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Analytical Method Development and Validation for the Quantitative Estimation of Favipiravir in API Form and Marketed Pharmaceutical Dosage Form by Using RP-HPLC

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Abstract
The present work includes a simple, economic, rapid, accurate and precise isocratic RP-HPLC method development for estimation of Favipiravir in bulk form and its marketed formulation. Estimation was done at 286nm which was found to be λ max of Favipiravir. The simple, selective, isocratic RP-HPLC method for Favipiravir was developed on Phenomenex Luna (C_{18}) RP Column; 250 mm x 4.6 mm, 5µm with a mobile phase of Phosphate Buffer (pH-4.6) and Methanol were taken in the ratio of 65:35% v/v at a flow rate of 1.0 ml/min and detection wavelength 286nm. The retention time of Favipiravir was found to be 4.783min. The developed method was validated successfully according to ICH Q2 (R1) guidelines. The chromatographic methods showed a good linear response with r2 values of 0.9995. The percentage relative standard deviation for method was found to be less than two, indicating that the methods were precise. The mean percentage recovery was for RP-HPLC method was 100.437%. From the results it could be concluded that both the developed method was specific, selective and robust. The method could be successfully applied for analysis of Bulk form and Marketed formulation of Favipiravir.

Keywords: Favipiravir, RP-HPLC, Method Development, Validation, ICH Guidelines

Introduction

Favipiravir is a member of the class of pyrazines that is pyrazine substituted by aminocarbonyl, hydroxy and fluoro groups at positions 2, 3 and 6, respectively. It is an anti-viral agent that inhibits RNA-dependent RNA polymerase of several RNA viruses and is approved for the treatment of influenza in Japan [1]. It has a role as an antiviral drug, an anticoronaviral agent and an EC 2.7.7.48 (RNA-directed RNA polymerase) inhibitor. It is a primary Carboxamide, a hydroxypyrazine and an organofluorine compound. Favipiravir is a pyrazinecarboxamide derivative with activity against RNA viruses. Favipiravir is converted to the ribofuranosyl triphosphate derivative by host enzymes and selectively inhibits the influenza viral RNA-dependent RNA polymerase. In 2014, Favipiravir was approved in Japan to treat cases of influenza that were unresponsive to conventional treatment [2]. Given its efficacy at targeting several strains of influenza, it has been investigated in other countries to treat novel viruses including Ebola and most recently, COVID-19. FAVIPIRAVIR is used in the case of Corona virus disease. Favipiravir 200 mg is the first oral antiviral medication under the trade name FabiFlu for the treatment of mild to severe COVID-19 cases, Glenmark Pharmaceutical launch this medication [3]. This medicine shows 80-88% efficacy in global clinical trials and it is currently used in Japan and UAE for the Covid-19 treatment. The IUPAC Name of Favipiravir is 5-fluoro-2-oxo-1H-pyrazine-3-carboxamide. The Chemical Structure of Favipiravir is shown in following figure-1.

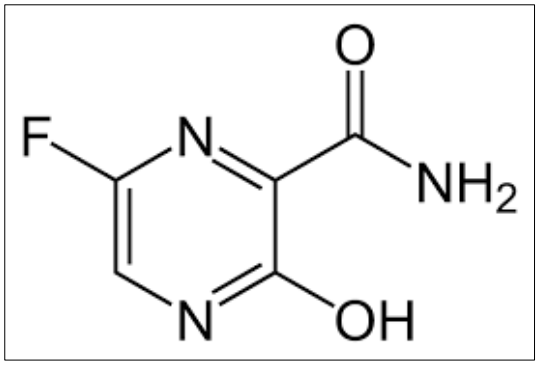

Fig 1: Chemical Structure of Favipiravir

Materials and Methods

Table 1: List of Instrument used

S. No.	Instruments/Equipments/Apparatus
1.	HPLC with Empower2 Software with Isocratic with UV-Visible Detector (Waters).
2.	ELICO SL-159 UV – Vis spectrophotometer
3.	Electronic Balance (SHIMADZU ATY224)
4.	Ultra Sonicator (Wensar wuc-2L)
5.	Thermal Oven
6.	Symmetry ODS RP C_{18},5µm, 15mm x 4.6mm i.d.
7.	P^H Analyzer (ELICO)
8.	Vacuum filtration kit (BOROSIL)

Table 2: List of Chemicals used

S.No.	Name	Specifications		Manufacturer/Supplier
		Purity	Grade	
1.	Doubled distilled water	99.9%	HPLC	Sd fine-Chem ltd; Mumbai
2.	HPLC Grade Water	99.9%	HPLC	Sd fine-Chem ltd; Mumbai
3.	Methanol	99.9%	HPLC	Loba Chem; Mumbai.
4.	Hydrochloric Acid	99.9	A.R.	Sd fine-Chem ltd; Mumbai
5.	Acetonitrile	99.9%	HPLC	Loba Chem; Mumbai.
6.	Sodium Hydroxide	99.9	A.R.	Sd fine-Chem ltd; Mumbai
7.	Ethanol	99.9	A.R.	Sd fine-Chem ltd; Mumbai
8.	Octanol	99.9	A.R.	Sd fine-Chem ltd; Mumbai

Development of analytical method

Selection of wavelength

The Standard & Sample Stock Solutions were prepared separately by dissolving standard & sample in a solvent in mobile phase diluting with the same solvent. (After optimization of all conditions) for UV analysis. It scanned in the UV spectrum in the range of 200 to 400nm. This has been performed to know the maxima of Favipiravir, so that the same wave number can be utilized in HPLC UV detector for estimating the Favipiravir [4]. The scanned UV spectrum is attached in the following page,

Sample & standard preparation for the uv-spectrophotometer analysis: 25 mg of Favipiravir standard was transferred into 25 ml volumetric flask, dissolved & make up to volume with mobile phase. Further dilution was done by transferring 1ml of the above solution into a 10ml volumetric flask and make up to volume with mobile phase [5].

Optimization of chromatographic conditions: The chromatographic conditions were optimized by different means. (Using different column, different mobile phase, different flow rate, different detection wavelength & different diluents for sample preparation etc.

Table 3: Summary of Process Optimization

Column Used	Mobile Phase	Flow Rate	Wave length	Observation	Result
Xterra ODS (C_{18}) RP Column, 150 mm x 4.6 mm, 5µm	Methanol: Acetonitrile: Water = 40:30:30	0.8ml/min	286nm	Very Low response	Method rejected
Zorbax ODS (C_{18}) RP Column, 250 mm x 4.6 mm, 5µm	Methanol: Acetonitrile = 70: 30	0.9ml/min	286nm	Low response	Method rejected
Develosil ODS (C_{18}) RP Column, 150 mm x 4.6 mm, 5µm	Acetonitrile: Methanol = 60: 40	1.0ml/min	286nm	Tailing peaks	Method rejected
Phenomenex Luna (C_{18}) RP Column, 250 mm x 4.6 mm, 5µm	Phosphate Buffer (pH-5.2): Methanol = 80:20	1.0ml/min	286nm	Resolution was not good	Method rejected
Phenomenex Luna (C_{18}) RP Column, 250 mm x 4.6 mm, 5µm	Phosphate Buffer (pH-3.8): Methanol = 55:45	1.0ml/min	286nm	Tailing peak	Method rejected
Phenomenex Luna (C_{18}) RP Column, 250 mm x 4.6 mm, 5µm	Phosphate Buffer (pH-4.6): Methanol = 65:35	1.0ml/min	286nm	Nice peak	Method accepted

Preparation of mobile phase: 650ml of prepared phosphate buffer and 350ml of HPLC Grade Methanol were mixed well and degassed in ultrasonic water bath for 15 minutes. The solution was filtered through 0.45 µm filter under vacuum filtration.

Method Validation

Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Results from method validation can be used to judge the quality, reliability and consistency of analytical results; it is an integral part of any good analytical practice [6,7,8].

1. Specificity: Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically, these might include impurities, degradants, matrix, etc [9]. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedure(s).

2. Accuracy: The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness [10].

3. Precision: The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility [11].

3.1 Repeatability: Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.

3.2 Intermediate precision: Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc.

3.3 Reproducibility: Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology).

4. Detection Limit: The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value [12].

5. Quantitation Limit: The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products [13].

6. Linearity: The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample [14].

7. Range: The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity [15].

8. Robustness: The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage [16].

Results and Discussion

Wavelength Selection:

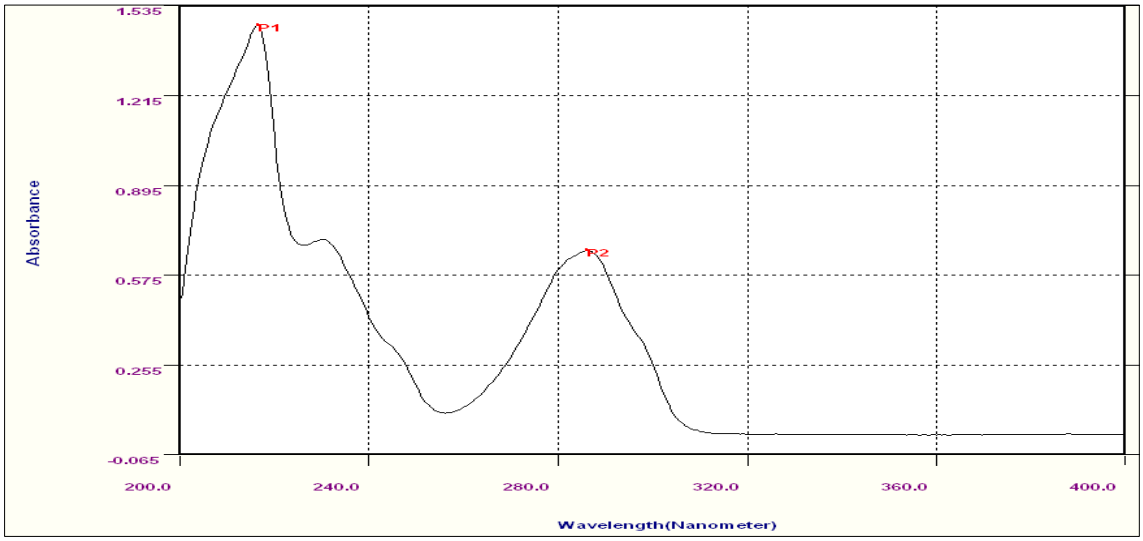

Fig 2: UV Spectrum for Favipiravir

Observation: While scanning the Favipiravir solution we observed the maxima at 286nm. The UV spectrum has been recorded on ELICO SL-159 make UV – Vis spectrophotometer model UV-2450.

Summary of optimized chromatographic conditions: The Optimum Chromatographic conditions obtained from experiments can be summarized as below:

Table 4: Summary of optimized Chromatographic conditions

Mobile phase	Phosphate Buffer (pH-4.6) : Methanol = 65:35%
Column	Phenomenex Luna (C_{18}) RP Column, 250 mm x 4.6 mm, 5µm
Column Temperature	Ambient
Detection Wavelength	286 nm
Flow rate	1.0 ml/ min.
Run time	10 min.
Temperature of Auto sampler	Ambient
Diluent	Mobile Phase
Injection Volume	20µl
Type of Elution	Isocratic
Retention time	4.783 minutes

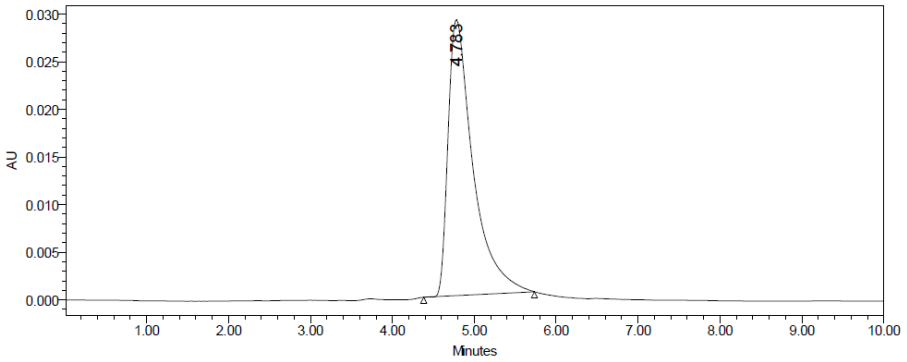

Fig 3: Chromatogram of Favipiravir in Optimized Condition

Final Observation: The selected and optimized mobile phase was Phosphate Buffer (pH-4.6): Methanol = 65:35% and conditions optimized were flow rate (1.0 ml/minute), wavelength (286nm), Run time was 10 mins. Here the peaks were separated and showed better resolution, theoretical plate count and symmetry [17]. The proposed chromatographic conditions were found appropriate for the quantitative determination of the drug.

Analytical method validation

1. System suitability test

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analysed constitute an integral system that can be evaluated as such. Following system suitability test parameters were established [18]. The data are shown in Table-5 & 6.

Table 5: Data of System Suitability Test

S.No.	Injection No.	RT	Area	USP Plate Count	USP Tailing
1	Injection 1	4.817	745236	6986	1.39
2	Injection 2	4.783	743652	6857	1.37
3	Injection 3	4.840	742587	6856	1.36
4	Injection 4	4.783	742946	6847	1.39
5	Injection 5	4.817	743654	6896	1.38
6	Injection 6	4.778	741698	6874	1.37
Mean			743295.5	6886	1.37666
SD			1199.773604		
%RSD			0.161412736		

Table 6: Data of System Suitability Parameter

S.No.	Parameter	Limit	Result
1	Retention Time	RT > 2	Azelnidipine= 4.778
2	Asymmetry	T ≤ 2	Azelnidipine= 1.35
3	Theoretical plate	N > 2000	Azelnidipine= 6859
4	Tailing Factor	T<2	Azelnidipine= 1.37

2. Linearity

To evaluate the linearity, serial dilution of analyte was prepared from the stock solution was diluted with mobile phase to get a series of concentration ranging from 60-140µg/ml. The prepared solutions were sonicated. From these solutions, 10µl injections of each concentration were injected into the HPLC system and chromatographed under the optimized conditions [19]. Calibration curve was constructed by plotting the mean peak area (Y-axis) against the concentration (X-axis).

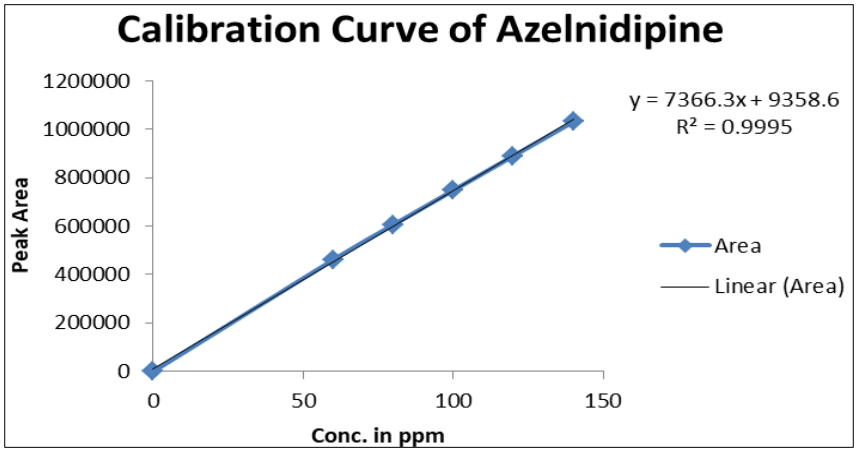

Fig 4: Calibration Curve of Azelnidipine

Table 7: Linearity Data for Azelnidipine

Conc. (µg/ml)	Area
0	0
60	461404
80	606157
100	748506
120	891041
140	1032196

3. Accuracy: The accuracy of the method was determined by recovery studies and the percentage recovery was calculated. The recoveries of Azelnidipine were found to be in the range of 99-102%. The proposed Liquid Chromatographic method was applied to the determination of Azelnidipine [20]. The results for Azelnidipine comparable with the corresponding labeled amounts.

Table 8: Shown Accuracy Observation of Azelnidipine

Accuracy	Amount Added	Amount Recovered	Peak Area	% Recovery	Mean Recovery
80%	80	80.798	604517	100.997	100.437%
	80	80.673	603598	100.841	
	80	80.756	604213	100.945	
100%	100	99.933	745471	99.933	
	100	100.083	746574	100.083	
	100	100.365	748652	100.365	
120%	120	120.290	895415	100.241	
	120	120.201	894762	100.167	
	120	120.442	896541	100.368	

4. Precision

Repeatability: The precision of each method was ascertained separately from the peak areas & retention times obtained by actual determination of six replicates of a fixed amount of drug. Azelnidipine (API) [21]. The percent relative standard deviation was calculated for Azelnidipine are presented in the table-9.

Table 9: Repeatability Data for Azelnidipine

S. No.	INJECTION	PEAK AREA
1	Injection 1	743826
2	Injection 2	745277
3	Injection 3	742506
4	Injection 4	747576
5	Injection 5	746715
6	Injection 6	741278
7	Average	744529.6667
8	SD	2440.4116
9	% RSD	0.32777

Intermediate Precision

The Intermediate Precision consists of two methods [22]: -

Intra Day: In Intra Day process, the 80%, 100% and 120% concentration are injected at different intervals of time in same day.

Inter Day: In Inter Day process, the 80%, 100% and 120% concentration are injected at same intervals of time in different days.

Table 10: Results of Intra-Assay & Inter-Assay

Conc. of Azelnidipine (API) (µg/ml)	Observed Conc. of Azelnidipine (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=6)	% RSD	Mean (n=6)	% RSD
80	80.096	0.487	79.685	0.688
100	100.074	0.968	100.057	0.789
120	120.056	0.847	120.016	0.698

Observations: The intra & inter day variation of the method was carried out for standard deviation & % RSD (% RSD < 2%) within a day & day to day variations for Azelnidipine revealed that the proposed method is precise.

5. Specificity

Specificity can be determined by comparing the chromatograms obtained from the drugs with the chromatogram obtained from the blank solution. Blank solution was prepared by mixing the excipients in the mobile phase without drug. Drug solutions were prepared individually and the sample containing one drug was also prepared. Now these mixtures were filtered by passing through 0.45 µ membrane filter before the analysis. In this observation no excipient peaks were obtained near the drug in the study run time. This indicates that the proposed method was specific [23].

The chromatograms representing the peaks of blank, Azelnidipine and the sample containing the one drug was shown in following figures respectively.

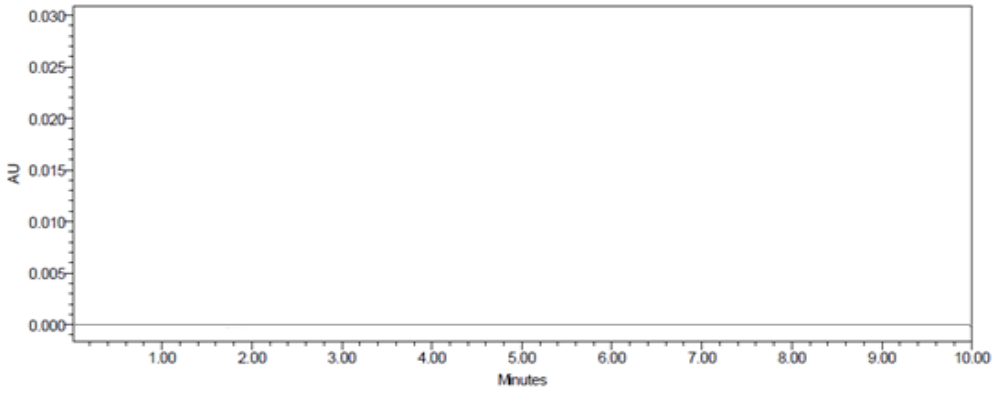

Fig 5: Chromatogram for Blank Solution

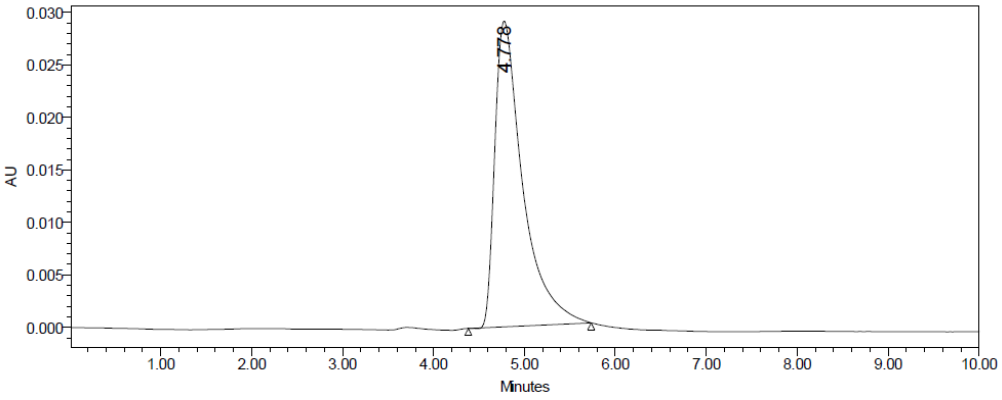

Fig 6: Chromatogram of Azelnidipine Standard Solution

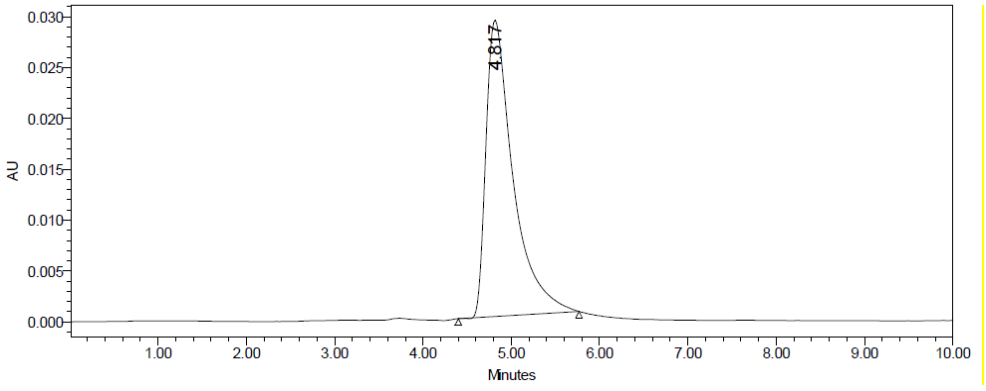

Fig 7: Chromatogram of Azelnidipine Sample Solution

Observation: In this test method blank, standard solutions were analyzed individually to examine the interference. The above chromatograms show that the active ingredient was well separated from blank and their excipients and there was no interference of blank with the principal peak. Hence the method is specific.

6. Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ parameter was evaluated by mistreatment the slope of line and variance obtained from accuracy studies. The detection limit (LOD) and quantization limit (LOQ) may be expressed as:

L.O.D. = 3.3(SD/S).

L.O.Q. = 10(SD/S)

Where, SD = Standard deviation of the response

S = Slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte [24-25].

The Minimum concentration level at which the analyte can be reliable detected (LOD) & quantified (LOQ) were found to be 1.469 & 4.454µg/ml respectively.

7. Method Robustness

Influence of small changes in chromatographic conditions such as change in flow rate 1.0 ml (± 0.1ml/min), Wavelength of detection 286 (±2nm) & organic phase content in mobile phase (±5%) studied to determine the robustness of the method are also in favour of (Table-11, % RSD < 2%) the developed RP-HPLC method for the analysis of Azelnidipine (API) [26-27].

Effect of variation of flow conditions

The sample was analyzed at 0.9ml/min and 1.1ml/min instead of 1ml/min, remaining conditions are same. 10µl of the above sample was injected and chromatograms were recorded.

Effect of variation of mobile phase organic composition

The sample was analyzed by variation of mobile phase i.e. Methanol: Phosphate Buffer was taken in the ratio and 40:60, 30:70 instead of 35:65, remaining conditions are same. 20µl of the above sample was injected and chromatograms were recorded [28].

Table 11: Results for Robustness

Parameter Used for Sample Analysis	Peak Area	Retention Time	Theoretical Plates	Tailing Factor
Actual Flow rate of 1.0 mL/min	742946	4.778	1.37	2896
Less Flow rate of 0.9 mL/min	698965	4.783	1.39	2986
More Flow rate of 1.1 mL/min	786598	4.817	1.42	2985
Less organic phase	732642	4.842	1.29	3102
More organic phase	702546	4.773	1.37	3247

8. Estimation of azelnidipine in pharmaceutical dosage form

Label Claim: 200mg

Each tablet contains: 200mg

Twenty Tablets were taken and the I.P. method was followed to determine the average weight. Above weighed tablets were finally powdered and triturated well. A quantity of powder equivalent to 25 mg of drugs were transferred to 25 ml volumetric flask, make and solution was sonicated for 15 minutes, there after volume was made up to 25 ml with same solvent. Then 10 ml of the above solution was diluted to 100 ml with mobile phase. The solution was filtered through a membrane filter (0.45 µm) and sonicated to degas [29]. The solution prepared was injected in five replicates into the HPLC system and the observations were recorded. The data are shown in Table-12.

ASSAY:

$$\text{Assay \%} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \text{Avg Wt.} = \text{mg}$$

Where:

AT = Peak Area of drug obtained with test preparation

AS = Peak Area of drug obtained with standard preparation

WS = Weight of working standard taken in mg

WT = Weight of sample taken in mg

DS = Dilution of Standard solution

DT = Dilution of sample solution

P = Percentage purity of working standard

Table 12: Recovery Data for Estimation Azelnidipine in Favivir Tablet

Brand Name of Azelnidipine	Labelled Amount of Drug (mg)	Mean (± SD) Amount (mg) found by the Proposed Method (n=6)	Assay % (± SD)
Favivir Tablet (Hetero Health Care)	200mg	199.385 (± 0.289)	99.425 (± 0.638)

Result & Discussion: The amount of drug in Favivir Tablet was found to be 199.385 (± 0.289) mg/tab for Azelnidipine & % Purity was 99.425%.

Stability Studies

The results of the strain studies indicated the specificity of the tactic that has been developed. Favipiravir was stable in oxidation and thermal stress conditions [30,31,32]. The result of forced degradation studies is given in the following table-13.

Table-13: Results of Forced Degradation Studies of Favipiravir API

Stress Condition	Time in hrs	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1 M HCl)	24Hrs.	92.406	7.594	100.0
Basic Hydrolysis (0.1 M NaOH)	24Hrs.	95.314	4.686	100.0
Wet heat	24Hrs.	93.241	6.759	100.0
UV (254nm)	24Hrs.	89.342	10.658	100.0
3 % Hydrogen peroxide	24Hrs.	90.355	9.645	100.0

Summary

To develop a precise, linear, specific & suitable stability indicating RP-HPLC method for analysis of Favipiravir, different chromatographic conditions were applied & the results observed are presented in previous chapters. Isocratic elution is simple, requires only one pump & flat baseline separation for easy and reproducible results. So, it was preferred for the current study over gradient elution. In case of RP-HPLC various columns are available, but here Phenomenex Luna (C18) RP Column, 250 mm x 4.6 mm, 5µm Column was preferred because using this column peak shape, resolution and absorbance were good. Detection wavelength was selected after scanning the standard solution of drug over 200 to 400nm. From the U.V spectrum of Favipiravir it is evident that most of the HPLC work can be accomplished in the wavelength range of 286 nm conveniently. Further, a flow rate of 1.0 ml/min & an injection volume of 20µl were found to be the best analysis. The result shows the developed method is yet another suitable method for assay and stability related impurity studies which can help in the analysis of Favipiravir in different formulations.

Conclusion

A sensitive & selective stability indicting RP-HPLC method has been developed & validated for the analysis of Favipiravir in bulk and pharmaceutical dosage form. Based on peak purity results, obtained from the analysis of samples using described method, it can be concluded that the absence of co-eluting peak along with the main peak of Favipiravir indicated that the developed method is specific for the simultaneous estimation of Favipiravir in the bulk and pharmaceutical dosage forms. Further the proposed RP-HPLC method has excellent sensitivity, precision and reproducibility. The specific Retention time for Favipiravir are found to be 4.783min. The tailing factor was found to be 1.37 with theoretical plates 6859 for Favipiravir. The %Recoveries was determined as 100.437% for Favipiravir in Accuracy. The %RSD in Repeatability is 0.327 with Intermediate Precision (Intra & Inter Day) are 0.767 & 0.725 for Favipiravir in Precision respectively. In Linearity, the correlation coefficient was found to be 0.9995 for Favipiravir. The LOD for Favipiravir was 1.469 and LOQ for Favipiravir are 4.454 respectively.

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