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Research Article

NOVEL ULTRA-PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR CONCURRENT ESTIMATION AND PHARMACOKINETIC ANALYSIS OF FAVIPIRAVIR AND MOLNUPIRAVIR IN RAT PLASMA

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ABSTRACT

Objectives: The objective of this study was to establish a rapid and sensitive ultra-performance liquid chromatography (UPLC) method for the simultaneous estimation of molnupiravir and favipiravir in rat plasma using nirmatrelvir as internal reference.

Methods: The separation was performed on Waters Acquity UPLC BEH C18 (100 mm × 2.1 mm, 1.7 µm) by isocratic elution with a buffer containing 1 mL of formic acid in 1 L of water and the mixer of two components such as buffer and acetonitrile in the ratio of 70:30 as mobile phase with flow rate was 0.3 mL/min at ambient temperature.

Results: Analysis was carried out within 3 min over a good linear concentration range from 100 ng/mL to 4000 ng/mL for both drugs of favipiravir (r^2=0.9999±0.018) and molnupiravir (r^2=0.9998±0.006). This method has been successfully applied, exploring favipiravir (3.33 mg/kg) and molnupiravir (3.33 mg/kg) with internal standard nirmatrelvir extracted from rat plasma using liquid–liquid extraction.

Conclusion: The drugs were stable throughout the stability studies according to US Food and Drug Administration guidelines, just because, the validated approach has successfully conducting to the pharmacokinetic studies of two drugs.

Keywords: Favipiravir, Molnupiravir, Ultra performance liquid chromatography, Validation, International Council for Harmonisation guidelines.

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INTRODUCTION

One example of an antiviral medicine is favipiravir, which is marketed under the trade name Avigan and others. Research on its potential as a treatment for SARS-CoV-2 [1,2] and other viruses is ongoing [3]. Similar to the investigational antiviral medications [4,5] T-1105 and T-1106, it is a derivative of pyrazinecarboxamide. Favipiravir is a modified pyrazine analog that may be used to treat influenza patients when the virus has developed resistance [6,7]. The antiviral medication specifically targets RdRp, an enzyme essential for the transcription and replication of viral genomes [8,9]. Favipiravir has the potential to treat avian influenza, prevent influenza A and B from replicating, and may be a different option for influenza strains resistant to neuramidase inhibitors. The use of favipiravir in the treatment of potentially fatal infections such as COVID-19, Ebola, and Lassa viruses is now under investigation [10,11]. Use during pregnancy may be harmful to the unborn child, according to the available data, results on four different kinds of animals indicated that it was teratogenic and embryotoxic [12]. One antiviral drug that blocks the reproduction of certain RNA viruses is molnupiravir, which is marketed under the trade name Lagevrio. This medication is given orally to those infected with the virus of SARS-CoV-2 to treat coronavirus disease 2019. Molnupiravir exerts its antiviral activity by generating errors in viral RNA replication. It functions as a prodrug of the synthetic nucleoside derivative N4-hydroxycytidine. The initial purpose of molnupiravir was to treat influenza. Molnupiravir is indicated for mild-to-moderate COVID-19 patients who have tested positive for SARS-COV-2 and have a risk factor for serious disease conditions. Do not use it if you are pregnant. There is insufficient human data regarding use during pregnancy to assess the risk to the mother or the fetus. Animal studies suggest that the medication may be harmful to developing fetuses. In the phase III MOVe-OUT trial, mild-to-moderate adverse effects were recorded, including diarrhea (2% of participants) [13,14], nausea (1% of participants) [15,16], and dizziness (1% of participants) [17,18]. The chemical structures of favipiravir (M.wt-157.104), molnupiravir (M.wt-329.31), and nirmatrelvir (IS, M.wt-499.535) are depicted in Figs. 1-3, respectively.

In the current study, ultra-performance liquid chromatography (UPLC) was employed to simultaneously quantify favipiravir and molnupiravir using rat plasma. Previously, there were no established procedures for quantifying the amounts of favipiravir and molnupiravir. Fostering a UPLC methodology for the making of favipiravir and molnupiravir has acquired interest than creating different techniques. Compared to high-performance liquid chromatography, UPLC provides superior separation and may, therefore, produce more information faster. Hence, UPLC techniques were applied for separating favipiravir and molnupiravir.

This method has shorter run time, greater precision, less expensive, strong linear calibration curves, and exceptional recovery rate as per US Food and Drug Administration (USFDA) standards [19]. A satisfactory pharmacokinetic study of favipiravir and molnupiravir was conducted using the bioanalytical assay.

EXPERIMENTAL

Chemicals and reagents

Acetonitrile (ACN), formic acid, and ultra-pure water were acquired from Merck Pharmaceuticals Ltd., positioned in Worli, Mumbai, India.

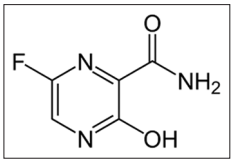

Fig. 1: Structure of favipiravir

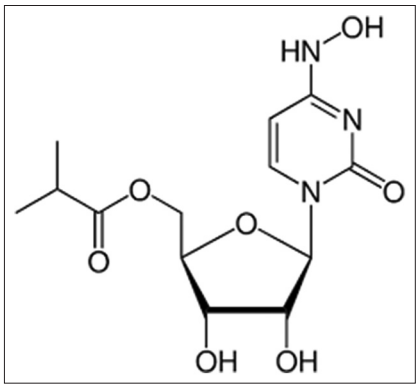

Fig. 2: Structure of molnupiravir

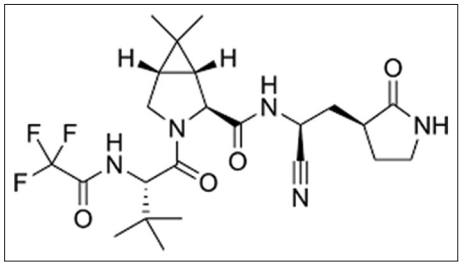

Fig. 3: Structure of nirmatrelvir

Favipiravir, molnupiravir, and nirmatrelvir (internal standard [IS]) were obtained from Zydus Cadila Healthcare Ltd., based in Ahmadabad. In addition, all reagents and materials were high-quality and conveniently accessible from a trusted vendor.

Equipment

This analysis was performed using waters company acquity model UPLC system equipped with a photodiode array (PDA) detector. Data were processed using Empower-2 software.

Preparation of calibration and quality control (QC) standards

Stock solutions of favipiravir and molnupiravir were used to produce concentrations of 2000 and 2000 ng/mL in the mobile phase. 100 ng/mL and 300, 500, 1000, 1500, 2000, 2500, 3000, and 4000 ng/mL each standards were developed for calibration purposes. Favipiravir and molnupiravir stock solutions were used to prepare these dilutions. For favipiravir and molnupiravir, the lower limit of quantification (LLOQ) was 100 ng/mL, medium QC (MQC) was 2000 ng/mL, and low QC (LQC) was 300 ng/mL, and both were measured in the same way. With the mobile phase and dilution procedure used, the IS stock solution (12000 ng/mL) was likewise processed. Until the samples were tested, all of the produced solutions were kept at a temperature between 2.0°C and 8.0°C.

Method of preparing a solution for plasma samples

Rat plasma specimens, 200 µL portions were mixed with 500 µL of IS working solution. Afterward, 300 µL of ACN was vigorously mixed for 15 min. After that, the samples were centrifuged for 15 min at a speed of 5000 revolutions/min. The resulting solution was separated into different portions, and the supernatant was carefully collected. It was then filtered using a 0.22 µ nylon syringe filter and transferred into a vial. Finally, the filtered solution was injected for UPLC analysis.

Animal parameters

In this study, three healthy white albino rats (weighing approximately 250–350 g) were obtained from Biological E Limited in Hyderabad, India. The Institute of Animal Ethics Committee accepted the protocol for the animal study (Reg. No: 1250/PO/RcBi/S/29/CPCSEA). The animal feed should be maintained at a temperature between 20°C and 26°C and a humidity level between 50% and 60%. All animals were fasted for an entire night before to the experiment and were given unlimited access to water. Favipiravir tablets and molnupiravir capsules were subjected to pharmacokinetic evaluation. Every rat received oral doses of 3.33 mg/kg for both molnupiravir and favipiravir. A blood sample volume of 0.3 mL was obtained at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4 h from the rat's body. Collected plasma centrifuge at 5000 rpm for 30 min. The supernatant was introduced into the chromatographic column, and the plasma specimens were kept at a temperature of 2–8°C until the analysis was completed.

Validation of bio-analytical method

Selectivity, matrix effect, and recovery

The study of selectivity was conducted by examining plasma samples from six lots of different rats to determine if there was any interference from unknown substances during the retention duration of favipiravir, molnupiravir, and the IS. By comparing the peak zone proportion in the post-extracted plasma sample from six distinct medication-free plasma samples and slick recovery samples, the effect matrix for favipiravir and molnupiravir was analyzed. The trials were conducted in triplicate, using six distinct batches of plasma, at MQC levels. The accuracy of the results, measured as the percentage coefficient of variation (CV), was deemed satisfactory if it was ≤15%. Six replicates were analyzed at each concentration of QC to ascertain the extraction efficiencies of favipiravir and molnupiravir. The level of recovery was determined through the comparison of the peak areas of non-extracted standards to the peak areas indicated in separate guidelines.

Dilution integrity and carry over

Dilution integrity must be demonstrated by spiking the matrix above the ULOQC with an analyte concentration and dilute using a blank matrix. Carry over describes the analyte that the chromatographic system retains after injecting a sample and discovers in later blank or unidentified samples.

Precision and accuracy

Replication analysis of QC specimens (n=6) was used to evaluate it at the lower quantification limit (LLOQ), LQC, MQC, and high QC (HQC) levels. With the exception of LLOQ, where it should be under 20%, the amount of CV should be below 15%.

Stability

By comparing the region response of the specimen made from the fresh stock solution with the area response of the analyte in the stability samples [20, 21], the stability of the stock solution was established. Six replicates were used for each dose in plasma stability studies, which were carried out at concentration levels of LQC and HQC. As per USFDA criteria, an analyte remains constant if the shift was below 15%. Benchtop stability of spiked rat plasma samples at room temperature was tested for 24 h. The stability of spiking rat plasma in auto sampler 2–8°C was tested for 24 h. Comparing the extract plasma samples that were injected right away with the samples that were re-injected at 2–8°C for 24 h after being stored in autosampler helped to determine the stability of the device. After comparing newly spiked QC samples with durability samples that had been frozen at −30°C and thawed 3 times, the durability of the freeze-thaw process was determined. Six aliquots from each of the LQC and HQC concentration ranges were utilized for the freeze-thaw stability assessment. For the long-term

stability assessment, the concentration after 24 h was compared with the initial concentration.

RESULTS AND DISCUSSION

Bioanalytical method development

To attain optimal chromatographic conditions, we evaluated various kinds of buffers with ACN as the mobile phase in varying proportions for both isocratic and gradient phases. At every trial, the composition of the mobile phase was adjusted to improve the resolution and reach appropriate retention times. Finally, 0.1% formic acid and ACN in isocratic mode at 70:30 v/v ratios was chosen as the mobile phase to maximize drug action. The optimization method used C18, C8, and CN-propyl stationary phases. Using acquity UPLC BEH C18 (100 mm × 2.1 mm, 1.7 µm) column with a PDA detector, we achieved satisfactory peak shapes for favipiravir and molnupiravir in several trials. Upon implementing the established conditions, we achieved retention intervals for favipiravir and molnupiravir of 0.836 min and 1.602 min, respectively, and 0.16, 0.41 as% CV of 6 replicate injections. It is noteworthy that the proposed approach appears to be highly specific. According to USFDA guidelines, the approach is now being validated. The chromatograms of Blank, Blank+IS, and Standard are depicted in Figs. 4-6.

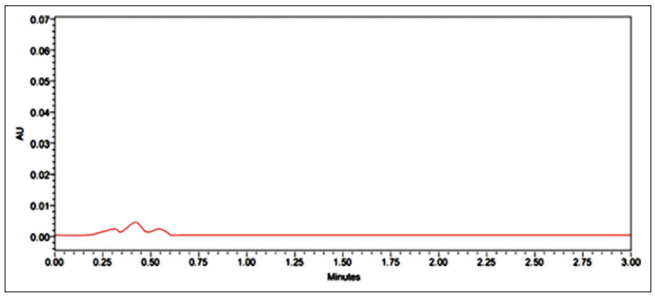

Fig. 4: Chromatogram of blank

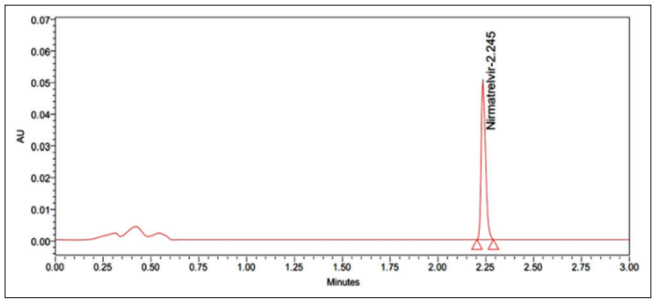

Fig. 5: Chromatogram of Blank+Internal standard

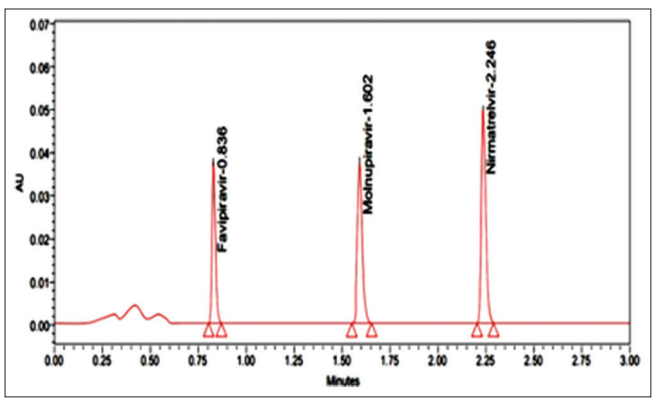

Fig. 6: Chromatogram of standard

Validation of bio analytical process

Matrix effect and recovery

The matrix effects of favipiravir and molnupiravir at LQC and HQC levels were 97.82, 98.01, and 97.02, 98.86%. Both drugs showed % CV of 0.22, 0.20 and 0.19, 0.20 at LQC and HQC levels. The results reveal that the matrix's influence on analyte ionization and internal specifications are in acceptable ranges. Favipiravir and molnupiravir had recovery rates of 97.53±0.14–98.84±0.10% and 96.66±0.20–98.82±0.16 at 300, 2000 ng/mL, and 3000 ng/mL concentrations in rat plasma. It reveals favipiravir and molnupiravir extraction efficiency. Table 1 shows details of the results.

Linearity, consistency, and precision

The area was relatively under focused in terms of adjustment norms at its height proportions. This approach yielded linearity ranges of 100–4000 ng/mL for favipiravir and 100–4000 ng/mL for molnupiravir. Figs. 7 and 8 shows favipiravir and molnupiravir calibration curves covering the linear concentration range with correlation coefficients above 0.9999 at varied QC levels. Favipiravir and molnupiravir linearity and correlation data are in Tables 2 and 3.

Accuracy and precision were optimized by merging all test data from several QC specimens. Accuracy values for favipiravir and molnupiravir QC samples were 96.15–98.58% and 96.15–98.87%, respectively. %CV was <5% for all samples at various doses. Quantification limits were met for all exactness and precision outcomes. The results are in Table 4.

Dilution integrity and carryover

Analyte matrix fixation should be spiked over the ULOQC to demonstrate dilution integrity, and this specimen should be diluted with blank matrix. At 2 × ULOQC, dilution integrity was assessed. Table 5 shows the details of the results.

Carryover refers to a system error that could have an impact on the sample's measured value. Using the following process, sample carryover on a UPLC system set up with waters acquity was assessed. Using the flow injection method on UPLC, a system blank injection volume of 5 µL was used for 0.1% formic acid and ACN (70:30). We can conclude from this approach that it did not influence the accuracy and precision of the suggested strategy. Sample carry-over results of both favipiravir and molnupiravir were LLQC (3.28%), ULQC (0.19%), and LLQC (4.47%), ULQC (0.84%) within the allowed limits. Carryover results in Table 6.

Re injection reproducibility

To confirm the system following hard product deactivation due to any instrumental disappointment, reproducibility of the reinjection was carried out during real subject sample analysis. The shift in LQC and HQC levels was <2.0, so during genuine subject specimen investigation, the group was re-infused due to instrument failure and samples were prepared and re-injected after 1 day. The % change at LQC and HQC levels was under 2.0%, so after 24 h batch can be re-injected.

Stability

Favipiravir and molnupiravir's benchtop stability was examined using a stock solution that was made and kept for 18 h at room temperature. For the auto sampler's stability, a stock solution which was stored at room temperature for an entire day produced dependable stability behavior within these circumstances. Freeze-thaw stability was evaluated on keeping the stock arrangement for 24 h at −28±5°C. Wet extract stability was evaluated onstoring the stock solution at 2–8°C for 18 h. Dry extract stability was observed by storing the stock at −20±3°C for 18 h. For short-term stability, the drugs were kept at 5±3°C for 1 week. For long-term stability, the stock was kept at -20±3°C for 28 days and then injected into the UPLC. Examine the stability outcomes of a recently prepared stock solution with a stock solution prepared 24 h prior. Our observations revealed that the percentage change in favipiravir and molnupiravir was 1.15% and 0.61%, respectively. This implies that the solutions remain stable for up to 24 h.

Table 1: Matrix variability and recovery (%) of favipiravir and molnupiravir in rat plasma

Analyte	Matrix	Matrix factor bias (%)		% RSD	Recovery (%)			
		Mean LQC	Mean HQC		Mean LQC	Mean MQC	Mean HQC	RSD
Favipiravir	Plasma	97.82	98.01	0.21	97.53	97.62	97.99	0.26
Molnupiravir	Plasma	97.02	98.86	0.19	96.66	97.31	98.69	0.24

Mean (n=6), RSD: Relative standard deviation, LQC: Low-quality control, HQC: High-quality control

Table 2: Linearity results of favipiravir and molnupiravir

Linearity	Favipiravir		Molnupiravir	
	Conc. (ng/mL)	Area response ratio	Conc. (ng/mL)	Area response ratio
1	100	0.039	100	0.038
2	300	0.114	300	0.112
3	500	0.192	500	0.188
4	1000	0.381	1000	0.374
5	1500	0.573	1500	0.560
6	2000	0.759	2000	0.742
7	2500	0.948	2500	0.926
8	3000	1.139	3000	1.115
9	4000	1.526	4000	1.492
Slope	0.000380		0.000372	
Intercept	0.000606		0.000355	
CC	0.99998		0.99998	

Table 3: Correlation results favipiravir and molnupiravir

Validation parameter	Favipiravir			Molnupiravir		
Quality control levels	Low	Middle	High	Low	Middle	High
QC Conc. (ng/mL)	300	2000	3000	300	2000	3000
Linearity range	100–4000 ng/mL			100–4000 ng/mL		
Correlation (r^2)	0.99998±0.012			0.99998±0.008		

Table 4: Precision and accuracy results favipiravir and molnupiravir

Matrix	Sample	Favipiravir		Molnupiravir	
		Precision mean accuracy (%)		Precision mean accuracy (%)	
		Intra-day	Inter-day	Intra-day	Inter-day
Plasma	LLQC	96.15	95.38	96.14	96.07
	LQC	97.73	97.32	97.01	97.02
	MQC	98.58	98.94	98.55	98.44
	HQC	98.08	97.16	98.87	97.16

Mean (n=6), LQC: Low-quality control, MQC: Medium-quality control, HQC: High-quality control

Table 5: Dilution integrity results favipiravir and molnupiravir

Analyte	Mean ULOQC conc.	Mean calculated conc.	% CV
Favipiravir	4000 ng/mL	4000.17 ng/mL	3.45
Molnupiravir	4000 ng/mL	4000.26 ng/mL	2.79

Mean (n=1)

Table 6: Carry over results favipiravir and molnupiravir

Concentration	% Mean carry-over	
	Favipiravir	Molnupiravir
Blank	0	0
LLQC	3.28	4.47
ULQC	0.19	0.84

Mean (n=1)

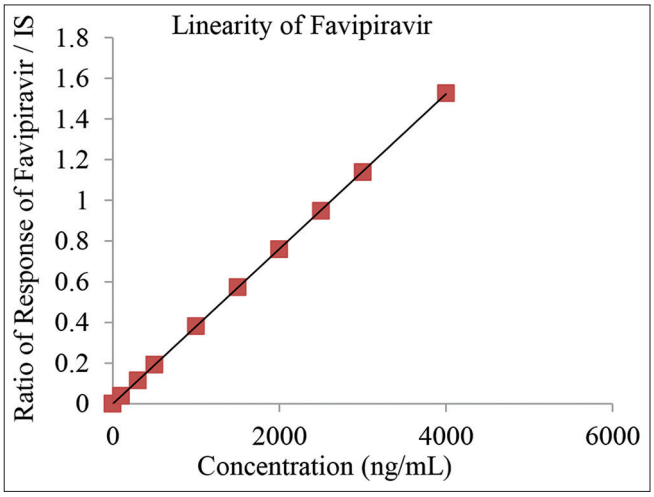

Fig. 7: Calibration plot of favipiravir

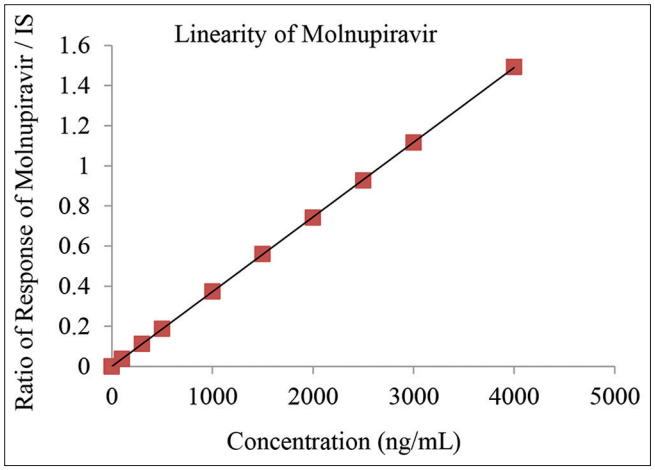

Fig. 8: Calibration plot of molnupiravir

Despite distinct conditions, both favipiravir and molnupiravir were stable in plasma at room temperature. Plasma specimens spiked with favipiravir and molnupiravir retained their stability under freezing and defrosting cycles of LQC, MQC, and HQC levels. Testing for long-term stability showed that favipiravir and molnupiravir both were stable for 24 h at a temperature of −30°C. The stability findings for favipiravir and molnupiravir are presented in Tables 7 and 8, respectively.

Pharmacokinetic study

To investigate the pharmacokinetic properties of favipiravir and molnupiravir, a market formulation dosage of 3.33 mg/1 kg of each drug was orally administered to rats. This allowed for the collection of mean plasma concentration-time profiles (Figs. 9 and 10). Favipiravir and Molnupiravir demonstrate significant differences in pharmacokinetic profiles upon oral administration. The samples were taken from the rat body at various time frames, such as 0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4 h after the drugs were administered. Following that, a sample being tested was made, injected into the chromatographic apparatus, and the results were recorded. The bioavailability of favipiravir and molnupiravir

Table 7: Stability results of favipiravir in plasma rats under different storage conditions

Stability	Storage conditions	Conc. level	Quantified concentration (ng/mL) (Mean±SD, n=6)	% RSD	Recovery (%)	Accuracy (% RE)
Benchtop stability	18 h at room temperature	LQC	8560±23.364	0.27	96.91	3.09
		MQC	58225±135.940	0.23	98.87	1.13
		HQC	86494±195.832	0.23	97.92	2.08
Auto sampler stability	24 h in auto sampler at room temperature	LQC	8524±45.598	0.53	96.50	3.50
		MQC	58004±301.459	0.52	98.50	1.50
		HQC	86452±240.395	0.28	97.87	2.13
Long-term stability	28 days at (−20±3)°C	LQC	7413±30.579	0.41	83.92	16.08
		MQC	49821±128.748	0.26	84.6	15.40
		HQC	75772±160.189	0.21	85.78	14.22
Freeze-thaw stability	24 h at (−28±5)°C then exposed to three freeze and thaw cycles	LQC	8544±18.715	0.22	96.72	3.28
		MQC	57755±162.302	0.28	98.07	1.93
		HQC	86231±130.913	0.15	97.62	2.38
Wet extract stability	18 h at 2–8°C	LQC	8527±17.784	0.21	96.53	3.47
		MQC	57361±149.380	0.26	97.41	2.59
		HQC	86265±138.926	0.16	97.66	2.34
Dry extract stability	18 h at (−20±3)°C	LQC	8528±13.515	0.16	96.54	3.46
		MQC	57334±98.289	0.17	97.36	2.3
		HQC	86174±107.645	0.12	97.56	2.44
Short-term stability	7 days at (5±3)°C	LQC	8134±16.729	0.27	96.76	3.24
		MQC	55490±178.208	0.25	97.26	2.74
		HQC	83448±158.768	0.19	97.58	2.42

Mean±SD (n=6), LQC: Low-quality control, MQC: Medium-quality control, HQC: High-quality control

Table 8: Stability results of molnupiravir in plasma rats under different storage conditions

Stability	Storage conditions	Conc. level	Quantified concentration (ng/mL) (Mean±SD, n=6)	% RSD	Recovery (%)	Accuracy (%RE)
Benchtop stability	18 h at room temperature	LQC	8341±17.960	0.22	96.62	3.38
		MQC	56347±148.538	0.26	97.90	2.10
		HQC	84800±189.580	0.22	98.23	1.77
Auto sampler stability	24 h in auto sampler at room temperature	LQC	8308±43.420	0.52	96.23	3.77
		MQC	56007±263.334	0.47	97.31	2.69
		HQC	84454±233.956	0.28	97.87	2.13
Long-term stability	28 days at (-20±3)°C	LQC	7267±19.343	0.27	84.18	15.82
		MQC	49704±185.918	0.37	86.36	13.64
		HQC	74707±161.305	0.22	86.54	13.46
Freeze-thaw stability	24 h at (−28±5)°C then exposed to three freeze and thaw cycles	LQC	8327±13.952	0.17	96.45	3.55
		MQC	55899±176.635	0.32	97.12	2.68
		HQC	84182±125.857	0.15	97.62	2.38
Wet extract stability	18h at 2–8°C	LQC	8321±16.120	0.19	96.38	3.62
		MQC	55574±130.091	0.23	96.56	3.44
		HQC	84230±108.600	0.13	97.57	2.43
Dry extract stability	18h at (−20±3)°C	LQC	8332±15.718	0.19	96.51	3.49
		MQC	55638±435.688	0.78	96.67	3.33
		HQC	84151±74.029	0.09	97.57	2.43
Short-term stability	7 days at (5±3)°C	LQC	7948±19.198	0.24	92.06	7.94
		MQC	54284±182.343	0.34	94.32	5.68
		HQC	80760±170.491	0.31	93.55	6.45

Mean±SD (n=6), LQC: Low-quality control, MQC: Medium-quality control, HQC: High-quality control

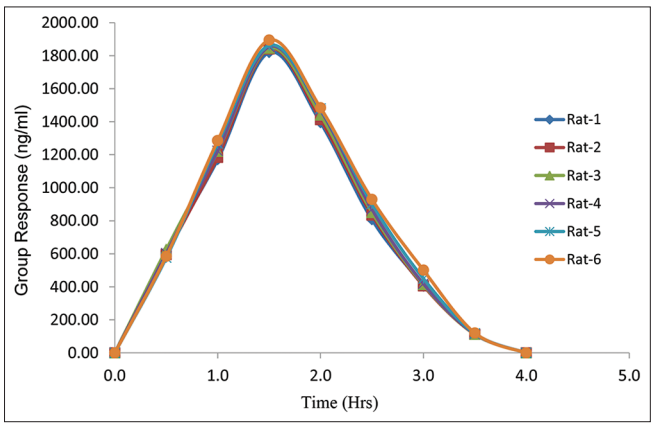

Fig. 9: Mean plasma concentration – time profile of favipiravir

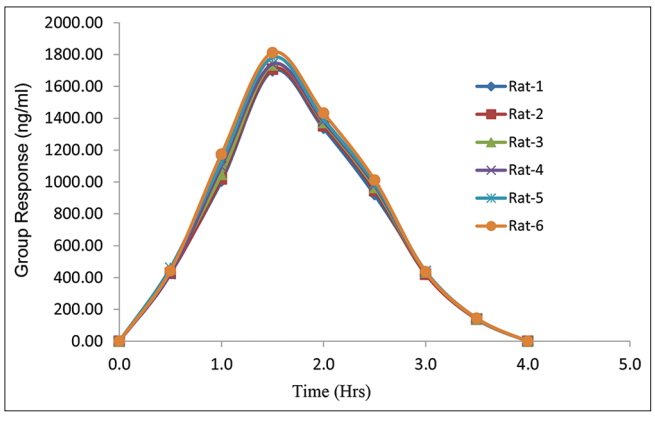

Fig. 10: Mean plasma concentration – time profile of molnupiravir

Table 9: Pharmacokinetic studies of favipiravir and molnupiravir

Pharmacokinetic parameters	Favipiravir	Molnupiravir
AUC_{0-t} (ng.h/mL)	4235	4057
C_{max} (ng/mL)	1852.309	1745.427
$AUC_{0-\infty}$ (ng.h/mL)	4235	4057
$T_{1/2}$ (h)	3.5	3.5
T_{max} (h)	1.5	1.5

$AUC_{0-\infty}$: Area under the curve extrapolated to infinity $AUC_{0-\text{lt}}$: Area under the curve up to the last sampling time C_{max}: The maximum plasma concentration, T_{max}: The time to reach peak concentration, $T_{1/2}$: Time the drug concentration

after intravenous injection was accurately calculated. The maximum plasma concentration (C_{max}) for favipiravir was 1852.309±0.425, and for molnupiravir, it was 1745.427±0.371. The time taken to reach C_{max} (T_{max}) was 1.5±0.2. The terminal rate constant (Kel) was determined using a semi-log plot of plasma concentration versus time, using the least square regression technique. The terminal half-life ($t_{1/2}$) was calculated as $0.693/K_{el}$ quotient. The values for AUC_{0-t} and $AUC_{0-\infty}$ were 4235 and 4057, respectively. These values were determined to be within the acceptable range. Table 9 shows the pharmacokinetic parameters of favipiravir and molnupiravir.

CONCLUSION

A novel UPLC approach was successfully designed and verified for the assessment of favipiravir as well as molnupiravir in rat plasma within a 3-min time frame. The absorption of favipiravir and molnupiravir after oral administration in rats was rapid, showing their pharmacokinetic behavior. The method outlined is rapid, robust, and replicable. It can be effectively used for pharmacokinetic research as well as to assess the studied analyte concentrations in biological fluids with a good linear concentration range and appropriate, accurate findings. These investigations are necessary to confirm our findings as a point of reference in the near future.

AUTHOR'S CONTRIBUTION

All authors are contributed equally.

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CONFLICT OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

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