RESEARCH ARTICLE

Stability Indicating RP-HPLC Method Development and Validation for Quantification of Favipiravir and its Related Substances

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ABSTRACT

Background: Favipiravir is an influenza antiviral medication. DEM Avigan is the commercial name for a pyrazine carboxamide derivative.

Aim: According to the guidelines framed by ICH - International Council for Harmonisation, the present method was developed and validated for the system suitability, specificity, linearity, precision, limit of quantification and limit of detection, accuracy, & robustness.

Results: All the results obtained were within the acceptable range. This method was used to better separate the peaks of Favipiravir and its three impurities. The retention times and squared correlation coefficient values of Impurity-1, Impurity-2, Favipiravir & Impurity-3 were obtained at 1.998, 3.223, 4.438 & 7.052 min and 0.9993, 0.9995, 0.9998 & 0.9997 respectively.

Conclusion: Thus, the results show that the suggested RP-HPLC method for separating three contaminants with Favipiravir was effective and may be utilised in routine pharmaceutical analysis and quality control. Forced degradation was carried out under different stress conditions thus proving as stability indicating as per ICH guidelines.

Keywords: Favipiravir, Related substances & RP-HPLC.

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INTRODUCTION

Favipiravir is an influenza antiviral medication. 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.[1-2] It was first approved by Toyama Chemical Co., Limited, Japan for treating resistant influenza cases. Favipiravir is being tested therapeutically for Lassa, Ebola & COVID-19. 5-fluoro-2-oxo-1H-pyrazine-3-carboxamide is the IUPAC name and $C_5H_4FN_3O_2$ is its chemical formula with a molecular weight of 157.104g/mol. The structure is depicted in figure 1. The IUPAC names of favipiravir impurity 1, impurity 2 & impurity 3 are 6-Fluoro-3-hydroxypyrazine-2-carboxylic acid, 5-Fluoropyrazin-2(1H)-one & 6-Fluoro-3,4-dihydro-4-hydroxy-3-oxopyridine-2-carboxamide respectively with molecular weights 158.1, 114.1 & 172.1 g/mol and the chemical formulae were represented as $C_5H_3FN_2O_3$, $C_4H_3FN_2O$ & $C_6H_5FN_2O_3$, respectively. The structures are shown in Figures 2-4 for the impurities. The drug is soluble in dimethyl sulfoxide & slightly water soluble. Favipiravir is basically the prodrug that passes via intracellular phosphorylation and ribosylation that later converts into active Favipiravir-ribofuranosyl-50-triphosphate (RTP). This complex binds to RNA and lead to inhibition of RNA-dependent RNA polymerase (RdRp) thus preventing the viral genome from being transcribed and replicated. According to studies, combining Favipiravir-RTP to the nascent strand of RNA and lowers the elongation & viral growth of RNA strand.[3-4] Toyama Chemical (a Fujifilm subsidiary) developed and manufactured it, and it received medical approval from Japan in 2014.[5] Fujifilm licensed it to Zhejiang Hisun Pharmaceutical Co.

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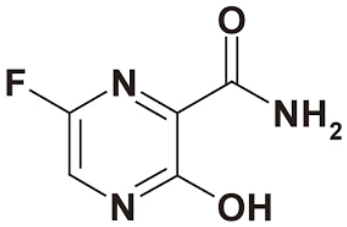

Figure 1: Favipiravir – Chemical structure

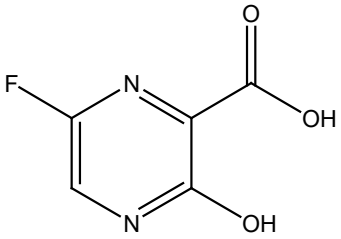

Figure 2: Impurity-1 – Chemical structure

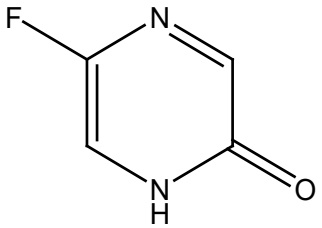

Figure 3: Impurity-2 – Chemical structure

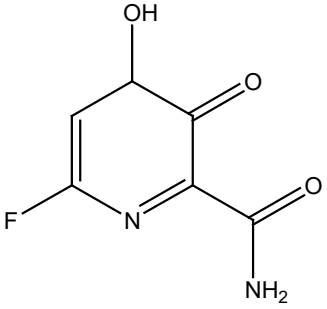

Figure 4: Impurity-3 – Chemical structure

in 2016.[6] Later it was converted into a generic drug in 2019.

Favipiravir is an antiviral medication that was allowed for the treatment of COVID-19 in numerous countries, including Russia, Japan, Turkey, Serbia& Thailand in crucial circumstances.[7-9] A fast meta-review of four trials done in September 2020 found that the drug has an improved radiological & clinical outcomes; nevertheless, no alterations in oxygen support accessories and mortality reduction were seen, looking forward for further robust clinical findings.[10-12]

According to a Cochrane Systematic review published in February 2024, there is no real benefit to using Favipiravir to treat COVID-19 in terms of mortality, admission to mechanical ventilation, or hospitalization, and it may not make a difference in adverse effects or serious adverse effects.[13] Clinical studies for big cohorts are under conducted as of May 2021. Favipiravir causes viral spread after 7 days and later leading to clinical improvement after 14 days. The results confirmed that Favipiravir has a high potential to treat COVID-19, especially for the people suffering with mild to severe illness. Further well-designed trials, including dosage and treatment duration assessments, are required to achieve definitive results.[14,15]

An extensive literature study reported a few analytical methods were validated for Favipiravir estimation along with its related substances by RP-HPLC.[16-26] The primary goal of this study was to develop & validate an RP-HPLC – Reverse phase High performance Liquid chromatographic method confined for separating and quantifying Favipiravir and its related substances in a single run. The method will be simple, accurate and economic with less run time which is to be engaged in routine quality control tests. The established method was moved for validation with the reference of ICH guidelines and used for routine quantitative analysis.[27]

MATERIALS AND METHODS

Chemicals and Solvents

Acetonitrile of HPLC grade was procured from Rankem, Haryana, India. Water used in this analysis was prepared with Millipore Milli Q Water purification system was employed. Analytical grade chemicals – Hydrochloric acid, 20% Hydrogen peroxide, Sodium hydroxide, Orthophosphoric acid. Methanol & formic acid were bought from E Merck Ltd. Mumbai, India.

Reference Drugs

The References samples were provided as a gift sample from Shree Icon Pharma laboratory, Vijayawada.

Instrumentation

Waters HPLC e 2695 module with 1525 HPLC binary pump Waters- 2998 detector Photo diode array, & Waters-2707 auto sampler. Data acquisition was done with Empower software. Electronic balance Sartorius was used for weighing the drugs. Digital pH meter make by Mestar company was used for all pH measurements. An ultrasonic bath from Unichrome was employed for sonicating the solutions. Hot air oven for thermal degradation 23000 model Ultraviolet chamber with a UV florescent lamp of 200 to 300 nm range was used for photodegradation studies.

Preparation of Solutions

Diluent

HPLC grade acetonitile and water were mixed in a ratio 50:50 and sonicated for 10 minutes.

Standard stock solution of Favipiravir

About 50 mg of favipiravir was taken into a volumetric flask of 10 and 7 mL of diluent was added and later subjected to sonication for 10 minutes. The volume was finally made up to mark with the diluent.

Favipiravir Sample stock solution

About 20 tablets of favipiravir (Famiflu-200) were powdered. 97.3 mg of powdered sample equivalent to 50 mg of the drug

was taken volumetric flask and diluent measuring 7 mL was mixed to it. Later, sonication was done till 30 minutes and volume was made upto the mark with the diluent.

Stock Solution of Impurity-A

About 5 mg of favipiravir impurity-1, impurity-2 was weighed accurately into 10 mL of volumetric flask and 7 mL of the diluent was added, sonictaed till 30 minutes. Final volume was prepared till marked using diluent.

Stock solution of Impurity-B

About 5 mg of favipiravir impurity-3 was weighed accurately to 10 mL of volumetric flask along with 7 mL of diluent. Later, the solution was subjected to sonication for 30 minutes and the volume was filled to the mark with diluent.

Impurity Stock Solution

About 1-mL stock solution of impurity-A & 3 mL stock solution of impurity-B was taken into a 10 mL volumetric flask & sonicated to 30 minutes, and final volume was added till the mark with diluent.

Spiked Standard Solution of Mixture

Transferred 5 mL of favipiravir standard stock & 5 mL impurity stock into a 50 mL volumetric flask & final volume was filled with diluent and further filtered with 0.45 µ filter paper.

Spiked Sample sSolution

Transferred 5 mL of favipiravir sample stock & 5 mL of impurity stock into a volumetric flask measuring 50 mL and finally, the volume was made marked using the same diluent. The solution was then filtered through 0.45 µ filter paper.

RESULTS AND DISCUSSION

Method Optimization

Method was optimized by performing various trial runs in HPLC. The trials performed were tabulated in Table 1.

Validation of the Developed Method

The optimized method was validated for below-mentioned parameters according to ICH [27] guidelines.

System suitability

This parameter ensures the working performance of an analytical system.

DISCUSSION

The plate count must be >2000, the tailing factor <2, and the resolution >2 as per ICH guidelines. The above results conferred that all the system parameters were within the acceptance limits.

Specificity

DISCUSSION

Interfering peaks were not seen in blank as well as in placebo at respective retention times indicating the developed method as specific.

Linearity

Table 1: Summary of chromatographic trial runs

Buffer	*Mobile phase*	*Column*	*Flow (mL min-1)*	*Diluent*	*Observation*
Water	Buffer + ACN 20+80	Waters- X-Bridge C_{18} (150 × 4.6 mm, 3.5 µ)	1.0	ACN + water (50:50)	Peaks were not separated
Water	Buffer + ACN 30+70	Waters- X-Bridge C_{18} (150 × 4.6 mm, 3.5 µ)	1.0	ACN + water (50:50)	Four peaks were eluted
Water	Buffer + ACN 30+70	Symmetry- C_{18} (150 × 4.6 mm, 3.5 µ)	1.0	ACN + water (50:50)	Peaks were not separated
Water	T/%B; 0/30, 10/50, 12/30, 17/30	Symmetry- C_{18} (150 × 4.6 mm, 3.5 µ)	0.5	ACN + water (50:50)	Peaks were not separated
Water	T/%B; 0/30, 10/50, 12/30, 17/30	Symmetry- C_{18} (150 × 4.6 mm, 3.5 µ)	0.5	ACN + water (50:50)	Less resolution
0.1%OPA	T/%B; 0/30, 2/60, 6/30, 9/30	Symmetry- C_{18} (150 × 4.6 mm, 3.5 µ)	0.5	ACN + water (50:50)	Peak shapes were not good
0.1%OPA	T/%B; 0/10, 7/50, 10/20, 14/20	Symmetry- C_{18} (150 × 4.6 mm, 3.5 µ)	0.5	ACN + water (50:50)	Fourth peak shape was not good
0.1%OPA	T/%B; 0/20, 10/70, 12/20, 18/20	Symmetry- C_{18} (150 × 4.6 mm, 3.5 µ)	0.5	ACN + water (50:50)	Fourth peak was not eluted
0.1%OPA	T/%B; 0/5, 5/30,10/50, 12/5, 18/5	Symmetry- C_{18} (150 × 4.6 mm, 3.5 µ)	0.5	ACN + water (50:50)	Less resoluion between impurity peaks
0.1%OPA	T/%B; 0/5, 4/70, 5/5, 7/5	Waters Phenyl Hexyl 150 × 4.6 mm, 3.5µm	0.5	ACN + water (50:50)	Four peaks were eluted
0.1%OPA	T/%B; 0/80,5/80, 10/20, 12/80, 18/80	Waters Phenyl Hexyl 150 × 4.6 mm, 3.5 µm	0.5	ACN + water (50:50)	This method were suitable for validation

*ACN: Acetonitrile, OPA: Orthophosphoric acid

Table 2: System suitability data of favipiravir

S. No.	*Parameter*	*Favipiravir*
1	Retention Time	4.429
2	Plate Count	52651
3	Tailing Factor	1.14
4	Resolution	3.67
5	%RSD	1.07

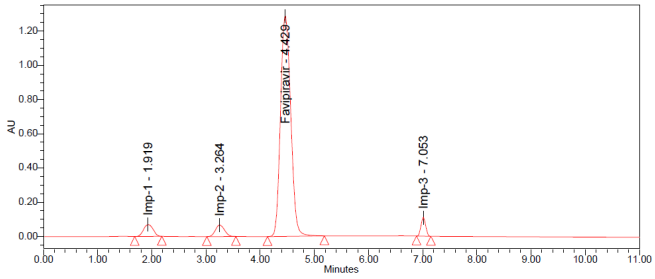

Figure 5: Chromatogram for standard solution

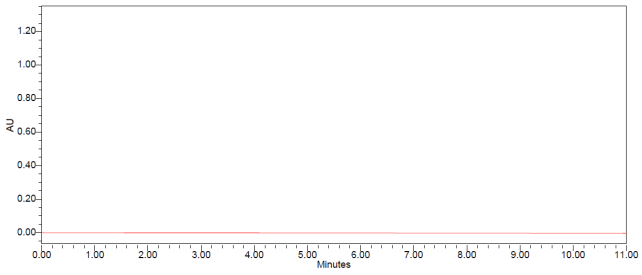

Figure 6: Blank chromatogram

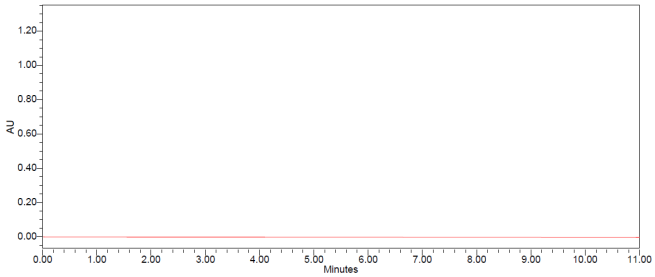

Figure 7: Placebo chromatogram

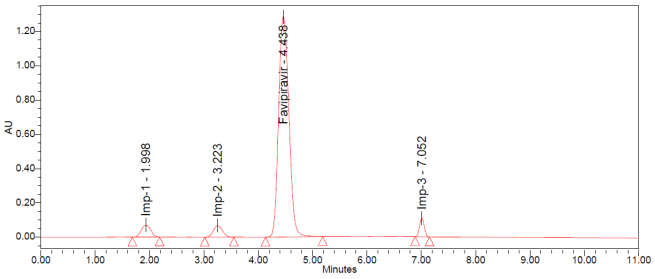

Figure 8: Optimized chromatogram

Precision

a) System precision:

b) Repeatability

Accuracy

The sample solutions were prepared at three levels using standard addition method. Infusions of injections were done in triplicate at each level.

Table 3: Results of Linearity

S. No	*Favipiravir*		*Imp-1*		*Imp-2*		*Imp-3*	
	Conc. in µg/mL	*Area*	*Conc. in µg/mL*	*Area*	*Conc. in µg/mL*	*Area*	*Conc. in µg/mL*	*Area*
1	50.00	1675421	0.50	88541	0.50	75402	1.50	64201
2	125.00	4206514	1.25	220320	1.25	185274	3.75	178549
3	250.00	8598754	2.50	422621	2.50	375402	7.50	336529
4	375.00	12720561	3.75	620218	3.75	546528	11.25	498652
5	500.00	16502364	5.00	842145	5.00	772547	15.00	652341
6	625.00	20874157	6.25	1038651	6.25	918248	18.75	805204
7	750.00	24889585	7.50	1194156	7.50	1107814	22.50	975325
Slope	33171.98		161512.23		148238.47		43003.07	
Intercept	91539.92		13274.97		1979.49		7475.54	
R^2	0.9998		0.9993		0.9995		0.9997	

Discussion: As the coefficient of correlation is not less than 0.999, the proposed method was linear.

Table 4: System precision data

S. No.	*Favipiravir*	*Impurity-1*	*Impurity-2*	*Impurity-3*
	Conc. (500 µg/mL) Area	*Conc. (5 µg/mL) Area*	*Conc. (5 µg/mL) Area*	*Conc. (15 µg/mL) Area*
Mean (n = 6)	16491312	846486	773622	651082
S.D	177053.770	3921.792	2113.459	937.676
%RSD	1.07	0.46	0.27	0.14

Table 5: Data of method precision

S. No	*Favipiravir area*	*Imp-1 area*	*Imp-2 area*	*Imp-3 area*
Average (n = 6)	16466312	844190	775998	654517
Std dev	186001.222	1699.161	2112.659	2537.186
%RSD	1.13	0.20	0.27	0.39

Discussion: The system precision was said to be passed as the precision limit was <2.

Table 6: Accuracy data of Favipiravir by RP-HPLC method

Amount spiked (n = 3)	*% Recovery*			
	Favipiravir	*Imp-1*	*Imp-2*	*Imp-3*
50%	99.6	100.0	100.0	99.5
100%	99.6	99.9	99.7	100.2
150%	99.9	99.9	99.9	99.0

Discussion: The obtained % Mean recovery was 99.7% for Favipiravir, 99.9% for Imp-1, 99.9% for Imp-2 and 99.6% for Imp-3 respectively.

Table 7: Robustness data of Favipiravir

Actual value	*Changed value*	*% RSD*			
		Favipiravir	*Imp-1*	*Imp-2*	*Imp-3*
1mL/min	0.8mL/min	0.13	0.03	0.05	0.08
	1.2mL/min	0.58	0.06	0.25	0.24
-	Org Minus	0.09	0.08	0.08	0.04
	Org Plus	0.51	0.06	0.07	0.1

Discussion: As there was no fluctuation seen in % RSD with the flow rate change and organic phase composition alteration, the method was called to be robust.

Table 8: Data of LoD & LoQ

Drug name	*LoD in µg/mL*	*LoQ in µg/mL*
Favipiravir	15	50
Imp-1	0.15	0.5
Imp-2	0.15	0.5
Imp-3	0.45	1.5

Table 9: Table showing degradation data of Favipiravir

Degradation parameter	*%Total impurities of favipiravir*	*Purity angle*	*Purity threshold*
Control	0.21	1.774	4.918
Acid	10.42	1.718	4.946
Alkali	10.37	1.625	4.854
Peroxide	10.66	1.726	4.924
Reduction	8.84	1.751	4.962
Thermal	6.72	1.736	4.974
Photo	5.93	1.732	4.915
Hydrolysis	0.88	1.748	4.964

Discussion: As the % of degradation is below 20. The validated method was also said as stability indicating.

Robustness

LOD and LOQ (µg/mL)

Degradation studies

The study was carried out by subjecting the solutions to different stress conditions and the analysis was carried out according to ICH [28] guidelines.

CONCLUSION

From the results obtained, it was concluded as the current developed method was simple, sensitive, reliable, accurate, precise, robust, linear over the concentrations range used. No interfering peaks were found in chromatogram runs for formulation samples. Shorter runtime inferred a good insight of analysis speed which enabled more samples in number analysed per unit time. Moreover, the method was a stability indicating one. Therefore, the method could be suggested foe the estimation of Favipiravir & its related substances in pharmaceutical formulations.

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