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Development and Validation of HPLC-UV Method for the Determination of Favipiravir in Human Plasma

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Abstract

Introduction. Coronavirus disease (COVID-19) is an acute infectious disease caused by SARS-CoV-2 (severe acute respiratory syndrome-related coronavirus 2). Favipiravir is a synthetic prodrug with antiviral activity used for the treatment of COVID-19. There are oral and parenteral dosage forms of favipiravir. Compared with oral administration, parenteral administration has some advantages. Developing a method for the determination of favipiravir in human blood plasma is necessary for performing the analytical part of clinical studies of favipiravir for parenteral administration as an infusion, studying pharmacokinetics, and choosing the optimal dosage of the drug.

Aim. The aim of this study is to develop and validate a method for quantitative determination of favipiravir in human plasma by high-performance liquid chromatography with ultraviolet detection (HPLC-UV) for pharmacokinetic studies.

Materials and methods. Determination of favipiravir in human plasma by HPLC-UV. The UV detection was set at 323 ± 2 nm. The samples were processed by methanol protein precipitation. Internal standard: raltegravir. Mobile phase: 0.1 % formic acid in water with 0.08 % aqueous ammonia (eluent A), 0.1 % formic acid in acetonitrile with 0.08 % aqueous ammonia (eluent B). Column: Phenomenex Kinetex®, C18, 150 × 4.6 mm, 5 µm. Analytical range: 0.25–200.00 µg/mL.

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

Conclusion. We developed and validated the method of quantitative determination of favipiravir in human plasma by HPLC-UV. The analytical range was 0.25–200.00 µg/mL in human plasma. The method could be applied in pharmacokinetics studies of favipiravir.

Keywords: favipiravir, COVID-19, plasma, HPLC-UV, 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 have developed and validated the analytical method. Natalia S. Bagaeva carried out statistical processing of the obtained results. Igor E. Shohin, Kira Ya. Zaslavskaya and Petr A. Bely carried out the organization of work in this direction. All the above authors participated in the discussion of the results in the format of scientific discussion.

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Разработка и валидация методики определения фавипиравира в плазме крови человека методом ВЭЖХ-УФ

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Резюме

Введение. Новая коронавирусная инфекция COVID-19 (Coronavirus Disease 2019) – это острое инфекционное заболевание, вызываемое вирусом SARS-CoV-2 (severe acute respiratory syndrome-related coronavirus 2). Фавипиравир – синтетический противовирусный препарат, применяемый для этиотропной терапии COVID-19. Выпускается в лекарственных формах для перорального и парентерального введения, при этом парентеральный путь введения фавипиравира имеет ряд преимуществ перед пероральным. Разработка и валидация методики количественного определения фавипиравира в плазме крови человека является необходимой процедурой для проведения аналитической части клинического исследования препарата фавипиравира для парентерального введения в виде инфузий, изучения его фармакокинетических параметров и выбора оптимальных доз препарата.

Цель. Целью исследования является разработка и валидация методики определения фавипиравира в плазме крови человека методом высокоэффективной жидкостной хроматографии с использованием с использованием ультрафиолетового детектора (ВЭЖХ-УФ) для дальнейшего изучения фармакокинетики.

Материалы и методы. Определение фавипиравира в плазме крови человека проводили методом ВЭЖХ-УФ при длине волны 323 ± 2 нм. В качестве пробоподготовки был использован способ осаждения метанолом. Внутренний стандарт: ралтегравир. Подвижная фаза: 0,1%-й раствор муравьиной кислоты в воде с прибавлением 0,08%-го аммиака (элюент А); 0,1%-й раствор муравьиной кислоты, 10%-й раствор воды в ацетонитриле с прибавлением 0,08%-го аммиака (элюент В). Колонка: Phenomenex Kinetex®, C18, 150 × 4,6 мм, 5 мкм. Аналитический диапазон методики: 0,25–200,00 мкг/мл.

Результаты и обсуждение. Разработанная методика была валидирована по следующим валидационным параметрам: селективность, калибровочная кривая, точность, прецизионность, степень извлечения, нижний предел количественного определения, перенос пробы, стабильность (стабильность исходных и рабочих стандартных растворов аналита и внутреннего стандарта; краткосрочная стабильность; стабильность при трехкратной заморозке-разморозке аналита; долгосрочная стабильность аналита в матрице).

Заключение. Разработана и валидирована методика определения фавипиравира в плазме крови человека методом ВЭЖХ-УФ. Подтвержденный аналитический диапазон методики составил 0,25–200,00 мкг/мл в плазме крови. Полученный аналитический диапазон позволяет применять разработанную методику для проведения фармакокинетических исследований препаратов фавипиравира

Ключевые слова: фавипиравир, COVID-19, плазма, ВЭЖХ-УФ, 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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ВВЕДЕНИЕ

New 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). This disease, first reported in China in December 2019, is the cause of the COVID-19 pandemic that is currently ongoing throughout the world [1]. According to the statistics, as of August 2022, about 578 million cases of SARS-CoV-2 virus infection were recorded in 228 countries of the world, of which more than 6 million were fatal. In Russia, more than 18.5 million cases have been registered to date, and the number of deaths has reached more than 380 thousand[1].

The emergence of new strains of the SARS-CoV-2 virus, a decrease in the rate of vaccination, as well as the abolition of previously existing restrictive measures and a reduction in measures aimed at reducing the spread of the new coronavirus infection can provoke an increase in the incidence and increase the burden on the healthcare system [2]. Currently, the distribution of the Omicron strain and its varieties is observed throughout the world. The danger of this strain and its substrains lies in the presence of mutations that cause increased transmissibility and allow avoiding immune response from vaccines and natural immunity [3, 4].

In the treatment of COVID-19, it is recommended that patients are administered with supportive pathogenetic, etiotropic, and symptomatic therapy. According to the latest version of the Temporary guidelines of the Ministry

[1] WHO Coronavirus (COVID-19) Dashboard. Available at: https://covid19.who.int/ Accessed: 07.08.2022.

of Health of the Russian Federation for prevention, diagnosis and treatment of a new coronavirus infection, several antiviral drugs are currently identified for the etiotropic therapy of COVID-19, including favipiravir[1].

Favipiravir is a synthetic antiviral drug, a purine nucleic acid analog, a selective RNA polymerase inhibitor that is active against a wide range of different RNA viruses [5, 6]. Favipiravir products for oral and parenteral administration are registered in Russia[2]. Favipiravir in tablet dosage form is used both in outpatient and inpatient settings, and parenteral favipiravir is used only in inpatient settings and is recommended for patients with mild to moderate COVID-19[3]. Favipiravir for parenteral administration is convenient for patients in the intensive care unit, as well as for patients who have lesions of the gastrointestinal tract or suffer from dysphagia. The parenteral route of administration has a high bioavailability and also helps to avoid interaction with food and the effects of digestive enzymes [7, 8].

The development and validation of a method for the quantitative determination of favipiravir in human plasma is a necessary procedure for conducting the analytical part of a clinical trial of favipiravir as infusions at various doses, studying pharmacokinetic parameters and selecting the optimal doses of the drug.

Currently, a number of works have been published in the literature on the development of methods for the quantification of favipiravir in order to conduct pharmacokinetic studies. To determine favipiravir in biological fluids, high-performance liquid chromatography with ultraviolet detection (HPLC-UV)], high-performance liquid chromatography with fluorescence detection (HPLC-FLD)], high performance liquid chromatography – tandem mass spectrometry (HPLC-MS/MS), as well as the method of ultra-performance liquid chromatography – tandem mass spectrometry (UPLC-MS/MS).

In the methods reviewed, the liquid-liquid extraction (LLE) method is mainly used as sample preparation, as well as the precipitation of blood plasma proteins with organic solvents (isopropanol, methanol, acetonitrile) (table 1).

After analyzing the methods for the quantification of favipiravir presented in the literature, it was concluded that there are no methods simultaneously with a simple sample preparation method and a wide analytical range, so it was decided to develop and validate a method that meets these requirements independently.

Due to the expected high levels of favipiravir in human plasma, HPLC-UV was selected as the method of choice for high-dose infusion formulations.

In this study, the development and validation of a method for determining favipiravir in human plasma by HPLC-UV was presented. The method of precipitation of blood plasma proteins with methanol was selected as sample preparation.

MATERIALS AND METHODS

Equipment

Chromatographic separation and detection were carried out on an LC-2040C high-performance liquid chromatograph (Shimadzu Corporation, Japan) with a built-in UV detector, a low-pressure four-component gradient pump, a degasser, an autosampler, a column thermostat and a controller. Primary data was processed using the LabSolutions software (Shimadzu Corporation, Japan).

Reagents and solutions

The following reagents were used in the work: methanol (Ultra Gradient HPLC-grade, J.T. Baker, Netherlands), acetonitrile (LC-MS grade, Biosolve, France; Ultra Gradient HPLC-grade, J.T. Baker, Netherlands), formic acid (class "98 % pure", PanReac, Spain), aqueous ammonia (class "for analysis", PanReac, Spain), demineralized water of class I. To prepare the stock reference solutions and working standard solutions, we used reference samples of favipiravir (JSC Biokhimik, Russia, assay 100.30 %) and raltegravir potassium (USP reference standard, USA, assay 99.10 %) were prepared.

The stock standard solution of favipiravir (FAV) and the internal standard (IS) of raltegravir (RAL) were prepared by dissolving an accurate weight of the substances in acetonitrile. Favipiravir working standard solutions were prepared by diluting stock solutions with acetonitrile to target plasma concentrations corresponding to levels 1–8, lower limit of quantification (LLOQ), low [L (low)], medium [M1 and M2 (middle 1, middle 2)] and high levels [H (high)] (table 2).

[1] Temporary Guidelines "Prevention, Diagnosis and Treatment of a New Coronavirus Infection (COVID-19). Version 15 (22.02.2022)" (approved by the Ministry of Health of Russia). Available at: http://www.consultant.ru/document/cons_doc_LAW_347896/ Accessed: 17.07.2022.

[2] GRLS (State Registry of Medicinal Products) – Ministry of Health of the Russian Federation. Available at: https://grls.rosminzdrav.ru/ Accessed: 17.07.2022.

[3] Temporary Guidelines "Prevention, Diagnosis and Treatment of a New Coronavirus Infection (COVID-19). Version 15 (22.02.2022)" (approved by the Ministry of Health of Russia). Available at: http://www.consultant.ru/document/cons_doc_LAW_347896/ Accessed: 17.07.2022.

Table 1. **Methods of favipiravir quantitative determination**

Analytical method (ionization source; ionization (+/–) if applicable)	Object	Sample preparation	Analytical range	Reference
HPLC-UV	Human plasma	Liquid-liquid extraction	3.10–60.00 μg/mL	[9]
HPLC-UV	Human plasma	Liquid-liquid extraction	25.00–80.00 ng/mL	[10]
HPLC-UV	Human plasma	Liquid-liquid extraction	0.20–3.20 μg/mL	[11]
HPLC-UV	Human plasma	Liquid-liquid extraction	0.10–100.00 μg /mL	[12]
HPLC-FLD	Human plasma	Protein precipitation by isopropanol	40.00–240.00 ng/mL	[13]
HPLC-MS/MS (electrospray; –)	Human plasma	Protein precipitation by methanol	100.00–20000.00 ng/mL	[14]
UPLC-MS/MS (electrospray; –)	Human plasma	Protein precipitation by acetonitrile	0.25–16.00 μg/mL	[15]
HPLC-MS/MS (electrospray; –)	Human serum	Protein precipitation by acetonitrile	0.048–50.00 μg/mL	[16]
HPLC-MS/MS (electrospray; +)			0.062–50.00 μg/mL	

Table 2. **Concentrations of favipiravir and raltegravir at calibration levels and quality control samples**

Level	Analyte concentration, μg/mL	IS concentration, μg/mL
	FAV	RAL
1	0,25	60,00
2	5,00	60,00
3	10,00	60,00
4	25,00	60,00
5	50,00	60,00
6	70,00	60,00
7	90,00	60,00
8	200,00	60,00
LLOQ	0,25	60,00
L	0,75	60,00
M1	40,00	60,00
M2	110,00	60,00
H	160,00	60,00

Samples of blank human plasma, as well as stock reference solutions and working standard solutions, were stored in a freezer at a temperature of –50 °C to –35 °C.

Sample preparation

To 200 μl of the sample placed in Eppendorf micro-centrifuge tubes 2 ml, 30 μl of the working solution of raltegravir BC was added, and 600 μl of methanol was precipitated. Then it was stirred on a Vortex shaker for 10 seconds and centrifuged at an acceleration of 15 000 g for 15 minutes. Then the supernatant was transferred into chromatographic vials and placed in the autosampler of the chromatograph for further analysis.

Conditions for chromatographic separation and detection

Chromatographic separation was performed on a Phenomenex Kinetex® column, 150 × 4.6 mm, 5 μm at a mobile phase flow rate (MF) of 1.0 ml/min. The volume of the injected sample was 10 μl, the temperature of the column thermostat was maintained at 40 °C.

The mobile phase was:

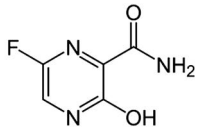
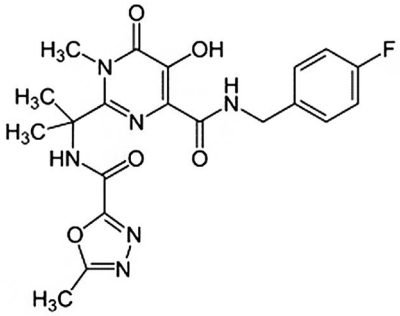
- *Eluent A*: 0.1 % formic acid in water with 0.08 % ammonia added (v/v).
- *Eluent B*: 0.1 % formic acid, 10 % water in acetonitrile with 0.08% ammonia (v/v).

The gradient elution mode presented in table 3 was used.

Table 3. **Gradient elution**

Time, min	Eluent A, %	Eluent B, %
0,00	96,0	4,0
1,00	96,0	4,0
2,80	85,0	15,0
5,00	0,0	100,0
7,00	0,0	100,0
7,50	96,0	4,0
9,00	96,0	4,0

Table 4. **Chemical and physical characteristics of the analyte and of the internal standard**

Name of substance	Favipiravir[1]	Raltegravir[2]
IUPAC name	6-fluoro-3-hydroxypyrazine-2-carboxamide	N-(4-Fluorobenzyl)-5-hydroxy-1-methyl-2-(2-{[(5-methyl-1,3,4-oxadiazol-2-yl)carbonyl]amino}-2-propanyl)-6-oxo-1,6-dihydro-4-pyrimidinecarboxamide
pKa	9,39	7,02
logP	0,49	1,30
Chemical structure		

Note. [1] Favipiravir. Drugbank. Available at: https://go.drugbank.com/drugs/DB12466/ Accessed: 17.07.2022.
[1] Raltegravir. Drugbank. Available at: https://go.drugbank.com/drugs/DB06817/ Accessed: 17.07.2022.

The retention time for favipiravir was about 3.8 minutes, for raltegravir about 5.5 minutes. The total run time of the UV detector was 9.0 minutes.

Detection was carried out at a wavelength of 323 ± 2 nm at a detection frequency of the detector signal of 5 Hz.

RESULTS AND DISCUSSION

Method development

The particularity of the development of a method for determining favipiravir in human plasma by HPLC-UV was a wide analytical range.

The detection wavelength within 323 ± 2 nm was selected based on the data on the absorption spectra of favipiravir in the UV region [17].

Based on the physicochemical properties of favipiravir, a chromatographic column Phenomenex Kinetex®, C18, 150 × 4.6 mm, 5 µm was selected. When using this column, it was possible to achieve the most complete separation of the matrix components.

Raltegravir was selected as the internal standard. This substance is similar to the analyzed substance in its structure and physico-chemical properties (table 4).

Methanol was selected as the precipitant, since it made it possible to achieve the most complete precipitation of blood plasma proteins and to obtain the optimal shape of chromatographic peaks.

Method validation

The bioanalytical method was validated on the basis of the Rules for conducting bioequivalence studies of drugs within the Eurasian Economic Union[1], as well as the EMA[2] and FDA[3] guidelines for the following parameters: selectivity, calibration curve, accuracy and precision, recovery, lower limit of quantification (LLOQ), sample carry-over, stability (stability of stock and working standard solutions; short-term ("benchtop" and "post-preparative") and long-term stability of the analyte in the matrix; stability at triple freezing-thawing).

Selectivity

To evaluate this parameter, we analyzed six samples of blank blood plasma, two samples of hemolytic blank plasma and two samples of blank plasma with

[1] Rules for conducting bioequivalence studies of drugs within the Eurasian Economic Union (approved by decision № 85 of the Council of the Eurasian Economic Commission dated 03.11.2016). Available at: https://docs.cntd.ru/document/456026107/ Accessed: of 07/17/2022.

[2] European Medicines Agency. Available at: https://www.ema.europa.eu/en/bioanalytical-method-validation/ Accessed: 17.07.2022.

[3] Food and Drug Administration. Available at: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/bioanalytical-method-validation-guidance-industry/ Accessed: 17.07.2022.

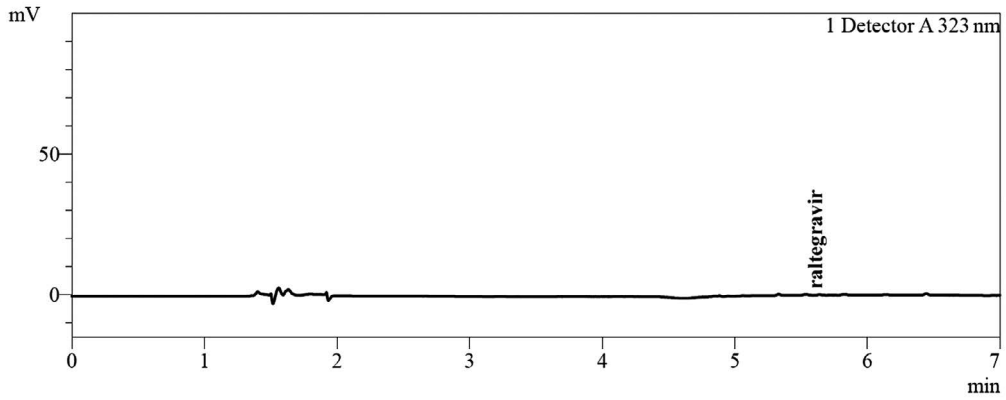


Figure 1. **Chromatogram of blank human plasma sample**

the increased lipid content, as well as samples with the addition of working standard solutions up to a favipiravir concentration of 0.25 μg/ml and a working solution of Raltegravir IS up to concentration of 60.00 μg/ml, which corresponds to the level of LLOQ (see table 2).

The chromatogram of a blank human plasma sample is shown in figure 1. Peak signals with retention times corresponding to the retention times of favipiravir and raltegravir do not exceed 20 % of the favipiravir signal at the LLOQ level and 5 % of the raltegravir signal, respectively.

Calibration curve

Eight samples of blank blood plasma were analyzed with the addition of raltegravir IS working solution to a concentration of 60.00 μg/ml and favipiravir working standard solutions to concentrations in the range of 0.25–200.00 μg/ml, corresponding to levels 1–8 (see table 2). According to the obtained concentration values, calibration graphs were plotted in the coordinates of the ratio of the favipiravir peak area to the area of the peak raltegravir on the ratio of the concentration of favipiravir to the concentration of raltegravir in plasma. Calibration graphs were linear (figure 2). The calibration curve equations and correlation coefficients (R) for the calibration curves in validation cycles № 1–3 are shown in table 5. The obtained values of the correlation coefficients are more than 0.999 and correspond to the normal values. The chromatogram of the calibration sample at level 8 (table 2) is shown in figure 3.

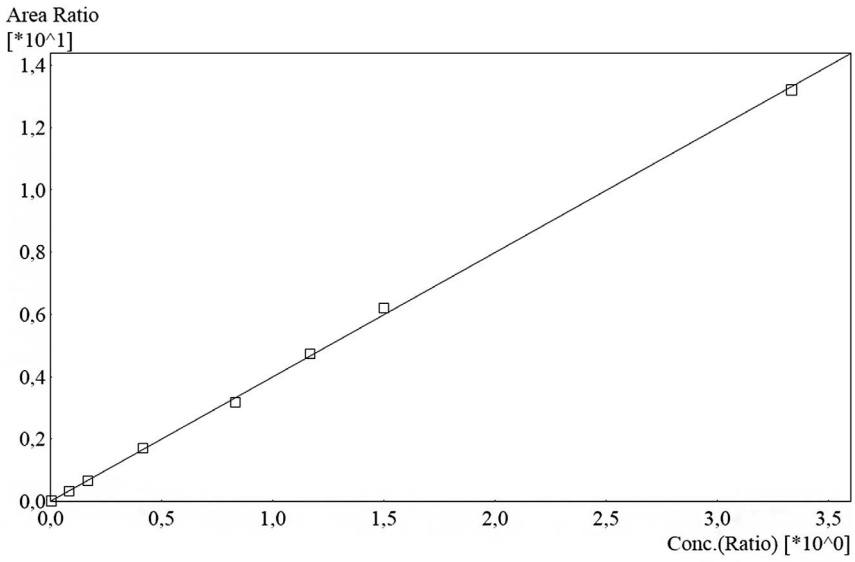


Figure 2. **Calibration curve**

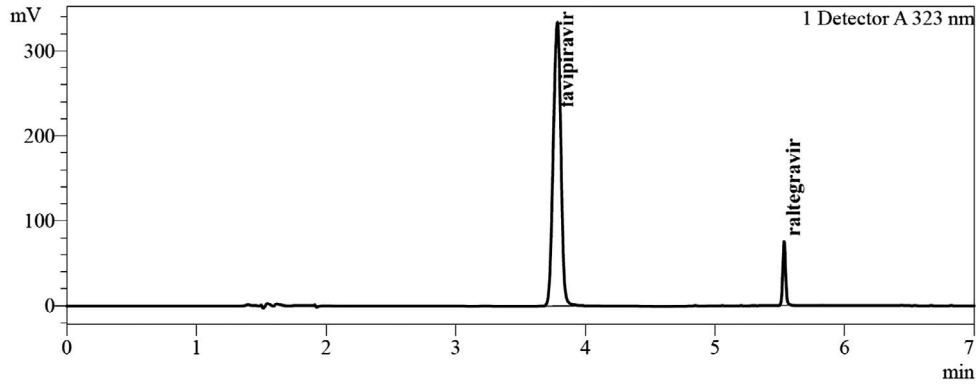


Figure 3. **Chromatogram of human blood sample with favipiravir at level 8**

Table 5. **Calibration equation and correlation coefficients**

№	Calibration equation	R
1	$y = 3{,}15469 \cdot x + 0{,}00327657$	0,9990149
2	$y = 3{,}21830 \cdot x + 0{,}00168604$	0,9992910
3	$y = 3{,}26573 \cdot x + 0{,}000615190$	0,9990230

Accuracy and Precision

To evaluate parameters "accuracy" and "precision", calibration plasma samples were analyzed corresponding to the levels LLOQ, L, M1, M2 and H, with a nominal concentration of favipiravir in the samples equal to 0.75 μg/ml, 40.00 μg/ml , 110.00 μg/ml and 160.00 μg/ml, respectively, and raltegravir IS concentration of 60.00 μg/ml (see table 2). Five samples were analyzed within three sequences (within a cycle, between two and between three cycles). The relative standard deviation (RSD, %) and the relative error (E, %) calculated for the obtained concentration values correspond to the normal values. The data are presented in table 6.

Recovery

To evaluate the "recovery" parameter, three samples prepared from blank plasma, hemolytic blank plasma and blank lipid-enhanced plasma were analyzed without affecting the recovery at levels L, M1, M2 and H (table 2), and also quality control samples prepared on various types of biological intact matrix to evaluate recovery. The average value of favipiravir recovery from various types of intact matrices is 100.36 %. The relative standard deviation of the calculated values of the degree of extraction of favipiravir from various types of intact matrices does not exceed 15 % and meets the acceptance criteria. The data are presented in table 7.

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

Injected (μg/mL)	RSD, %			E, %		
	(*n* = 5)	(*n* = 10)	(*n* = 15)	(*n* = 5)	(*n* = 10)	(*n* = 15)
0,25	6,09	6,20	6,03	0,97	–2,10	–0,42
0,75	1,24	1,74	5,70	–5,98	–6,16	–2,65
40,00	0,08	5,36	4,90	4,87	–0,20	–1,71
110,00	0,06	1,20	1,39	6,05	7,28	6,55
160,00	0,04	3,02	4,35	5,24	2,30	–0,13

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

Biological matrix	Recovery FAV, %			
	L	M1	M2	H
Blank plasma	91,12	111,11	98,18	103,97
	93,91	111,14	98,25	103,96
	90,26	111,20	98,18	103,97
Hemolyzed blank plasma	88,66	104,29	103,68	102,97
	88,74	103,99	103,54	102,94
	85,85	104,01	103,44	103,03
Lipemic blank plasma	91,95	100,62	105,77	102,50
	89,80	100,70	105,42	102,51
	94,70	100,58	105,56	102,42
Average	100,36			
SD	6,58			
RSD	6,56			

Lower limit of quantification

The lower limit of quantification of the method was determined based on calibration curve data, accuracy, and precision. The minimum concentration of favipiravir in the plasma within the analytical range for which favipiravir can be quantified with the values of the relative standard deviation and the relative error of no more than 20 % was taken as the LLOQ of the method. The LLOQ of favipiravir in human plasma was 0.25 μg/ml. The chromatogram of blood plasma with the content of favipiravir at the LLQL level is shown in Figure 4.

Stability

Three samples were analyzed to evaluate benchtop and post-preparation short-term stability, triple freeze-thaw stability, stability of stock and working standard solutions (when stored for 112 days at –50 to –35 °C), long-term stability (when stored for 112 days at –50 °C to –35 °C) of the analyte at levels L and H (table 2). The relative errors calculated for the concentration values meet the acceptance criteria (table 8).

Sample carry-over

To evaluate the "sample carry-over" parameter within validation cycles № 1–3, a sequential analysis of calibration samples at level 8 (table 2) and samples of intact blood plasma was carried out. On chromatograms of samples of blank plasma, the values of favipiravir and raltegravir areas met the acceptance criteria.

CONCLUSION

A method for favipiravir quantification in human plasma by HPLC-UV was developed and validated. The validated analytical range of the method was 0.25–

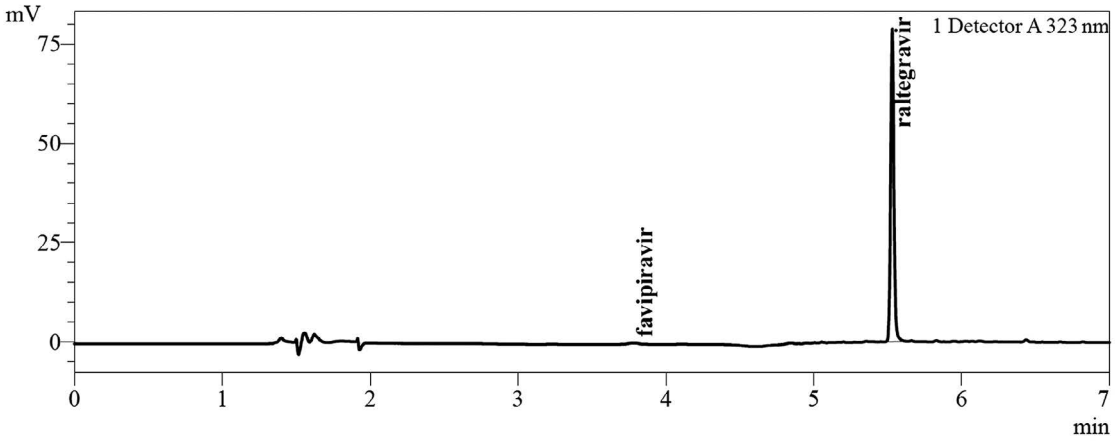


Figure 4. **LLOQ plasma sample chromatogram**

Table 8. **Stability assessment**

Type of stability	Time and storage conditions	Average value of E, %	
		L	H
Bench-top stability	Analyzed freshly prepared; stored at 20 ± 5 °C	–6,12	6,31
Postpreparative stability	48 hours at 4 °C	–4,31	–0,70
Freeze-thaw stability	36 hours at –42.5±7.5 °C and 6 hours at 20 ± 5 °C	–10,72	–13,31
Long-term stability	112 days at –42.5 ± 7.5 °C	–4,88	3,13
Stock solution stability	112 days at –42.5 ± 7.5 °C	–7,31	–10,67
Work solution stability	112 days at –42.5 ± 7.5 °C	–7,66	–10,58

200.00 μg/mL in human plasma. This analytical range allows applying the developed method for conducting pharmacokinetic studies of favipiravir products.

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