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Original Article

A Green Eco-friendly Analytical Method Development, Validation, and Stress Degradation Studies of Favipiravir in Bulk and Different Tablet Dosages Form by UV-spectrophotometric and RP-HPLC Methods with their Comparison by Using ANOVA and *in-vitro* Dissolution Studies

Subhadip Chakraborty, Sumanta Mondal*

Department of Pharmaceutical Chemistry, GITAM School of Pharmacy, GITAM (Deemed to be University), Visakhapatnam, Andhra Pradesh, INDIA.

ABSTRACT

Favipiravir is an antiviral drug with significant and widespread antiviral action. Favipiravir was crucial in the contest against the COVID-19 pandemic because of how well it treated the SARS-CoV-2 virus. It is well known that contemporary pharmaceutical analysis establishes green, stability-indicating analytical procedures. The current study aimed to develop and assess UV-spectrophotometric (zero order, first order, area under the curve) and RP-HPLC methods for estimating favipiravir in its pharmaceutical dose form, comparing them using ANOVA and an *in-vitro* dissolution analysis. A green solvents composition of methanol, ethanol, and water (25:35:40 v/v/v) is used for analysis as a mobile phase and diluent. Method A is a simple zero-order spectrophotometric method for determining favipiravir at 236 nm, and the correlation coefficient in the linearity study was found to be 0.9962, LOD, and LOQ are 0.18 and 0.55 µg/mL. Method B is a first-order spectrophotometric method for determining favipiravir at 227 nm, and the correlation coefficient in the linearity study was found to be 0.9964, LOD, and LOQ are 0.64 and 1.96 µg/mL. Method C is an area under the curve spectrophotometric method for determining favipiravir at 230 to 243 nm, and the correlation coefficient in the linearity study was found to be 0.9986, LOD, and LOQ are 0.32 and 0.96 µg/mL. Method D is the RP-HPLC method for the determination of favipiravir at the retention time of 7.216 min, a flow rate of 0.80 mL/min, column temperature of 25°C, at 236 nm, Isocratic mode, and the correlation coefficient in the linearity study was found to be 0.9996, LOD, and LOQ are 0.52 and 1.56 µg/mL. All developed methods demonstrated good repeatability and recovery with %RSD < 2. The proposed established methods were assessed using one-way ANOVA. It was revealed that the $F_{calculated}$ value was lower than the $F_{tabulated}$ value, with no discernible variation in the assay results. Studies on stress degradation show that oxidation and acid degradation mostly impact favipiravir solutions. The Analytical Eco-scale verified that these methods are the greenest and most environmentally friendly, enabling the suggested approach to use an effective green analytical methodology to measure favipiravir extensively. Phosphate buffer (pH 6.4) was the best dissolution medium after analysis of the favipiravir dissolution study in several dissolution media.

Keywords: Favipiravir, Green, UV-spectrophotometric, HPLC, Avigan, Fluguard, Degradation study, *In-vitro* dissolution studies.

Correspondence:
Dr. Sumanta Mondal
Associate Professor and NSS Programme Officer (Unit-IX), School of Pharmacy, GITAM (Deemed to be University), Visakhapatnam-530045, Andhra Pradesh, INDIA.
Email: mondalresearch@gmail.com

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INTRODUCTION

The relationship between viruses, medications, and health has a far longer and more nuanced history. Sir Martinus Willem Beijerinck was the first to identify the "virus" cause of the *Tobacco mosaic* in 1898. Louis Pasteur and Edward Jenner established the first vaccines to prevent viral illnesses.[1] Several viruses continuously and frequently affect the world, resulting in the question of survival. In the year 2019, the most dangerous and harmful virus came, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the result is a highly contagious infectious disease.[2]

In the worldwide pandemic situation, favipiravir (Figure 1) was introduced as an antiviral medicine for controlling fever

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due to the coronavirus after several case studies.[3] Favipiravir (6-fluoro-3-hydroxy-2-pyrazinecarboxamide) is a synthetic prodrug first identified by evaluating the antiviral efficacy of chemical compounds active against the influenza virus in Toyoma Chemicals' chemical library,[4] a highly effective broad spectrum and has demonstrated a significant level of antiviral efficacy against several RNA viruses.[5] The mechanism action of favipiravir has been observed in two ways by induction of lethal mutagenesis and by RNA chain termination and incorporation of T-705 in the viral RNA strand.[6] Favipiravir (prodrug) is a purine base analog that undergoes intracellular phosphoribosylation to become active favipiravir ribofuranosyl-5B-triphosphate (favipiravir-RTP), which can prevent SARS-CoV, MERS-CoV, and other RNA virus infections by directly inhibiting viral transcription and replication.[7] In pharmacokinetics studies, favipiravir shows significant bioavailability (>94%), a modest volume of distribution, and a 54% protein binding rate (10e20 L). After a single dosage, it achieves C_{max} in less than 2 hr, whereas T_{max} and half-life rise with further doses.[8] Patients with mild to severe COVID-19 receive 1800 mg of favipiravir twice daily on day one; mild to moderate COVID-19 patients receive a maximum of 800 mg twice daily for up to 14 days, and metabolites are eliminated by the renal. Pharmacodynamics reported that 54% of plasma protein was bound, acting as a prodrug, and undergoing intracellular ribosylation and phosphorylation. In contrast, the known adverse effects include diarrhoea, a drop in neutrophil counts, increased blood uric acid, and transaminases.[9,10]

Various analytical techniques have been reported for quantifying favipiravir by UV-spectrophotometric and RP-HPLC methods. H.M. Marzouk *et al.*, have developed a novel stability-indicating HPLC-DAD method for determining favipiravir, a potential antiviral drug for COVID-19 treatment; application to degradation kinetic studies and *in-vitro* dissolution profiling.[11] Comparison of HPLC and UV-spectrophotometric methods for quantification of favipiravir in pharmaceutical formulations.[12] RP-HPLC and UV analyses of favipiravir in bulk and pharmaceutical dosage form for the development, validation, and forced degradation stability-indicating investigation and prosecution.[13] Favipiravir, one of the COVID-19 antiviral regimens, was determined using green micellar solvent-free HPLC and spectrofluorimetry.[14] The analytical method developed by design reported method, analytical performance, and greenness evaluation of five multi-level design models utilized for impurity profiling of favipiravir, a promising COVID-19 antiviral drug.[15] Multivariate optimization for determination of favipiravir, a SARS-CoV-2 molecule, by the reverse-phase liquid chromatographic method using a QbD approach.[16] Application to spiked human plasma using bioanalytical reported methodologies and experimental design methodologies for creating a spectrofluorimetric method for determination of favipiravir, a prospective COVID-19 virus treatment agent.[17]

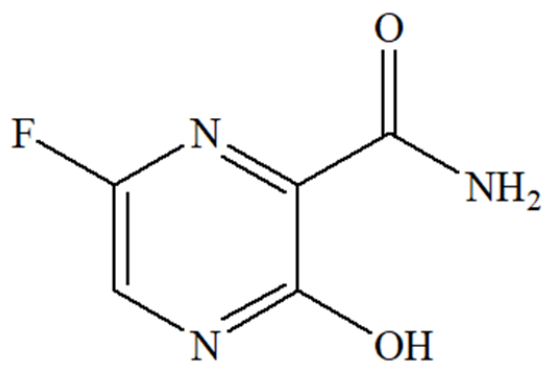

Figure 1: Chemical structure of favipiravir.

According to the previously reported analytical methods, our mainly aimed is to develop green, robust, rapid, sensitive, selective, linear, and precise different types of UV-spectrophotometric and RP-HPLC methods for the determination of favipiravir, determine the variance between the proposed methods by ANOVA, and *in-vitro* dissolution studies. The method was validated as per the United States Pharmacopeia[18] and ICH guidelines.[19] The proposed green analytical method developed by the appropriate selection of green, less toxic eco-friendly solvents is an enormous challenge because better separation and quantification analysis is much more important. This study uses green solvents as social, economic, environment friendly, eco-toxicity, hazard-free, and easily possible waste management. As per ICH Q2(R1) guidelines,[20] linearity, accuracy, precision, specificity, Limit of Detection (LOD), and Limit of Quantification (LOQ) are performed and utilized in determining the drug content of the favipiravir in various pharmaceutical products. *In-vitro* dissolution studies help to measure the extent and rate of solution formation from the favipiravir dosage form (Avigan-200 mg and Fluguard-200 mg).[21] ANOVA (one-way analysis of variance) is a data analysis technique determining the variance between the different proposal methods.[22]

MATERIALS AND METHODS

Materials

Mylan Laboratories Ltd., Hyderabad, India, provided favipiravir bulk powder as a kind gift. Favipiravir was purchased from the local pharmacy using the branded tablets avigan (200 mg) and fluguard (200 mg). All chemical reagents of high analytical grade were employed in the investigation. HPLC-grade water, methanol, and ethanol were procured from GlaxoSmithKline Pharmaceuticals Limited in Mumbai, India. We received sodium hydroxide, hydrogen peroxide, and hydrochloric acid from Gujarat, India's Ideal Chemicals Pvt. Ltd.

Instrumentation

Shimadzu 1800 UV spectrophotometer was used for this analysis, with 1 cm matched quartz cells for all measurements and UV probe 4.2 series software. HPLC system with a Swinnex type

filter (pore size = 0.45 µm), Waters 1525 binary pump, Waters 484 variable UV absorbance detector, and autosampler coupled to a symmetrical C_{18} column (4.6 mm x 150 mm, 3 µm spherical particles). Study the tablet dissolving profile using the 0.45 µm Nylon disc filter, TDT-08L, and USP type 2 rotating paddle apparatus from Electrolab. The investigation employed a digital analytical balance (Mettler Toledo, India), an ultrasonic sonicator (Spectra Lab, India), and validated borosilicate glass pipettes, volumetric flasks, and beakers.

Selection of mobile phase based on the green solvent solubility and stability studies

The drug's solubility and stability at 25°C were evaluated using a variety of green solvents. 10 mL of volumetric flasks containing various solvents and buffers, including water, ethanol, methanol, acetonitrile, phosphate buffer saline pH 7.2, phosphate buffer pH 5.5, 5.6, 7.2, and 7.4 were each given 10 mg of the drug. Comparatively, the drug observed throughout the experiment was completely soluble in water, ethanol, methanol, and acetonitrile but not in buffers. To conduct additional studies on solution stability, prepare 10 µg/mL solutions with each of the solvents mentioned above and continuously examine the samples using UV-visible spectroscopy at 2, 4, 6, 8, and 12 hr. Consequently, the mixture of methanol, ethanol, and water (25:35:40 v/v/v) was chosen as a mobile phase for the preparation of solutions based on the solvent effect, UV-spectrum, absorbance, and interference investigation. Zero-hazardous solvents, such as water, were initially chosen as an eco-friendly development method. Still, individual water could have performed better and was less stable than the mixture of methanol, ethanol, and water in the stock solution.

Preparation of mobile phase

HPLC-grade methanol, ethanol, and water (25:35:40 v/v/v) are used to prepare the mobile phase. The selected mobile phase was mixed well and degassed in an ultrasonic water bath for 20 min, and then the resulting solution was filtered through a 0.45µm filter under vacuum filtration.

Preparation of solutions for UV Spectrophotometry and HPLC

Favipiravir was accurately weighed at 10 mg and then transferred to a volumetric flask with a 10 mL capacity. The solute was first dissolved in the mobile phase to make the standard stock solution with a concentration of 1,000 µg/mL. This solution was then further sonicated and diluted with the mobile phase to the desired level. To achieve the working standard with a concentration of 100 µg/mL, further, dilute the previously prepared standard stock solution with the mobile phase. The solutions with the requisite concentrations for procedures A, B, C, and D were prepared by dilution with the mobile phase made from the working standard.

Different methods of development

Growing energy demand and the need to reduce environmental impact and increase energy security. Green chemistry is also called sustainable chemistry, and basically, this is focused on the design of products and processes that eliminate the use and generation of hazardous substances. So our research provides how green chemistry helps in methods of development by using a green solvent in the UV spectrophotometric and RP-HPLC methods for favipiravir in bulk, and different tablet dosages followed by ICH guidelines, and the greenness profile of the developed UV spectrophotometric and RP-HPLC techniques was evaluated using Analytical Eco-scale, which proved the greenness of the method concerning solvent, chemicals, energy consumed, and waste produced.[23-24]

Method A (Zero order spectrophotometric method)

The simplest way to run various analyses is using the principle of UV-spectroscopy. A blank solution for the mobile phase was maintained. From 200 to 400 nm, samples were recorded. Following the linearity analysis, the λ_{max} was confirmed to be 236 nm.

Method B (First-order spectrophotometric method)

The approach can recover unresolved band spectra with both qualitative and quantitative data. The mobile phase was kept as a blank solution. Spectra between 200 and 400 nm were measured. The zero-order spectra were transformed into first-order derivative spectra (delta lambda 8, scaling factor 1) using the inbuilt software of the instrument. After interpreting the data for linearity, the λ_{max} was found to be 227 nm.

Method C (Area under the curve spectrophotometric method)

Effectively solves the broad spectrum with the methodology is two effective points on the mixed spectrum are directly proportional to the concentration of the spectral component of interest. A reference solution was preserved for the mobile phase. Samples were captured between 200 and 400 nm. Using UV probe software-2.42, the spectra between 230 and 243 nm were recorded. The area versus concentration data was used to conduct the linearity assessment.

Method D (RP-HPLC method)

In the reverse-phase, HPLC is used to determine and separate most classes of chemical compounds by using polar aqueous solvents for the mobile phase and no-polar components like $C_{18}H_{37}$ or C_8H_{17} for the stationary phase.

Optimization of chromatographic detection for RP-HPLC method

Chromatographic conditions were optimized to establish a routine analysis of favipiravir, with excellent technique reproducibility and analytical throughput. Numerous columns were used for this analysis, like the Inertsil ODS, C_{18}, C_8, Kromasil nonpolar, and cyano columns. In contrast, symmetry C_{18} column (4.6 mm x 150 mm, 3 µm spherical particles), with methanol, ethanol, and water in the ratio of 25:35:40 (v/v/v), were used to achieve the best resolution, appropriate run duration, short retention time, nice peak shape, and enhanced responsiveness. Robustness studies revealed that using symmetry C_{18} columns from various vendors and a slight modification in the composition of the mobile phase had no impact on the analysis. As shown in Figure 2, the chromatogram of the favipiravir reference standard had a retention time of 7.216 min, a constant flow rate of 0.80 mL/min, at a column temperature of 25°C, at 236 nm, isocratic mode, and a 10 min run-time.

Method validation

The developed method was validated according to the ICH guidelines (ICH Q2R1) for linearity, specificity, precision, accuracy, robustness, the limit of detection, and quantification.[20]

Linearity

Linearity is an analytical method that produces test results directly proportional to the concentration of analyte present in the test sample. A plethora of solutions was made for the standard calibration curve based on Breer's Lambert law for methods A, B, and C at 2-12 µg/mL and for method D at 20-60 µg/mL.

Precision

Precision is the analytical method, indicating the analytical procedure's repeatability. When a procedure is exposed to many samplings of a homogenous sample, precision is defined as the degree of agreement between individual test findings. Six concentrations of 8 µg/ mL (methods A, B, C) and 40 µg/mL (method D) of standard drug solution are evaluated for intraday and interday precision, and variations are investigated. The drug concentrations were evaluated on different consecutive days in the intermediate precision investigation, demonstrating the laboratory variation on different days. The percentage RSD was calculated.

Accuracy

Accuracy is the analytical method investigating the closeness of test results obtained and the true value. By adding an adequate amount of favipiravir standard stock solution to the sample solution, accuracy was evaluated at three different concentration levels (50%, 100%, and 150%). The recovery was ascertained by measuring the percent recovery and determining the amount of drug in triplicate preparations at each concentration level.

Robustness

Robustness is a measure of its capacity to stay unaffected by little, but deliberate changes in analytical process parameters indicate its consistency over time. It was performed by altering the wavelength (±2 nm) in the UV-spectrophotometric technique and the flow rate (±0.1 mL/min), mobile phase ratio, and temperature (±3°C) in the RP-HPLC method. Still, there was no apparent difference in the results within ICH guidelines. The samples were validated six times.

Sensitivity

The Limit of Quantification and Limit of Detection were used as parameters in the sensitivity calculation. LOD refers to the lowest analyte concentration in a sample that can be detected but not fundamentally quantified. The lowest level at which an analyte may be measured with acceptable accuracy and precision is known as the LOQ.

The following formulae were used to calculate LOD and LOQ.

LOD = 3.3× standard deviation of response/slope of the calibration curve

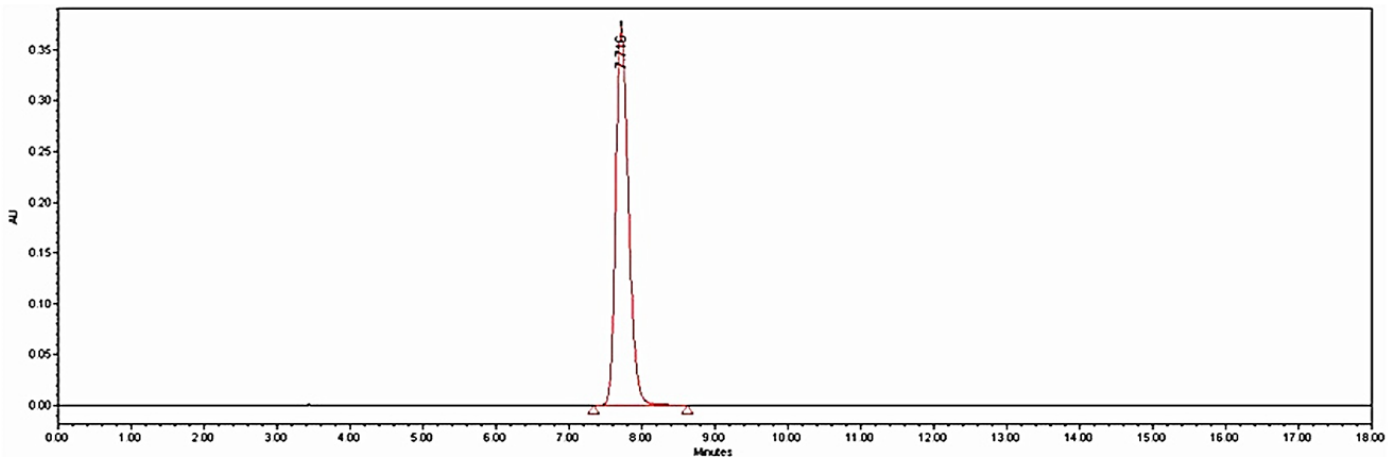

Figure 2: Optimised chromatogram of favipiravir.

LOQ = 10× standard deviation of response/slope of the calibration curve

Analysis of commercial dosage form

To analyze various commercial tablet dosage forms based on the efficacy and pharmacokinetics study evaluation, chose two distinct manufacturer tablets and carried out the assay to determine the amount of drug present in the dosage forms. The 200 mg brand-name avigan and fluguard tablets of favipiravir (20 tablets) were compared in this dissertation. For evaluation, 20 tablets are accurately energised and weighed. The powdered tablet containing the equivalent of 10 mg of favipiravir was weighed and placed into a volumetric flask with a capacity of 10 mL. The mobile phase was added to the mark and sonicated, and all solutions were filtered. Measure both tablet brands by contrasting them to the reference drug.

Stress degradation studies

Since it impacts the drug product's safety and effectiveness, the chemical stability of pharmaceutical compounds is a major source of concern. The FDA and ICH guidelines state the requirement of stability testing data to understand how a drug's substance and product quality change with time under various environmental factors. Knowledge of the molecule's stability helps select the proper formulation and package and provide proper storage conditions and shelf life, which is essential for regulatory documentation. So, we also emphasize stress degradation studies as specified in regulatory guidelines.[25]

Oxidation stress degradation studies

The 1 mL of favipiravir stock solution was combined with 1 mL of 35% hydrogen peroxide, diluted with mobile phase up to 10 mL, and left at room temperature for 90 min. The reference solution underwent the same conditions without adding 35% hydrogen peroxide. The test solution was sufficiently diluted to provide test solutions with concentrations of 10 µg/mL for Methods A, B, C, and 50 µg/mL for Method D. At last; the samples were analyzed using UV-spectroscopy, and HPLC to calculate the percentage of degradation.

Acid stress degradation studies

The 1mL of favipiravir stock solution was mixed with 1mL of 1N hydrochloric acid, and the volume was filled off with mobile phase to 10 mL and maintained at room temperature for 90 min. The same conditions were applied to the reference solution without adding acid. The test solution was neutralized with NaOH and diluted adequately to get a test solution of 10 µg/mL for methods A, B, and C, whereas 50 µg/mL for method D. The samples were also scanned in the UV-spectroscopy and HPLC and calculated the percentage of degradation.

Alkali stress degradation studies

In addition, 1mL of 1N NaOH was added to 1mL of favipiravir stock solution. The volume was then filled to 10 mL with mobile phase and left at room temperature for 90 min. Additionally, the reference solution was treated under identical circumstances without adding NaOH. Further, the solution was diluted to provide test solutions with 10 µg/mL concentrations for methods A, B, C, and 50 µg/mL for method D. To calculate the percentage of deterioration, the samples were further scanned using UV-spectroscopy and HPLC.

Dry heat stress degradation Studies

The standard drug solution was kept in an oven at 80°C for 48 hr to assess dry heat degradation. Developed the test solutions at 10 µg/mL for methods A, B, C, and 50 µg/mL for method D. The reference solution underwent the same procedures without the sample being subjected to heat. The samples' deterioration percentages were also estimated after the samples were scanned employing UV-spectroscopy and HPLC.

Photolytic stress degradation studies

The sample solution was exposed to UV light at 365 nm for 48 hr in a UV chamber to test the drug's photolytic stability. Developed the test solutions at 10 µg/mL for methods A, B, C, and 50 µg/mL for method D. The reference solution was also subjected to the same circumstances but without exposure to the UV light. After the samples were scanned using UV and HPLC, the degree of deterioration was also recorded.

In-vitro dissolution test

A crucial tool for comprehending product behaviour, conducting bioavailability/bioequivalence studies, and developing new formulations is establishing and analyzing dissolution profiles.[26] The United States of Pharmacopoeia type two rotating paddle dissolution equipment was used to dissipate the 200 mg of avigan and fluguard tablets, both of which are branded with the favipiravir drug. Both brands of tablets with the equivalent amount of pharmaceutical formulated drug dosage were placed into 900 mL of various dissolution mediums like acetate buffer (pH 4.4 and 5.0), 0.067M phosphate buffer (pH 5.4), chloride buffer (pH 2.0), and phosphate buffer (pH 2.8, 3.0, 3.5, 5.5, 5.8, 6.4, 7.0, and 7.2). The dissolution medium is maintained at 37 ± 0.5°C and performed at different speeds at 25-50 rpm as per the USP general specification.[21] Samples were collected at different time intervals, 10, 20, 30, 40, 50, and 60 min, and filtered the solutions by a 0.45µm nylon disc filter. All solutions are analyzed, and the maximum drug release profile is identified with excellent sink conditions.

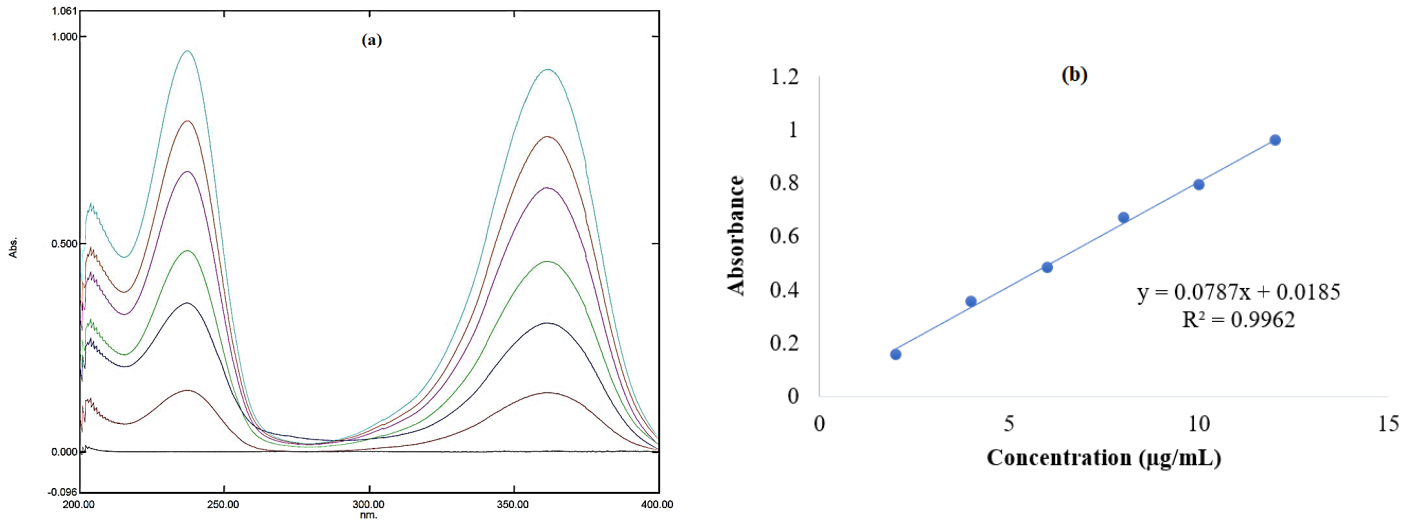

Figure 3: Spectrum of favipiravir for method A (Zero order spectrophotometric method) at (2-12 µg/mL): (a) Overlay spectrum (b) Calibration curve.

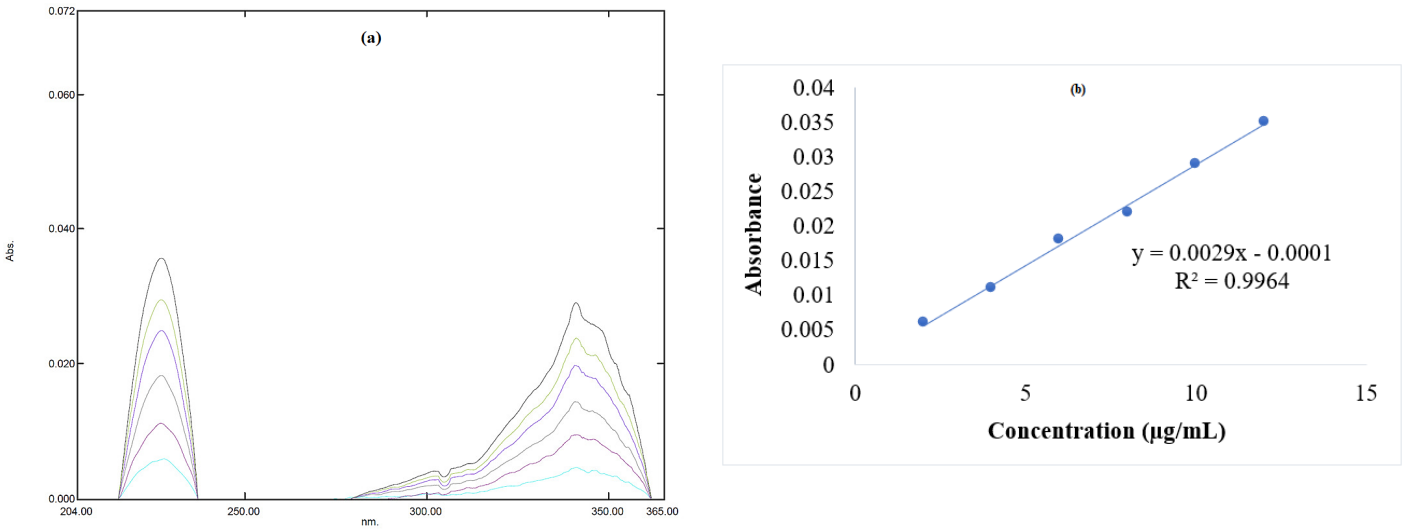

Figure 4: Spectrum of favipiravir for method B (First-order spectrophotometric method) at (2-12 µg/mL): (a) Overlay spectrum (b) Calibration curve.

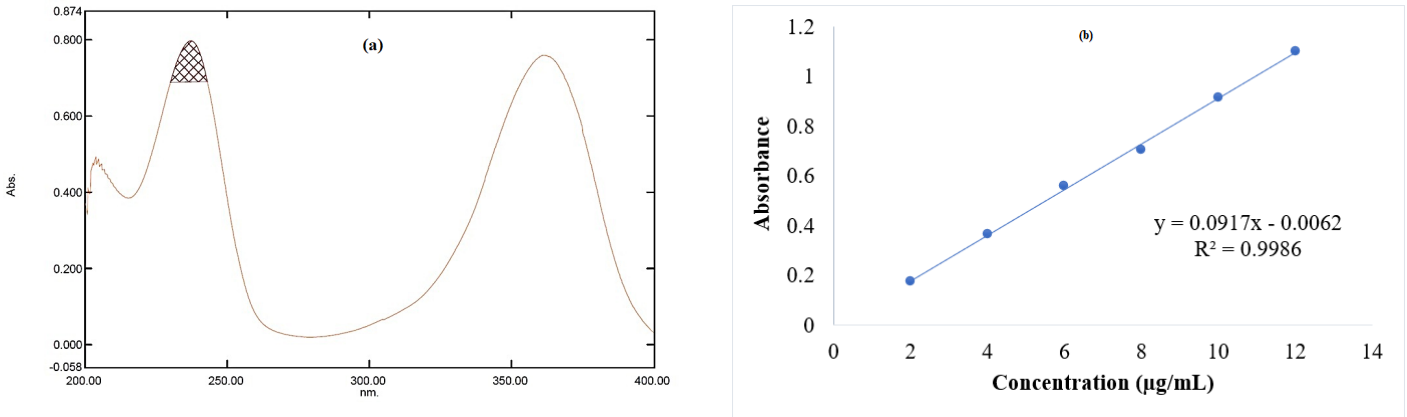

Figure 5: Spectrum of favipiravir for method C (Area under the curve spectrophotometric method): (a) AUC of 10 µg/mL spectrum (b) Calibration curve (2-12 µg/mL).

Analysis of variance (ANOVA)

ANOVA is a statistical method to determine the variance between the results produced by different newly developed methods in the research of favipiravir ($p < 0.05$) by the market formulation assay data studies.[22]

RESULTS

Linearity

In Linearity studies, calibration curves were graphed in a concentration range of 2-12 µg/mL for Methods A, B, C, and 20-60 µg/mL for Method D. The linear regression equation of method A is y = 0.0787x + 0.0185 with a correlation coefficient of 0.9962 (Figure 3), Method B is y = 0.0029 x - 0.0001 with a correlation coefficient of 0.9964 (Figure 4), Method C is y = 0.0917 x - 0.0062 with a correlation coefficient of 0.9986 (Figure 5), Method D is y = 74520x + 51916 with a correlation coefficient of 0.9996 (Figure 6).

Precision

When RSD in precision studies was less than 2%, the suggested procedure had acceptable reproducibility. The performance of intraday and interday precision and the percent RSD for the response of six replicate measurements in methods A, B, C, and D were within the acceptable ranges. Results from the intraday and interday precision studies are summarized in Tables 1, 2, and 3.

Accuracy

The percentage of recovery values in the accuracy studies demonstrates that the proposed method is accurate and that interference response exists. Three replicate measurements using different methods A, B, C, and D, showed that the percent recovery was within the allowed ranges (Table 4 and 5).

Robustness

All the parameters were passed with no notable changes. The percent RSD was within the acceptable range (Table 6).

Assay

The commercially available avigan (200 mg) and fluguard (200 mg) formulations of favipiravir assay were carried out, and the purity percentage was assessed by methods A, B, C, and D. Neither substantial variation was found during the percentage purity analysis. The interpretation findings for the marketed tablets of favipiravir are depicted in Table 7.

Table 1: Intraday precision for methods A, B, and C of favipiravir.

Sl. No.	Conc. (µg/mL)	Method A	Method B	Method C	%RSD		
		Absorbance		Area	Method		
					A	B	C
1	8	0.675	0.022	0.702	0.38%	0%	0.62%
2	8	0.671	0.022	0.704			
3	8	0.674	0.022	0.701			
4	8	0.672	0.022	0.703			
5	8	0.678	0.023	0.713			
6	8	0.672	0.022	0.703			

Method A (Zero order spectrophotometric method), Method B (First-order spectrophotometric method), Method C (Area under the curve spectrophotometric method).

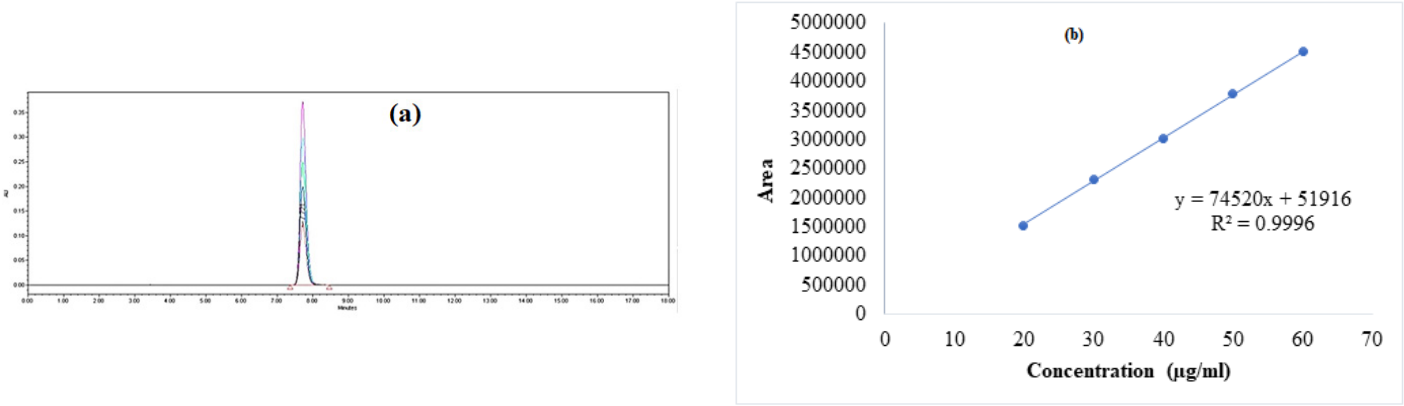

Figure 6: Chromatogram of favipiravir for method D (RP-HPLC method) at 20-60 µg/mL: (a) Overlay chromatogram (b) Calibration curve.

Table 2: Interday precision for methods A, B, and C of favipiravir.

Sl. No.	Conc. (µg/mL)	Method A	Method B	Method C	%RSD		
		Absorbance		Area	Method A	Method B	Method C
1	8	0.660	0.022	0.690	1.19%	0%	1.44%
2	8	0.663	0.022	0.685			
3	8	0.680	0.023	0.712			
4	8	0.661	0.022	0.704			
5	8	0.665	0.022	0.692			
6	8	0.674	0.022	0.701			

Method A (Zero order spectrophotometric method), Method B (First-order spectrophotometric method), and Method C (Area under the curve spectrophotometric method).

Table 3: Intraday and Interday precision for method D (RP-HPLC method).

Sl. No.	Conc. (µg/mL)	Intraday precision area	Interday precision area
1	40	3123692	3026345
2	40	3024531	3126543
3	40	3024451	3089760
4	40	3060237	3130953
5	40	3100137	3055778
6	40	3132137	3123457
%RSD		1.56%	1.39%

Table 4: Favipiravir accuracy observations for methods A, B, and C.

Level	Conc. (µg/mL)	Amount of drug added (µg/mL)		Amount recovered (µg/mL)			% Recovery		
		Pure	Formulation	Method			Method		
				A	B	C	A	B	C
50%	3	2	1	2.96	3.01	2.93	1.69%	0.57%	0.70%
	3	2	1	2.92	3.01	2.97			
	3	2	1	3.02	2.98	2.94			
100%	6	2	4	6.03	6.02	6.04	0.48%	0.49%	0.59%
	6	2	4	5.98	6.05	6.02			
	6	2	4	5.98	5.99	5.97			
150%	9	2	7	8.99	9.01	9.07	0.33%	0.28%	0.83%
	9	2	7	9.03	8.97	8.99			
	9	2	7	8.97	9.02	8.92			

Method A (Zero order spectrophotometric method), Method B (First-order spectrophotometric method), Method C (Area under the curve spectrophotometric method).

Table 5: Favipiravir accuracy observations for methods D (RP-HPLC method).

Level	Conc. (µg/mL)	Amount of drug added (µg/mL)		Amount recovered (µg/mL)	% Recovery
		Pure	Formulation		
50%	15	10	5	15.03	0.37%
	15	10	5	15.10	
	15	10	5	14.99	
100%	30	10	20	29.89	0.16%
	30	10	20	29.97	
	30	10	20	29.98	
150%	45	10	35	44.96	0.12%
	45	10	35	44.88	
	45	10	35	44.86	

Table 6: The favipiravir robustness data for several approach techniques using UV and HPLC.

Method	Condition	%RSD
A	Wavelength 234 nm	0.121
	Wavelength 238 nm	0.123
B	Wavelength 229 nm	0.23
	Wavelength 225 nm	0.24
C	Wavelength 244 nm to 231nm	0.56
	Wavelength 242 nm to 229 nm	0.53
D	Flow rate 0.6 0mL/min	0.64
	Flow rate 1mL/min	0.61
	Mobile phase methanol: ethanol: water 30:25:45 (v/v/v)	0.24
	Mobile phase methanol: ethanol: water 20:40:40 (v/v/v)	0.40
	Temperature 27°C	0.87
	Temperature 33°C	0.64

*Mean of six observations.Method A (Zero order spectrophotometric method), Method B (First-order spectrophotometric method), Method C (Area under the curve spectrophotometric method), and Method D (RP-HPLC method).

Sensitivity

In the LOD analysis, the detection limits for methods A, B, C, and D were 0.18, 0.64, 0.32, and 0.52 µg/mL, while the quantitation limits were 0.55, 1.96, 0.96, and 1.56 µg/mL. Table 8 displays the relevant LOD and LOQ values for favipiravir.

Stress degradation studies

Studies on stress degradation were carried out under various stressful conditions, but no significant degradation was observed. The highest degradation percentage was observed in oxidation stress tribunals, where methods A, B, and C observed 18.56, 20.63, and 14.39% of degradation, respectively. However, method D showed 15.82% degradation at 8.261 min of retention time (Figure 7).

Studies on acid stress degradation indicated that methods A, B, and C exhibited 9.21, 10.34, and 10.40% degradation, respectively. In contrast, method D showed a degradation percentage of 8.60% at 8.247 min of retention time (Figure 8).

In investigations on alkali stress degradation, it was revealed that methods A and C exhibited degradation rates of 1.64 and 1.75%, respectively. In contrast, method D had a degradation rate of 2.7% at 8.246 min of retention time. Method B, however, showed no indications of deterioration (Figure 9).

Less percentage of degradation was observed in dry heat stress degradation studies, with method A finding 0.12% and method D finding 0.23% at 7.961 min retention time. However, the analysis period saw no degradation for methods B or C (Figure 10).

Regarding photolytic stress degradation, methods A and C showed degradation percentages of 1.14, and 0.65; however, methods B showed no degradation at all, and method D showed a degradation percentage of 0.04% at 7.960 min retention time (Figure 11).

The validation parameters are summarized in Table 9, while Table 10 summarizes the desired outcome of stress degradation studies.

In-vitro Dissolution Test

The favipiravir commercial tablets were used in the dissolution investigation, and the findings indicate that pH and rpm affect how quickly the drug is released from the tablet. Compared to other dissolution mediums, phosphate buffer pH 6.4 had a higher favipiravir solubility and a higher drug dissolution percentage. The US-FDA states that less than 85% of drug release indicates an unsatisfactory outcome.[21] After assessing the highest drug release profile and excellent sink conditions, phosphate buffer pH 6.4 at

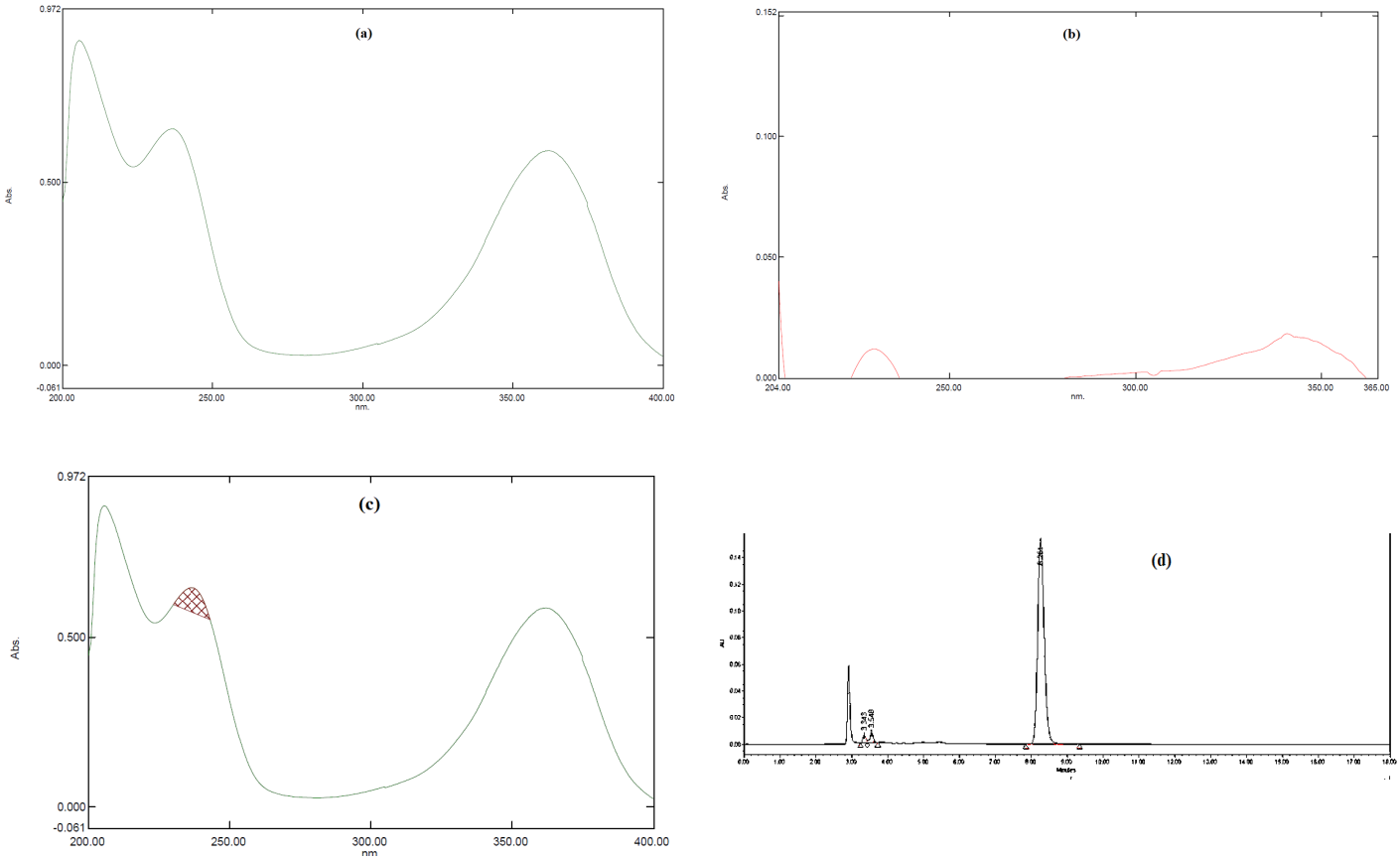

Figure 7: The oxidative stress degradation studies spectrum and chromatograms for methods A, B, C, and D.

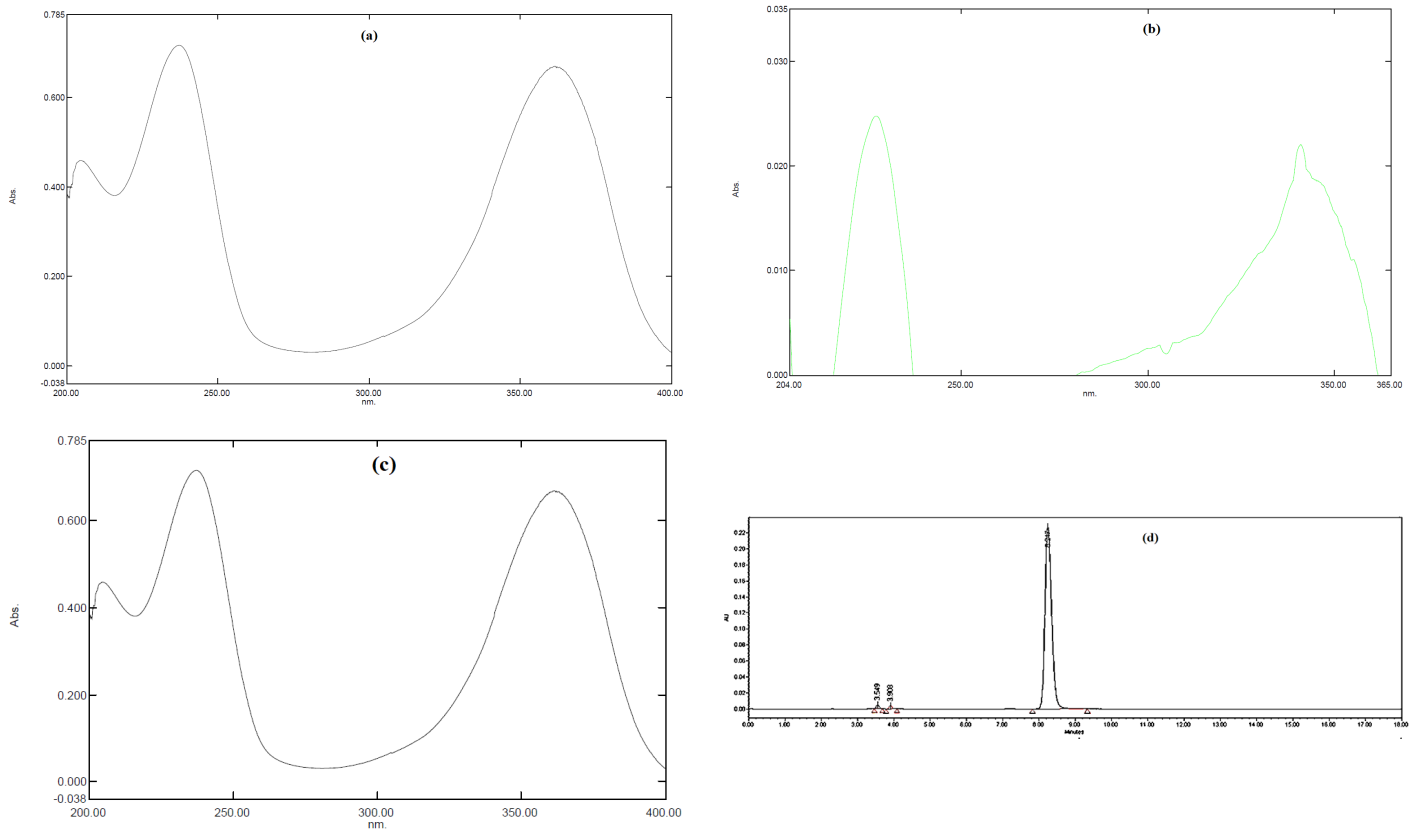

Figure 8: The acid stress degradation studies spectrum and chromatograms for methods A, B, C, and D.

Table 7: Assay data for the commercially available favipiravir formulations (Avigan and Fluguard) using UV and HPLC techniques.

Drug and label claim	Amount estimated (mg/tab)				Purity (% w/w) ± S.D, (%RSD)			
	Method				Method			
	A	B	C	D	A	B	C	D
Avigan (200 mg)	199 ± 0.52	199 ± 0.44	199 ± 0.22	199 ± 0.42	99.66 ± 0.26 (0.26%)	99.59 ± 0.22 (0.22%)	99.63 ± 0.11 (0.11%)	99.72 ± 0.20 (0.20%)
Fluguard (200 mg)	199 ± 0.25	199 ± 0.30	199 ± 0.18	198 ± 0.68	99.57 ± 0.20 (0.20%)	99.64 ± 0.15 (0.15%)	99.70 ± 0.09 (0.001%)	99.43 ± 0.34 (0.34%)

*Mean of three observations.Method A (Zero order spectrophotometric method), Method B (First-order spectrophotometric method), Method C (Area under the curve spectrophotometric method), and Method D (RP-HPLC method).

Table 8: Employing UV and HPLC techniques, the sensitivity assessments (LOD and LOQ) of favipiravir.

Method	LOD (µg/mL)	LOQ (µg/mL)
Method A	0.18	0.55
Method B	0.64	1.96
Method C	0.32	0.96
Method D	0.52	1.56

*Mean of three observations.Method A (Zero order spectrophotometric method), Method B (First-order spectrophotometric method), Method C (Area under the curve spectrophotometric method), and Method D (RP-HPLC method).

Table 9: Overview of favipiravir UV-spectrophotometric and RP-HPLC validation parameters.

Parameters	Method A	Method B	Method C	Method D
λ_{max}	236 nm	227 nm	230-243 nm	_
Linearity (µg/mL)	2-12 µg/mL	2-12 µg/mL	2-12 µg/mL	20-60µg/mL
Regression coefficient	R^2 = 0.9962	R^2 = 0.9964	R^2 = 0.9986	R^2 = 0.9996
Regression equation (y=mx+c)	y = 0.0787x + 0.0185	y = 0.0029x - 0.0001	y = 0.0917x - 0.0062	y = 74520x + 51916
Intra-day precision (% RSD)	0.38%	0%	0.62%	1.56%
Inter-day precision (% RSD)	1.19%	0%	1.44%	1.39%
Robustness (% RSD)	0.121-0.123	0.23-0.24	0.53-0.56	0.24-0.87
LOD (µg/mL)	0.18	0.64	0.32	0.52
LOQ (µg/mL)	0.55	1.96	0.96	1.56
Analytical Eco-scale Score	94	94	94	92

37°C and 50 rpm were selected as a suitable dissolution media. The findings of the dissolution study are depicted in Figure 12.

.Statistical Comparison of Different Types of Methods by One-Way Analysis of Variance (ANOVA)

The A, B, C, and D methods were contrasted using a one-way analysis of variance (ANOVA). Since the $F_{calculated}$ value was lower than the $F_{tabulated}$ value and the *p*-value was 0.976523, it was concluded that there was no discernible difference between the methods. Table 11 displays the findings from the ANOVA analysis.

Evaluation of Greenness Proposed Methods

Evolution must occur before a method may be deemed environmentally benign. The methodologies that can be used to assess greenness in analytical green chemistry include the Analytical Eco-Scale (AES), Green Analytical Procedure Index (GAPI), National Environmental Methods Index (NEMI), and Analytical Greenness Metric Approach (AGREE).[27] The Analytical Eco-Scale (AES) technique can assess the green methods by calculating the analytical eco-scale total score, which indicates the level of hazards, level of toxic solvents, eco-friendly level, presence of toxic substances, manufactured waste, and energy consumed. According to the formula (Analytical Eco-scale = 100-total penalty points), the AES technique determines the analytical methods' Eco-scale score and penalty points.[28]

The proposed methods are developed by a series of eco-friendly solvents like methanol, ethanol, and HPLC grade water, and observed a better chromatogram and spectrums. Compared to

Table 10: The desired outcome of favipiravir stress degradation studies employing UV-spectrophotometric and RP-HPLC approaches.

Degradation Condition	Method A	Method B	Method C	Method D	% Degradation			
	Absorbance		Area		Method			
					A	B	C	D
Oxidation	0.645	0.023	0.678	3191385	18.56%	20.63%	14.39%	15.82%
Acid	0.719	0.026	0.814	3464969	9.21%	10.34%	10.40%	8.60%
Alkali	0.779	0.029	0.898	3689021	1.64%	0%	1.75%	2.7%
Dry Heat	0.791	0.029	0.914	3782462	0.12%	0%	0%	0.23%
Photolytic	0.783	0.029	0.908	3789589	1.14%	0%	0.65%	0.04%

Table 11: One-way ANOVA statistical comparison of various favipiravir executives' techniques.

Source of Variation	SS	d_f	MS	$F_{calculated}$	*P*-value	$F_{tabulated}$
Between Groups	0.013337	3	0.004446	0.06332	0.976523	6.591382
Within Groups	0.28085	4	0.070213			
Total	0.294188	7				

Table 12: Methods developed based on an evaluation of greenness.

Reagents and Instruments	Method			
	A	B	C	D
Water	0	0	0	0
Methanol	4	4	4	4
Ethanol	2	2	2	2
Filtration	0	0	0	0
Occupational hazard	0	0	0	0
HPLC	0	0	0	1
UV spectroscopy	0	0	0	0
Waste	0	0	0	0
Total penalty point	8	8	8	9
Analytical eco-scale total score	94	94	94	92

*Analytical Eco-scale score, ideal method if = 100, excellent >75, acceptable > 50, inadequate < 50.

methanol, and ethanol, water is the most eco-friendly, with zero toxic levels. Hence, the use of water gets a green method but only used water not observed good separation and a good spectrum for that used a less portion of ethanol and methanol which is less toxic as compared to others highly toxic and expensive solvents like acetonitrile, benzene, ortho-phosphoric acid. Based on the solvent's penalty point by analytical eco-scale, calculate the toxicity level, and demonstrate that the proposed methods A, B, C, and D are showing more eco-score like 94, 94, 94, and 92 (Table 12).[29-32] After comparing the eco-score values of other reported methods[11-14] with the proposed methods, it declares that the proposed methods are ecologically friendly.[33]

DISCUSSION

Throughout the whole lifecycle of pharmaceutical manufacturing, from dispensing to waste disposal, there is a persistent risk of chemical hazards. Favipiravir tablets have been prescribed worldwide for the first-line treatment of influenza, which can potentially target other viral infections. UV-spectrophotometric and RP-HPLC Methods are eco-friendly and valid based on the eco-scale score and ICH guidelines.[20] The eco-scale score of developed methods is < 92.00, which indicates the methods are more significantly eco-friendly with the environment and level of safety.[27] An overlay spectrum and calibration curve show the linearity of the developed method, and the R^2 value is more than 0.99, which indicates the efficacy of the among methods. In the linearity studies, concentration, absorbance, and area are equally and significantly increased depending on each other; based on

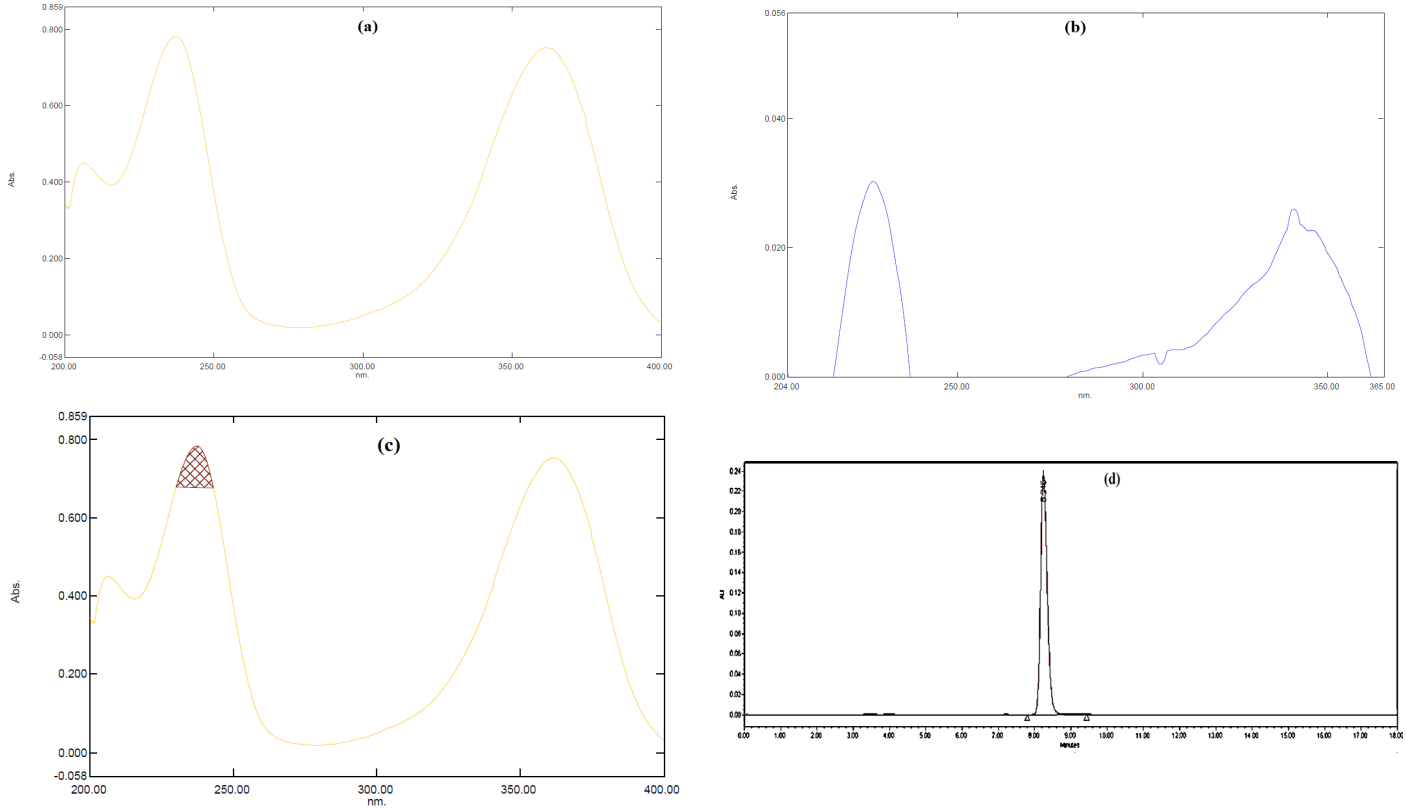

Figure 9: The alkali stress degradation studies spectrum and chromatograms for methods A, B, C, and D.

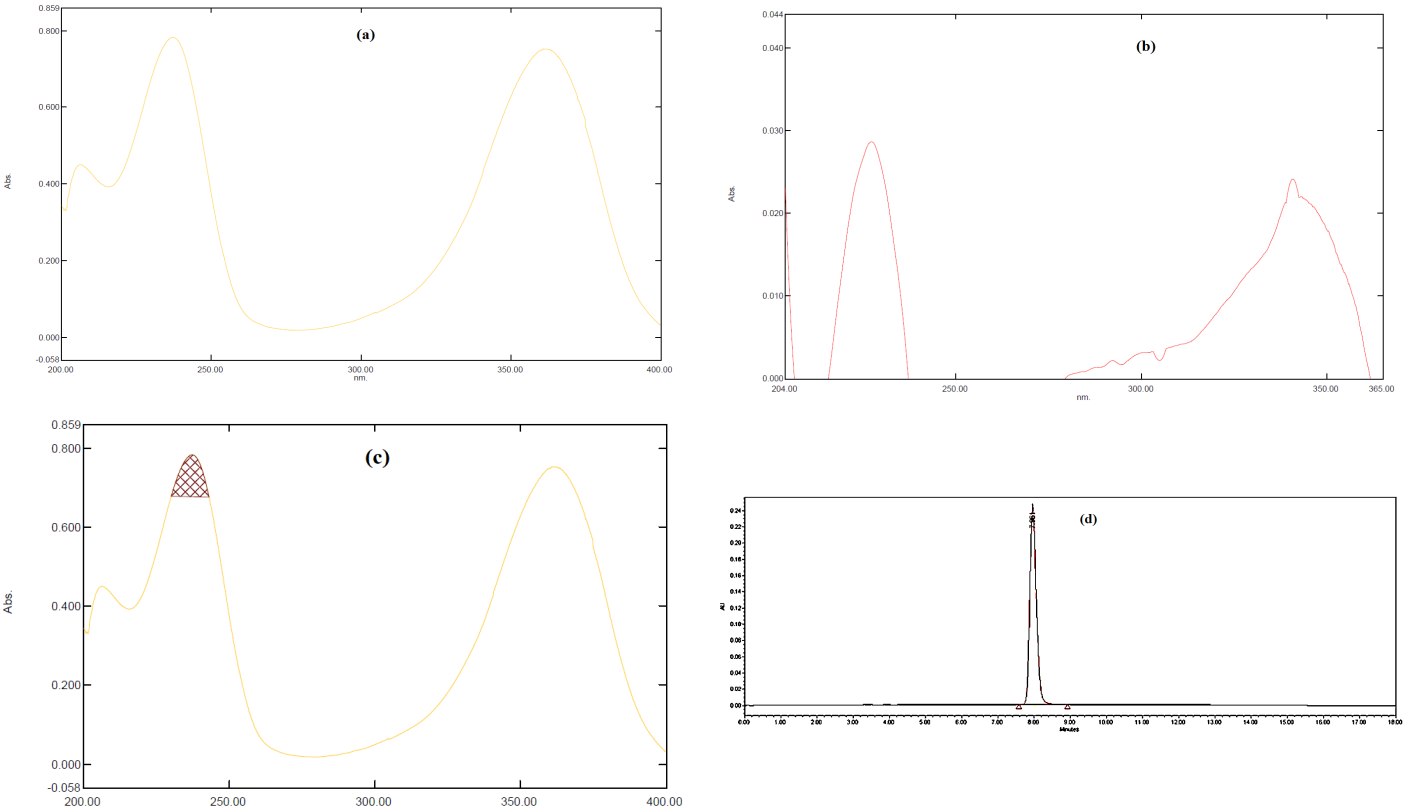

Figure 10: The thermal stress degradation studies spectrum and chromatograms for methods A, B, C, and D.

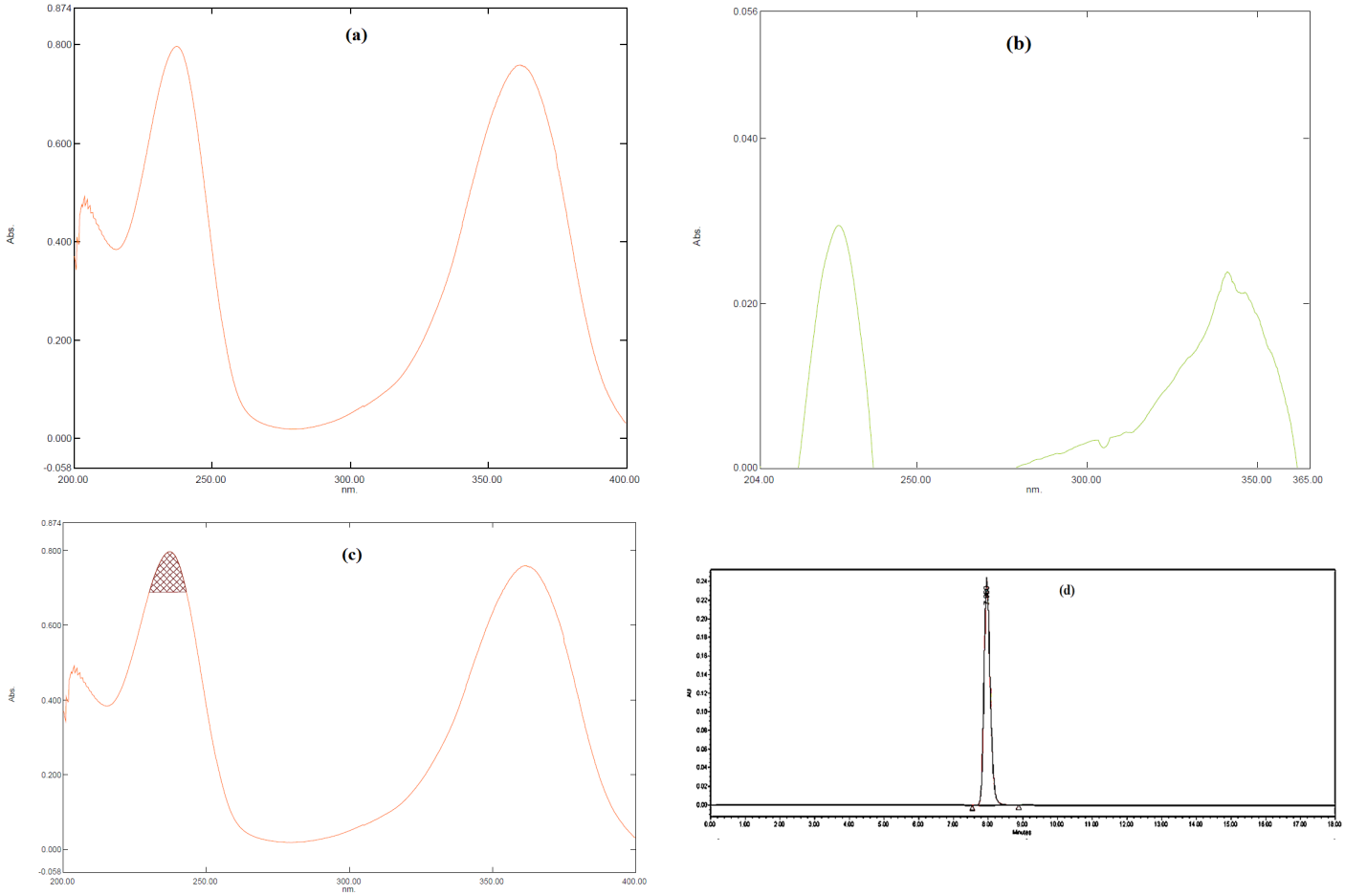

Figure 11: The photolytic stress degradation studies spectrum and chromatograms for methods A, B, C, and D.

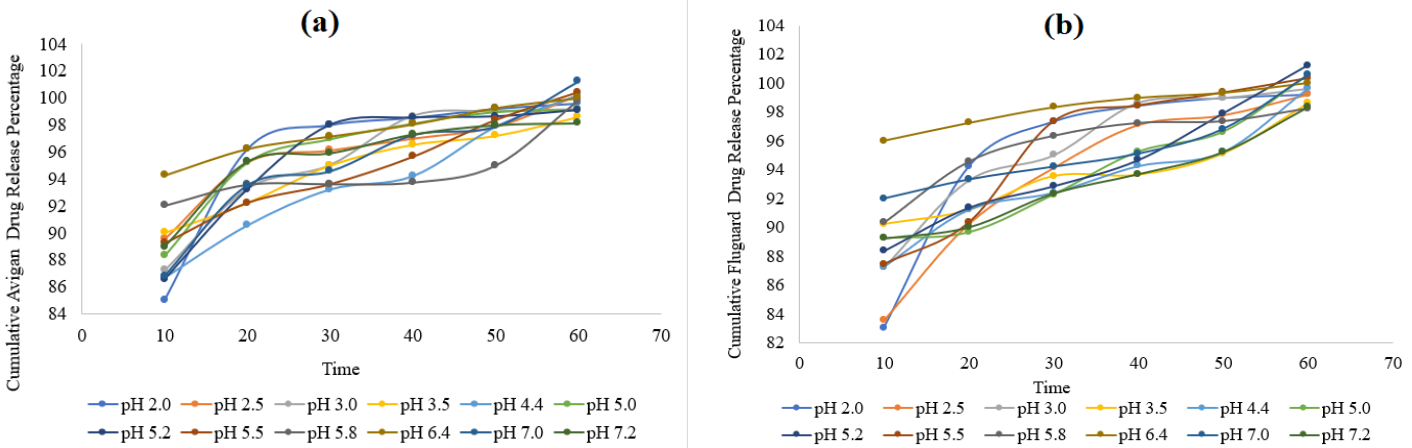

Figure 12: RP-HPLC technique dissolution profiles for (A) Avigan and (B) Fluguard. Statistical Comparison of Different Types of Methods by One-Way Analysis of Variance (ANOVA).

this character, all developed methods follow the Beer-Lambert law. Accuracy is the best indication of systematic errors; three samples with three different drug concentrations were assessed to ensure the accuracy of the newly developed methods. The mean percent recovery at various working strengths was > 98.00. Precision was evaluated by repetitive intra-day determination of drug solution at different time intervals within the day. At the same time, inter-day precision was determined by conducting the same procedure by two analysts on different days. The % of RSD values of intra and inter-day precisions studies is less than 2%. The detection limit indicates the minimum amount of analyte in a sample that could be detected but not necessarily

precisely quantified. Quantitation limits express the quantitation of parameters for quantitative assay for low levels of compounds in sample matrices and the useful for identifying impurities or product degradation. They indicate the lowest amount of the analyte in a sample that can be determined with acceptable specifications. Studies on stress degradation were carried out under various stressful conditions, but no significant degradation was observed. In the ANOVA test, there was no discernible difference between the methods.[22] Phosphate buffer pH 6.4 at 37°C and 50 rpm were selected as a suitable dissolution media for the dissolution of favipiravir products.

CONCLUSION

The proposed "green" analytical spectroscopic and RP-HPLC methods are uncomplicated, quick, accurate, precise, robust, sensitive, selective, and stability-indicating. The proposed methods have been suitable for the routine analysis of the standard drug and commercial products of favipiravir. Different spectroscopic methods show significant results with a linear calibration curve. The RP-HPLC method can separate the favipiravir using green mobile solvents at minimal retention time. By ANOVA statistical tool evaluated the results of the proposed methods are significantly not produced any variance. *In-vitro* dissolving studies show that phosphate buffer (pH 6.4) is the optimum dissolution media and observed a linear drug release rate at 37°C and 50 rpm; this study is beneficial for monitoring the characterization of favipiravir drug release from commercial formulations.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

AES: Analytical Eco-Scale; **AGREE:** Analytical Greenness Metric Approach; **ANOVA:** Analysis of Variance; **API:** Active Pharmaceutical Ingredient; **C_{max}:** Peak Plasma Concentration; **DAD:** Diode-Array Detection; **GAPI:** Green Analytical Procedure Index; **HPLC:** High-Performance Liquid Chromatography; **ICH:** International Council for Harmonisation; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification; **MERS-CoV:** Middle East Respiratory Syndrome; **NEMI:** National Environmental Methods Index; **QbD:** Quality by Design; **RNA:** Ribonucleic Acid; **RP-HPLC:** Reverse Phase High-performance Liquid Chromatography; **RSD:** Relative Standard Deviation; **SARS-CoV-2:** Severe Acute Respiratory Syndrome Coronavirus; **T_{max}:** Time to Reach the Maximum Concentration; **US-FDA:** United States Food and Drug Administration; **USP:** United States Pharmacopoeia; **UV:** Ultraviolet; **R^2:** Coefficient of Determination; **λ_{max}:** Maximum Wavelength.

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