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Research Article

Quantitative Analysis of Favipiravir by HPLC: Development and Validation

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Favipiravir is a broad-spectrum oral antiviral agent and has been approved for the treatment of COVID-19 infection cases. It inhibits a protein known as RNA polymerase, which transcribes and replicates the viral RNA genome, causing the spread of the infection. The current study aimed to develop and validate a new analytical method utilizing HPLC in accordance with international requirements (ICH and FDA). The chromatographic conditions used to achieve good resolution and reproducibility were a mixture of acetonitrile and 0.1% phosphoric acid buffer in the ratio of 60 : 40 v/v as the mobile phase. The flow rate was 1.0 mL/min, the wavelength (λ) was determined at 250 nm, and a retention time was approximately 3 minutes for favipiravir. The HPLC analysis was performed on the Dionex 300 system equipped with a Phenomenex C8 (250 cm 4.6 mm) 5 μm column. The total runtime was 6.0 min. The findings indicated that the method had been validated satisfactorily. Across the concentration range of 0.10–0.75 mg/ml, the calibration curve revealed a linear relationship. The accuracy of the current method was to be 99.2%. The limit of detection (LOD) and limit of quantification (LOQ) were 0.004 and 0.013 ppm, respectively. The standard and sample solution repeatability tests revealed that the procedure was precise and within acceptable ranges. The RSD% for the determination of precision was <2%. The results for robustness and solution stability were within acceptable limits. Finally, the new method provided an excellent result for all analytical method validation parameters and met the acceptance criteria. In addition, the new approach has a short run time and a retention time of around 4 minutes.

1. Introduction

Favipiravir (T-705) is a purine nucleic acid analog developed by Toyama Chemical in Japan, and it is an antiviral drug tested in multiple clinical trials [1]. The chemical name of favipiravir is 6-fluoro-3-hydroxyproline-2-carboxamide. In 2014, it was approved in Japan as an alternative treatment option for influenza virus infections. Favipiravir has been licensed in several countries for the treatment of patients with mild to moderate COVID-19 disease. Favipiravir (Figure 1) is an RNA-dependent RNA polymerase inhibitor. It is activated in the cell in its phosphoribosylated form (favipiravir-RTP) and inhibits viral RNA polymerase activity [2].

When other influenza virus medicines are inefficient or insufficient, favipiravir is indicated for outbreaks caused by new or re-emerging influenza viruses (highly contagious respiratory infections, influenza), and it is also indicated for prophylaxis from influenza virus [3, 4].

The usual adult favipiravir dosage is 1600 mg twice daily in the morning and evening on day one and 600 mg twice daily in the morning and evening for the next four days to complete the administration period of five days [5, 6]. Favipiravir is available in several countries and is manufactured by many different companies, including TOYAMA CHEMICAL CO., LTD's Avigan 200 mg FCT and Optimus Pharma Private Limited's Araflu 400 mg FCT, which is

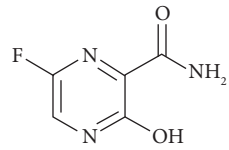

Figure 1: Chemical structure of favipiravir.

manufactured by Optimus Pharma Private Limited and distributed by Biocon Biologics India Limited. Given the therapeutic relevance of favipiravir, our research group intends to develop a powder for an oral suspension dosage form as a single dose in a foil laminate sachet. The new dosage has many advantages in improving patient medication adherence and treatment outcomes and is easily administered to children with COVID-19 [7].

Validation of analytical methods confirms that various HPLC analytical procedures produce accurate and reproducible results; it is an essential stage in the development of new dosage forms since it gives evidence about accuracy, linearity, precision, detection, specificity, robustness, and quantitation limits. According to the ICH guideline, "the objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose." Therefore, it is now required in the process of developing pharmaceutical dosage forms to submit validation data to the drug product registration authority. Guidelines for analysis method validation include ICH and USP guidelines [8, 9].

A few methods for determining favipiravir in bulk pharmaceuticals and pharmaceutical preparations have been found after a review of the literature [10, 11]. The development of a reliable analytical method is a critical step in the process of developing a new formula. The study's objective was to develop a simple, fast, and accurate HPLC analytical method to quantify the amount of favipiravir in an oral suspension dosage form. The method was validated according to ICH standards by evaluating its selectivity, specificity, linearity, precision, accuracy, detection limit, quantification limit, and robustness.

2. Materials and Methods

2.1. Instrumentation. The instruments used in this study were supplied by Jerusalem Pharmaceuticals Company. HPLC system DIONEX Ultimate 3000 was used to develop and validate methods. The HPLC was equipped with a diode array detector, Phenomenex C8 (25 cm × 4.6 mm, 5 μm) column, automatic injector, and a computer equipped with the analysis software Chromeleon Version: 7.2. Analytical balance types AS 60/220 R2 and Precisa XT 220A, Nylon filter (pore size 0.45 μm and 0.22 μm) from Merck and pH Meter type Metrohm 691 were used.

2.2. Chemicals and Reagents. Jerusalem Pharmaceuticals Company provided all pharmaceutical-grade substances and materials used in this research. The reagents and chemicals utilized were all of HPLC analytical grade. The following materials and reagents were used in this study: favipiravir, orthophosphoric acid 85% (Merck), sodium hydroxide (Merck), hydrochloric acid 35% (Merck), acetonitrile (Fischer), and hydrogen peroxide 30% (Sun pharm).

2.3. Preparation of Mobile Phase and Favipiravir Solutions

2.3.1. Mobile Phase. 1 ml orthophosphoric acid 85% was transferred to 1000 ml water to prepare the mobile phase. This solution was diluted with 600 mL of acetonitrile. The mobile phase was filtered through 0.45 m membrane filters and degassed for 10 minutes using sonication.

2.3.2. Standard Solution. A precisely weighed quantity of favipiravir (100 mg) was dissolved in 50 ml of the mobile phase to produce a standard solution, and then, 5 mL of the resulting solution was diluted to 20 mL by the same solvent to produce a standard solution of favipiravir (0.5 mg/ml).

2.3.3. Sample Solution. An accurately weighed amount of favipiravir (equivalent to 100 mg) was transferred to a 50 ml volumetric flask. 40 ml of mobile phase was added to the volumetric flask and shaken well, and then, the volume was completed by the mobile phase. Next, 5 ml of the stock solution was transferred to a 20 ml volumetric flask, and the remaining volume was filled with the mobile phase to achieve a concentration of 0.5 mg/ml. The prepared solution was filtered through 0.45 μm membrane filters.

2.4. Chromatographic Conditions. The analysis was performed using a Dionex 3000 HPLC instrument. The experiments were carried out on a C8, 5 m, 250 × 4.6 mm analytical column, and the detection wavelength was 250 nm. The column's operating temperature was set to 25°C. The injection volume was set to 10 μL. The flow rate was set to 1.0 mL/min, and the run lasted 6 minutes.

2.5. Method Validation. The method was validated in compliance with the USP general chapter 1225, ICH Q2 (R1), and FDA guidelines [8, 9, 12].

2.5.1. Specificity. The method can be considered specific when the intended analyte can be determined without any interference by other excipients in the tested sample [13]. The method's specificity was evaluated by the following:

(1) Placebo Interference. The goal of this test is to show that placebo components will not have an undue impact on the results by injecting 10 μl solutions of standard, sample, blank, and placebo separately.

(2) Forced Degradation. Experiments on forced degradation utilizing favipiravir and favipiravir powder for oral suspension were carried out to verify the assay. Studies on forced degradation are crucial because they offer the knowledge to develop a stability-indicating analytical method. From the sample solution (0.5 mg/ml of favipiravir

WS), specific quantities were transferred to a 200 ml volumetric flask. Then, the volume was titrated to 200 ml using different solutions, as shown in Table 1.

2.5.2. Linearity. The linearity of the analytical method is determined, as is its capacity to provide test results that are directly proportional to the concentration of the analyte over a specific range. [14] Six standard solutions within the concentration range were utilized to prepare the calibration standards. For the preparation of the solutions, the standard stock solution was diluted with the mobile phase in the following favipiravir concentrations: 0.1, 0.25, 0.4, 0.5, 0.6, and 0.75 mg/ml, which correspond to 20%, 50%, 80%, 100%, 120%, and 150% of the target concentration, respectively. To confirm the linearity, three injections of six different concentrations were employed.

2.5.3. Sensitivity. Favipiravir's limit of detection (LOD)/ limit of quantitation (LOQ) was identified by analyzing various favipiravir solutions. The LOD and LOQ of favipiravir were calculated as $\mathrm{LOD} = 3.3\,\sigma/s$ and $\mathrm{LOQ} = 10\,\sigma/s$, where σ is the standard deviation of the y-intercepts of the regression line and s is the slope of the calibration line [15].

2.5.4. Accuracy. The n analytical method accuracy is expressed as the closeness between the expected value and the value discovered. It was calculated through the calculation of the percent recovery (R%) of the analyte recovery. In this instance, to assess the accuracy of the developed method, three concentration levels (80%, 100%, and 120%), i.e., 0.4, 0.5, and 0.6 mg/ml, and three samples from each concentration were injected.

2.5.5. Precision. In the current research, system precision and method precision were assessed. Ten observations of the standard solution at 100% concentration levels were performed on the same day to evaluate system precision. Six assay results from the same day's sample solution at 100% concentration were used for method precision. To verify repeatability, the RSD of the given results was determined.

2.5.6. Ruggedness (Intermediate Precision). On a different day, a different analyst performed a ruggedness investigation on six test sample solutions using different equipment and a different column. The assay and relative standard deviation (RSD) were calculated based on the obtained results.

2.5.7. Robustness. The method's robustness was established by introducing minor and purposeful modifications to the experimental parameters. The modifications include flow rates of the mobile phase ±0.1 mL/min and wavelength ±2 nm. The data obtained for each case were evaluated by calculating the % RSD and percentage of the recovery.

2.5.8. Filter Compatibility. The effect of utilizing filters in the analytical procedure (nylon filter 0.45 m and 0.22 m) on the assay results was investigated. The current research evaluated filter leachability and absorbance [16].

(1) Leachability. Filters used to clarify samples must not affect the UV/HPLC spectra/chromatogram at the measuring wavelength. Leachable chemicals must also not compromise the quantitative integrity of the dissolved API.

The filter leachability was calculated according to the following equation:

$$\text{Filter Leachability} = \left(\frac{\text{Filtered blank response}}{\text{Average of the three standard response}}\right) * 100\%. \quad (1)$$

(2) Adsorbance. There should be no absorbed API material on the surfaces of the filters used to clean the test sample solutions. The magnitude of this adsorption must be tested, and the filter saturation point must be identified and represented in the test technique.

The recovery percentage value was calculated according to the following equation:

$$\%\ \text{Recovery} = \left(\frac{\text{Filtered standard or sample response}}{\text{Unfiltered standard response}}\right) * 100\%. \quad (2)$$

3. Results and Discussion

3.1. Optimization of Chromatographic Conditions. Numerous chromatographic conditions were applied to establish a suitable HPLC assessment of favipiravir, and optimal chromatographic conditions were developed. Chromatographic conditions include detection wavelength, mobile phase composition, buffers, buffer strength and pH, and flow rate. A series of experiments were carried out to optimize the chromatographic conditions by varying the ratio of acetonitrile to 0.1% phosphoric acid buffer using a Phenomenex C8 (250 cm 4.6 mm) 5 μm column. The following were the optimized chromatographic conditions: mobile phase consisting of acetonitrile and phosphoric acid buffer in the ratio 60 : 40 v/v with a flow rate of 1 mL/min, injection volume 10 μl, run time 6 min, and column temperature 25°Cat

Table 1: Solutions for forced degradation studies under stressful conditions.

#	Sample solution conc. (mg/ml)	Sample weight (mg)	Stress condition	Total volume (ml)
1	0.5	225	—	200
2	0.5	225	0.5 M NaOH & heat in water bath @ 50°C, 15 minutes	200
3	0.5	225	0.5 M HCl & heat in water bath @ 70°C, 15 minutes	200
4	0.5	225	6% H2O2 & heat in water bath @ 70°C, 15 minutes	200
5	0.5	225	Heat in water bath @ 70°C, 15 minutes	200
6	0.5	225	Under UV light for 24 hrs	200

wavelength (λ) 250 nm. In addition, favipiravir was eluted, forming a sharp symmetrical shape, with good resolution with a retention time of approximately 4 minutes (Figure 2).

3.2. Specificity

3.2.1. Placebo Interference. Table 2 demonstrates that the placebo constituents did not significantly influence the results because the placebo, standard, and sample solutions were prepared at nominal concentrations and absorbance measurements were performed at 250 nm for the nominal, standard, and placebo solutions. Placebo interference should be less than 2%. In order to calculate the placebo interference percentage, the following formula was used [17]:

$$\text{Interference \%} = 100 * \left(\frac{A_P}{A_{\text{St}}}\right), \tag{3}$$

where A_P is the absorbance of the placebo and A_{St} is the absorbance of the standard.

3.2.2. Forced Degradation. The results of the forced degradation investigation are presented in Table 3. The method can identify potential degradants. Favipiravir peak purity was NLT 980, with degradation ranging from 0.1 to 6.1%. No coeluting peaks were observed in the retention time of the favipiravir interference. This result showed that the analyte peak was pure, confirming the specificity of the method (Figure 3).

$$\text{\% Degradants} = \frac{\text{Area of degradation peak in stressed sample X 100\%}}{\text{Area of favipiravir in unstressed sample}}. \tag{4}$$

3.3. Linearity. Linearity was evaluated by constructing a calibration curve from a series of favipiravir concentrations and calculating the correlation coefficient (R^2). For all concentrations, the % RSD was less than two (Table 4). The correlation coefficient (R^2) was 9995, indicating a good fit between the data and the regression line (Figure 4).

3.4. Sensitivity: Limit of Detection (LOD)/Limit of Quantitation (LOQ). The LOD is defined as the minimal quantity of analyte identified in a sample, whereas the LOQ is defined as the minimum amount of analyte that can be quantitatively determined in a sample [15, 18]. The method showed an LOD of 0.004 ppm and LOQ of 0.013 ppm for favipiravir (Table 5).

3.5. Accuracy. The RDS values were in the 0.03–0.30% range, as shown in Table 6. The percentage recovery and % RSD results were within the acceptable limits of 98.0%–102.0% and not more than 2.0%, respectively [17], indicating the method's applicability for routine drug analysis.

3.6. Precision. The method's precision is defined as "the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions." It is typically expressed as the relative standard deviation. As shown in Table 7, the findings of both the system and the method precision indicated that the method was precise within acceptable ranges. In addition, the RSD values were within acceptable ranges. For the RSD, acceptable precision was less than 2.0%.

3.7. Ruggedness (Intermediate Precision). Six oral suspension test samples were prepared using favipiravir powder. The samples were produced by a different analyst, analyzed on a different instrument with a different column on a different day, and then injected into the HPLC system in accordance with the test method. The % assay was calculated, and the results are listed in Table 8. The results obtained for the intermediate precision were excellent since the absolute difference RSD (all) was 1.5%.

3.8. Robustness. As described in Section 2.5.7, the analytical method robustness was verified by investigating the effect of slight changes in HPLC conditions on the system suitability parameters of the proposed method. The robustness testing findings revealed that a minor change in the method conditions, such as the flow rate and wavelength, is robust within acceptable ranges. In all modifications, the % SD was maintained at less than 2.0% (Table 9).

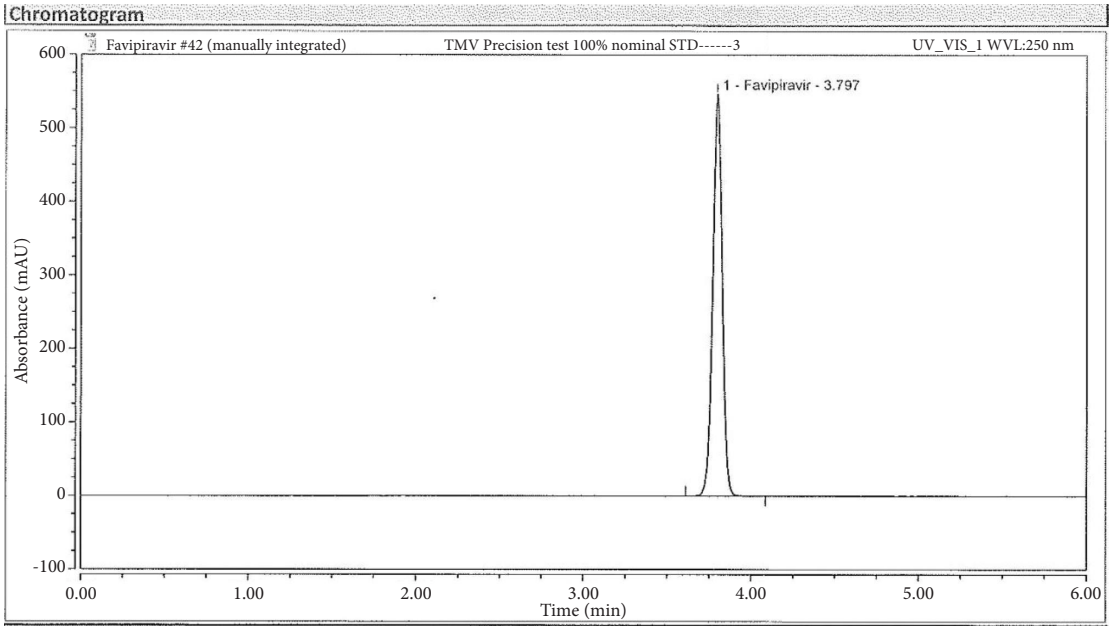

Figure 2: Optimized chromatogram showing favipiravir peak at 3.8 min.

Table 2: Interference data and results for favipiravir.

Favipiravir conc. (mg/ml)	Avg $ABS_{Placebo}$	Avg ABS_{St}	Avg ABS_{Sa}	Interference (%)
0.50	0	36.804	36.94	0.00
Acceptance criteria			NMT 2%	

Table 3: Forced degradation study results under stressful conditions.

Forced degradation of favipiravir			
Reagent added/stress condition	Favipiravir		Degradants (%)
	Recovered (%)	Peak purity	
0.5 M NaOH & heat in water bath @ 50°C, (15) min	92.6	1000	0.1
0.5 M HCl & heat in water bath @ 70°C, (15) min	88.2	1000	6.11
6% H2O2 & heat in water bath @ 70°C, (15) min	82.9	1000	0.5
Heat in water bath @ 70°C, 15 minutes	94.6	1000	0.1
Under UV light for 24 hours	94.7	1000	0.1

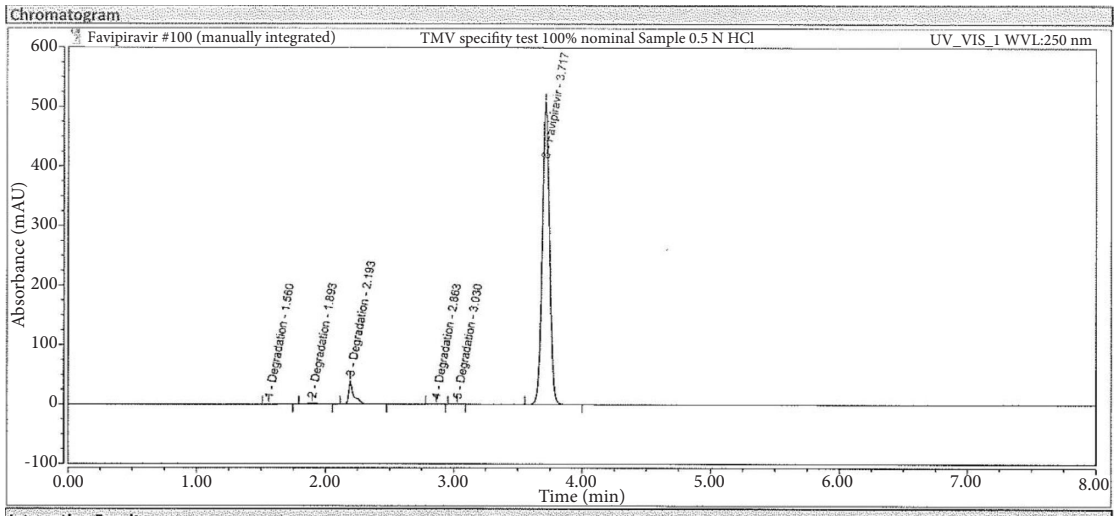

Figure 3: Chromatogram of forced degradation using 0.5 N HCl.

Table 4: Linearity data and range for favipiravir.

Linearity of favipiravir							
Conc (%)	Conc. (mg/ml)	1st area	2nd area	3rd area	Av area	SD	RSD (%)
20	0.1	7.664	7.523	7.662	7.6	0.081	1.1
50	0.25	19.186	19.158	19.115	19.2	0.036	0.2
80	0.4	31.835	31.811	31.866	31.8	0.028	0.1
100	0.5	39.635	39.658	39.643	39.6	0.012	0.0
120	0.6	47.346	47.233	47.325	47.3	0.060	0.1
150	0.75	58.509	58.639	58.323	58.5	0.159	0.3

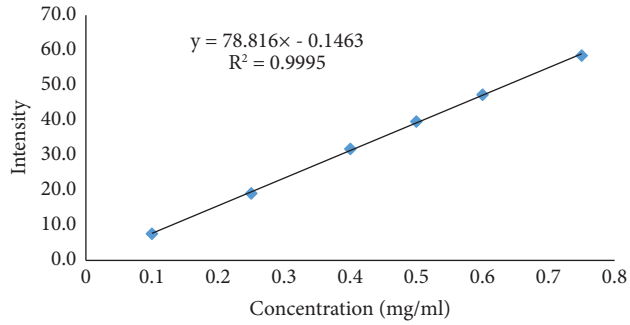

Figure 4: Favipiravir calibration curve.

Table 5: Favipiravir sensitivity showing LOD and LOQ.

Conc (%)	Conc. (ppm)	Injection area repetition 1	Injection area repetition 2	Injection area repetition 3	Average area
5.0	0.0250	0.018	0.019	0.018	0.018
12.5	0.0625	0.046	0.045	0.047	0.046
25.0	0.1250	0.093	0.094	0.092	0.093
50.0	0.2500	0.185	0.186	0.187	0.186
100.0	0.5000	0.374	0.373	0.373	0.373
150.0	0.7500	0.562	0.558	0.56	0.560
200.0	1.0000	0.746	0.747	0.747	0.747
STEYX		0.001	0.001	0.001	0.001
Slope		0.7480	0.7464	0.7474	0.747
LOQ (ppm)		0.0156	0.0151	0.0084	0.0130
LOD (ppm)		0.0051	0.0050	0.0028	0.0043

Table 6: Accuracy result of favipiravir.

Conc. (mg/ml)	Area of sample	% accuracy	Recovery (mg/ml)	Area of standard	Conc. (%)
80	28.973	100.6	0.3864	29.055	
80	28.71	99.7	0.3829	28.661	
80	28.761	99.9	0.3836	28.659	80
AV	28.8	100.1		28.792	
SD	0.1	0.5	0.1	0.5	
RSD (%)	0.5	0.5	0.1	0.5	
100	36.098	99.2	0.4760	36.2	
100	35.963	98.8	0.4742	36.4	
100	35.91	98.6	0.4735	36.6	100
AV	36.0	98.9		36.4	
SD	0.1	0.3	0.1	0.3	
RSD (%)	0.3	0.3	0.3	0.3	
120	43.3	98.7	0.5686	44.0	
120	43.319	98.8	0.5688	43.8	
120	43.24	98.6	0.5678	43.8	120
AV	43.3	98.7		43.9	
SD	0.0	0.03	0.0	0.03	
RSD (%)	0.1	0.03	0.1	0.03	

Table 7: Precision results for favipiravir.

Sample #	Nominal standard area	Nominal sample area
1	36.877	36.941
2	36.967	36.133
3	36.958	36.779
4	36.884	36.963
5	36.952	36.999
6	36.788	37.009
Average	36.9	36.804
SD	0.1	0.3
RSD	0.2	0.9

Table 8: Ruggedness results.

Sa. #		Area of standard	% assay	
			Area of sample	%
Analyst 1	1	36.877	36.941	100.1%
	2	36.967	36.133	97.9%
	3	36.958	36.779	99.7%
	4	36.884	36.963	100.2%
	5	36.952	36.999	100.3%
	6	36.788	37.009	100.3%
	Avg	36.9	36.804	99.7%
	RSD	0.2%	0.9%	0.9%
Analyst 2	1	36.407	36.679	98.6%
	2	36.491	36.292	97.6%
	3	36.390	37.384	100.5%
	4	36.512	35.963	96.7%
	5	36.432	36.680	98.6%
	6	36.479	35.743	96.1%
	Avg	36.5	36.457	98.0%
	RSD	0.1%	1.6%	1.6%
	Absolute difference RSD (all)	—	—	1.5% For both analysts

Table 9: Robustness results of favipiravir.

Changed parameter	Actual average peak area ± SD ($n = 6$)	RSD (%)
Wavelength + (248 nm)	41.678 ± 0.06	0.1
Wavelength – (252 nm)	32.993 ± 0.04	0.1
Flow rate + (1.1 ml/min)	33.662 ± 0.06	0.2
Flow rate – (0.9 ml/min)	41.105 ± 0.16	0.4

Table 10: Filter leachability results.

No	Standard reading	Filtered blank reading
1	36.372	0
2	36.257	0
3	36.37	0
Average	36.333	0
RSD (%)	0.18	0.00

**Acceptance criteria: leachability results should be less than or equal to 0.5% of the mean response value of the 100% standard solution.

Table 11: Filter absorbance results.

#	Nylon 0.45 μm			Nylon 0.22 μm		Centrifuge	
	Standard area	Sample area	% assay	Standard area	Sample area	Standard area	Sample area
1	36.877	36.755	99.5	36.679	99.3432	37.457	101.4504
2	36.967	37.119	100.5	36.696	99.38924	37.422	101.3556
3	36.958	37.125	100.6	36.731	99.48404	37.413	101.3312
4	36.884	37.109	100.5	36.744	99.51925	37.499	101.5641
5	36.922	37.027	100.3	36.7125	99.43393	37.44775	101.4253
SD	0.05	0.18	0.49	0.03	0.08	0.04	0.11
RSD (%)	0.13	0.5	0.49	0.08	0.08	0.10	0.104

Table 12: Filter absorbance results according to volume.

Sample no	100% of nominal standard			100% of nominal spiked sample		
	Filtrated total volume (ml)	Area	Recovery (%)	Filtrated total volume (ml)	Area	Recovery (%)
1	1	34.593	95.1	1	34.055	93.7
2	2	35.34	97.2	2	34.837	95.8
3	3	36.429	100.2	3	35.938	98.8
4	4	36.426	100.2	4	35.896	98.7
5	5	36.405	100.1	5	35.902	98.7
6	6	36.421	100.2	6	35.887	98.7
Average		35.8386				

Determine the saturation point that achieves recovery value at least within 98%–102% after 3 ml according to the results.

3.9. Filter Compatibility. Tables 10–12 summarize the results of the filter compatibility studies, which show that either a 0.22 m or a 0.45 m filter can be utilized for the analytical method without affecting the results.

4. Conclusion

The current study developed and validated a favipiravir analytical method utilizing a Phenomenex C8 (25 cm, 4.6 mm, and 5 m) column. The mobile phase was a buffer mixture of 0.1% (v/v) orthophosphoric acid in purified water and acetonitrile in a 40/60 (v/v) ratio, with a flow rate of 1 ml/min. Favipiravir was monitored at a wavelength of 250 nm using a PDA detector. The method was validated according to ICH guidelines for all parameters including linearity, precision, accuracy, repeatability, filter compatibility, and degradation. According to the ICH criteria, all validation parameters were within the acceptable ranges. The validated method was successfully applied to determine favipiravir in powder form for oral suspension formulations. The advanced RP-HPLC methods are precise, accurate, sensitive, robust, and reproducible for the quantitative estimation of favipiravir bulk and its impurities. Therefore, pharmaceutical industries can use this method for the QC analysis of favipiravir.

Data Availability

The data supporting the conclusions of the study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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