Spectrophotometric determination of favipiravir in presence of its acid hydrolysis product

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Abstract

Favipiravir (FAV) has been approved as an antiviral drug used in pandemic corona virus to treat covid-19. It has an amide moiety susceptible to hydrolysis and degradation in acid medium. Therefore, four simple, sensitive, and accurate stability indicating spectrophotometric methods have been developed for the determination of FAV in presence of its acid induced degradation product. The first method describes direct determination of FAV at 323 nm. Dual wavelength method was the second developed one for FAV quantitation by recording the absorbance difference at 322.7 and 270 nm. The third method involves using first derivative peak to peak amplitude at 338.0 and 308.0 nm, while difference spectrophotometry was the fourth suggested method, and it was based on recording the spectral changes at 361.3 nm as pH changes. The obtained calibration curves were linear over 4.0–22.0 μg/mL. Accuracy of the suggested procedures ranged from 99.11 to100.06, while precision results were from 0.80 to1.68. The developed methods were used to determine FAV in pure powdered form, laboratory-prepared mixtures with their degradation product, and pharmaceutical formulation without interference from its acidic degradation product. The greenness was assessed based on GAPI and ACREE metric and was found to be compatible and in reconciliation with green analytical chemistry concepts.

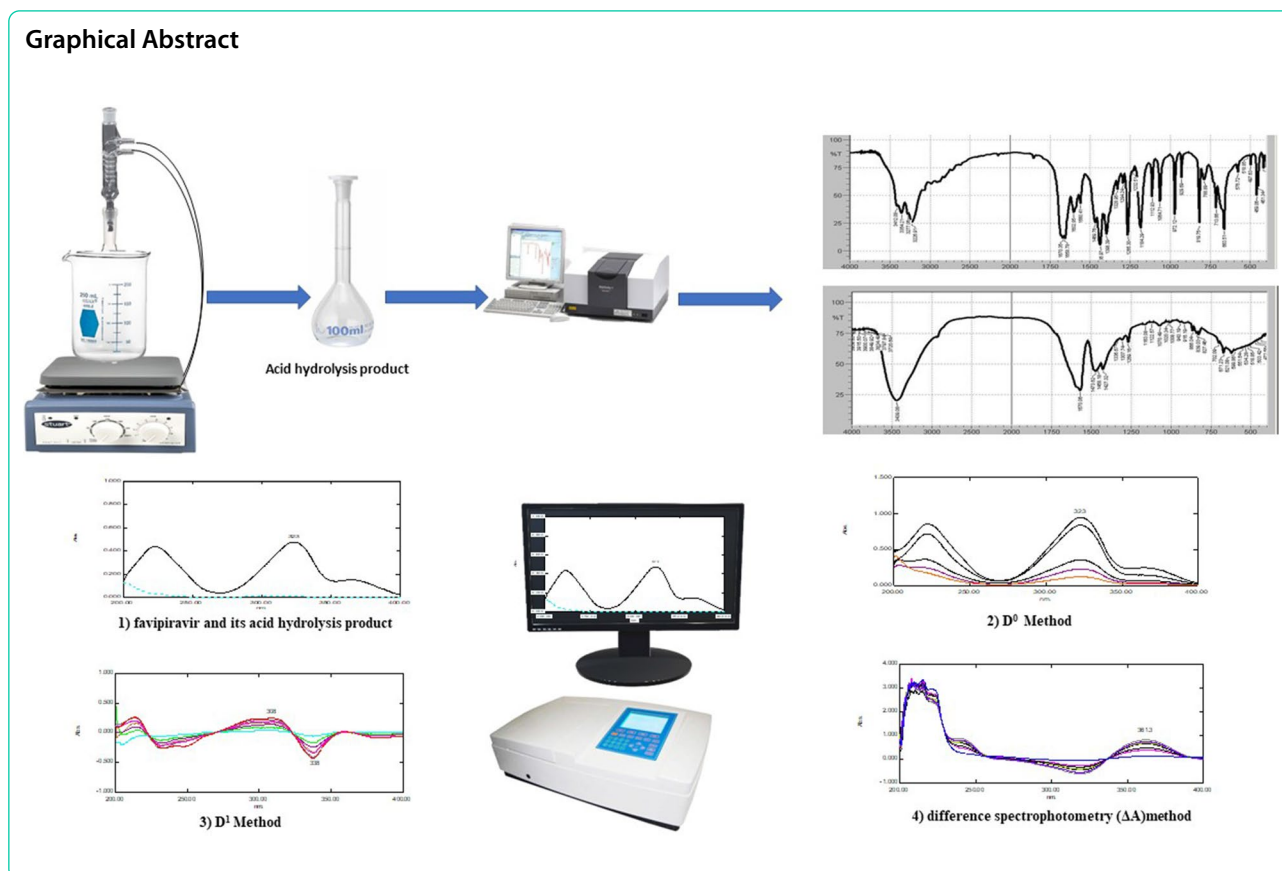**Keywords** Favipiravir, Acid-induced degradation, Spectrophotometry, Pharmaceutical formulation

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Graphical Abstract

Acid hydrolysis product

1) favipiravir and its acid hydrolysis product

2) D⁰ Method

3) D¹ Method

4) difference spectrophotometry (ΔA)method

Introduction

Globally, there have been more than four hundred million confirmed cases of COVID-19, including about 6 million deaths, reported to the World Head Organization (WHO). COVID-19 (coronavirus disease 2019) is a disease caused by a virus known as SARS-CoV-2. It spreads from infected person to another through breathing out droplets and small particles which contain the virus. Many trials have been done to solve this problem and treat patients. One of the most important and effective drugs that have been discovered and used to treat COVID-19 is Favipiravir (FAV). It was approved to treat novel viruses including Ebola and most recently, COVID-19. FAV is (5-fluoro-2-oxo-1*H*-pyrazine-3-carboxamide), Fig. 1. It is a synthetic purine base analog prodrug that is converted to active favipiravir ribofuranosyl-5B-triphosphate (FAV-RTP) by intracellular phosphoribosylation [1], This active form binds to and hinders RNA dependent RNA polymerase (RdRp), which finally blocks viral transcription and replication.

Stability study of drugs is considered one of the most important ways to confirm drug stability and is a regulatory requirement as well. Forced degradation must be the first step in method development. Hydrolysis is one of the major mechanisms for degradation of biodegradable materials [2]. Identification of primary degradation products as well as unknown impurities is then performed.

The literatures showed several analytical methods for the quantitative determination of FAV including spectrophotometry [3], spectrofluorimetry [4–6], HPLC and LC-UV [7–20], HPTLC [21, 22] and electrochemical methods [23–27] in pharmaceutical formulations or plasma.

In this paper, four spectrophotometric methods were applied for determination of FAV in the presence of its acid-induced degradation product (FAV deg.) in pure form and in pharmaceutical dosage form. The applied methods are Direct, Dual wavelength (DW), difference spectrophotometry (ΔA) and first derivative peak to peak (D¹). The developed methods are simple, accurate, validated, cost-effective, and can be used for the determination of FAV in the presence of (FAV deg.).

Experimental

Instrumentation

Spectrophotometric measurements were performed using Shimadzu 1601 spectrophotometer (Tokyo, Japan), a double beam UV–Vis spectrophotometer assisted with
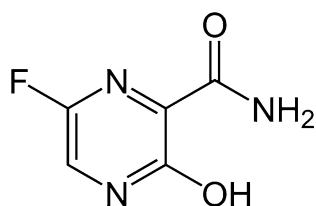
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Fig. 1 Chemical structure of favipiravir

UV Probe software (version 2.51). Scanning was done at 200–400 nm range, with 0.1 nm intervals. PH meter (Jenway) was used for pH adjustment. UV-lamp with short wavelength (254 nm; Deuterium, USA). IR Spectrophotometer: Shimadzu 435 (Kyoto, Japan), sampling was undertaken as potassium bromide disks.

Materials and reagents
All reagents and solvents utilized during the experiment were of analytical grade. Methanol and Hydrochloric acid (Sigma-Aldrich, Germany while Sodium hydroxide was obtained from VWR Chemicals, US. FAV pure drug $(100.03 \pm 0.61\%)$ was kindly supplied from Eva Pharm Co. (Cairo, Egypt). Piravafi® tablets (batch no.2132642; Marcyrl Pharmaceutical Industries, Egypt) containing 200.0 mg FAV per tablet were purchased from local market.

Stock standard solution preparation
A stock standard solution of FAV (100.0 µg/mL) was prepared by dissolving 10.0 mg of FAV in 100.0 mL methanol.

Acid-induced degradation solution preparation
Acidic degradation of FAV was done to study its stability, according to International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guideline [28], 25 mg of FAV was dissolved in 25.0 mL 1.0 N HCl, refluxed for 1 h in water bath at 100 °C then neutralization was done by 2.0 N NaOH, the volume was completed with a 100.0 ml volumetric flask to obtain 250.0 µg/mL.

Complete degradation was confirmed using the TLC developing system composed of ethyl acetate–methanol-ammonia (2:4:0.1) by volume, Additional file 1: Fig. S1.

IR sample preparation
For Favipiravir, about 150 mg of the KBR salt was weighted, poured into the mortar then Favipiravir was added, just a small amount on the tip of a spatula. Mixing Favipiravir with KBR salt in a good manner using pestle to ensure obtaining homogenous powder and to avoid obtaining a pellet too sticky which may cause a difficulty in getting it out of that pellet press. Next, add a small amount of homogenous powder into pellet press which consist of three pieces" the short bolt, the large bolt and then the doughnut." Put the solid sample to be pressed into the pellet to give a thin layer covering the surface of the metal; not little or large quantity to obtain desired pellet not thick or fine which may make a pellet not hold up when pressed it. Support the bottom of the die set into the pellet press. Pressing the solid sample done between the two bolts about five to ten seconds to confirm completely pressing. Opening pellet up, taking the middle part with transparent layer of powder formed after pressing. That transparent nature was important because IR is going to have to pass through that pellet, to get some kind of absorbency inside the machine. That's explain why it must not be too thick Additional file 2: Fig. S2.

For FAV degradation, after acidic degradation of Favipiravir and neutralization with NaOH, salt and water were formed. Evaporation was done using rotavap after that residue was washed with methanol and evaporated twice to obtain finally the degradation product in a pure solid form, applying on it the same previous procedure as Favipiravir.

Procedure
Construction of the calibration curve.
Direct spectrophotometric method
FAV working solutions (4.00–22.00 µg/mL) were prepared by accurately transferring aliquot from the stock standard solution using methanol as a diluent. Scanning the prepared solutions was done from 200.0 to 400.0 nm. The quantitative determination of FAV was done at 323.0 nm. The calibration curve was constructed by plotting the absorbance versus the corresponding concentration.

Dual wavelength spectrophotometric method (DW)
Using the previously scanned spectra, a calibration curve was constructed by correlating the absorbance difference between 322.7 and 270.0 nm to the corresponding FAV concentrations. At the selected wavelengths FAV deg. has zero difference in absorbance.

First derivative (D1) peak to peak spectrophotometric method
Using zero order spectra, a manipulation operation was done to obtain their D^1 derivative spectra with 10 scaling factor and $(\Delta\lambda = 4)$. A calibration curve was constructed from D^1 spectra by plotting sum of FAV peak amplitudes
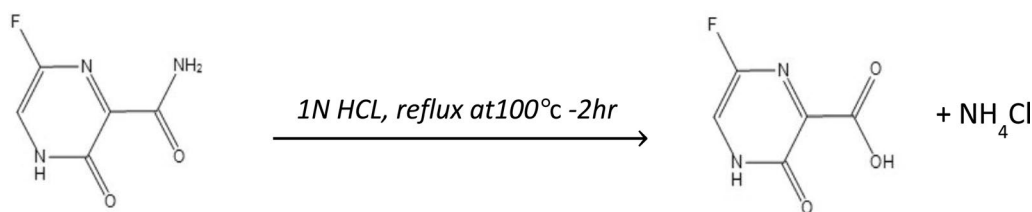
Scheme1: Suggested acidic degradation pathway of Favipiravir

at 338.0 and 308.0 nm versus its corresponding concentrations over a range of 4.0–22.0 µg/mL.

Difference spectrophotometric method (ΔA)

In this method, serial dilutions with 1.0 N NaOH were measured against the same drug concentrations in 1.0 N HCL as a blank, the calibration curve was constructed by recording the absorbance of FAV working solutions diluted with 1.0 N NaOH against the same concentrations of FAV diluted with 1.0 N HCl at 361.3 nm in a range of 4.0–22.0 µg/mL.

Analysis of laboratory prepared mixtures

Mixtures containing different ratios of FAV and FAV Deg. were prepared, then the absorption spectra of these mixtures were recorded. Then the procedures were completed as previously discussed for Direct, DW, D^1 and ΔA. The concentration of FAV was calculated using the corresponding regression equation for each method.

Application to a pharmaceutical dosage form

Ten tablets of Piravafi ® were accurately weighed and grinded into fine powder after removing their coats. A quantity of powdered tablets equivalent to 10.0 mg of FAV was accurately weighed and transferred to a 100 mL volumetric flask, 30 mL of methanol were added, sonicated for 15 min, completed to the specified volume with methanol to obtain the final concentration 100 µg/ml then, filtered through a 0.45 um membrane filter. Then the procedures were completed as explained under Construction of the calibration curves.

Results and discussion

Acid hydrolysis of FAV

Reviewing the literature, some stability studies described the acidic, alkaline, oxidative, thermal, and photolytic degradation of FAV [8, 17–19]. These studies confirmed that the drug is more liable to acidic degradation. The presence of an amide moiety could be the reason for FAV degradation in acid medium. A reported mechanism suggested the formation of carboxylic acid and ammonium

salt of amides on degradation by acid hydrolysis [29] as shown in scheme 1.

IR spectroscopy was utilized to confirm the suggested mechanism. Unsubstituted amides, -CO-NH$_2$, were reported to have two NH$_2$ stretch bands near 3350 cm^{-1} and 3180 cm^{-1}. In addition, the C=O of an amide has stretch band at about 1680–1640 cm^{-1} as well as an NH$_2$ deformation band at about 1640–1620 cm^{-1}. Identification of carboxylic acid salts can be done by the presence of two nearly equivalent bonds in -CO$_2^-$ group which are intermediate between C=O and C-O. These bonds have a characteristic stretch bands at 1650–1540 and 1450–1360 cm^{-1} and these values vary for different positive counterion [30].

The IR chart of intact FAV shows two NH$_2$ stretch bands at 3354 and 3226 cm^{-1}. Meanwhile the stretch band of C=O at 1670 cm^{-1}, and the NH$_2$ deformation band at 1658 cm^{-1} are clearly noticed (Fig. 2a). On the other hand, Fig. 2b represents the IR chart of FAV deg. in which NH$_2$ stretch bands are not found, thus verifying the cleavage of NH$_2$ group. Moreover, two bands specific for carboxylate group at 1570 and 1473 cm^{-1} can be noticed. Therefore, the suggested acid hydrolysis mechanism was confirmed.

Spectrophotometric methods

Absorption spectra of FAV and FAV deg. (Figure. 3) showed a partial interference from FAV deg. Therefore, the main goal of this research is to develop simple, selective, cost-effective, and eco-friendly UV-spectrophotometric methods for assessment of FAV in presence of FAV deg. The developed methods include D^0, DW, D^1 and ΔA.

Direct spectrophotometric method (D0)

It is an important, smart, and easy method for determination of the concentration of FAV in presence of FAV deg. Figure 4 presents D^0 spectra of FAV over the range of 4.0–22.0 µg/mL.

Dual wavelength spectrophotometric method (DW)

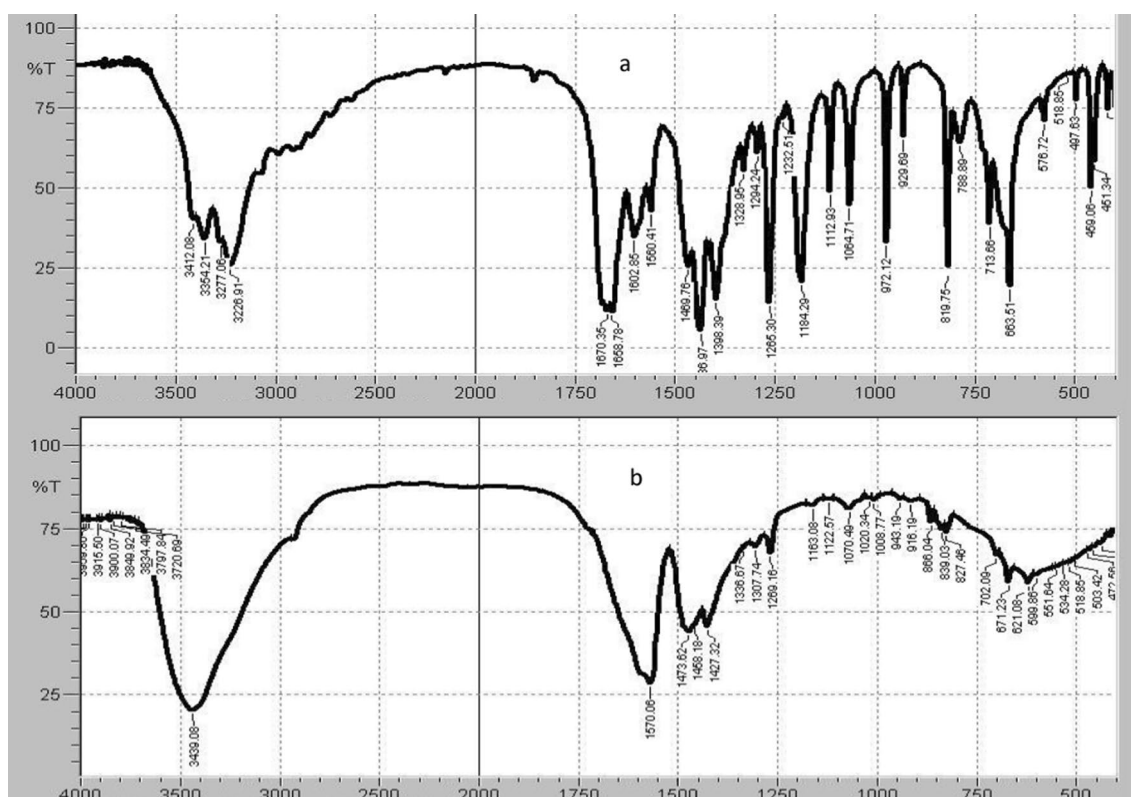DW is an accurate, easy method, with no need for software programs which make it simple to be applied. For

Fig. 2 IR spectrum of (**a**) Favipiravir, (**b**) acid-induced degradation product

the elimination of this overlapping, two wavelengths in the mixture spectra were chosen in a way that there is an absorbance difference between the chosen wavelengths directly proportional to FAV concentration while for FAV degradation the difference equals to zero. FAV concentration was calculated using the corresponding regression equation, Table 1.

First derivative (D1) peak to peak spectrophotometric method

This method is simple, easy to apply for resolving FAV and FAV deg. This method basically depends on the derivatization of the stored spectra of FAV&FAV deg. D^1 spectra of FAV and FAV deg. is presented in Fig. 5.

Difference spectrophotometric method (ΔA)

Another simple, easy, and accurate method was applied for determination of concentration of FAV in presence of FAV deg. This method was based on the recording induced effect of pH on absorption spectra of FAV at 361.3 nm where FAV has higher absorbance in NaOH than HCl (Fig. 6). Table 1 shows the linear regression equation of the suggested method.

Favipiravir contains a phenolic group which is considered as weak acid so can react with a base as NaOH

which is strong base, forming sodium phenoxide and water. Sodium phenoxide is a moderately strong base increase pH leading to bathochromic shift [31, 32]. Sodium phenoxide contains negative charge on oxygen atom. The delocalization of negative charge on the oxygen atom over the aromatic ring (conjugation) as compared to the lone pairs which results in a lower energy gap between HOMO and LUMO which mean more stabilization leading to higher wavelength (bathochromic shift) as they are inversely proportional to each other that's explain and summarize why change in wavelength maxima to 361.3 nm.

Methods validation

According to ICH guidelines [33], the proposed UV methods were suggested to confirm methods validation.

Linearity and range

The linearity of the suggested methods was assessed by analyzing five concentrations of FAV over a range of 4.0–22.0 μg/mL. Absorbance was recorded by scanning three replicates of each concentration at 200–400 nm using methanol as a blank in the first three methods, while the last method using drug in1.0N HCL as blank. The results
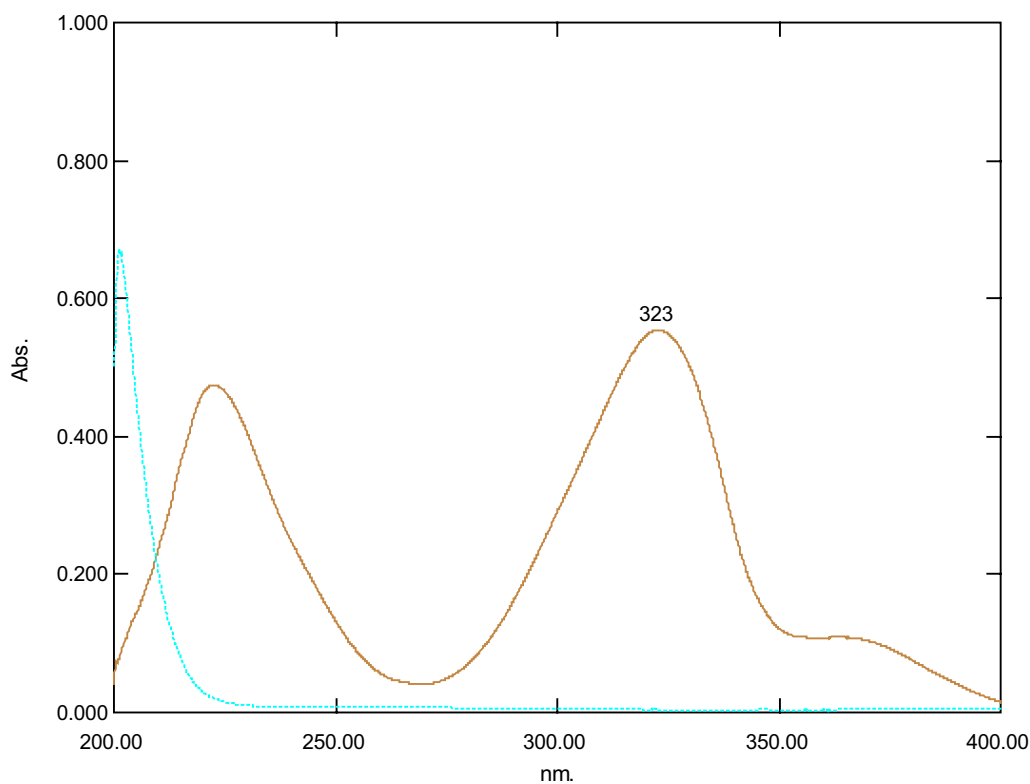
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Fig. 3 Absorption spectra of 10 µg of favipiravir (—) and 10 µg of its acid-induced degradation product (- - -)
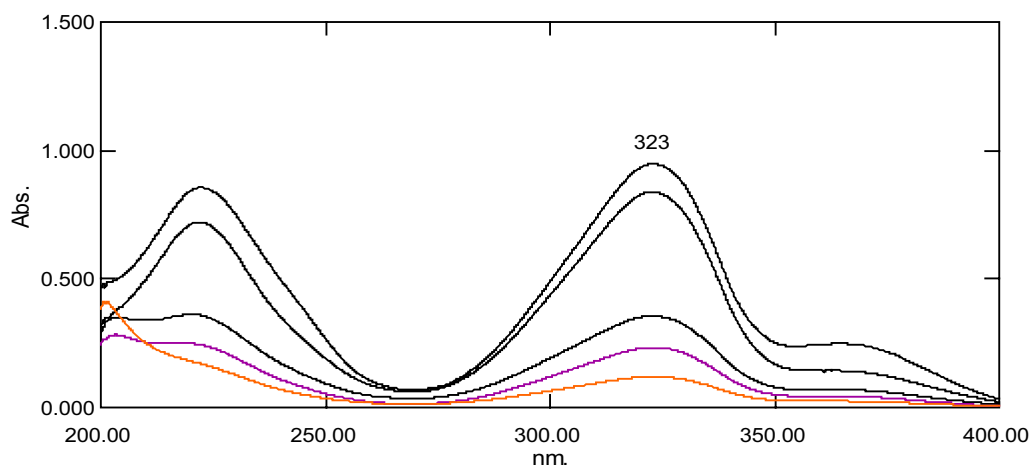

Fig. 4 Absorption spectra of laboratory prepared mixtures of favipiravir (2–18 µg/mL) and its acidic degradation product (2–18 µg/mL) in methanol at 323 nm

showed good linear relationships according to the computed correlation coefficient values listed in Table 1.

Limit of detection [LOD] and limit of quantification [LOQ]

Values of LOD and LOQ were computed to confirm and evaluate method sensitivity (Table 1). LOD and LOQ were calculated using following equations:

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Table 1 Regression method validation parameters data of the proposed spectrophotometric methods for favipiravir determination

Parameter	Methods			
	Zero order	DW	D^1	ΔA
Linearity range (µg/mL)	4.00–22.00	4.00–22.00	4.00–22.00	4.00–22.00
Slope	0.0567	0.0526	0.0443	0.0471
Intercept	0.0756	0.0696	0.0876	0.0159
Determination coefficient (r^2)	0.9995	0.9998	0.9999	0.9997
Accuracy (mean ± SD)	100.06 ± 1.52	99.47 ± 0.77	100.01 ± 1.11	99.11 ± 1.17
LOD (µg/mL)	0.578	0.347	0.301	0.483
LOQ (µg/mL)	1.929	1.158	1.003	1.612
Intra-day precision RSD%[a]	0.802	1.051	0.811	0.949
Inter-day precision RSD%[a]	1.131	1.144	1.027	1.053

[a] Average of RSDs of three concentrations in triplicate analysis, the concentrations were as follows (10.0, 14.0, 18.0 µg/mL)
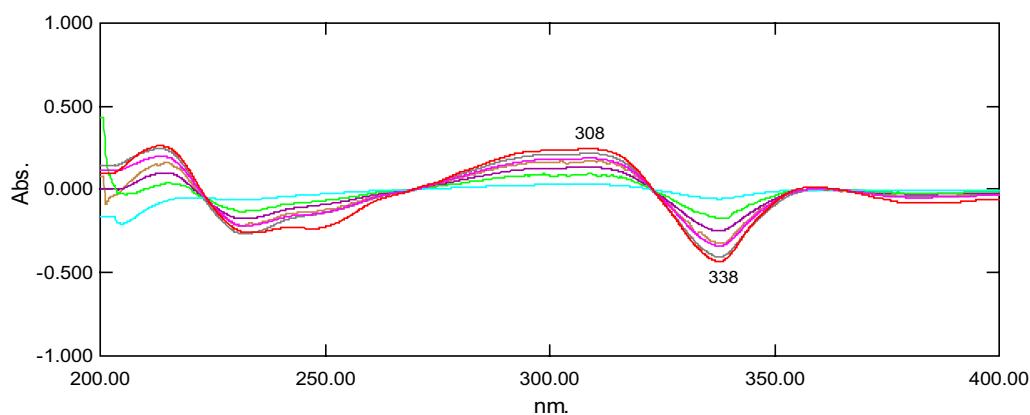
Fig. 5 First derivative spectra for mixtures of favipiravir (2–18 µg/mL) and its acidic degradation product (2–18 µg/mL)
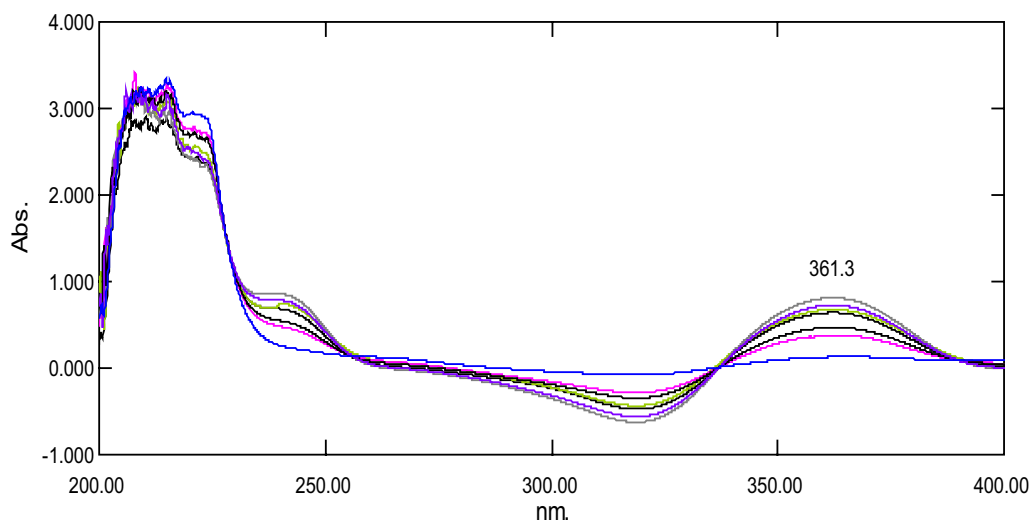
Fig. 6 Laboratory prepared mixtures of favipiravir (2–18 µg/mL) and its acidic degradation product (2–18 µg/mL) in sodium hydroxide against hydrochloric acid

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$$LOD = (3.3 * SD)/S$$

$$LOQ = (10 * SD)/S$$

where SD is the standard deviation of y-intercept and S is the slope of the calibration graph.

Accuracy and precision

The accuracy of our methods was evaluated by applying these methods for determination of five different concentrations of FAV in triplicate. FAV concentrations were calculated from the corresponding regression equations of the developed methods after that percentage recoveries determination. Results obtained were within the acceptable limits.

Intra-day and inter-day precision were studied by analysis of three different concentrations of FAV 3 times on the same day and on three consecutive days, respectively. The percentage relative standard deviation (RSD%) values were calculated, and satisfactory results were obtained (Table 1).

Specificity

Different ratios of laboratory mixtures of FAV and FAV deg. were prepared within their linearity range and analyzed to evaluate method specificity. Results confirmed that FAV can be quantified in presence of FAV deg. without any interference from degradation product up to 60% degradation product in laboratory mixture (Table 2).

Application of these methods to pharmaceutical dosage forms

The developed methods were valid for the quantitative determination of FAV in pharmaceutical formulations. Results of the suggested procedures showed high recoveries % as summarized in Table 3.

Statistical analysis

Statistical comparison of the results obtained from the developed and reported method [8] was performed, where Student's t-test and variance ratio F-test showed no significant difference between the two methods as shown in (Table 3) v. In addition, the proposed methods were compared using a one-way ANOVA test. Results showed no significant variations between the methods as the calculated F-values did not exceed the critical one (Table 4).

Greenness assessment

The greenness of the four developed spectrophotometric methods were assessed using the Green Analytical Procedure Index (GAPI) and AGREE metric. GAPI is represented with three colors: green, yellow and red. It deals with and takes into consideration all parts of the process from sample collection to final products and wastes. It indicates hazards and safety of reagent and solvents used [34]. AGREE is another method used to green assessment.it is a clock shaped which divided into 12 sections each one represents one factor of assessment. It also contains three colors green, yellow and red. Score range from 0 to 1 [35].

The developed methods were assessed using both the GAPI and AGREE tools (Table 5).

Comparing the results shown in Tables 1 and 5, the first three methods were greener, but the first method (zero-order) was the most accurate and simplest one.

Table 2 Determination of favipiravir in presence of its acid-induced degradation product in laboratory prepared mixtures with proposed spectrophotometric methods

Conc (μg/mL)		Direct	DW	D¹	ΔA
FAV	Deg	Recovery%[a]			
18.00	2.00	99.90	100.40	99.27	100.25
16.00	4.00	100.70	100.71	99.97	99.47
14.00	6.00	99.47	100.16	98.94	98.58
10.00	10.00	99.75	99.92	100.81	99.77
8.00	16.00	100.88	99.48	99.77	105.07
6.00	14.00	116.87	123.76	130.02	120.97
2.00	18.00	172.48	166.92	194.80	152.76

[a] Average of three determinations

Table 3 Statistical analysis of proposed and reported methods for the analysis of Pirafavi® tablets
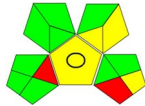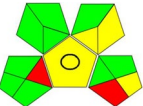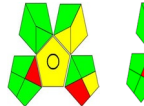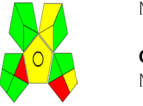
Parameters	Reported method	Zero order	DW	D¹	ΔA
Mean recovery %	102.35	102.03	100.01	99.41	102.42
Variance	17.88	13.67	9.33	11.37	51.99
n	5	7	6	6	7
Students t-test	–	0.135 (1.895)[a]	1.033 (2.262)[a]	1.160 (2.262)[a]	0.023 (2.228)[a]
F-test	–	1.31 (5.19)[a]	1.91 (6.16)[a]	1.57 (6.16)[a]	2.91 (5.19)[a]

[a] Tabulated t- and F- values

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Table 4 One-way ANOVA results for determination of favipiravir using the proposed spectrophotometric methods

Source of variation	Sum of squares	Degree of freedom	Mean of squares	F-value	P-value	Critical F
Between groups	42.11713	4	10.52928	0.462519	0.762489	2.776289
Within groups	546.362	24	22.76508			
Total	588.4791	28				

Table 5 Green assessments of the developed spectrophotometric methods

	Zero order	DW	D^1	ΔA
AGREE	0.9	0.9	0.79	0.81
GAPI				

Conclusion

The developed methods were simple, rapid, precise, accurate, and easy to apply for routine analysis in the laboratory. They were successfully used to determine FAV in presence of up to 60% FAV deg. The developed procedures were in good agreement with each other and with the reported method. The greenness of validated methodologies was assessed and compared with each other showed that zero-order was the most simple, accurate and green method.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13065-023-01046-6.

Additional file 1: S1. Separation of A) FAV and B) acid-induced degradation product using mobile phase ethyl acetate–methanol-ammonia (2:4:0.1, v/v).

Additional file 2: S2. Calibration curves (a) Zero-order method (b) DW (c) D1 (d) ΔA Linearity range (4-22 μg/ml).

Availability of data and materials
Data will be available on request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
All authors declare that they have no competing interests.

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