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HPLC_UV Method for Quantification of Favipiravir in Pharmaceutical Formulations

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Background: After the break, out of corona virus at 2019 it was named as SARS_COVID19 and researches on virus and its therapy started. The pandemic of corona caused many deaths all over the world as its novelty, hardened the treatment process. Available anti-viral medications used and tested for the treatment and at the same time, many researchers started to work on these medications. Mentioned drugs were tested for their effectiveness, with different methods of testing.
Objective: HPLC_UV Method for Quantification of Favipiravir in Pharmaceutical Formulations.
Method*:* One of the frequently used methods, HPLC, was used to quantify anti-viral drugs in their dosage form or in human or animal plasma. Favipiravir is one of the drugs of interest in those researches. Favipiravir, which is also known as T-705, is a newly invented drug compared to other antivirals. It is used in different cases of Ebola and influenza pandemics but not officially available in any pharmacopeia yet. After the pandemic of corona Favipiravir rose an attention to its therapeutically potentials against novel viruses. As it is not used widely, the resistance of viruses against Favipiravir is less. After many clinical trials, it was approved as a therapeutic choice for mild to moderate SARS_COVID19 patients whom are conscious and have the ability to swallow the tablet. Afterwards many researches for quantifying the drug with different analytical methods started. The purpose was to quantify the drug in its tablet dosage form or in human spiked plasma and even in animal blood samples.
Results and Conclusion: The results of these researches could help the producer companies, research clinics and at the very critical point, the final goal could be saving lives of patients. Hereby in this thesis project, an overall information on Favipiravir and the HPLC_UV analytical researches on this drug are mentioned.

Keywords: Favipiravir, HPLC_UV, Pharmaceutical Formulations, validation

INTRODUCTION

In December 2019, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which is the cause coronavirus disease 2019 (COVID-19), was identified in Wuhan, China, and considered as a pandemic by the World Health Organization (WHO) on March 11th, 2020 [1].

By October 2021, about 242 million cases of COVID-19 were reported worldwide, with 4.9 million deaths. The increase in COVID-19 cases worldwide required an urgent, effective therapeutic treatment. In line with this, the effects of different existing anti-viral drugs on COVID-19 were tested on in in vivo and in vitro models, and clinical cases [2-4].
Active therapeutic alternatives are urgently needed as a rising COVID-19 pandemic and possible effects on global health [4-6]. Many medications such as chloroquine, arbidol, remdesivir, and favipiravir are currently undergoing clinical trials in several countries to assess their effectiveness and safety in treating coronavirus disease [7-10]. So far, there is no gold standard for the treatment of COVID-19 since there is not enough evidence [11, 12].
One of the weapons designated by the Japanese researchers is an antiviral medication named as “Favipiravir”. Favipiravir sold under the brand name “Avigan” in the market is also named and known as T-705[13]. Avigan was first produced by the “Fujifilm Toyama” chemical company in Japan (2014) as a treatment of novel and reemerging influenza. Favipiravir was first designated/discovered when the Japanese researchers were working on antiviral agents for seasonal influenza treatment. This newly discovered drug was proven to have resistance and inhibition against almost 53 types of influenza viruses during the years [14, 15]. As it is necessary in nowadays lives to find a way against the emerging and novel viruses, the resistance of the available drugs is being tested over years and against different viruses. Favipiravir has shown to have resistance against many viruses in comparison with other anti-influenza drugs [16, 17]. The drug is a newly invented one and there are so many doubts and questions about its effectiveness or adverse effects and interactions, but it has a significant property. The significance of the drug is that there is a lack of resistant viruses against Favipiravir [13]. As an efficacy factor it was approved that Favipiravir can resist against viruses and used in form of a single type of drug as a treatment and protection in an influenza pandemic [18].
Favipiravir is a small molecule with three subunits in its structure (-F, -OH, -CONH2). It has a lower molecular weight in comparison with other nucleoside analogue antivirals. The hydroxyl group (-OH) makes the molecule acidic and become tautomerized to a keto group which with the help of intracellular enzymes transforms the prodrug to its active form. The increasing emergence and high necessity for effective antivirals, results in many new researches on pharmaceutical aspects of this class of drugs. A fast, low cost and green method synthesis of pharmaceutical products is an important matter that makes the drug desirable [19, 20]. Many synthetic pathways for Favipiravir production were suggested starting from 2000. Purpose of each new synthesis is to reduce the steps of the process and reducing the cost of materials used [21, 22].
There are many analytical methods for examining the analytes in order to determine their different aspects in a wide variety of researches. When there are problems using a method, new ways will come day by dat. After years of research progress on analytical methods, high performance liquid chromatography (HPLC) is derived from formerly used column chromatography [23-25].
The new era of pharmaceutical science is investigating available and newly invented drugs. The basic and important role of this investigation is carried out by many analytical methods one which is HPLC. HPLC can be considered as an important tool for pharmaceutical processes. High performance liquid chromatography is used in three different stages for discovering new drug, quantifying process of old and new drugs. In order to fulfill the mentioned processes, determining the best and effective method development in each stage is

important. High performance liquid chromatography is widely used for non-pharmaceutical and pharmaceutical products in order to decrease their cost and time consumption. For this purpose, in pharmaceutical products, HPLC is for separating and quantifying of the API, any impurities or degradants [26-28].

MATERIALS AND METHODS

Favipiravir is a novel antiviral drug, which is not officially available in any pharmacopoeia. This issue is because of the lack of enough in vitro, in vivo and chromatographic research results. The drug needs to be investigated in different fluids and circumstances using different quantification and qualification methods. As mentioned in the second part of introduction (2. HPLC part), high performance liquid chromatography is one of the most useful means of investigating pharmaceutical products. Regarding the novel Favipiravir medication, some of HPLC researches using this drug are mentioned below.

I. HPLC_UV method for quantification of Favipiravir in pharmaceutical formulations

To inquire the Favipiravir efficacy on Covid_19 patients and quality control in pharmaceutical formulations, the isocratic HPLC_UV method has been performed. Using analytical grade chemicals without further purification, these chemicals were chosen. Potassium dihydrogen phosphate, ortho-phosphoric acid, HPLC-grade acetonitrile, deionized water purified using Millipore system, Favipiravir powder and Favipiravir tablets. Afterwards stock standard solution and sample solution were prepared using mentioned chemicals. The mobile phase consisted of potassium dihydrogen phosphate 50mM (pH 2.3) plus acetonitrile (90:10 v/v). The mixture was filtered and degassed through a 0.45 µm filter. Used column was Inertsil ODS-3V C18 (4.6 mm ×250 mm×5.0µm). The column was thermostatic at 30 ºC. The flow rate of mobile phase was 1 ml/min with the total run time of 15 minutes. For determination of ƛ max, UV spectrophotometer named as Shimadzu UV-1800 was used between 200 and 800 nm ranges. Validity of the method was performed through ICH guidelines of validation. The results obtained as a linear relationship in the range of 10_100 µg/ml between peak area and Favipiravir concentration. Sensitivity of the method obtained by finding the LOD of 1.20 µg/ml and LOQ of 3.60µg/ml. precision which is the inter day and intraday RSD value for peak area and retention time were less than 0.4% and 0.2%. accuracy, specifity and robustness were also checked and confirmed that this method could be a sensitive, precise, accurate, specific and robust method for quantification of Favipiravir. The obtained statistical data and chromatograms are shown on table (1) and figure (1) respectively [13, 29, 30].

Table 1. Statistical data (calibration curve, FVP)

Parameter	Value
Linearity range (mg mL^1)	10–100
Slope	49.122
Intercept	82.598
Correlation coefficient	0.9999
Lack of fit F	
	2.90
P	0.0516
SE of intercept	6.9024

SD of intercept	15.4340
LOD/LOQ (mg mL[1])	1.20/3.60

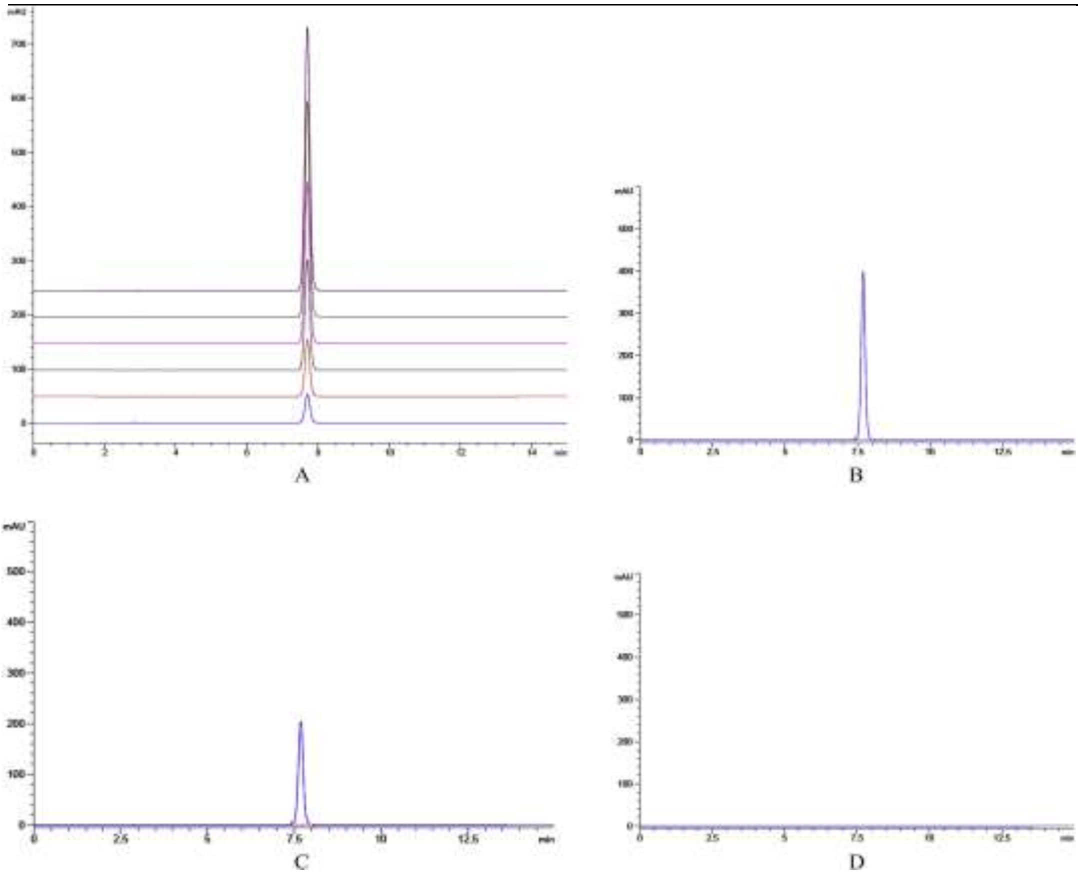


Figure 1. (A) Overlay chromatogram (standard solutions, 10–100 mg mL1, λ: 323 nm). (B) Chromatogram (standard solution, 80 mg mL1, λ: 323 nm). (C) Chromatogram (sample solution, 40 mg mL1, λ: 323 nm). (D) Chromatogram (Blank solution, λ: 323 nm)

II. Comparison of HPLC and UV spectrophotometric methods for quantification of Favipiravir in pharmaceutical formulations

With the growing attention on Favipiravir due to the Covid-19 pandemic situation, this analytical research compared two frequently used analytical methods for quantification of pharmaceutical products. In this experiment, the chemicals used were same in order to compare the results on idle circumstances. Chromatographic method performed on an Agilent 1260 series liquid chromatography, which had UV detector, a quaternary pump a vacuum degasser, a column oven and a Chemstation software. The used chemicals were sodium acetate, glacial acetic acid, acetonitrile, deionized water and Favipiravir tablets. The standard solution and sample solution were prepared using these chemicals. Sodium acetate solution 50mM (pH 3.0 adjusted using glacial acetic acid) and acetonitrile used as mobile phase had the flow rate of 1.0 ml/min. the mobile phase was degassed and filtered by a 0.22 µm filter. The column was an Inertsil ODS-3 C18 column reached to the temperature of 30 ºC. The total time of the experiment under mentioned conditions were 10 min. The wavelength at which the

UV detector had detected Favipiravir was 227nm. Method validation method was imitating ICH method resulted the following results shown in table (2,3) [31-33].

Table 2. Results of HPLC of Favipiravir

Parameter	**Spectrophotometric Method**	**Liquid Chromatographic Method**
Concentration Range (µg mL^{-1})	10-60	10-60
Limit of detection and quantification (µg mL^{-1})	1.4/4.3	0.4/1.1
Slope	0.0419	47.143
Standard Error of Slope	0.00052	0.2700
Intercept	0.1122	16.941
Standard Error of Intercept	0.0073	2.2000
Correlation Coefficient	0.9996	0.9999
Standard deviation of Residuals	1.00	0.29

Spectrophotometric method Precision parameters			**Liquid chromatographic method**	
	Absorbance*	**R.S.D. (%) ***	**Peak Area***	**R.S.D. (%) ***
Repeatability	1.375	0.364	1431.17	0.198
Intermediate Precision	1.372	0.372	1430.03	0.204

*(n = 5 for repeatability; n = 15 for Intermediate precision); R.S.D. (%) = Percentage Relative Standard Deviation.

Table 3. Recovery tests data.

Methods	**Level of drug taken**	**Mean percent recovery***	**R.S.D. (%) ***	**S.E.**
Spectrophotometric method	80	100.45	0.4781	0.0762
	100	99.83	0.5140	0.2963
	120	100.19	0.5814	0.3350
Liquid Chromatographic method	80	100.20	0.4574	0.2646
	100	100.14	0.2908	0.1683
	120	99.87	0.4329	0.3350

*(n = 3); R.S.D. (%) = Percentage Relative Standard Deviation n; S.E. = Standard Error.

Robustness study data.

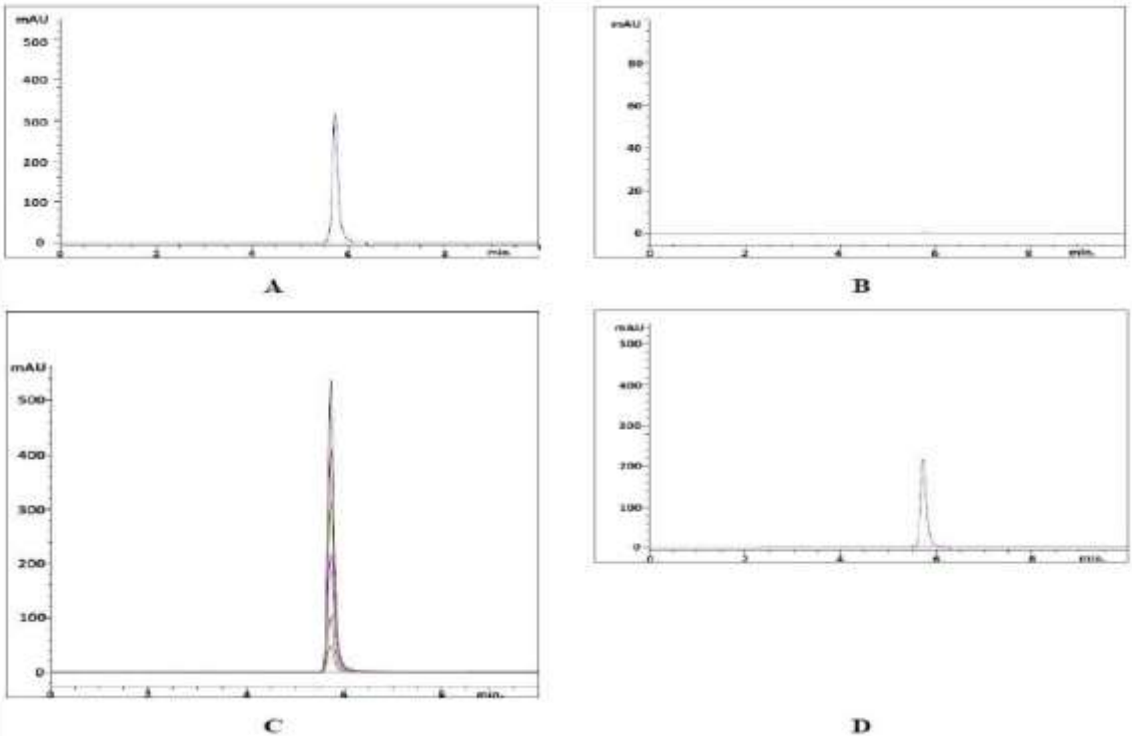


Figure 2. (A) Chromatogram of standard Favipiravir (60-µg mL-1). (B) Chromatogram of blank solution. (C) Overlay chromatogram (Standard solutions, 10-60 µg mL-1). (D) Chromatogram of sample solution (40-µg mL-1).

III. Green micellar solvent-free HPLC and spectrofluorometric determination of Favipiravir as one of COVID-19 antiviral regimens

For controlling quality of Favipiravir in available tablet forms and in spiked human plasma samples, this research had taken place. It is double-sided research in which both chromatographic and spectrofluorimetric results are obtained and compared to each other. Both analytical procedures were eco-friendly, assessed by GAPI, and AGREE metrics. The chromatographic HPLC was a reversed phase, isocratic process. The C18-RP was the column type and as the mobile phase a solvent free mixture of Brij-35 0.02 M, SDS 0.15 M and disodium hydrogen phosphate 0.02 M was used. The used system type was Agilent HPLC-1200. After completing the process and method validation procedure the table (8) shows results of this method. A comparison between this method and two other ones shown in table (4,5) [34-36].

Table4. 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 ($\mu g\ mL^{-1}$)	0.004	0.985
LOQ ($\mu g\ mL^{-1}$)	0.011	2.986

Table 5. 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

IV. *A validated high performance liquid chromatographic method for quantification of Favipiravir by PDA detector*

Considering the lack of research on Favipiravir anti-viral medication, which had shown to be effective against COVID-19, was the purpose behind this research. An isocratic HPLC using Shimadzu Prominence-I, LC-2030 C at total run time of 8.0 minutes were done successfully. The column used was Shim-Pack GISTC18 thermostated at 30 ºC and the mobile phase running through the column with flow rate 1.0ml/min was mixture of 10 mM potassium dihydrogen ortho phosphate buffer (pH 4.0) and acetonitrile (90:10 v/v). UV detection between 200-800 nm submitted the detection wavelength of Favipiravir mixed with mobile phase at 315 nm. Method validation was according to ICH Q2 (RI) method of validation. The obtained results are on the chromatograms shown in figure3 [18, 37, 38].

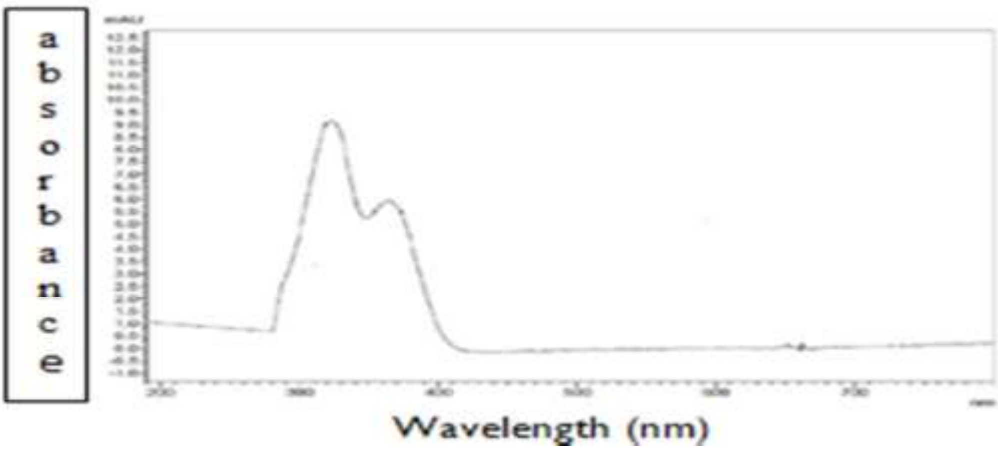
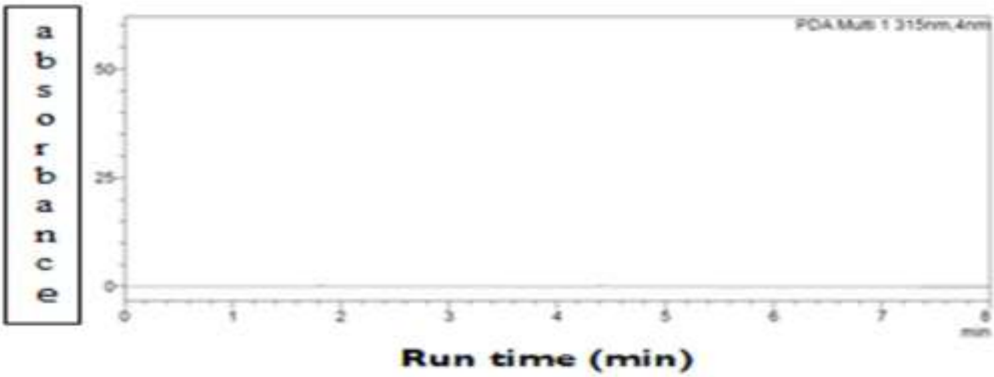
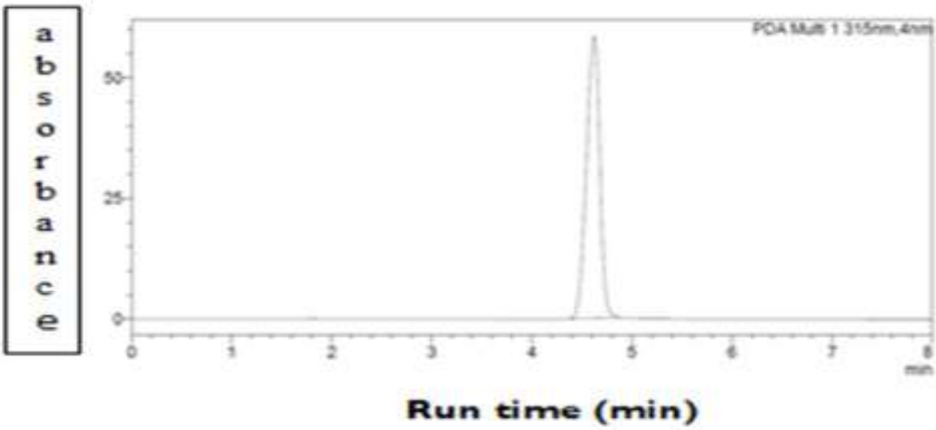


Figure3.λmax (315 nm) of UV Spectrum (standard solution, 40µg/ml)



Chromatogram of blank



Chromatogram of standard

V. Experimental design approach for development of spectrofluorimetric method for determination of Favipiravir; a potential therapeutic agent against COVID-19 virus: application to spiked human plasma

A spectrofluorimetry method using Favipiravir done for determination of this drug. The purpose of rapidity, robustness, sensitivity and green eco-friendly procedure was achieved. The fluorimetry proceeded using BOX BEHNKEN design and a comparison chromatographic method (HPLC), also run. The HPLC was on a Dionex Ultimate 3000 RS system. The instrument connected to Chromelon system. The column was Inertsil ODS-3. Using ethanol, methanol,acetonitrile, 0.2 M acetate buffer the process completed and the results of validation are shown in tables below [39-41].

Table 6. Linearity regression data for FAV using the proposed spectrofluorimetric method.

Parameter	Value
Linearity range (ng/mL)	40–280
slope	3.0128
SE of slope	0.0154
Intercept	1.0068

SE of Intercept	2.5939
Correlation coefficient (r)	0.9998
SE of estimation	3.5587

Table 7. Evaluation of accuracy for the determination of FAV.

Drug	conc. taken (ng/mL)	conc. found (ng/mL)*	% Recovery	Mean % recovery ± SD
FAV	40	40.25	100.63	100.54 ± 0.53
	60	60.73	101.22	
	120	120.21	100.18	
	240	241.94	100.81	
	280	279.66	99.88	

* Mean of five determinations.

Table 14 Evaluation of the precision of the proposed method for the determinate on of FAV.

Drug	Intra day			Inter day		
	conc. taken (ng/mL)	conc. found (ng/mL)*	%RSD	conc. taken (ng/mL)	conc. found (ng/mL)*	%RSD
FAV	60	60.28 61.03 60.89	0.66	60	60.27 61.07 61.25	0.85
	120	119.80 120.00 120.85	0.46	120	120.21 121.11 121.56	0.57
	240	243.12 241.41 241.29	0.42	240	240.63 242.26 242.91	0.49

VI. Development and validation of a method for quantification of Favipiravir as COVID-19 management in spiked human plasma

In order to qualify Favipiravir as a therapy option of Covid-19 this HPLC method which is simple, economic, with high accuracy and precision has proceeded. The method was an isocratic one using Acyclovir as an internal standard. The mobile phase was a mixture of methanol + acetonitrile and phosphate buffer (pH 3.1) all in proportion of 30:10:60 %, v/v/v respectively. The flow rate was 1 ml/min. After validating, the method using US-FDA guidelines the results are shown in figure 4 ,5 below [39,42-48].

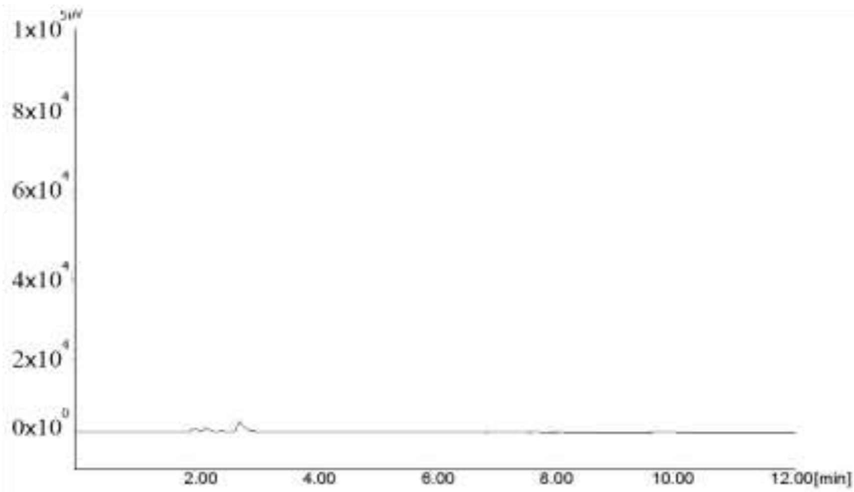


Figure 4. Representative chromatogram of blank plasma extracted with dichloromethane.

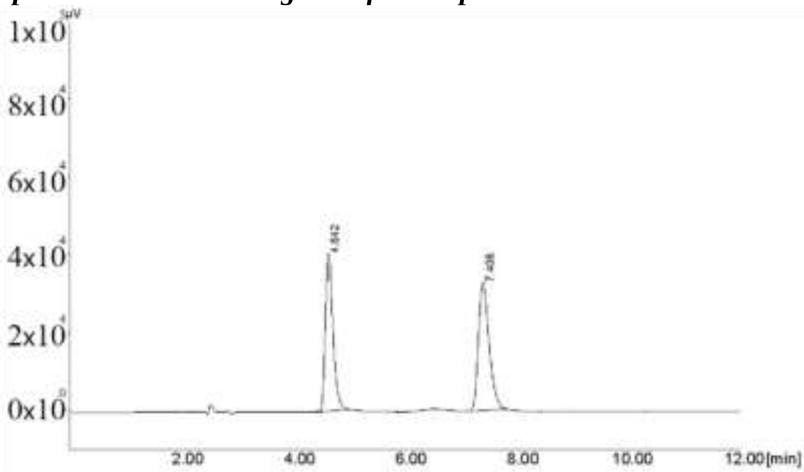


Figure 5. Representative chromatogram of FVIR and ACVR (IS) extracted in dichloromethane. (FVIR RT: 7.40 min; IS RT: 4.64 min).

RESULTS and CONCLUSION

With a proper investigation through the researches done on Favipiravir medication, it could be concluded that this medication has many aspects, which need more investigation and analysis. As an anti-viral drug, T-705 is efficient on most of viral human infecting viruses because of its novelty and broad spectrum. The medication is not officially available on any pharmacopoeia but it is used world wild when a viral crisis like Covid-19 pandemic happens. Favipiravir usefulness has shown to be on mild to moderate SARS-Covid-19 patients whom can swallow the tablet. The medication has no other medicinal form other than tablets yet so it cannot be used on witless patients. This medication can be used only with a governmental permission during a viral outbreak.

Emergence need for Favipiravir quality control tests in pharmaceutical dosage forms and in vivo studies, rose numerous analytical studies on this matter. High performance liquid chromatography is one of the mostly used analytical chemistry methods. Many types of HPLC performed on Favipiravir with different conditions resulted in valuable information. Yet there is need for more investigation. Some methods were just HPLC method considering different aspects during the procedure while some were comparison of HPLC method with other analytical methods like spectrofluorimetry. The first group of researches observed the HPLC process in spiked human plasma, animal models or pharmaceutical products with differences in their mobile phase, column, run time, isocratic or gradient type etc.

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