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Original Article

Development and Validation of a Sensitive, Fast and Simple LC-MS/MS Method for the Quantitation of Favipiravir Pure and Tablet Dosage Forms

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ABSTRACT

Objectives: The analytical method development and validation for the determination of Favipiravir (FVPR) in pure and tablet dosage forms by LC/MS-MS Technique. **Materials and Methods:** A simple LC-MS/MS method was developed for the determination of a new antiviral drug, FVPR in pharmaceutical formulations the separation process was conducted using a Waters X-Bridge Phenyl Hexyl column (150x4.6 mm, 3.5 µm). The elution method employed was isocratic, involving a buffer solution consisting of 1 mL of Formic acid in 1 Litter of water. The mobile phase consisted of a mixture of two components: the aforementioned buffer and Acetonitrile in a 60:40 v/v ratio. The elution was maintained at a flow rate of 1 mL/min, as well as the entire process was carried out at room temperature. The proposed method was validated according to the International Conference on Harmonization (ICH) guidelines. The established method found better outcomes. **Results:** The linearity graph was found in the range of 2-40 $ngmL^{-1}$, and the correlation coefficient value (R2) obtained was found to be 0.9997. The limit of detection and limit of quantification were 4.044 $ngmL^{-1}$ and 12.253 $ngmL^{-1}$, respectively. Tremendous recovery outcomes were observed and found to be 99.49%, 100.09.0% and 100.21% for the FVPR at 150% upper, 100% middle and 50% lower concentrations, respectively. For studying Favipiravir, an electrospray ionization source was employed, with ion pairs of mass analysis at m/z 560.28→158.63 for Favipiravir and m/z 607.33→193.48 for the Internal Standard (IS), which was Zanamivir in this case. **Conclusion:** All obtained outcomes were complying with the ICH guidelines. The developed method was simple, unique, accurate, robust, precise, and reproducible for the determination of FVPR in tablet formulation. The method is novel and could be adopted in the formulation industry.

Keywords: LC-MS/MS, Favipiravir, Zanamivir, Validation, Analytical method.

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INTRODUCTION

Favipiravir was first used against SARS-CoV-2 in Wuhan at the very epicentre of the pandemic. Favipiravir is aPyrazine carboxamide imitative with activity in opposition to RNA viruses. Favipiravir is changed to the Ribofuranosyl tri phosphate imitative by host enzymes and particularly obstructs the influenza viral RNA-dependent RNA polymerase.

Favipiravir, also recognized as Avigan, serves as an antiviral medication employed for treating Influenza in Japan. Ongoing research is exploring its potential as a remedy for various viral infections. It belongs to the pyrazinecarboxamide analogue class, similar to investigational antiviral agents like T-1105 and T-1106. Notably, evidence suggests that its use during pregnancy could harm the developing infant, as teratogenic and embryotoxic effects have been observed across four animal species. In Japan, Favipiravir has obtained approval for addressing influenza; however, its application is currently limited to emerging influenza strains associated with more severe illness, rather than seasonal influenza.[1,2]

Over the past few decades, only a handful of articles have tackled the determination of favipiravir using LC-MS/MS. Challenges such as lengthy runtime, intricate sample preparations, and the costliness of mobile phases were encountered in prior methods. Nonetheless, our newly developed approach adheres to USFDA guidelines and boasts a shorter runtime, heightened precision, reduced expenses. Our bioanalytical assay has proven successful

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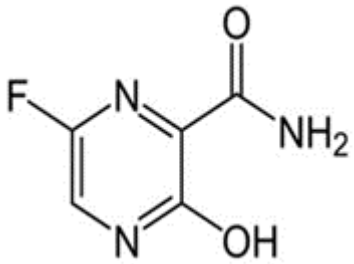
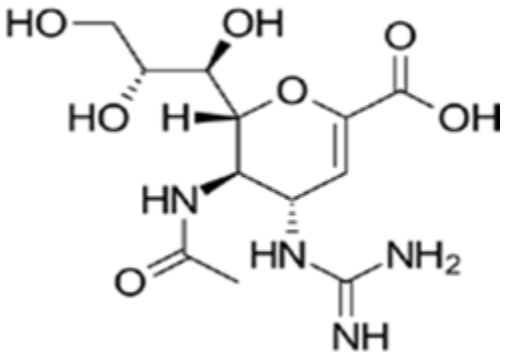
in pharmacokinetic studies of Favipiravir. Notably, despite these achievements, a groundbreaking method for determining Favipiravir remains absent.

The current study is crafted with the aim of thoroughly investigating and validating a precise MS/LC strategy for accurate quantification of Favipiravir within rat plasma. Additionally, the study seeks to evaluate the pharmacokinetics of this drug subsequent to intravenous administration of test extracts in rat subjects.



The chemical structure of Favipiravir[3]



The chemical structure of Zanamivir[4]

MATERIALS AND METHODS

Favipiravir, along with Zanamivir used as the internal standard, was procured from Zydus Cadila in Ahmedabad. Acetonitrile of LCMS Grade with a purity of 99.99%, Milli Q water, and HPLC-grade formic acid with a purity of at least 99.0% was a gift sample from Merck (India) Situated, Mumbai.[5]

Instruments and Conditions

The QTRAP 5500 triple quadrupole mass spectrometer (SCIEX) and the Waters Alliance Model LC system were used to create a bioanalytical test. The Waters X-Bridge Phenyl Hexyl column (150x4.6 mm, 3.5 m) was used to produce chromatographic separation using an isocratic technique at room temperature. A 60:40 v/v mixture of buffer and acetonitrile flowed through the mobile phase at a rate of 1.0 mL/min. 5 min of total runtime and a 10 L injection volume were used. Mass spectrometry was carried out in Multiple Reaction Monitoring (MRM) mode, monitoring the ion pairs of mass: m/z 560.28→158.63 for analyze and m/z 607.33→193.48 for the Internal Standard (IS). For more detailed information on the instrumentation requirements, the results are shown in Table 1.

Top of Form

Experiment

Stock Preparedness, Calibration and Quality Control Specimens

Inventory answers have been prepared using Favipiravir at an awareness of 120 ng/mL and Zanamivir at a concentration of 120 pg/mL. These solutions have been dissolved in a mixture of Formic acid (0.1%) and Acetonitrile (60:40, v/v). The linearity of the calibration curve changed into established inside the variety of 2-40 ng/mL for Favipiravir. For the calibration and nice control samples, the aforementioned inventory answers have been diluted and then mixed with clean plasma. The calibration set blanketed 8 one-of-a-kind concentrations: 2, 5, 10, 15, 20, 25, 30, and 40 ng/mL of Favipiravir. In addition, first-rate manipulate (quality controls) samples were organized with concentrations of two. 2 ng/mL (decrease restrict of quantification-LLOQ), 10 ng/mL (Lower great manipulate-LQC), 20 ng/mL (Medium satisfactory manipulate-MQC), and 30 ng/mL (high great control-HQC) of Favipiravir. All of the prepared specimens were stored at a temperature of -20°C and have been delivered lower back to ambient temperature earlier than undergoing a complete investigation.[6,7]

Preparation of Solution for Plasma Samples

To prepare the samples, two hundred µL aliquots of rat plasma sample have been combined with 500 µL of the Internal preferred (IS) running answer. Subsequent this, 300 µL of Acetonitrile turned into brought and the mixture changed into vortexed for 15 min. The samples have been then centrifuged at 5500 rpm for 15 min, ensuing inside the separation of the supernatant answer. This supernatant become cautiously accrued, divided, and subjected to filtration the usage of a zero. 45 µ nylon syringes filter right into a vial. The filtered solution was then injected into the UPLC gadget for analysis.[8]

RESULTS

Selectivity, Matrix Effect and Recovery

Selectivity was assessed by analyzing plasma samples from six batches of dissimilar rats to check for any interference from unidentified substances during the retention times of Favipiravir and the Internal Standard (IS). The impact matrix for Favipiravir was calculated by comparing the proportion of peak areas in post-extracted plasma samples from six distinct drug-free plasma samples and spiked recuperation samples. This experiment was performed in triplicate, using six different sets of plasma samples at the Medium Quality Control (MQC) level, with an acceptable

accuracy (percent coefficient of variation, CV) of ≤15%.To determine the extraction efficiencies of Favipiravir, the process was repeated six times at each QC concentration.[9,10]

Dilution Integrity

To assess dilution integrity, a matrix with an analyte concentration above the Upper Limit of Quantification (ULOQC) was spiked and then diluted with blank matrix. This process ensures that the dilution procedure maintains the accuracy and precision of the analysis.[11]

Carry Over

Carry over refers to the presence of a substance retained by the chromatographic system from a previous sample injection, which subsequently appears in subsequent blank or unknown samples.[12]

Bioanalytical Method Development

In this approach, Electrospray Ionization (ESI) was chosen due to its significantly higher reactivity compared to Chemical Ionization by Atmospheric Pressure (APCI). For quantification of favipiravir ions, the Multiple Reaction Monitoring mode was employed. The ion pair scan for Favipiravir produced an intense reaction at m/z 158.63. Similarly, the internal standard, Zanamivir, exhibited high-intensity daughter ions of $[M+H]^+$ at m/z 159.63 and m/z 333.65, with an ion pair scan at m/z 332.65. Interestingly, favipiravir exhibited a robust positive ion response mode, outperforming its ion-negative mode counterpart. This finding underscores the efficacy of the selected positive ionization mode for detecting and analyzing Favipiravir ions. The result was shown in Figure 1.

To establish the foremost chromatographic conditions, a scientific approach turned into followed, related to the evaluation of various buffer mixtures with Acetonitrile as the cell phase in each isocratic and gradient modes. Each trial changed the cellular section composition to improve decision and attain suited retention times. In the long run, a mobile segment comprising 0.1% formic acid and ACN in an isocratic mode at a 60:40 v/v ratio turned into selected. This mixture yielded the highest reaction for the chosen drugs.[13]

During the optimization system, various desk bound stages inclusive of C_{18}, C_8, and CN-propyl have been tested. The X-Bridge Phenyl Hexyl column, with dimensions of 150 mm x 4.6 mm and particle length three. 5 µ, connected to a PDA detector, exhibited favourable top shapes for Favipiravir. The cell phase turned into brought at a drift fee of 1 mL/min. Below those conditions, the retention instances for Favipiravir and Zanamivir have been determined as 2.818 min and 3.919 min, respectively.[14]

The method demonstrated excellent precision, with a % Coefficient of Variation (% CV) of 0.45 from six replicate injections, indicating high specificity of the proposed strategy. The ongoing method has undergone validation in accordance with the regulations set by (USFDA). For further information on Favipiravir, additional articles can be found in the references provided below.[15] The results are shown in Figure 2.

Matrix Effect and Recovery

The rat plasma was examined for their impact on Favipiravir, revealing no significant effects. The results suggest that both the ionization and internal standards remained consistent across various plasma sources, falling within acceptable limits. Recovery assessment for Favipiravir was conducted at low (10 ng/mL) and (30 ng/mL) concentration levels in rat plasma. These findings highlight the extraction efficiency of favipiravir.

Linearity, Precision and Consistency

The region exhibiting high response levels adhered closely to the calibration standards. The method's linearity range for favipiravir

Table 1: Optimized LCMS Conditions.

LC parameters		MS parameters	
LC	**Waters Alliance**	**MS**	**Sciex QTRAP 5500**
Isocratic step mobile	ACN: Formic acid 0.1% in water 40:60 v/v.	Ionization source	Drying gas: N_2 gas Drying stream: 5 mL/min. Pressure: 55 psi.
	Stream: 1 mL/min.		Source temp: 550°C
	Volume of injection:10 µL.		Capillary voltage:5500 V.
Waters X-Bridge Phenyl Hexyl	150 mm	Collision cell gas	High purity of Nitrogen.
	4.6 mm ID	Mode	MRM
	3.5 µm PS		
Analyte	Favipiravir	Favipiravir MRM transitions.	m/z-560.28 m/z158.63 CE-15V.
Internal standard	Zanamivir	Zanamivir MRM transitions.	m/z-607.33 m/z 193.48 CE-15V.

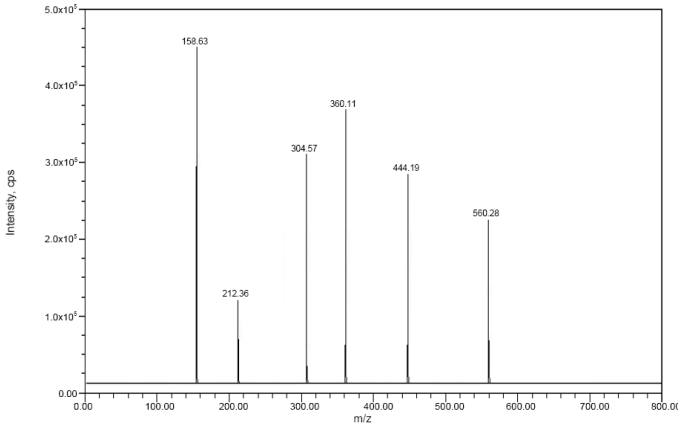


Figure 1a: MS Spectra of Favipiravir.

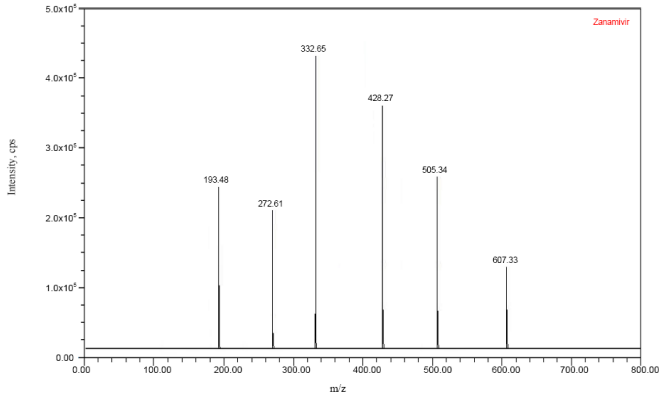


Figure 1b: MS Spectra of Zanamivir (IS).

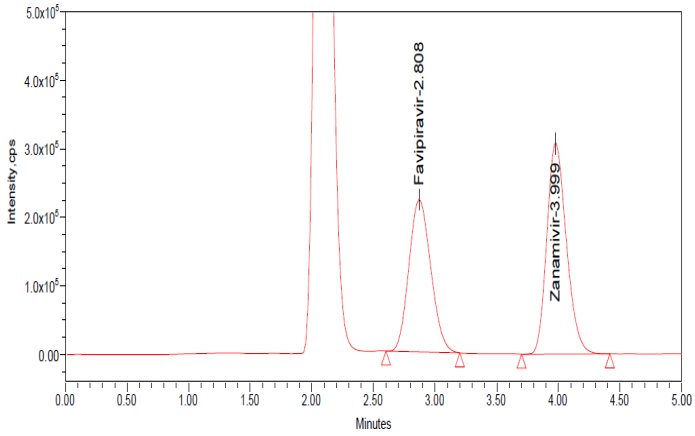


Figure 2a: Chromatogram of System precision.

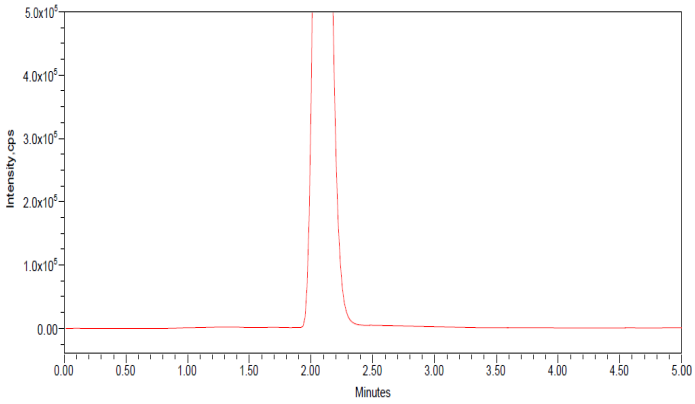


Figure 2b: Chromatogram of blank.

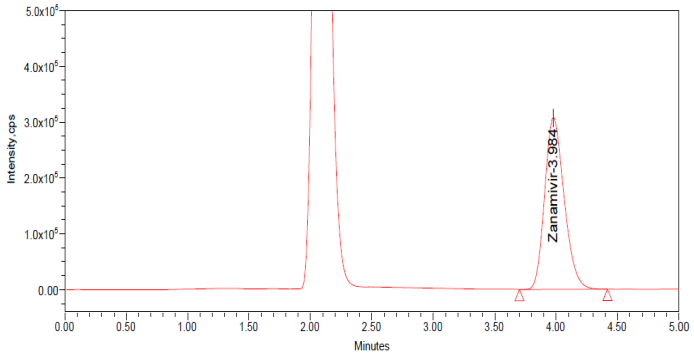


Figure 2c: Blank rat plasma spiked with internal standard.

spanned from 2-40 ng/mL. Calibration curves were generated across this linear concentration range, yielding correlation coefficients exceeding 0.9997 for Favipiravir at various Quality Control (QC) levels. The linearity and correlation outcomes for Favipiravir can be observed in Tables 3 and 4, with the respective calibration curves displayed in Figure 3.

Precision and accuracy were meticulously monitored through the amalgamation of diverse QC specimen test outcomes. The specific findings for quality control samples of Favipiravir exhibited a range of 96.6% to 101.75%. Furthermore, the Coefficient of Variation (%CV) for Verapamil and Trandolapril remained below 5% across all concentration levels for quality control samples.[16] notably; all precision and accuracy outcomes fell within the established quantification threshold. Elaborated results can be perused in Table 2.

Dilution Integrity and Carry Over

Dilution integrity was established by introducing the analytic matrix into the Upper Limit of Quantification (ULOQC) and subsequently diluting this sample with a blank matrix. This assessment of dilution reliability was carried out at twice the ULOQC level (40 ng/mL for Favipiravir). The Coefficient of Difference (%CV) for Favipiravir maintained itself well within the acceptable limits, thereby confirming the dependability of the procedure.

Carryover, a form of method error capable of impacting the considered value of a sample, was investigated through the following procedure using LC/MS system. Employing the injection technique, a machine blank injection volume of 10 µL containing 0.1% Formic acid and Acetonitrile (60:40) was administered on the Waters Z-spray triple quadruple mass detector. Through this methodology, it can be deduced that the accuracy and precision of the proposed approach remained unaltered.[17]

The carryover assessment for Favipiravir samples yielded the following results: LLQC (4.21%) and ULQC (0.22%) for one set, and LLQC (3.69%) and ULQC (1.54%) for another set, all of

Table 2: Precision and accuracy results of Favipiravir in rat plasma.

LLOQ	$0.251x10^5$	$0.255x10^5$	0.0172	96.6%	6.92
LQC	$1.255x10^5$	$1.248x10^5$	0.0142	101.75%	1.14
MQC	$2.529x10^5$	$2.534x10^5$	0.0210	100.12%	0.83
HQC	$3.742x10^5$	$3.749x10^5$	0.0246	99.48%	0.66

Table 3a: Results of Dilution Integrity.

Analyte	ULOQC Conc.	Calculated Conc.	%CV
Favipiravir	40 ng/mL	40.23 ng/mL	3.67

Table 3b: Results of carry over.

Concentration	% of Carry over
	Favipiravir
Blank	0
LLOQC	3.69
ULOQC	1.54

which fell within acceptable limits. Comprehensive information regarding the carryover effects is presented in Table 3.

Re-Reproducibility

In the course of analyzing actual subject samples, the reproducibility of re-injections was conducted to validate the system's performance subsequent to the dissolution of the solid Dosage form due to instrumental malfunction. The observed shifts in levels at the Lower Quality Control (LQC) and Higher Quality Control (HQC) concentrations remained below 2.0. Consequently, during the examination of genuine subject specimens, in cases where instrument malfunction occurred, the sample group was re-infused following the preparation of new samples. These re-injected samples, administered after a 24 hr interval, exhibited a percentage change of under 2.0% at both LQC and HQC levels. This underscores that, in instances of instrument failure during authentic specimen analysis, the lot can be re-injected following a 24 hr interval.[18,19]

Stabilities

The benchtop stability evaluation of Favipiravir encompassed various storage conditions. For the stock solution, it was stored at room temperature for 18 hr. Auto sampler stability was tested by storing the stock solution in the auto sampler at room temperature for 24 hr, revealing consistent stability behaviour under these circumstances.[20]

Freeze-thaw stability assessment involved storing the stock solution for 24 hr at (-28±5)ºC. Wet extract stability was determined by storing the stock solution for 18 hr at 2-8ºC, while dried up extort stability involved storage for 18 hr at (-20±3)ºC. In short-term stability evaluations, the drugs were stored for 7 days at (5±3)ºC. Long-term stability investigations extended to storing the stock solution for 28 days at (-20±3)ºC before injecting it into the LC-MS/MS system. Comparing the stability outcomes of freshly prepared stock solutions with those prepared before 24 hr, a percentage change of 1.59% for Favipiravir was observed. This indicates that the solutions remain stable for up to 24 hr. Under room temperature conditions, Favipiravir exhibited stability in plasma across different scenarios.[21] The assessment revealed that the stability of Favipiravir was unaffected by multiple cycles of freezing and thawing in plasma specimens at LQC, MQC, and HQC levels. Additionally, the long-term stability analysis demonstrated that Favipiravir remains stable at a sub-zero temperature of -30ºC for up to 24 hr. The comprehensive stability results for Favipiravir are presented in Table 4.

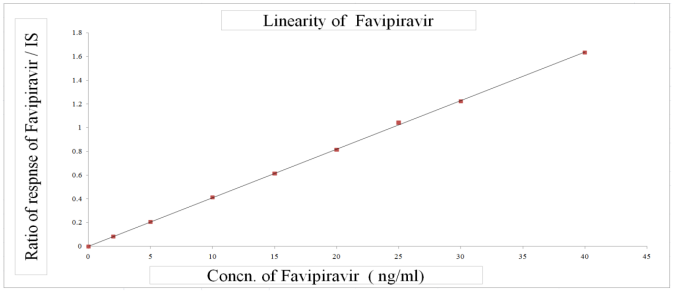


Figure 3: Calibration plot of Favipiravir.

DISCUSSION

The selected method demonstrated effective chromatographic separation and successful mass transitions, utilizing a concentration of acetonitrile and 0.1% formic acid buffer (60:40 v/v) to account for its volatility, a requirement in mass spectrometry analysis. An X-bridge phenyl hexyl column (150 mm x 4.6 mm, 3.5 µ) was maintained at 30ºC to ensure column efficiency. The linear regression model was employed to determine the best-fit linearity curve for the relationship between chromatographic response and concentration. The precision and accuracy data for Favipiravir assessed both within the same day and across different days, fell within acceptable limits. Benchtop stability experiments were performed by leaving standard stock solutions of Favipiravir and Zanamivir (IS) on the bench for up to 18 hr. Comparisons with newly prepared stock solutions at LQC and HQC levels indicated % accuracy within the 85-115% range. Long-term stability evaluations spanned 28 days, with initial concentrations compared to those of LQC and HQC levels.

Table 4: Stability results of Favipiravir in plasma under different storage conditions.

Stability	Storage condition	Conc. level	Measured conc (ng/mL) (Mean±SD, *n*=6)	% RSD	% Recovery
Bench top stability.	18 hr at room temp.	10	10.265±2.6	3.75	100.95
		20	20.415±1.4	1.46	99.56
		30	30.239±0.8	1.03	100.06
Auto sampler stability.	24 hr in auto sampler at room temp.	10	10.742±1.4	2.64	99.32
		20	20.165±3.3	1.11	101.47
		30	30.298±2.3	0.82	99.58
Long term stability.	28 days at (-20±3)°C.	10	10.295±2.6	0.72	83.77
		20	20.547±2.8	0.32	85.84
		30	30.269±4.2	0.13	84.67
Freeze thaw stability.	24 hr at (-28±5)°C.	10	10.243±2.1	1.63	99.16
		20	20.857±3.5	0.62	100.52
		30	30.547±4.3	0.68	99.38
Wet extract stability.	18 hr at 2-8°C.	10	10.458±2.2	2.5	98.84
		20	20.432±3.8	1.31	101.44
		30	30.196±5.7	0.91	100.09
Dry extract stability.	18 hr at (-20±3)°C.	10	10.274±2.6	1.72	99.01
		20	20.539±2.8	1.36	101.12
		30	30.623±5.1	0.87	100.04
Short term stability.	7 days at (5±3)°C.	10	10.765±1.7	4.62	91.26
		20	20.334±0.9	0.31	96.33
		30	30.754±2.6	0.2	96.05

The % accuracy in these assessments also remained within the acceptable 85-115% limits.

The reported analytical methods for the determination of FVPR were less sensitive, and takes 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, the LOD and LOQ values were found to be 4.044 $ngmL^{-1}$ and 12.253 $ngmL^{-1}$, respectively and the linearity range was found between 50 $ngmL^{-1}$ to 200 $ngmL^{-1}$ for the five different concentrations (R2=1.0). The results of the solution stability studies were found well within the limit. The recovery and assay data were found to be acceptable and better than the 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 the industries for the routine analysis of FVPR. The proposed method overcomes most of the limitations of the reported methods. This proposed method is cost-effective. The total run time of the method was much less. Hence the method is reliable for the rapid analysis of FVPR and can reproduce the accurate and precise results for the formulation samples also.

Stability studies were conducted to assess the impact of various conditions. Freeze thaw stability tests, involving freezing at -30°C and subsequent thawing for 3 cycles, exhibited results within the acceptable range of 85-115%. Autosampler sample stability assessments conducted at 20°C for 70 hr, at both LQC and HQC concentration levels, displayed mean % accuracy within the 85-115% limits.

CONCLUSION

An efficient, highly sensitive, and economical bio-analytical approach for the LC-MS/MS assessment of Favipiravir in rat plasma has been developed and effectively implemented. The majority of the formulations of an antiviral drug have 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 the method by the LC-MS/MS instrument. The established method was highly sensitive, reproducible and rugged. Above all, all the parameters outcomes were complying with ICH guidelines. Thus, the proposed LC-MS/MS method has exposed the determination of FVPR in bulk and formulations.

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CONFLICT OF INTEREST

The authors declare that there was no conflict of interest.

ABBREVIATIONS

LC-MS/MS: Liquid Chromatography Mass Spectrometry; **ICH:** International Conference on Harmonisation; **DPs:** Degradant Products; **MSn:** Multi Stage Mass Fragmentation Studies; **BTK:** Bruton Tyrosine Kinase; **MCL:** Mantle Cell Lymphoma; **RP-HPLC:** Reverse Phase High Performance Liquid Chromatography; **aOH:** Sodium Hydroxide; **HCl:** Hydrochloric Acid; **MeOH:** Methanol; **ACN:** Acetonitrile; **H_2O_2 :** Hydrogen Peroxide; **mg:** Milligram; **mL:** Milliliter; **ESI:** Electrospray Ionization; **CUR:** Curtain Gas; **IS:** Ion spray Voltage; **GS1 and GS2:** Ion source gas; Polytetrafluoroethylene; **LOD:** Limit of Detection; **LOQ:** Limit of Quantitation; **MRM:** Multiple Reaction Monitoring; **R.S.D:** Relative Standard Deviation; **SD:** Standard Deviation.

SUMMARY

As per the findings of the study for the chosen drug, sample recoveries in all formulations were in line with the claims made on each drug's label, and the suggested LC-MS/MS method method was found to be accurate, sensitive, quick, and affordable for the detection of Favipiravir Pure and Tablet Dosage Forms pharmaceutical formulations. Satisfactory findings were obtained after validating the suggested approach in accordance with guidelines of ICH and comparing the acquired values with the standard values. As a result, the technique can be used to determine the Favipiravir Pure and Tablet Dosage Forms.

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