Bioanalytical LC-MS Method Development and Validation of Favipiravir

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KEYWORDS

Favipiravir, Pharmacokinetics, Bioanalysis, LQC, Middle Quality Control, HQC.

ABSTRACT:

Favipiravir (FVP) is a broad-spectrum antiviral that selectively inhibits viral RNA-dependent RNA polymerase first trialled for the treatment of influenza infection. It has been shown to be effective against a number of RNA virus families including arenaviruses, flaviviruses and enteroviruses. A liquid chromatography tandem mass spectrometry method for the quantification of FVP in rat plasma has been developed and validated. The total chromatographic runtime is 5.0 min with retention time for Favipiravir at 2.808 min and for internal standard Remdesivir is 3.999 min. respectively. The method is validated over a dynamic linear range of 2.00 – 40.00 ng/mL for Favipiravir with a correlation coefficient of R^2 0.99978. The developed bioanalytical method was validated according to USFDA guidelines and all the validation parameters were within the acceptable limit. this method was found to be an accurate, precise, sensitive, and rapid method for the determination of Favipiravir in LC-MS by using Remdesivir as an internal standard. The work shows less run time while compared with other. According to the guidelines, the intra-batch and inter-batch precision (%CV) across three levels (LQC, MQC and HQC) are less than 15%. A battery of stability studies was performed on the method, and the results were found to be within the assay variability limits throughout the entire process. Under this optimised method, the pharmacokinetic study of Favipiravir in rat plasma was successful. Furthermore, a simple precipitation method for biological sample pre-treatment was developed that was simpler, more efficient, and less expensive than previously reported methods. This study prove that the method could be used for a variety of exploratory and other preclinical studies.

INTRODUCTION

Favipiravir is an influenza antiviral medication.[1] DEM Avigan is the commercial name for a pyrazine carboxamide derivative. It's also being researched as a potential treatment for a variety of other viral infections.[2] It was first approved for therapeutic use in resistant cases of influenza by Toyama Chemical Co., Ltd. in Japan.[3] Favipiravir is being tested for the treatment of life-threatening infections like Ebola, Lassa, and now COVID-19.[4,5,6]

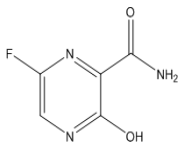

Figure 1: Chemical structure of Favipiravir[7]

IUPAC name[8]	: 5-fluoro-2-oxo-1H-pyrazine-3-carboxamide
Chemical formula	: $C_5H_4FN_3O_2$
Molecular weight	: 157.104 g/mol
Appearance[9]	: Light yellow to yellow solid
Solubility[10]	: Soluble in organic solvents such as DMSO and slightly soluble in water
^{pk}a	: 5.1

Favipiravir is a prodrug that passes through intracellular ribosylation and phosphorylation to become the active Favipiravir-ribofuranosyl-50-triphosphate (RTP). Favipiravir-RTP binds to and inhibits RNA-dependent RNA polymerase (RdRp), preventing the viral genome from being transcribed and replicated.

The method of action of Favipiravir is distinct from current influenza antivirals, which largely prevent virus entry and exit from cells. According to certain research, incorporating Favipiravir-RTP into a nascent RNA strand reduces RNA strand elongation and viral growth. Purine analogues have also been reported to impair Favipiravir antiviral efficacy, suggesting a competition for RdRp binding between Favipiravir-RTP and purine nucleosides.[11] Favipiravir has a nearly full bioavailability of 97.6 percent. The suggested dosing regimen for Favipiravir has a mean Cmax of 51.5 µg/mL.[12] The Cmax was reduced when Favipiravir was given in a single dosage with meals. When Favipiravir is given at a greater dose or in multiple doses, it appears that irreversible inhibition of aldehyde oxidase (AO) develops, reducing the influence of meals on Cmax.[13]The Half-Life: 2 to 5.5 hours.Favipiravir is metabolised extensively, and the metabolites are eliminated in the urine. The antiviral medicine is hydroxylated to the inactive metabolite T705M1 by aldehyde oxidase and to a lesser extent by xanthine oxidase.[14]Favipiravir's metabolites are largely removed through the renal route of elimination.

Favipiravir inhibits a variety of influenza viruses, including seasonal strains A (H1N1), A (H3N2), and influenza B; the A (H1N1) pandemic virus; highly pathogenic avian influenza virus A (H5N1) isolated from humans; A (H1N1) and A (H1N2) isolated from pigs; and A (H2N2), A (H4N2), and A (H5N2) isolated from poultry (H7N2).WHO shortlisted by Favipiravir as one of the drugs for trials in Ebola virus outbreak in 2014.

MATERIALS AND METHODOLOGY

Instruments used

Waters 2695 HPLC with high-speed autosampler, column oven, and degasser, and SCIEX QTRAP 5500 Mass Spectrometer with Empower-2 software were used for chromatography.

Chemicals and reagents

Biocon Laboratories in Bangalore provided the Favipiravir reference samples. Merck Chemical Division, Mumbai, provided LCMS Grade Acetonitrile, LCMS Grade Methanol, and all other chemicals. Throughout the investigation, HPLC grade water was used, which was obtained using the Milli-Q water purification system.

Stock solution preparation

In 1000 mL of HPLC grade water, one mL of formic acid was transferred and mixed well. The solution was then filtered through 0.45 µ filter paper and sonicated for 30 minutes to degas it. Formic acid buffer and acetonitrile was mixed in 60:40 ratio. The solution was then sonicated for 30 minutes after being filtered through 0.45 µ filter paper. This mobile phase was used as a diluent. 5mg Favipiravir working standard was accurately weighed and transferred to a 10 mL volumetric flask, and further diluted to volume with diluent. 0.1 mL of the same solution was taken and diluted with diluent to make 10 ml. Then, 0.2 mL of the aforementioned solution was transferred to a 10 mL volumetric flask and diluted to 100 ng/mL concentration using diluent.

500 µL of the standard stock solution was transferred to a 2 mL centrifuge tube. This was mixed with 200 µL of plasma, 500 µL of internal standard, 300 µL of acetonitrile, and 500 µL of diluent, then centrifuged at 4000 RPM for 20 minutes. The supernatant liquid was then filtered and transferred to an HPLC vial.

Transferred 5 mg of Remdesivir working standard into a 10 mL volumetric flask and diluted to the desired concentration with diluent. A further 0.1 mL was taken and diluted with diluent to make 10 mL. The concentration of 120 ng/mL was obtained by diluting 0.24 mL to 10 mL with diluent.

Five commercially available Favipiravir tablets (each containing 200 mg of Favipiravir) fine powder was weighed using an analytical balance. A few mL of diluent was added and sonicated for 10 minutes to dissolve the powdered sample weight of 65mg, which is equivalent to 5mg of Favipiravir, and the full volume was made up with the diluent. Then, using diluent, dilute 0.1 mL of the aforesaid solution to 10 mL. 0.2mL of this solution was further diluted to 10 mL with diluent.

500 µL of the standard sample stock solution was transferred to a 2 mL centrifuge tube. This was mixed with 200 µL of plasma, 500 µL of internal standard, 300 µL of acetonitrile and 500 µL of diluent, then centrifuged at 4000 RPM for 20 minutes. The supernatant liquid was then filtered and transferred to an

HPLC vial. Calibration curve standards were generated at 2.00 ng/mL, 5.00 ng/mL, 10.00 ng/mL, 15.00 ng/mL, 20.00 ng/mL, 25.00 ng/mL, 30.00 ng/mL and 40.00 ng/mL and centrifuged at 4000 RPM for 15–20 minutes. The supernatant solution was collected and injected into the chromatograph using an HPLC container. The QC samples were prepared using the above-mentioned procedure and contained Favipiravir concentrations of the lower limit of quantification quality control (LLOQQC) 2.00 ng/mL, low-quality control (LQC) 10.329 ng/mL, middle-quality control (MQC) 20.565 ng/mL and high-quality control (HQC) 30.548 ng/ml.

Extraction and isolation

Centrifuged and treated plasma samples were labelled according to respective time intervals. 300 µL of diluent were added to 200 µL of plasma and thoroughly mixed. 500 µL acetonitrile was added to the vortex cyclo mixture and centrifuged at 4000 RPM for 15–20 minutes to precipitate all the proteins. The supernatant solution was collected and injected into the chromatograph using an HPLC container. Except for 30 samples each of LQC and HQC, which were transferred for storage in cell frost deep freezer (temp range: -17°C to -27°C) for the generation of long-term stability at -22°C ± 5°C, the calibration curve standards and QC samples were logged in ultra-low temperature deep freezer (temp range: -55°C to -75°C). These samples were utilised in experiments.

Methodology for analysis

Although bioanalytical methods for identifying Favipiravir in human plasma have been published, the reported methods have a lower quantification limit. For bioequivalence and pharmacokinetic research, a lower LOQ is better since it allows for more accurate quantification of the drug concentration profile with a smaller sample amount. The purpose of this study is to develop and validate a simple, selective, sensitive, fast, robust, and repeatable assay method for determining Favipiravir concentrations in plasma samples. HPLC with MS detection was chosen as the method of choice for developing a simple and easy to apply Favipiravir test in rat plasma for pharmacokinetic investigation. The extraction method, chromatographic parameters and the mass parameters are to be optimized carefully to achieve the best results. Initially, a mobile phase of ammonium formate and acetonitrile in various combinations was tested, but there was a low response. The formic acid: water (20:80 v/v) and formic acid: methanol (20:80 v/v) mobile phases produce a better response, but the peak shape is unsatisfactory. A mobile phase containing 0.1 percent formic acid in water and acetonitrile was tested in various combinations. For both the drug under investigation and the IS, a mobile phase containing 0.1 percent formic acid in water in conjunction with acetonitrile (60:40 v/v) produced the best signal and a significant improvement in peak shape. Short-length columns, such as Symmetry Shield RP 18 (50mm x 2.1 mm, 3.5 µm), Inertsil ODS-2V (50mm x 4.6 mm, 5 µm), Hypurity C18 (50mm x 4.6 mm, 5 µm) and Hypurity Advance (50mm x 4.0 mm, 5m), X-Bridge phenyl column (150 x 4.6 mm, 3.5 µm), Zorbax Eclipse Plus C 18, (2.1mm x 50 mm, 3.5 µm) were tried during the method development. The best signal and peak shape were achieved with the X-Bridge phenyl column, 150 x 4.6mm, 3.5 µm. It produced acceptable peak shapes for Favipiravir and IS. The run time was decreased to 5.0 minutes by using a flow rate of 1 mL/min without a splitter. Drug and IS were both eluted with a shorter time at 4.0 min. When a considerable matrix impact is possible, using stable isotope- labelled analogue medicines as an internal standard for an LC-MS analysis is beneficial. Remdesivir was shown to be the best option in this investigation. The temperature in the column oven was room temperature. For better ionisation and chromatography, the injection volume of a 10 µL sample is utilised. Figures 3.2 to 3.6 represent the trial chromatograms. The chromatograms were recorded after injecting blank (plasma solution), linearity solutions, and sample solutions into the chromatograph. The Favipiravir peak area was counted. The linearity plot was used to calculate the concentration of Favipiravir in the plasma sample.

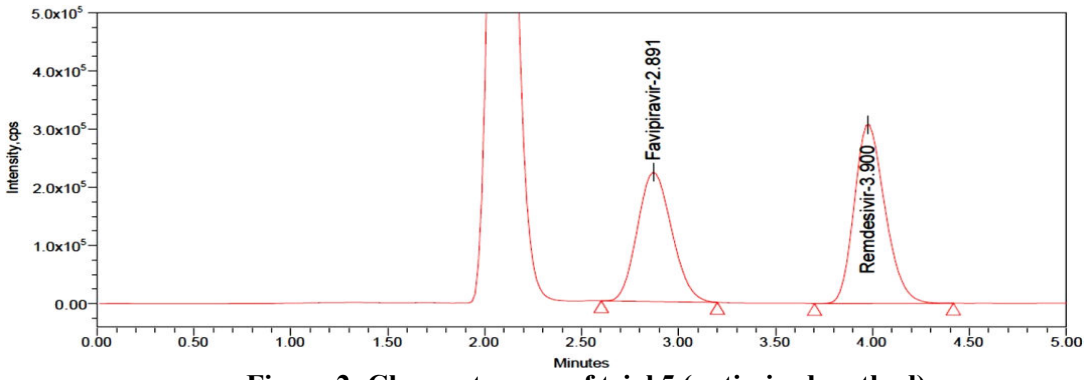

Figure 2: Chromatogram of trial 5 (optimized method)

Table 1: Optimized chromatographic conditions

Parameter	Optimized Conditions
Mobile Phase	Formic acid buffer and acetonitrile in the ratio 60:40 v/v
Flow rate	1.0 mL/min
Column	X-Bridge phenyl column, 150 x 4.6mm, 3.5µm.
Column temperature	25^{0C}
Injection volume	10 µL
Run time	5 min
Diluent	Formic acid buffer and acetonitrile in the ratio 60:40

Optimised mass parameters

The mass parameter optimization was carried out by injecting Favipiravir and IS solutions directly into the mass spectrometer's ESI source. Other parameters such as the nebulizer and heater gases, as well as declustering potential (DP), entrance potential (EP), and collision energy (CE) were optimised to achieve a better spray shape, which resulted in better ionisation and droplet drying, resulting in protonated ionic Favipiravir and IS molecules. From its parent ion mass spectra, a CAD product ion spectrum for Favipiravir and IS generated high-abundance fragment ions of m/z (amu) 158.63 and m/z (amu) 602.33 respectively In positive ion electrospray ionisation interface mode, the mass spectrometer was operated. To quantify the chosen medication, the multiple reactions monitoring mode was used.

Table 2: Optimized mass spectrometric conditions

Collision energy (CE)	15 V for the selected drug and IS
Ion spray voltage	5500 V
Source temperature	550^{0}c
Drying gas temperature	120-250°C
Collision gas, nebulizing gas	Nitrogen
Drying gas flow stream	5 L/min
Declustering potential	40 V
Entrance potential	10 V
Exit potential	7 V
Dwell time	1 Sec
Type of ionization	Positive mode of electro spray ionization

Results and Discussion

In this investigation, a positive ionisation LC-MS assay was designed and assessed. The complete scan mass spectrum of Favipiravir and the internal standard Remdesivir in the positive MRM was shown in Figures 3 and 4. Finally the linearity, accuracy, precision, sensitivity, selectivity, and recovery studies were used to assess the reliability of the method. The LC-MS approach was successfully validated to meet the approval requirements of the Food and Drug Administration's industrial guidance for bioanalytical method validation.[17]

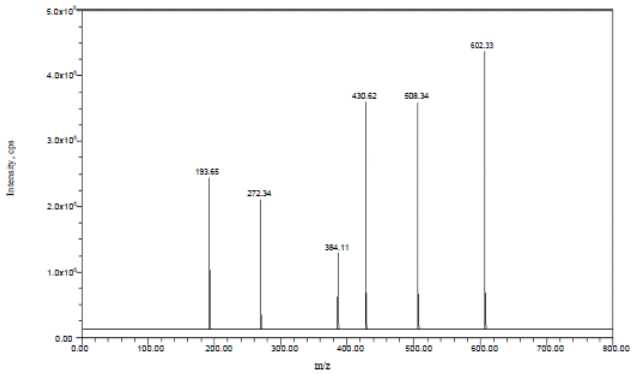

Figure 3: Parent ion mass spectrum of Favipiravir

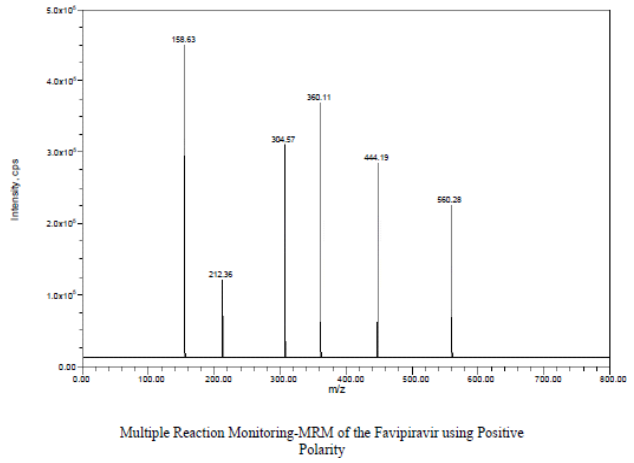

Figure 4 Parent ion mass spectrum of Remdesivir

System suitability

The coefficient of variation (CV) for the response ratio of drug and internal standard, as well as the retention time of drug and IS, were calculated after six consecutive injections of the reference standard solution containing Favipiravir at MQC level concentration and IS at working concentration level. Table 3 summarizes the findings. The percent CVs for Favipiravir and IS area ratios were 0.17 and 0.88, respectively, and the percent CVs for Favipiravir and IS retention time were 0.11 and 0.09 respectively. Because the acceptance requirements for the percent CV of the retention time (RT) of the analyte and internal standard should be ≤ 2.00 percent, the developed method passed the system suitability test. The percent CV of the analyte's area ratio and internal standard should be ≤ 5.00 %.

Table 3: System suitability results of Favipiravir

Sample Name MQC (20 ng/mL)	Analyte Area(cps)	Analyte RT(min)	ISTD* Area (20 ng/mL)	ISTD RT (min)	Area Ratio
MQC-1	$2.505x10^5$	2.808	$3.071x10^5$	3.999	0.8157
MQC-2	$2.503x10^5$	2.802	$3.089x10^5$	3.995	0.8103
MQC-3	$2.510x10^5$	2.800	$3.050x10^5$	3.992	0.8230
MQC-4	$2.503x10^5$	2.806	$3.029x10^5$	3.990	0.8263
MQC-5	$2.507x10^5$	2.804	$3.031x10^5$	3.998	0.8271
MQC-6	$2.514x10^5$	2.801	$3.035x10^5$	3.994	0.8283
Mean	$2.507x10^5$	2.804	$3.051x10^5$	3.995	0.822
SD	0.00434	0.00308	0.02443	0.00344	0.00723
%CV	0.17	0.11	0.80	0.09	0.88

ISTD*-Internal Standard.

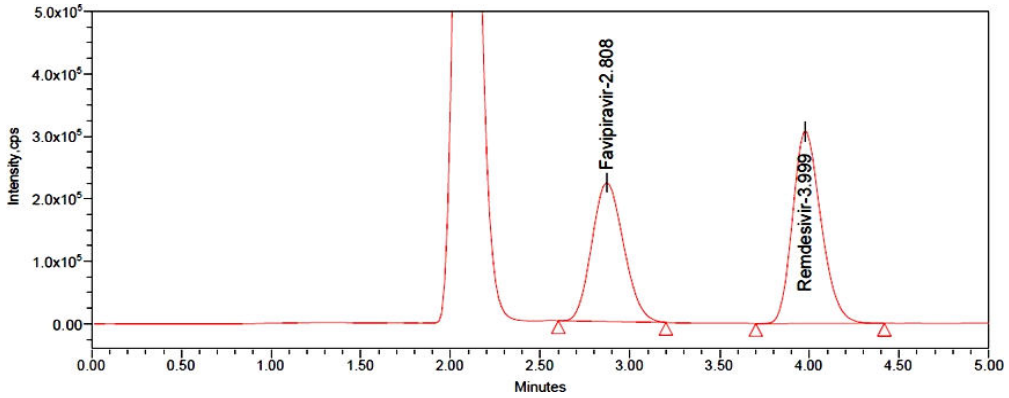

Figure 5: Chromatogram of system suitability

Auto sampler carryover

For both unextracted and plasma extracted samples, the injector carryover impact was assessed. It was evaluated by injecting reconstitution solution after the maximum concentration aqueous standard (Aqueous ULOQ) and extracted standard blank after the extracted ULOQ standard, as well as aqueous and extracted LLOQ samples. In comparison to the response of the extracted LLOQ samples, there was no carry over effect at the RTs of Favipiravir and IS in aqueous standard and in extracted standard blank. The analyte peak area in a blank sample after ULQC must be less than 20% of the peak area of the LLOQ to qualify for carryover. As a result, the proposed approach was shown to be free of potential carry-over effects.

Table 4: Autosampler carryover of Favipiravir

Sample ID	Peak Area		% Recovery	
	Drug	ISTD	Drug	ISTD
Un Extracted Samples				
RS	0	0	N/A	N/A
HQC			0.00	0.00
RS	0	0		
LLOQ			N/A	N/A
Extracted Samples				
Std Blk	0	0	N/A	N/A
HQC			0.00	0.00
Std Blk	0	0		
LLOQ			N/A	N/A

Specificity and screening of biological matrix

The response of extracted LLOQ samples of analyte processed with internal standard is compared with that of extracted blank plasma. Specificity was determined using six different random blank plasma samples and six different Favipiravir samples at LLOQ level processed with IS for the proposed method. The retention times of Favipiravir and IS in plasma blank samples showed no interfering peaks, indicating that the percent interference for both the analyte and the internal standard was zero. As a result, the proposed method was specific, and the percent of interference was within acceptable limits, i.e., the response of interfering peaks in STD Blank at the retention time of analyte should be less than 20% of that in LLOQ, and the response of interfering peaks in STD Blank at the retention time of analyte should be less than 5% of that in LLOQ.

Table 5: Specificity and screening of biological matrix of Favipiravir

S. No.	Sample ID	Intensity(cps)		% Interference		Pass/ Fail
		Drug	ISTD	Drug	ISTD	
1.	Std Blk 1	0	0	0	0	Pass
2.	LLOQ 1m(2ng/mL)	$0.250x10^5$	$3.010x10^5$	0	0	Pass
3.	Std Blk 2	0	0	0	0	Pass
4.	LLOQ 2 (2 ng/mL)	$0.253x10^5$	$3.014x10^5$	0	0	Pass
5.	Std Blk 3	0	0	0	0	Pass
6.	LLOQ 3 (2 ng/mL)	$0.261x10^5$	$3.012x10^5$	0	0	Pass
7.	Std Blk 4	0	0	0	0	Pass
8.	LLOQ 4 (2 ng/mL)	$0.258x10^5$	$3.019x10^5$	0	0	Pass
9.	Std Blk 5	0	0	0	0	Pass
10.	LLOQ 5 (2 ng/mL)	$0.252x10^5$	$3.015x10^5$	0	0	Pass
11.	Std Blk 6	0	0	0	0	Pass
12.	LLOQ 6 (2 ng/mL)	$0.255x10^5$	$3.016x10^5$	0	0	Pass

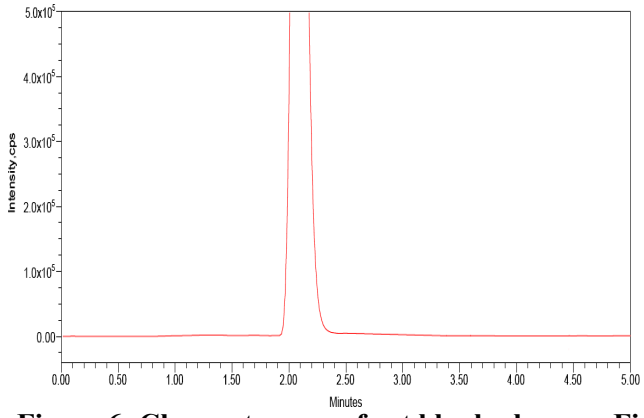

Figure 6: Chromatogram of rat blank plasm

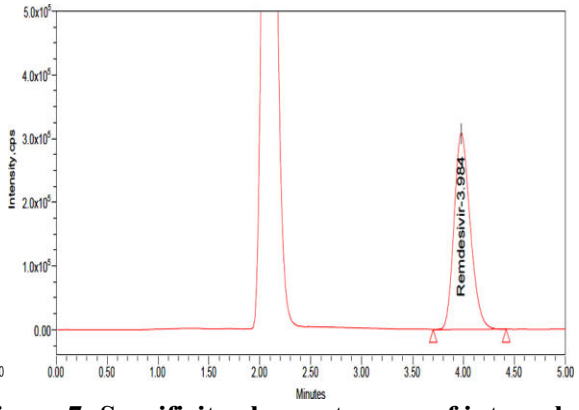

Figure 7: Specificity chromatogram of internal standard

Sensitivity

The proposed approach was used to prepare six independent plasma samples containing LLOQ of Favipiravir. These six values mean concentration and percent RSD were calculated. Using the calibration equation, the mean percentage accuracy of six independent samples was calculated and determined to be 100.91 percent, with a percent CV value of 1.012. As a result, the suggested method's lowest calibration

concentration was deemed the lowest limit of quantification (LLOQ) and the method was deemed sensitive. The acceptance criteria for sensitivity is percent mean accuracy of LLOQ should be within 80.00-120.00 percent, and the percent CV (precision) should be less than 20.00 percent.

Table 6: Sensitivity results of Favipiravir

Replicate Number	LLOQ
	Nominal Concentration(ng/mL)
	2.24
	Analyte peak area
1	0.257x105
2	0.255x105
3	0.251x105
4	0.254x105
5	0.251x105
6	0.251x105
N	6
Mean	0.253x105
SD	0.00256
% CV	1.012
% Mean Accuracy	100.91%

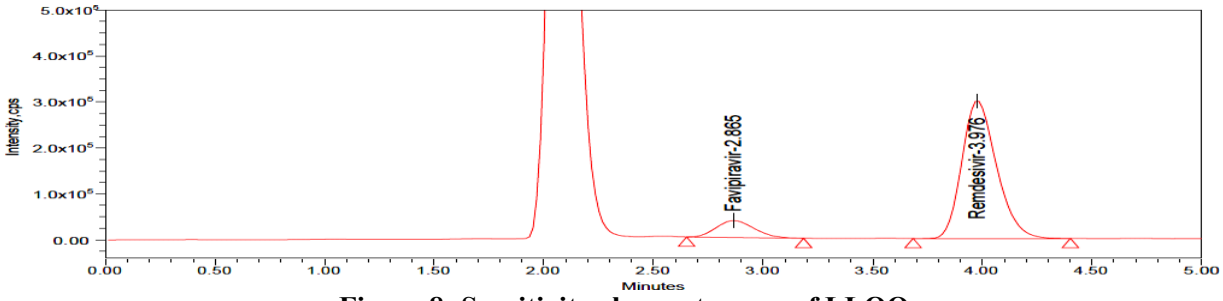

Figure 8: Sensitivity chromatogram of LLOQ

Matrix effect

The post extraction spike method' was used to determine the matrix effect. Six sets of blank matrix were spiked at HQC and LQC levels after post extraction, and the responses of post extracted plasma samples were compared to analytes from neat samples at equivalent concentrations. Using chromatographically screened rat plasma, the matrix effect proposed approach was evaluated. Back estimated concentrations of HQC and LQC levels had a mean percent accuracy of 99.66 and 100.27 percent, respectively. Because the results met the acceptance criterion of 85.00-115.00 percent, the present approach did not demonstrate any ionization effects.

Table 7: Matrix effect results of Favipiravir (HQC-30 ng/mL, LQC-10 ng/mL)

S. No	Plasma Lot No.	HQC	LQC
		Nominal concentration (ng/mL)	
		30.42	10.43
		Analyte peak area	
1.	Lot 1	$3.753x10^5$	$1.260 x10^5$
		$3.759 x10^5$	$1.260x10^5$
		$3.761 x10^5$	$1.264 x10^5$
2.	Lot 2	$3.705 x10^5$	$1.235 x10^5$
		$3.720 x10^5$	$1.277 x10^5$
		$3.746 x10^5$	$1.288 x10^5$
3.	Lot 3	$3.753 x10^5$	$1.210 x10^5$
		$3.771 x10^5$	$1.205 x10^5$
		$3.700 x10^5$	$1.201 x10^5$
4.	Lot 4	$3.711 x10^5$	$1.259 x10^5$
		$3.791 x10^5$	$1.288 x10^5$
		$3.726 x10^5$	$1.287 x10^5$
5.	Lot 5	$3.780 x10^5$	$1.205 x10^5$
		$3.798 x10^5$	$1.308 x10^5$
		$3.783 x10^5$	$1.298 x10^5$
6.	Lot 6	$3.720 x10^5$	$1.266 x10^5$
		$3.740 x10^5$	$1.243 x10^5$
		$3.751 x10^5$	$1.277 x10^5$
N		18	18
Mean		$3.748x10^5$	$1.257x10^5$
SD		0.02982	0.03386
%CV		0.80	2.69
%Mean Accuracy		99.66%	100.27%

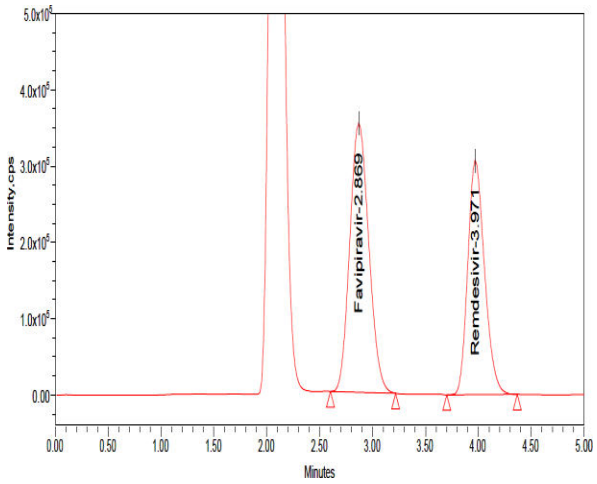

Figure 9: Matrix effect chromatogram of HQC

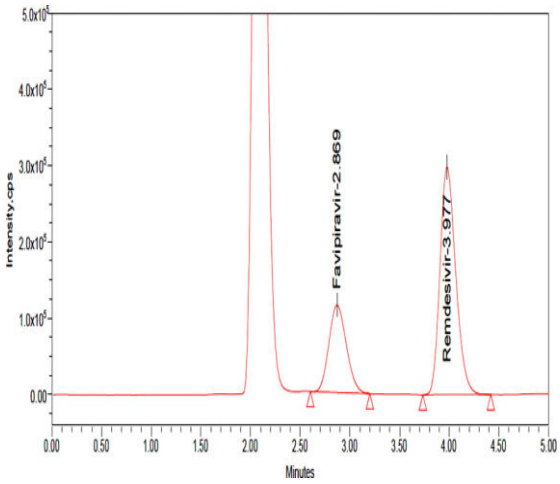

Figure 10: Matrix effect chromatogram of LQC

Linearity

With the use of eight calibration standards, calibration curves for Favipiravir were constructed over a concentration range of 2.00 ng/mL to 40.00 ng/mL. The peak area ratio method of the analyte to that of IS was used to quantify the samples. 0.99978 was the mean correlation coefficient. The ratios of peak area to plasma concentrations were displayed. The calibration curve was revealed to be linear, with the best fit consistently > 0.99. During validation, the calibration curve standards percent accuracy ranged from 95.81 to 101.32 percent. As a result, the suggested approach was shown to be linear over the above concentration range, with a regression coefficient of >0.99 and a percent accuracy for a calibration standard lying between the acceptability limit.

Table 8: Back-calculated standard curve data for Favipiravir in rat plasma

Final conc. in ng/mL	Response	Area response ratio
0	0	0.0
2.00	0.25	0.083
5.00	0.625	0.207
10.04	1.255	0.414
15.03	1.88	0.615
20.00	2.501	0.815
25.03	3.13	1.042
29.99	3.75	1.222
40.08	5.012	1.632
Slope	0.0411	
Intercept	0.00101	
R^2 Value	0.9998	

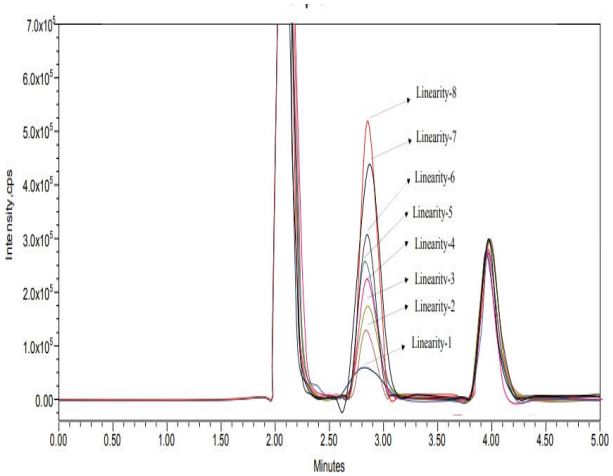

Figure 11: Linearity overlay chromatogram

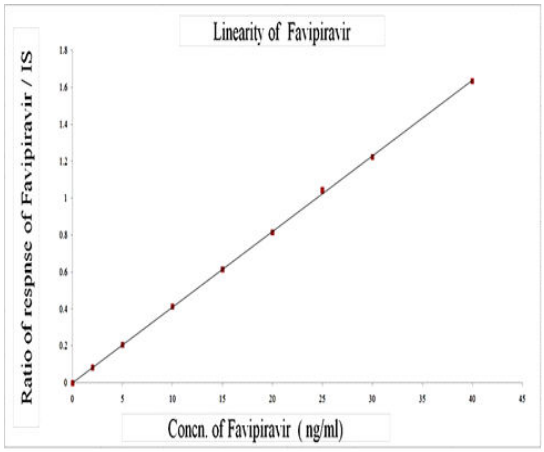

Figure 12: Calibration plot for concentration v/s Area ratio of Favipiravir

LoD and LoQ

The limit of detection (LoD) and limit of quantitation (LoQ) was calculated based on signal to noise ratio. The results were represented in table 9 and the chromatograms for LoD and LoQ were represented in Figure 13 and 14.

Table 9: LoD and LoQ Results

Drug Name	LoD (S/N) Value	LoQ (S/N) Value
Favipiravir	4	25

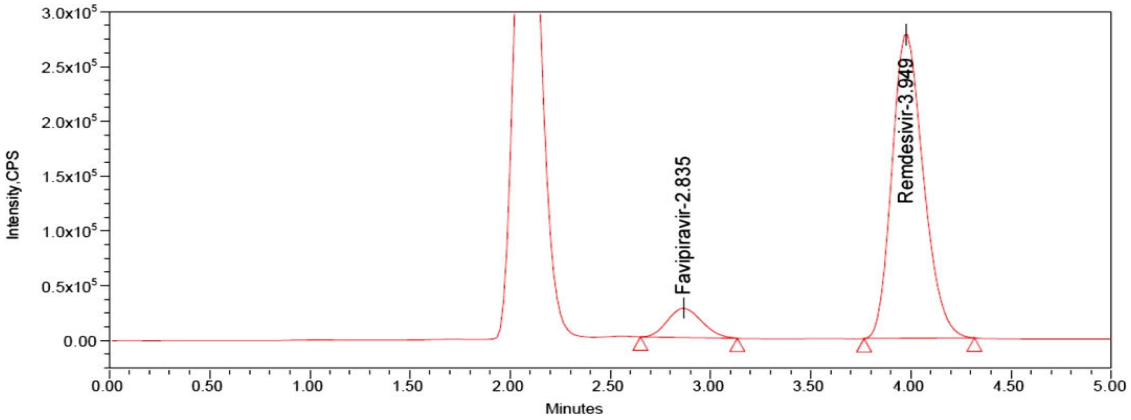

Figure 13: LoD chromatogram

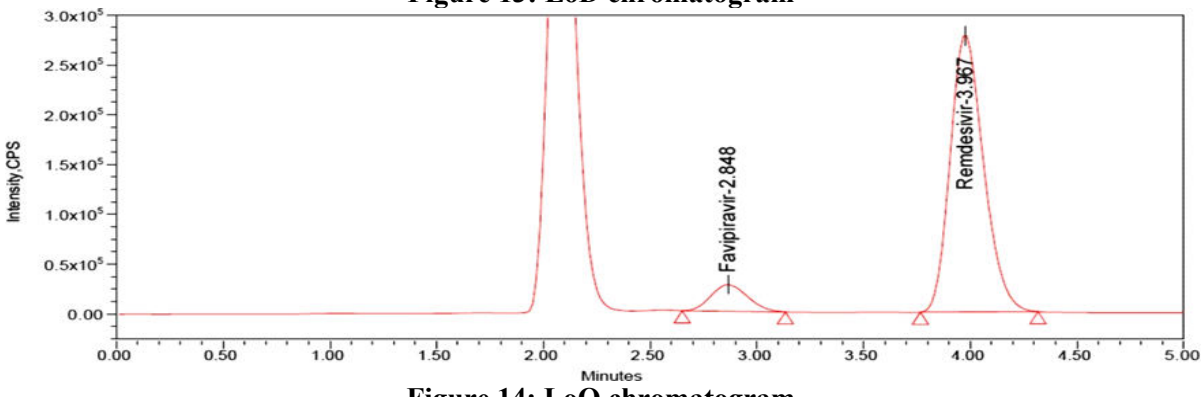

Figure 14: LoQ chromatogram

Precision and accuracy

Six replicates containing Favipiravir were analysed at three different QC levels to determine the intra-assay precision and accuracy. Analyzing the three levels of QC samples on independent runs determined the inter-assay precision. The suggested method's percent mean accuracy varied from 98.33 percent to 101.36 percent, and the precision (percent CV) for LQC, MQC, and HQC was 0.68 to 2.89 percent. Table 10 summarized the findings, whereas Figure 15 depicted the chromatogram.

Table 10: Calculated concentrations obtained for precision and accuracy batches

S.NO	Concentration (ng/mL)		
	LQC	MQC	HQC
1	10.04	20.26	29.99
2	9.77	19.99	30.14
3	9.62	20.70	30.16
4	10.37	20.18	30.20
5	9.88	20.10	29.85
6	10.26	20.51	25.03
Mean	9.99	20.29	29.23
SD	0.289	0.267	2.061
% CV	2.89	1.31	7.05

Table 11: Intra and inter-run precision and accuracy for Favipiravir in rat plasma

Parameter		Concentration (ng/mL)		
		LQC (10)	MQC (20)	HQC (30)
atch-1 (n=6)	Intra-run	9.99	20.29	29.23
	SD	0.289	0.267	2.061
	% CV	2.89	1.31	7.05
	% Accuracy	98.33	98.42	101.36
r-Batch (n=6)	Inter-run mean	9.88	20.40	29.75
	SD	0.233	0.183	0.201
	% CV	2.36	0.90	0.68
	% Accuracy	98.55%	98.77%	98.6%

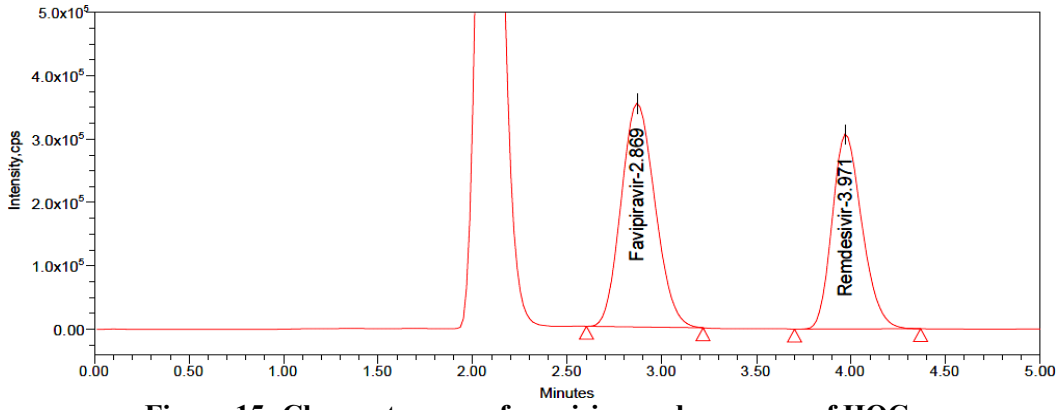

Figure 15: Chromatogram of precision and accuracy of HQC

Recovery of analyte

Extraction efficiency (analyte recovery from a sample matrix) is a comparison of analytical response from a quantity of analyte added to that calculated from the sample matrix. Because of Favipiravir basic structure, extraction was done with a mobile phase solvent. Six duplicates of LQC, MQC, and HQCs were used to assess analyte and IS recovery. Favipiravir mean areas in extracted samples were compared to Favipiravir mean areas in post-extracted samples. Similarly, mean IS areas in extracted middle quality control samples were compared to mean IS areas in post extracted middle control samples. It inferred that Favipiravir average recovery rate was 100.6 percent and the average IS recovery at the concentration used was 99.02 percent. The results showed that the extraction efficiency for Favipiravir using the protein precipitation method was satisfactory, consistent, and concentration independent.

Table 12: Recovery of the analyte of Favipiravir at HQC level

Replicate Number	HQC (30 ng/mL)		
	Extracted Response	Unextracted Response	Matrix Factor
1.	$3.784x10^5$	$3.860x10^5$	0.9803
2.	$3.786x10^5$	$3.889x10^5$	0.9735
3.	$3.782x10^5$	$3.801x10^5$	0.9950
4.	$3.731x10^5$	$3.807x10^5$	0.9800
5.	$3.748x10^5$	$3.813x10^5$	0.9830
6.	$3.701x10^5$	$3.831x10^5$	0.9661
N	6	6	6
Mean	$3.755x10^5$	$3.834x10^5$	0.980
SD	0.03485	0.03455	0.00968
%CV	0.93	0.90	0.99
%Mean Recovery	99.85%	101.95%	-

Table 13: Recovery of the analyte of Favipiravir at MQC level

Rep No.	MQC (20 ng/mL)		
	Extracted Response	Unextracted Response	Matrix Factor
1.	$2.504x10^5$	$2.601x10^5$	0.9627
2.	$2.533x10^5$	$2.633x10^5$	0.9620
3.	$2.505x10^5$	$2.671x10^5$	0.9379
4.	$2.515x10^5$	$2.600x10^5$	0.9673
5.	$2.514x10^5$	$2.603x10^5$	0.9658
6.	$2.567x10^5$	$2.673x10^5$	0.9603

N	6	6	6
Mean	$2.523x10^5$	$2.630x10^5$	0.959
SD	0.02394	0.03467	0.01081
%CV	0.95	0.95	1.13
%Mean Recovery	100.63%	104.90%	-

Table 14: Recovery of the analyte of Favipiravir at LQC level

Rep No.	**LQC (10 ng/mL)**		
	Extracted Response	**Unextracted Response**	**Matrix Factor**
1.	$1.231x10^5$	$1.297x10^5$	0.9491
2.	$1.209x10^5$	$1.293x10^5$	0.9350
3.	$1.200x10^5$	$1.293x10^5$	0.9281
4.	$1.237x10^5$	$1.302x10^5$	0.9501
5.	$1.281x10^5$	$1.307x10^5$	0.9801
6.	$1.283x10^5$	$1.304x10^5$	0.9839
N	6	6	6
Mean	$1.240x10^5$	$1.299x10^5$	0.954
SD	0.03516	0.00589	0.02300
%CV	2.84	0.45	2.41
%Mean Recovery	98.92%	103.62%	-

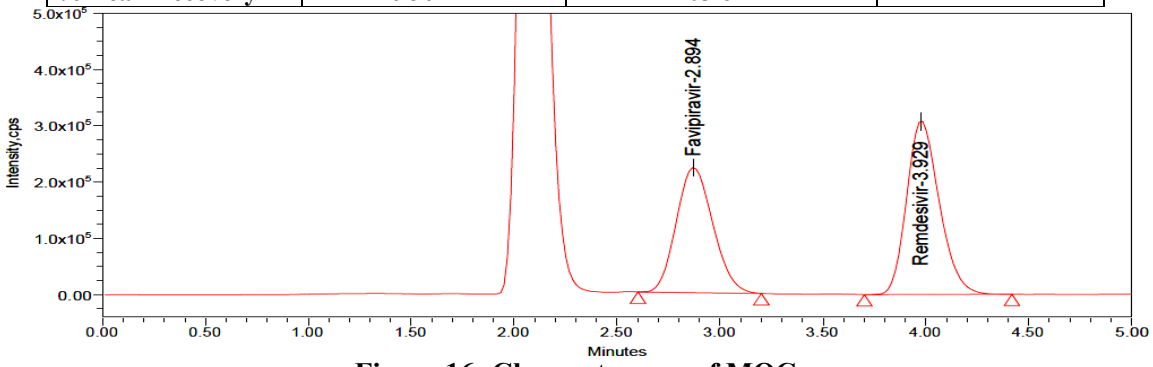

Figure 16: Chromatogram of MQC

Table 15: Internal standard results of Remdesivir (30 ng/mL)

S. No.	**Un Extracted Area Ratio**	**Extracted Area Ratio**
1.	$3.043x10^5$	$3.030x10^5$
2.	$3.050x10^5$	$3.027x10^5$
3.	$3.091x10^5$	$3.065x10^5$
4.	$3.097x10^5$	$3.000x10^5$
5.	$3.082x10^5$	$3.056x10^5$
6.	$3.076x10^5$	$3.004x10^5$
N	6	6
Mean	$3.073x10^5$	$3.030x10^5$
SD	0.02199	0.02640
% CV	0.72	0.87
%Mean Recovery	100.72%	99.31%

Ruggedness on precision accuracy

Six replicates were performed at the HQC, MQC, and LQC levels using various columns (the same make and model) by different analysts using the same equipment to test ruggedness on precision accuracy. The % mean accuracy at HQC, MQC and LQC were found to be 99.10, 101.9 and 98.68 % and the %CV for Favipiravir was found to be 0.68- 2.36 %. Hence it passed the ruggedness on precision accuracy.

Table 16: Ruggedness on precision accuracy results of Favipiravir

P& A ID	Acquisition Batch ID	HQC (30 ng/mL)	MQC (20 ng/mL)	LQC (10 ng/mL)
		Nominal Concentration (ng/mL)		
		30.627	20.385	10.269
		Analyte peak area		
Different Column		$3.777x10^5$	$2.522x10^5$	$1.255x10^5$
		$3.716x10^5$	$2.540x10^5$	$1.285x10^5$
		$3.721x10^5$	$2.555x10^5$	$1.202x10^5$
		$3.708x10^5$	$2.560x10^5$	$1.219x10^5$
		$3.717x10^5$	$2.563x10^5$	$1.228x10^5$
		$3.720x10^5$	$2.590x10^5$	$1.233x10^5$
N		6	6	6
Mean		$3.727x10^5$	$2.555x10^5$	$1.237x10^5$
SD		0.02516	0.02293	0.02924
% CV		0.68	0.90	2.36
% Mean Accuracy		99.10%	101.9%	98.68%

Stability studies

Favipiravir stability in plasma was tested using six replicates of QC samples at low, medium, and high levels. Drug-free plasma was spiked with appropriate volumes of Favipiravir standard solutions to create the samples. Bench-top stability, auto- sampler stability, freeze-thaw stability, wet extract stability, dry extract stability, short term stability, and long-term stability were all evaluated.

Bench top stability

The spiked quality control samples bench top stability was tested for an 8-hour period at room temperature. Acceptance requirements are that at least 67 percent of QC samples must pass an acceptance limit of 85 – 115 % mean accuracy. The mean accuracy at LQC, MQC and HQC samples of Favipiravir have a bench top stability of 98.6, 98.57 and 98.60 percent and % CV of LQC, MQC and HQC samples were found to be 3.75, 1.46 and 1.03 respectively. Table 17 summarizes the findings.

Table 17: Bench top stability of Favipiravir

S.No	LQC (10 ng/mL)	MQC (20 ng/mL)	HQC (30 ng/mL)
1	10.36	20.72	30.20
2	10.31	20.00	29.64
3	9.58	20.02	29.67
4	9.64	20.42	30.32
5	9.66	20.06	30.33
6	10.24	20.02	30.09
Mean	9.96	20.21	30.04
% CV	3.75	1.46	1.03
% Nominal	10	20	30
% Stability	98.62%	98.57%	98.6%

Auto sampler stability

By storing the processed quality control samples in an auto sampler maintained at 15°C for 12 hours, the auto sampler stability of the processed quality control samples was assessed. Acceptance requirements are that at least 67 percent of QC samples must pass an acceptance limit of 85 – 115 % mean accuracy. The mean accuracy at LQC, MQC and HQC samples of Favipiravir in auto sampler stability were found to be 98.58, 98.59 and 98.58 percent, % CV of HQC, LQC and MQC samples were found to be 1.64, 0.63 and 0.68 respectively. Table 18 summarises the findings of auto sampler stability.

Table 18: Auto sampler stability of Favipiravir

S.No	LQC (10 ng/mL)	MQC (20 ng/mL)	HQC (30 ng/mL)
1	9.66	20.64	29.76
2	9.64	20.26	29.68
3	10.28	20.16	29.60
4	10.18	20.44	30.22
5	10.04	20.40	29.84
6	10.11	20.10	29.65
7	10.36	20.25	29.54
8	9.96	20.10	29.84
9	9.74	20.06	30.15
10	10.20	20.42	30.25
11	9.62	20.74	30.09
12	9.66	20.02	29.80
13	10.04	20.35	29.73
14	9.59	20.25	29.99
15	9.76	20.67	29.84
16	9.76	20.70	30.06
17	10.01	20.21	29.93
18	9.58	19.97	29.56
19	10.00	20.40	30.18
20	10.15	20.42	29.80
21	10.28	20.09	29.54
22	10.32	20.49	30.23
23	9.64	20.18	29.92
24	9.91	20.08	30.33
Mean	9.94	20.31	29.9
% CV	2.64	1.11	0.82
% Nominal	10	20	30
% Stability	98.58%	98.59%	98.58%

Method of Application for Pharmacokinetic Studies

Six male Wistar rats weighing 180-220 g were used for the pharmacokinetic studies. The animals were kept in ventilated cage with adequate supply of feed and water for 7 days before starting the experiments. Rats were kept on overnight fasting prior to dosing. Pharmacokinetic study of Favipiravir was conducted in rats (n = 6). The protocol was approved by the local Independent Ethics Committee. Each rat was treated with Favipiravir at a dose equivalent to 200 mg based on body weight in a single dose and was administered by oral gavages and collected the blood samples at different time intervals like 0.5, 1, 2, 3, 4, 5, 6,7, and 8-hours post-dose in six different rats. The protein precipitation extraction method was used

to isolate Favipiravir in rat plasma. For this, 200 µL of plasma sample (respective concentration) were added into labelled polypropylene tubes and vortexed briefly after that 500µL of acetonitrile was added and vortexed for approximately 10 min followed by centrifugation at 4000 RPM at 20°C. Supernatant from each sample was transferred to labelled via tube and evaporated at 40°C until dryness. These samples were reconstituted with 500µL of acetonitrile and vortexed briefly and then transferred the sample into autosampler vials for injection. The pharmacokinetic parameters of Favipiravir were computed. The Cmax (maximum plasma concentration) and Tmax (time to maximum plasma concentration) were obtained directly from the plasma time profile curve. Pharmacokinetic parameters of Favipiravir were calculated using WinNonlin (Version 5.2) software package. The stability of the study samples was established by incurred sample reanalysis (ISR). The linear trapezoidal rule was used to estimate all other pharmacokinetic parameters like AUC 0-t, AUC 0-∞, t1/2, C_{max} T_{max} and clearance (CL) are summarized in table 19.

Table 19: Pharmacokinetic data (Time Vs Mean plasma concentration)

Time intervals (Hr)	Favipiravir Concentration (ng/mL)
0.5	2.181
1	5.389
2	8.972
3	14.417
4	17.434
5	14.002
6	10.179
7	5.793
8	1.635

Table 20: Pharmacokinetic parameters of Favipiravir

Pharmacokinetic parameters	Favipiravir
$^{AUC}0$-t	85ng /mL.h
^{C}max	18.6 ng/mL
$^{AUC}0$-∞	85 ng/mL.h
^{T}max	4.0 Hour
$^{t}1/2$	8.0 Hours
CL	1.5 mL/min/kg

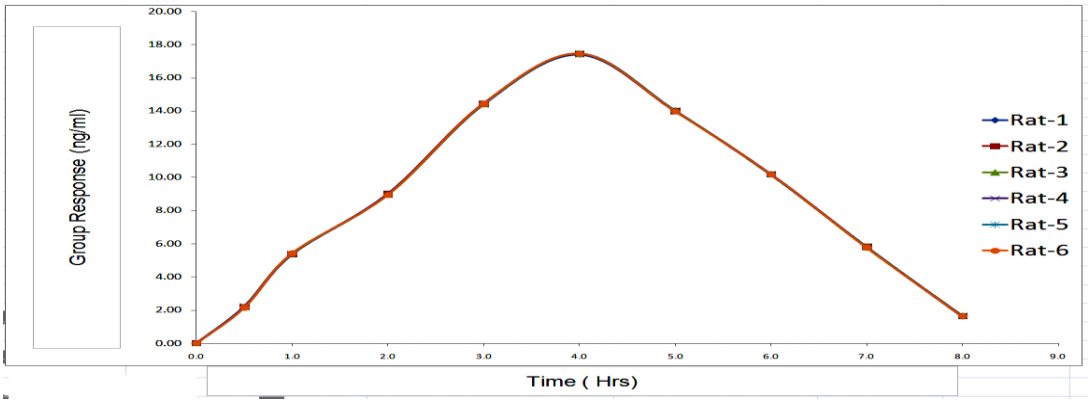

Figure 17: Mean plasma concentration Vs Time (hrs) plot of Favipiravir in rat plasma by LC-MS

Summary and Conclusion

An accurate, precise, sensitive, and rapid method for the determination of Favipiravir in LC-MS by using Remdesivir as an internal standard. The work shows less run time while compared with other work articles. The total chromatographic runtime is 5.0 min with retention time for Favipiravir at 2.808 min and for internal standard Remdesivir is 3.999 min. respectively. The method is validated over a dynamic linear range of 2.00 – 40.00 ng/mL for Favipiravir with a correlation coefficient of R^2 0.99978. The developed bioanlytical method was validated according to USFDA guidelines and all the validation parameters were within the acceptable limit. In pharmacokinetics studies with male wistar rats, the method was successfully used to quantify Favipiravir. According to the guidelines, the intra-batch and inter-batch precision (%CV) across three levels (LQC, MQC and HQC) are less than 15%. A battery of stability studies were performed on the method, and the results were found to be within the assay variability limits throughout the entire process. Under this optimised method, the pharmacokinetic study of Favipiravir in rat plasma was successful. Furthermore, a simple precipitation method for biological sample pre-treatment was developed that was simpler, more efficient, and less expensive than previously reported methods. Favipiravir plasma concentrations were measured, and the main pharmacokinetic parameters were determined. This study prove that the method could be used for a variety of exploratory and other preclinical studies.

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