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ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF FAVIPIRAVIR BY USING RP-HPLC

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Abstract : The Coronaviruses disease-2019 outbreak all over the world. Hence there is need to develop drug for the treatment of this ailment. In this case, Favipiravir is antiviral drug was approved for new and re-emerging use and its shown potent in-vitro activity against SARs COV-2. A simple, specific, rapid, accurate, precise and robust RP-HPLC method was to developed. Separation was carried out by chromasil 60-5-CN column (150×4.6mm, 5um), the mobile phase was mixture of 50mM potassium dihydrogen phosphate (pH 3.2) and methanol (90:10 v/v) at a flow rate 1.0ml/min. Column temperature was 30°C with detection at 231 nm by UV detector and 10 min run time. The linearity was observed in the concentration range 20-100 µg/ml (r^2= 0.9996) and RT was found to be 3.6 ±0.25 min. The method was validated according to ICH guidelines and value of linearity, precision, robustness, limit of detection, limit of quantitation, specificity and recovery was found to be within limit. Hence proposed method has been used in routine quality control for quantification of Favipiravir in pharmaceutical formulation.

Keywords: Antiviral drug, Favipiravir, COVID-19, HPLC, validation

1. Introduction

In Wuhan, China at the end of 2019, COVID-19 was started and it declared a global pandemic by World Health Organization (WHO) on 12- March-2020 [1]. The outbreak of severe acute respiratory syndrome corona virus-2 (SARs COV-2) infection and its caused corona virus disease(COVID-19), it affect the all countries and regions around the world and spread rapidly [2].

Their is need to develop drug for the treatment of this ailment. There are several drug tried to used in the treatment of COVID-19, such as Chloroquine, Hydroxychloroquine, Ribovirin, Interferon, Lopinavir, Ritonavir, Remdesivir and Favipiravir. FVP is antiviral drug which received the emergency approval use in the treatment of influenza and COVID-19 disease [3].

It has primary discovered and assessing the antiviral activity in the chemical library of Toyama Chemical against the influenza virus [4]. Favipiravir (T-705) is synthetic prodrug, Chemically, 6-fluro-3-hydroxy pyrazine-2-carboxamide, an analog of pyrazine (fig-1) [2] and its is converted into its ribofluranosyl 5'-triphosphate metabolite, then introduce in the growing RNA stand. Mechanism action of FVP, it bind and inhibit the RNA dependent RNA polymerase which ultimately prevent viral genome RNA transcription and

replication [5]. FVP has also used some life threatening infection such as a Ebola, Lassa Fever and rabies. Hence Favipiravir is a shows potential drug for the treatment of COVID-19, and it also minimizes the hospital stay and need of mechanical ventilation [6].

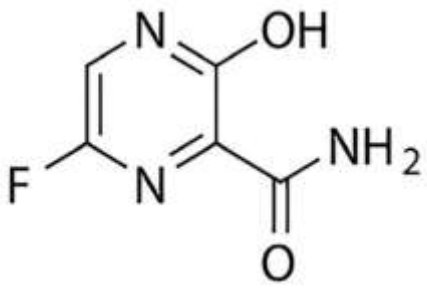

Figure 1: Structure of Favipiravir

Literature survey reveals that, there are few published HPLC method available for estimation of FVP in pharmaceutical dosage form [2,7]. and also favipiravir is not found in any official pharmacopoeia, hence there is need for the the development and validation of HPLC method.

The aim of the current work has to develop simple, specific, rapid, accurate, precise and robust method for estimation of favipiravir in pharmaceutical dosage form and develop method validate according to ICH guidelines. Moreover, the proposed method involves the use of water as diluent which match with green chemistry requirement.

2. Materials and methods

2.1 Chemicals and Reagents

Analytical grade chemicals were used such as, potassium dihydrogen phosphate (Merck Life Science Pvt Ltd), ortho-phosphoric acid (≥85%, Thermo Fisher Scientific India Pvt Ltd), and HPLC-grade methanol (Merck Life Science Pvt Ltd) and water (S D Fine-Chem Limited) were used. Bulk powder of favipiravir and fabiflu tablets (400 mg) were obtained from Lee Pharma Ltd, Hyderebad and Glenmark Pharmaceuticals Ltd respectively.

2.2 Preparation of 50mM Phosphate buffer Solution

Dissolved 6.8 gm of potassium dihydrogen phosphate in 1000 ml of water. Adjusted pH 3.2 (±0.05) with ortho phosphoric acid (OPA).

2.3 Preparation of standard stock solution

Stock solution was prepared by accurately weighed 100 mg of favipiravir standard into a 100 ml volumetric flask, added 40 ml of water, shake and sonicated to dissolve the content, made up the volume with water and filtered through 0.45 micron membrane filter. The stock solution was further diluted with water to obtain the required concentration of standard solution (20-100 µg/ml).

2.4 Preparation of sample solution

Ten tablet were accurately weighted and triturated to a fine powder. Weighed accurately powder equivalent to 100 mg of favipiravir was taken into a 100 ml volumetric flask. Added 40 ml of water, shake for 5 min and sonicated for 30 min and made up the volume with water, and filtered through 0.45 micron membrane filter. From above solution pipetted out 3 ml of filtrate to 50 ml volumetric flask, made up the volume with water, shaken and collected the sample after discarding the first few ml of solution.

2.5 Chromatography Condition

The HPLC system consisted autosampler with Model No LC-2010 AHT (SHIMADZU). The detector consisted of an ultraviolet (UV)-visible spectrophotometry and data were integrated using LC Solution, version-1.25, SPINCO BIOTECH PVT LTD.

Chromatographic separation was carried out with chromasil 60-5-CN column (150 X 4.6 mm, 5 µm). The mobile phase used for isocratic elution was prepared by mixing phosphate buffer: methanol (90:10 v/v). prior to using, the mobile phase was filtered through 0.45 µm membrane filter and degassed by ultrasonication. The flow rate was 1.0 ml/min, column temperature 30°C, the injection volume was 20 µl, and detection at 231 nm using a UV detector.

3. Results and Discussion

3.1 Determination of λmax

The standard solution of favipiravir was scanned in the wavelength range of 200-800 nm on a UV-Visible Spectrophotometer (SHIMADZU UV-1900i Spectrophotometer) from this, wavelength corresponding to maximum absorbance (λ max) was found to be 231nm, as shown in Fig.2.

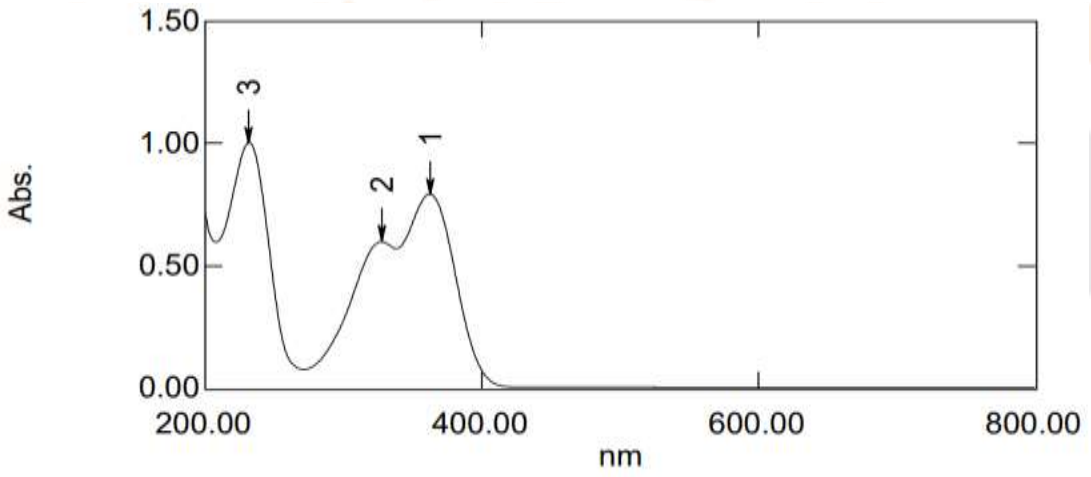

Figure 2: UV Spectra of Favipiravir

3.2 Method Development

For the method development, several preliminary trial runs were taken on the pre-validated RP-HPLC system by varying the concentration of mobile phase and change in the flow rate of mobile phase to get the optimized parameters.

Table 1: **Summary of parameters change during method development**

Trial No	Mobile Phase Composition	Flow rate	Temperature	Retention Time
1	Water: Methanol (50:50)	1 ml/min	30 ˚C	1.35 min
2	Water: Methanol (70:30)	1 ml/min	30 ˚C	1.50 min
3	Water: Methanol (90:10)	1 ml/min	30 ˚C	1.60 min
4	Water: Methanol (90:10)	0.8 ml/min	25 ˚C	2.18 min
5	Potasssium Dihydrogen phosphate: Methanol (90:10)	1 ml/min	30 ˚C	3.84 min

From the above table it was observed that, trail 1-4 comprises of water: methanol as mobile phase in different ratios. The retention time (RT) of the favipiravir peak was observed in the range of 1.3 to 2.3 min. But, this RT can not be considered because of the presence of solvent peak in the range of 1-3 min. So, the trial 5 was developed with change in the mobile phase composition. Retention time was shifted to 3.8 min which will considered as RT of Favipiravir. And Favipiravir peak being well shaped and symmetrical using this system and they provide stronger theoretical plate (>2000) and peak tailing factor (<2.0). Collectively, the best chromatographic condition were achieved using on isocratic mobile phase comprising 50 mM potassium dihydrogen phosphate (pH 3.2): methanol (90:10 v/v) at a flow rate of 1.0 ml/min on an chromasil 60-5-CN column (150×4.5mm, 5 um). The analysis was conducted at 30℃ and detected at 231 nm by using UV detector and runtime is 10 min. Under this chromatographic condition favipiravir eluted at RT 3.6 ±0.25 min. They having lots of advantage such as good chromatographic peak shape, enhance column efficiency, low column pressure and it is economic.

Hence, trail 5 was considered as optimized trail from the all the trails which has been developed properly and validated on prevalidated RP-HPLC system as per ICH guidelines.

3.4 Method Validation [8]

The validation of developed method has been performed as per ICH guidelines, validation of analytical procedure: Q2 (R1). The validation parameters such as system suitability, linearity, accuracy, specificity, precision, the limit of detection (LOD), the limit of quantification (LOQ) and robustness were performed.

3.4.1 System suitability

The system suitability was determined by five replicate injection of favipiravir at a concentration of 40 µg/ml. The system suitability criteria should be fulfill, when % RSD is less than 2.0%, number of therotical plate is greater than 2000, and tailing factor is less than 2.0, as shown in Table 2.

3.4.2 Specificity

The specificity was determined by analyzing the blank, placebo, standard and sample solution. They ensure that there was no interference from the excipients present in the formulation and diluents and no peak was detected close to the retention time of favipiravir and standard are match with sample, this proved the high degree of specificity of the method. The chromatogram of specificity studies are shown in figure 4 to 7.

3.4.3 Linearity and Range

Linearity is the capability to obtain test results that are directly proportional to the concentration of the analyte. Linearity was determined by three injections of five different standard solution in the concentrations range of (20, 40, 60, 80 and 100 µg/ml). The mean peak areas were plotted against concentrations and linearity was determined using the calibration curve. The correlation coefficient, slope and intercept was determined. In general, a value of correlation coefficient (r^2) > 0.998 is considered as the evidence of linear relationship. From the regression analysis, a linear equation was obtained: y = 49872x + 43430, and correlation coefficient (r^2) was found to be 0.9996, in the concentration range of 20-100 µg/ml as shown in Fig.3. This indicated a linear relationship between the concentration of analyte and peak area. The chromatogram of linearity studies are shown in figure 8 to 12.

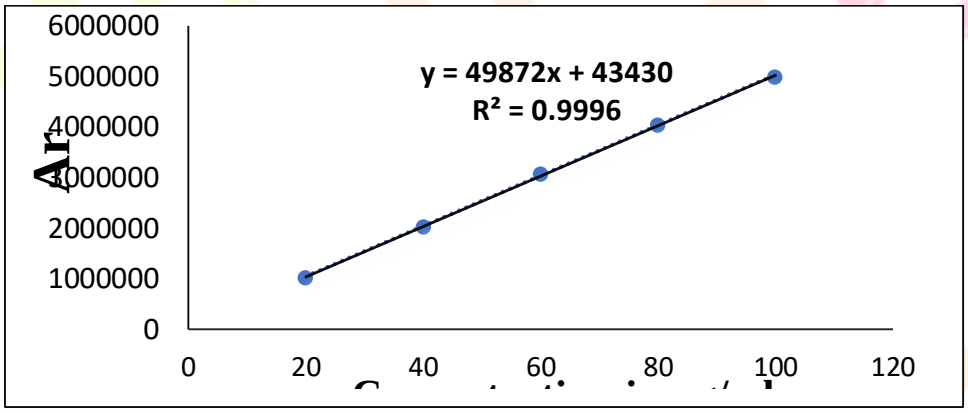

Figure 3: Calibration curve for Favipiravir

3.4.4 Precision

System precision and method precision of the proposed methods was determined by several measurements of standard solution and sample solution, respectively. System precision was determined by five replicated injection of the standard solution at the 100% concentration levels. Method precision was determined by six assay evaluation of the sample solution at the 100% concentration levels. Intraday and interday precision study was performed by analyzing standard solution at three different concentration 40, 60, and 80 µg/ml on same day but in different time and on three different consecutive day respectively. The results of precision was found within limit, hence method are precise. The results are shown in table 3.

3.4.5 Ruggedness

Ruggedness of the method was determined by using different analyst and different instrument. The % RSD was found within limit, hence method is robust.

3.4.6 Accuracy (Recovery)

The accuracy of the method expresses the closeness between the expected value and the true value. Accuracy mainely performed at three levels 80%, 100% & 120% of the working concentration level for favipiravir (60 ppm). each level prepared in triplicates. Results of % recovery was found to be at all three levels in the range of 99.33–101.27% and % RDS values were in the range of 0.32–0.50% as shown in table 4. i.e. results of % recovery and % RSD were within the acceptable limits from 98.0% to 102.0% and not more than 2.0%, respectively, which indicates the method is accurate.

3.4.7 Limit of detection (LOD) and limit of quantification (LOQ)

LOD is the lowest amount of analytes in the sample which can be detected, but not essentially quantified under the stated investigational conditions. LOQ is the lowest amount of analytes in the sample which can be quantified with acceptable limit of precision and accuracy. These two parameters were determine by signal-to-noise ratios of 3:1 and 10:1 and calculated by using the formula LOD = 3.3×SD/S and LOQ = 10×SD/S, where SD = standard deviation of response (peak area) and S = slope of the calibration curve. The LOD and LOQ of favipiravir were found to be 1.36 µg/ml and 4.13 µg/ml respectively.

3.4.8 Robustness

Robustness of the method was determined by applying minor and deliberate changes in the experimental parameters, for eg change in column temperature (±5°C), flow rate (±0.2 ml/min), wavelength (±3 nm), mobile phase composition (±5%) and mobile phase pH (±2%). change was made to evaluate its effect on the method. The data obtained was evaluated by calculating % RSD and percent of recovery. The results was shown no marked changes in the chromatograms as shown in table 5, it indicated that the method are robust.**3.4.9 Solution Stability**

The stability of standard and sample solutions was performed over a 26 h period and % RSD of the peak area and retention time were evaluated. The % RSD for peak area (n=4) and retention time (n=4) was found to be 0.1711% and 0.1708% respectively. The results shown, there are no major changes in active ingredient concentration have been found in the sample solution. Results are shown in table 6. This indicated standard and sample solution are stable over period of time.

3.4.10 Application of the method

The developed and validated method has been applied for estimation of favipiravir in marketed tablet formulation. In this, single-injection of blank, five replicated injection of standard solution and duplicate injection of sample solution were injected on the system. The % assay of favipiravir samples was calculated and results are shown in table 7.

Chromatogram

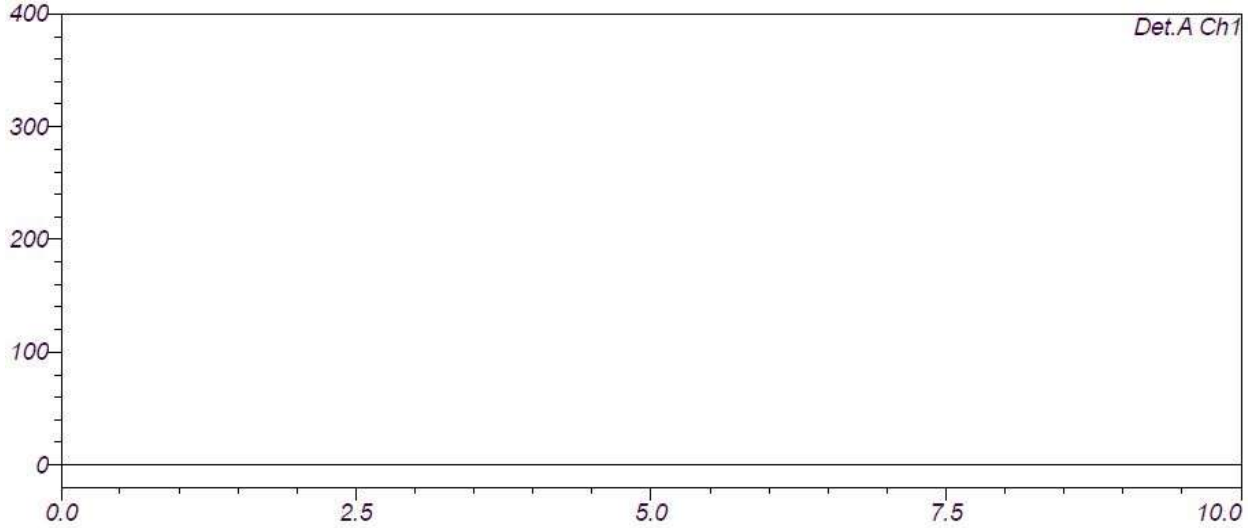

Figure 4: Chromatogram of Blank

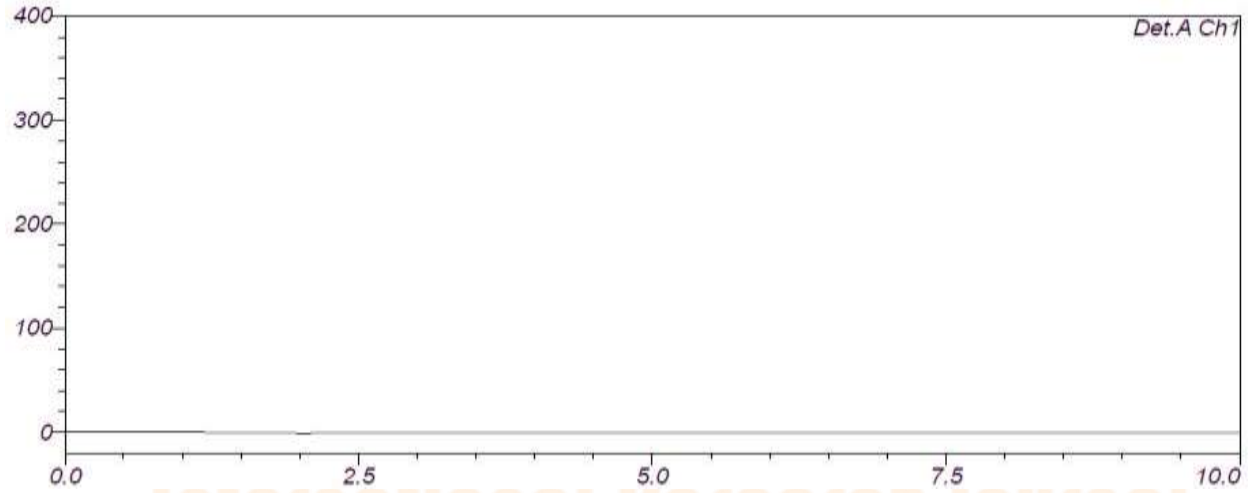

Figure 5: Chromatogram of Placebo

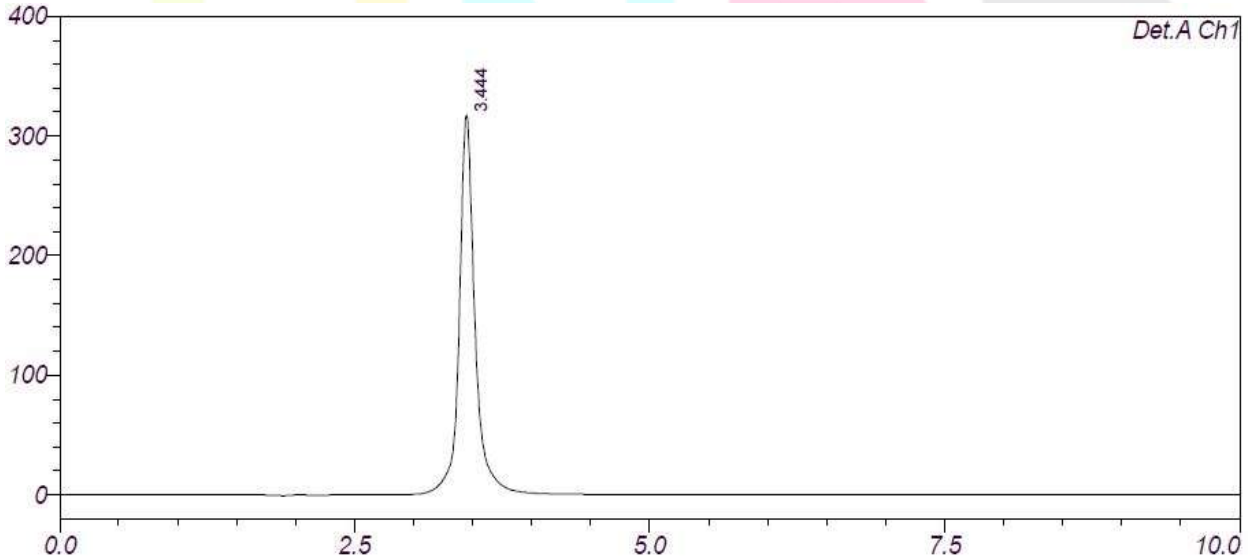

Figure 6: Chromatogram of Standard

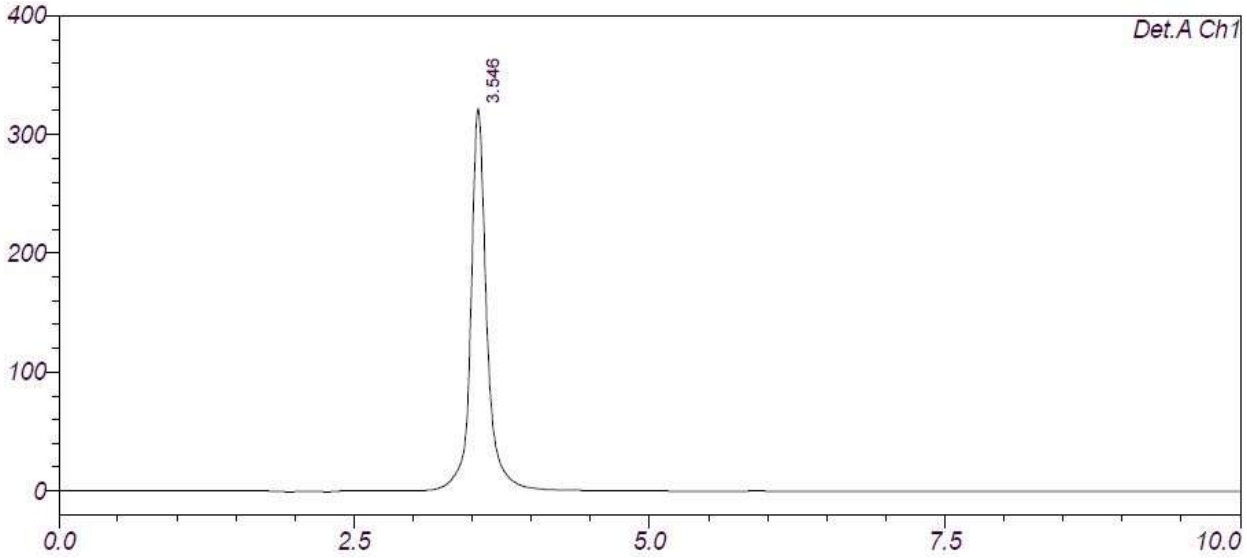

Figure 7: Chromatogram of Sample

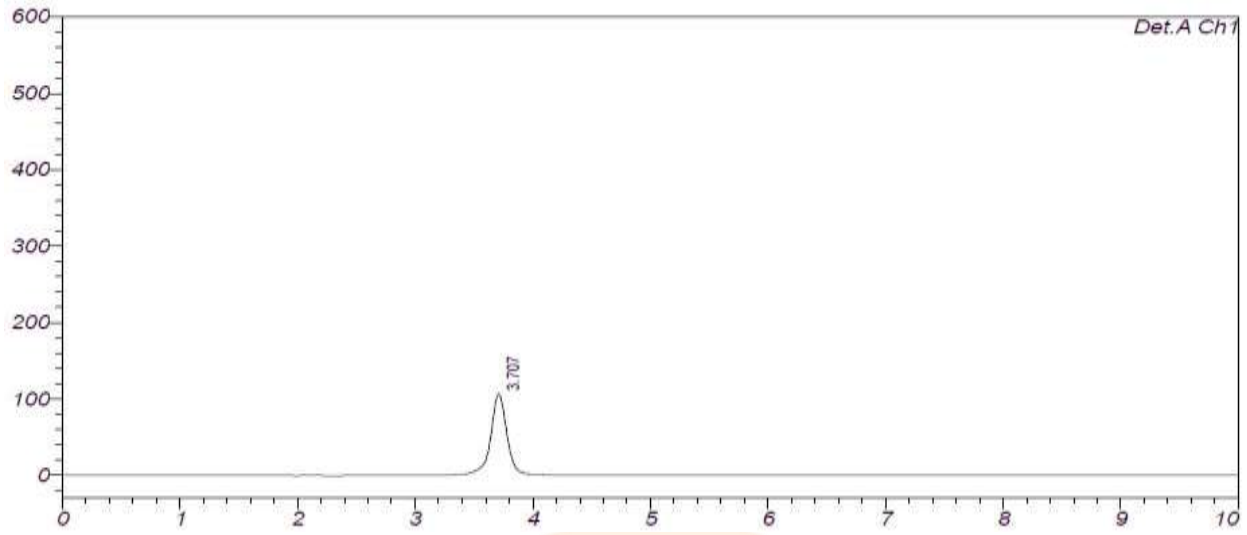

Figure 8: Chromatogram of Linearity at 20 µg/ml

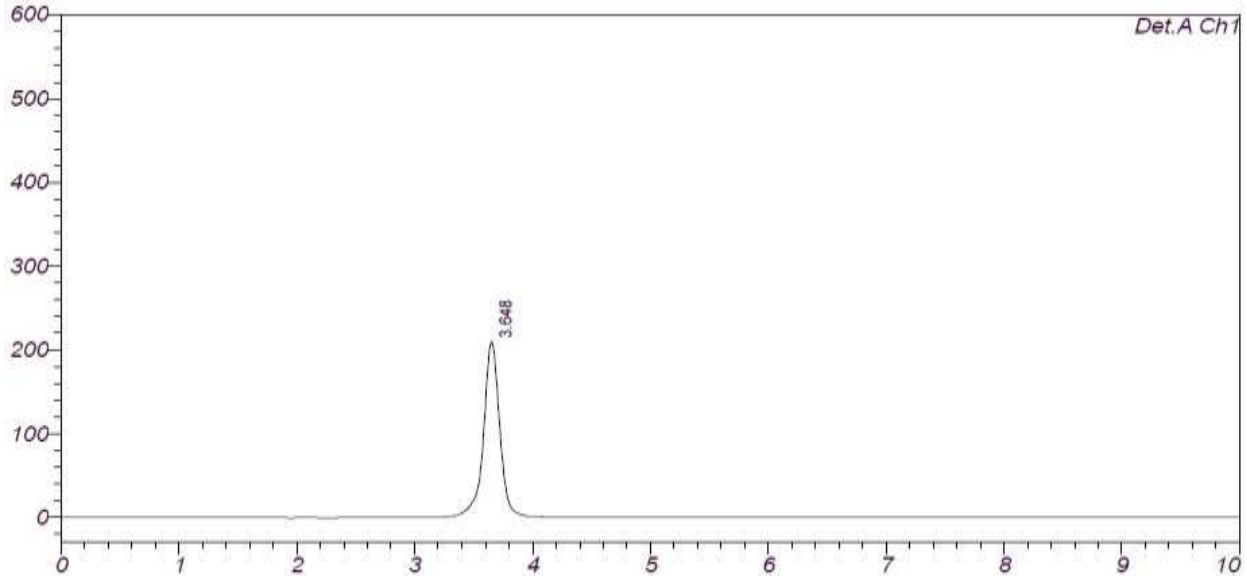

Figure 9: Chromatogram of Linearity at 40 µg/ml

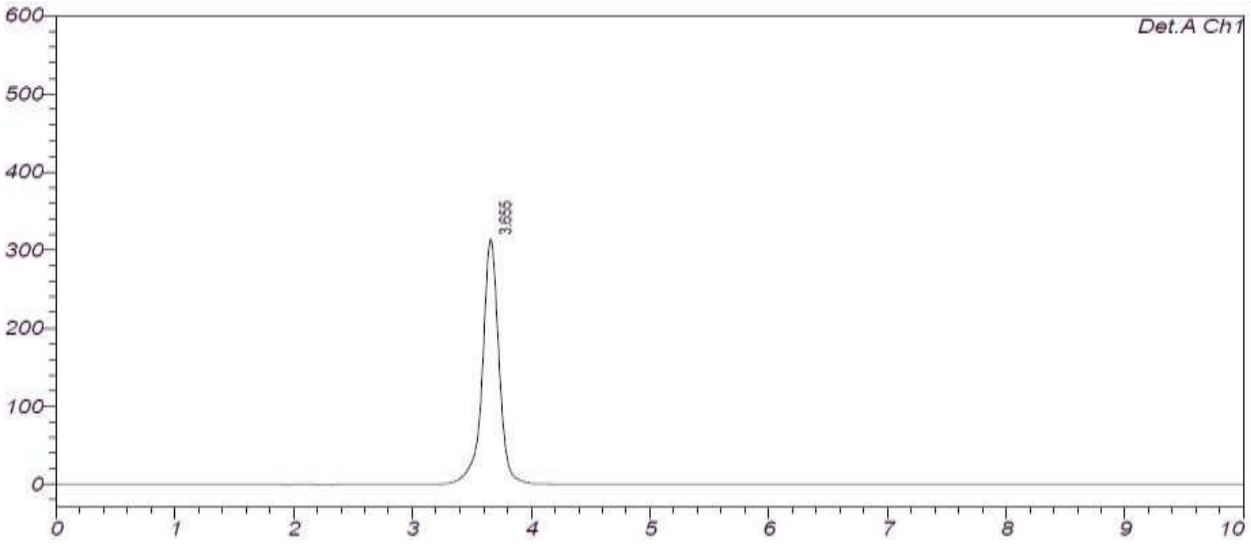

Figure 10: Chromatogram of Linearity at 60 µg/ml

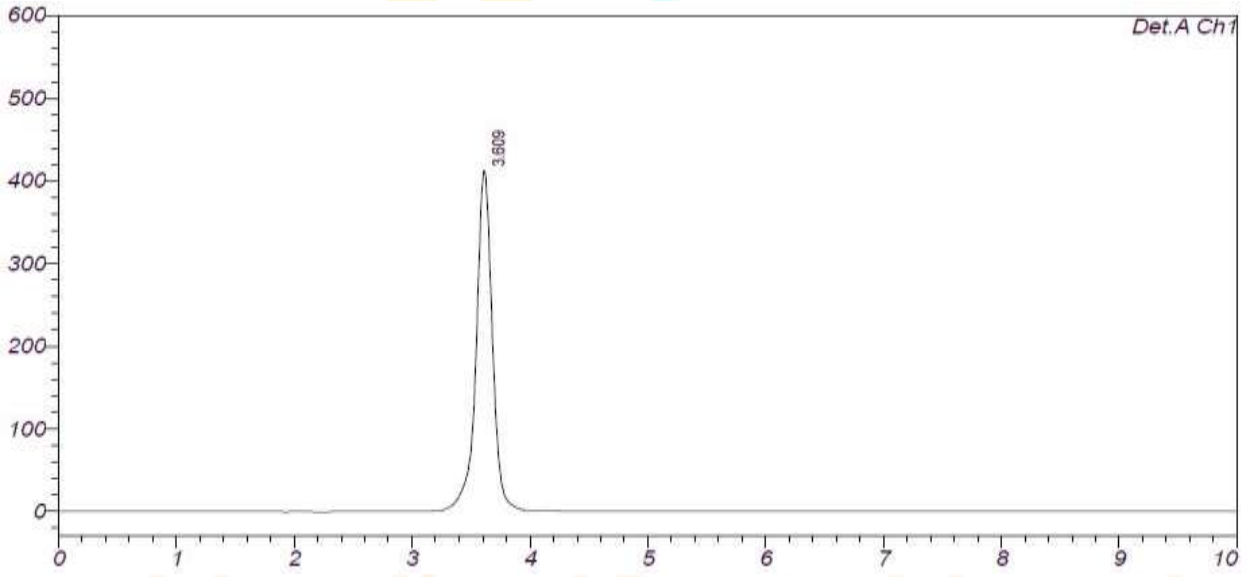

Figure 11: Chromatogram of Linearity at 80 µg/ml

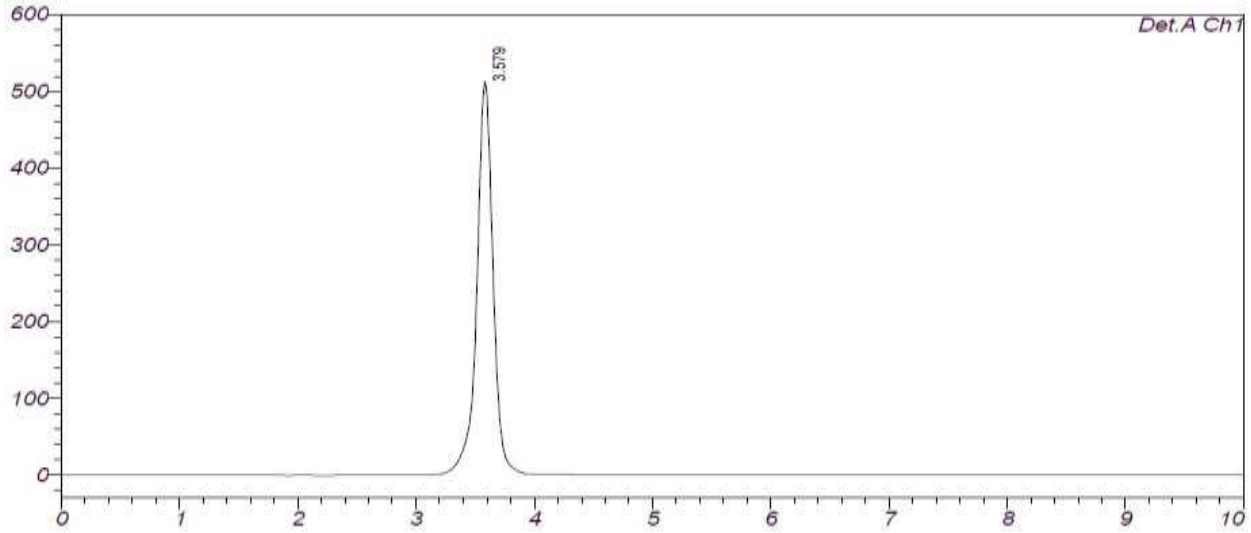

Figure 12: Chromatogram of Linearity at 100 µg/ml

Table 2: System suitability parameters

Parameter	Result
Retention Time (RT)	3.668
Theoretical plate number	25833.15
Tailing Factor	0.934
% Relative Standard Deviation (RSD)	1.01%

able 3: Precision study for proposed HPLC method (n=3)

Std.Conc (µg/ml).	Intraday precision			Interday precision		
	Amount found (%)	Peak area RSD (%)	Retention time RSD (%)	Amount found (%)	Peak area RSD (%)	Retention time RSD (%)
40	99.12	1.19	1.45	99.66	1.05	1.31
60	97.63	1.07	1.35	98.76	1.09	1.6
80	97.51	0.97	1.26	98.23	0.67	1.43

Table 4: Accuracy study for proposed HPLC method (n=3)

Spiked level (%)	Amount added (µg/ml)	Amount recovered (µg/ml)	Recovery (%)	Average (%)	SD	RSD (%)
80	48	48.79	101.64	101.27	0.324	0.32
	48	48.41	100.65			
	48	48.64	101.33			
100	60	60.09	100.15	99.54	0.498	0.50
	60	59.73	99.55			
	60	59.36	98.93			
120	72	71.81	99.73	99.33	0.438	0.44
	72	71.67	99.54			
	72	71.08	98.72			

Table 5: Robustness data of the proposed HPLC method (n=3)

Parameter	%RSD of peak area	%ASSAY
Wavelength (Normal 231nm)		
230nm	0.15	98.75
232nm	0.27	99.38
Flow Rate (Normal 1.0ml/min)		
0.9 ml/min	1.02	99.67
1.1 ml/min	0.51	98.58
pH of Mobile phase (Normal 3.2)		
3.1	0.24	99.48
3.3	0.09	99.68

Table 6: Standard solution stability (60 µg/ml)

Time(h)	%RSD of peak area	%RSD of RT	%ASSAY
0 (Initial)	0.92	0.06	99.15
8	0.09	0.05	98.69
20	0.21	0.04	98.83
26	0.04	0.02	98.66

Table 7: Method application results

Formulation	Label claim (mg)	Amount of drug(mg)	% Assay
Fabiflu tablet	400	396.24	99.06

4. Conclusion

In the current research, a simple, rapid and sensitive analytical method was developed and validated for the estimation of favipiravir and the developed method was validated in accordance with ICH guidelines. The result of recovery study was satisfactory and show that there is no interference of excipients in tablet dosage form. The mobile phase is simple to prepare and economical and drug is also stable for long period of time. In addition, the main features of the developed method are short run time and retention time around 3.6 ±0.25 min. The method is robust enough to reproduce accurate, reliable, precise results and cost effective. Also the % assay was determined on developed, it found within limit, hence it can be useful for routine quality control analysis of favipiravir.

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