RESEARCH ARTICLE

Liquid Chromatographic Approach for Analysis of Favipiravir–A Repurpose Drug for COVID-19

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ABSTRACT

Favipiravir is a potential repurpose moiety to treat COVID-19 by depletion of virus load in infectious patients. To analyze and separate Favipiravir with remarkable efficiency, X-Bridge C8 column (150 x 4.6 mm, 5 µ) and a solvent phase of 0.1% TEA and acetonitrile (40:60 v/v) with 1-mL/min flow rate were used. The eluted favipiravir and possible degradants were detected at 225 nm. Further, the process was validated by using ICH (Q2R1) guidelines to ensure the method's suitability in the pharmaceutical sector. The RT of Favipiravir was observed at 3.7 min with good linearity of 2 to 30 µg/mL. %RSD of both system and method precision was assessed in the series of 0.32 to 0.98. The mean percentage recovery of Favipiravir was in the range of 99.0–100.4%. The limit of detection (LoD) and limit of quantification (LoQ) were assessed to be 0.024 and 0.084 µg/mL for favipiravir. The outcomes confirmed that the projected approach was economical, insightful, simple and precise with better sensitivity. Investigation of Favipiravir in the incidence of a variety of stressed or forced degradation environments ensures stability indicating quality of the developed approach.

Keywords: Favipiravir, Forced degradation, Stability indicating, X-Bridge C8 column, Sensitivity.

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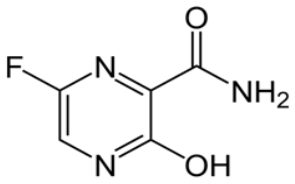
INTRODUCTION

The exceptionally high potential for SARS-CoV-2 to spread generated a global catastrophe with major barriers to diagnosis, treatment, and prevention.[1] One of the most difficult parts of the pandemic has been the lack of a specific antiviral medicine identified for the treatment of COVID-19, despite the aggressive search for an efficient and permanent cure[1]. Repurposing current antiviral drugs for COVID-19 treatment has become the norm.[1-3] Favipiravir, one of the repurposed antiviral agent, competitively inhibits the RNA replicase enzyme leading to termination of genome replication of RNA virus. Initially, in 2014 it was developed to treat influenza virus infections.[4] Recently, WHO approved favipiravir as an emergency therapeutic agent in the coronavirus infection such as COVID 19.[4-6] It is an effective and potent therapeutic drug using in ebola virus infection (Figure 1).[1]

A thorough examination of the literature showed that only three RP-HPLC methods have been used to analyze favipiravir in bulk and tablet form.[7-9] Various difficulties or disadvantages were identified in previous liquid chromatographic methods, including longer retention time and lower sensitivity.[7] Aside from that, no stability representing the RP-HPLC process was discovered during a thorough review of the literature.[8-9] A stability indicating RP-HPLC process with effective separation



Molecular Formula	:	$C_5H_4FN_3O_2$
Molecular Weight	:	157 g\mol
IUPAC Name	:	5-fluoro-2-oxo-1H-pyrazine-3-carboxamide
Appearance	:	White powder
Pka	:	5.1

Figure 1: Molecular structure and basic properties of favipiravir.

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of Favipiravir and possible degradants with remarkable resolution was designed and tested to fill that gap.

MATERIALS AND METHODS

Materials

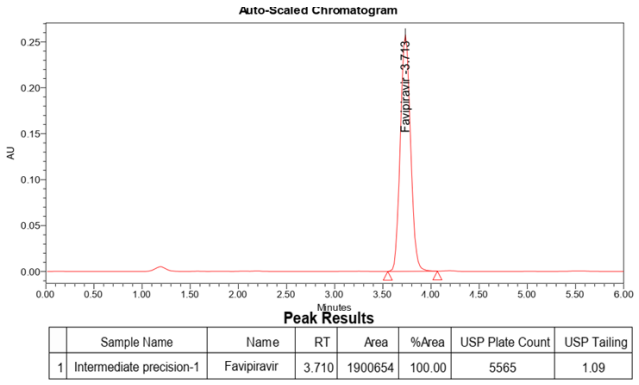
Favipiravir pure drug was procured from Shree icon Labs, Vijayawada. The chemicals and reagents used in the present study were procured from Avntar chemicals Pvt Ltd, India. High-quality borosilicate glassware was used to prepare the solutions.

Preparation Standard Solution (20 µg/mL)

About 20 mg of favipiravir API powder was properly weighed and placed into 100 mL volumetric flask, dissolved with diluents (Acetonitrile: TEA (40:60 v/v)) to obtain a solution with 200 µg/mL. In 5 mL of the resultant solution further diluted to 50 mL to obtain a solution consisting of 20 µg/mL of favipiravir represented as 100% level concentrations.

Preparation of Sample Solution (20 µg/mL)

The tablet powder equal to 20 mg of favipiravir powder was precisely weighed and placed into 100 mL volumetric flask, dissolved with diluents (Acetonitrile: TEA (40:60 v/v)) to obtain a solution with 200 µg/mL. 5 mL of the resultant solution further diluted to 50 mL to obtain a solution consisting of 20 µg/mL of Favipiravir.



Peak Results

	Sample Name	Name	RT	Area	%Area	USP Plate Count	USP Tailing
1	Intermediate precision-1	Favipiravir	3.710	1900654	100.00	5565	1.09

Figure 2: Optimized method chromatogram representing Favipiravir RT at 3.71 minutes.

Method Development

Method development was done by doing various trials (Table 1) with dissimilar ratios of movable (solvent) phases, various kinds of columns and changes in flow rate. Trial-10 was considered the optimized method for separating Favipiravir in both formulation and bulk with a retention time (RT) of 3.7 minutes (Figure 2).

Method Validation

ICH Q2 (R1) specifications were taken in to consideration for validation of the current approach.[10,11]

System Suitability

This has been ascertained by analyzing standard solution (100% level) in six repetitive samplings and further evaluation of parameters like %RSD, plate count (N) and peak asymmetry (T) were done from the obtained chromatograms.

Linearity

A standard linear plot was constructed between stated concentrations and respective responses by performing serial volumetric dilutions of the stock solution (200 µg/mL) for the 2 to 30 µg/mL range. The R^2 value from linear plot was used to confirm the linearity of the approach.

Accuracy

The usual addition method was employed to conduct the accuracy experiments. Standard levels of 50, 100, and 150% were spiked in triplicate to separate individual sample sets. Favipiravir's %accuracy was calculated for each level, and the average %accuracy (n = 3) and %RSD were found.

Precision

It serves as a general indicator of repetition and reproducibility. Method's system and method precision were tested by analysis of standard and sample in 6 replications, respectively. To verify the accuracy of the system and the procedure, respectively, the %RSD for the generated peak regions and percent assay were evaluated.

Specificity

When the favipiravir is successfully identified using the specified approach in the incidence of additional compounds but

Table 1: Various trials

Trial. No	Column type	Mobile phase	Observation
1	Inertsil, C18 (250 × 4.6 mm, 5 µ)	0.1% Formic acid: ACN (30:70 v/v)	Unknown peak was identified
2	Inertsil, C18 (250 × 4.6 mm, 5 µ)	0.1% Formic acid: ACN (40:60 v/v)	Tailing effect observed
3	X-Bridge phenyl (150 × 4.6 mm, 3.5 µ)	0.1% Formic acid: ACN (50:50 v/v)	Asymmetric shape peak
4	X-Bridge pheny (150 × 4.6 mm, 3.5 µ)	0.1% OPA: ACN (40:60 v/v)	Less plate count, More Tailing
5	X-Bridge phenyl (150 × 4.6 mm, 3.5 µ)	0.1% OPA: ACN (35:65 v/v)	Two peaks were observed
6	X-Bridge C8 (150 × 4.6 mm, 3.5 µ)	0.1% OPA: ACN (30:70 v/v)	Broad peak
7	X-Bridge C8 (150 × 4.6 mm, 3.5 µ)	0.1% TEA: ACN (20:80 v/v)	Peak tailing
8	X-Bridge C8 (150 × 4.6mm, 3.5 µ)	0.1% TEA: ACN (25:75 v/v)	Base line was bad, Un known peaks formed
9	X-Bridge C8 (150 × 4.6 mm, 3.5 µ)	0.1% TEA: ACN (30:70 v/v)	Peak tailing
10	X-Bridge C8 (150 × 4.6 mm, 3.5 µ)	0.1% TEA: ACN (40:60 v/v)	Peak with symmetric shape

Flow rate- 1-mL/min., Wavelength- 225nm, and Volume of injection -10 µL.

Table 2: Results of system suitability test for Favipiravir

Injection No.	*RT (Min.)*	*Peak area*	*Plate count*	*Tailing factor*
1	3.714	1891923	5634	1.1
2	3.717	1895508	5623	1.1
3	3.72	1887820	5580	1.1
4	3.722	1891040	5600	1.1
5	3.725	1888217	5558	1.09
6	3.729	1893460	5567	1.09
MEAN		1891328	5593.6	1.096
SD		2981.24		
% RSD		0.16		

Table 3: Linearity data of Favipiravir

S. No	*%Level*	*Favipiravir*		
		Concentration (µg/mL) (n=3)	*Peak area*	*%RSD*
1	10	2.00	184880	0.95
2	25	5.00	459113	1.62
3	50	10.00	896209	0.57
4	75	15.00	1256347	1.28
5	100	20.00	1834505	1.34
6	125	25.00	2178517	1.10
7	150	30.00	2655617	0.79
Correlation coefficient (R2)			0.9991	

without any interference, the specificity of the process is shown. During the procedure, 0.1 mL of each distinct solution for the blank, standard, sample, and placebo with standard had to be added in turn. The recorded chromatograms were used to determine whether or not there were interferences at the RT of favipiravir's with the aforementioned solutions. The exploration was strengthened by correlating the RT of the degradants generated from various degradation solutions with the RT of favipiravir in fresh Favipiravir's standard solution to determine the interferences from degradants towards Favipiravir.

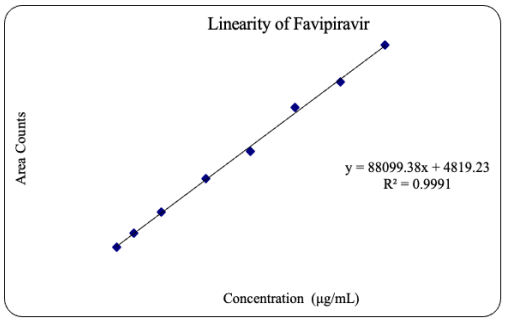


Figure 3: Calibration curve of Favipiravir.

Sensitivity

LoD and LoQ were determined by use of the standard deviation approach.

$$LoD = 3\sigma/S \text{ and } LoQ = 10\ \sigma/S$$

σ is SD of the intercept (n=3)

S is slope of the linear plot (n=3)

Robustness

The method's robustness was ascertained by faintly altering the method conditions such as flow rate (± 0.1 mL/min), mobile system ratio (± 1 mL) and maximum absorption wavelength (± 2 nm). The %RSD value was computed to considerable system suitability parameters upon injecting standard concentration in three replicates.

Forced Degradation Studies

The stability demonstrating nature of the current method has been proven by using forced degradation studies (Table 8).[12-14]

Acidic Degradation

Equal portions of 1N HCl and stock solutions of standard Favipiravir were mixed homogenously and refluxed on water bath at 60°C for 60 minutes. The resultant solution cooled at room temperature and neutralized with NaOH (1N). Further dilution process has been made to attain a solution of 20 µg/mL of Favipiravir. In the same way 1N NaOH and 10% H_2O_2 alkali were added separately with solutions of to perform alkali and oxidative degradation. 10 mL of favipiravir stock solution has been sited in the temperature controlling unit (80°C/75% RH) and UV chamber (254 nm, dark control) for 24 hours to conduct thermal and photodegradation studies. The resultant solutions were further diluted with mobile phase to attain a solution of 20 µg/mL of favipiravir.

About 10 mL of stock solution was treated with 10% Sodium bisulfate and reflex for 30 minutes at 60°C. After 24 hours,

Table 4: Results of the %recovery of Favipiravir

% Level	*Amount added (µg/mL)*	*Amount recovered (µg/mL)*	*%Recovery*	*%*Recovery ± SD*	*%RSD*	*Acceptance limit*
	10	9.89	98.9			
50	10	9.9	99.0	99.0 ± 0.15	0.15	
	10	9.92	99.2			
	20	20.18	100.9			
100	20	20.1	100.5	100.8 ± 0.26	0.26	100 ± 2 %
	20	20.2	101.0			
	30	30.2	100.7			
150	30	30.1	100.3	100.4 ± 0.22	0.22	
	30	30.08	100.3			

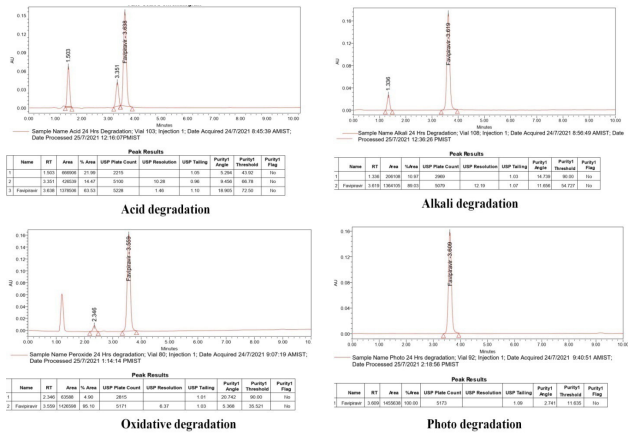


Figure 4: Chromatograms of various degradation conditions.

20 µg/mL of Favipiravir solution was prepared from above resultant solution by making dilution. Similarly, neutral degradation was performed by mixing 10mL stock solution with 10 mL of water and sonicate for 10 minutes. After 24 hours, 20 µg/mL of Favipiravir solution was prepared from the above resultant solution by making dilution. The %degradation of favipiravir in all the degradation solutions was assessed for 24 hours with an interval timing of 6 hours.

Assay of Marketed Dosage Forms

Assay of the Favipiravir in the marketed formulation was assessed by analyzing standard and sample solutions of 20 µg/mL of favipiravir one after another.

Table 5: Precision results of Favipiravir

Concentration	*Injection No.*	*Peak area*	*%Assay*
20 µg/mL	1	1920847	101.6
	2	1867995	98.8
	3	1893999	100.1
	4	1907151	100.8
	5	1913482	101.2
	6	1902710	100.6
	MEAN	1901031	100.5
	SD	18594.63	0.985
	% RSD	0.98	0.98

RESULTS AND DISCUSSION

Method Validation

The results mentioned in Table 2 confirmed the approach's system suitability as of ICH guidelines. The tailing factor, plate count and % RSD values were assessed to be ≤2, ≥2000, ≤2, respectively.

The R^2 value accomplished for linearity concentration ranges from 2 to 30 µg/mL of Favipiravir was 0.999 (Table 3 and Figure 3). The Mean %recovery of favipiravir in various spiked solutions (50, 100, and 150%) observed to be 100% ± 2 limits (Table 4). Which remarkably reveals the linearity and accuracy of the created method. The %RSD of repeated samplings of 100% level favipiravir solution in both precisions were computed to be ≤ 2 (Table 5). No Interference has been

Table 6: Results of robustness of Favipiravir

Variation of parameter		*Favipiravir (n = 3)*			
		Peak area (Mean ± SD)	*%RSD*	*Plate count*	*Tailing*
Mobile phase ratio (± 1)	41:59	2136563.3 ± 2744.2	0.13	6843.6	1.11
	61:39	2147810 ± 133330	0.62	6840	1.12
Flow rate (± 0.1 mL)	0.9 mL	2275929 ± 5048.0	0.22	5650	1.09
	1.1 mL	2274250 ± 3961.0	0.17	562	1.09
Wavelength (± 2 nm)	227	1629862 ± 3134.6	0.19	5511	1.07
	223	1632475 ± 588.27	0.03	5554.6	1.09

Table 7: %Degradation of favipiravir in various forced degradation conditions

	%degradation						
	Acid	*Alkali*	*Oxidative*	*Photo*	*Thermal*	*Reduction*	*Neutral*
Control	0.4	0.4	0.4	0.4	0.4	0.4	0.4
0	2.9	3.4	2.4	3	3.4	2.1	2.1
6	4.3	4.5	3.6	4.9	5	4.3	4.4
12	9.6	9.2	8.8	9	11.7	8.4	8.4
18	16.2	16.6	14.3	16.7	17	16.1	15
24	26.7	27.9	24.6	23	25.1	26.1	24.2

Table 8: %Assay of the marketed tablets of Favipiravir

Drug name	*Solution*	*RT (Min.)*	*Peak Area*	*Tailing*	*Plate count*	*Assay ± SD (%w/w) (n=6)*	*%RSD*
Favipiravir	Standard	3.714	1900654	5540	1.09	98.4 ± 0.45	0.46
	Test	3.717	1897351	5468	1.12		

Tablet average weight - 247mg, Label claim: 200 mg

found at RT of favipiravir in all the above-stated solutions in the specificity procedure represents the high specificity of the current approach towards the analysis of favipiravir. The LoD and LoQ were assessed to be 0.024 and 0.084 µg/mL for Favipiravir, which illustrates the sensitivity of the projected method. No significant changes have been seen in system suitability results by intentional alteration of method parameters (Table 6).

Degradation Studies

From the mentioned chromatograms and their parameters data (Figure 4, Table 7), favipiravir's degradation was around 20%. From the FD studies it was noticed that the % degradation of favipiravir was high in acidic (26.7%), alkali (27.9%) and peroxide (24.6%) degradation studies as compared with remaining stress conditions. The peak purity of the obtained degradants and favipiravir were high due to the higher values of purity thresholds than the purity angles. Hence, the stated method consider to be stability representing to evaluate the stability of favipiravir API and tablet form.

The reported RP-HPLC methods had longer RT (7.5 and 4.6 minutes) and were less sensitive. Few of the reported methods were not stability representing. In the anticipated method, shorter RT for Favipiravir (3.7 minutes) was observed with high sensitivity (0.024 µg/mL LoD and 0.084 µg/mL LoQ). FD studies were conducted by using the stated approach to assess the stability of the favipiravir.

CONCLUSION

An effective, economical, sensitive, precise and simple RP-LC procedure with higher responsiveness has been developed for the study of Favipiravir in pure powder and its tablet forms. Investigation of Favipiravir under a variety of FD environments confirms the stability indicating the quality of the process. The expected method competently separated Favipiravir and possible degradants with acceptable resolution making the method specific. The proposed method has a shorter RT for Favipiravir. Hence, the projected method has considerable recognition in the pharmaceutical sector.

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AUTHORS' CONTRIBUTIONS

All the authors were involved equally in all stages of the research work and manuscript preparation.

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