Favipiravir Determination in Pharmaceutical Formulation *via* HPLC Chromatographic Approach

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ABSTRACT: *Favipiravir is a broad-spectrum antiviral drug and it has increasing interest regarding its potential use in COVID-19 therapy. In the present work, a simple, fast, precise Reverse-Phase High-Performance Liquid Chromatographic (RP-HPLC) technique was developed for the quantification of favipiravir (FVP) from pharmaceutical formulation. Moreover, spectral and chromatographic behavior of FVP was investigated. The developed method was performed on using an ACE 5 C18 column (250 mm × 4.6 mm, 5 µm), with the mobile phase composition 10 mM phosphate buffer (pH = 2.5): methanol (80:20, v/v) at a flow rate 0.6 mL/min. The method validation parameters, such as linearity, precision, accuracy, and robustness, were determined. The recovery yields for accuracy were between 99.9 and 101.4 % for three concentrations. The linearity range was determined between 0.5 and 100 µg/mL with regression coefficient (R^2) 0.99998. The limit of detection and limit of quantification values were evaluated as 0.02 µg/mL and 0.05 µg/mL, respectively. The precision of the method was evaluated in inter-day and intra-day precision studies with a relative standard deviation of less than 2%. The method robustness was investigated using the alteration of flow rate, detection wavelength, and mobile phase ratio. Moreover, the effect of the pH and mobile phase ratio on the capacity factors were analyzed and the pKa value of the FVP was determined chromatographically as 5.03 ± 0.02.*

KEYWORDS: *Favipiravir, HPLC, Method validation, Acid Dissociation Constant*

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 [2,3,4]. Previous studies have pointed to the importance of antiviral therapy in COVID-19, which reduces SARS-CoV-2 replication and prevents or decreases severe symptoms or death [4,5]. Favipiravir (FVP), 6-Fluoro-3-hydroxypyrazine-2-carboxamide, is a broad-spectrum antiviral drug, and differently from other anti-influenza drugs, it is able to inhibit RNA-dependent

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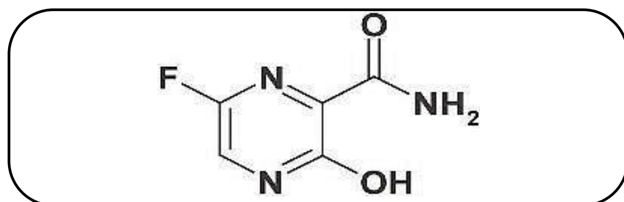

Fig. 1: Chemical Structure of FVP.

RNA polymerase of RNA-viruses without the inhibition of cell DNA and RNA [6,7,8,9] and its chemical structure is given in Fig. 1.

There has been increasing attention on FVP due to its therapeutic use during COVID-19 [10,11,12,13]. The act of FVP was defined as a nucleotide analog and the results lethal mutagenesis by slowdown viral RNA synthesis. Despite the fact that many studies have been conducted regarding FVP effects and metabolism, there have been few studies about its determination [12,14]. Furthermore, some of molecular docking studies were performed in order to better understand interaction mechanism between protein and anti-viral drugs, including Favipiravir, potential antiviral drugs and natural products [15-18].

However, these previous studies pointed out the importance of the increasing interest to develop a simple, fast, and precise method for the determination of FVP from pharmaceutical formulations and biological samples [12,14,19-21]. *Acquavia et al.* [21] have been reported developed analytical methods, sample preparation techniques, and the method validation parameters commonly used in the last decades for quantification of antiviral drugs including COVID-19 drugs in biological fluids and tissues. *Mehaged et al.* [14] developed a spectrofluorometric method for the determination of FVP from pharmaceutical formulation with a linear range 0.04-0.28 ppm drug concentration in alkaline media because of higher fluorescence intensity. On the other hand, some electrochemical techniques have been investigated for the determination of FVP from pharmaceutical formulations and real samples [22, 23]. Moreover, *Bulduk* [19] validated a RP-HPLC method for the determination of FVP from pharmaceutical products for a 90:10 (1 v:v) Phosphate Buffer (I=50mM, pH =2.3): Acetonitrile mobile phase, with LOD and LOQ values 1.2 and 3.6 ppm respectively with 15 minutes run time. *Prasanthi et al.* have reported that a simple, sensitive RP-HPLC methods determination of pyrazinamide in biological material e.g. a strong chromophore at 242 nm [24].

Favipiravir has different protonation constants [21, 25,26] and therefore, ionization of FVP plays a key role on the chromatographic and spectroscopic behavior of the drug. *Megehad et al.* [14] were the firsts to take in account the pH and solvent role on the fluorescence emission intensity of FVP. However, the correlation between fluorescence behavior and ionization constant was not investigated. In the present study different spectral and chromatographic behaviors were correlated with ionization constant of drug. In the best of our knowledge, experimental studies were not previously done on this issue. In the present study, the solvent effect on chromatographic and spectral behavior of FVP is studied. After optimization of pH and solvent ratio, a new, fast, sensitive and simple RP-HPLC method validated to determinate FVP. Moreover, the acid dissociation constant of the drug was evaluated as 5.03±0.02 by chromatography. The FVP used herein was kindly gifted from Atabay Pharmaceutical Fine Chemicals Inc. (Istanbul, Turkey).

EXPERIMENTAL SECTION

Chemicals and Reagents

All of the chemicals were analytical grade and were employed without further purification. HPLC-grade methanol and hydrochloric acid (HCl) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The sodium hydroxide (NaOH), sodium dihydrogen phosphate (NaH_2PO_4), and disodium hydrogen phosphate (Na_2HPO_4) were purchased from Merck (Darmstadt, Germany). The water is used as the mobile phase and to prepare the solutions was Ultrapure water, which was purchased from Tekkim (Turkey).

Instrumentation

A Cary 100 spectrophotometer (Agilent Technologies, Santa Clara, CA, USA), which was equipped with jacketed cell holders and allowed the temperature to be controlled to within ±0.1 °C was used to perform the spectral measurements. A glass syringe connected to a Mitutoyo micrometer screw (Mitutoyo America Corp., Aurora, IL, USA) was used to add an increasing amount of drug to the spectrophotometric cell.

An Oheaus Starter 5000 pH meter (Ohaus Corp., Parsippany, NJ, USA), equipped with a combined glass electrode, was used to perform the pH measurements. The HPLC analyses were performed on a 1220 Infinity

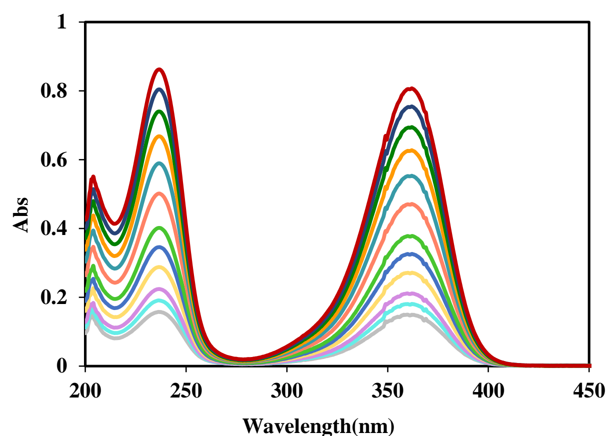

Fig. 2: Absorption spectra of different concentrations of FVP up to 0-8.44x10^{-5} M in, T = 25°C, [NaCaC]= 0.01, pH = 7.0.

LC system (Agilent Technologies), equipped with a gradient pump, degasser, and a UV detector controlled by Chem32 software (Agilent Technologies) for data acquisition.

Chromatographic Conditions

Chromatographic separations were carried out at room temperature using an ACE 5 C18 column (250 mm × 4.6 mm, 5 μm column) (Advanced Chromatography Technologies, Aberdeen, Scotland). The water: methanol solvent composition was used as the mobile phase. The dependence on the capacity factor on the mobile phase ratio and pH level was investigated. The method validation studies were performed with the following mobile phase composition: pH = 2.5, 10 mM Phosphate Buffer System (PBS): methanol (80:20, v/v) at a flow rate 0.6 mL/min. The sample injection was performed through a fixed sample loop with a volume of 20 μL.

Standard Solution Preparation

As a first step, 25 mg of the standard drug was weighed. The drug is completely soluble in water and dissolved in 25 mL ultrapure water. The solution was sonicated for about 30 min. The stock solution was stored at 4 °C.

Sample solution preparation

Favicovir (Atabay Pharm.) containing 200 mg Favipiravir was obtained from the local pharmacy. Twenty tablets were weighed and the average weight for each tablet was calculated. The tablets were crushed into a powder using a pestle and mortar, and then an amount equivalent to one tablet was weighed and transferred into a 250 mL volumetric

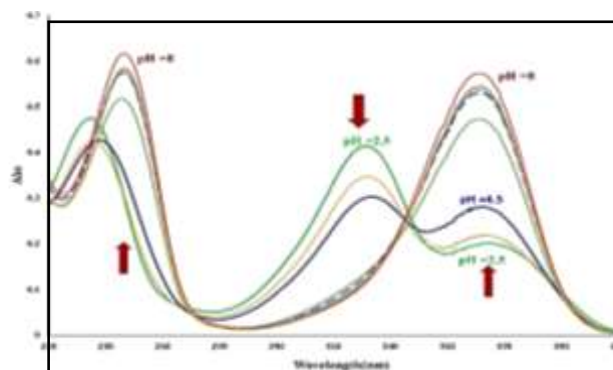

Fig. 3. The absorbance spectrum of FVP in different pH at 25 ◦C, I =10 mM.

sonicated for 30 min. The solution was then filtered through a 0.45-μm membrane filter and stored in 4 °C.

RESULTS AND DISCUSSIONS

In the spectral observation, the absorption spectrum of the FVP was recorded in the presence of different drug concentrations at a pH = 7.0. These observed spectrums are given in Fig. 2, where it can be seen that the spectrums of the two absorbance bands were observed with maximum absorbance values that were 236 and 360 nm, respectively. The molar absorptivity coefficient for these mentioned wavelengths was calculated as ε_{240} = 9843.9 1/M.cm and ε_{360} = 9600.6 1/M.cm, respectively, at 25 °C.

The effect of the pH level on the spectral behavior of the FVP was also investigated. As seen in Fig. 3, with the decrease in the pH values, a hypochromic effect was observed for the absorbance band, with a maximum absorbance value at 360 nm, while a new absorbance band occurs with a maximum absorbance value at 320 nm. Moreover, the other absorbance band, with a maximum absorbance value at 236 nm, also displayed a hypochromic shift to 220 nm with a hypochromic effect. Concerning the UV spectrum of FVP given in Fig. 2, we assumed that the two observed electronic bands are related to $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ electronic transitions where, consistent with literature reports [27].

The correlation between the absorbance change at 320 and 360 nm, with pH values from 2.5 to 8, is also demonstrated in Fig. 4-1.

On the other hand, the chromatographic behavior of the FVP was also investigated for the pH range of the buffer, from 2.5 to 6.5, with 0.5 data intervals, which can be seen in Fig. 4-2. The obtained results demonstrated that

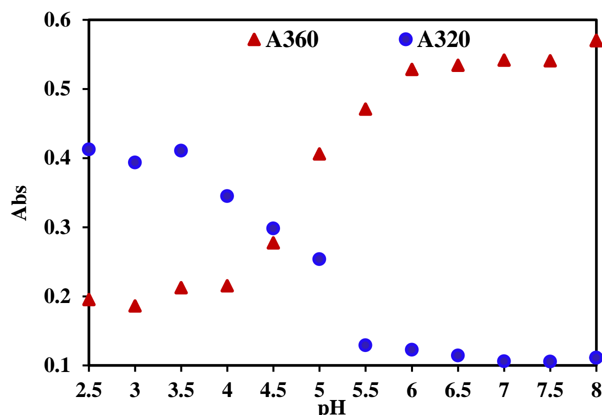

Fig. 4-1: The Absorbance values and pH correlation.

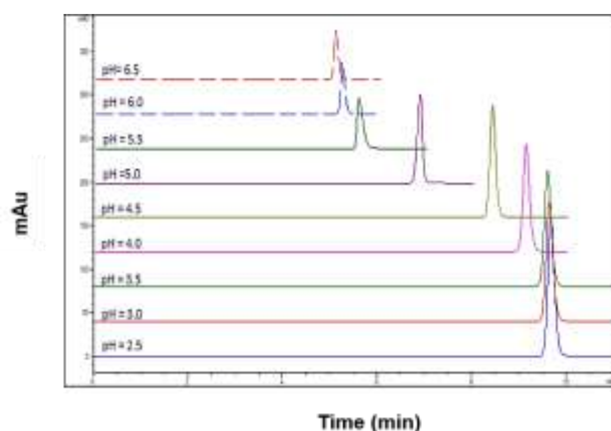

Fig. 4-2: The RP-HPLC chromatograms of FVP at the different pH values, 80:20 (10 mM Buffer: Methanol), 0.6 mL/min, at 320 nm.

the retention time of the FVP increased with the decrease in the pH level. This behavior could be explained by the higher pH values that the FVP presents in its ionic form and the decrease in the column retention.

In order to determinate pKa value of Favipiravir chromatographically the capacity factors of drug have been determinated at different pH values. The capacity factors (k') have been calculated according Eq. (1) where t_R is retention time of Favipiravir and t_0 is the dead time.

$$k' = \frac{t_R - t_o}{t_o} \tag{1}$$

The calculated capacity factors plotted versus of the mobile phase pH values and as given in Fig. 5, between pH and k' sigmoidal correlation have been obtained. The inflection point of the mentioned plot corresponds to pKa values of Favipiravir and has been evaluated by fitting plot with sigmoid equation shown below (Eq. (2)) [28] using a non-linear least square program (SigmaPlot).

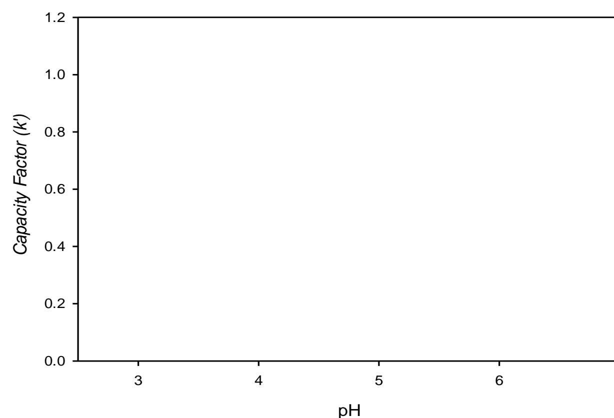

Fig. 5: The capacity factor of FVP, 80:20 (10 mM Buffer: Methanol), at 320 nm.

The calculated parameters of Eq. (2) were given in supplementary materials Table 1.

$$y = a + \frac{b}{1 + exp\left(-\frac{x - c}{d}\right)} \tag{2}$$

We have found pKa= 5.03 for FVP by chromatographic technique. In the literature there are only few theoretical studies on Favipiravir protonation constant [26]. To our knowledge there are no experimental determinations of protonation constant of FVP. Therefore, the present one is first experimental study about this issue. *Acquavia et al.* [22] and Pharmaceuticals and Medical Devices Agency (PMDA) [25] reported pKa = 5.1 for FVP associated to hydroxyl group deprotonation/protonation. *Silva* [26] reported for the pKa of FVP the values ~ 5.1, ~ 7.4 and ~ 9.8 by Density Functional Theory (DFT) calculations with the SMD continuum solvation model. Moreover, the protonation-deprotonation scheme of Favipiravir has been reported previously by Silva which were attributed with enol-ketone tautomerism [26]. The stability of Favipiravir at different pH values has been studied. For this purpose, Favipiravir solutions have been prepared at pH 2.5; 3.0; 4.0; 4.5; 5.0; 6.0; 6.5 and 7.0. The UV-Vis spectra of the solutions were recorded for 8 hours with 2-hour time interval. The absorbance vs. time graphs have been done for all pH values and are shown in Fig. 6. The spectra recorded at different times for all pH are given in the supplementary materials.

The mobile phase solvent ratio is known as another parameter that effects the capacity factor of an analyte. Therefore, the chromatographic and spectroscopic behavior of the FVP was studied for the different organic

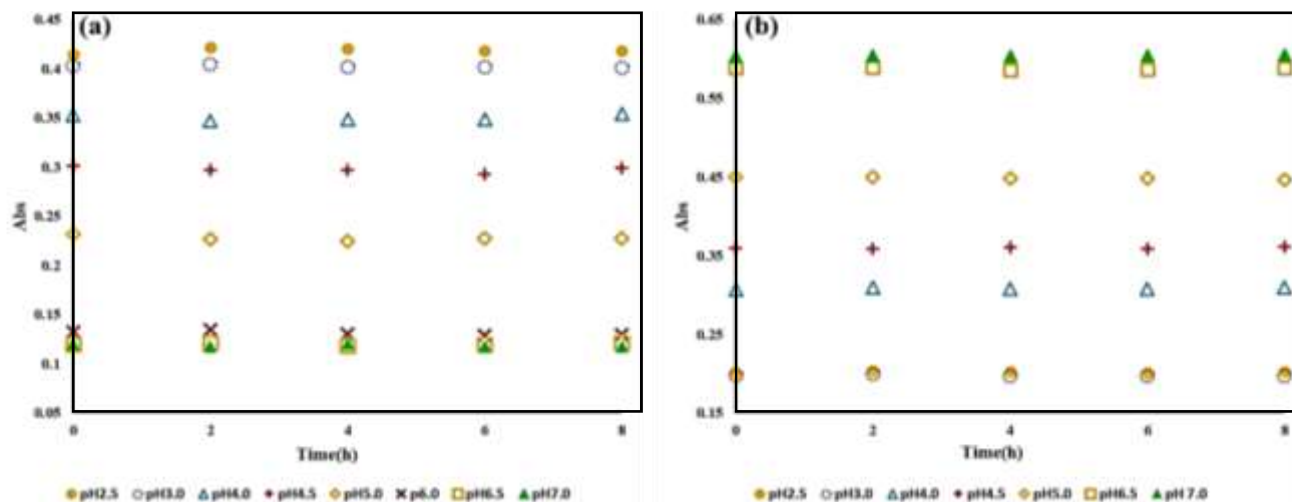

Fig.6: a) The time dependence of Abs values at 320nm, b) The time dependence of Abs values at 360nm; at different pH values between 0-8 hours, [FVP] = 6.3 ×10^{-5} M, T = 25°C, I= 0.01 M, pH = 2.5,3.0,4.5, 5.0,6.0,6.5,7.0.

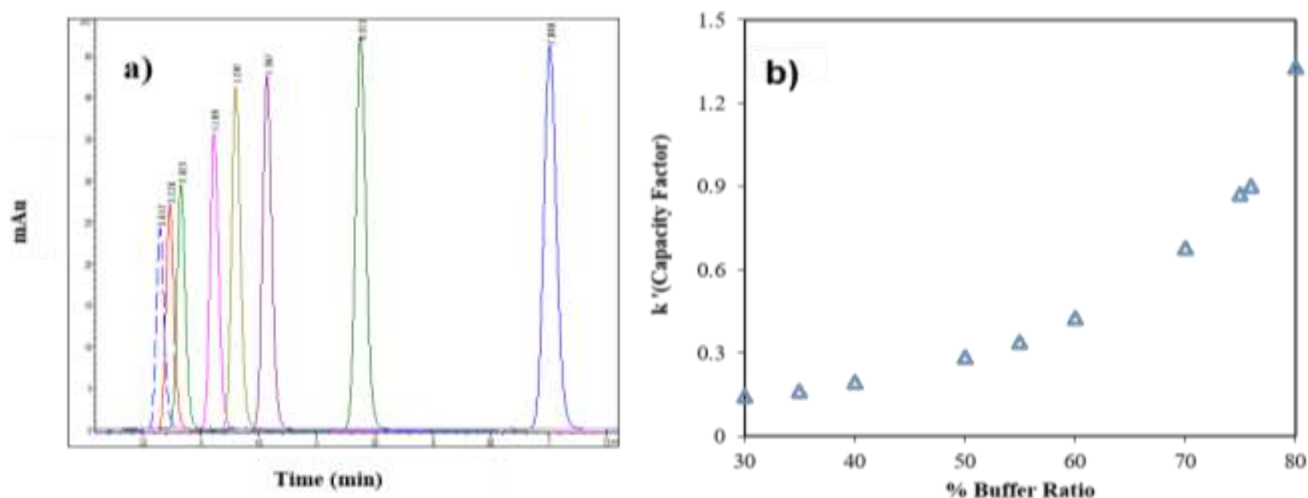

Fig. 7: a) The chromatogram of the FVP, b) The capacity factor of FVP; in the different buffer ratio; pH=2.5 (I=0.010 mM PBS) at 360 nm with 0.8 mL/min.

solvent ratios of the mobile phase at a constant temperature and pH level. The effect of the buffer ratio on the chromatographic behavior of the FVP is given in Fig. 7, which indicates that the augmentation on the buffer ratio of the mobile phase resulted in an exponential enhancement in the retention time and the capacity factor.

Moreover, the absorbance spectrum of the FVP was recorded by altering methanol ratio of the mobile phase. As given in Fig. 8, the increase in the methanol ratio was caused by the occurrence of a new absorbance band, with a maximum absorbance value 360 nm, while also exhibiting a hypochromic effect at 320 nm.

According to the obtained results, the mobile phase buffer pH value and mobile phase composition were optimized as pH 2.5 and 80:20, respectively (v/v, buffer: methanol). Observed absorbance spectrums indicated the

In the above-mentioned conditions the system suitability parameters as like theoretical plate number was evaluated more than 2000, the tailing factor calculated as 1.2 (< 2) and the capacity factor determined about 1.2 and subsequently, the method validation parameters were investigated.

Linearity studies were performed at two different wavelengths, respectively, as 320 nm and 360 nm, and the calibration graphs are given in Fig. 9. The regression equation at 320 nm was more suitable for the quantitative analysis of the FVP, which had the higher slope value.

Table 1. Linearity parameters of the validated method.

Regression equation	y=74.8×+2.033
Standard error of slope	0.4
Regression coefficient (R^2)	0.99998
Linearity Range(µg/mL)	0.5-100
LOD (µg/mL)	0.02
LOQ(µg/mL)	0.05

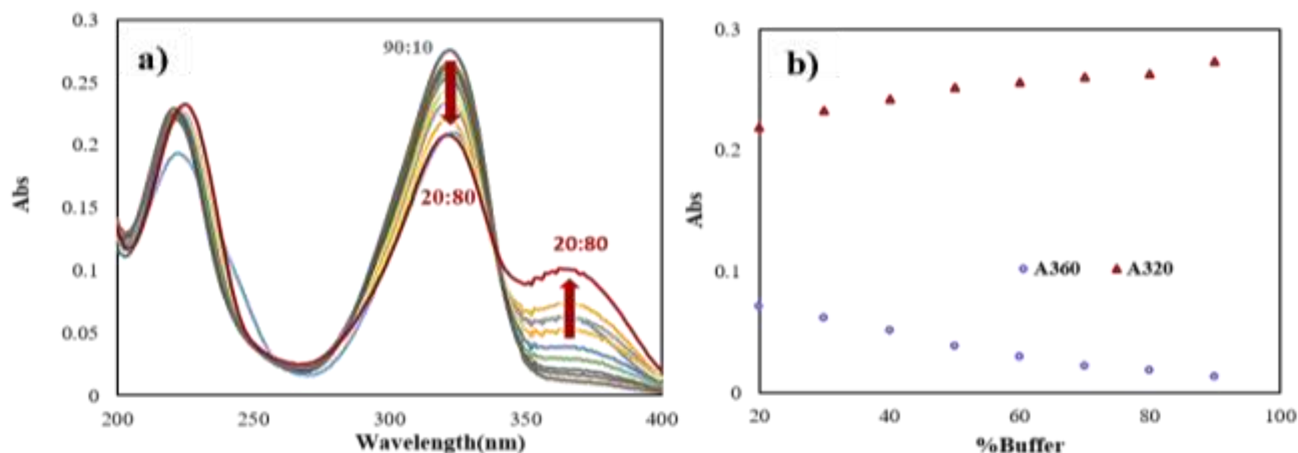

Fig. 8.a) The absorbance spectrum of FVP in the presence of difference mobile phase ratio, pH=2.5, T=25 ◦C, I =10 mM(PBS). b) The absorbance values and buffer ratio correlation.

$$\%Recovery = \frac{|FVP\ Amount\ of\ Spiked\ Sample\ - FVP\ Amount\ of\ Sample\ |}{Added\ FVP} \times 100 \quad (3)$$

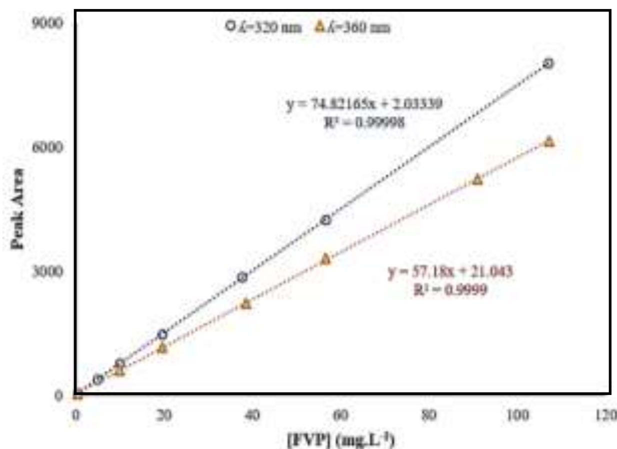

Fig. 9: The calibration graph was obtained for 10 mM phosphate buffer (pH=2.5): Methanol (80:20, v/v) system 0.6 mL/min.

The calibration standard solution was prepared from the stock drug (1000 µg/mL) solution for 7 different concentrations, ranging between 0.5 and 100 ppm and the linearity between peak area and drug concentration were determined. Six calibration curves were performed and the observed linearity parameters at 320 nm are given in Table 1.

The accuracy of the proposed method was investigated using the standard addition method. The sample solutions were spiked by a known amount of the FVP and the measurements were taken of the samples both with and without standard addition. The recovery yield was calculated using Eq. (3).

The measurements were taken for 3 different concentrations on the same day, with 3 replicated samples for each concentration level, and the obtained results are given in Table 2.

The recovery yields of the FVP for three concentrations were estimated between 99.9% and 101.4%, indicating that the accuracy of the validated method was acceptable. Moreover, the precision of the validated method was investigated by performing intra-day and inter-day precision studies for the three concentrations of FVP. In the case of the inter-day precision for each concentration, 3 solutions were prepared by the same analyst in the same laboratory on the same day and the determined drug amounts with the relative standard deviations results are given in Table 3.

Table 2: %Recovery of the FVP for the different spiked drug amounts.

S. No.	Added (mg)	Founded (mg)	%Recovery
1.	20	19.9±0.1	99.7±0.5
2.	20	20.2±0.1	101±0.5
3.	20	19.8±0.1	99.1±0.5
Mean		19.9±0.1	99.9±0.5
%RSD		1.0	1.0
1.	50	50.0±0.1	100.0±0.2
2.	50	50.1±0.1	100.2±0.2
3.	50	49.9±0.1	99.8±0.2
Mean		50±0.1	100±0.2
%RSD		0.1	0.1
1.	80	81.3±0.15	101.7±0.2
2.	80	80.5±0.14	100.7±0.2
3.	80	81.5±0.15	101.8±0.2
Mean		81.1±0.14	101.4±0.2
%RSD		0.3	0.4

Table 3. Inter-day repeatability of FVP determination by RP-HPLC.

Exp. No.	15 (mg)	30 (mg)	45 (mg)
1.	15.1±0.1	29.7±0.1	44.2±0.1
2.	14.7±0.1	29.7±0.1	44.3±0.1
3.	14.7±0.1	29.4±0.1	44.4±0.1
Mean	14.8±0.1	29.6±0.1	44.3±0.1
%RSD	1.4	0.6	0.2

On the other hand, the intra-day studies were performed in the same laboratory by the same analyst on 7 consecutive days for three concentrations and the obtained results are demonstrated in Table 4.

According to the %RSD values that are presented in Tables 3. and Table 4. for the inter-day and intra-day precision values that were smaller than 2, the precision of validated method was suitable.

Robustness of the validated method was tested altering mobile phase ratio for different 2 drug level, buffer pH. Moreover, even if optimum flow rate of mobile phase was determination 0.6 mL/min because of the higher capacity factor. Also, the mobile phase rate from 0.6 mL/min to 0.8 mL/min was tested in order to test method robustness. The observed results are given in Table 5. with %RSD values lower than 2 that confirmed suitability of robustness for the developed method.

Table 4: Intra-day repeatability of FVP determination by RP-HPLC.

Day No.	15 (mg)	30 (mg)	45 (mg)
1.	15.0±0.1	29.8±0.1	44.2±0.1
2.	15.3±0.1	29.9±0.1	44.8±0.1
3.	15.2±0.1	30.0±0.1	44.8±0.1
4.	15.3±0.1	29.9±0.1	45.2±0.1
5.	14.9±0.1	29.8±0.1	45.4±0.1
6.	15.0±0.1	30.0±0.1	45.4±0.1
7.	15.1±0.1	29.9±0.1	44.3±0.1
Mean	15.1±0.1	29.9±0.1	44.9±0.1
%RSD	1.0	0.3	1.1

Table 5. The method robustness parameters

Parameter	Added (mg)	Founded (mg)	SD	%RSD
Mobile Phase Ratio 79:21 (Buffer: Methanol)	15	15.2	0.2	1.3
	100	100.4	0.4	0.4
Flow rate 0.8 mL/min	15	15.1	0.1	0.7
Buffer pH = 2.4	100	99.3	0.7	0.7

CONCLUSIONS

In order to the quantification of FVP from pharmaceutical products a suitable HPLC method has been developed and validated. During the method development studies, the effect of the mobile phase buffer pH and the mobile phase solvent ratio on the chromatographic and spectroscopic behavior of FVP were investigated. The correlation between lower mobile phase pH and higher capacity factor of the FVP was explained with the pKa values of the drug that was determined as 5.03 ± 0.02. In the validation studies, the obtained results proved the suitability of the developed method for the linearity, sensitivity, precision, and accuracy.

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