.84	4.51	3.84
800	3.39	2.80	3.66	5.96	–5.40	5.96	7.75	–1.15	–5.16	1.22	0.48	1.22

Recovery

Three samples were prepared from blank blood plasma, hemolyzed plasma and hyperlipidemic blank plasma were analized at QC at L, M1, M2 and H levels (see Table 2) without taking into account the recovery, as well as QC samples prepared on various types of biological blank matrix to assess the recovery. The average nirmatrelvir recovery from various types of blank matrices was 93.85 %, ritonavir recovery was 108.51 %. The RSD of the calculated nirmatrelvir recovery from various types of blank matrices is 11.04 %, ritonavir – 14.64 % (Table 9).

Matrix effect

To evaluate the effect of the biological matrix on the quantification of nirmatrelvir and ritonavir, samples were analyzed with the addition of mixed working standard solutions without taking into account the biological matrix, as well as samples prepared on blank plasma without taking into account the influence of the recovery of the analyzed substances and IS from the biological matrix. The matrix effect was evaluated at the L and H levels (see Table 2). For promethazine IS, the matrix effect was calculated at the level of 1 ng/mL. Based on the data obtained, the IS-normalized matrix factor was calculated (Table 10).

Stability

Three samples were analyzed to evaluate bench-top and post-preparative short-term stability, triple freeze-thaw stability, stability of stock standard solutions and working standard solutions (when stored

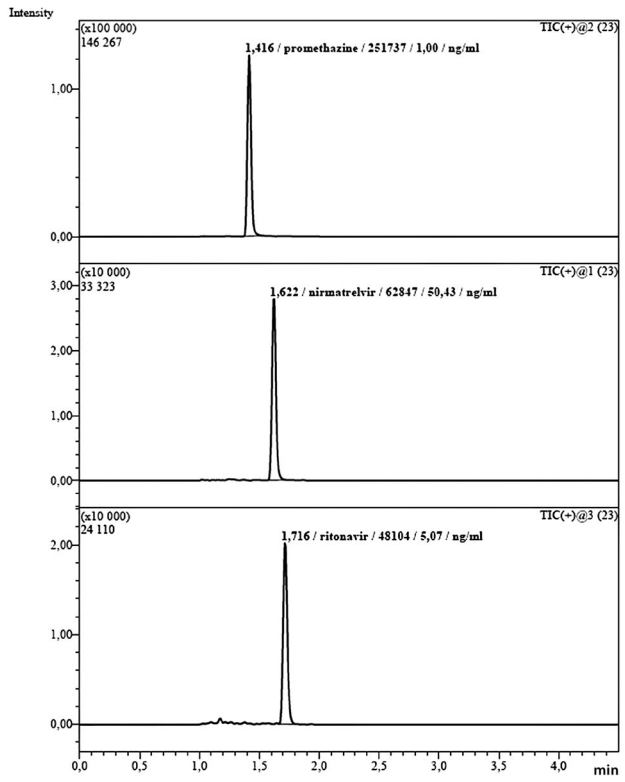


Figure 5. Chromatogram of plasma sample (NIRM 50 ng/mL, RIT 5 ng/mL)

for 17 days at a temperature of –50 to –35 °C), long-term stability of the analyte at levels L and H (see Table 2). Long-term stability was evaluated twice: an interim evaluation was carried out when stored for 17 days at a temperature of –50 to –35 °C, as well as an additional evaluation when stored for 38 days at a temperature of –50 to –35 °C, since the minimum period for evaluation of the type of stability should correspond to the period of storage of samples from the time of collection at the clinical center until the analysis of the last sample in the analytical phase of the study. The results of the stability evaluation are given in Table 11.

Table 10. Calculation of IS-normalized matrix factors of nirmatrelvir and ritonavir

	Blank plasma		Hemolyzed blank plasma		Lipemic blank plasma	
	Normalized Mf					
	L	H	L	H	L	H
NIRM						
Average	1.20	0.95	1.16	0.81	1.09	0.81
RSD, %	4.24	9.81	4.16	5.37	3.55	5.53
RIT						
Average	1.95	1.44	1.67	1.18	1.32	1.17
RSD, %	4.22	5.39	9.95	14.12	7.57	11.09

Table 9. Calculation of nirmatrelvir and ritonavir recovery at levels L, M1, M2, H

Biological matrix	Recovery NIRM, %				Recovery RIT, %			
	L	M1	M2	H	L	M1	M2	H
Blank plasma	86.10	73.60	102.49	80.51	83.77	117.96	123.33	76.04
	85.51	74.87	100.13	93.18	87.48	118.53	123.03	89.66
	91.20	73,61	109.31	91.72	92.75	126.56	135.12	89.80
Hemolyzed blank plasma	96.99	90.96	80.22	113.04	98.72	129.86	107.27	124.46
	88.04	98.16	82.05	105.03	87.55	132.14	105.21	118.66
	87.51	92.60	90.58	106.67	83.20	122.07	114.86	117.33
Lipemic blank plasma	95.96	98.21	98.71	103.32	110.55	108.96	120.82	103.36
	99.93	107.23	89.55	94.42	106.70	118.63	106.27	94.57
	94.43	114.49	94.10	94.22	98.25	131.23	104.67	97.01
Average	93.85				108.51			
SD	10.36				15.89			
RSD	11.04				14.64			

Table 11. **Stability assessment**

Type of stability	Time and storage conditions	Average value of E, %			
		NIRM		RIT	
		L	H	L	H
Bench-top stability	Analyzed freshly prepared; stored at 20 ± 5 °C	4.63	8.56	13.73	12.70
Post-preparative stability	24 hours at 4 °C	–10.35	–11.45	9.62	–1.44
Freeze-thaw stability	36 hours at –42.5 ± 7.5 °C and 6 hours at 20 ± 5 °C	2.67	3.23	3.23	–2.57
Long-term stability	17 days at –42.5 ± 7.5 °C	3.16	4.69	2.67	2.65
	38 days at –42.5 ± 7.5 °C	1.10	4.89	–2.47	–2.06
Stock solution stability	17 days at –42.5 ± 7.5 °C	0.37	4.29	–1.76	–0.93
Work solution stability	17 days at –42.5 ± 7.5 °C	–0.01	5.56	–2.36	–2.65

Carryover

Within validation cycles № 1–3, a sequential analysis of calibration samples at level 8 (see Table 2) and samples of blank plasma was carried out. The results of the carryover are presented in Table 12.

CONCLUSION

A method for the quantification of nirmatrelvir and ritonavir in human plasma by HPLC-MS/MS was developed and validated.

The confirmed analytical range of the method was 50–10,000 ng/mL for nirmatrelvir and ritonavir in the range of 5–1000 ng/mL for ritonavir in blood plasma. This method was used as part of the analytical phase of the study in order to investigate the pharmacokinetic parameters of the combined drugs nirmatrelvir and ritonavir.

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Table 12. **Assessment of carry-over effect**

№	Sample	Area			Area ratio, %		
		NIRM	RIT	PROM	NIRM	RIT	PROM
1	LLOQ	65110	56522	275624	–	–	–
	Blank plasma	0	4358	0	0.00	7.71	0.00
2	LLOQ	66863	66863	312152	–	–	–
	Blank plasma	0	1714	0	0.00	2.56	0.00
3	LLOQ	63158	77307	318969	–	–	–
	Blank plasma	0	0	0	0.00	0.00	0.00

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