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Simultaneous Determination of Major Molnupiravir Metabolite (β-D-N4-hydroxycytidine) and Favipiravir in Human Plasma by HPLC-MS/MS

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Abstract

Introduction. Coronavirus disease 2019 (COVID-19) is an infectious disease caused by the SARS-CoV-2 virus (severe acute respiratory syndrome-related coronavirus 2). COVID-19 is now expected to stay with us for many years as a recurring disease. Molnupiravir and favipiravir are oral antiviral drugs with anti-RNA polymerase activity. The Russian Health Ministry has approved molnupiravir and favipiravir for the treatment 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 β-D-N4-Hydroxycytidine and favipiravir in human blood plasma. The method could be applied in pharmacokinetic study of molnupiravir and favipiravir.

Aim. The aim of this study is to develop and validate a HPLC-MS/MS bioanalytical method for the determination of β-D-N4-Hydroxycytidine and favipiravir in human plasma.

Materials and methods. The determination of β-D-N4-Hydroxycytidine and favipiravir in human plasma by HPLC-MS/MS. The samples were processed by 0.1 % formic acid in acetonitrile. Internal standard: promethazine. Mobile phase: 0.01 mol/L Ammonium formate buffer solution (Eluent A), 0.1 % formic acid and 0.08 % aqueous ammonia in water/acetonitrile 10 : 90 (Eluent B). Column: Shim-pack GWS C18, 150 × 4.6 mm, 5 µm. Analytical range: 50.00–10000.00 ng/mL for β-D-N4-Hydroxycytidine, 250.00–20000.00 ng/mL for favipiravir in human plasma. Ionization source: electrospray ionization. Detection conditions: 260.00 m/z → 82.10 m/z, 260.00 m/z → 111.00 m/z, 260.00 m/z → 127.95 m/z (β-D-N4-Hydroxycytidine); 156.15 m/z → 65.95 m/z, 156.15 m/z → 85.00 m/z, 156.15 m/z → 113.10 m/z (favipiravir); 285.05 m/z → 198.05 m/z (promethazine).

Results and discussion. This method was validated by selectivity, suitability of reference standard, 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 β-D-N4-Hydroxycytidine and favipiravir in human plasma was developed and validated. The analytical range was 50.00–10000.00 ng/mL for β-D-N4-Hydroxycytidine, 250.00–20000.00 ng/mL for favipiravir in human plasma. This method was applied to investigate the pharmacokinetics of molnupiravir and favipiravir.

Keywords: β-D-N4-Hydroxycytidine, NHC, molnupiravir, favipiravir, 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 основного метаболита молнупиравира (β-D-N4-гидроксицитидина) и фавипиравира в плазме крови человека методом ВЭЖХ-МС/МС

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Резюме
Введение. Новая коронавирусная инфекция [Coronavirus Disease 2019 (COVID-19)] – острое инфекционное заболевание, вызываемое вирусом SARS-CoV-2 (Severe acute respiratory syndrome-related coronavirus 2), которое продолжает представлять серьезную опасность для здоровья. Молнупиравир и фавипиравир – противовирусные препараты с анти-РНК-полимеразной активностью, одобренные Министерством здравоохранения Российской Федерации для лечения COVID-19. Разработка и валидация методики совместного определения метаболита молнупиравира β-D-N4-гидроксицитидина и фавипиравира в плазме крови человека является необходимой процедурой для проведения аналитической части клинического исследования с целью дальнейшего изучения фармакокинетики.
Цель. Целью исследования является разработка и валидация методики совместного определения β-D-N4-гидроксицитидина и фавипиравира в плазме крови человека методом высокоэффективной жидкостной хроматографии с тандемным масс-селективным детектированием (ВЭЖХ-МС/МС) для дальнейшего изучения фармакокинетики молнупиравира и фавипиравира.
Материалы и методы. Определение β-D-N4-гидроксицитидина и фавипиравира в плазме крови человека проводили методом ВЭЖХ-МС/МС. В качестве пробоподготовки был использован способ осаждения белков 0,1 % раствором муравьиной кислоты в ацетонитриле. Внутренний стандарт: прометазин. Подвижная фаза: аммонийно-формиатный буфер 0,01 моль/л (Элюент А), 0,1 % муравьиной кислоты, 10 % воды в ацетонитриле с прибавлением 0,08 % аммиака (Элюент В). Колонка: Shim-pack GWS C18, 150 × 4,6 мм, 5 мкм. Аналитический диапазон методики: 50,00–10000,00 нг/мл для β-D-N4-гидроксицитидина, 250,00–20000,00 нг/мл для фавипиравира в плазме крови. Источник ионизации: электроспрей. Условия детектирования: 260,00 m/z → 82,10 m/z, 260,00 m/z → 111,00 m/z, 260,00 m/z → 127,95 m/z (β-D-N4-гидроксицитидин); 156,15 m/z → 65,95 m/z, 156,15 m/z → 85,00 m/z, 156,15 m/z → 113,10 m/z (фавипиравир); 285,05 m/z → 198,05 m/z (прометазин).
Результаты и обсуждение. Разработанная методика была валидирована по следующим параметрам: селективность, пригодность стандартного образца, эффект матрицы, калибровочная кривая, точность, прецизионность, степень извлечения, нижний предел количественного определения перенос пробы, стабильность.
Заключение. Разработана и валидирована методика совместного определения β-D-N4-гидроксицитидина и фавипиравира в плазме крови человека методом ВЭЖХ-МС/МС. Подтвержденный аналитический диапазон методики составил 50,00–10000,00 нг/мл для β-D-N4-гидроксицитидина и 250,00–20000,00 нг/мл для фавипиравира в плазме крови. Полученный аналитический диапазон позволяет применять разработанную методику для проведения фармакокинетических исследований комбинированных препаратов молнупиравира и фавипиравира.

Ключевые слова: β-D-N4-гидроксицитидин, NHC, молнупиравир, фавипиравир, 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 novel coronavirus infection [Coronavirus Disease 2019 (COVID-19)] is an acute infectious disease caused by the SARS-CoV-2 virus (Severe acute respiratory syndrome-related coronavirus 2), which continues to pose a serious health hazard. It is known that the emergence of new variants of coronavirus SARS-CoV-2 leads to an increase in the incidence of COVID-19 [2]. One of the most recent subvariants of the omicron strain of the coronavirus is the Kraken strain [2, 3]. Although the pathogenicity of the Kraken has changed slightly compared to other omicron variants [2], it is important to continue developing new drugs to treat COVID-19 in order to be prepared for the emergence of new variants of the SARS-CoV-2 virus with various pathogenicity.

Currently, according to version № 17 of the temporary guidelines of the Ministry of Health of the Russian Federation on the prevention, diagnosis and treatment of a new coronavirus infection for the etiotropic therapy of COVID-19, it is recommended to prescribe antiviral drugs such as molnupiravir and favipiravir[1].

Molnupiravir (({2R,3S,4R,5R)-3,4-Dihydroxy-5-[4-(hydroxyamino)-2-oxopyrimidine-1(2H)-yl]oxo-lan-2-yl}methyl)(2-methylpropanoate) is an antiviral drug with anti-RNA polymerase activity. Molnupiravir is a prodrug

[1] Temporary guidelines "Prevention, diagnosis and treatment of a new 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: 05.02.2023.

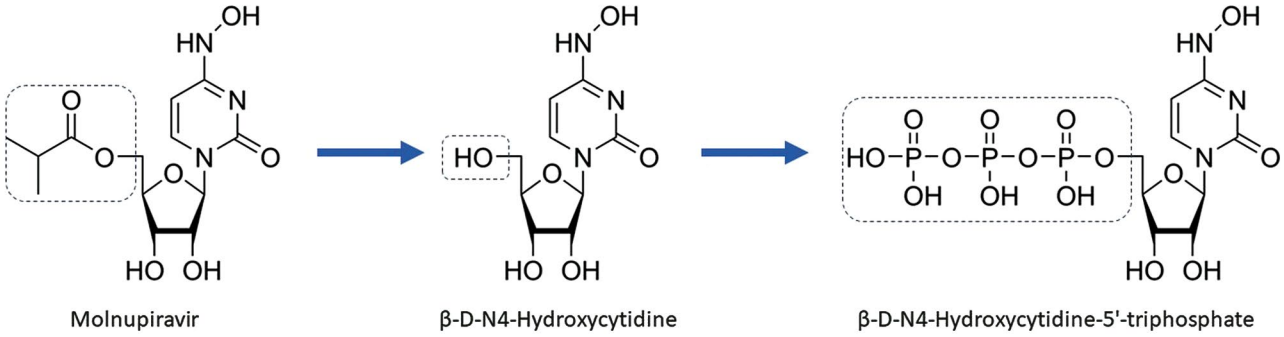

Figure 1. Molnupiravir metabolism

that in the blood plasma under the action of esterase is metabolized to an analogue of ribonucleoside [β-D-N 4-Hydroxycytidine]. Then β-D-N4-hydroxycytidine is phosphorylated by kinase to form the active ribonucleoside triphosphate, which, in turn, is embedded in the RNA of the virus causing the accumulation of mutations and suppression of viral replication [4–6]. The scheme of metabolic transformations of molnupiravir is presented in figure 1. The drug has oral bioavailability, so it can be prescribed to outpatients in the early stages of the COVID-19 disease [5, 6]. In Russia, molnupiravir is recommended for the treatment of mild or moderate COVID-19 in adults, including cases of the increased risk of progression of COVID-19 to severe course, and not requiring additional oxygen therapy. For drug therapy of COVID-19, molnupiravir should be initiated as soon as possible after diagnosis[1].

Favipiravir (5-fluorine-2-oxo-1H-pyrazine-3-carboxamide, FAV) is a synthetic drug with a direct antiviral action against viral RNA polymerase. The mechanism of action of favipiravir is similar to that of molnupiravir. Similar to molnupiravir, favipiravir is a prodrug that, after phosphorylation, becomes active and inhibits viral RNA polymerase [7, 8]. In Russia, favipiravir preparations for oral and parenteral administration[2] are registered.

It is known that molnupiravir and favipiravir can be co-administered since both drugs have a high bioavailability when taken orally [7]. It is also noted that when co-administered with favipiravir, molnupiravir can be used in a lower dose [7, 9].

A number of new studies have now been published to determine molnupiravir metabolite β-D-N4-hydroxycytidine, as well as favipiravir in human biological fluids, in order to investigate pharmacokinetic parameters. To determine β-D-N4-hydroxycytidine in biological fluids, the methods of high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) are used, as well as ultra-performance liquid chromatography – tandem mass spectrometry (UPLC-MS/MS). To determine favipiravir, the methods of high performance liquid chromatography with ultraviolet detection (HPLCUV), high-performance liquid chromatography with fluorescence detection (HPLC-FLD)] are used, as well as HPLC-MS/MS and UHPLC-MS/MS.

Among the methods for determination of β-D-N4-hydroxycytidine, the method of protein precipitation with acetonitrile, as well as ultrafiltration, is used as a sample preparation; among the methods for determination of favipiravir – liquid-liquid extraction (LLE), as well as precipitation of plasma proteins with various organic solvents (table 1).

Up to date, no methods for the co-determination of β-D-N4-hydroxycytidine and favipiravir in human biological matrices have been published in the scientific literature, so it was decided to develop and validate a method for the co-determination of this combination of substances independently. Earlier, we had already developed a method for determination of favipiravir in the blood plasma by HPLC-UV in order to study the pharmacokinetics of favipiravir with parenteral administration [15]. This study provides the development and validation of a method for the co-determination of β-D-N4-hydroxycytidine and favipiravir in human plasma by HPLC-MS/MS for oral administration.

[1] Temporary guidelines "Prevention, diagnosis and treatment of a new 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: 05.02.2023.

[2] GRLS – Ministry of Health of the Russian Federation. Available at: https://grls.rosminzdrav.ru/ Accessed: 05.02.2023.

Table 1. **Bioanalytical methods of β-D-N4-Hydroxycytidine and favipiravir quantitative determination**

Analytical method (ionization source; ionization (+/–) if applicable)	Object	Sample preparation	Analytical range	Reference
NHC				
HPLC-MS/MS (electrospray; +)	Human plasma	Protein precipitation by acetonitrile	20.0–10000.0 ng/mL	[10]
HPLC-MS/MS (electrospray; -)	Human plasma, salvia	Protein precipitation by acetonitrile	2.50–5000.00 ng/mL	[11]
UPLC-MS/MS (electrospray; +)	Human plasma	Ultrafiltration	1.00–5000.00 ng/mL	[12]
FAV				
HPLC-UV	Human plasma	Liquid-liquid extraction	3.10–60.00 μg/mL	[13]
HPLC-UV	Human plasma	Liquid-liquid extraction	0.10–100.00 μg /mL	[14]
HPLC-UV	Human plasma	Protein precipitation by methanol	0.25–200.00 μg /mL	[15]
HPLC-FLD	Human plasma	Protein precipitation by isopropanol	40.00–240.00 ng/mL	[16]
HPLC-MS/MS (electrospray; –)	Human plasma	Protein precipitation by methanol	100.00–20000.00 ng/mL	[17]
UPLC-MS/MS (electrospray; –)	Human plasma	Protein precipitation by acetonitrile	0.25–16.00 μg/mL	[18]

MATERIALS AND METHODS

Equipment

For chromatographic separation and detection, a high-performance liquid chromatograph Nexera XR with a gradient pump, a column and sample thermostat, a degasser, an autosampler, an automatic sample feeder to an autosampler, a high-pressure flow switching valve and a tandem mass spectrometric detector LCMS-8040 (triple quadrupole) were used. Source data were processed with LabSolutions software (Ver. 5.91, Shimadzu Corporation, Japan).

Reagents and solutions

During the study, the following reagents were used: acetonitrile (HPLC Super Gradient grade, Macron, Poland); methanol (chemically pure grade, LLC "TH CHIMMED", Russia); formic acid (98 % pure grade, PanReac, Germany & AppliChem, Spain); aqueous solution of ammonia 30% (for analysis, ACS, PanReac, Germany & AppliChem, Spain); ammonium formate "eluent additive for LC-MS, LiChropur™, >99.0 %", Sigma-Aldrich, USA); demineralized water (I grade of purity).

For the preparation of stock standard solutions (SSS) and working standard solutions (WSS), reference standards (RS) of β-D-N-N4-hydroxycytidine (JSC "Biochemist", Russia, assay of 93.00 %), favipiravir (JSC "Biochemist", Russia, assay of 100.40 %), as well as promethazine hydrochloride (USP reference standard, France, assay of 99.90 %) were used. β-D-N4-hydroxycytidine and favipiravir, as well as the internal standard (IS) of promethazine (PROM) were prepared by dissolving the accurate weighs of the substances in methanol. The concentration of β-D-N4-hydroxycytidine was 200,000.00 ng/ml, favipiravir – 400,000.00 ng/ml, promethazine – 100,000.00 ng/ml.

Mixed working standard solutions of β-D-N4-hydroxycytidine and favipiravir were prepared by diluting β-D-N4-hydroxycytidine and favipiravir with methanol

until plasma concentrations of calibration levels № 1 to 8 and quality control levels (QC) were obtained: lower limit of quantification (LLOQ), low (L), middle (M1 and M2)] and high levels (high) (table 2). Working standard solution of promethazine internal standard was prepared by dilution of promethazine stock standard solution with methanol to obtain plasma concentrations of 256.10 ng/ml.

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

Sample preparation

In the Eppendorf centrifuge microtube, an aliquot of a sample was transferred, a working standard solution of promethazine IS was added, then plasma proteins were precipitated with 0.1 % solution of formic acid in acetonitrile. The sample was then stirred on a vortex shaker for 10 seconds, centrifuged for 15 min with an acceleration of 15,000 g, the supernatant was transferred to chromatographic vials, and the vials were placed in the chromatographic autosampler. Figure 2 shows a sample preparation scheme.

Conditions of chromatographic separation and detection

- Column: Shim-pack GWS C18, 150 × 4.6 mm, 5 µm.
- Precolumn: Phenomenex SecurityGuardTM Cartridges C18, 4 × 3.0 mm, 5 µm.
- Thermostat temperature: 40 °C.

Table 2. **β-D-N4-Hydroxycytidine and favipiravir concentrations at calibration levels and quality control samples**

Level	Analyte concentration, ng/ml		IS concentration, ng/ml
	NHC	FAV	PROM
1	50,00	250,00	256,10
2	250,00	500,00	256,10
3	500,00	1000,00	256,10
4	1000,00	3000,00	256,10
5	3000,00	6000,00	256,10
6	6000,00	10000,00	256,10
7	8000,00	15000,00	256,10
8	10000,00	20000,00	256,10
LLOQ	50,00	250,00	256,10
L	150,00	750,00	256,10
M1	2000,00	4000,00	256,10
M2	5000,00	12000,00	256,10
H	7500,00	16000,00	256,10

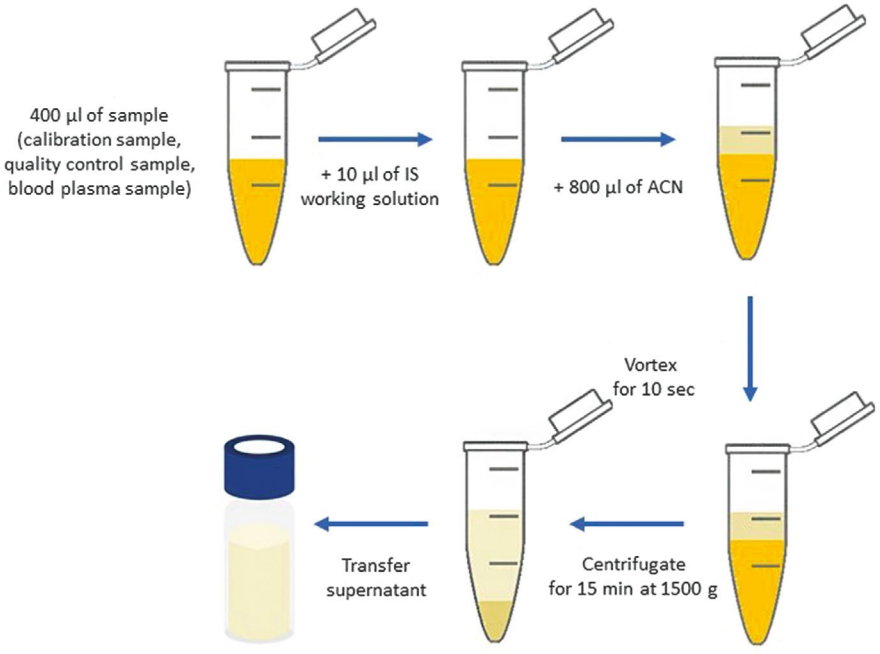

Figure 2. **Sample preparation**

- Mobile phase: Eluent A: ammonium-formate buffer 0.01 mol/L (by volume); Eluent B: 0.1 % formic acid, 10 % water in acetonitrile with the addition of 0.08 % ammonia (by volume).
- Gradient by the mobile phase composition and flow rate of the mobile phase are presented in table 3.

Table 3. Gradient elution

Time, min	Eluent A, %	Eluent B, %	Flow rate, mL/min
0,00	94,00	6,00	1,70
0,90	94,00	6,00	1,70
0,95	0,00	100,00	1,70
1,00	0,00	100,00	1,00
1,40	0,00	100,00	1,00
1,50	94,00	6,00	1,00
3,10	94,00	6,00	1,00
3,20	94,00	6,00	1,70
4,50	94,00	6,00	1,70

- Sample injection volume: 20 µl.
- Run time by mass spectrometric detector: 0.00–4.50 min.
- Ionization source: electrospray.
- Parameters of the ionization source: spray gas 3 l/min, drying gas 20 l/min, heating unit 400 °C, desolvation line 200 °C.
- Capillary stress, ionization mode and detection conditions are presented in table 4.

Table 4. Ion-source parameters and detection conditions

	Ion mode, capillary voltage, kV	Detection conditions, m/z
NHC	+5,0	260,00 → 82,10 260,00 → 111,00 260,00 → 127,95
FAV	–5,0	156,15 → 65,95 156,15 → 85,00 156,15 → 113,10
PROM	+4,5	285,05 → 198,05

RESULTS AND DISCUSSION

Method development

Molnupiravir in human plasma is metabolized to β-D-N4-hydroxycytidine, which does not bind to plasma proteins [4–6]. Due to that, the main metabolite β-D-N4-hydroxycytidine is quantified in human plasma.

As an internal standard for β-D-N 4-hydroxycytidine and favipiravir, promethazine ((RS)-N,N,α-trimethyl-10H-phenothiazine-10-ethanamin was selected. Promethazine has a structure and physicochemical properties similar to analytical substances (table 5).

Table 5. Chemical and physical characteristics of the analytes and IS

	pKa	log P	Molecular weight
NHC[1]	12.55	–2,70	259,218
FAV[2]	9,39	0,49	157,104
PROM[3]	9,05	4,29	284,419

Note. [1] N4-Hydroxycytidine. Drugbank. Available at: https://go.drugbank.com/drugs/DB15660. Accessed: 05.02.2023.

[2] Favipiravir. Drugbank. Available at: https://go.drugbank.com/drugs/DB12466. Accessed: 05.02.2023.

[3] Promethazine. Drugbank. Available at: https://go.drugbank.com/drugs/DB01069. Accessed: 05.02.2023.

For the analysis, the conditions of mass spectrometric detection were used, allowing to obtain peaks of β-D-N4-c, favipiravir and promethazine with the highest intensity. For that, while developing the method, fragments obtained at different impact energies were analyzed.

For the chromatographic separation of the combination of substances, based on their physicochemical properties, the column Shimpack GWS C18, 150 × 4.6 mm, 5 µm was selected.

As a sample preparation, variants of precipitation of plasma proteins with acetonitrile, acidified acetonitrile solution, methanol and trifluoroacetic acid were considered. As a result, 0.1 % solution of formic acid in acetonitrile was selected as a precipitator, since only when it was used, the most complete precipitation of plasma proteins occurred, and the optimal form of chromatographic peaks was provided.

Method validation

The developed bioanalytical method was validated in accordance with the Rules for conducting bioequivalence studies within the Eurasian Economic Union[1] based

[1] Rules for conducting drug bioequivalence studies within the Eurasian Economic Union (approved by Decision № 85 of the Council of the Eurasian Economic Commission of 03.11.2016). Available at: https://docs.cntd.ru/document/456026107/ Ссылка активна на 05.02.2023.

on the rules of the EMA[1] and FDA[2]. guidelines. The method was fully validated for the following parameters: selectivity, reference standard suitability, calibration curve, accuracy and precision, recovery, lower limit of quantification (LLOQ), sample carry-over, stability (stability of stock reference solutions and working standard solutions; short-term stability of the analyte in the matrix ("desktop" and "post-preparative"), long-term stability of the analyte in the matrix; stability with triple freezing-thawing). 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 %
Suitability of reference standard	Zero calibrators: the analyte ≤20 % LLOQ, calibration level 8 no IS ≤5 % IS
Calibration curve	R ≥ 0,99; –15 % ≤ E ≤ 15 %, except –20 % ≤ E ≤ 20 % at LLOQ
Accuracy (inter-day, intra-day)	–15 % ≤ E ≤ 15 % , except –20 % ≤ E ≤ 20 % at LLOQ
Precision (inter-day, intra-day)	RSD ≤ 15 %, except RSD ≤ 20 % at LLOQ
LLOQ	RSD ≤ 20 %, –20 % ≤ E, % ≤ 20 %
Spike recovery	RSD ≤ 15 %
Matrix effect	IS-normalized matrix factors of the analytes: RSD ≤ 15 %;
Stability	–15 % ≤ E, % ≤ 15 %
Carry-over effect	Blank samples: the analyte ≤20 % LLOQ, the IS response ≤5 %

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

Selectivity

Six different samples of blank plasma, hemolyzed blank plasma and hyperlipidemic blank plasma were used, in which the absence of the analytes was proved, as well as samples with the addition of working standard solutions up to concentrations of the LLOQ level (see table 2). A chromatogram of a sample of human blank plasma is shown in figure 3.

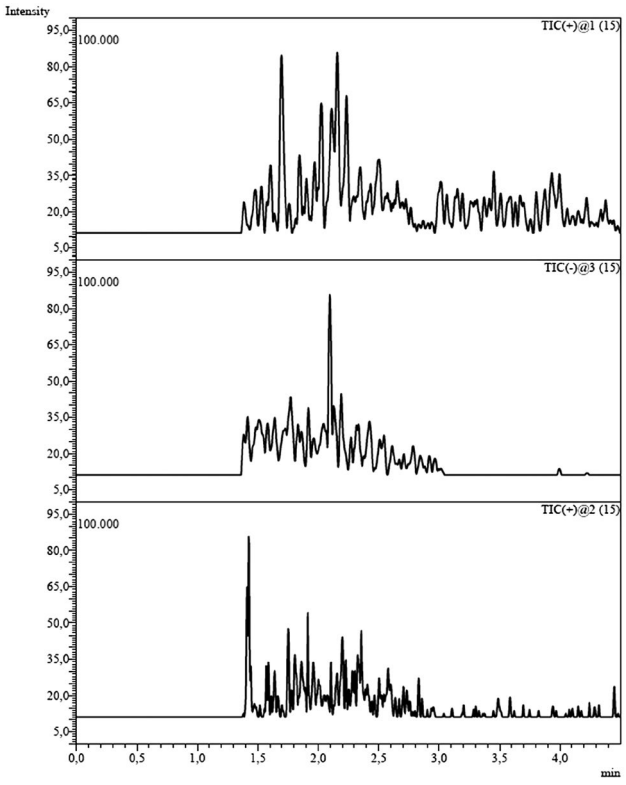

Figure 3. Chromatogram of blank human plasma sample

Reference standard suitability

A zero sample prepared with the addition of the IS solution (PROM 256.10 ng/ml), a sample prepared from blank plasma with the addition of mixed WSS (NHC 10,000.00 ng/ml, FAV 20,000.00 ng/ml) and without the addition of IS, a quality control sample at the LLOQ level (NHC 50.00 ng/ml, FAV 250.00 ng/ml) was analyzed. A chromatogram of the sample to evaluate RS suitability is given in Figure 4.

Calibration curve

To evaluate this parameter, eight samples of blank plasma were analyzed with the addition of working standard solution of promethazine IS to a concentration of 256.10 ng/ml and mixed WSS of β-D-N 4-hydroxycytidine and favipiravir to concentrations of β-D-N 4-hydroxycytidine in the range of 50.00-10,000.00 ng/ml, and favipiravir in the range of 250.00-20,000.00 ng/ml. Based on the obtained values, calibration plots were constructed in the coordinates of the ratio of the peak area of the analyte to the peak area of the IS from the ratio of the analyte concentration to the IS concentration in the blood plasma.

[1] European Medicines Agency. Available at: https://www.ema.europa.eu/en/bioanalytical-method-validation/ Accessed: 05.02.2023.

[2] Food and Drug Administration. Available at: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/bioanalytical-method-validation-guidance-industry/ Accessed: 05.02.2023.

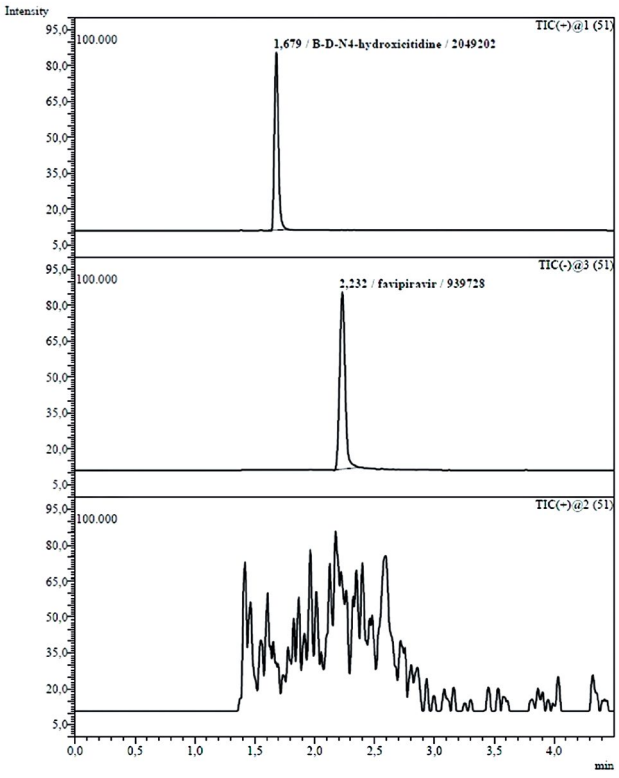

Figure 4. Chromatogram of sample without IS (NHC 10 000.00 ng/mL, FAV 20 000.00 ng/mL)

Calibration graphs were linear. The calibration curve equations and correlation coefficients (R) for calibration graphs in validation cycles № 1–4 are shown in table 7. The obtained values of the correlation coefficients are >0.99.

Accuracy and precision

Calibration samples of blood plasma corresponding to the quality control levels of LLOQ, L, M1, M2 and H were analyzed (see table 2). The analysis was carried out in 4 sequences of 5 sample injections for each concentration level of β-D-N 4-hydroxycytidine and favipiravir. The study was carried out for 1–4 sequences (intra- and inter-cycle). The relative standard deviation (RSD, %) and relative error (E, %) were calculated for the obtained values; at the inter-cycle level, data obtained during 1–3 sequences (inter-cycle, $n = 15$), as well as for 1–4 sequences (inter-cycle, $n = 20$) were used. The data on accuracy and precision of the method for determination of β-D-N4-hydroxycytidine and favipiravir between 1–4 sequences ($n = 20$) are presented in table 8.

Table 8. Accuracy and precision of β-D-N4-Hydroxycytidine and favipiravir determination ($n = 20$)

Введено (мкг/мл) Injected (μg/mL)	NHC		ФАВ FAV	
	RSD, %	E, %	RSD, %	E, %
LLOQ	10,95	–0,58	9,82	–0,01
L	5,17	0,76	8,44	–5,23
M1	2,66	0,32	5,13	–6,34
M2	4,83	–4,52	5,26	–6,46
H	4,19	–6,39	4,70	–5,32

Lower limit of quantification

As LLOQ of the method, the minimum concentration of β-D-N4-hydroxycytidine and favipiravir in the blood plasma in the analytical range was adopted, which may be quantified with RSD and E values not exceeding 20 %: 50.00 ng/ml for β-D-N4-hydroxycytidine and 250.00 ng/ml for favipiravir. A chromatogram of blood plasma containing analytical substances at the LLOQ level is presented in figure 5.

Table 7. Calibration equation and correlation coefficients

№	NHC		FAV	
	Calibration equation	R	Calibration equation	R
1	0,0357194 · *x* + 0,00290660	0,9987935	0,00746999 · *x* + 0,000430450	0,9992086
2	0,0438155 · *x* + 0,00249078	0,9996536	0,00819751 · *x* + 0,000837929	0,9985260
3	0,0390813 · *x* + 0,00274801	0,9980786	0,00721237 · *x* – 3,78162e – 007	0,9987659
4	0,0310063 · *x* + 0,00444778	0,9977221	0,00300419 · *x* + 0,000701577	0,9965709

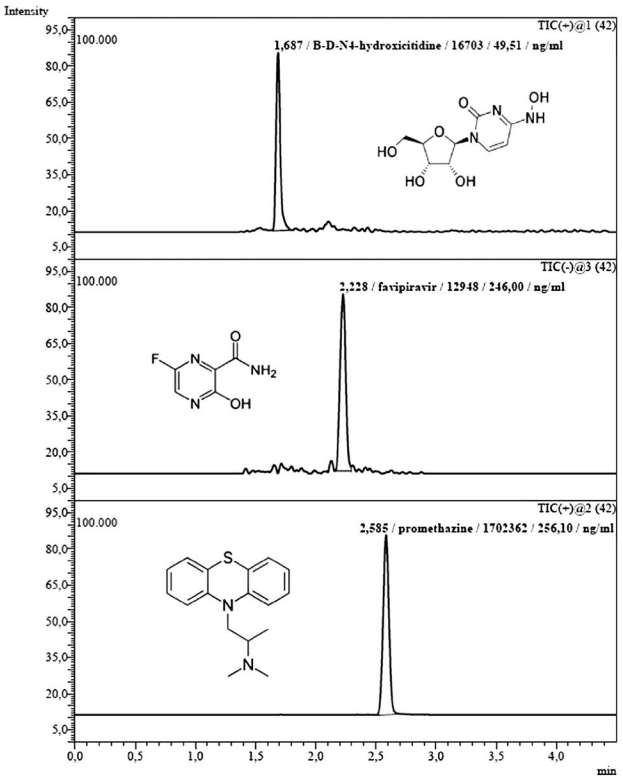

Figure 5. Chromatogram of plasma sample (NHC 50.00 ng/mL, FAV 250.00 ng/mL)

Recovery

Three samples prepared from blank plasma, hemolyzed blank plasma and hyperlipidemic blank plasma without the recovery effect at L, M1, M2 and H levels were analyzed (see table 2), as well as QC samples prepared on various types of biological blank matrix to evaluate the recovery. The mean value of β-D-N4-hydroxycytidine recovery from various types of blank biological matrices is 91.37% (RSD = 10.20 %), favipiravir – 91.96 % (RSD = 12.75 %). The data are presented in table 9.

Matrix effect

To evaluate the effect of the biological matrix on the quantification of β-D-N4-hydroxycytidine and favipiravir, samples with the addition of mixed working standard solutions without the effect of the biological matrix, as well as samples prepared on blank plasma not considering the effect of the recovery of the analytes and IS from the biological matrix, were analyzed. The matrix effect was evaluated at levels L and H of the analytical concentration ranges of β-D-N4-hydroxycytidine and favipiravir (see table 2). For promethazine IS, the matrix effect was calculated at 256.10 ng/ml. The data are presented in table 10.

Table 9. Calculation of β-D-N4-Hydroxycytidine and favipiravir recovery at levels L, M1, M2, H

Biological matrix	Recovery NHC, %				Recovery FAV, %			
	L	M1	M2	H	L	M1	M2	H
Blank plasma	79,51	100,45	95,72	88,93	71,89	92,79	97,00	83,87
	83,72	98,75	105,48	93,07	73,16	98,42	107,71	95,30
	80,08	108,52	111,36	86,89	67,18	108,35	106,65	83,95
Hemolyzed blank plasma	87,34	98,58	90,01	94,65	76,19	100,41	104,01	91,37
	77,66	101,50	91,09	92,27	75,67	105,71	98,33	92,51
	86,66	91,25	96,63	97,40	85,67	91,36	104,00	97,25
Lipemic blank plasma	75,06	95,62	95,43	80,90	70,24	101,50	104,37	79,89
	74,45	97,28	93,27	86,42	85,22	101,08	95,25	88,36
	78,59	96,27	99,72	78,75	87,81	98,78	105,51	83,78
Average	91,37				91,96			
RSD	10,20				12,75			

Table 10. **Calculation of IS-normalized matrix factors of β-D-N4-Hydroxycytidine and favipiravir**

Biological matrix	NHC		FAV	
	Normalized Mf			
	L	H	L	H
Blank plasma	0,77	1,10	1,00	1,26
	0,79	1,05	0,96	1,16
	0,80	1,05	1,09	1,16
	0,76	1,04	0,97	1,12
	0,80	1,06	0,97	1,22
Hemolyzed blank plasma	0,84	1,04	1,03	1,21
	0,70	0,95	1,02	1,15
	0,83	0,93	0,99	1,06
	0,72	0,85	0,95	0,99
	0,72	0,95	0,83	1,07
Lipemic blank plasma	0,70	1,09	1,01	1,29
	0,78	1,12	0,99	1,33
	0,73	1,07	0,93	1,13
	0,79	0,99	0,90	1,12
	0,79	1,07	0,91	1,14
Average	0,78	1,05	0,98	1,19
RSD, %	6,70	10,31	6,45	9,59

Stability

Five samples were analyzed to evaluate desktop and post-preparative short-term stability, stability in triple freezing-thawing, stability of stock and working standard solutions (when stored for 24 days at temperature of –50 to –35 °C), long-term stability of the analyte at L and H levels (Table 2). Long-term stability was evaluated twice: an intermediate evaluation was carried out when stored for 24 days at temperature of –25 to –15 °C and –85 to –65 °C, as well as an additional evaluation when stored for 59 days at temperature of –25 to –15 °C and –85 to –65 °C, since the minimum period for evaluation of this type of stability should correspond to the period of sample storage from the beginning of sampling in the clinical site until the completion of the analysis of the last sample of the analytical study stage. The results of the stability evaluation are presented in table 11.

Sample carry-over

To evaluate the "sample carry-over" parameter in validation cycles № 1 to 3, the successive analysis of calibration samples at level 8 (see table 2) and samples of blank plasma was performed. The results of the sample carry-over evaluation are given in table 12.

CONCLUSION

The method for the co-quantification of β-D-N4-hydroxycytidine and favipiravir in human plasma by HPLC-MS/MS was developed and validated. The con-

Table 11. **Stability assessment**

Type of stability	Time and storage conditions	Average value of E, %			
		NHC		FAV	
		L	H	L	H
Bench-top stability	Analyzed freshly prepared; stored at 20 ± 5 °C	–10,97	–7,53	–2,54	1,63
Postpreparative stability	78 hours at 4 °C	–3,55	–4,99	–8,58	–5,68
Freeze-thaw stability	36 hours at –42.5 ± 7.5 °C and 6 hours at 20 ± 5 °C	4,66	–9,66	–1,86	–14,70
Long-term stability	24 days at –20 ± 5 °C	7,02	–1,63	–8,82	–11,89
	24 days at –75 ± 10 °C	9,81	0,44	–12,22	–11,94
	59 days at –20 ± 5 °C	–12,68	–8,29	–11,87	–13,03
	59 days at –75 ± 10 °C	–10,61	–5,71	–10,42	–11,41
Stock solution stability	24 days at –42.5 ± 7.5 °C	–2,23	–3,43	–9,62	–10,42
Work solution stability	24 days at –42.5 ± 7.5 °C	8,15	–1,37	–7,11	–10,01

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

№	Sample	Area			Area ratio, %		
		NHC	FAV	PROM	NHC	FAV	PROM
1	LLOQ	17042	12405	1758043	–	–	–
	Blank plasma	1886	0	0	11,07	0,00	0,00
2	LLOQ	16204	11618	1348471	–	–	–
	Blank plasma	0	0	14723	0,00	0,00	1,09
3	LLOQ	15746	12016	1581170	–	–	–
	Blank plasma	0	0	16587	0,00	0,00	1,05

firmed analytical range of the method was 50.00–10,000.00 ng/mL for β-D-N4-hydroxycytidine and 250.00–20,000.00 ng/mL for favipiravir in the blood plasma. This analytical range allows using the developed method for pharmacokinetic studies of combined preparations of molnupiravir and favipiravir.

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