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RESEARCH ARTICLE

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Simultaneous quantitation of favipiravir and its hydroxide metabolite in human plasma and hamster matrices using a UPLC–MS/MS method

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Abstract

Favipiravir, a broad-spectrum RNA-dependent RNA polymerase inhibitor, is currently being evaluated in preclinical and clinical studies for the treatment of various infectious diseases including COVID-19. We developed an ultra-performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS) assay for the quantification of favipiravir and its hydroxide metabolite (M1), in human and hamster biological matrices. Analytes were separated on an Acquity UPLC HSS T3 column (2.1 × 100 mm, 1.8 µm) after a simple protein precipitation with acetonitrile. The mobile phase consisted of water and methanol, each containing 0.05% formic acid. Experiments were performed using electrospray ionization in the positive and negative ion mode, with protonated molecules used as the precursor ion and a total run time of 6 min. The MS/MS response was linear over the concentration ranges from 0.5–100 µg/ml for favipiravir and 0.25–30 µg/ml for M1. Intra- and inter-day accuracy and precision were within the recommended limits of the European Medicines Agency guidelines. No significant matrix effect was observed, and the method was successfully applied to inform favipiravir dose adjustments in six immunocompromised children with severe RNA viral infections. In conclusion, the UPLC–MS/MS assay is suitable for quantification of favipiravir over a wide range of dosing regimens, and can easily be adapted to other matrices and species.

Abbreviations: µL, Microliter; 13C, Carbon-13; 15N, Nitrogen-15; 2H, Deuterium; APHM, Assistance Publique Hôpitaux de Marseille; BID, Bis In Die; BRC, Biomedical Research Center; Co, Compagny; COVID-19, Coronavirus Disease; CS, Calibration Standards; CV, Coefficient of Variation; EBOV, Ebola Virus; EDTA, Ethylenediamine Tetra-Acetic Acid; EMA, European Medicines Agency; ES-, Electrospray ionization in the negative; ES+, Electrospray ionization in the positive; eV, Electronvolt; HPLC-UV, High-performance liquid chromatography- Ultraviolet; HQC, High Quality Control; HSS T3, High Speed Steel, reversed phase C18 line; INSERM, Institut National de la Santé et de la Recherche Médical; IRD, Institut de la Recherche et du Développement; IS, Internal standard; IUPAC, International Union of Pure and Applied Chemistry; LC-MS/MS, Liquid Chromatography/tandem Mass Spectrometry; LLOQ, Lower Limit of Quantification; LQC, Low Quality Control; m/z, Mass-to-charge ratio; M1, Favipiravir hydroxide; MF, Matrix factor; mL, Milliliter; MQC, Medium Quality Control; MRM, Multiple Reaction Monitoring; NIHR, National Institute of Health Research; NPH, Non-human primates; OC, Degree Celsius; PK, Pharmacokinetics; PMDA, Pharmaceuticals and Medical devices Agency; QC, Quality control; RNA, Ribonucleid Acid; SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus 2; SD, standard Deviation; SS, Stock Solution; tid, three times daily; UCL, University College London; UCLH, University College London Hospitals; UK, United Kingdom; UPLC-MS/MS, Ultra-Performance Liquid Chromatography/tandem mass Spectrometry; UPLC-TQD IVD, Triple quadrupole mass spectrometry; USA, United States of America; UVE, Unité des Virus Emergents; V, Volt.

KEYWORDS
favipiravir, lung homogenate, metabolite M1, plasma, UPLC–MS/MS

1 | INTRODUCTION

Favipiravir is an oral antiviral drug approved for human use in Japan to treat resistant influenza virus diseases (Furuta et al., 2013). Favipiravir is an RNA-dependent RNA polymerase inhibitor targeting the polymerase of a wide range of RNA viruses. The repurposing of favipiravir has been studied in the treatment of various viral infections including hemorrhagic fever and COVID-19. Favipiravir showed strong antiviral efficacy *in vitro* and in small-animal models of several viruses responsible for hemorrhagic fever, including Ebola virus (EBOV) (Oestereich et al., 2014; Smither et al., 2014). During the last EBOV epidemic in Guinea Conakry (West Africa), several therapeutic approaches were explored but none could demonstrate a significant reduction in mortality, including favipiravir (Sissoko et al., 2016). However, two recent preclinical studies showed the efficacy of higher doses of favipiravir in non-human primates for the treatment of EBOV disease (Bixler et al., 2018; Guedj et al., 2018). A dose of 180 mg/kg b.i.d. intravenously led to a 60% survival in infected animals vs. 0% in untreated macaques. These preclinical studies concluded that target trough plasma concentrations of favipiravir of around 70–80 μg/ml were necessary against EBOV. Plasma concentrations of this range have never been achieved at the dose regimens used in humans. Therefore, studies are required to investigate both the pharmacokinetics and the safety of high doses of favipiravir in humans. Recently, favipiravir has been proposed in therapeutic approaches during the SARS-CoV-2 outbreak because of its *in vitro* potential antiviral efficacy (Chen et al., 2020; Watanabe et al., 2020). It has also been used on a compassionate basis for the treatment of life-threatening infections owing to other RNA viruses in immunocompromised hosts (Lumby et al., 2020; Ruis et al., 2018). Variable clinical observations suggest that further preclinical studies in animal models are required for a good understanding of the relationship between exposure levels and pharmacological effect (Irie et al., 2020; Thammathiwat et al., 2021).

Favipiravir's pharmacokinetics (PK) is nonlinear and complex. The large variability in drug concentrations is partly because favipiravir inhibits aldehyde oxidase, the main enzyme involved in its metabolism, which converts favipiravir in an inactive oxidative metabolite T-705 M1 excreted by the kidney (Madelain et al., 2016). Self-inhibition of its own metabolism increases the favipiravir/M1 plasma concentration ratio after chronic dosing as reported in preclinical studies in mice (PMDA, 2014). Moreover, expeditious tissue distribution with rapid uptake and clearance of favipiravir has also been reported, which may explain the secondary decrease in favipiravir plasma exposure seen by repeated dosing over time during treatment. Therefore, the investigation of the PK of favipiravir would benefit from measuring both favipiravir and M1 levels, as this would more thoroughly assess the activity of aldehyde oxidase. This may allow improved understanding of the nonlinearity of favipiravir PK and help determine the dose required to achieve the target plasma concentration for EBOV disease. The concentration assay of these two compounds is possible by a high-performance liquid chromatography (HPLC)-UV method (PMDA, 2014). A comparative study of this reference method with high-performance liquid chromatography/tandem mass spectrometry (LC–MS/MS) showed concordance of the analytical performances of the favipiravir assay (Madelain et al., 2017). However, this study highlighted a slight overestimation and underestimation of low and high concentrations of favipiravir, respectively, with a lower limit of quantification (LLOQ) of 5 μg/ml. The use of internal isotopic standard (IS) of types ^{13}C, ^{15}N and ^{2}H makes it possible to improve the sensitivity of LC–MS/MS methods and remains essential for accurate quantification in both biological matrices and different species (Landvaster & Tyburski, 2015). The aim of this study is to provide a sensitive bioanalytical LC–MS/MS method to support further preclinical and/or clinical research on favipiravir. We developed a UPLC–MS/MS method, which is easy to use to quantify wide concentration ranges of favipiravir and its M1 metabolite in plasma and lung tissue. We fully validated the assay in human plasma and in hamster biologic matrices.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Favipiravir and M1 whose structures are shown in Figure 1, were supplied by Fujifilm Toyama Chemical Co. (Toyama-shi, Japan). Isotopic favipiravir $^{13}C^{15}N$ obtained from AlsaChim (Illkirch-Graffenstaden, France) was used as an IS for both tested compound because of the similarity of their chemical structures. Methanol LC–MS hypergrade was provided by E. Merck (Darmstadt, Germany). UPLC-grade water was produced with a Milli-Q academic system (Millipore, Bedford, MA, USA). Acetonitrile LC–MS grade was obtained from Fisher Scientific (Loughborough, UK) and formic acid from Sigma-Aldrich (Stenheim, Germany). Blank plasma collected in a lithium heparinate tube from healthy donors was kindly supplied by the French Blood Bank ("Etablissement Français du Sang", Marseille, France).

2.2 | Liquid chromatographic and mass spectrometric conditions

An Acquity UPLC–TQD IVD tandem mass spectrometer (Waters, Milford, MA, USA) was used to perform the assays. Chromatographic separation was achieved on an Acquity UPLC HSS T3 column (1.8 μm, 2.1 × 100 mm, Waters), maintained at 50°C.

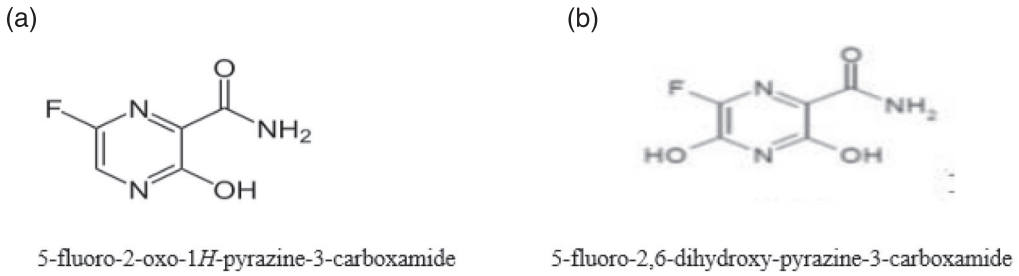

FIGURE 1 Structure and IUPAC name of favipiravir (a) and favipiravir hydroxide (b).

TABLE 1 MS/MS parameters: compound retention times, precursor and product ions, cone voltage and collision energy for favipiravir and M1.

Compound	Retention times (min)	Parent > daughter ions (*m/z*)	Cone voltage (V)	Collision energy (eV)
Favipiravir (ES+)	3.48	158.05 > 113	23.0	18.0
	3.48	158.05 > 141	23.0	14.0
M1 (ES−)	1.59	172 > 155	30.0	15.0
Favipiravir ^{13}C (ES+)	3.47	160 > 113	25	20.0

The mobile phases consisted of water + formic acid 0.05% as aqueous phase (buffer A) and methanol + formic acid 0.05% as organic phase (buffer B). The gradient elution was performed at a flow rate of 0.4 ml/min, as follows: 100% buffer A from 0.0–4.0 min, 20% buffer A from 4.0–4.5 min and 100% buffer A from 4.5–6 min.

Experiments were performed using electrospray ionization in the positive (ES+) and negative (ES−) ion mode. The protonated molecules were used as the precursor ion for the MS/MS experiment and the most suitable product ion was selected. Drug retention times, parent and daughter ions, cone voltage and collision energy were optimized for both compounds and IS and are presented in Table 1. Data were processed using Mass Lynx software NT (version 4.1, Waters).

2.3 | Preparation of stock, calibration standards and quality control samples

Stock solutions were prepared in UPLC-grade water and in acetonitrile, respectively, for favipiravir and M1 to obtain a final concentration of 1 mg/ml. Aliquots of stock solutions (SS) were then stored at −35°C. The SS were further diluted in UPLC-grade water to obtain working solutions at 10 and 100 µg/ml. Calibration standards (CS) and quality control (QC) samples were prepared extemporaneously by dilution of various volumes of working solutions and SS in blank human and hamster matrices to reach a final volume of 1000 µl of plasma. Nominal concentrations of 0.5, 1, 5, 10, 25, 50 and 100 µg/ml of favipiravir and 0.25, 0.5, 2, 5, 10, 20 and 30 µg/ml of M1 were used for CS levels. Nominal concentrations for QC samples were for low (LQC), medium (MQC) and high (HQC) levels 0.75, 50 and 80 and 0.75, 10 and 25 µg/ml for favipiravir and M1, respectively.

2.4 | Blood and tissues sample preparation

For animal samples, blood and lung tissues from 4–5-week-old female Syrian hamsters (provided by Janvier Labs) were collected immediately after euthanasia. These hamsters were those uninfected and untreated from a recent study assessing the efficacy of favipiravir against SARS-CoV-2 and organs were harvested as described (Madelain et al., 2017). Briefly, the lungs were washed in a 0.9% sodium chloride solution, weighed and then crushed using a tissue lyser machine (Retsch MM400) in 0.9% sodium chloride solution. Supernatant media were centrifuged and stored at −80°C. Blood was harvested in 100 µl of 0.5 M EDTA and centrifuged, then plasma was stored at −80°C until analysis.

The extraction method was based on a simple protein precipitation procedure for both analytes in human plasma as well as in hamster plasma and tissue samples. Briefly, 500 µl of acetonitrile or ice-cold acetonitrile containing the isotopic IS (1.5 µg/ml) was added to 50 µl of plasma sample or lung homogenate samples, respectively. After vortex mixing thoroughly for 2 min, the samples were centrifuged at 10,000*g* for 10 min. A 500 µl volume of supernatant was then transferred into glass tubes and evaporated at 50°C under gentle nitrogen flow for approximately 10 min. The dry residue was reconstituted with 500 µl of UPLC-grade water; 50 µl of each sample was injected into the UPLC–MS/MS system for analysis.

2.5 | Bioanalytical method validation

Validation of the assay was performed in accordance with the 2012 European Medicines Agency (EMA) guidelines and the International Standards Organization 15189 guidelines (EMA, 2011).

2.5.1 | Linearity

Calibration standards were prepared and analyzed in six independent runs for each compound. Calibration curves were fitted using the quadratic regression and applying a $1/x$ weighting. To obtain acceptable linearity, deviations of the mean calculated concentrations for each CS over six runs had to be within ±15% of nominal concentrations except for the LLOQ, for which a ±20% range was accepted. At least 75% of calibration standards had to fulfil these criteria. The regression coefficient must be >0.99 for each analytical run.

2.5.2 | Precision and accuracy

Intra-assay precision and accuracy were determined by analyzing six replicates of each spiked QC sample and the LLOQ (i.e. 0.25 µg/ml for M1 and 0.50 µg/ml for favipiravir) in a single assay for each matrix. Inter-assay precision and accuracy were determined by analyzing one sample of each spiked QC per day, including the LLOQ, over six different days. Intra- and inter-assay precisions were expressed as the coefficient of variation (CV) and could not exceed 15%. Accuracy was calculated as the percentage deviation from the nominal concentration and was acceptable if within ±15%. For the LLOQ, the precision and accuracy had to be <20%.

2.5.3 | Selectivity and specificity

Interferences from endogenous compounds at the retention times of both analytes and IS were investigated by analyzing eight different blank human plasma and blank plasma or lung tissue homogenate samples from six different animals. To further investigate potential interferences, for clinical applications, plasma from patients hospitalized in our institution and treated with different drugs that may be concomitantly administered (i.e. antibiotics, antifungal, antiviral, antiretroviral, anticancer) were analyzed. The peak areas of blank or patient samples were compared with those of favipiravir, M1 and IS-spiked plasma and tissue homogenate samples at the LLOQ level.

2.5.4 | Matrix effect

The matrix effect was evaluated at two concentrations on seven different batches of blank human plasma and on blank hamster plasma and lung homogenates from six different animals. The concentrations studied were the LQC (0.75 µg/ml) and HQC (80/25 µg/ml) levels. The matrix factor (MF) was determined by dividing the peak area obtained in the presence of matrix by the peak area obtained in absence of matrix at the same concentration. The MF was calculated for favipiravir, M1 and the IS. Then the IS-normalized MF (i.e. the ratio between analyte and IS MF) was calculated for both analytes. The matrix effect was considered acceptable if the coefficient of variation of the IS-normalized MF across the different batches was ≤15%.

2.5.5 | Carryover

Instrumentation carryover was explored by injecting three blank samples after samples spiked at 100 and 200 µg/ml for favipiravir and at 25 and 50 µg/ml for M1.

2.5.6 | Dilution integrity

Dilution integrity was performed by analyzing six replicates of favipiravir and M1 samples spiked at concentrations of 200 and 50 µg/ml, respectively, using 4- and 10-fold dilutions. The limits of acceptance for bias and precision were ±15%.

2.5.7 | Stability

Both analytes' stability in human plasma was investigated for 24 h in the sample injector at 4°C (short-term stability), for three freeze–thaw cycles (freeze–thaw stability) and for 2 months at −35°C in the freezer (long-term stability). Deviation of peak area and/or concentration between the conditions assessed should not exceed 15%.

2.6 | Clinical application

To evaluate the validity of the proposed bioanalytical method and its high selectivity for clinical application, we analyzed plasma concentrations in patients treated for various RNA viral infections.

The Drugs and Therapeutics Committee at Great Ormond Street Hospital approved compassionate treatment with favipiravir in six immunocompromised, pediatric patients with severe RNA viral infections. After obtaining informed consent, 1 ml EDTA blood samples were collected approximately 8 h after dose administration and plasma was isolated. Plasma samples were shipped on dry ice to the Pharmacokinetic and Toxicology Department of Timone University Hospital for measurement of favipiravir and M1 trough levels.

3 | RESULTS

3.1 | Chromatographic and mass spectrometric conditions optimization

Both analytes were optimized prior to their quantification by UPLC–MS/MS as previously described. The favipiravir was monitored using two transitions both using ES+ ion mode, although M1 was more sensitive using the ES− ion mode. For favipiravir, the first multiple reaction monitoring transition (m/z 158.05 > 113) was used for confirmation and the second (m/z 158.05 > 141) for quantification (Table 1). The acquisitions setting was set as follows: source temperature, 150°C; desolvation temperature, 400°C; desolvation gas flow, 1,000 L/h; cone gas flow, 50 L/h; and collision gas flow (argon),

0.2 ml/min. Total run time was 6 min. Typical representative chromatograms of extracted blank plasma and spiked plasma samples for favipiravir and M1 are shown in Figure 2.

3.2 | Bioanalytical method validation

3.2.1 | Linearity

Assays showed an excellent linearity over the studied calibration range from 0.50 to 100 µg/ml for favipiravir and from 0.25 to 30 µg/ml for M1 using quadratic regression with a $1/x$ weighting. The regression equations obtained were $y = -0.0000000891\,x^2 + 0.159x + 0.00509$ ($r^2 = 0.9985$) for favipiravir (m/z 158 → 141) and $y = 0.000334x^2 + 0.0528x + 0.000336$ ($r^2 = 0.9993$) for M1 (m/z 172 → 155) ($n = 6$). Precision and accuracy of calibration levels ranged, respectively, from 0.89 to 6.74% and from −1.78 to 2.53% for favipiravir, and from 0.87 to 8.05% and from −3.33 to 2.67% for M1. The LLOQs were established respectively, at 0.50 µg/ml for favipiravir and 0.25 µg/ml for M1 with precision and accuracy of 4.99% and −3.67% for favipiravir and 10.6% and −2.00% for M1, respectively. The LLOQ's inter-day precision and accuracy were 2.30 and 1.67% for favipiravir and 4.91 and 1.33% for M1, respectively. Precision and accuracy were within the acceptance limits (<20%).

3.2.2 | Precision and accuracy

The intra- and inter-day assay performances for favipiravir and its metabolite M1 are summarized in Table 2 for human plasma and in

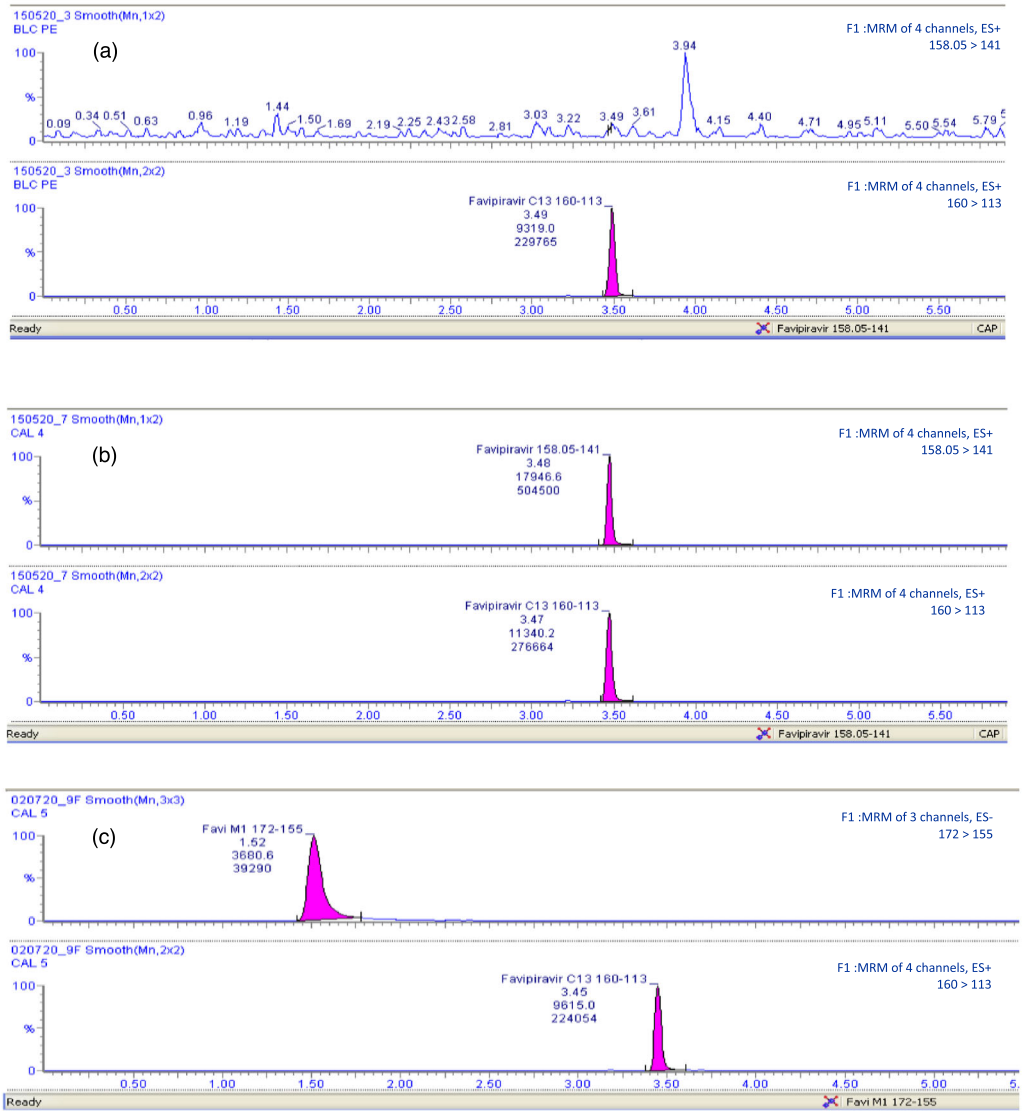

FIGURE 2 Extracted individual chromatograms of blank plasma sample (a), plasma spiked with favipiravir at 10 µg/ml (b) and M1 at 10 µg/ml (c). All samples are spiked with isotopic favipiravir $^{13}C^{15}N$ (IS).

TABLE 2 Intra- and inter-day precision and accuracy for favipiravir and M1 in human plasma ($n = 6$).

Compound	Favipiravir			M1		
Concentration (μg/ml)	0.75	50	80	0.75	10	25
Intra-day						
Mean (μg/ml)	0.75	48.0	79.5	0.71	10.9	24.8
SD (μg/ml)	0.02	1.63	2.69	0.02	0.22	0.92
Precision (CV, %)	2.45	3.39	3.38	2.89	2.04	3.73
Accuracy (%)	−0.22	−3.93	−0.58	−5.56	9.83	−0.97
Inter-day						
Mean (μg/ml)	0.75	50.6	81.6	0.71	10.5	24.9
SD (μg/ml)	0.01	1.11	3.12	0.04	0.72	1.53
Precision (CV, %)	1.12	2.19	3.82	5.16	6.81	6.12
Accuracy (%)	−0.67	1.21	1.99	−5.56	4.97	−0.19

Abbreviations: CV, coefficient of variation; SD, standard deviation.

TABLE 3 Intra- and inter-day precision and accuracy for favipiravir and M1 in hamster plasma and lung homogenates ($n = 6$).

		Favipiravir			M1		
Compound concentration (μg/ml)		0.75	50	80	0.75	10	25
Intra-day							
	Precision (CV, %)	4.54	2.96	0.74	11.1	4.32	2.88
Plasma							
Lung homogenate	Accuracy (%)	−3.50	0.40	11.9	−0.30	−1.80	−3.80
	Precision (CV, %)	6.56	2.03	2.80	5.83	3.11	4.78
	Accuracy (%)	−9.50	−8.40	9.20	11.0	13.2	2.20
Inter-day							
	Precision (CV, %)	2.37	5.13	2.94	12.3	7.63	4.70
Plasma							
Lung homogenate	Accuracy (%)	8.30	11.3	9.00	−5.30	0.40	−3.40
	Precision (CV, %)	5.56	4.08	2.97	5.39	10.9	3.99
	Accuracy (%)	−4.00	2.70	0.30	−9.90	0.40	−9.80

Table 3 for hamster plasma and lung tissue. Performance values met the current guidelines (<15%) for both compounds for the three QC concentrations.

3.2.3 | Selectivity and specificity

The specificity and selectivity of the method were validated on both human and hamster biological samples. No co-eluting peak areas that were >20% of the LLOQ response for both favipiravir and M1 and >5% of the IS response were observed. Furthermore, no significant interference with drugs that may be concomitantly co-administrated was observed at the retention times of favipiravir, M1 and IS (data not shown). The representative overlay chromatograms with blank matrix and spiked matrix samples at the LQC concentrations are shown in Figure 3.

3.2.4 | Matrix effect

The matrix effect was evaluated by comparing the peak area between plasma and lung homogenate extracts spiked at LQC and HQC levels and the corresponding standard working solutions at the same concentrations. Coefficients of variation for both concentrations were all <15% as shown in Table 4, confirming the absence of matrix effect in human plasma and hamster biological matrices.

3.2.5 | Instrument carryover

No carryover was detected after injection of a higher concentration corresponding to 1.5- and 2-fold the upper calibration levels of favipiravir (200 μg/ml) and M1 (50 μg/ml), respectively. Blank plasma

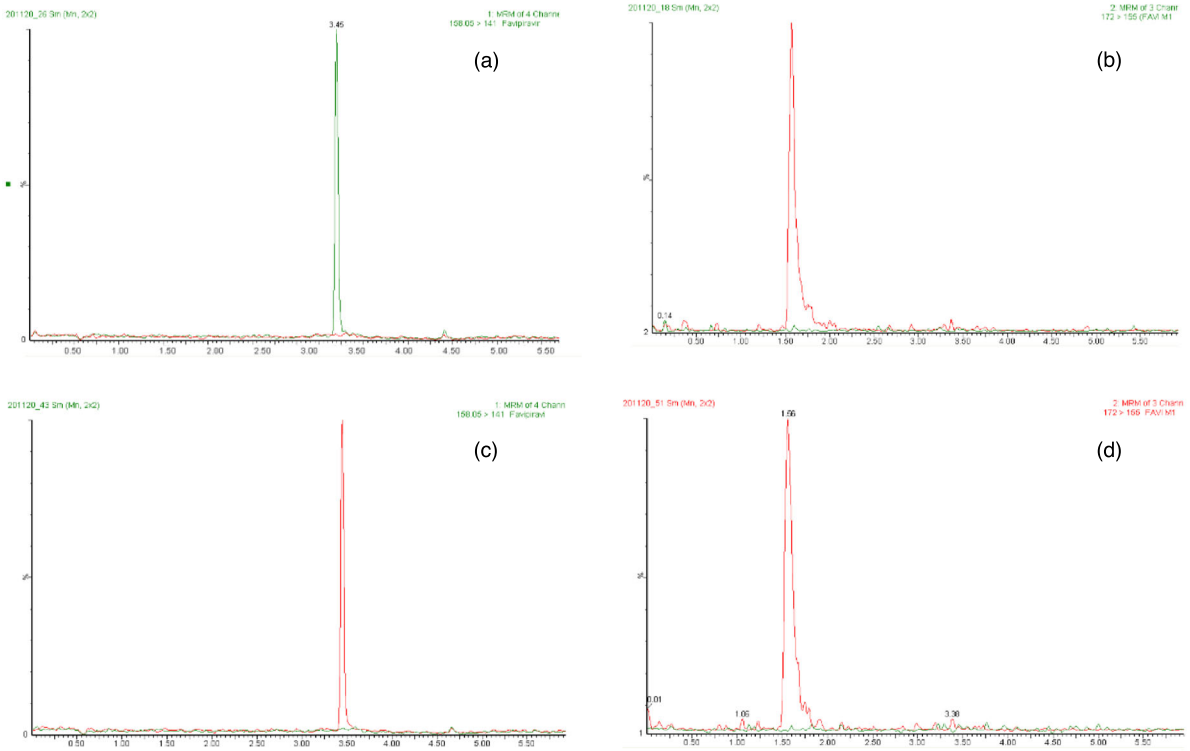

FIGURE 3 Representative multiple reaction monitoring ion-overlay chromatograms (blank matrix and matrix samples spiked at low quality control levels) for favipiravir and M1, respectively in (a, b) hamster plasma and (c, d) lung tissue.

TABLE 4 Matrix factor and IS-normalized matrix factor for favipiravir and M1 in human plasma, hamster plasma and hamster lung homogenate ($n = 6$).

	Favipiravir			M1		
	Human plasma	Hamster plasma	Hamster lung homogenate	Human plasma	Hamster Plasma	Hamster lung homogenate
MF (CV%)	0.97 (4.7%)*	1.15 (3.1%)*	1.08 (3.3%)*	1.06 (5.4%)*	1.03 (3.7%)*	1.04 (8.7%)*
	0.99 (1.1%)**	1.00 (1.7%)**	1.00 (1.3%)**	1.01 (2.0%)**	0.97 (3.1%)**	0.98 (1.6%)**
IS-normalized MF (CV%)	0.94 (4.7%)*	1.07 (3.1%)*	1.06 (3.3%)*	1.03 (5.4%)*	1.01 (3.7%)*	1.04 (8.7%)*
	1.01 (1.1%)**	1.00 (1.7%)**	1.02 (1.3%)**	1.03 (2.0%)**	0.99 (3.1%)**	1.00 (1.7%)**

Abbreviations: CV, coefficient of variation; MF, matrix factor.
*LQC level (i.e. 0.75 μg/ml for favipiravir and M1).
**HQC level (i.e. 80 μg/ml for favipiravir and 25 μg/ml for M1).

chromatograms did not show signal >20% of the LLOQ at the favipiravir and M1 retention times, or >5% at the IS retention time.

3.2.6 | Dilution integrity

After 4- and 10-fold dilution of six spiked plasma samples, dilution integrity was confirmed for both compounds, allowing accurate quantification of samples that exceed the upper limit of the calibration curve.

3.2.7 | Stability

The mean percentage changes of the peak area of calibration and QC samples ranged from −9.7 to 7.8% and from 2.7 to 17.6% for favipiravir and M1 respectively, after 24 h in the sample injector at 4°C. Table 5 summarizes the stability results from different conditions. Both analytes were found to be within ±15% of the nominal concentrations for the three QC levels with a good precision, indicating that this method offers satisfactory stability and is suitable for large-scale sample analysis.

TABLE 5 Stability of favipiravir and M1 in human plasma ($n = 16$).

Condition	Favipiravir			M1		
	Nominal concentration (μg/ml)	Mean ± SD (μg/ml)	Precision (%)	Nominal concentration (μg/ml)	Mean ± SD (μg/ml)	Precision (%)
Stored at − 35°C for 2 months	0.75	0.74 ± 0.02	3.39	0.75	0.73 ± 0.07	9.30
	50	49.7 ± 2.28	4.58	10	11.2 ± 0.44	4.00
	80	81.6 ± 2.86	3.51	25	27.4 ± 3.22	11.8
Three freeze–thaw cycles	0.75	0.74 ± 0.03	4.60	0.75	0.79 ± 0.05	6,00
	50	48.4 ± 2.09	4.30	10	11,6 ± 0.30	2.60
	80	80.0 ± 1.86	1.90	25	26.7 ± 1.62	6.10

Abbreviation: SD, standard deviation.

TABLE 6 Favipiravir and M1 plasma trough levels in pediatric patients ($n = 6$).

Infection	Dose	Favipiravir (μg/ml)	M1 (μg/ml)	M1/F ratio	Treatment adjustment	Clinical outcome
Norovirus chronic enteritis	400 mg	148	7.99	0.054	Continued unchanged	Improvement
Astrovirus CNS infection	200 mg	23.8	8.90	0.374	Dose increase	Stabilization
	400 mg	154	6.04	0.039	Continued unchanged	Improvement
RSV URTI	200 mg	10.1	1.44	0.143	Discontinued	Immune reconstitution
Enterovirus endephalitis	200 mg	81.1	3.40	0.042	Continued unchanged	Improvement
RSV URTI	200 mg	9.7	1.39	0.143	Dose increase	Improvement
Sapovirus chronic enteritis	200 mg	14	2.03	0.146	Dose increase	No change
	400 mg	50	4.4	0.088	Continued unchanged	Improvement

Abbreviations: CNS, central nervous system; F, favipiravir; RSV, respiratory syncytial virus; URTI, upper respiratory tract infection.

3.3 | Clinical application

The developed and validated method was successfully applied to the determination of favipiravir and M1 concentrations for six pediatric patients. The results are shown in Table 6. All patients received favipiravir 200 or 400 mg three times daily. Blood samples were collected at a mean time (± standard deviation) of 8 h 6 min (±1 h 6 min) after the last drug intake. All concentrations were above the quantification limits for both favipiravir and M1. The M1/favipiravir ratio was calculated and shows the dose-dependent effect on the auto-inhibition of metabolism.

4 | DISCUSSION

The nonlinear and complex pharmacokinetics of favipiravir and the potential impact of specific pathophysiological characteristics requires an evaluation of its pharmacokinetics for each viral infection studied in both humans and animal models. Variability related to aldehyde oxidase polymorphism is difficult to predict and using the M1 metabolite concentration may be useful to improve therapeutic management in a severe infectious context.

By simultaneously quantifying favipiravir and its metabolite M1, the assay we developed and validated in both animal and human matrices limits analytical variability with the potential to enhance the robustness of the data obtained in preclinical and clinical pharmacokinetic studies. The method proved to be accurate and sensitive over the calibration range without overestimating low concentrations and underestimating high concentrations using a low amount of biological sample, a simple precipitation and a total run time of 6 min. Moreover, our analytical method was validated with respect to linearity, precision, accuracy with a lower sensitivity than previously reported, allowing determination of drug levels in small animals such as hamsters and in organs. In addition, the use of deutered favipiravir as the IS for both compounds makes it possible to control for matrix effects that may be encountered between different species and between biological matrices. Recently, we successfully applied this method, firstly in hamster models assessing the efficacy of high doses of favipiravir against SARS-CoV-2 infection (Driouich et al., 2021; Kaptein et al., 2020) and secondly in a monkey model, in which the method could easily be transposed (Marlin et al., 2022). Furthermore, our method can also be applied to inform dose adjustments using patient samples, as shown in this report for the treatment of immunocompromised children with severe RNA viral infections receiving favipiravir on a compassionate basis. This was particularly helpful as no pediatric PK data is available for children. Their treatment was started at dose regimens used in EBOV trials in children (Bouazza et al., 2015), but we found that this resulted in low plasma concentrations of favipiravir associated with

high M1/favipiravir ratios reflecting increased metabolism compared with adult patients. The favipiravir dose regimen was increased in a number of patients, resulting in higher plasma concentrations.

Although two recent studies reported the use of both simple and rapid LC–MS/MS assays (Eryavuz et al., 2021; Morsy et al., 2021), our method brings additional advantages in terms of clinical and preclinical applications. First, our method allows for the evaluation of high doses of favipiravir, which have been proposed for the treatment of EBOV disease (Nguyen et al., 2017) and which are planned in the upcoming phase 1 Favidose trial for the evaluation of the pharmacokinetics and the tolerance of high-dose favipiravir (EudraCT no. 2019-000377-23). In addition, pharmacokinetics/pharmacogenetics relationships will be assessed by determining M1 and genetic polymorphism of aldehyde oxidase. Secondly, our method allows for the quantification of favipiravir (±M1) in human and animal biological matrices such as lung tissue, and is extrapolable to other tissues of interest depending on the tropism of the virus studied. This may contribute to addressing biodistribution, and may be of interest when exploring the potential of favipiravir as a broad-spectrum antiviral. Indeed, although its effectiveness against SARS-CoV-2 is limited, favipiravir may have a potential role in the treatment of other viral infectious diseases such as Lassa fever (Lingas et al., 2021; Rosenke et al., 2018).

In conclusion, we have developed a simple, rapid, highly specific, and reproducible UPLC–MS/MS method for the simultaneous quantitation of favipiravir and its metabolite M1 in human plasma as well as in hamster plasma and lung tissue. This method is easily transposable to various biological matrices, using the selectivity and specificity process of validation, and will be useful for future preclinical evaluation of favipiravir in other species, such as mice or monkeys.

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CONFLICT OF INTEREST STATEMENT

There is no conflict of interest to disclose.

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