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Determination of Favipiravir in Human Blood Plasma by HPLC-MS/MS

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Abstract

Introduction. Favipiravir is one of the most well-known broad-spectrum drugs against many RNA viruses, including the severe acute respiratory syndrome virus 2 [severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)]. Due to its structure, favipiravir is embedded in the RNA of the virus and blocks its further replication in the cell of the human body. Favipiravir is also included in the list of vital and essential medicines, which confirms the importance for Russian healthcare of this drug in the fight against common RNA viruses. We have already published bioanalytical methods for determining favipiravir in human blood plasma by high-performance liquid chromatography with an ultraviolet detector (HPLC–UV) in order to study the pharmacokinetics of favipiravir with parenteral administration (the analytical range of the technique was 0.25–200.00 μg/ml for the dosage of favipiravir 400 mg in 1 vial of lyophilizate for the preparation of concentrate for the preparation of solution for infusions) and by HPLC with tandem mass-selective detection (HPLC-MS/MS) in order to study the pharmacokinetics of β-D-N4-hydroxycytidine and favipiravir in their joint determination in blood plasma with oral administration (the analytical range of the technique was 250.00–20000.00 ng/ml for the dosage of favipiravir 400 mg in 1 tablet). The expectation of low favipiravir's concentrations (the dosage of favipiravir in the drugs in question is 200 mg in 1 tablet in this study) and, in this regard, the expansion of the range by reducing the value of the lower limit of quantitative determination (LLOQ) used in this study necessitates the development of another method. Therefore, this study is given the development and validation of a method for determining favipiravir in human blood plasma by HPLC-MS/MS with an analytical range of 50.00–15000.00 ng/ml.

Aim. The aim of this study is to develop a method for quantitative determination of favipiravir in human blood plasma by HPLC-MS/MS for further for further researches of pharmacokinetics and bioequivalence of drugs.

Materials and methods. In the process of sample preparation, a method of proteins precipitation with methanol was used. A solution labeled with stable isotopes of favipiravir-13C3 was used as an internal standard, the mobile phase was a 0.1 % solution of formic acid in water (eluent A) and methanol (eluent B). Chromatographic column – Phenomenex Kinetex C18, 100×3.0 mm. The determination of favipiravir in human blood plasma was carried out by HPLC using a tandem mass spectrometric detector with a triple quadrupole. The analytical range for favipiravir is 50.00–15000.00 ng/ml in human blood plasma.

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

Conclusion. A method of quantitative favipiravir's determination in human blood plasma by HPLC-MS/MS with a confirmed analytical range of 50.00–15000.00 ng/ml in human blood plasma has been developed and validated. This method allows using it for the analytical part of pharmacokinetics and bioequivalence studies of drugs containing favipiravir in order to expand their range in the domestic pharmaceutical market.

Keywords: favipiravir, human blood plasma, validation, COVID-19, validation, HPLC-MS/MS, pharmacokinetics, bioequivalence

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 Komarov, Olga A. Archakova, Dana S. Shchelgacheva and Polina A. Karpova participated in the development and validation of the bioanalytical methodology. Polina K. Karnakova was preparing samples for work. Natalia S. Bagaeva was responsible for the statistical processing of the received data. Igor E. Shokhin, Kira Ya. Zaslavskaya and Petr A. Bely Bely provided organization of the study. All the above authors participated in the discussion of the obtained results in the form of a scientific discussion.

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Определение фавипиравира в плазме крови человека методом ВЭЖХ-МС/МС

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Резюме

Введение. Одним из наиболее известных препаратов широкого спектра действия против многих РНК-вирусов, в том числе и вируса тяжелого острого респираторного синдрома 2 [severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)] является фавипиравир. За счет своей структуры фавипиравир встраивается в РНК вируса и блокирует его дальнейшую репликацию в клетке организма человека. Также фавипиравир указан в перечне жизненно необходимых и важнейших лекарственных препаратов, что подтверждает значимость для российского здравоохранения этого лекарственного средства в борьбе с распространенными РНК-вирусами. Нами уже были опубликованы биоаналитические методики определения фавипиравира в плазме крови методом высокоэффективной жидкостной хроматографии с ультрафиолетовым детектором (ВЭЖХ-УФ) с целью изучения фармакокинетики фавипиравира при парентеральном введении (аналитический диапазон методики составил 0,25–200,00 мкг/мл для дозировки фавипиравира 400 мг в 1 флаконе лиофилизата для приготовления концентрата для приготовления раствора для инфузий) и методом ВЭЖХ с тандемным масс-селективным детектированием (ВЭЖХ-МС/МС) с целью изучения фармакокинетики β-D-N4-гидроксицитидина и фавипиравира при их совместном определении в плазме крови при пероральном введении (аналитический диапазон методики составил 250,00–20000,00 нг/мл для дозировки фавипиравира 400 мг в 1 таблетке). Ожидание низких значений концентраций фавипиравира (в данном исследовании дозировка фавипиравира в рассматриваемых лекарственных средствах составляет 200 мг в 1 таблетке) и, в связи с этим расширение диапазона за счет снижения значения нижнего предела количественного определения (НПКО), применяемого в данном исследовании, обуславливает необходимость разработки другой методики. Поэтому в настоящем исследовании освещается разработка и валидация методики определения фавипиравира в плазме крови человека методом ВЭЖХ-МС/МС с аналитическим диапазоном 50,00–15000,00 нг/мл.

Цель. Разработка и валидация методики определения фавипиравира в плазме крови человека методом ВЭЖХ-МС/МС для дальнейшего исследования фармакокинетики и биоэквивалентности лекарственных средств фавипиравира является целью настоящего исследования.

Материалы и методы. В процессе пробоподготовки использовался способ осаждения белков метанолом. Раствор меченного стабильными изотопами фавипиравира-13С3 применялся как внутренний стандарт, подвижной фазой служили 0,1%-й раствор муравьиной кислоты в воде (элюент А) и метанол (элюент В). Хроматографическая колонка – Phenomenex Kinetex C18, 100 × 3,0 мм. Определение фавипиравира в плазме крови человека проводили методом ВЭЖХ посредством тандемного масс-спектрометрического детектора с тройным квадруполем. Аналитический диапазон для фавипиравира – 50,00–15000,00 нг/мл в плазме крови человека.

Результаты и обсуждение. Проведение валидации разработанной методики было осуществлено по следующим параметрам: селективность, калибровочная кривая, точность, прецизионность, эффект матрицы, перенос пробы, степень извлечения, НПКО, стабильность (стабильность исходных и рабочих стандартных растворов аналита; краткосрочная стабильность; стабильность при трехкратной заморозке-разморозке; долгосрочная стабильность аналита в матрице).

Заключение. Разработана и валидирована методика количественного определения фавипиравира в плазме крови человека методом ВЭЖХ-МС/МС с подтвержденным аналитическим диапазоном 50,00–15000,00 нг/мл в плазме крови человека. Данная методика позволяет использовать ее для аналитической части исследований фармакокинетики и биоэквивалентности лекарственных средств, содержащих фавипиравир, с целью расширения их ассортимента на отечественном фармацевтическом рынке.

Ключевые слова: фавипиравир, плазма, COVID-19, валидация, ВЭЖХ-МС/МС, фармакокинетика, биоэквивалентность

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 и П. А. Карпова участвовали в разработке и валидации биоаналитической методики. П. К. Карнакова занималась подготовкой проб к работе. Н. С. Багаева отвечала за статистическую обработку полученных данных. И. Е. Шохин, К. Я. Заславская и П. А. Белый обеспечивали организацию исследования. Все вышеуказанные авторы участвовали в обсуждении полученных результатов в форме научной дискуссии.

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ВВЕДЕНИЕ

Initially, favipiravir was studied as a potential means of treatment for severe infections caused by various RNA viruses, including influenza, arenaviruses (Junin, Machupo and Pichinde), phleboviruses (Rift Valley fever, Punta Toro), hantaviruses (Maporal, Dobrava and Prospect Hill), flaviviruses (yellow fever and West Nile virus), enteroviruses (polio virus and rhinoviruses), alphavirus, paramyxovirus, respiratory syncytial virus, and noroviruses. However, with the emergence of the COVID-19 pandemic, attention shifted to exploring favipiravir's efficacy against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections [1, 2].

Favipiravir functions as a prodrug, converting into its active form, ribosyl triphosphate, once inside the human body's cells. Classified as a pyrazine derivative, this active form inhibits viral RNA synthesis by integrating into viral RNA through the enzyme RNA dependent RNA polymerase (RdRp), ultimately causing mutations in the virus's daughter RNAs (Figure 1) [3–5].

The pharmacokinetic profile of favipiravir reveals a rapid absorption phase, with maximum concentration (C_{max}) attained within 2 hours post-oral administration in human blood plasma. Subsequently, the drug undergoes a swift elimination phase, characterized by an elimination half-life ($t_{1/2}$) ranging from 2 to 5.5 hours. Favipiravir undergoes metabolism in the liver cytosol, primarily catalyzed by aldehyde oxidase, and to a lesser extent, xanthine oxidase. This metabolic process yields an inactive metabolite, T-705M1, which is then excreted via the kidneys [5]. The main adverse reactions of the drug are teratogenicity and embryotoxicity, which preclude its use in pregnant or presumably pregnant women. Additionally, favipiravir has been associated with gastrointestinal adverse reactions, including vomiting, diarrhea, nausea, duodenal ulcer, and functional dyspepsia [6, 7].

The inclusion of favipiravir in the list of vital and essential medicines underscores its importance in Russian healthcare for combating common RNA viruses[1].

[1] Order of the Government of the Russian Federation dated October 12, 2019 No. 2406-r "On approval of the list of vital and essential drugs for medical use for 2023, the list of drugs for medical use, including drugs for medical use prescribed by decision of doctors commissions of medical organizations, a list of medications intended to provide persons with hemophilia, cystic fibrosis, pituitary dwarfism, Gaucher disease, as well as the minimum range of medications necessary to provide medical care". (as amended on March 30, 2022). Available at: http://www.consultant.ru/document/cons_doc_LAW_335635/ Assessed: 07/15/2023.

This recognition has spurred extensive research efforts, as evident from the plethora of studies published on the determination of favipiravir in various biological fluids. These studies aim to elucidate the pharmacokinetic behavior and bioequivalence of the drug using advanced analytical techniques such as ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS), high-performance liquid chromatography with ultraviolet detection (HPLC-UV), and high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) [8].

The abovementioned bioanalytical methods for detecting favipiravir using HPLC with various detectors have limitations, including low sensitivity, lengthy analysis times, and the requirement for large sample volumes of biological matrices. Sample preparation techniques such as solid-phase extraction, protein precipitation with organic solvents, precipitation followed by dilution, and liquid-liquid extraction (LLE) have been employed, utilizing biological matrices such as human blood plasma, blood serum, and human breast milk (Table 1).

However, the absence of methods employing stable isotope-labeled favipiravir (favipiravir-13C3) as an internal standard (IS) for detection in human blood plasma, forms a notable gap in the literature. To address this, we have previously developed HPLC-UV and HPLC-MS/MS methods for the determination of favipiravir in human blood plasma, targeting different analytical ranges suitable for parenteral and oral administration, respectively. Specifically, our HPLC-UV method exhibited an analytical range of 0.25–200.00 mcg/ml for a favipiravir dosage of 400 mg per vial in lyophilizate form for infusion preparation [11]. Additionally, our HPLC-MS/MS method enabled the joint determination of β-D-N4-hydroxycytidine and favipiravir, with an analytical range of 250.00–20.000.00 ng/ml for a favipiravir dosage of 400 mg in tablet form [13]. The current study presents a unique challenge, as lower plasma concentrations of favipiravir are anticipated due to a reduced dosage of 200 mg per tablet. Therefore, we aimed to develop and validate a novel HPLC-MS/MS method with an extended analytical range of 50.00–15,000.00 ng/ml, suitable for pharmacokinetic and bioequivalence studies of such a dosage.

A simple and cost-effective protein precipitation method with methanol was employed for sample preparation; the mobile phase consisted of eluent A – a 0.1 % formic acid solution in water (by volume) – and eluent B, methanol.

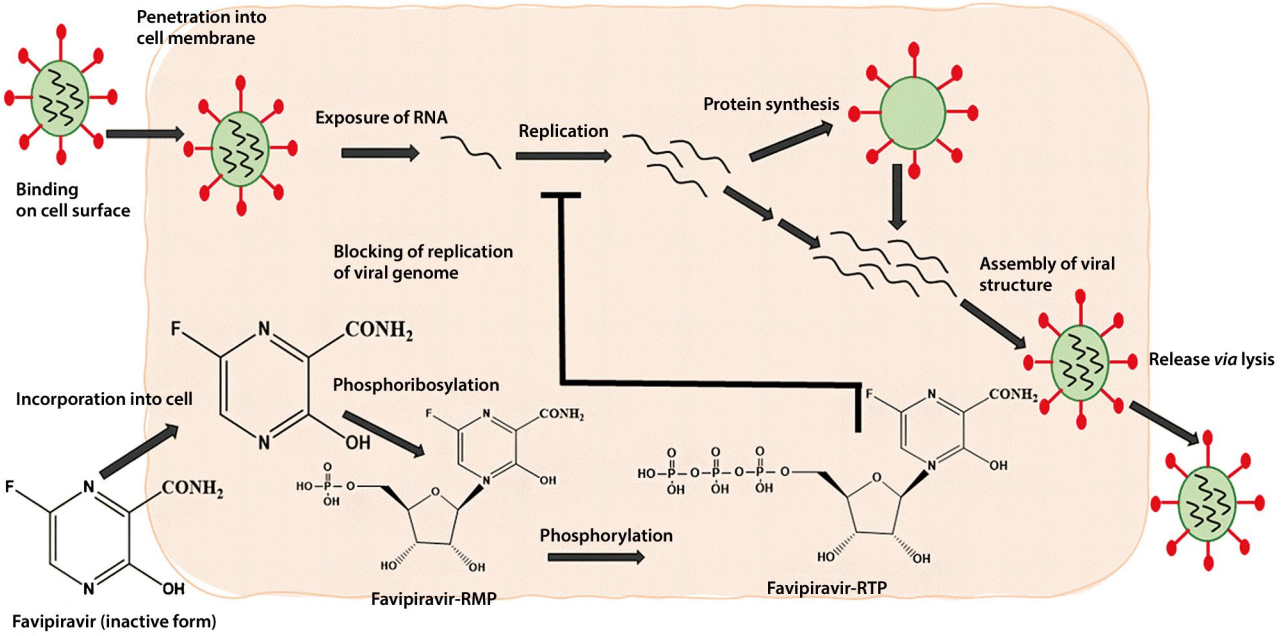

Figure 1. **Schematic representation of the mechanism favipiravir's action [6]**

Table 1. **Bioanalytical methods of favipiravir quantitative determination**

Analytical method	Object	Sample preparation's notes	Analytical range	Reference
HPLC-UV	Human plasma, human breast milk	Solid-phase extraction	0.50–20.00 μg/mL	[9]
HPLC-UV	Human plasma	Protein precipitation of proteins with trifluoroacetic acid and subsequent LLE	0.10–100.00 μg/mL	[10]
HPLC-UV	Human plasma	Protein precipitation with methanol	0.25–200.00 μg/mL	[11]
UPLC-MS/MS	Human plasma	Protein precipitation with acetonitrile	50.00–10000.00 ng/mL	[12]
HPLC-MS/MS	Human plasma	Protein precipitation with 0.1 % formic acid solution in acetonitrile	250.00–20000.00 ng/mL	[13]
HPLC-MS/MS	Human serum	Protein precipitation with acetonitrile	0.048–50.000 μg/mL	[14]
HPLC-MS/MS	Human plasma	Protein precipitation with acetonitrile followed by dilution	500.00–50000.00 ng/mL	[15]
UPLC-MS/MS	Human plasma	Protein precipitation with acetonitrile	0.50–100.00 μg/mL	[16]
HPLC-MS/MS	Human serum	Protein precipitation with acetonitrile	1.60–600.00 μg/mL	[17]

MATERIALS AND METHODS

Equipment

Chromatographic separation and determination were performed on a Shimadzu Nexera XR high-performance liquid chromatograph coupled with a triple quadrupole tandem mass spectrometric detector, the LCMS-8040 (Shimadzu Corporation, Japan). The system was equipped with a thermostat for maintaining column and sample temperature stability, a vacuum degasser, a high-pressure flow line switching valve (FCV-20AH2), an autosampler, and an automatic sample feeder device (Rack Changer II). Data acquisition and processing were accomplished using the LabSolutions software (version 5.91) (Shimadzu Corporation, Japan) [8, 18].

Reagents and solutions

The following reagents were employed in the study:

- acetonitrile (class "C.P.", LLC "Chimmed Group", Russia);
- formic acid (class "for analysis", PanReac, Spain);
- methanol (class "C.P.", LLC "Chimmed Group", Russia);
- demineralized water, purity class I.

Additionally, the substances favipiravir (JSC "Biokhimik", Russia, content 100.40 %) and favipiravir-13C3 (Toronto Research Chemicals Inc., Canada, content 98.42 % HPLC purity, and 98.20 % isotopic purity) were utilized for stock and working solutions preparation.

The standard stock solution of favipiravir was prepared by dissolving an exact quantity of the substance in acetonitrile of the "C.P." class. Working standard solutions were subsequently prepared by diluting aliquots of the standard stock solution with the same solvent, as detailed in Tables 2 and 3.

Table 2. Concentrations of favipiravir and favipiravir-13C3 at calibration levels

Level	Favipiravir concentration, ng/mL	Favipiravir-13C3 concentration, ng/mL
1	50.00	1283.74
2	100.00	
3	500.00	
4	1000.00	
5	2500.00	
6	5000.00	
7	10000.00	
8	15000.00	

Similarly, a working standard solution of the internal standard (IS) favipiravir-13C3 was prepared by dissolving an exact amount of the standard sample in acetonitrile of the "C.P." class. Intact blood plasma samples, standard stock and working standard solutions were stored at temperatures ranging from –50 to –35 °C in a freezer.

Sample Storage and Preparation

For sample preparation, 200 µl of the respective sample (calibration samples (CS), quality control samples (QC), and intact blood plasma samples) was placed in Eppendorf-type microtubes, to which 10 µl of the working standard solution of IS and 400 µl of precipitant were added. The mixture was then vortexed for 10 seconds and centrifuged at an acceleration of 15,000 g for 15 minutes. Following centrifugation, the supernatant was carefully transferred to chromatographic vials and placed in the trays of the Rack Changer II chromatograph dispenser.

Table 3. Concentrations of favipiravir and favipiravir-13C3 at quality control (QC) samples

Level	Favipiravir concentration, ng/mL	Favipiravir-13C3 concentration, ng/mL	Notes
LLOQ	50.00	1283.74	LLOQ – lower limit of quantification. L – low level of concentration. M1 and M2 – middle levels of concentration. H – high level of concentration
L	150.00		
M1	3000.00		
M2	7500.00		
H	12000.00		

Chromatographic Conditions:

- *Chromatographic column*: Phenomenex Kinetex C18, 100 × 3.0 mm, 5 µm.
- *Pre-column*: Phenomenex SecurityGuard™ Widepore C18 4 × 3.0 mm.
- *Column thermostat temperature*: 40 °C.
- *Mobile phase*: 0.1 % formic acid solution in water (by volume) (eluent A) and methanol (eluent B).
- *Flow rate*: 0.85 ml/min.
- *Gradient composition of the mobile phase*: Refer to Figure 2.
- *Injection volume*: 10 µl.
- *Retention time of favipiravir and favipiravir-13C3*: approximately 1.2 minutes.
- *Chromatogram running time*: 0.0–3.75 minutes.
- *Electrospray emitter voltage, ionization mode, and determination conditions*: Refer to Table 4.

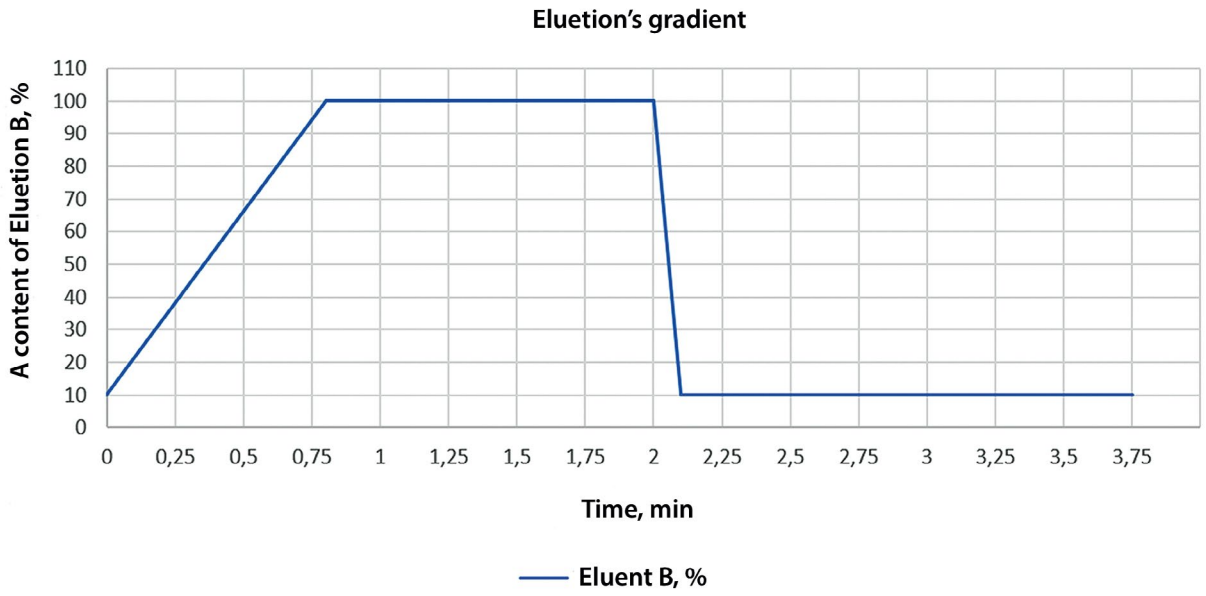

Figure 2. **Eluetion's gradient**

Table 4. **Mass-spectrometric conditions of favipiravir and favipiravir-13C3**

	Detection conditions	Electrospray capillar voltage	Ion mode
FAV	156.20 → 113.10 m/z 156.20 → 65.00 m/z 156.20 → 58.20 m/z	–4.9 kV	Negative
FAV-13C3	159.15 → 115.05 m/z 159.15 → 86.10 m/z		

RESULTS AND DISCUSSION

Development of the Bioanalytical Method

The selection of the internal standard (IS) was a crucial step in the development process, and it was based on finding a compound with structural and physicochemical properties similar to those of the analyte, favipiravir. To achieve this, favipiravir-13C3, labeled with stable isotopes, was chosen as the IS due to its close resemblance to the analyte.

Mass spectrometric detection conditions were optimized by identifying the precursor and daughter ions of favipiravir and favipiravir-13C3 formed in the negative ionization mode at various collision energies. The voltage in the optical system of the mass spectrometric detector was also adjusted to enhance signal intensity. An electrospray ionization source was utilized.

Following the development of mass spectrometric detection conditions, we focused on selecting a suitable chromatographic column and optimizing the mobile phase composition and gradient to achieve efficient separation of favipiravir and favipiravir-13C3. The chosen chromatographic conditions resulted in retention times of approximately 1.2 minutes for both substances, enabling a time-efficient analysis. This also ensured effective separation from matrix components and yielded optimal chromatographic peak shapes for the analyte and IS.

Methanol was selected as the precipitant after evaluating various sample preparation methods described in the literature. Methanol provided the most complete precipitation of human plasma proteins, simplifying the sample preparation process while adhering to regulatory documentation requirements.

Validation of the Bioanalytical Method

The developed bioanalytical method for the determination of favipiravir in human blood plasma was validated in accordance with the guidelines of the Eurasian Economic Union[1], the European Medicines

[1] Rules for conducting bioequivalence studies of medicines within the framework of the Eurasian Economic Union (approved by decision No. 85 of the Council of the Eurasian Economic Commission dated November 3, 2016). Available at: https://docs.cntd.ru/document/456026107. Assessed: 15.07.2023.

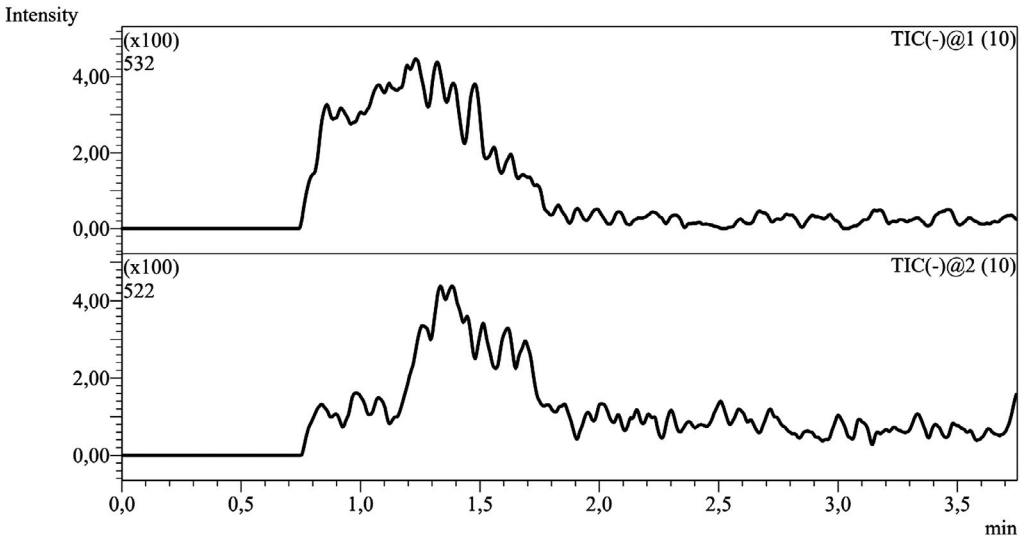

Figure 3. **Blank human blood plasma sample chromatogram (example)**

Agency (EMA)[1], and the U.S. Food and Drug Administration (FDA)[2] for bioequivalence studies. The validation parameters included selectivity, calibration curve, matrix effect, accuracy, and precision at the lower limit of quantitative determination (LLQD), low (L), medium-low (M1), medium-high (M2), and high (H) concentration levels (Table 3). The validation was conducted both within and between cycles, assessing additional parameters such as the degree of extraction (DE), sample transfer, LLQD, and stability (stability of the stock and working standard solutions of the analyzed substance; short-term stability ("desktop" and "post-preparation"); stability during triple freezing-defrosting; long-term stability of the analyte in the matrix) [8, 18].

Selectivity

To evaluate selectivity, intact, hyperlipidemic intact, and hemolyzed intact blood plasma samples obtained from various sources ($n = 6$) were analyzed, along with samples spiked with the working standard solution of favipiravir and the working standard solution of IS (Table 2).

Chromatograms of intact blood plasma samples were examined to ensure that peak signals corresponding to the retention times of favipiravir and favipiravir-13C3 did not exceed the permissible acceptance criteria: no more than +20 % of the signal at the LLQD level and no more than +5 % of the IS signal, respectively. An example chromatogram of an intact blood plasma sample is presented in Figure 3.

Calibration Curve

Calibration samples ($n = 8$) were prepared by merging intact blood plasma with both working standard solutions of favipiravir and the working standard solution of favipiravir-13C3 to achieve concentrations corresponding to levels 1–8 (Table 2).

Calibration graphs were constructed by plotting the ratio of the peak area of favipiravir to the peak area of favipiravir-13C3 against the ratio of their respective concentrations in human blood plasma. An example of a calibration graph is shown in Figure 4.

The calibration curves exhibited a linear relationship, with correlation coefficients meeting the prescribed criteria (at least 0.99). Deviations of calibration sample concentrations from their nominal values were calculated using the linear dependence equation and found to be within acceptable limits: no more than +20 % for level 1 (LLQD) and no more than +15 % for levels 2–8.

[1] European Medicines Agency. Available at: https://www.ema.europa.eu/en/bioanalytical-method-validation. Assessed: 15.07.2023.

[2] Food and Drug Administration. Available at: https:// www.fda.gov/regulatory-information/search-fda-guidancedocuments/bioanalytical-method-validation-guidanceindustry. Assessed: 15.07.2023.

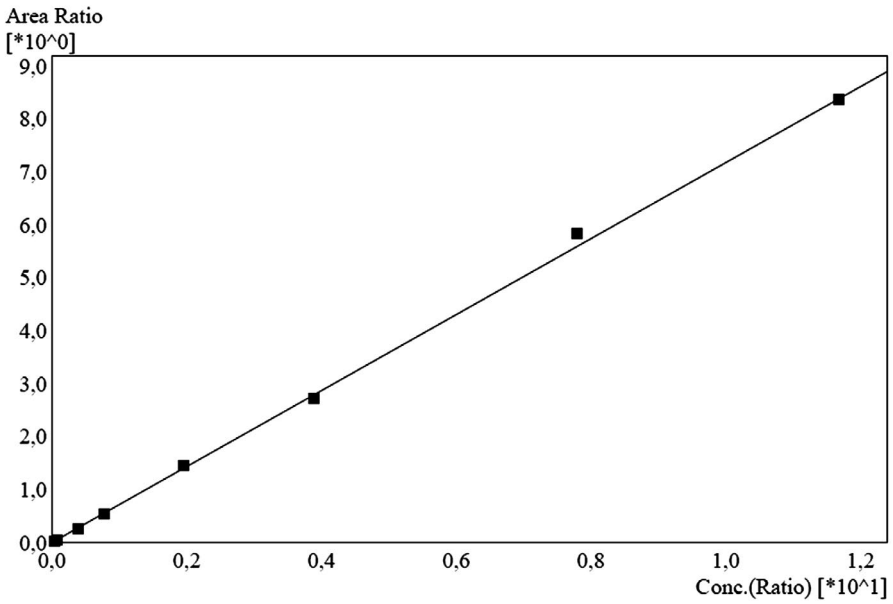

Figure 4. **Calibration curve (example)**

Evaluation of the Matrix Effect

To assess the matrix effect, samples of intact, hyperlipidemic intact, and hemolyzed intact blood plasma were prepared without considering the influence of the degree of extraction from intact blood plasma ($n = 6$) and the influence of intact blood plasma ($n = 6$) at low (L) and high (H) concentration levels (Table 3). The matrix effect was calculated for favipiravir-13C3 at a concentration level of 1283.74 ng/ml.

The relative standard deviation (RSD, %) of the matrix factor normalized by the internal standard (IS) did not exceed 15 % at the L and H levels (Table 5), indicating an acceptable matrix effect.

Accuracy and Precision

Quality control (QC) samples corresponding to the lower limit of quantitative determination (LLOQ), L, M1, M2, and H concentration levels were analyzed (Table 3). This evaluation was performed by injecting each QC sample five times at each of the five concentration levels within three validation cycles.

Accuracy and precision were assessed both within and between cycles. The values of relative standard deviation (RSD) and relative error (E, %) were calculated from the discovered concentrations of favipiravir. The obtained data met the acceptance criteria: RSD and E values did not exceed 15 % for levels L, M1, M2, and H, and they did not exceed 20 % for the LLOQ level (Table 6).

Table 5. **Calculation of the favipiravir matrix factor at L and H levels, normalized by the favipiravir-13C3 matrix factor for samples prepared from diverse types of intact plasma**

Type of biological matrix	Level of QC	Injected, ng/mL)	RSD, %
Human blank plasma	L	150.00	5.30
	H	12000.00	4.07
Human hemolyzed blank plasma	L	150.00	12.02
	H	12000.00	2.77
Human lipemic blank plasma	L	150.00	7.48
	H	12000.00	3.01

Evaluation of the Degree of Extraction

To evaluate the degree of extraction (DE), three samples prepared from intact, hyperlipidemic intact, and hemolyzed intact blood plasma were analyzed without considering the influence of DE from intact blood plasma at levels L, M1, M2, and H (Table 3). Additionally, QC samples prepared on various intact matrices were assessed. The data obtained, as presented in Table 7, showed that the RSD of the calcu-

Table 6. **Accuracy and precision of favipiravir determination procedure (inter-day, intra-day 1, intra-day 2)**

Level	Injected, ng/mL	RSD, %			E, %		
		Inter-day (n = 5)	Intra-day 1 (n = 10)	Intra-day 2 (n = 15)	Inter-day (n = 5)	Intra-day 1 (n = 10)	Intra-day 2 (n=15)
LLOQ	50.00	17.69	18.64	16.07	–15.72	–4.48	–7.31
L	150.00	3.27	5.80	6.55	–7.87	–5.24	–4.24
M1	3000.00	1.28	7.17	7.14	2.86	–0.57	1.68
M2	7500.00	1.50	5.98	6.62	–2.40	–7.26	–5.34
H	12000.00	2.74	3.06	3.28	–0.99	0.93	1.96

lated DE values of favipiravir from different types of biological matrices did not exceed the prescribed limit of +15 %.

Table 7. **Calculation of favipiravir recovery at L, M1, M2, H levels from the different types of biological matrix**

Type of biological matrix	Favipiravir recovery %			
	L	M1	M2	H
Blank human blood plasma	91.97	95.45	96.44	101.65
	81.25	98.17	98.59	97.86
	107.76	99.24	103.92	105.00
Hemolyzed blank human blood plasma	106.62	103.53	99.67	103.70
	94.74	108.69	100.09	108.04
	82.84	111.02	106.43	106.52
Lipemic blank human blood plasma	111.23	100.92	105.13	114.77
	104.22	110.49	100.73	114.32
	115.28	109.36	98.87	116.44
Average	99.60			
RSD, %	4.90			

Sample Transfer

During the sequential analysis of calibration samples corresponding to favipiravir concentration level 8 (Table 2) and intact plasma samples, no peaks corresponding to the retention times of favipiravir and the internal standard (IS) were identified on the chromatogram of intact plasma samples. This indicated effective sample transfer without carryover effects.

Determination of the Lower Limit of Quantitative Determination (LLQD)

The LLQD of the method was established based on the calibration curve, accuracy, and precision data. The LLQD represents the lowest concentration of favipiravir in blood plasma within the analytical range where the analyte can be quantified with RSD and E values not exceeding +20%. The developed method demonstrated an LLQD of 50.00 ng/ml. An example chromatogram of a blood plasma sample with favipiravir content at the LLQD level is shown in Figure 5.

Stability Assessment

The stability of favipiravir was confirmed at concentration levels L (150.00 ng/ml) and H (12,000.00 ng/ml) under various conditions, including triple freezing-thawing, short-term stability ("desktop" and "post-preparation"), stability of stock and working standard solutions (stored at temperatures from –50 to –35 °C for 47 days), and long-term stability of favipiravir in human blood plasma (stored at –50 to –35 °C for 47 days).

Relative error calculations for the obtained concentrations of favipiravir confirmed the stability of the analyte under these conditions, with relative errors within +15 % of the corresponding control values at the L and H levels (Table 3).

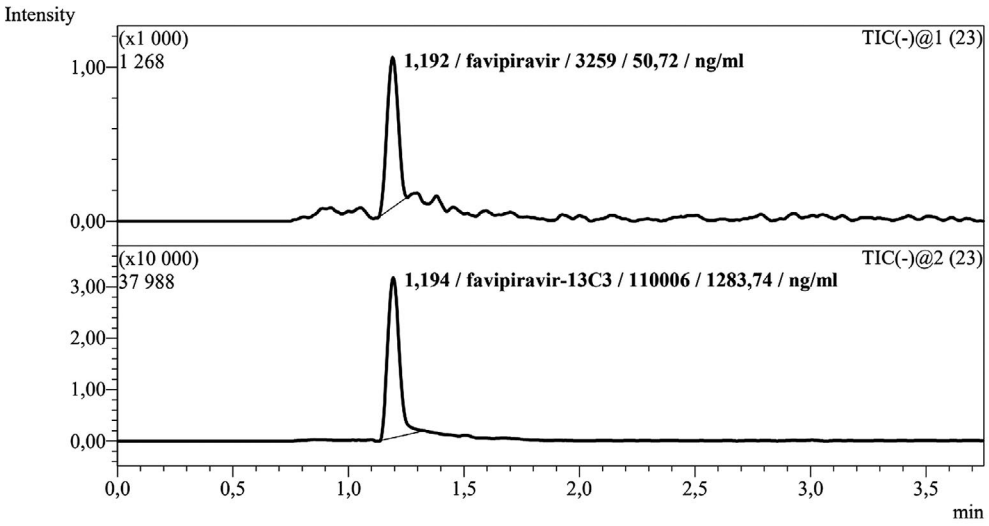

Figure 5. Chromatogram of human blood plasma with favipiravir content at the level of LLOQ (example)

CONCLUSION

In conclusion, a quantitative bioanalytical method for the determination of favipiravir in human blood plasma using HPLC-MS/MS has been successfully developed and validated. The method exhibits a wide analytical range of 50.00–15,000.00 ng/ml in blood plasma. This method can be reliably employed for pharmacokinetic and bioequivalence studies of drugs containing favipiravir, contributing to the expansion of their assortment in the domestic pharmaceutical market.

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