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RESEARCH ARTICLE

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Favipiravir (SARS-CoV-2) degradation impurities: Identification and route of degradation mechanism in the finished solid dosage form using LC/LC–MS method

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Abstract

Favipiravir finished dosage was approved for emergency use in many countries to treat SARS-CoV-2 patients. A specific, accurate, linear, robust, simple, and stability-indicating HPLC method was developed and validated for the determination of degradation impurities present in favipiravir film-coated tablets. The separation of all impurities was achieved from the stationary phase (Inert sustain AQ-C18, 250 × 4.6 mm, 5-μm particle) and mobile phase. Mobile phase A contained KH_2PO_4 buffer (pH 2.5 ± 0.05) and acetonitrile in the ratio of 98:2 (v/v), and mobile phase B contained water and acetonitrile in the ratio of 50:50 (v/v). The chromatographic conditions were optimized as follows: flow rate, 0.7 mL/min; UV detection, 210 nm; injection volume, 20 μL; and column temperature, 33°C. The proposed method was validated per the current International Conference on Harmonization Q2 (R1) guidelines. The recovery study and linearity ranges were established from the limit of quantification to 150% optimal concentrations. The method validation results were found to be between 98.6 and 106.2% for recovery and $r^2 = 0.9995–0.9999$ for linearity of all identified impurities. The method precision results were achieved below 5% of relative standard deviation. Forced degradation studies were performed in chemical and physical stress conditions. The compound was sensitive to chemical stress conditions. During the study, the analyte degraded and converted to unknown degradation impurities, and its molecular mass was found using the LC–MS technique and established degradation pathways supported by reaction of mechanism. The developed method was found to be suitable for routine analysis of research and development and quality control.

KEYWORDS
degradation impurities, favipiravir, LC/LC–MS, related substances, SARS-CoV-2, stability indicating

1 | INTRODUCTION

The 2019 coronavirus pandemic refers to the development of the SARS-CoV-2 virus. Before it became widely known, most people were

Abbreviations used: HCl, hydrochloric acid; ICH, International Conference on Harmonization; LOD, limit of detection; LOQ, limit of quantification; NaOH, sodium hydroxide; PDA, photo diode array; RSD, relative standard deviation; R_T, retention time.

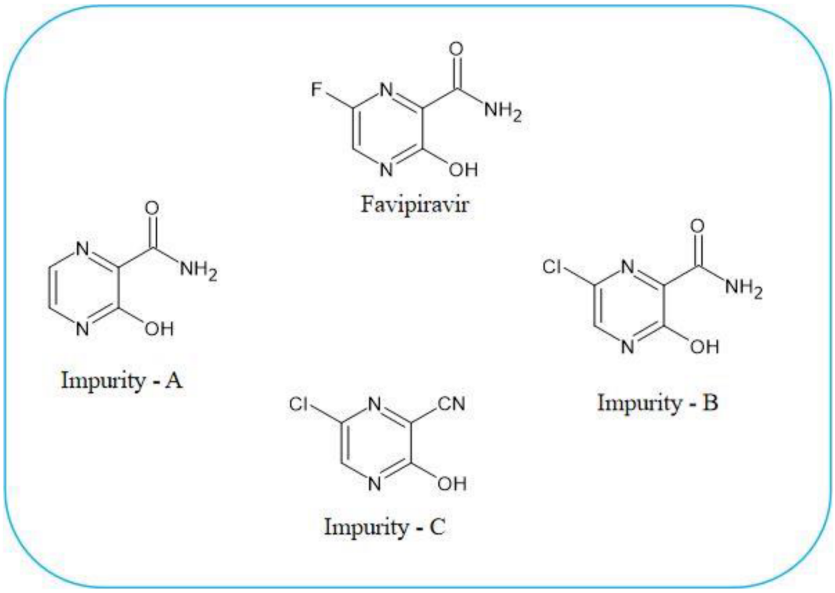


FIGURE 1 Chemical structures of favipiravir and its impurities

infected with this illness with mild to moderate symptoms. Pyrazine carboxamide is a derivative that exhibits antiviral activity against RNA viruses. It is known as favipiravir. The drug can help lower the severity of symptoms caused by SARS-CoV-2 in humans. It has also been shown to reduce the duration of illness in some people. The chemical name of favipiravir is 6-fluoro-3-oxo-3,4-dihydro-2-pyrazinecarboxamide, its molecular formula is $C_5H_4FN_3O_2$, and its mass is 157.104 amu. The chemical structure of favipiravir is shown Figure 1. The development of the process for favipiravir production was carried out by identifying the impurities, namely impurities A, B, and C.

A literature survey revealed that no stability-indicating method has been reported yet for the quantitative determination of favipiravir impurities. A few analytical methods were found; those various experiments were performed in human plasma using LC–MS/MS and HPLC techniques (Balu & Paresh, 2021; Eryavuz et al., 2021; Hailat et al., 2021; Ibrahim et al., 2021; Katharina et al., 2021; Marzouk et al., 2022; Mikhail et al., 2021; Mohamed et al., 2021; Nakayama & Ryo, 2021; Nazifa Sabir Ali et al., 2021; Nippes et al., 2021; Pallavi & Kamalkishor, 2021; Ramarao & Abhinandana, 2021; Rezk et al., 2021; Srinivas, et al., 2021) (Table 1). The literature does not report any HPLC method that accurately and quantitatively assesses the relative and degradation impurities of favipiravir. It is therefore necessary to develop a new stability-indicating method for the determination and estimation of favipiravir and its related impurities. The mass of major degradation impurities was identified using LC–MS techniques. Therefore, a reproducible, stability-indicating LC method was developed to determine favipiravir and its impurities. This method was successfully validated according to the current International Conference on Harmonization (ICH) guidelines. The proposed method can be used to estimate the known and degradation impurities of favipiravir in the finished dosage and drug substance. In addition, it is user friendly and cost effective.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Favipiravir standard (99.9% purity) and its related known impurities (impurities A, B, and C) were provided by Laurus Labs (Hyderabad, India). Favipiravir film-coated tablets (marketed sample) were provided by GITAM University, Hyderabad, India. A more compatible buffer was selected for this research. Potassium dihydrogen phosphate was purchased from Merck KGaA (Darmstadt, Germany). Orthophosphoric acid and acetonitrile (HPLC grade) were purchased from JT Baker (Avantor Performance Materials India Limited, Gurgaon, India), and Water was of Milli-Q grade. To identify the primary compound behavior in different stress reagents such as acid, base, and peroxide solution, forced degradation studies were performed. For these studies, we purchased concentrated hydrochloric acid (HCl) solution, sodium hydroxide (NaOH) pellets, and peroxide solution (AR grade) from Rankem Chemicals (Mumbai, India).

2.1.1 | Equipment and software

Liquid chromatographic method development and analysis were performed on an RP-HPLC (Waters Alliance e2695 HPLC System, Milford, MA, USA) equipped with a quaternary pump and a photodiode array (PDA) detector. Data acquisition and processing were performed using Empower-3 software (Waters Corporations, Milford, MA, USA). The weighing was performed on an XP4002S precision balance, XP205 Delta Range analytical balance, AX205 Delta Range analytical balance, or MX5 micro balance (Mettler Toledo, Columbus, OH, USA). The pH was measured using a SevenMulti pH meter (Mettler Toledo).

TABLE 1 Comparison of existing and proposed methods

Sample name/details	Mobile phase/pump mode	Column	Observations/dis advantages	References
Quantification of favipiravir as COVID-19 management in spiked human plasma	Methanol/acetonitrile/20 mM phosphate buffer pH 3.1 (30:10:60 v/v/v), isocratic mode	Symmetry C18-(250 × 4.6 mm, 5 μm)	1. This method did not explain the degradation study 2. It is helpful for bioanalytical samples, not for the finished product and API samples	Mohammad Hailat et al., 2021
Quantification of favipiravir in human plasma: application to a bioequivalence study	Mobile phase A: 10 mM ammonium formate + 0.1% formic acid, B: methanol/gradient mode	Acquity UPLC HSS C18 (100 × 2.1 mm, 1.8 μm)	3. This method did not explain the degradation study 4. It is helpful for bioanalytical samples, not for the finished product and API samples	Mamdouh R. Rezk et al., 2021
Quantification of COVID-19 drug favipiravir by a two-dimensional isotope dilution LC–MS/MS method in human serum	Mobile phase A: water, B: acetonitrile: formic acid (99.9:0.01, v/v); gradient mode	HP column (30 × 2.1 mm, 20 M, Waters) online solid-phase extraction	5. This technical method is helpful for bioanalytical samples of seven repurposed COVID-19 drugs 6. It is not helpful for finished product and API samples	Katharina Habler et al., 2021
This method has been developed for green micellar solvent-free HPLC and spectrofluorimetric determination of favipiravir as one of COVID-19 antiviral regimens	Mobile phase consisting of 0.02 M Brij-35, 0.15 M Sodium Dodecane Sulfonate, and 0.02 M disodium hydrogen phosphate adjusted to pH 5.0, isocratic mode	VDSpher 150 C18-E column (5 μm, 250 × 4.6 mm)	7. This method has been developed for green micellar solvent-free 8. It is not a useful method for finished product analysis	Ibraam E. Mikhail et al., 2021
Development and validation of a sensitive, fast, and simple LC-MS/MS method for the quantitation of favipiravir in human serum	Mobile phase A: 0.1% formic acid in water, B: 0.1% formic acid in methanol; gradient mode	Phenomenex C18 column (50 × 4.6 mm, 5 μm, 100 Å)	9. This was developed for human plasma, not for finished products	Duygu Eryavuz Onmaz et al., 2021
Developed method Mobile phase A: 10 mM KH_2PO_4 buffer (pH 2.5):acetonitrile (98:2) v/v. Mobile phase B: acetonitrile:water (50:50) v/v. Column: Inert sustain AQ-C18 (250 × 4.6 mm, 5 μm) at 33°C. Diluent: water:acetonitrile (98:2) v/v. Flow rate is 0.7 mL/min, injection volume is 20 μL, and linear gradient program was used for the development. These exert more influence on the determination of favipiravir and its related impurities in the finished dosage forms and API. Analytical scientists can operate it as it is very convenient and easy. This method can be used for the determination of impurities in favipiravir API, finished product routine sample, and stability sample analysis.				Proposed method

Ultrasonic baths used were Branson 8510 (Emerson Electric, St. Louis, MO, USA). Photostability was measured in a Suntest XLS + xenon test instrument (Atlas Material Testing Technology, part of Ametek, Mount Prospect, IL, USA).

2.2 | Chromatographic conditions

Chromatographic separation was achieved from a suitable stationary phase (Inert sustain AQ-C18, 250 × 4.6 mm, 5-μm particle), and gradient program was set as time/%B: 0.0/0, 40/30, 60/55, 62/00, and 70/00. Mobile phase A consisted of phosphate buffer (pH 2.5) and acetonitrile in the ratio of 98:2 (v/v), and mobile phase B comprised acetonitrile and water in the ratio of 50:50 (v/v). The measurements were performed as follows: injection volume, 20 μL; flow rate, 0.7 mL/min; column temperature, 33°C; and UV detection, 210 nm. The diluent was prepared using water and acetonitrile in the ratio of 98:2 (v/v). All impurities were well separated in these conditions.

2.3 | LC–MS conditions

The LC–MS studies were performed using a Waters TQD triple Quadrupole mass spectrometer [12–15]. The source voltage was kept at 5000 V and capillary temperature at 400°C. Mass range was kept at *m/z* 90–500 in positive ionization mode. Mobile phase A consisted of 0.01 M ammonium acetate (pH 2.5) and acetonitrile in the ratio of 98:2 (v/v), and mobile phase B consisted of acetonitrile and water in the ratio of 1:1 (v/v).

2.4 | Standard solution preparation (0.5%, with respect to sample concentration)

Favipiravir standard 60 mg was transferred into a 200-mL volumetric flask; then 120 mL of the diluent was added, sonicated for a few minutes, and made up to volume with the diluent; 5 mL of this solution was transferred into a 100-mL volumetric flask and made up to volume with the diluent. For the final concentration (0.5%), 5 mL of this solution was transferred into a 50-mL volumetric flask, and the remaining volume was diluted with the diluent.

2.5 | Impurity stock solution preparation

About 25 mg of impurities A, B, and C was accurately weighed and transferred into a 200-mL volumetric flask; then 120 mL of the diluent was added, sonicated for a few minutes, and made up to volume with the diluent. For further dilution, 5 mL of this solution was transferred into a 200-mL volumetric flask and made up to volume with the diluent.

2.6 | Sample solution preparation (0.3 mg/mL concentration)

Prepared 0.3-mg/mL concentration of the sample solution, such as 750 mg of favipiravir-equivalent sample powder (1031 mg), was transferred into a 250-mL volumetric flask; a sufficient amount of the diluent (120 mL) was added and sonicated for 20 min, and the remaining volume was diluted with the diluent. Then the sample solution was filtered through a 0.45-micron filter. For further dilution, 5 mL of the filtered sample solution was transferred into a 50-mL volumetric flask and then made up to volume with the diluent.

2.7 | Placebo solution preparation (without favipiravir API)

About 281.25 mg of favipiravir placebo powder was transferred into a 250-mL volumetric flask; a sufficient amount of the diluent (120 mL) was added and sonicated for 20 min, and the remaining volume was diluted with the diluent. Then the sample solution was filtered through a 0.45-micron filter. For Further dilution, 5 mL of the filtered sample solution was transferred into a 50-mL volumetric flask and made up to volume with the diluent.

2.8 | Spiked sample solution preparation (0.1% specification level)

Prepared 0.3-mg/mL concentration of the sample solution, such as 750 mg of favipiravir-equivalent sample powder (1031.51 mg), was transferred into a 250-mL volumetric flask; a sufficient amount of the diluent (120 mL) was added and sonicated for 20 min, and 6 mL of the impurity stock solution was transferred into a sample solution. Then the remaining volume was diluted to volume with the diluent. For further dilution, 5 mL of the filtered sample solution was transferred into a 50-mL volumetric flask and made up to volume with the diluent.

3 | RESULTS AND DISCUSSIONS

3.1 | Method development and optimization

favipiravir's related impurities were identified in the finished dosage form using the LC system. First, however, we gathered more critical information on the compound from the literature: p*Ka* ~5.1, solubility polarity (in water: 8.7 μg/mL), melting point (187–193°C), hygroscopic nature (nonhygroscopic), and polymorphism (two polymorphs available) of the molecule. Based on the nature of the compound, reversed-phase chromatographic technique was selected. For LC development, mobile phase plays a significant role. Its pH 2.5 was optimized based on the compound p*Ka* value. Generally, compound polarity is an essential character in the stationary phase; per polarity

of the compound a suitable stationary phase (C18) was chosen. Based on the available information, few experiments were carried out. The diluent chosen according to the compound's solubility (98:2, v/v) was water/acetonitrile. Recovery up to high-level concentration was achieved well. The determined UV max of all described compounds depended on the PDA spectral graph. Based on the spectra of the known and degradation impurities, we finalized wavelength at 210 nm and achieved good mass balance. All experimental results were reviewed. For more details, see Table 2 and Figures 2–5. Per the final experimental results conclusion, one appropriate method was selected for validation. As part of the method validation studies, forced degradation experiments were performed using the LC method and a PDA detector. The LC–MS technique was used to identify the mass value of two unknown degradation impurities found during forced degradation studies. The liquid chromatography with mass spectrometer used with Electrospray positive ionization source with required nebulization gas, drying gas with source temperature. The mass of degradation impurities were identified in MS Scan mode, and

TABLE 2 Method development trails

Number of experiments	Method details	Column	Results and observations	Method status
Experiment 1	Mobile phase A: 0.1% Orthophosphoric acid, mobile phase B: acetonitrile; flow rate: 1.0 mL/min, injection volume: 10 µL, UV: 225 nm; a linear gradient program was created; run time: 50 min	Inertsil ODS 3V (250 × 4.6 mm, 5 µm)	1. In this experiment, impurity A eluted at the void 2. Method needs to be optimized to retain impurity A; for more details, see Figure 2	Rejected
Experiment 2	Mobile phase A: 10 mM phosphate buffer (pH 2.5):acetonitrile (98:2, v/v), mobile phase B: acetonitrile and water (50:50, v/v); flow rate: 0.7 mL/min, injection volume: 20 µL, UV: 220 nm; a linear gradient program was created; run time: 90 min	Zorbax SB C8 (150 × 4.6 mm, 3.5 µm)	3. In this trial, impurity B merged with the main peak 4. Impurity A eluted at the void 5. Unknown impurity eluted at impurity C; for details see Figure 3	Rejected
Experiment 3	Mobile phase A 10 mM phosphate buffer (pH 2.5):acetonitrile (98:2, v/v), mobile phase B: acetonitrile and water (50:50, v/v); flow rate: 0.7 mL/min, injection volume: 20 µL, UV: 210 nm; a linear gradient program was created; run time: 60 min	Inert sustain AQ-C18 (250 × 4.6 mm, 5 µm)	6. Unknown impurity eluted at impurity C 7. The method needs to be optimized for excellent separation of all impurities	Rejected
Experiment 4	For the mobile phase, refer to experiment 3, which changed only a linear gradient program; run time: 70 min	Inert sustain AQ-C18 (250 × 4.6 mm, 5 µm)	8. All impurities are well separated 9. All impurities possess good peak shapes 10. No interference was observed at known impurities and degradation impurities; for details see Figure 4	Approved

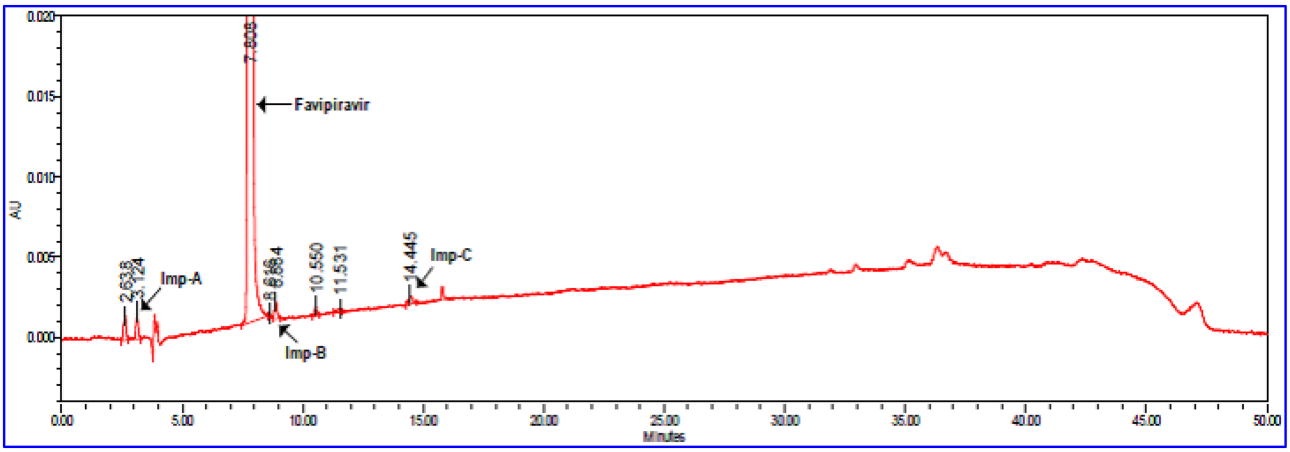


FIGURE 2 Spiked sample chromatogram in trial 1 experiment

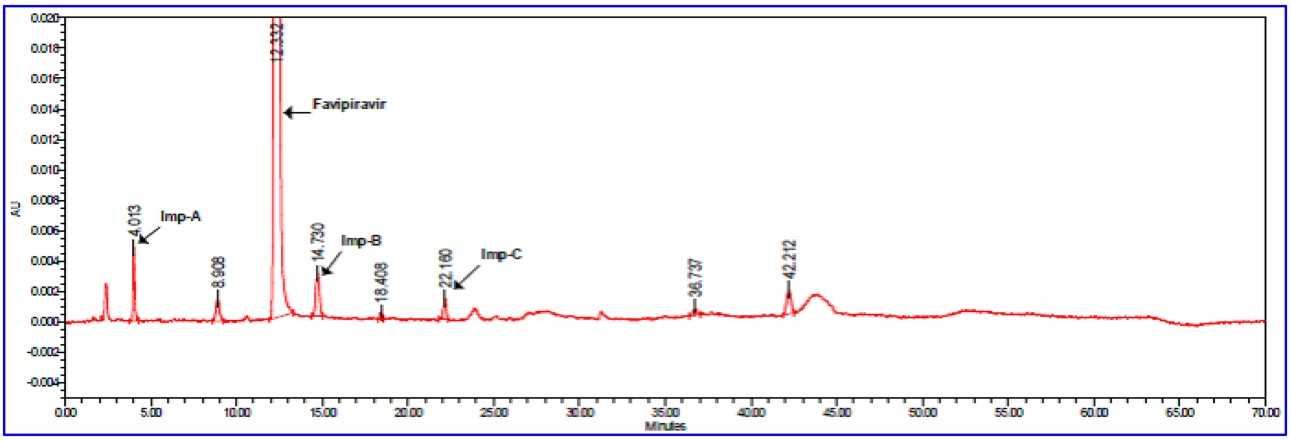


FIGURE 3 Spiked sample chromatogram in trial 2 experiment

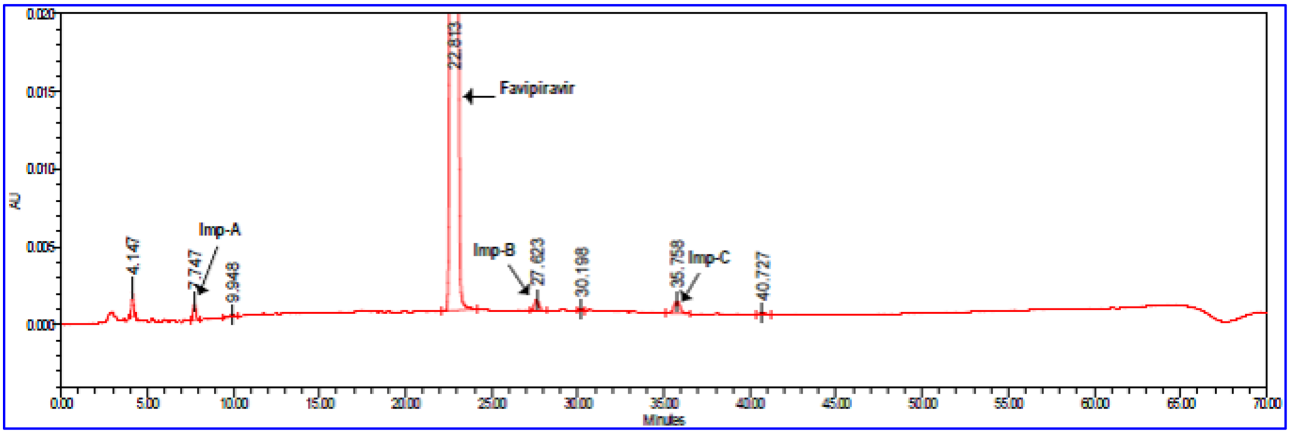


FIGURE 4 Spiked sample chromatogram in trial 3 experiment

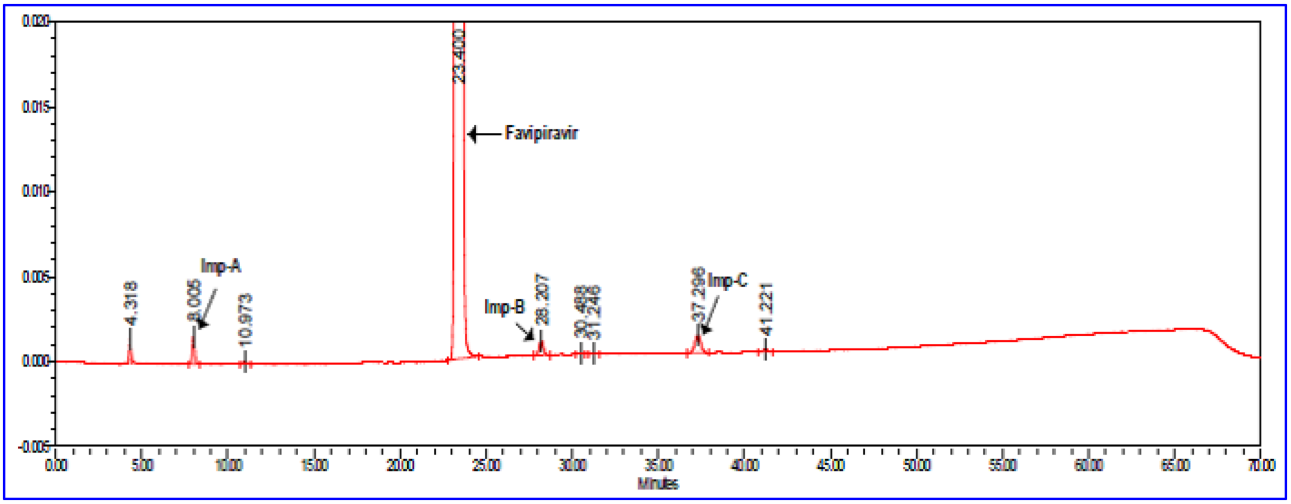


FIGURE 5 Spiked sample chromatogram in final method

the structures of major degradation impurities were identified. The proposed method was validated per the current ICH guidelines (ICH Q2 (R1), 2005).

3.2 | Analytical method validation

The described method was validated per current ICH Q2 (R1), 2005 guidelines. The significant analytical parameters were as follows: specificity, accuracy, linearity, precision, detection and quantification of the method, and ruggedness [ICH Q2(R1), 2005; ICH Q1B, 1996; ICH Q3B (R2), 2006].

3.3 | Specificity

3.3.1 | Interference test

Specificity parameter plays a significant role in the method finalization, which can identify interference at the retention time of known and degradation impurities (Konduru et al., 2021; Subramanian et al., 2020). To identify the interference test, verification samples, such as blank, placebo, test sample, and all impurities-spiked sample solutions, were injected in the current LC method. All chromatography data were reviewed, no interference was observed at the retention time of known and degradation impurities, and peaks of favipiravir and known impurities were found to be spectrally pure.

3.4 | Forced degradation studies

This study plays a significant role in identifying the stability-indicating method and could identify the molecular behavior in the different stress reagents. Any LC method must undergo this study to be reviewed by the U.S. Food and Drug Administration, the European Union, and other regulatory bodies. Forced degradation studies confirm that the method is stability indicating in nature. Before the experiment was started, we obtained molecule solubility and polarity information and some stress reagent concentrations and conditions from regulatory guidelines (Blessy et al., 2014; Murthy et al., 2013; Saranjit et al., 2013).

3.5 | Acid hydrolysis degradation sample

Prepared acid hydrolysis degradation sample, such as 750 mg of favipiravir-equivalent sample powder (1031 mg), was transferred into a 250-mL volumetric flask, and 25 mL of 0.01 N HCl solution was added and kept on a benchtop for 48 h. Then, 100 mL of the diluent was added, sonicated for 20 min, and made up to volume with the diluent. Finally, the degradation sample solution was filtered through a 0.45-micron filter.

For further dilution, 5 mL of the filtered sample solution was transferred into a 50-mL volumetric flask and then made up to volume with the diluent. In acid hydrolysis, two unknown degradation impurities were found at retention time 4.152 and 4.382 min. The LC–MS technique identified the mass value of degradation impurities and found the predicted molecular structures using a mechanism route. The results are presented in Table 3 and Figures 6, 10, 11, and 12.

3.6 | Base hydrolysis degradation sample

Prepared base hydrolysis degradation sample, such as 750 mg of favipiravir-equivalent sample powder (1030 mg), was transferred into a 250-mL volumetric flask; 25 mL of 0.1 N NaOH solution was added

TABLE 3 Forced degradation results

Serial number	Stress conditions	Percentage of assay	Percentage of single max (unknown impurities)	Percentage of degradation impurities	Mass balance	Peak purity
1	As such sample	99.2	0.07	0.5	na	Pass
2	Acid hydrolysis degradation sample	85.2	11.1	13.7	99.2	Pass
3	Base hydrolysis degradation sample	90.1	9.9	10.9	101.3	Pass
4	Peroxide degradation sample	89.5	5.71	9.3	99.1	Pass
5	Water hydrolysis degradation sample	94.3	3.8	4.2	98.8	Pass
6	Thermal degradation sample	98.9	Not Detected	0.7	99.9	Pass
7	Humidity degradation sample	99.1	Not Detected	0.56	100.0	Pass
8	UV light degradation sample	98.3	Not Detected	0.88	99.5	Pass

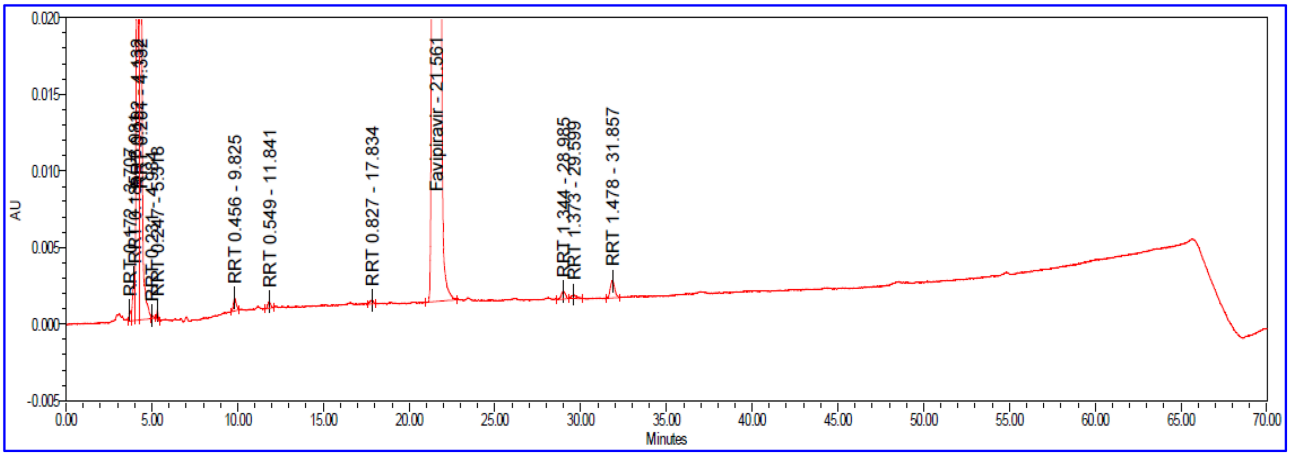


FIGURE 6 Acid hydrolysis degradation sample chromatogram

and kept on a benchtop for 5 days. Once the actual period of time was over, 100 mL of the diluent was added, sonicated for 20 min, and then made up to volume with the diluent. Finally, the degradation sample solution was filtered through a 0.45-micron filter.

For further dilution, 5 mL of the filtered sample solution was transferred into a 50-mL volumetric flask and then made up to volume with the diluent. When the degradation data were reviewed, one unknown degradation impurity was found at R_T 11.816 min. The LC–MS technique identified the impurity mass value and found a molecular structure with proper reaction mechanism, and the results are presented in Table 3 and Figure 7.

3.7 | Peroxide degradation sample

Prepared peroxide degradation sample, 750 mg of favipiravir-equivalent powder (1032 mg), was placed in a 250-mL volumetric flask; 25 mL of 0.05% hydrogen peroxide was added and kept on a benchtop for 5 days. Once the interval had passed, 100 mL of the diluent was added, and the mixture was sonicated for 20 min. The diluent was then made up to volume with the diluent, and the degradation sample solution was filtered through a 0.45-micron filter.

For further dilution, 5 mL of the filtered sample solution was transferred into a 50-mL volumetric flask and then made up to volume with the diluent. When the degradation data were reviewed, two unknown degradation impurities were observed at R_T 4.101 and 4.386 min. The LC–MS technique identified mass values of these impurities and found molecular structures with proper reaction mechanisms; the results are presented in Table 3 and Figures 8, 10–12.

3.8 | Water hydrolysis degradation sample

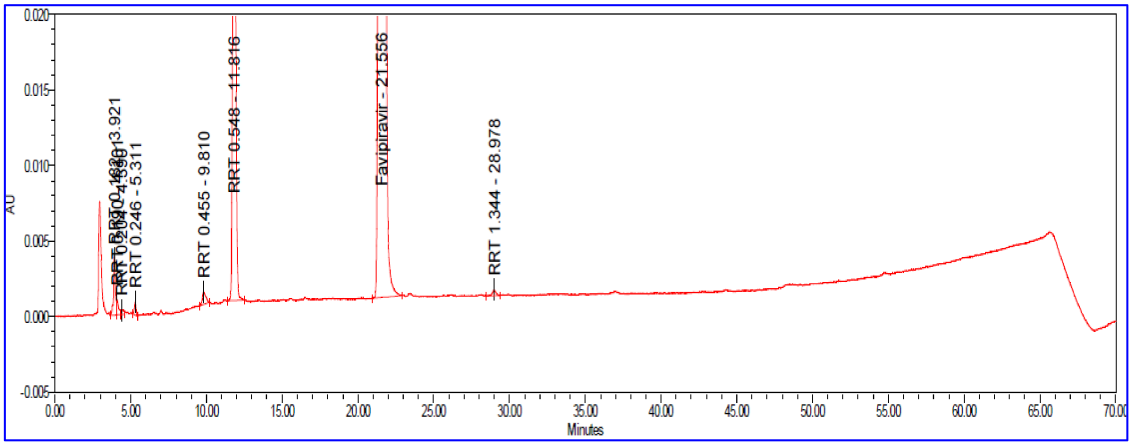
Prepared water hydrolysis test sample, 750 mg of favipiravir-equivalent powder (1031 mg), was placed in a 250-mL volumetric flask; 25 mL of water was added and kept on a benchtop for 3 days. Then, 100 mL of the diluent was added and sonicated for 20 min. The diluent was then made up to volume with the diluent, and the degradation sample solution was filtered through a 0.45-micron filter.

For further dilution, 5 mL of the filtered sample solution was transferred into a 50-mL volumetric flask and then made up to volume with the diluent. After the degradation data were reviewed, two unknown degradation impurities were observed at R_T 4.102 and 4.334 min. The mass value of the degradation impurities was identified using the LC–MS technique, and the results are presented in Table 3 and Figures 9, 10, 11, and 12.

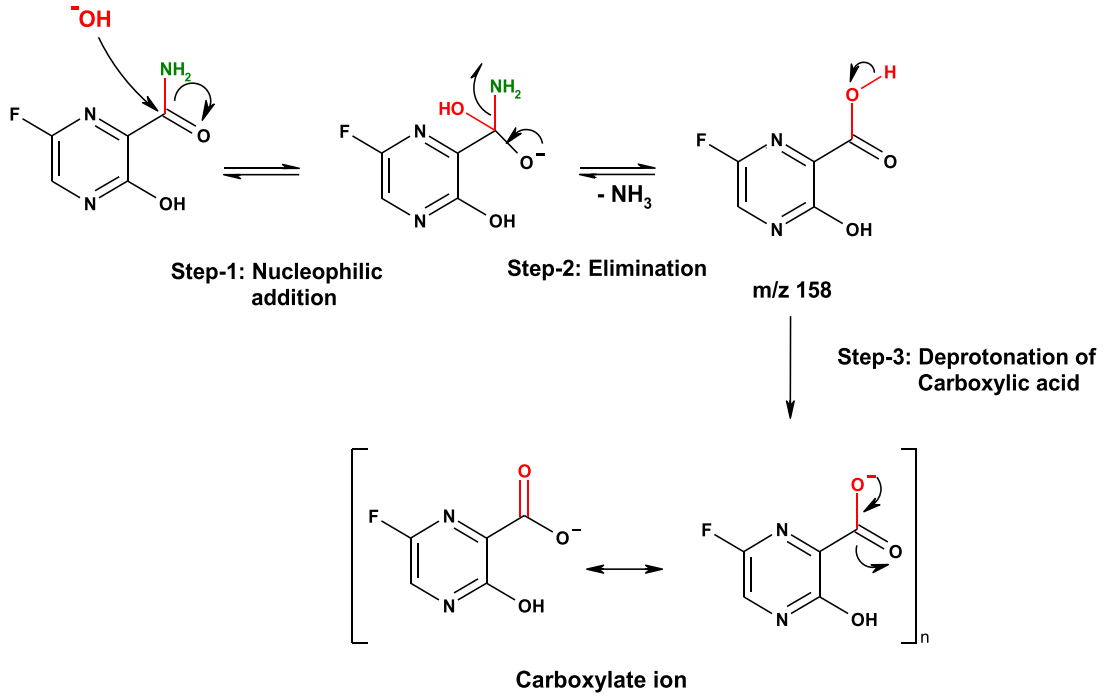
Based on the chemical degradation study results, this compound is very sensitive to acid, base, peroxide, and water hydrolysis conditions.

3.9 | Solid-state degradation studies

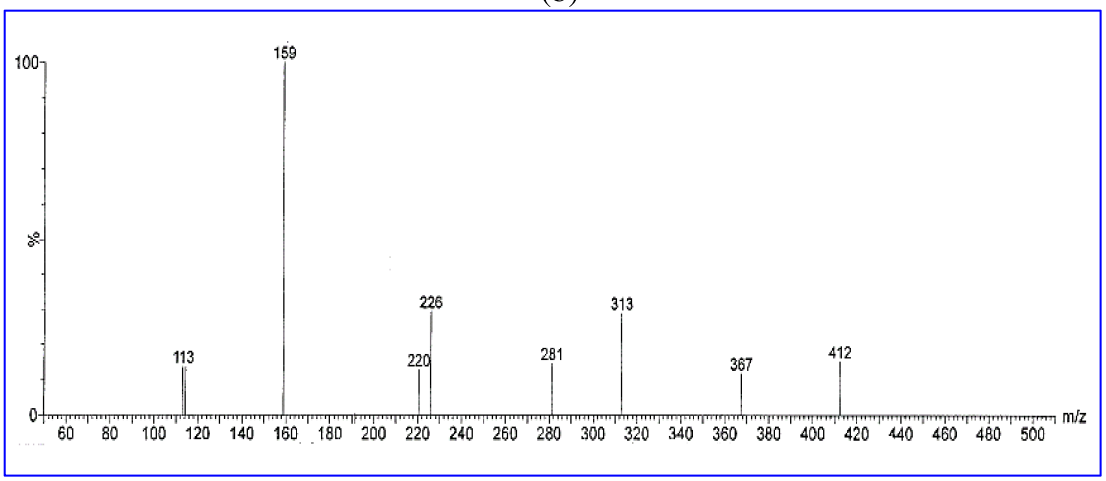
Physical degradation study plays an essential role in degradation study. It could identify the degradation pathway of impurities formed in stability studies. Solid-state degradation includes heat, humidity, and light stress studies. Before experimental testing, the samples were kept in respective storage conditions. Therefore, the drug product samples were stored in a photostability chamber and were exposed to 200 Wh/m^2 of UV light and 1.2 million lux hours of visible light (per ICH Q1B, UV 200 Wh/m^2, 1.2 million lux hours) in humidity conditions (85% Relative Humidity) for 7 days and exposed to heat (105°C) for 10 days. After time intervals, the samples were removed, and the degradation solutions were prepared and immediately injected into the LC instrument. No major degradation impurities were observed in all degradation sample chromatograms; good results were achieved within limits. The results show that the compound is stable at heat, humidity, and light environmental conditions. The results are presented in Table 3.



(a)



(b)



(c)

FIGURE 7 (a) Base hydrolysis degradation sample chromatogram, (b) route of degradation mechanism in hydrolysis condition, and (c) degradation impurity mass spectrum

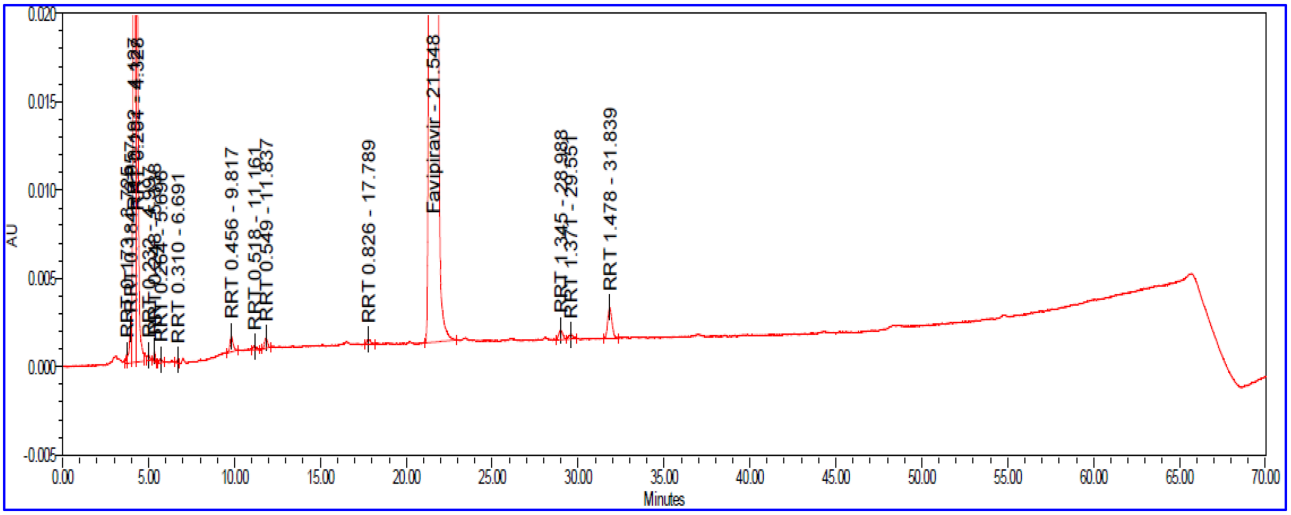


FIGURE 8 Peroxide degradation sample chromatogram

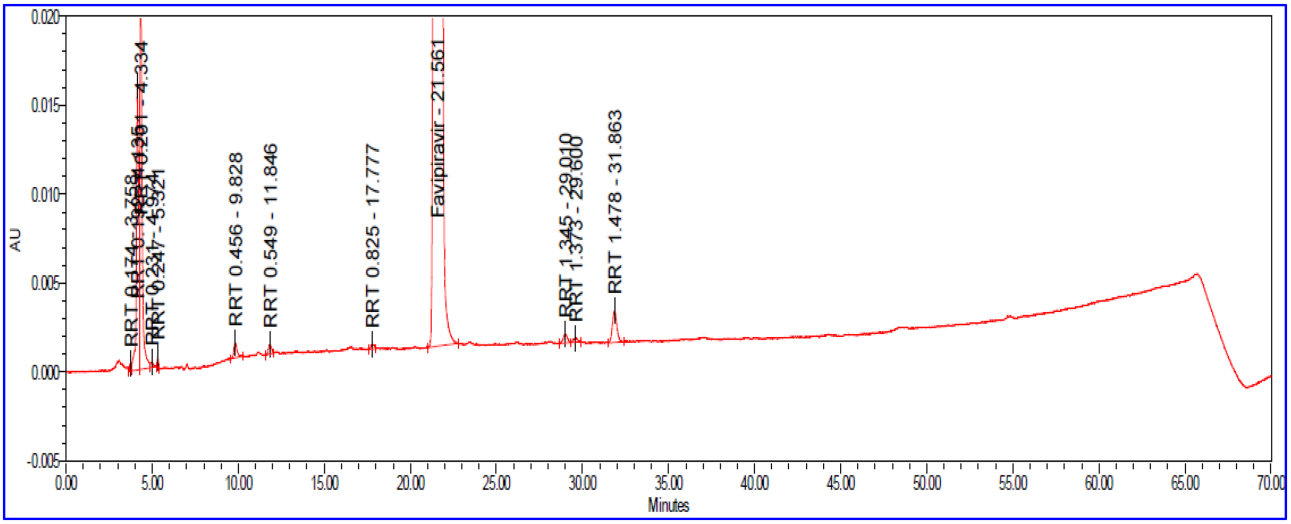


FIGURE 9 Water hydrolysis degradation sample chromatogram

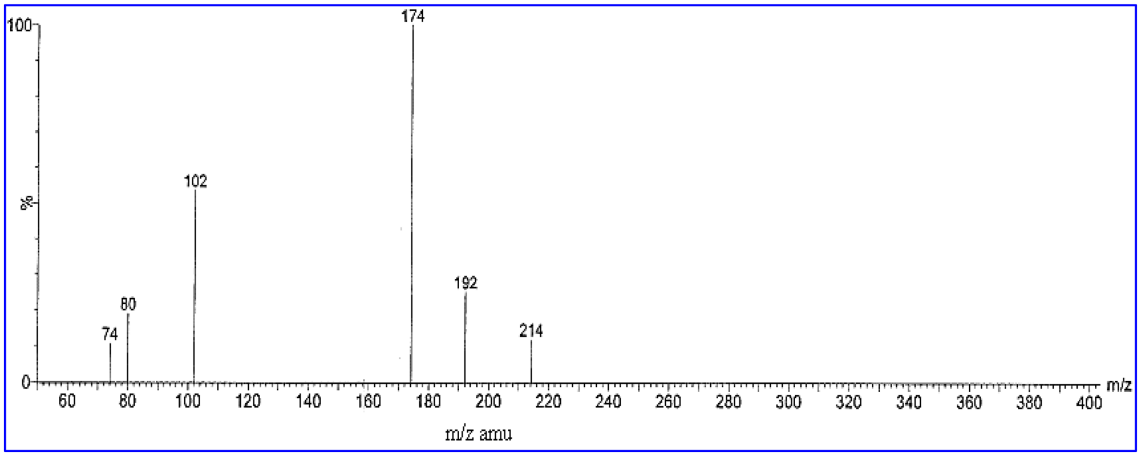


FIGURE 10 Mass fragmentation of oxidation degradation peak 1

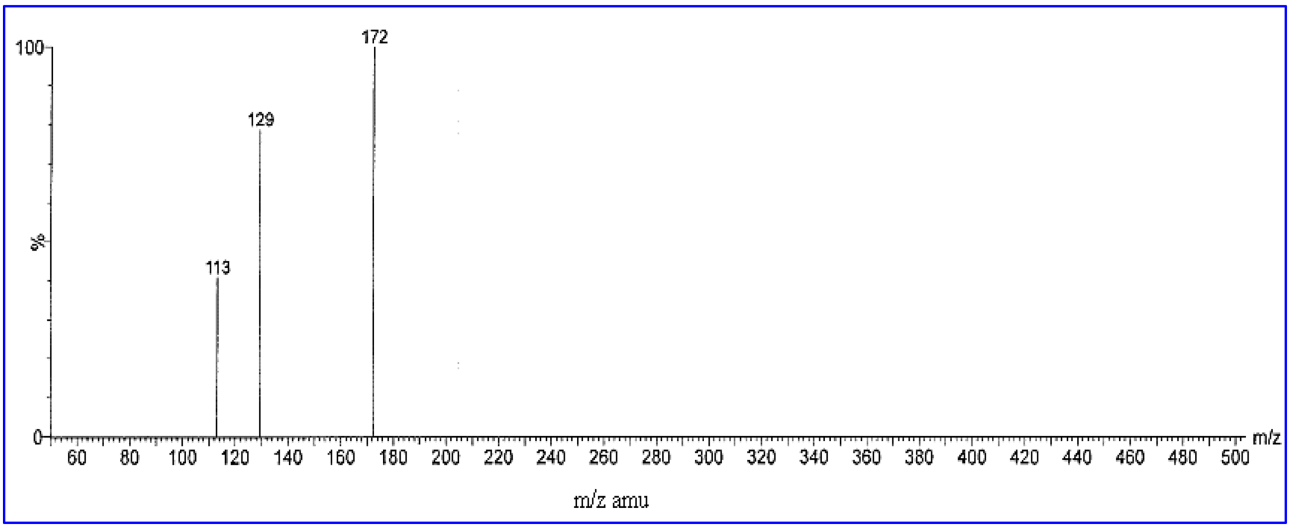
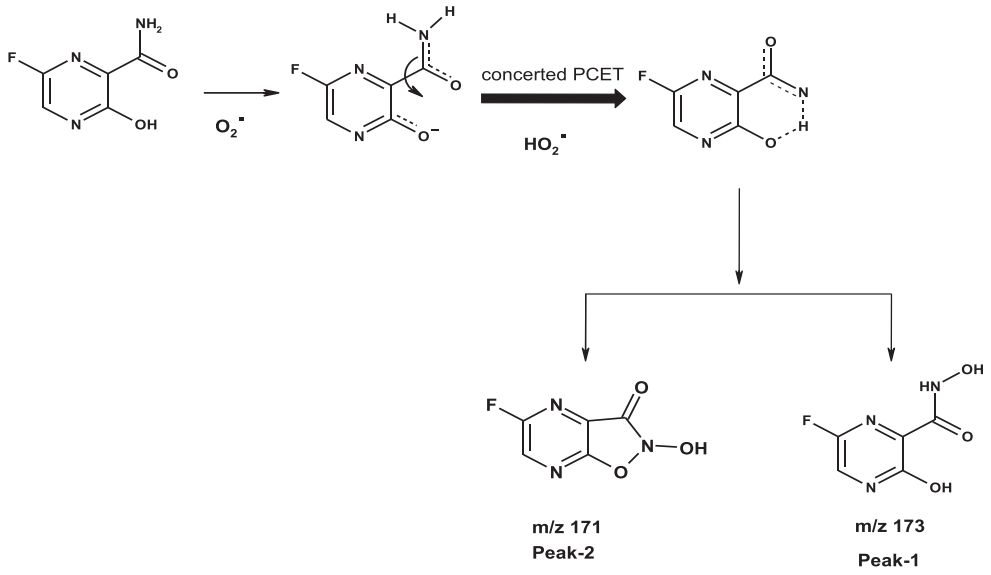


FIGURE 11 Mass fragmentation of oxidation degradation peak 2

FIGURE 12 The route of degradation reaction occurred in acid, water, and oxidation environment conditions



3.10 | Precision

The current method's precision was established through repeatability and ruggedness. This experiment could find the reproducibility results of target impurities. Repeatability was determined by injecting six freshly prepared drug product sample solutions containing 0.3 µg/mL of each known impurity on the same day, and their recoveries were observed. Ruggedness was determined by injecting six freshly prepared sample solutions containing the same concentration of each known impurity on different days and using different LC instruments by different scientists. The overall percentage relative standard deviation (RSD) values for each <3% confirm that the developed test method's precision is good and well suited to different laboratory conditions, and the results are presented in Table 4 and Figures 13 and 14.

3.11 | Accuracy

This study was tested by the standard addition method by spiking the known impurities at the limit of quantification (LOQ) level: 50, 100, and 150% of the specification concentrations, that is, 0.09, 0.15, 0.3, and 0.45 µg/mL with respective test concentrations. Each spiked sample determination was carried out thrice, and the corresponding data are presented in Table 4 and Figures 15 and 16. The recovery of impurities at four levels (LOQ: 50, 100, and 150%) was 98.2–105.1%

TABLE 4 Method validation results

Parameters	Details	Acceptance criteria	Results			
System suitability	$n = 6$ (favipiravir standard 0.5%, with respect to sample concentration)	Tailing factor for favipiravir should not be more than 1.5 Plate count for favipiravir should not be less than 5000 %RSD of favipiravir peak from six injections of standard solution should not be more than 2.0%	USP tailing: 1.04 %RSD: 0.35 USP plate count: 15,632			
Specificity	$n = 1$, each individual injection for blank, standard, sample, and standard spiked with sample at specification level	Each peak should elute at a different retention time	There are no peak interferences with each other. All of the peaks eluted at different retention times			
Precision (day 1)	$n = 6$ (six determinations at specification level spiked with sample)	RSD should be <5%	Impurity A (1.58%)	Impurity B (2.52%)	Impurity C (1.85%)	
Precision (day 2)	$n = 6$ (six determinations at specification level spiked with sample)	RSD should be <5%	Impurity A (1.88%)	Impurity B (2.24%)	Impurity C (1.56%)	
Accuracy	$n = 12$ (three determinations each one at LOQ, 50, 100, and 150% levels spiked with sample)	Recovery at each impurity should be >80–120%; RSD should be <10%	Concentrations	Impurity A (results %)	Impurity B (results %)	Impurity C (results %)
			LOQ	105.6	106.2	104.8
			50%	103.8	102.8	102.9
			100%	99.8	101.3	100.6
			150%	98.9	99.2	98.6
			%RSD	3.13%	2.87%	2.65%
Linearity	$n = 6$ (six concentration levels in the range LOQ to 150%)	Correlation coefficient should be >0.999	r^2	Impurity A	Impurity B	Impurity C
				0.9995	0.9999	0.9997
LOD	Lowest detectable concentration: 0.027 μg/mL	Signal-to-noise ratio: ~3		Impurity A	Impurity B	Impurity C
				2.94	2.98	3.1
LOQ	Lowest detectable concentration: 0.09 μg/mL	Signal-to-noise ratio: ~10		Impurity A	Impurity B	Impurity C
				10.25	11.1	10.67
Robustness	Flow variation [0.7 mL/min1 (actual), 0.6 mL/min (low level), and 0.8 mL/min (high level)]	% Standard RSD should be <5%	Actual (1.82%)	Low level (2.1%)	High level (1.72%)	
	Column temperature [33°C (actual), 31°C (low level), and 35°C (high level)]		Actual (1.89%)	Low level (2.5%)	High level (1.78%)	
	Mobile phase variations [pH 2.5 (actual), 2.3 (low level), and 2.7 (high level)]		Actual (1.79%)	Low level (2.3%)	High level (1.98%)	

Note: LOD, limit of detection; LOQ, limit of quantification; RSD, relative standard deviation.

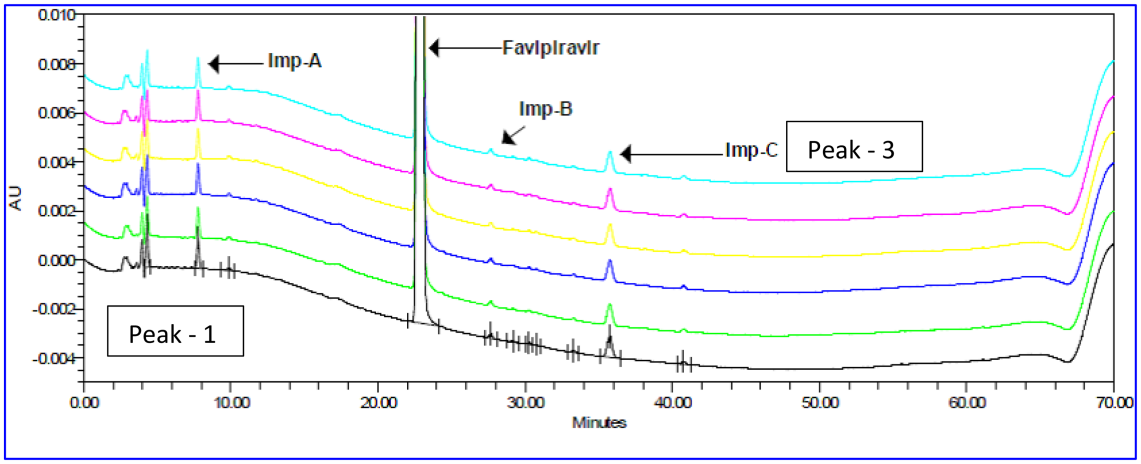


FIGURE 13 Overlay chromatogram of precision solutions for impurities A and C

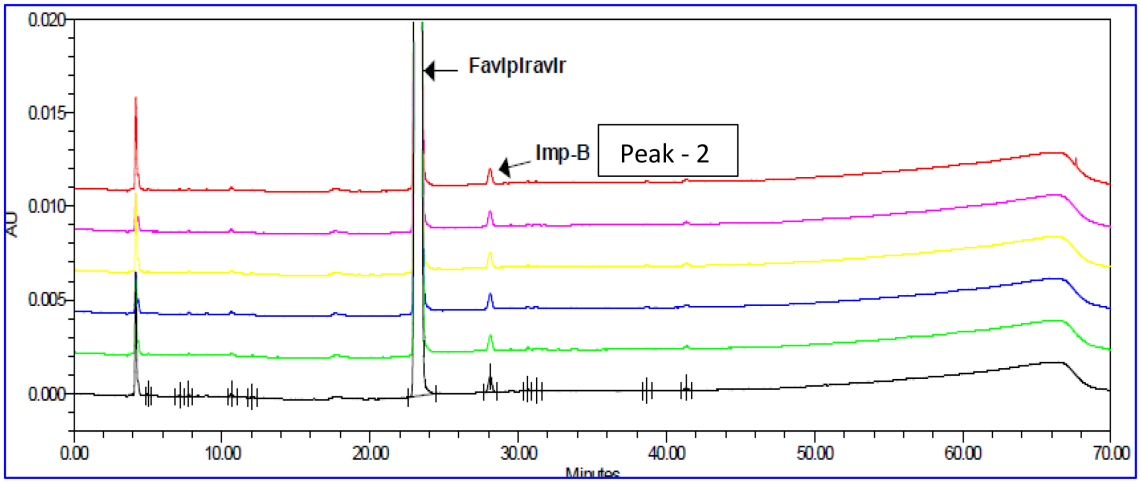


FIGURE 14 Overlay chromatogram of precision solutions for impurity B

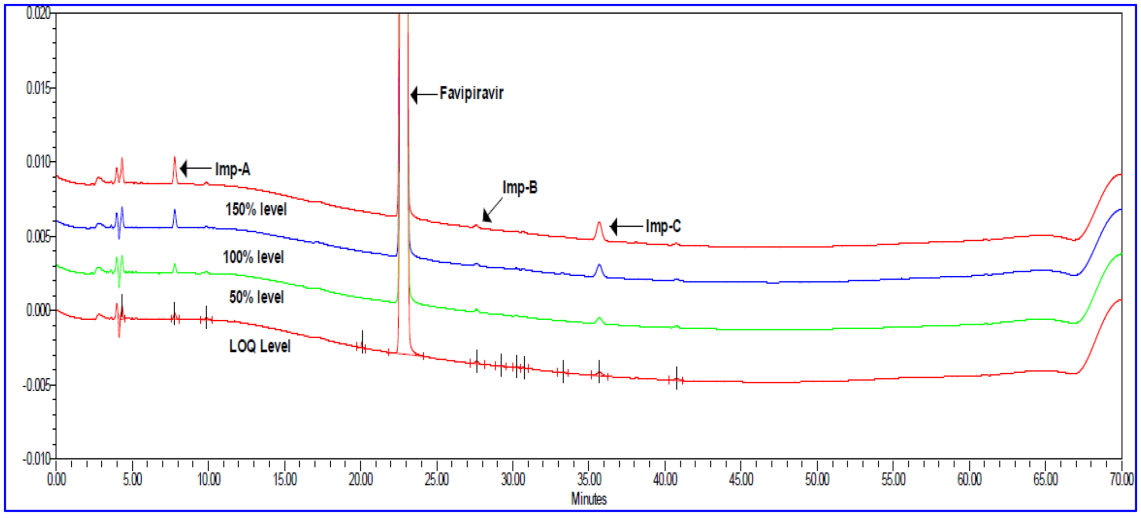


FIGURE 15 Overlay chromatogram of accuracy solutions for impurities A and C

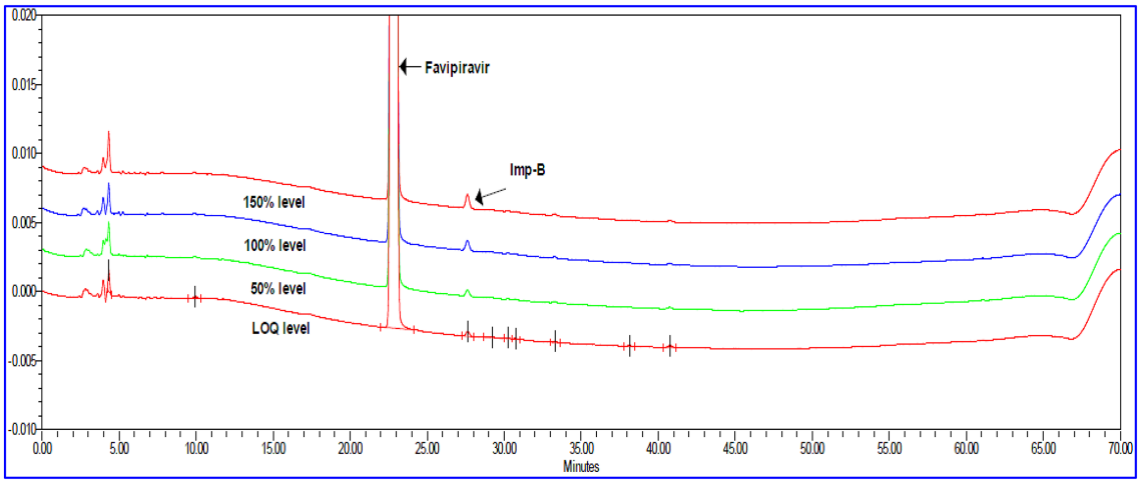


FIGURE 16 Overlay chromatogram of accuracy solutions for impurity B

TABLE 5 Solution stability results

Favipiravir solution stability						
Spiked solution at 2–8°C	**Impurity A**	**Impurity B**	**Impurity C**	**Impurity at RRT 0.19**	**Major unspecified impurity**	**Total impurity**
Initial	0.210	0.206	0.245	0.078	0.085	0.82
Day 1	0.207	0.201	0.244	0.088	0.086	0.83
Difference	−0.003	−0.005	−0.001	0.010	0.001	0.002
Day 2	0.205	0.198	0.242	0.096	0.088	0.83
Difference	−0.005	−0.008	−0.003	0.018	0.003	0.005
Spiked solution at RT	**Impurity A**	**Impurity B**	**Impurity C**	**Impurity at RRT 0.19**	**Major unspecified impurity**	**Total impurity**
Initial	0.210	0.206	0.245	0.078	0.085	0.82
After 1 h	0.208	0.206	0.245	0.082	0.085	0.83
Difference	−0.002	0.000	0.000	0.004	0.000	0.00
After 2 h	0.207	0.205	0.244	0.088	0.086	0.83
Difference	−0.003	−0.001	−0.001	0.010	0.001	0.01
After 4 h	0.205	0.204	0.243	0.098	0.087	0.84
Difference	−0.005	−0.002	−0.002	0.020	0.002	0.01
After 5 h	0.204	0.203	0.241	0.122	0.087	0.86
Difference	−0.006	−0.003	−0.004	0.044	0.002	0.03

Note: Spiked solution was stable for 4 h at room temperature i.e. 25 °C. and 2 days at 2–8°C; acceptance limit: NMT 0.04%.

with an RSD of <3.0%, which is consistent with the required range of 80.0–120.0% and an RSD limit of 10.0%.

3.12 | Linearity

This experiment shows the ability of the method range from the low to high levels. Thus, all known impurities (impurities A, B, and C) were injected at different concentrations between the LOQ (0.09 μg/mL) and 200% (0.60 μg/mL) of the specification. The calibration curve was obtained by drawing a graph between the peak areas and concentration of all impurities at 0.09, 0.15, 0.24, 0.30, 0.36, and 0.60 μg/ mL. The slope, intercept, and correlation coefficient values obtained by linear least square regression analysis are presented in Table 5.

3.13 | Determination of LOD and LOQ

This study plays an essential role in the related substances method development, from which the method of sensitivity could be identified. The limit of detection (LOD) and LOQ were calculated using the

signal-to-noise ratio method. The LOQ and LOD solutions were prepared from the impurity standard stock solution. In the process, the concentrations of all impurity solutions were decreased successively to obtain an LOQ, such that the yield signal-to-noise ratio was 10.5 for impurity A, 10.8 for impurity B, and 10.9 for impurity C. The LOD was calculated using the formula LOD = LOQ/3.3, and the LOQ solution was diluted thrice, and the LOD solution was injected into the HPLC instrument. The experimental and theoretical LOD values are comparable, which is good. The LOQ and LOD for all known impurities were 0.09 and 0.027 μg/mL, respectively. The results are presented in Table 4.

3.14 | Solution stability

This study is more beneficial to analytical scientists. Thus, the standard and spiked sample solutions should confirm how many hours they are stable at refrigerator and room temperatures. The stability study was evaluated by injecting the standard and spiked sample solutions at 2–8°C and room temperature 25°C. At refrigerator condition, target samples were injected at initial, 24, and 48 h, yielding results within limits. At room temperature, as the impurities are sensitive, this study was performed at initial, 1, 2, 4, and 5 h. The results are presented in Table 5.

3.15 | Robustness

This study could achieve method sensitivity. The optimized test method was examined by intentional changes in flow rate, column temperature, and mobile phase pH. The actual flow rate of the mobile phase was 0.7 mL/min, which was changed (0.6–0.8 mL/min). The effect of column temperature on analysis was 31 and 35°C. The optimized mobile phase pH was 2.5, which was changed by ±0.2. Standard and spiked samples were injected into the LC instrument. All the aforementioned parameters did not show any significant differences in identified impurities. The experimental data are presented in Table 4.

4 | CONCLUSION

A high-sensitive, accurate, linear, specific, and robust analytical method was developed and validated to determine favipiravir degradation and identified impurities in the tablet dosage form. The mass values of degradation impurities were identified using the LC–MS technique. The proposed method has been validated per current ICH guidelines. The forced degradation studies identified three unknown degradation impurities in the presence of acid, base, water, and oxidation conditions. Therefore, it is sensitive to chemical stress conditions. Per solid-state degradation study results, no degradation occurred. The molecule was found to be stable at heat, humidity, and UV light conditions. According to method validation results, this method is typically able to estimate favipiravir impurities in the finished dosage forms and drug substances. Now in this pandemic situation, this drug product is more helpful to treat SARS-CoV-2 patients. Therefore, in the present pandemic situation, this research work contributes more to developing the efficient drug product of favipiravir. Therefore, for quality products, this method can be used. The current method is cost effective and simple for quality control analysis.

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DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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