Method Development and Validation of An Analytical Method for Determination of Favipiravir in Pure Form and Marketed Pharmaceutical Dosage Form by RP-HPLC

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ABSTRACT

A novel, the simple, specific, accurate, precise method was developed and validated for the estimation of Favipiravir by RP-HPLC in bulk and marketed formulation. A High-Performance Liquid Chromatography WATERS Alliance 2695 separation module, Software: Empower 2, 996 PDA Detector with Inertsil ODS C18 (4.6mm x 250mm, 5μm) column, with mobile phase composition of Methanol: Acetate Buffer (pH-4.2) (40:60% v/v) was used. The flow rate of 1.0 ml min^{-1} and effluent was detected at 323 nm. The retention time of Favipiravir was 3.388 minutes. Linearity was observed over a concentration range of 60-140 ng ml^{-1}. The Limit of detection and limit of quantification was found to be 1.5 ng ml^{-1} and 4.5 ng ml^{-1} respectively. The accuracy of the proposed method was determined by recovery studies and found to be 98% to 102%. Then a method was validated in terms of linearity, accuracy, precision, (repeatability, intermediate precision) specificity (by assay), robustness, and system suitability. Thus, the validated method is can be successfully applied to routine analysis for regulating the quality. It also should be used for analytical research purposes.

INTRODUCTION

Coronavirus disease (COVID-19): CO' stands for corona, 'VI' for virus, and 'D' for disease. Formerly, this disease was referred to as '2019 novel coronavirus or '2019-nCoV.' The COVID-19 virus is a new virus linked to the same family of viruses as Severe Acute Respiratory Syndrome (SARS) and some types of the common cold. A highly contagious respiratory disease caused by the SARS-CoV-2 virus. SARS-CoV-2 is thought to spread from person to person through droplets released when an infected person coughs, sneezes, or talks. It may also be spread by touching a surface with the virus on it and then touching one's mouth, nose, or eyes, but this is less common. The most common signs and symptoms of COVID-19 are fever, cough, and trouble breathing. Fatigue, muscle pain, chills, headache, sore throat, runny nose, nausea or vomiting, diarrhea, and a loss of taste or smell may also occur. The signs and symptoms may be mild or severe and usually appear 2 to 14 days after exposure to the SARS-CoV-2 virus. Some people may not have any symptoms but are still able to spread the virus. Most people with COVID-19 recover without needing special treatment. But other people are at higher risk of serious illness. Those at higher risk include older adults and people with serious medical problems, such as heart, lung, or kidney disease, diabetes, cancer, or a weak immune system. Serious illnesses may include life-threatening pneumonia and organ failure. Research is being done to treat COVID-19.

Favipiravir (6-fluoro-3-hydroxy-2-pyrazine carboxamide) is an anti-viral agent that selectively and potently inhibits the RNA-dependent RNA polymerase (RdRp) of RNA viruses. Favipiravir was discovered through screening chemical library for anti-viral activity against the influenza virus by Toyama Chemical Co., Ltd. Favipiravir undergoes an intracellular phosphoribosylation to be an active form, favipiravir-RTP (favipiravir ribofuranosyl-5'-triphosphate), which is recognized as a substrate by RdRp, and inhibits the RNA polymerase activity. Since the catalytic domain of RdRp is conserved among various types of RNA viruses, this mechanism of action underpins a broader spectrum of anti-viral activities of favipiravir. Favipiravir is effective against a wide range of types and subtypes of influenza viruses, including strains resistant to existing anti-influenza drugs. Of note is that favipiravir shows anti-viral activities against other RNA viruses such as arenaviruses, bunya viruses, and filo viruses, all of which are known to cause fatal hemorrhagic fever. These unique anti-viral profiles will make favipiravir a potentially promising drug for specifically untreatable RNA viral infections.
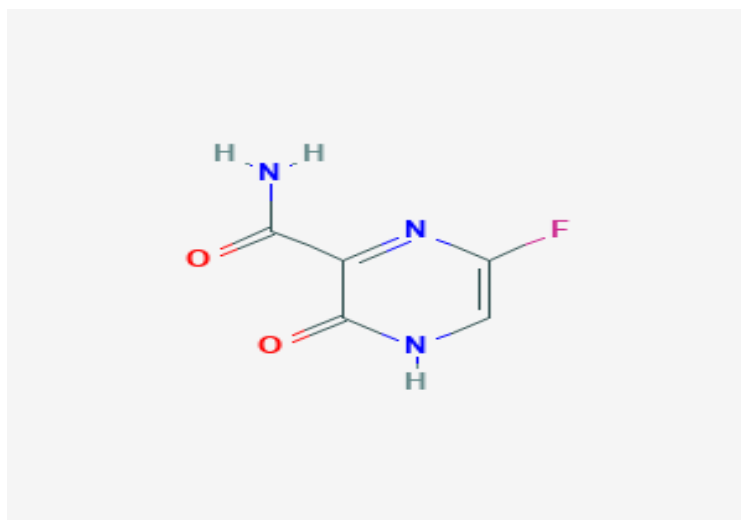
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Figure No. 1: Favipravir

Molecular Formula: $C_5H_4FN_3O_2$

Molecular Weight: 157.10 gm/mol.

Melting Point: 187^0C to 193^0C

Solubility: Slightly soluble in water

pKa: 5.1

Table No. 1: Marketed Drug Formulation

Drug Name	Brand Name	Dose	Manufacturer
Favipiravir	FluGuard[400]	400mg	Sun Pharma
Favipiravir	Fabiflu	400mg	Glenmark

MATERIALS AND METHODS

Instruments used:

The liquid chromatographic system used was WATERS, software: Empower 2, Alliance 2695 separation module 996 PDA detectors, Inertsil-C18 ODS (250 x 4.6 mm, 5 µ) column, P^H meter Lab India, Ultrasonicator-Denver.

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Chemicals used:

A gift sample of favipiravir was procured from Sura Labs, Hyderabad, HPLC grade water, methanol, acetonitrile, and Potassium dihydrogen phosphate were purchased from MERCK laboratories, Mumbai.

Method Development

Preparation of standard solution:

10 mg of Favipiravir working standard was accurately weighed and transferred into 10ml of clean dry volumetric flasks, about 7ml of methanol is added, sonicated, and volume was made up to the mark with Methanol. Further 1ml of the above Favipiravir stock solution was pipetted into a 10ml volumetric flask and diluted up to the mark with methanol.

Preparation of Sample Solution:

The equivalent weight of one tablet was crushed and 10 mg equivalent weight of favipiravir was transferred into a 10ml clean dry volumetric flasks. 7mL of diluent was added and sonicated to dissolve it completely. The volume was made up to the mark with the same solvent.

Mobile Phase Optimization:

Initially, the mobile phase tried was methanol: water with varying proportions. Finally, the mobile phase was optimized to Methanol and Acetate Buffer in proportion 40:60 v/v respectively.

Optimization of Column:

The method was performed with various C18 columns like the ODS column, Xterra, and AltimaC18 column. Inertsil ODS C18 (4.6mm x 250mm, 5μm) was found to be ideal as it gave good peak symmetry and resolution at 1.0ml/min flow.

Method development was initiated using liquid chromatographic system WATERS, software: Empower 2, Alliance 2695 separation module. 996 PDA detectors, Inertsil-C18 ODS (250 x 4.6 mm, 5 μ) column. Initially varied concentrations of methanol: water were used and was finally optimized by methanol and acetate buffer in the ratio of 40:60 v/v, Column Inertsil - ODS C_{18} (250 x 4.6 mm, 5μ), flow rate 1 ml/min, detection wavelength at 323 nm. The

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retention time of favipiravir in the optimized chromatogram was found to be 3.338 min. The Chromatogram is shown in **figure-2.** The developed method was validated for specificity, accuracy, precision, linearity, LOD & LOQ as per the ICH guidelines.

Method Validation:

System suitability: A Standard solution was prepared by using Curcumin and Piperine working standards as per the test method and was injected in replicates five times into the HPLC system. The system suitability parameters like theoretical plates, tailing factor, resolution were evaluated from standard chromatograms.

The standard and sample solutions were injected five times and peak areas of injections were measured in HPLC. The % RSD for the area of five replicate injections was found to be within the specified limits. The results were given in table-2.

Three replicate injections of standard and sample solutions were injected and the assay was calculated by using the formula:

% ASSAY =

$$\frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

Linearity: A Series of solutions were prepared using the favipiravir working standard at concentration levels from 60ppm to 140 ppm of the target concentration. Each sample solution was injected into the HPLC system in replicates and the peak areas were measured. A graph was plotted with peak areas vs concentrations and the r2 value was calculated. The results were shown in table – 3.

Accuracy: The accuracy of the newly developed method was evaluated by recovery studies at three different levels equivalent to 50,100&150%. At each level, the target concentration was spiked in triplicates and the amount recovered was calculated the percentage recovery at each level was calculated and reported in table-4.

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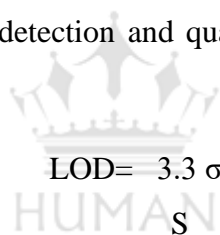
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Precision:

Repeatability:

The standard solution was injected five times and the peak area for all five injections was measured. The % RSD for the area of five replicate injections was found to be within the specified limits. The results were given in table -5.

Intermediate precision:

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different days by maintaining the same conditions. The standard solution was injected six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits. The results were given in table –6.

Limit of Detection and Limit of Quantification:

From the linearity data, the limit of detection and quantification were calculated using the following formulae.

$$LOD = \frac{3.3\,\sigma}{S}$$

σ = standard deviation of the response

S = slope of the calibration curve of the analyte

$$LOQ = \frac{10\,\sigma}{S}$$

The values were given in table -

Robustness: A study was conducted to determine the effect of variation in flow rate, change in mobile phase composition, and detection of wavelength. A standard solution prepared as per the test method was injected into the HPLC system using flow rates, 1.0 ml/min and1.2 ml/min. The same studies were also performed by varying mobile phase composition and detection wavelength. The system suitability parameters were evaluated and reported in table 7.

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RESULTS AND DISCUSSION

Method development:

Optimized Chromatogram (Standard)

Mobile phase ratio   : Methanol: Acetate Buffer (pH-4.2) (40:60 v/v)

Column   : Inertsil ODS C18 (4.6mm x 250mm, 5μm)

Column temperature   : 35ºC

Wavelength   : 323 nm

Flow rate   : 1.0 ml/min

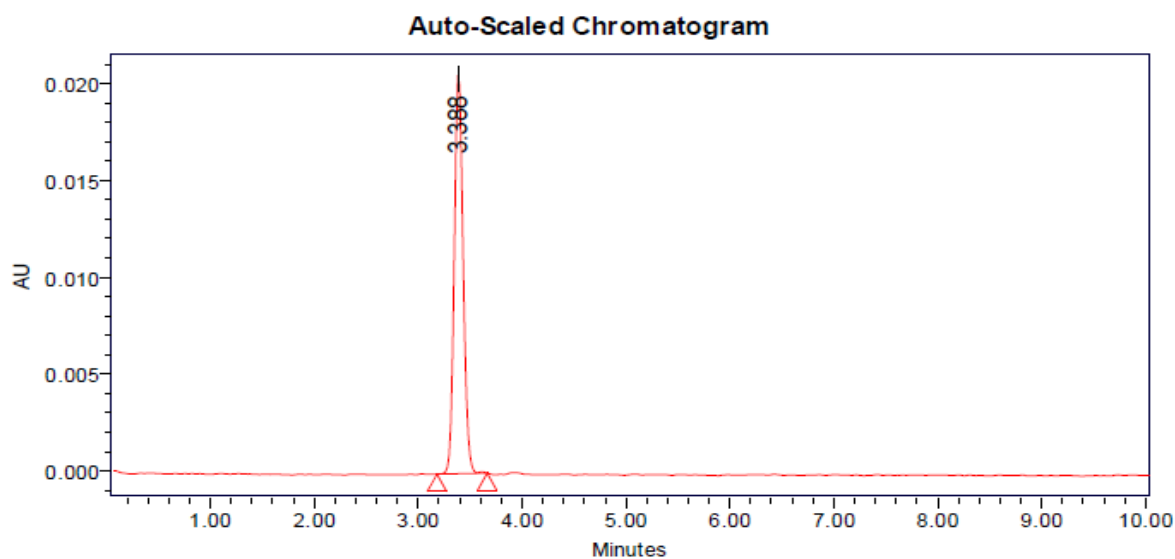Injection volume   : 10 μL

Run time   : 10 min

Figure No. 2: Optimized Chromatogram (Standard)

Table No. 2: Data of optimized chromatogram

S. No.	Name	RT	Area	Height	USP Tailing	USP Plate Count
1	Favipiravir	3.388	145867	32546	1.76	8457

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Method Validation:

System suitability: The theoretical plates are more than 2000 and the tailing factor is less than 2 in each injection for both the analytes. The values were within the acceptance criteria.

Table No. 3: Results of System Suitability for Favipiravir

S. No	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Favipiravir	3.398	145965	32653	8475	1.78
2	Favipiravir	3.324	146857	32785	8495	1.79
3	Favipiravir	3.349	145985	32598	8492	1.80
4	Favipiravir	3.388	146697	32695	8463	1.76
5	Favipiravir	3.364	145982	32675	8458	1.77
Mean			146380.25			
Std. Dev.			462.762			
% RSD			0.316137			

Linearity: A graph was plotted with peak areas vs concentration and the correlation coefficient was calculated. The r^2 values were found to be 0.999 which was within the limits confirms the linearity of the method.

Table No. 4: Linearity data of Favipiravir

Concentration µg/ml	Average Peak Area	Statistical Analysis	
60	85784	Slope	1358
80	112564	y-Intercept	2288
100	139867	Correlation Coefficient	0.999
120	165248		
140	189586		

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Accuracy: Three target concentrations 50%, 100%, 150% were prepared concerning target assay and injected into the HPLC system in triplicates. At each spike level, the mean recovery values are between 98 to 102 % which were in agreement with the acceptance criteria. The recovery values indicate the method is accurate.

Table No. 5: Accuracy data for Favipiravir

% Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	70031.67	50	49.884	99.768%	
100%	138413.33	100	100.239	100.239%	99.86%
150%	205138	150	149.374	99.582%	

Precision: Repeatability was performed in five replicate injections and the % RSD of the peak areas was calculated. The % RSD for the peak areas of five standard injections was found to be0.183536which was within the limits.

Intermediate precision was also performed on two different days the % RSD for the peak areas of six standard injections was found to be 0.119311 which was in agreement with acceptance criteria.

Table No. 6: Data of repeatability for Favipiravir

S. No.	Peak name	Retention time	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Favipiravir	3.397	145865	32652	8547	1.78
2	Favipiravir	3.390	145874	32541	8498	1.78
3	Favipiravir	3.384	145842	32564	8547	1.77
4	Favipiravir	3.378	145869	32548	8572	1.77
5	Favipiravir	3.364	145265	32569	8569	1.78
Mean			145743			
Std.dev			267.4911			
%RSD			0.183536			

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Table No. 7: Data of Intermediate precision for Favipiravir

S. No	Peak Name	RT	Area (μV*sec)	Height (μV)	USP Plate Count	USP Tailing
1	Favipiravir	3.371	147856	32685	8569	1.79
2	Favipiravir	3.376	147584	32654	8574	1.79
3	Favipiravir	3.382	147965	32685	8654	1.78
4	Favipiravir	3.359	147523	32654	8542	1.79
5	Favipiravir	3.333	147854	32689	8571	1.78
6	Favipiravir	3.341	147856	32784	8534	1.79
Mean			147773			
Std. Dev.			176.3088			
% RSD			0.119311			

Specificity: There is no interference observed in the blank. The chromatograms of Standard and Sample were identical with the same retention time.
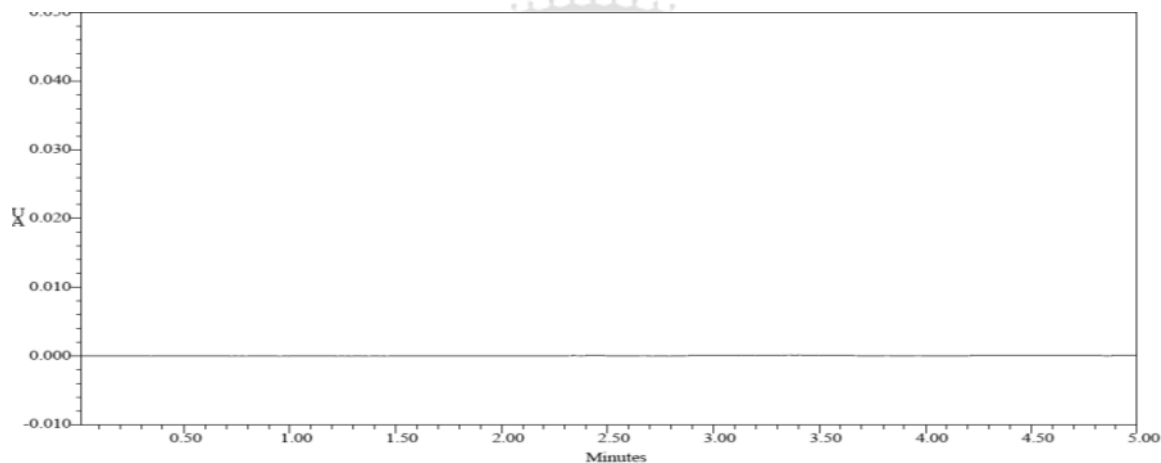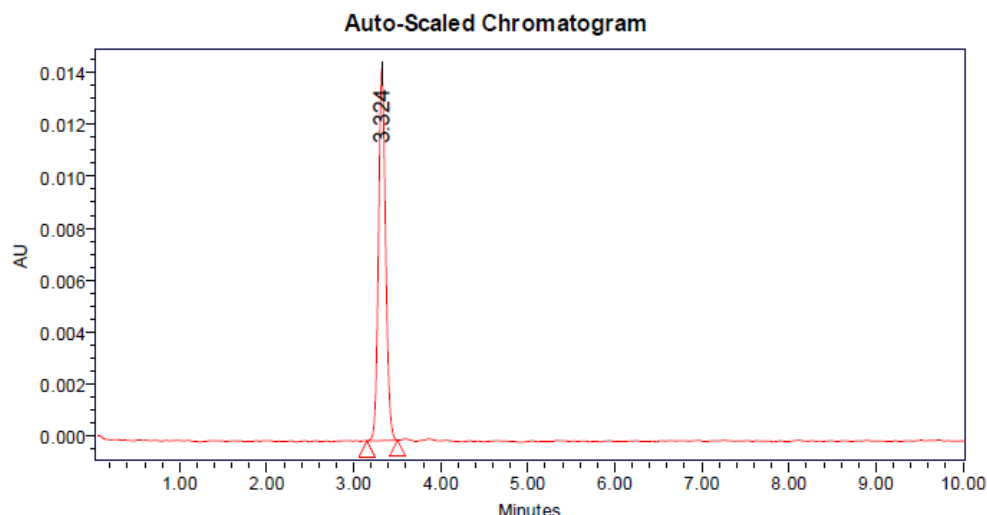

Figure No. 3: Chromatogram of blank

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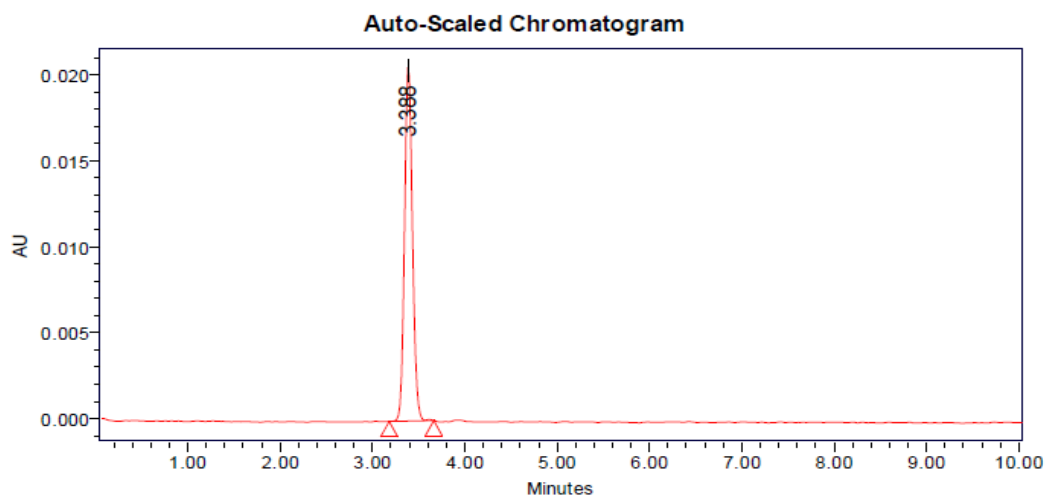
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Figure No. 4: Chromatogram of standard

Figure No. 5: Chromatogram of sample

Limit of Detection and Quantitation (LOD and LOQ): The LOD and LOQ of favipiravir were found to be 1.54μg/ml and 4.56μg/ml respectively. The results indicate that the method was sensitive.

Robustness: The robustness was performed for the flow rate variations from 0.9 ml/min to 1.1ml/min and mobile phase ratio variation from the more organic phase to less organic phase ratio for Favipiravir. The method is robust only in less flow condition and the method is robust even by a change in the Mobile phase ±5%. The standard and samples of Favipiravir were injected by changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count.

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Table No. 8: Robustness data of favipiravir

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0 mL/min	145867	3.388	8457	1.76
Less Flow rate of 0.9 mL/min	146854	3.595	8152	1.74
More Flow rate of 1.1 mL/min	135262	3.122	7985	1.73
More organic phase (about 5 % Increase in Methanol)	143652	3.119	8142	1.72
Less organic phase (about 5 % decrease in Methanol)	142546	3.545	7985	1.75

CONCLUSION

A Rapid and Precise RP-HPLC method was developed and validated for the quantification of favipiravir in bulk as well as in tablet dosage form. Chromatography was carried out on Inertsil-ODS C18 (250 x 4.6mm, 5µm) column. The method was optimized using a mixture of methanol: acetate buffer (40:60) as the mobile phase at a flow rate of 1.0ml/min, the detection was carried out at 323nm. The retention time of favipiravir was found to be 3.388. The method was linear in the concentration range of 60-140µg/ml. The method was precise since the % RSD values of peak areas were found to be below " 2". The % recovery values for both the analytes were found to be "99.86" indicating the method was accurate. The specificity of the method was assessed by injections of standard, sample, and blank solutions separately and the chromatograms were recovered. The LOD& LOQ values were 1.5 and 4.5 µg/ml respectively. There are very few methods reported on the estimation of favipiravir. Therefore it was contemplated to develop a simple RP-HPLC method for the routine analysis of favipiravir. The results of validation were in agreement with acceptance criteria. This indicates that the method is suitable and can be adopted for regular quality control analysis.

ACKNOWLEDGEMENT

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REFERENCES

1. https://pubchem.ncbi.nlm.nih.gov/compound/Favipiravir#section=Structureshttps://pubchem.ncbi.nlm.nih.gov/compound/Favipiravir#section=Structureshttps://pubchem.ncbi.nlm.nih.gov/compound/Favipiravir#section=Structures.

2. Lingabathula, S., & Jain, N, Stability Indicative And Cost Effective Analytical Method Development And Validation Of Favipiravir And Peramivir In Bulk And Pharmaceutical Dosage Form By Using RP-HPLC. *International Journal of Applied Pharmaceutics*: (2021):*13*(4), 265-271.

3. Ibraam E. Mikhail, Heba Elmansi, Fathalla Belal, Adel Ehab Ibrahim, Green micellar solvent-free HPLC and spectrofluorimetric determination of favipiravir as one of COVID-19 antiviral regimens; Microchemical Journal:2021:165:106-129.

4. Mamdouh R. Rezk, Kamal A. Badr, Naglaa S. Abdel-Naby, Magy M. Ayyad, A novel, rapid and simple UPLC–MS/MS method for quantification of favipiravir in human plasma: Application to a bioequivalence study; Biomedical Chromatography:2021:35(7):1125-1138.

5. *Ibrahim Bulduk,* HPLC-UV method for quantification of favipiravir in pharmaceutical formulations; Acta Chromatographica:2021:209-215.

6. Mohammad Hailat1, Israa Al-Ani 2, Mohammed Hamad 3 , Zainab Zakareia 4 and Wael Abu Dayyih, Development and Validation of a Method for Quantification of Favipiravir as COVID-19 Management in Spiked Human Plasma; Molecules : 2021:26:1-11.

7. Maria A. Acquavia, Luca Foti, Raffaella Pascale, Antonia Nicolò, Vincenzo Brancaleone, Tommaso R.I. Cataldi, Giuseppe Martelli, Laura Scrano, and Giuliana Bianco, Detection and quantification of Covid-19 antiviral drugs in biological fluids and tissues; Talanta.: 2021; 224: 232.

8. Zeshan Aqeel, J Preston, Phil Koerner, HPLC Column Screening for Favipiravir on Six Different 100 x 4.6 mm Columns; Phenomenex, Inc. :2020:1-2.

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