HPLC-UV method for quantification of favipiravir in pharmaceutical formulations

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Received: August 5, 2020 • Accepted: August 9, 2020

Published online: August 31, 2020

Acta Chromatographica

33 (2021) 3, 209–215

DOI:

10.1556/1326.2020.00828

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REVIEW PAPER

ABSTRACT

Favipiravir (FVP), a pyrazine analog, has shown antiviral activity against a wide variety of viruses. It is considered to be worth further investigation as a potential candidate drug for COVID-19. It is not officially available in any pharmacopoeia. A rapid, simple, precise, accurate, and isocratic high performance liquid chromatography (HPLC) method has been developed for routine quality control of favipiravir in pharmaceutical formulations. Separation was carried out by C18 column. The mobile phase was a mixture of 50 mM potassium dihydrogen phosphate (pH 2.3) and acetonitrile (90:10, v/v) at a flow rate of 1 mL min⁻¹. The ultraviolet (UV) detection and column temperature were 323 nm, and 30 °C, respectively. The run time was 15 min under these chromatographic conditions. Excellent linear relationship between peak area and favipiravir concentration in the range of 10–100 µg mL⁻¹ has been observed (r^2 , 0.9999). Developed method has been found to be sensitive (limits of detection and quantification were 1.20 µg mL⁻¹ and 3.60 µg mL⁻¹, respectively), precise (the interday and intraday relative standard deviation (RSD) values for peak area and retention time were less than 0.4 and 0.2%, respectively), accurate (recovery, 99.19–100.17%), specific and robust (% RSD were less than 1.00, for system suitability parameters). Proposed method has been successfully applied for quantification of favipiravir in pharmaceutical formulations.

KEYWORDS

favipiravir, antiviral, HPLC, method, development, validation

INTRODUCTION

Chinese-borne coronavirus disease (COVID-19) spread rapidly and became an epidemic, affecting almost all countries and regions around the world. COVID-19 case death rate ranges from 1% to 7% according to the reports of World Health Organization (WHO). It caused all people in the world to change their lifestyle. It still threatens the entire World [1]. Since the outbreak of the COVID-19 began to affect the world, countries have implemented different treatment methods.

Active therapeutic alternatives are urgently needed as a rising COVID-19 pandemic and possible effects on global health [2]. Many medications such as chloroquine, arbidol, remdesivir, and favipiravir are currently undergoing clinical trials in several countries to assess their effectiveness and safety in treating coronavirus disease [3, 4]. So far, there is no gold standard for the treatment of COVID-19 since there is not enough evidence [5].

Favipiravir (6-fluoro-3-hydroxypyrazine-2-carboxamide) is an analog of pyrazine (Fig. 1). Favipiravir (FVP) is an antiviral drug that was initially developed for influenza by Toyama Chemical. It selectively inhibits the RNA polymerase of RNA viruses, thus preventing viral reproduction. It displays antiviral activity against alpha-, filo-, bunya-, arena-, flavi-, and noroviruses [6, 7], as well as being active against the influenza virus.

After a pilot trial by Zhongnan Hospital of Wuhan University has found a better recovery rate in COVID-19 patients in the favipiravir group compared to the arbidol group [8], FVP is considered to be worth further investigation as a potential candidate drug for this disease.

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Fig. 1. Chemical structure (favipiravir)

According to the literature search, there are two published high performance liquid chromatography (HPLC) methods for determining FVP assay and impurities in active pharmaceutical ingredients [9, 10]. In both of these methods, a gradient HPLC mode was used for chromatographic separation and the run time was 60 min. FVP is not officially available in any pharmacopoeia and there is still a need for validated HPLC methods to determine FVP in pharmaceutical formulations.

EXPERIMENTAL

Chemicals

Analytical grade chemicals were used without further purification in this study. Potassium dihydrogen phosphate (99.5–100.5%, Sigma–Aldrich), ortho-phosphoric acid ($\geq 85\%$, Sigma–Aldrich), and HPLC-grade acetonitrile ($\geq 99.9\%$, Sigma–Aldrich) were used. Deionized water was purified by a Milli-Q system (Millipore) with conductivity lower than $18.2 \mu\text{S cm}^{-1}$. FVP bulk powder and tablets (favicovir, 200 mg) were obtained from Atabay Pharmaceuticals and Fine Chemicals Inc (Istanbul, Turkey).

Stock standard solution

One hundred milligram pure drug was accurately weighed, dissolved in about 30 mL of deionized water and transferred to a 100 mL volumetric flask. Then the volume was completed to 100 mL with deionized water to obtain 1 mg mL^{-1} of stock solution. The resulting stock solution was sonicated and filtered through a $0.45 \mu\text{m}$ filter. The stock solution was further diluted with deionized water to obtain the required concentration of standard solutions ($10\text{--}100 \mu\text{g mL}^{-1}$) before being injected into the system for analysis.

Sample solution

Ten FVP tablets were accurately weighed and transferred to a dry and clean mortar, then ground into a fine powder. Next, tablet powder equal to 250 mg FVP was transferred to a volumetric flask of 250 mL. About 100 mL deionized water was added and this flask was attached to a rotary shaker for 10 min. to completely disperse the ingredients. The mixture was sonicated for 30 min, diluted to volume with deionized water to give a solution containing $1,000 \mu\text{g mL}^{-1}$ and then filtered through a $0.45 \mu\text{m}$ filter.

Determination of λ_{max}

Standard solution ($40 \mu\text{g mL}^{-1}$) was subjected to scanning between 200 and 800 nm on a ultraviolet (UV) spectrophotometer (Shimadzu UV-1800 spectrophotometer). λ_{max} was obtained from the UV spectrum of standard solution.

Chromatographic conditions

Chromatographic analysis was performed on a column of Inertsil ODS-3V C18 ($4.6 \text{ mm} \times 250 \text{ mm}$, $5.0 \mu\text{m}$). The mobile phase consisted of potassium dihydrogen phosphate 50 mM (pH 2.3) and acetonitrile (90:10, v/v). The mobile phase was filtered and degassed through a $0.45 \mu\text{m}$ membrane filter before use and then pumped at a flow rate of 1 mL min^{-1} . The column has been thermostated at 30°C . The run time was 15 min under these conditions.

Method validation

The analytical method validation has been performed as per ICH guidelines of Validation of Analytical Procedure: Q2 (R1) [11, 12]. The validation parameters such as system suitability, linearity, the limit of detection (LOD), the limit of quantification (LOQ), accuracy, specificity, precision, and robustness were addressed.

Linearity

Standard calibration has been prepared using six standard solutions within the concentration range of $10\text{--}100 \mu\text{g mL}^{-1}$. In optimized chromatographic conditions, each standard solution was chromatographed for 15 min three times. Least squares linear regression analysis of the average peak area versus concentration data were used to evaluate the linearity of the method.

Specificity/selectivity

Selectivity is the ability of the analytical method to produce a response for the analyte in the presence of other interference. The selectivity of the method was tested by comparing the chromatograms obtained for FVP standard, tablet, and

Fig. 2. UV spectrum (standard solution, $40 \mu\text{g mL}^{-1}$)

blank solutions. The parameters retention time and tailing factor were calculated in order to prove that the method chosen was specific.

Limit of detection and limit of quantification

These values were determined using the standard error (s) and slope of the regression line (m) as shown in following equations:

$$\text{LOD} = 3.3 \cdot s/m$$

$$\text{LOQ} = 10 \cdot s/m$$

Precision

Precision was analyzed by calculating variations of the method in intraday (repeatability performed by analyzing standard solution on the same day) and inter-day (repeatability carried out by analyzing standard solution on three different days). Precision study was performed by injecting six times of standard solution at three different concentrations, 20, 40, and 60 $\mu\text{g mL}^{-1}$ on the same day and three consecutive days.

Accuracy

Recovery studies were conducted by the standard addition technique to confirm the accuracy of the proposed method. In this method, 80, 100 and 120% of three different levels of pure drug were added to the previously analyzed sample solutions, and favipiravir recovery was calculated for each concentration.

Robustness

A robustness analysis was performed to determine the impact of minor yet systematic differences in chromatographic conditions. The modifications include different flow rates of the mobile phase ($\pm 0.1 \text{ mL min}^{-1}$), acetonitrile ratio in the mobile phase ($\pm 1\%$) and column temperatures ($\pm 2^\circ\text{C}$). After each change, System suitability parameters were checked by injecting the sample solution into the chromatographic system and the results were compared with those under the original chromatographic conditions.

Analysis of marketed formulations

Four milliliter of above prepared sample solution has been transferred into a volumetric flask of 100 mL and filled the mark with deionized water to prepare at the concentration of 40 $\mu\text{g mL}^{-1}$ sample solution. This sample solution was filtered using 0.45 μm filter and then analyzed.

Solution stability

The stability of sample and standard solutions was monitored over a 24 h period. For this, standard and sample solutions were injected into the system at 8 h periods, and the peak area and retention time were evaluated. During the stability study, standard solutions have been stored at ambient temperature (25°C) and protected from light.

RESULT AND DISCUSSION

Determination of λ_{max}

The wavelength corresponding to maximum absorbance (λ_{max}) was determined as 323 nm from the UV spectrum of standard solution (Fig. 2).

Method development

Several preliminary studies were conducted to optimize the chromatographic conditions for the quantification of FVP. Mobile phases consisting of several buffer systems were tried at the beginning of the study; they could not meet the required system parameters. Then only potassium dihydrogen phosphate buffer system was tested without using organic modifiers, long analysis times were obtained. Different acetonitrile solution ratios were investigated to obtain optimum conditions. The acetonitrile ratio was determined as 10% against 50 mM potassium dihydrogen phosphate solution ($\text{pH} = 2.3$) due to the favipiravir peak being well shaped and symmetrical using this system. Eventually, it was found that the mobile phase consisting of 50 mM potassium dihydrogen phosphate ($\text{pH}: 2.3$ with ortho-phosphoric acid) and acetonitrile (90:10, v/v) provided stronger theoretical plates ($>2,000$) and peak tailing factor (<1.0). Mobile phase running at different flow rates ($0.5\text{--}1.5 \text{ mL min}^{-1}$) and containing mixtures of organic solvents and phosphate buffers, with ionic strengths and pH ranges were tested. Collectively, the best chromatographic conditions were achieved using an isocratic mobile phase comprising 50 mM potassium dihydrogen phosphate ($\text{pH} = 2.3$)-acetonitrile (90/10, v/v) at a flow rate of 1.0 mL min^{-1} on an Inertsil ODS-3V C18 column ($4.6 \text{ mm} \times 250 \text{ mm}$, $5.0 \mu\text{m}$) that was kept at 30°C . The analysis was conducted at 30°C , which offers a lot of advantages such as good chromatographic peak shape, enhanced column efficiency, and low-column pressure, in addition to being economic. The eluate was monitored using a UV detector set at 323 nm. Under the chromatographic conditions FVP were eluted at retention times 7.696. The tablet solution was analyzed for 60 min to ensure that there were no matrix components

Fig. 3. Calibration curve (λ : 323 nm)

Table 1. Statistical data (calibration curve, FVP)

Parameter	Value
Linearity range ($\mu\text{g mL}^{-1}$)	10–100
Slope	49.122
Intercept	82.598
Correlation coefficient	0.9999
Lack of fit	
<i>F</i>	2.90
<i>P</i>	0.0516
SE of intercept	6.9024
SD of intercept	15.4340
LOD/LOQ ($\mu\text{g mL}^{-1}$)	1.20/3.60

remaining in the column for much longer under the specified conditions. However, continuing the analysis after 15 min will increase both the analysis time and the cost. Overlapping peaks were not observed to overlap in samples

from sample analyses injected into the system consecutively with 15 min of analysis time. Due to all these, the analysis time was determined as 15 min.

METHOD VALIDATION

Linearity

The stock standard solution of FVP was diluted appropriately with deionized water to obtain standard solutions within the concentration range of 10–100 $\mu\text{g mL}^{-1}$. Each standard solution was injected three times into the HPLC system under the above-mentioned chromatographic working conditions. Linearity of the proposed method has been estimated at 6 concentration levels in the range of 10–100 $\mu\text{g mL}^{-1}$ by regression analysis. The calibration curve was developed by plotting average peak area versus standard

Fig. 4. (A) Overlay chromatogram (standard solutions, 10–100 $\mu\text{g mL}^{-1}$, λ : 323 nm). (B) Chromatogram (standard solution, 80 $\mu\text{g mL}^{-1}$, λ : 323 nm). (C) Chromatogram (sample solution, 40 $\mu\text{g mL}^{-1}$, λ : 323 nm). (D) Chromatogram (Blank solution, λ : 323 nm)

Table 2. Precision data

Std. conc. $\mu\text{g mL}^{-1}$	Intraday precision			Interday precision		
	Found conc. (6) $(\mu\text{g mL}^{-1})$	Peak area RSD (%)	Retention time RSD (%)	Found conc. (6) $(\mu\text{g mL}^{-1})$	Peak area RSD (%)	Retention time RSD (%)
20	20.02	0.178	0.015	19.92	0.195	0.034
40	40.35	0.066	0.004	40.08	0.082	0.012
60	60.24	0.041	0.002	60.10	0.064	0.025

Table 3. Recovery data

Spiked level (%)	Amount added $(\mu\text{g mL}^{-1})$	Amount recovered $(\mu\text{g mL}^{-1})$	Recovery (%)	Average (%)	SD	RSD (%)
80	32	31.78	99.31	99.96	0.157	0.157
	32	31.74	99.19			
	32	31.84	99.50			
100	40	39.79	99.48	99.93	0.180	0.180
	40	39.75	99.38			
	40	39.89	99.73			
120	48	48.02	100.04	99.75	0.125	0.125
	48	48.08	100.17			
	48	47.96	99.92			

concentration (Fig. 3). The correlation coefficient, slope, and intercept of the regression line were determined using the least squares method. The relation between mean peak area Y ($n = 3$) and concentration, X expressed by equation $Y = a + bX$, was linear. Values of slope, intercept, and correlation coefficient (r) were 49.122, 82.598 and 0.9999, respectively as shown in Table 1. Lack of fit test was performed to evaluate linearity. In the Lack of fit test, P value (0.0516) greater than 0.05 indicates that the data satisfies the linearity condition. Overlay chromatogram of FVP standard solutions (10–100 $\mu\text{g mL}^{-1}$) was demonstrated in Fig. 4A.

Specificity/selectivity

The chromatogram of FVP standard solution has been given in Fig. 4B. There is only one peak at the retention time of 7.696 min. The chromatogram of the tablet solution has been given in Fig. 4C. There is only one peak at the retention time of 7.696 min in this chromatogram. There are no other peaks caused by excipients and additives in this chromatogram. The chromatogram of the mobile phase has also been given in Fig. 4D. There are no other peaks caused by contents of the mobile phase in this chromatogram. This indicates that the analytical method is specific. The parameters retention time and tailing factor were calculated in order to prove that the method chosen was specific. Retention time, theoretical plate number, and peak tailing factor values were 7.696, 13798, and 0.920, respectively. All of the values were within the accepted level.

Precision

Precision study was performed by injecting six times of standard solution at three different concentrations, 20, 40,

and 60 $\mu\text{g mL}^{-1}$ on the same day and three consecutive days. The precision data were given in Table 2. All RSD values for retention time and peak area for selected FVP concentrations were less than 0.5 and 2.0%, respectively. In this case, the method is precise and can be used for our intended purpose.

Accuracy study

A known quantity of standard solution has been added to the sample solutions previously analyzed at three different levels (80%, 100% and 120%). The amount recovered for favipiravir has been calculated for three concentration. The recovery data were summarized in Table 3. Percent RSD values for all analyses were less than 2% indicating that excipients found in pharmaceutical formulations do not interfere and analytical method is very accurate.

Table 4. Robustness data

Condition	Variation	Assay(%)	SD	RSD(%)
Mobile phase flow rate (1.00 mL min^{-1})	0.90 mL min^{-1}	99.86	0.60	0.60
	1.10 mL min^{-1}	99.94	0.62	0.62
Acetonitrile ratio in mobile phase (10%)	9%	100.12	0.67	0.67
	11%	99.96	0.71	0.71
Column temperature (30 °C)	28 °C	99.96	0.34	0.34
	32 °C	100.05	0.32	0.32

Table 5. Standard solution stability (40 µg mL⁻¹)

Time (h)	Peak area	Mean	SD	RSD (%)	Retention time (min)	Mean	SD	RSD (%)
8	2013.5	2019.7	5.5	0.275	7.594	7.596	0.009	0.115
	2021.3				7.606			
	2024.2				7.589			
16	2025.6	2020.0	5.2	0.257	7.597	7.599	0.015	0.192
	2019.2				7.614			
	2015.3				7.585			
24	2012.2	2018.1	6.5	0.323	7.610	7.597	0.011	0.150
	2025.1				7.588			
	2017.1				7.594			

Table 6. Method application results

Formulation	Label claim (mg)	Amount of drug (mg)	% Assay ± SD
Favicornir tablet	200	200.35	100.18 ± 0.38

Robustness

The results showed that the change in flow rate and mobile phase concentration had little effect on the chromatographic behavior of FVP. The small change in the mobile phase flow rate and acetonitrile content have a small impact on the retention time of FVP. The change in the column temperature did not have a significant effect on the method. The results of this study, expressed as % RSD, were presented in Table 4.

Solution stability

The stability of sample and standard solutions was monitored over a 24 h period. For this, standard and sample solutions were injected into the system at 8 h periods, and the peak area and retention time were evaluated. No changes in standard concentrations have been observed over a period of 24 h. The % RSD for peak area ($n = 3$) was 0.275% and the value for retention time ($n = 3$) was 0.12% for standard solution. The results have been demonstrated in Table 5. No major changes in active ingredient concentration have also been found in the tablet solution.

Application of the method to the marketed tablets

The developed and validated method has been applied successfully for determination of FVP in pharmaceutical formulations. The result of assay of the marketed tablet of favipiravir is shown in Table 6. The results obtained are closely related to the amount indicated on the labels of the tablets. This shows that the method for content evaluation is useful.

CONCLUSIONS

A very quick, cost-effective, precise and accurate HPLC method for the determination of FVP has been developed

and validated in compliance with ICH guidance Q2. Besides the short run time (15 min), retention time (7.696) and flow rate of mobile phase (1 mL min⁻¹) made the method attractive because these features save analysis time and cost. Potassium dihydrogen phosphate, used as a general purpose buffer, has many interesting properties. The most important of these features are good buffering capacity in the selected pH range, easy availability, low toxicity and cost, and greatly improved separation ability without column degradation. In short, this method is sensitive, selective, reproducible and rapid for favipiravir in bulk and tablets. The accuracy and precision are within reasonable limits, the maximum of quantification is as small as 3.60 µg mL⁻¹ and finally analytical method is reliable and robust.

ACKNOWLEDGEMENTS

The author would like to thank to Atabay Pharmaceuticals and Fine Chemicals Inc. for providing pure drug reference sample and supporting this work.

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