Green micellar solvent-free HPLC and spectrofluorimetric determination of favipiravir as one of COVID-19 antiviral regimens

Ibraam E. Mikhail [a], Heba Elmansi [a], Fathalla Belal [a], Adel Ehab Ibrahim [b],*

[a] *Pharmaceutical Analytical Chemistry Department, Faculty of Pharmacy, Mansoura University, Mansoura 35516, Egypt*
[b] *Pharmaceutical Analytical Chemistry Department, Faculty of Pharmacy, Port-Said University, Egypt*

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ABSTRACT

Quality control (QC) of pharmaceutical products requires fast, sensitive as well as economic methodologies in order to provide high through output at low cost which are the main aspects considered by such economic facilities. Meanwhile, the ecological impacts must be considered by researchers to minimize the hazardous effects of research laboratories. Favipiravir (FAV) is an antiviral agent recently approved for treatment of COVID-19 infections during 2020 pandemic crisis, so the size of its production by international pharmaceutical corporations evolved dramatically within the past few months. Two novel simple, sensitive, and green methods were developed and validated for FAV determination based on solvent-free micellar LC and spectrofluorimetry techniques. To improve FAV native fluorescence, several factors were studied including solvent type, buffering, pH and added surfactants. The best sensitivity for FAV fluorescence was obtained in Britton-Robinson buffer (pH 4) at 436 nm after excitation at 323 nm within concentration range of 20–350 ng mL^{-1}. Another HPLC method was validated using C18-RP (5 μm, 250 × 4.6 mm) stationary phase and solvent-free mobile phase consisting of (0.02 M Brij-35, 0.15 M SDS, and 0.02 M disodium hydrogen phosphate, pH 5.0) isocratically eluted at a flow rate of 1 mL min^{-1} and detection wavelength of 323 nm. LC method was validated across concentration range of 10–100 μg mL^{-1} and FAV eluted in 3.8 min. The methods were validated according to the FDA guidelines and were applied successfully for determination of FAV in its marketed tablet dosage forms and in spiked human plasma samples. The proposed methods are eco-friendly since they are typically based on biodegradable reagents in aqueous solvent-free phases, which was proven by their assessment on two recent greenness metrics (GAPI and AGREE) to prove their eco-friendly properties.

1. Introduction

In December 2019, the WHO was alerted by the Chinese government of pneumonia hospitalized cases with unidentified cause. These cases were identified later as COVID-19 in which the causative virus was SARS-Cov-2. In March 2020, WHO declared Coronavirus infection outbreak as pandemic. By the end of June 2020, the number of reported cases worldwide exceeded 10 million cases with hundreds of thousands of deaths [1]. Symptoms in mild cases were fatigue, fever and dry cough, however in severe infections failure of kidney and respiratory system occurred [2]. The virus caused severe acute respiratory syndrome (SARS) for about 16% of the infected cases after 5 days of exposure [3]. The high mortality rates caused by COVID-19 (about 1–2%), endeavoured global scientists to discover antiviral agents that can suppress the viral spread and enhance patients' recovery [3]. Since the process for

approval of a new drug for human use is complex and comprises multiple stages to establish safety data and discover potential risks, the easiest and fastest way was to try FDA previously approved antivirals. Several antivirals were clinically tried including lopinavir/ritonavir combination, favipiravir, umifenovir, remdesivir and tocilizumab.

Favipiravir, FVP (Structure Fig. 1) is a potent antiviral agent that inhibits viral RNA-polymerase. FVP has chemical formula ($C_5H_4FN_3O_2$), pKa value 5.1 and logP values 0.25 & 0.49[4]. FVP was approved first in Japan for treatment of Influenza. It's a prodrug which must be activated first in-vivo by a phosphoribosylation step into the active form which inhibits viral protein synthesis. Several Clinical trials were made to test the efficacy of FVP in Corona virus infections and declared that FVP was found to enhance viral clearance and improve chest CT [3,5,6]. The side effects associated with the use of FAV were found mild and manageable. Recently, FVP was approved in several countries for treatment of

COVID-19 infections, including Italy, Japan, Russia, Egypt, India, KSA, UAE and Turkey [7].

Pharmaceutical good manufacturing practices (GMP) require monitoring the active pharmaceutical ingredient (API) within quality control (QC) laboratories throughout the multiple steps of production. These steps include the screening of API as raw material, as intermediate during in-process control, after packaging and for stability testing throughout the product's shelf-time. Such QC multiple analyses necessitates the presence of fast, green and economic analytical methodologies to provide high throughput, decrease analysis-cost and minimize the environmental impact.

The use of fluorescence spectroscopy has several advantages including high selectivity, specificity, sensitivity and fast outcome. Depending on 2 wavelengths for both excitation and emission improves method's selectivity. The sensitivity of fluorescence is much higher than absorption spectroscopy since it doesn't compare the intensity to a reference beam; it measures the intensity directly against low background. The absence of mobile phase preparation and conditioning steps required for chromatographic techniques increase the tool's output.

Micellar liquid chromatography is another green tool for chromatographic techniques [8]. The use of mixed micellar mobile phases proved enhanced column's separation efficiency, decreased the analytes' retention time and eliminated the need for an environmentally hazardous organic solvents for elution [9]. The mixture of the anionic surfactant, sodium dodecyl sulphate (SDS), and the non-ionic polyoxyethylene-23-lauryl ether surfactant (Brij-35) has been recently replacing the organic solvents within the recent past few years [9–12].

Literature review revealed few methods for determination of FAV. Five reported studies focusing mainly on the drug's pharmacokinetics and pharmacodynamics, developed liquid chromatographic methods for determination of FAV in plasma [13–17]. Two of these reported studies used the same HPLC/UV-detection methodology with isocratic elution by mobile phase composed of 0.1 M triethylammonium phosphate buffer (pH 6.5) and acetonitrile [14,17], however, no validation details was mentioned, no chromatograms and no data about retention time. One report didn't give any information about the used chromatographic conditions except that a conventional HPLC with UV-detection was used [13]. The other two methods used gradient HPLC techniques which will be discussed later. An article written in Chinese was reported for determination of FAV and its related substances [18]. Another recent HPLC method was reported for determination of FVP in pharmaceutical dosage forms [19]. Finally, during preparing the present manuscript, a recent spectrofluorometric method for determination FAV was reported [20]. However, some drawbacks will be discussed forward in details when comparing the proposed study to the reported work.

The aim of the proposed research is to develop a green LC tool that uses solvent-free mobile phase and a conventional, sustainable and economic HPLC technique for determination of FAV in pure form, bulk powders and in formulated dosage forms. Another aim was to make use all advantages of spectrofluorimetric techniques to develop another sensitive and fast tool for FAV determination.

Since claiming greenness of any analytical methodology is not enough, the proposed methods were assessed against two recent greenness metrics, the green analytical procedure index (GAPI) and AGREE tools [21,22]. Another aim for this study is to provide a mini-review about FAV determination methodologies, and a thorough comparison to some previously reported methods to evaluate their greenness aspects and differences between used metrics.

2. Experimental

2.1. Instruments

Fluorescence measurements were performed using Cary Eclipse fluorescence spectrophotometer equipped with Xenon flash lamp (Agilent technologies, USA). Universal laboratory centrifuge, model Sigma 2-16P from Sigma Laborzentrifugen GmbH was used for centrifugation of plasma extracts.

Chromatographic procedures were performed using Agilent HPLC-1200 system (Agilent Technologies, USA) consisting of a solvent pump, auto-sampler, connected to column compartment and UV detector (models; G1311A, G1329A, G1316A and G1314A, respectively) was used. pH was adjusted using a pH Meter model 713 (Metrohm, Switzerland). VDSpher 150® C18-E column (5 µm, 250x4.6 mm) was purchased from VDS Optilab Chromatographie Technik, GmbH, Germany.

2.2. Materials and reagents

De-ionized water produced in-house by Millipore water purification system was used during preparation of solutions and mobile phase all over the study.

Organic solvents; methanol (MeOH), ethanol (EtOH), acetonitrile (ACN), acetone, and n-propanol, were all HPLC grades and purchased from Fisher Scientific distributer in Egypt. Cetrimide, SDS, cremophor, Brij-35and Tween-80 were purchased from Sigma-Aldrich, Germany.

Borax, boric acid, phosphoric acid, disodium hydrogen phosphate, sodium dihydrogen phosphate, sodium hydroxide and hydrochloric acid were all analytical grades and were purchased from El-Nasr Pharmaceutical Chemicals, Cairo, Egypt.

Plasma sample was purchased from the National Egyptian Blood Bank and was freezed at −20 °C until used after gentle thawing.

FAV pure raw materials were kindly supplied by a national pharmaceutical company (EIPICo., Egypt). Pharmaceutical dosage forms Avipiravir® tablets (lot. No. 2008230, 200 mg FAV per tablet) was supplied by EVA Pharma Co., Cairo, Egypt.

2.3. Analytical conditions

The relative fluorescence intensity (RFI) was measured at 436 nm (emission wavelength) using 323 nm as excitation wavelength and smoothing factor of 20 (Fig. 1).
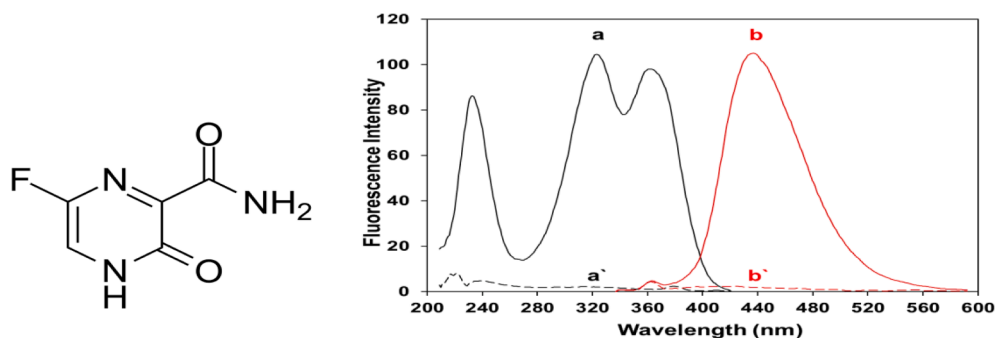

Fig. 1. Chemical Structures and Spectrofluorometric excitation and emission spectrum of FAV.

Chromatographic analysis was performed by isocratic elution using mobile phase consisting of 0.02 M Brij-35, 0.15 M SDS, and 0.02 M Disodium hydrogen phosphate adjusted to pH 5.0 using phosphoric acid. Flow rate was 1 mL min^{-1} on VDSpher-150® C18-E column and detection wavelength was adjusted at 323 nm. The sustainability of the method was enhanced by recycling the mobile phase between chromatographic runs. Also the system was purged after each injected sequence for 15 min using a mixture of water:MeOH (1:1) to remove bonded surfactants from the surface of stationary phase.

2.4. Validation standards

For spectrofluorometric analysis, accurately measured volumes (10, 25, 50, 100, and 175 μL) of FAV standard solution (20.0 μg mL^{-1}) were quantitatively transferred to a set of 10 mL volumetric flasks. Britton-Robinson buffer of pH 4, was completed to the mark to adjust the final volumes so that the final concentrations of FAV were (20, 50, 100, 200, and 350 ng mL^{-1}).

Linearity was established throughout the developed LC-method by injecting 20.0 μL volume of six standard concentrations (5, 20, 40, 60, 80, and 100 μg mL^{-1}). Standards were prepared by dilution of FAV stock solution (1.0 mg mL^{-1} in a solvent mixture of methanol: water 1:1) using the mobile phase.

For testing accuracy of the proposed methods, 3 different quality control standards within the linearity ranges were prepared by dilution of stock standards in the same way used for each technique. Concentrations of QC standards were (20, 100, and 350 ng mL^{-1}) for spectrofluorometric method, and (10, 50, and 75 μg mL^{-1}) for the chromatographic method. Each QC standard was tested in triplicate.

Precision testing was performed on the same QC standards by calculating percentage recoveries three different times within the same day (Intra-day) and within three different days (Inter-day) to evaluate repeatability and intermediate precisions.

2.5. Analysis of dosage forms

A quantity of finely pulverized Avipiravir® tablets equivalent to 200 mg FAV was transferred into a 100 mL volumetric flask and the volume was made up to the mark with ethanol. The contents of the flask were sonicated for 10 min., and then filtered. For chromatographic analysis, 2 mL of the filtrate was diluted volumetrically to 100 mL using the mobile phase (40.0 μg mL^{-1}).

For spectroscopic analysis, 1 mL of the filtrate was diluted volumetrically to 100 mL using ethanol (20.0 μg mL^{-1}). Different volumes of the tablet extract were accurately transferred into a series of 10 mL volumetric flasks. The procedure described for preparation of validation standards (section 2.4) was followed and the drug content per tablet was calculated using the corresponding regression equation.

2.6. Determination of FAV in spiked human plasma

The developed spectrofluorimetric method was applied for determination of FAV in human plasma within range of 200–3500 ng mL^{-1}. 1 mL of human plasma were placed into a series of centrifugation tubes and spiked with stock standard solution of FAV to obtain drug plasma concentrations of 0.2, 0.5, 1, 2, and 3.5 μg mL^{-1}. Plasma extraction was performed by the addition of 3.0 mL acetonitrile to each tube to precipitate plasma proteins, samples were vortex-mixed for 1 min, and then centrifuged for 15 min at 3000 rpm. The supernatants were aspirated carefully into beakers and evaporated to dryness at room temperature. The residues were reconstituted with 1 mL of ethanol, transferred into a series of 10 mL volumetric flasks, and BRB buffer, pH 4, was added to the mark to give a final drug concentration for measurement ranging from 20 to 350 ng mL^{-1}. Blank plasma experiments were performed simultaneously. The RFIs after subtracting the plasma blank readings were plotted versus the final concentrations of the drug to construct the calibration graph. The corresponding regression equations and correlation coefficients were derived.

3. Results and discussion

3.1. Method development

3.1.1. Spectrofluorimetric analysis
The sensitivity of fluorescence spectroscopy is affected by several factors. FAV has a remarkable native fluorescence (Fig. 1). Factors affecting the intensity of its native fluorescence were evaluated in order to optimize the developed methodology.

3.1.2. Effect of solvent
The polarity of the diluting solvent certainly affects fluorescence intensity, so different diluting solvents were tried, acetonitrile, ethanol, water, methanol, and 2-propanol. Ethanol and water had the maximum intensities of fluorescence with nearly similar RFIs. For more ecological safety, water was chosen as diluting solvent (Fig. 2).

3.1.3. Effect of water soluble surfactants
The addition of surface active agents (SAA) can affect the fluorescent quantum yield. So, different water soluble SAA, anionic (SDS), cationic (cetrimide), and non-ionic (Tween-80 and cremophor) surfactants were added separately to water as a diluting solvent (at concentration 1%, w/v). The free aqueous solvent had the highest intensity of fluorescence. FAV native fluorescence was slightly decreased by the added SAAs, which was the highest upon addition of cetrimide (Fig. 2). So no surfactant was added to the aqueous solvent during the validation study.

3.1.4. Effect of pH and buffer volume
The pH of the diluting medium affects the fluorescence quantum yield because of affecting the ionization state of the analytes under study [23]. The RFI (Fig. 3) was studied across pH range of 2.6–9.0, using BRB universal buffer. FAV demonstrated the highest intensity at pH 4.0 with noticeable solution turbidity above pH 6.0. Buffer of pH 4.0 was chosen for the experimental conditions. The effect of relative volume of BRB added per 10 mL of aqueous solvent was tested. RFI of FAV increased with increasing the buffer volume up to 10 mL (Fig. 3). So, the validation study was performed by dilution of the drug solution under study with BRB.

3.1.5. Chromatographic Analysis:
For HPLC technique, several factors affect the elution of analytes. The effect of stationary phase, together with composition and pH of the mobile phase were studied on elution of FAV.

3.1.6. Choice of stationary phase
FAV has low logP values, 0.25 & 0.49 [4], indicating high polarity of its molecule. This low polarity resulted in its low retention on C18-reversed stationary phase (RP). However, the use of normal silica stationary phases is less favoured relative to RP from the ecological perspective, since normal phase chromatography requires large amounts of hazardous organic solvents [24]. The use of organic solvents in normal phase chromatography is also less economic than aqueous mobile phases used in RP-chromatography. So, C18-RP was the primary choice.

3.1.7. Mobile phase pH
The pH of the mobile phase affects the ionization state of the resolving analytes. pH of the mobile phase was altered within range of 2.8 – 6.5, since FAV has weak acidic pKa value of 5.1 in order to keep the molecule non-ionized and enhance its retention on RP-C18 phase. The best retention was obtained on adjusting the pH of the mobile phase at pH 5.0.
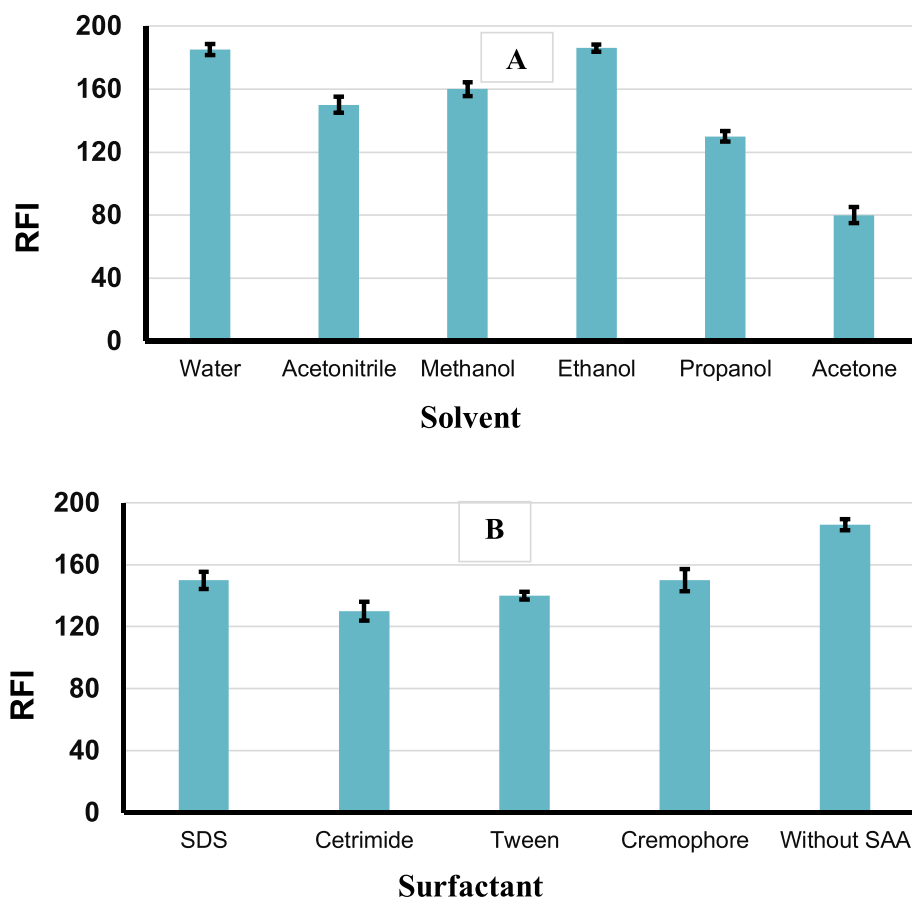
Fig. 2. Solvent (A) and SAA type (B) effects on the intensity of fluorescence of FAV (200.0 ng mL^{-1}).

3.1.8. Mobile phase composition

To overcome the low retention of FAV on C18-reversed stationary phase, the addition of Brij-35/SDS mixture to the aqueous mobile phase can enhance its retention behaviour. Brij-35 is an ethoxylated fatty alcohol which when adsorbed on the C18-RP, has the capability of altering its polarity while remaining neutral. Meanwhile, SDS monomers render reversed stationary phase surface into negatively charged one [25]. The overall polarity of C18 surface is altered and the only trial needed would be to adjust the ratios of Brij-35 relative to SDS in the mobile phase to obtain the best combination. Several ratios of Brij-35/SDS were prepared and used the combination, which introduced the best retention time and best column efficiencies, was chosen.

3.2. Method validation

The optimized methodologies were validated according to FDA guidelines [26].

3.2.1. Specificity

Fluorescence spectroscopic techniques have high specificity resulting from their dependence on two spectral wavelengths, excitation and emission. Selectivity and specificity of developed fluorescent spectroscopic methods are confirmed by comparing blank solvent spectra to those from pure FAV solution (Fig. 1). For HPLC methodology, the specificity was confirmed by injecting pure FAV solution and solution containing tablet dosage form. As shown in Fig. 4, excipients from tablet dosage forms had no interference with FAV peak.

3.2.2. Linearity and range

Calibration curve was constructed by plotting the average responses obtained versus the concentration of linearity standards tested. Linearity

and regression equations for the developed methodologies are presented in (Table 1). Results obtained indicate linear responses across validated concentration ranges.

3.2.3. Limits of detection (LOD) and quantification (LOQ)

LODs and LOQs were calculated from the slope (S) and standard deviation (σ) of the calibration curve. LODs were calculated corresponding to values of (3.3σ/S) and LOQs were corresponding to values of (10σ/S). Results shown in (Table 1) indicate the sensitivities of the proposed methods.

3.2.4. Accuracy and precision

Accuracy was tested by calculating percentage recoveries for the prepared quality control solutions prepared at low, medium and high concentration levels within the calibration ranges. Repeatability and Intermediate precisions were tested by repetition of analysis of the quality control solutions within the same day (Intra-day) and at three different days (Inter-day). The obtained results are presented in Table 2, with acceptable percentage recoveries and standard deviations.

3.3. Applications

The proposed analytical methods were used for determination of FAV in its marketed Avipiravir® tablets. The percentage of FAV relative to labelled content (200 mg per tablet) was calculated using the validated methods. Student's *t*-test and F-test were used to evaluate statistical differences compared to those obtained by the supplier's method. The dosage form supplier uses a HPLC method using C18 RP column (5 μm, 250x4.6 mm) and mobile phase composed of 0.01 M potassium dihydrogen phosphate buffer pH 4.0 and acetonitrile (9:1, v/v) at 210 nm detecting wavelength. Results shown in (Supporting information
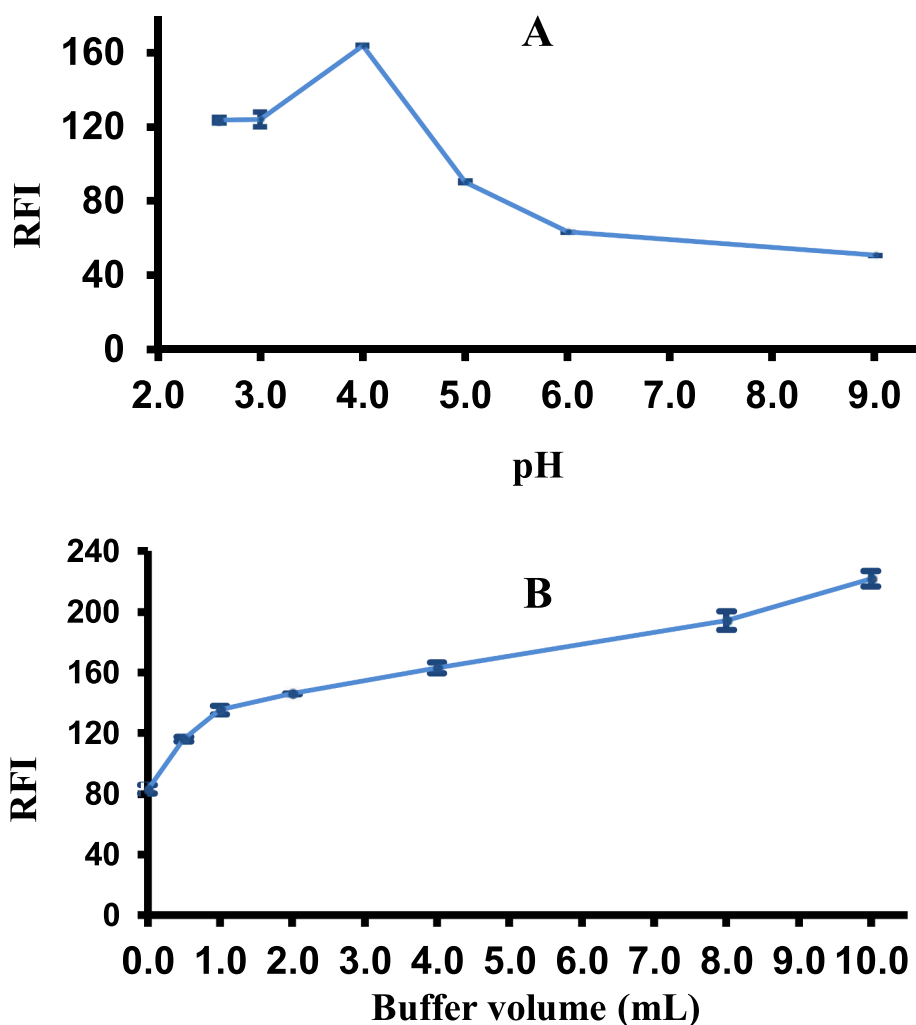
Fig. 3. Buffer pH (A) and volume added (B) effects on the intensity of fluorescence of FAV (200.0 ng mL^{-1}).

Table 1) indicate that there was no significant difference between both validated methods and the reported supplier's methodology.

After oral absorption, FAV has short half-life. FAV reaches its peak blood concentration within 2 h then it's removed by either of 2 pathways. Either FAV undergoes extensive metabolism by aldehyde oxidase and to a lesser extent by xanthine oxidase into inactive metabolite to be excreted in urine, or it's absorbed by the virus where undergo ribosyalation and phosphorolyation steps intracellularly into the active form which inhibits viral RNA [27]. However, tracing the drug during this short half-life in blood is crucial to identify viral elimination kinetics during different infections and supply information for proper dosing frequencies. The proposed spectrofluorimetric method was applied for determination of FAV in spiked human plasma within range of (200–3500 ng mL^{-1}) as described in the experimental part. The method was found linear with linearity equation (Y = 0.776X + 1.721) and correlation coefficient (r = 0.999). Blank plasma was determined to check possible interferences and results proved no interference from endogenous matrices. Results of spiked plasma (Table 3) revealed acceptable recovery percentages between actual and found concentrations.

3.4. Comparison to reported analytical methods

Although a recent fluorescence spectroscopic method was recently reported for determination of FAV [20], the proposed study has some superiority in a number of points. Although the cited authors reported

that the average plasma C$_{max}$ was (22.01–36.24 µg mL^{-1}) according to the Pharmaceuticals and Medical Devices Agency (PMDA) [20], their working range in plasma was only 6.00–24.00 µg mL^{-1}, compared to the range covered by the proposed method, 0.2–350.0 µg mL^{-1}. Moreover; some reports showed that the steady state C$_{max}$ of the drug for some case studied patients was 4.43 µg mL^{-1}, which falls outside the working range of the reported paper [28]. The reported working plasma concentration range was (48–192 ng mL^{-1}) [20], however; this mentioned range was related to the final diluted concentrations following the extraction step not the actual plasma concentration. The proposed study covers wider working ranges both in plasma and in bulk powder determination, 20.0–350.0 ng mL^{-1}, compared to reported method (40–280 ng mL^{-1}). Secondly, the proposed study is more sensitive with lower LOD and LOQ (3.6 and 10.9 ng mL^{-1}) compared to (9.4 and 28.6 ng mL^{-1}) in the reported method. A final point, the proposed validated methodologies were applied in determination of the drug in its marketed tablet dosage forms and the results were statistically compared to each other to find any significant differences. However, the reported study was applied on a laboratory prepared mixture with the addition of assumed excipients, instead of the commercial formulation, and the results obtained were compared to a reference method which was missed from citing within the reported manuscript.

The assessment of ecological impact for newly developed analytical methodologies became an important aspect during method development in order to give concise and objective evaluation for future comparisons between reported methodologies. Several assessment metrics were
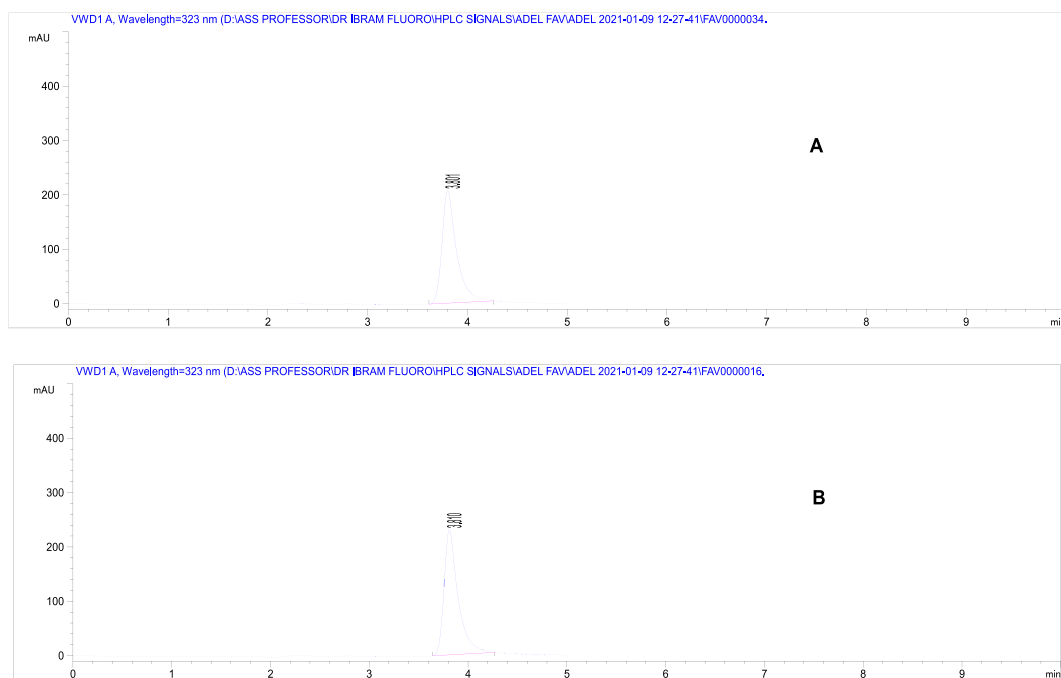
Fig. 4. Chromatograms showing FAV separation in (A) Avipiravir® tablet dosage form and in (B) pure form (60 μg mL^{-1}).

Table 1
Linearity and regression statistical results for determination of FAV using the proposed fluorescent spectroscopy and liquid chromatographic methods.

Parameter	Fluorescence Spectroscopy	HPLC method
Linearity range (μg mL^{-1})	0.02–0.35	10–100
Linearity equation	$Y = 0.86\,X + 13.91$	$Y = 41.89\,X - 18.00$
Correlation coefficient (r^2)	0.999	0.999
Standard Error	0.8	1.0
LOD (μg mL^{-1})	0.004	0.985
LOQ (μg mL^{-1})	0.011	2.986

Table 2
Accuracy and precision results for determination of FAV using the proposed method.

Standard concentration	Accuracy (Recovery %)	Intra-day precision*	Inter-day precision*
(ng mL^{-1})	Fluorescent Spectroscopy method		
20.0	99.1	102.6 ± 1.6	101.1 ± 1.6
100.0	102.2	98.8 ± 0.2	102.1 ± 2.1
350.0	99.9	100.7 ± 0.4	101.9 ± 0.5
(μg mL^{-1})	Liquid Chromatography method		
10.0	100.7	100.1 ± 3.0	100.5 ± 2.9
50.0	99.2	99.7 ± 0.9	99.5 ± 1.0
75.0	100.9	100.1 ± 2.0	99.8 ± 2.6

Table 3
Application of the proposed spectrofluorimetric method for determination of FAV in spiked human plasma.

Spiked plasma conc. (ng mL^{-1})	Found conc. (ng mL^{-1})	%Recovery	RSD%
200.0	188.5	94.2	7.6
500.0	484.4	96.9	1.2
1000.0	1019.0	101.9	1.5
2000.0	2024.6	101.2	0.9
3500.0	3483.2	99.5	0.9

developed lately, among those metrics the analytical eco-scale [29] and the Green Analytical Procedure Index (GAPI) [21] have been utilized extensively. AGREE assessment tool was reported recently [22]. GAPI is composed of 15 pentagrams, each coloured (red, yellow or green) to assess its environmental impact, where red represent bad impact, yellow for intermediate and green for safe and low environmental hazards [30]. AGREE provides a clock-shaped graph with perimeter divided into 12 parts based on the 12 principles of Green Analytical Chemistry [31]. Each division corresponds to one principle at colour scale (red-yellow-green) to evaluate the agreement of the analytical procedure to green analytical chemistry (GAC) principle. The heart of the AGREE graph has an overall assessment colour together with an overall assessment figure into a scale of 0 to 1.

The proposed methods were assessed using both the GAPI and AGREE tools. Table 4 shows a through comparison between the proposed methodologies and reported methods that had introduced actual reported data about their validation studies. All analytical methodologies show 2 red zones on GAPI and their corresponding parts 1 & 3 on AGREE. Such red zones appeared due to the off-line sampling and transport to QC laboratories which occur mandatory due to the separation between pharmaceutical production and QC sites. Evaluating the proposed and the reported spectrofluorimetric methods (on GAPI and AGREE) showed the same outcomes. The yellow pictograms on GAPI represent the medium impacts during sample storage and waste disposal. The same overall AGREE scores obtained by both methods was the highest followed by that for the proposed LC methodology.

The proposed LC method showed superiority over the other reported chromatographic techniques (Table 4) relative to retention time, run time, and the use of organic solvents. The proposed LC method is greener on both GAPI and AGREE since it is solvent-free, uses water as diluting solvent for samples that requires no special treatment except simple dilution. No organic waste is generated.

Despite the energy required by a spectrofluorometer is the lowest among other analytical instrumentation (less than 0.1kWh) compared to HPLC (about 1.5kWh) [29], LC is more common in the pharmaceutical QC and research laboratories. The main advantage of spectrofluorimetric technique lies in its high sensitivity (nano-scale) similar to UHPLC techniques coupled with MS/MS detection, but at much simple, fast, economic and at lower energy consumption.

Table 4

Comparison of the proposed analytical methods to chosen reported methodologies.

	Proposed spectroscopic method	Reported method [20]	Proposed LC method	Reported method [18]	Reported method [19]	Reported method [16]
Technique	Fluorescent Spectroscopy	Fluorescent Spectroscopy	Micellar HPLC-UV	HPLC-DAD	HPLC-UV	HPLC-UV
Linearity range ($\mu g\ mL^{-1}$) and application	Bulk powder (0.03-0.35) Plasma (0.20-3.50)	Bulk powder (0.04-0.28) Plasma (6.00-24.00)	Bulk powders only $10.00 - 100.00$	Bulk powders only Range not clear; "Article in chinese"	Bulk powders only $10.00 - 100.00$	Plasma only Range not clear; "No validation data"
Organic Solvent	ACN for plasma extraction Free for dilution	MeOH for plasma extraction Free for Dilution	Totally Free	Gradient elution using ACN	Isocratic elution using 10% ACN	Gradient elution using MeOH
Run time	NA	NA	4 min.	60 min.	8 min.	21 min.
Column	NA	NA	C18-RP	C18-RP	C18-RP	C18-RP
GAPI assessment						
AGREE assessment						

A disclosing comment on both recent metrics has to be mentioned. Both tools provided the best simple software for assessing analytical methodologies among other older metrics. GAPI covers each step within the analytical procedure including sample collection, preservation and transport, which is not totally covered by AGREE. AGREE did not consider the health hazards of used reagent as considered by GAPI. However, AGREE provides a numerical estimating overall value that facilitates the overall view of the comparison. AGREE also considers the method through output which is a crucial parameter by considering number of samples analysed per analytical run and per 1 h time which was not considered by GAPI. If GAPI could be modified to include such overall numerical value in the core pictogram and consider amount of samples measured hourly, that would an advance in this assessment target.

4. Conclusion

In the proposed study, two analytical methods were developed and validated for determination of favipiravir using fluorescence spectroscopy and solvent-free HPLC. The two methods were applied successfully for determination of the drugs under study in their marketed dosage forms. The spectrofluorimetric technique was applied in determination of the drug in spiked human plasma, as well. The methods proved to be sensitive, fast and economic. The validated methodologies were assessed on recent green metrics and were compared to previously reported methods and were found simpler, superior in sensitivity and more eco-friendly.

CRediT authorship contribution statement

Ibraam E. Mikhail: Methodology, Validation, Writing. **Heba Elmansi:** Investigation, Writing, Validation. **Fathalla Belal:** Project administration, Supervision, review and editing. **Adel Ehab Ibrahim:** Methodology, Validation, Writing.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2021.106189.

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