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ORIGINAL ARTICLE

# LC-MS/MS Method Development and Validation for Determination of Favipiravir Pure and Tablet Dosage Forms

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## ABSTRACT

**Objectives:** Analytical method development and validation for determination of favipiravir (FVPR) in pure and tablet dosage forms by liquid chromatography with tandem mass spectrometry/mass spectrometry (LC-MS/MS) technique.

**Materials and Methods:** A simple LC-MS/MS method was developed for determination of a new antiviral drug, FVPR in pharmaceutical formulations. The stationary phase employed was a Shim pack GISS, $C_{18}$ (100 mm × 2.1 mm, 1.9 µm) column and mobile phase used in pump A was 10.0 mM ammonium acetate and in pump B methanol was used. The gradient program was used with fixed mobile phase flow rate at 0.4 mL $min^{-1}$. Total run time was 5.0 min. The proposed method was validated according to International Conference on Harmonization (ICH) guidelines. The established method found better outcomes.

**Results:** The linearity graph was found in the range of 50-200 µg/mL and the correlation coefficient value ($R^2$) obtained was found to be 1.0. The limit of detection (LOD) and limit of quantification (LOQ) were 4.044 µg/mL and 12.253 µg/mL, respectively. Tremendous recovery outcomes were observed and found to be 101%, 99.0%, and 99.5% for FVPR at 150% upper, 100% middle, and 50% lower concentrations, respectively.

**Conclusion:** All outcomes obtained comply with ICH guidelines. The developed method was simple, unique, accurate, robust, precise, and reproducible for determination of FVPR in tablet formulation. The method is novel and could be adopted in formulation industry.

**Key words:** Favipiravir, LC-MS/MS, method development, method validation, quantification

## INTRODUCTION

Favipiravir (FVPR) is an antiviral drug used for the treatment of all three types of influenza A, B, and C.[1] The International Union of Pure and Applied Chemistry (IUPAC) name of FVPR is 6-fluoro-3-hydroxy-2-pyrazine carboxamide with molecular formula $C_5H_4FN_3O_2$ (Figure 1). This is a pyrazine carboxamide derivative. The melting point is about 187 ˚C to 193 ˚C. It is sparingly soluble in water, but completely soluble in organic solvents such as ethanol, dimethyl sulfoxide, and dimethylformamide. FVPR is a prodrug that goes through intracellular ribosylation and phosphorylation into adynamic form of FVPR ribofuranosyl-5'-triphosphate, inhibits viral RdRp (RNA-dependent RNA polymerase) and, can bind, transcript, replicate the viral genome, and thereby inhibit the viral RNA polymerase.[2-4] Despite being vital against influenza, it was also revealed that FVPR exhibits antiviral activity against alpha-, filo-, bunya-, arena-, flavi-, and noroviruses and currently coronavirus disease-2019 (COVID-19).[5,6]

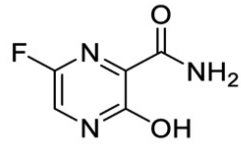


**Figure 1.** Structure of FVPR
FVPR: Favipiravir

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Literature survey revealed that few analytical methods are reported for determination of FVPR. A chromatographic separation method using high performance liquid chromatography (HPLC) with a runtime of 60 min was reported.[7,8] A liquid chromatography with tandem mass spectrometry/mass spectrometry (LC-MS/MS) method reported for the bioanalysis of antiviral drug FVPR in human plasma.[9] Quantification of FVPR in pharmaceutical formulations by HPLC-ultraviolet (UV) method, in which the total run time showed 15 min and limit of detection (LOD) and limit of quantification (LOQ) concentrations were in mg/mL.[10] HPLC and spectrofluorimetric methods were developed by Mikhail et. al.[11] for determination of FVPR. In the HPLC method, FVPR peak was eluted at 4.0 min and total run time was 10.0 minutes.[11] A pharmacokinetics method was developed by Nguyen et al.[12] for estimation of FVPR in Ebola-infected patients. The spectrofluorimetric method has been developed by Megahed et al.[13] for determination of FVPR and quantified in human plasma. An LC-MS/MS method was reported for determination of multiple antiviral drugs.[14] Another LC-MS/MS method was reported for quantification of FVPR in human plasma.[15,16] A RP-HPLC method was reported for determination of FVPR in spiked human plasma.[17] LOQ of reported method was 0.72 µg/mL, where the linearity range was 0.2 µg/mL to 3.2 µg/mL. In the proposed method, the results were obtained in µg/mL. Another LC-MS method has been developed for the assay of FVPR in human plasma, LOQ of the method was found to be at 80 µg/mL, where the linear range reported was between 80 µg/mL to 30000 µg/mL.[18] The sensitivity of this method is quite more than the proposed method. An LC/LC-MS method[19] was reported in the literature for the determination of FVPR. This method is quite different from the proposed method. In this method, the route of degradation mechanism and degradation impurities are studied. LOD and LOQ of the method were 0.09 µg/mL and 0.027 µg/mL, respectively. The results of the reported methods are tabulated in Table 1.

**Table 1. Comparison of the statistical data of the reported methods and proposed methods**

| Ref. no. | Analytical method | Results | Remarks |
|---|---|---|---|
| 7 | HPLC | LOD - 0.2 µg/mL<br>LOQ - NA | Analyzed related substances of FVPR |
| 8 | HPLC | LOD - 0.2 µg/mL<br>LOQ - NA | Analyzed related substances of FVPR |
| 9 | LC-MS/MS | LOD - NA<br>LOQ - 100 µg/mL | FVPR determined in human plasma |
| 10 | HPLC-UV | LOD - 1.20 µg/mL<br>LOQ - 3.60 µg/mL | Different mobile phase used, mixture of 50 mM potassium dihydrogen phosphate (pH 2.3) and acetonitrile (90:10, *v/v*) |
| 11 | HPLC and spectrofluorimetric | LOD - 0.985<br>LOQ - 2.986 | FVPR determined in human plasma samples<br>Mobile phase used as 0.02 M Brij-35, 0.15 M sodium dodecyl sulfate, and 0.02 M disodium hydrogen phosphate, pH 5.0 |
| 12 | Parmacokinetics | LOD - NA<br>LOQ - NA | Other than HPLC-UV method developed |
| 13 | Spectrofluorimetric | LOD- 9.44 µg/mL<br>LOQ- 28.60 µg/mL | Other than HPLC-UV method developed |
| 14 | LC-MS/MS | LOD - 25990 µg/mL<br>LOQ - NA | FVPR determined in human serum |
| 15 | LC-MS/MS | LOD- NA<br>LOQ- 60 µg/mL | FVPR identified in human plasma |
| 16 | LC-MS/MS | LOD - NA<br>LOQ - 0.062 µg/mL | FVPR estimated in human serum |
| 17 | LC-MS/MS | LOD - NA<br>LOQ - 0.72 µg/mL | FVPR spiked in human plasma |
| 18 | LC-MS/MS | LOD - NA<br>LOQ - 80 µg/mL | FVPR determined in human plasma |
| 19 | LC-MS/MS | LOD - 0.09 µg/mL<br>LOQ - 0.027 µg/mL | Determine impurity of FVPR and degradation rout mechanism |

NA: Not available, HPLC: High-performance liquid chromatography, LOD: Limit of detection, LOQ: Limit of quantification, FVPR: Favipiravir, LC-MS/MS: Liquid chromatography with tandem mass spectrometry, UV: Ultraviolet

Keeping the drawbacks of the reported LC-MS methods in mind, we developed and validated the LC/MS-MS method for determination of FVPR in pure and tablet dosage forms. In the proposed LC-MS/MS method, total run time was 5.0 min and the FVPR peak was eluted at 1.9 min. LOD and LOQ concentrations were found in $\mu g/mL^{-1}$ concentrations. Hence, the proposed method is more sensitive than other reported methods. These results clearly indicate that the established method is simpler, accurate, reproducible, and robust than the reported methods.

## MATERIALS AND METHODS

*Instruments*

Shimadzu prominence HPLC and LCMS-8045 instruments were used for the proposed method development and validation for determination of FVPR. HPLC instrument consisted of a deuterium lamp as the source of light, a UV detector, a quaternary pump, and an auto-injector. MS/MS system used was Shimadzu LCMS-8045, which achieves both high sensitivity and ultra-high-speed detection, outfitted by a heated electrospray ionization (ESI) probe. LCMS-8045 has maximum sensitivity in its category, which is designed to maximize sensitivity and minimize contamination by high-temperature heating block, heated ESI probe, drying gas, and heated desolvation line. The Lab Solutions software was used for the analysis and interpretation of data. All these factors provide robust instrumentation for the determination of FVPR.

*Chemicals and reagents*

More than 98% purity of FVPR pure drug was provided by Karnataka Antibiotics and Pharmaceuticals Ltd. (Bengaluru, India) as a gift sample. FVPR tablets (label claim, 200 mg, commercial name-Avigan-200 mg, manufacturer-Dr. Reddy's Lab Ltd., India) were commercially purchased from local medical shops. HPLC-grade methanol and ammonium acetate were procured from Merck Ltd. (India). HPLC-grade ultrapurified water by Millipore purifier instrument was employed in the study. The stationary phase used was Shim-pack GISS, column ($C_{18}$, 2.1 x 100 mm, and 1.9 µm) was obtained from Shimadzu Ltd. (Japan).

*Mobile phase preparation, standard stock solution and dilutions*

The mobile phase consisted of 0.1 M ammonium acetate buffer of pH 6.5 in pump A and methanol in pump B. The standard stock solution of FVPR was prepared by dissolving accurately weighed 100 mg of FVPR into a 100 mL standard volumetric flask and made up to the mark with mobile phase. The prepared standard stock solution was of the concentration of 1000 µg/mL. From the above stock solution 1.0 mL was pipetted out into another 1000 mL standard volumetric flask and made up to the mark with the mobile phase. The concentration of resulting working standard solution was 10 µg/mL. Similarly, working standard solutions of different concentrations of FVPR were prepared from least to maximum dilutions to examine the parameters of interest such as linearity, accuracy, recovery, LOD, and LOQ of the proposed method. For the assay analysis, the test sample weights were taken according to the standard equivalent and the following formula was used for the determination of test sample weights:

$$\text{Sample weight} = \frac{\text{Standard weight x Average weight of 20 tablets}}{\text{Label claim of 1 tablet}}$$

*Chromatographic conditions*

In method development, chromatographic conditions play an important role. The mobile phase consists of 10 mM ammonium acetate buffer of pH 6.5 in pump A and methanol in pump B, followed by a gradient program, as in Table 2. The stationary phase used was a Shim-pack GISS column. The flow rate of mobile phase was fixed at 0.4 mL/min. Column oven temperature was kept at 40 °C and wavelength of detection was fixed at 323 nm throughout the method development and validation. Sample injection volume was fixed at 10 µL. With these chromatographic conditions, FVPR sharp peak was eluted at 1.9 min. Total run time was fixed at 5.0 min.

*Mass spectroscopy conditions*

MS/MS system used was Shimadzu LC-MS-8045 consisting of heated ESI probe high-temperature gas supplements the nebulizer gas which improves the the desolvation efficiency. This facilitates the ionization of various compounds. High-voltage power supply for polarity switching, which assists fork ultrafast scan speed (30,000 u/s) and maintains a polarity switching time of 5 ms. High-speed acquisition benefits the laboratory by reducing run times for increased throughput and shortening the method development time. The system is designed to be robust. The heated desolvation line, high-temperature heating block, heated ESI probe, drying gas, and center optics all proceed to minimize contamination and maximize sensitivity. Lab Solutions software was used to analyze the complete method development and validation for the determination of FVPR and offers the latest features designed to streamline workflows and allow analysis to be started without long hours of method establishment.

**Table 2. Mobile phase gradient program**

| Time in minutes | Pump A (10 mM ammonium acetate of pH 6.5) | Pump B (methanol) |
|---|---|---|
| 0.01 | 90 | 10 |
| 2.00 | 40 | 60 |
| 3.10 | 90 | 10 |
| 5.00 | 90 | 10 |

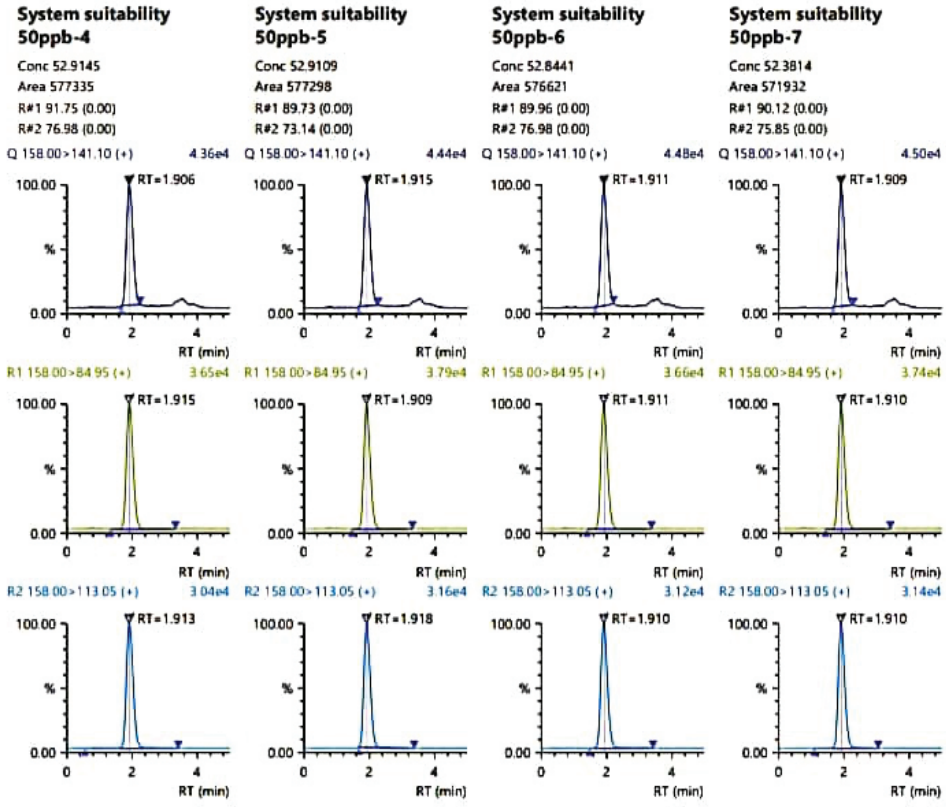


**Figure 2.** LC chromatograms of FVPR
LC: Liquid chromatography, FVPR: Favipiravir

The mass spectrometer with ESI probe operated in positive polarity, the data acquisition and processing were performed using Lab Solutions software. The distinguishing working conditions were as follows: nebulization gas flow was fixed at 3 L min, heating gas flow was kept constant at 10 L min, interference temperature was fixed at 150 °C, heat block temperature was kept constant at 300 °C, and drying gas flow was fixed at 10 L min. These conditions were maintained for mass spectrometer throughout the method development and validation process.

## RESULTS

*Method development*

Mobile and stationary phases play an important role in the proposed method development and validation for determination of FVPR. The mobile phase was balanced by analyzing different trials with various mixtures of solution A (pump A) and solution B (pump B) followed by gradient time programs. FVPR sharp peak was eluted after various trials in mobile phase ratios with a gradient time program. The expected peak was not eluted suitably after analyzing different ratios of pump A and B mobile phases. Hence, pump A mobile phase was replaced by 10 mM ammonium acetate of pH 6.5 and methanol in pump B. The wavelength of detection was fixed at 323 nm. Gradient time program was fixed as shown in Table 2. Under these conditions, FVPR sharp peak was eluted with a good baseline

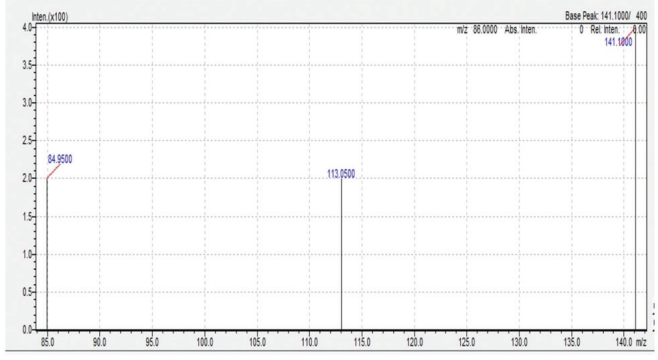


**Figure 3.** Mass spectrum of FVPR
FVPR: Favipiravir

in chromatograms as in Figure 2. In the mass spectrum, three peaks were observed *m/z*: 84.95, *m/z*: 113.05, and *m/z*: 141.1 (Figure 3). Hence, for the whole method development and validation, pump A was used for 10 mM ammonium acetate of pH 6.5 and pump B for methanol followed by the gradient time program as mentioned in Table 1. With these various trials, good peaks were observed in both chromatogram as well as mass spectrum and approximate fragment structures (ionized ion fragments) found and revealed in Figure 4. All the parameters of the proposed method were in compliance with the International Conference on Harmonization (ICH) guidelines.[20]

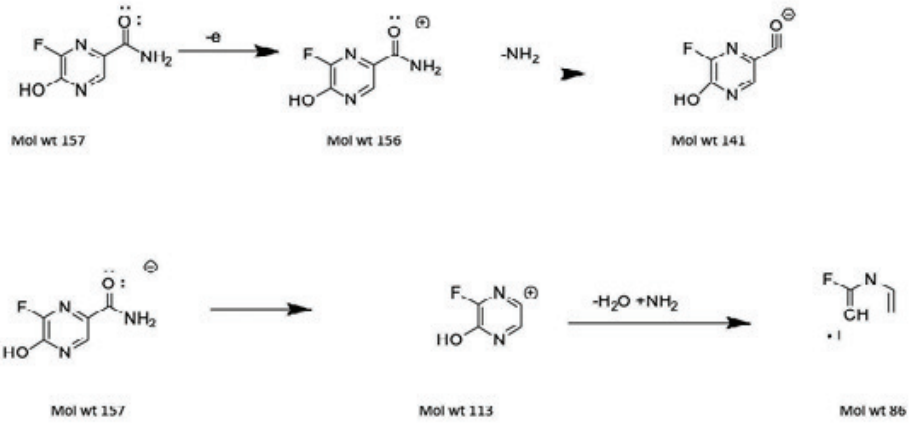


**Figure 4.** Fragmentation pattern of FVPR
FVPR: Favipiravir

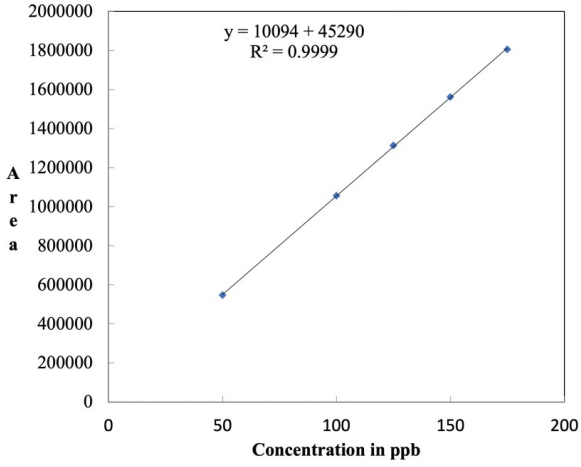


**Figure 5.** Linearity graph of FVPR
FVPR: Favipiravir

*Method validation*

*Linearity*

In the proposed LC-MS/MS method, five concentrations between 50 and 200 µg/mL standard solutions of FVPR were injected and examined. The regression significance was found suitable ($R^2$: 1.0). Y= bX+C equation was used for determination of $R^2$ values. The linearity graph of FVPR was designed by different areas against different concentrations of FVPR solutions. The resultant graph revealed a straight line for the FVPR as shown in Figure 5. The outcomes indicated that the method could be analyzed at various concentrations. Hence the developed an LC-MS/MS method is supposed to be validated. The results are presented in Table 3.

*Precision*

In the proposed method, precision data were found to be excellent and in accordance with ICH guidelines. The outcomes were found to be precise and well within the range. On the same day and on different days, six separately spiked standard solutions and test solution were analyzed repetitively for the precision parameter. The intra- and inter-day performances were examined and outcomes revealed that there were not many deviations in the obtained results. The percentage of relative standard deviation of the test solution of six individual assay outcomes was found to be less than 2.0%. Therefore, it can be concluded that the developed LC-MS/MS method is precise. The results are revealed in Table 3.

**Table 3. Precision, LOD, LOQ, and linearity outcomes**

| Parameters | Results | Limit |
|---|---|---|
| **Precision** | | |
| Intraday | 0.09 RSD% | NMT - 2.0% |
| Interday | 0.05 RSD% | NMT - 2.0% |
| LOD | 4.044 µg/mL | - |
| LOQ | 12.253 µg/mL | - |
| **Linearity** | | |
| Range | 50-200 µg/mL | - |
| Slope (b) | 10122 | - |
| Intercept (c) | 42721 | - |
| The correlation coefficient ($R^2$) | 1.000 | $R^2$ - above 0.995 |
| Standard error of intercept | 5063.3606 | - |
| Standard deviation of intercept | 12402.650 | - |

LOD: Limit of detection, LOQ: Limit of quantification, RSD: Relative standard deviation, NMT: Not more than

*The LOD and LOQ*

Several methods for determining detection and quantification limits are described in ICH guideline.[20] These include visual assessment, signal-to-noise *(S/N)* ratio calculations, response standard deviation calculations, and calibration curve slope calculations.

**Table 4. Recovery and assay results**

| Parameter | Brand and label claim/table | Amount found mg/ tablet | Concentrations in % | Assay, % | Recovery, % | RSD% | Limit |
|---|---|---|---|---|---|---|---|
| **Assay (spiking FVPR)** | Avigan 200 mg | 201.0 | 100 | 100.5 | - | 0.02 | 98.0-102.0% |
| **Recovery** | - | - | 50 | - | 100.1 | 0.51 | |
| | | | 100 | - | 101.5 | 0.72 | |
| | | | 150 | - | 101.2 | 0.64 | |

RSD: Relative standard deviation, FVPR: Favipiravir

**Table 5. Robustness data of FVPR**

| Parameters | Actual | Low | High |
|---|---|---|---|
| Flow variation | 0.4 mL/min | 0.3 mL/min | 0.5 mL/min |
| Column temperature (°C) | 40 | 38 | 42 |
| RSD% | 0.9 | 1.1 | 1.3 |

RSD: Relative standard deviation, FVPR: Favipiravir

LOD and LOQ in the current study were determined using the third approach and were based on the 3.3 x (σ/m) and 10 x (σ/m) criteria, respectively. σ stands for the standard deviation of y-intercept of the regression line and m for the slope of the calibration curve. LOD and LOQ of the proposed method were found to be 4.044 µg/mL and 12.253 µg/mL, respectively. These results indicated that the method was very sensitive for the determination of FVPR. The results are tabulated in Table 3.

*Recovery*

In the recovery parameter, data was accomplished by three different concentration solutions of FVPR; lower, middle, upper, and blank was spiked at 50%, 100%, and 150% against the standard solution. The results obtained fulfilled the ICH guidelines. Hence, the established method was excellent. The standard formula was used to calculate outcomes. The data of the recovery parameter was found satisfactory as shown in Table 4. The limit of recovery range accepted is 98-102%. The obtained outcomes were well within the range for all three different concentrations. Therefore, the recovery parameter indicated that the proposed method can be used in industry.

*Specificity*

Standard procedures were used for the assay of FVPR. The clear and separated peak was found in liquid chromatography and in the case of mass spectrometer there were three peaks eluted at *m/z* 84.95, *m/z* 113.05, and *m/z* 141.1, respectively. When injected these solutions separately, the consistent retention time and *m/z* obtained for both the standard (working standard) as well as test solution (formulation) were found to be between 98.0% and 102.0%. Thus, assay data were complying with the ICH guidelines. It was also observed that there was no probable excipient peak interference for the determination of FVPR. The following excipients were used for the specificity parameter: microcrystalline cellulose, starch, magnesium stearate, lactose monohydrate, micropowder silica gel, and magnesium sulfate. These excipients did not interfere during the assay of FVPR using the LC-MS/MS method. Therefore, the proposed method revealed specificity for FVPR assay. Results of the assay were found to be satisfactory and are displayed in Table 4.

*Robustness studies*

The robustness parameter contains deliberate changes in the developed method. The known concentration of the standard solution of FVPR was injected at different conditions, *i.e.*, column oven temperature was changed from 40 °C to 35 °C and 45 °C and flow rate variation in the mobile phase ranging from 0.3 mL/min to 0.5 mL/min. The results are exhibited in Table 5. The acquired outcomes were satisfactory and comply with the ICH guidelines. There were no many deviations in the overall results. Hence, the established method can be used under varying conditions. Thus, the established LC-MS/MS method is robust.

*Solution stability*

Solution stability of FVPR was studied up to 48 h by keeping the solutions at 8 °C. To study this parameter, the standard (50 µg/mL) as well as the test solutions from 0 h to 48 h were injected. The obtained assay results were found to be 101.5%, 101.1%, 99.8%, and 99.0% for the 0, 12, 24, and 48 h, respectively. On observing these data, it can be concluded that there was no much deviation in the area and the calculated assay values up to 0 h to 48.0 h. Since the FVPR solution stability results were found stable up to 48 h, the developed LC-MS/MS is stable for determination of FVPR.

## DISCUSSION

The critical literature survey (Table 1) exposed that there were not many analytical methods on LC-MS/MS for estimating FVPR in bulk and formulations. Most of LC-MS/MS methods report the analysis of FVPR in biomatrix including plasma and body fluids. Further, some LC methods used different stationary and mobile phases for determination of FVPR in bulk and formulation forms. The reported analytical methods for the determination of FVPR were less sensitive and took more time for the analysis. Hence, it was planned to develop a highly sensitive, simple, reproducible, rugged, and robust analytical method for the determination of FVPR in pure and pharmaceutical formulations. In the proposed method, LOD and LOQ values were found to be 4.044 µg/mL and 12.253 µg/mL, respectively, while linearity range was found between

50 µg/mL to 200 µg/mL for five concentrations ($R^2$: 1.0). The results of solution stability studies were found to fit well within the limit. Recovery and assay data were found to be acceptable and better than literature methods. The developed method was highly sensitive, simple, accurate, rugged, reproducible, and robust. The proposed method is novel and exclusive, which can be employed in industries for the routine analysis of FVPR. The proposed method overcomes most of the limitations of the reported methods. The proposed method is cost-effective. Total run time of the method was much less. Hence, the method is reliable for the rapid analysis of FVPR and can reproduce accurate and precise results for the formulation samples as well.

## CONCLUSION

Majority of the formulations of an antiviral drug has analytical methods for their determination, such as LC-MS/MS, HPLC, UPLC, and UV-spectroscopic methods. FVPR is an antiviral drug used to prevent COVID-19 and other influenza. We developed an analytical method and validated it by LC-MS/MS instrument. The established method was highly sensitive, reproducible, and rugged. Above all, all parameters outcomes were complying with the ICH guidelines. Thus, the proposed LC-MS/MS method exposed the determination of FVPR in bulk and formulations.

*Ethics*

**Ethics Committee Approval:** There is no requirement for ethical approval.

**Informed Consent:** Not applicable.

**Peer-review:** Externally peer-reviewed.

*Authorship Contributions*

Concept: M.D.H., N.I., Design: N.I., H.A., Data Collection or Processing: H.A., N.I., Analysis or Interpretation: H.A., N.I., Literature Search: N.I., B.C.Y., Writing: M.D.H., N.I.

**Conflict of Interest:** No conflict of interest was declared by the authors.

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