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RP-HPLC Method for Determination of Favipiravir (RdRp of RNA Viruses) in Pharmaceutical Dosage Form

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Abstract

A simple, sensitive, precise, accurate, rapid, and reproducible HPLC method was developed and validated for the determination of Favipiravir (RdRp of RNA viruses) in pharmaceutical dosage form. Good quality chromatographic separation of Favipiravir (FAV) was conceded out by using Nucleosil C_{18} column (4.6 mm i.d., X 250 mm., 5 µm particle size) (based on 99.999 % ultra-high purity silica) using mobile phase consisting acetonitrile: methanol: HPLC Grade water (50:40:10 % v/v) at flow rate of 1 ml/minute. The λmax of the Favipiravir was found to be 365 nm. The retention time of Favipiravir was found to be 2.794 min and the calibration curve was linear function of drug in the conc. range of 10-50 µg/mL (r^2 = 0.9998). The LOD and LOQ were found to be 1.042995 µg/mL and 3.160591 µg/mL respectively. The recovery (accuracy) studies were performed and the percentage recovery was found to be 99.34 - 99.42 %. Percentage assay of Favipiravir tablets were found to be 99.85 %. Infact the % RSD values for all validation key parameters were less than 2 %. Thus, the developed method found to be fruitfully practicable for the determination of Favipiravir in quality control analysis in pharmaceutical formulations.

Keywords: RP - HPLC, Favipiravir, Validation, ICH guidelines.

Introduction

The chemical name for FAV is 6-fluoro-3-hydroxypyrazine-2-carboxamide. It has molecular formula $C_5H_4FN_3O_2$ and a molecular weight of 157.104 g/mol. FAV is an antiviral used to manage influenza, and that has the potential to target other viral infections (1). Literature Survey, it is revealed that the drug has been estimated by UV Spectroscopy (2) UPLC MS/MS (3), LC-MS/MS (4,5), HPLC (6-8) But only a few UV Spectroscopic methods and Liquid Chromatography analyses have been reported for the determination in pharmaceutical dosage forms. In general view, HPLC has been proven to be useful in diagnostic purposes and the pharmaceutical industry. The aim and Objective of the present work was to develop and validate a rapid, precise, and sensitive for FAV in pharmaceutical dosage form. The chemical structure of FAV is shown in (Figure 1).

Materials and Methods

Chemicals and Reagents

Sample of FAV was gifted from Hetero Labs Ltd., Hyderabad, India. HPLC grade Methanol, HPLC grade Acetonitrile, HPLC grade Water, AR grade Triethylamine, AR grade Sodium Hydroxide and AR grade ortho phosphoric acid were purchased from Merck specialties Pvt. Ltd., Mumbai, India and AR grade potassium dihydrogen phosphate purchased from Glaxo SmithKline Pharmaceuticals Ltd., Mumbai. Other excipients were prepared in our laboratory.

Instrumentation

For the detection of sample using UV, ELICO SL-210 spectrophotometer, having 1

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cm matched quartz cells, was used for all spectral and absorbance measurements, and solutions were prepared in methanol, acetonitrile and HPLC grade water (50:40:10). For HPLC, the chromatographic system used consists Agilent technologies-1260 series with G1311C Quat pump VL, Thermo scientific C_{18} column, 1260 series with G1511D DAD VL. having diode array detector was used for higher data quality for more confidence. The data that was acquired, was processed by utilizing EZ chrome elite software.

Method Development and Optimization of Chromatographic Conditions

For HPLC development, a variety of mobile phases containing HPLC grade water, acetonitrile, methanol in different ratios with or without buffers, and also various flow rates were performed. A good symmetrical peak was found when the mobile phase containing of a mixture of acetonitrile: methanol: HPLC grade water (50:40:10% v/v).

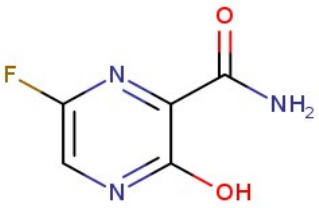

Fig 1. Chemical Structure of FAVIPIRAVIR

Selection of Detection Wavelength

The UV spectrum of diluted solutions of various concentrations of FAV in methanol was recorded by using UV spectrophotometer. The wavelength of maximum absorbance was scanned over a range of 200 - 400 nm and the UV overlay absorption spectrum is represented in (Figure 1a). The maximum absorbance was found at 365 nm.

Preparation of the Mobile Phase

Mobile phase consisting a mixture of acetonitrile: methanol: HPLC grade water (50:40:10 % v/v) was prepared and filtered through Whatman's filter paper and degassed by sonication.

Preparation of Standard Stock Solution

Exactly weighted 100 mg of FAV was transferred into 100 ml of volumetric flask, dissolved and diluted up to the mark with mobile phase to get stock solution containing

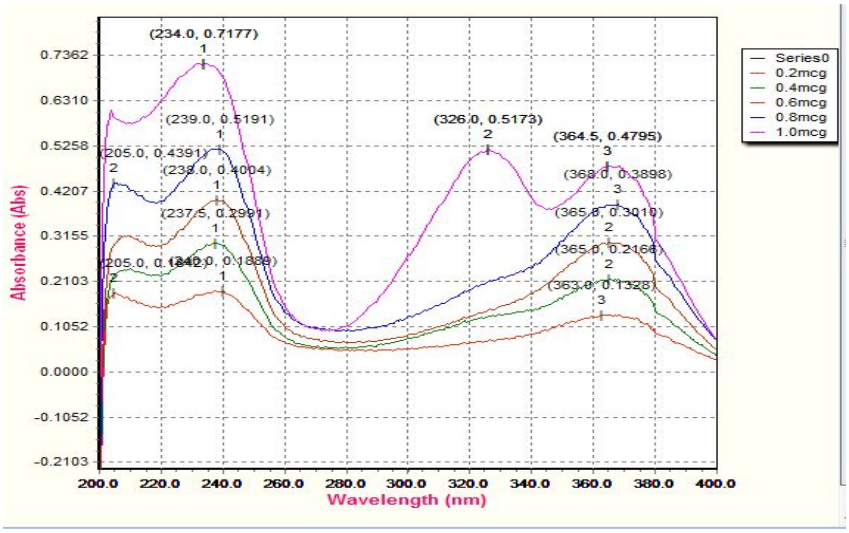

Fig 1a. Overlay Spectrum of FAV for UV Method

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1.0 mg/mL of FAV. Aliquots from stock solution were diluted with mobile phase to attain the calibration standard solutions over the range of 10, 20, 30, 40, 50 µg/mL FAV for high pressure liquid chromatographic method (n=5). As a matter of fact, the calibration curve was constructed by plotting the peak area on Y- axis and concentrations on X-axis.

Preparation of Sample Solution

For the assay twenty tablets containing FAV were precisely weighed and was shifted into a cleaned and dry mortar and ground to a fine powder and mixed homogenously. An exactly weighed amount of powder equivalent to 100 mg of FAV was taken and transferred into a 100 ml volumetric flask. The drug was extracted with mobile phase. The mixture was sonicated for 10 minutes and volume was filled up to mark and filtered through whatman filter paper and degassed by sonication. From this stock solution 4.0 ml was transferred to 100 ml volumetric flask and diluted with the mobile phase to obtain an intermediate solution of 40 µg/mL of FAV. Indeed, this solution was filtered through whatman filter paper, degassed by sonication and 20 µL was injected in chromatographic system. Infact sample solution was injected in to the developed high performance liquid chromatographic system and area of the peak was measured and percent assay was determined.

Method Development Optimization

The optimized HPLC conditions of several mobile phases with different compositions have been tested to develop an optimized chromatographic conditions like tailing factor, peak shape, and the number of theoretical plates as shown in (Table 1). For the selection of the mobile phase, primarily acetonitrile: methanol: HPLC grade water has been tested for different compositions. Eventually, the gradient mode and mobile

Table1. Optimized Chromatographic Conditions for FAV

Parameter	Chromatographic conditions
Instrument	Agilent SPD 20A prominence UV- Vis detector LC-20AT prominence liquid chromatograph, 1260 Quat Pump VL,1260 Diode Array Detector
Column	Nucleosil C18 column (4.6 mm i.d., X 250 mm., 5 µm particle size) (based on 99.999% ultra high purity silica)
Detector	1260 Diode Array Detector.
Mobile phase	ACN: MeOH: HPLC Grade Water (50: 40:10 v/v)
Flow rate	1 mL/minute
Detection wave length	UV at 365 nm
Run time	12 minutes
Temperature	Ambient temperature (25^{o}C)
Volume of injection loop	20 µL
Retention time (R_t)	2.794
Theoretical plates [th.pl] (Efficiency)	6051
Theoretical plates per meter [t.p/m]	12102
Tailing factor (asymmetry)	1.20

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phase containing a mixture acetonitrile: methanol: HPLC grade water (50:40:10% v/v) at a flow rate of 1 ml/ minute was found to be satisfactory and proper system suitability parameters obtained as shown in Table 2.

***Method Validation* (9,10,11,12,13)**

Validation is the process of establishing documented evidence, which provides a high degree of assurance, that a specific activity will consistently produce desired results or products, meeting its pre-determined specifications and quality characteristics. The method was validated as per ICH guidelines (14).

System Suitability

System suitability parameters can be defined as tests to ensure that the method can generate results of acceptable accuracy and precision. The requirements for system suitability are usually developed after method development and validation has been completed. The system suitability parameters like theoretical plates, retention time, tailing factor, were studied and found satisfactory.

Specificity

Specificity of the method was evaluated by assessing whether excipients and other additives that are usually present in pharmaceutical formulations of FAV do not interfered with the peaks of the analyte under optimum conditions as shown in Table 3.

Linearity

The linearity of the method was determined at five concentration levels ranging from 10-50 µg/ml for FAV. Evaluation of the drug was performed with a PDA detector at 365.0 nm peak area was recorded for all the peaks. The correlation coefficient value of FAV was found to be 0.9998 as shown in Table 4.

Accuracy (Recovery Studies)

The accuracy of the method was evaluated by standard addition method. Recovery tests were carried out by analyzing drug with different compositions. Known amounts of standard drugs were added to pre analyzed sample at three different levels 10%, 30%, 50 % and the mixed standard solutions were analyzed in triplicate at every level as per suggested

Table 2. System suitability parameters

System suitability parameters	FAV
Tailing factor (T)*	1.20
Number of theoretical plates	6051
Theoretical Plates per meter (N)*	1,21,020
Retention time*	2.794 minutes
Peak area*	702331
SD for peak area	4.324349662
% RSD for peak area	0.000615712
*Average of six determinations, SD = Standard deviation, RSD = relative standard deviation.	

Table 3. Results of Specificity Study for FAV

Name of the Solution	Retention Time (t_R) Minutes
Mobile phase (blank)	No interference at RT of analyte peak
Placebo	No interference at RT of analyte peak
FAV 50 µg/mL (sample)	2.793 minutes

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method. The accepted limits are 98 % - 105 % and all observed data are within the required range which indicates good recovery values and hence the accuracy of the method developed.

Precision

Method precision was determined by performing assay of sample under the tests of repeatability (Intraday precision) and intermediate precision (Interday precision) performed during three consecutive days by three different analysts, at different working concentrations. The percent relative standard deviation (% RSD) was calculated which is within the acceptable criteria of not more than 2.0.

Robustness

The robustness of an analytical procedure is the measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. For the determination of a method's robustness, parameters such as variation in detector wavelength are varied within a realistic range and the quantitative influence of the variables is determined. If the influence of the parameter is within a previously specified tolerance, the parameter is said to be within the method's robustness range. The absorbance was

Table 4. Linearity Data of FAV by HPLC

S. No	Concentration (µg/m)	Retention Time (RT) Minutes	Peak Area, mV.s.
1	0	-	0
2	10	2.793	147397
3	20	2.793	281672
4	30	2.794	429690
5	40	2.793	69493
6	50	2.793	702331

Table 4a. The Summary Output of ANOVA Study of FAV

SUMMARY OUTPUT								
Regression Statistics								
Multiple R	0.99985							
R Square	0.9997							
Adjusted R Squ	0.99959							
Standard Error	4448.22							
Observations	5							
ANOVA								
	df	SS	MS	F	Significance F			
Regression	1	1.95E+11	1.95E+11	9873.01	2.25E-06			
Residual	3	5.9E+07	2E+07					
Total	4	1.95E+11						
	Coefficien	Standard	t Stat	P-value	Lower 95%	Upper 95%	Lower 95.	Upper 95.0%
Intercept	6809.9	4665.33	1.45968	0.24049	-8037.254	21657.05	-8037.3	21657.1
X Variable 1	13976.9	140.665	99.363	2.25E-06	13529.231	14424.55	13529.2	14424.5

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measured and assay was calculated as shown in Table 5.

Linearity and Range

A graph of peak area versus concentration (µg/mL) were plotted for FAV at concentration range between 10-50 µg/ml. The linear regression equation and correlation coefficient (r2) were y = 14074x + 3242.8 and 0.9998 respectively.

LOD and LOQ

LOD

Limit of Detection is the lowest concentration in a sample that can be detected, but not necessarily quantified under the stated experimental conditions.

LOQ

The limit of quantitation is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy.

Analysis FAV in Tablet Formulation

FAV standard solution and FAV sample solution were prepared. The samples were analyzed using the developed chromatographic method and the % content of the FAV in the tablets was estimated. The % content of the FAV was calculated using the following formula.

AT/AS x WS/25 x 9/100 x 250/5 x 100/5 x P/100 x 100/LC

Where, AT = Peak area response of FAV in the chromatogram obtained from the test solution.
AS = Average peak area response of FAV in the chromatograms obtained from replicate injections of standard solution.
WS = Weight of FAV standard taken in mg in standard solution.
P = % Purity of FAV standard.
LC = Label claim of FAV in mg per tablet.

Results and Discussion

The objective of the proposed work was to develop some new, sensitive and

Table 5. Robustness Results of FAV

S No	Parameter	optimized	Used	Retention time (t_R), min	Plate count$^{\$}$	Peak asymmetry$^{\#}$	Remark
1.	Change in flow rate (±0.2 mL/minute)	1.0 mL/min	0.8 mL/min	2.896	9,054	1.20	*Robust
			1.0 mL/min	2.794	8,998	1.21	*Robust
			1.2 mL/min	2.673	8,834	1.3	*Robust
2.	Detection wavelength (±1 nm)	365 nm	364 nm	2.794	8,790	1.1	Robust
			365 nm	2.794	8,998	1.6	Robust
			366 nm	2.794	8,890	1.2	Robust
3.	Mobile phase composition (Acetonitrile :Methanol:HPLC Grade water)	50:40:10 v/v	45:45:10 v/v	2.684	8,634	1.8	*Robust
			50:40:10 v/v	2.794	8,998	1.4	*Robust
			40:40:20 v/v	2.854	8,713	1.22	*Robust
Acceptance criteria (Limits): $^{\#}$Peak Asymmetr (AS) < 1.5, $^{\$}$ Plate count (PC) > 2000, * Significant change in Retention time							

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selective analytical method for the determination of FAV and to validate the developed method according to the ICH guidelines and applying the same for its estimation in marketed formulation. FAV is a purine analogue and is incorporated in place of guanine, adenine and inhibits viral replication. Literature survey reveals that very few analytical methods have been reported for the determination of FAV in bulk and pharmaceutical dosage form. Hence, on the basis of literature survey it was thought to develop a rapid, precise, accurate, simple, specific and reliable RP-HPLC methods for routine analysis of FAV in bulk and pharmaceutical dosage forms. In HPLC method, the conditions were optimized to obtain an adequate separation of eluted compounds. Initially, various mobile phase compositions were tried to separate title ingredients. The objective of this study was to develop a rapid and sensitive RP-HPLC method for the analysis of FAV in bulk drug and pharmaceutical dosage form by using the most commonly employed C_{18} column with UV-detection. Initially, various mobile phase compositions were tried (numerous trials were performed) to elute the drug. From all the trials finally awesome reproducibility results, good peak shape, minimal peak tailing and short runtime were identified when mobile consisting of Acetonitrile: Methanol: HPLC Grade water in the proportion of (50:40:10 v/v) and 1 mL/minute flow rate was selected. The retention time for FAV was found to be 2.794 min. The calibration was linear in the concentration range of 10 – 50 µg/mL for FAV. Calibration graph of FAV is shown in Fig 2, and standard chromatograms of FAV are shown in Fig 3 – 3d. Specificity of the chromatographic method was tested by injecting sample concentration prepared from marketed formulation. The response was compared with that obtained from the standard drug. The chromatogram confirms the presence of FAV at 2.794 minutes without any interference. Precision study was determined to find out intra-day and inter-day variation in the test methods of FAV for 3 times on the same day. The % RSD of the intra-day and inter day precision obtained was <2 which indicates that the proposed method is precise. Method validation following ICH guidelines indicated that the developed method had high sensitivity with LOD of1.0429 µg/mL and LOQ of 3.1605 µg/mL. The robustness of an analytical method was determined by analysis of aliquots from homogenous lots by differing physical parameters such as change in flow

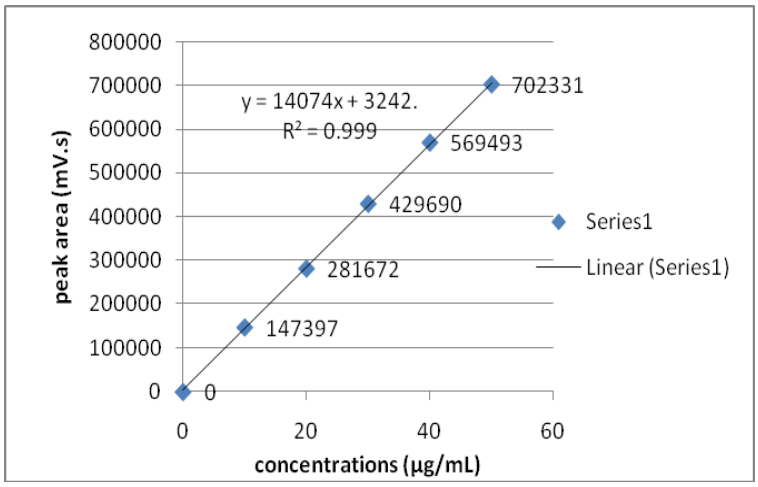

Fig 2. Calibration Graph of FAV by RP- HPLC

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rate to 1.0 ± 0.2 mL and changing detection wavelength 365 nm ± 1 nm and mobile phase composition change to 50:40:10 v/v ± 5 v/v. The obtained values were given in Table 6. These values indicated that the method was quite robust. The proposed method was validated in accordance with ICH parameters and was applied for analysis of the same in

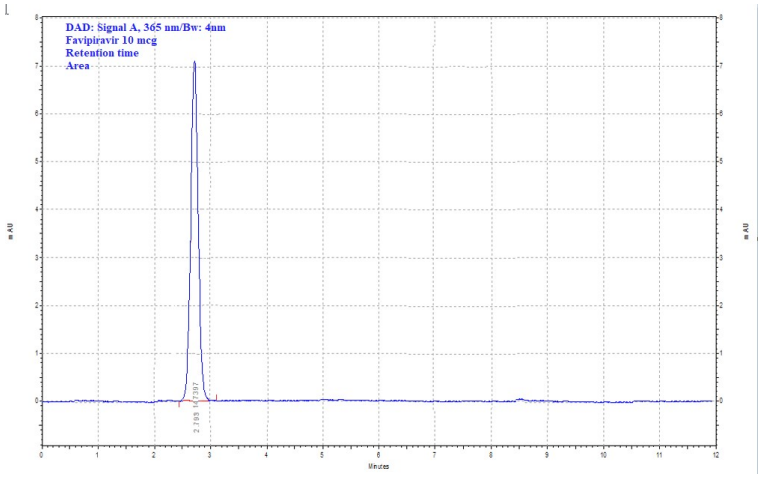

Fig 3. Standard Chromatogram of FAV (10 µg/mL)

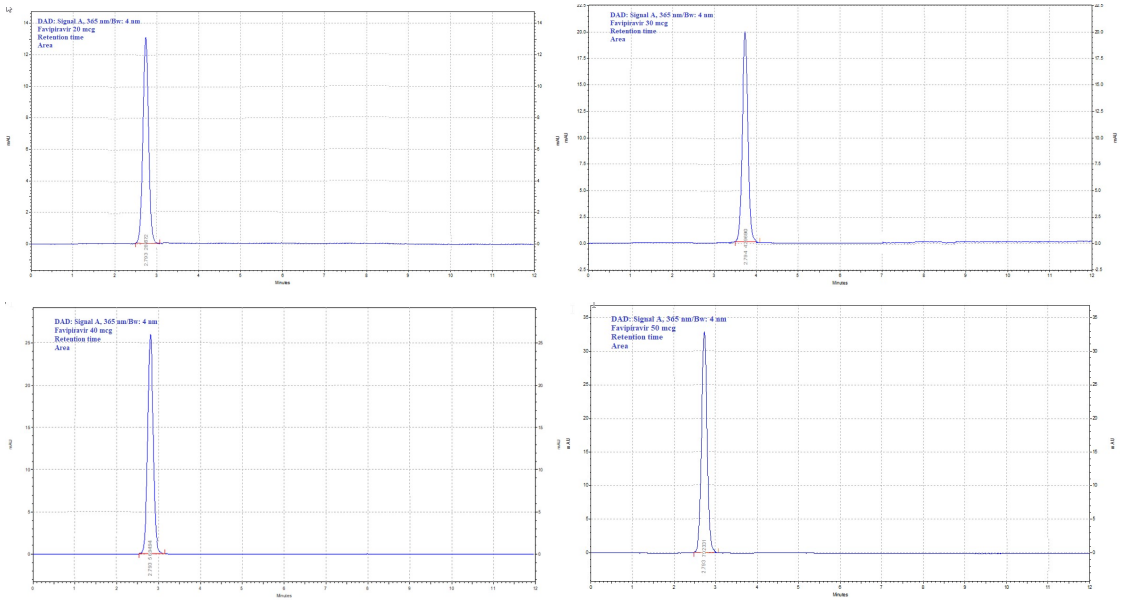

Fig 3. Standard Chromatogram of FAV (A) (20 µg/mL), (B) (30 µg/mL), (C) (40 µg/mL), (50 µg/mL)

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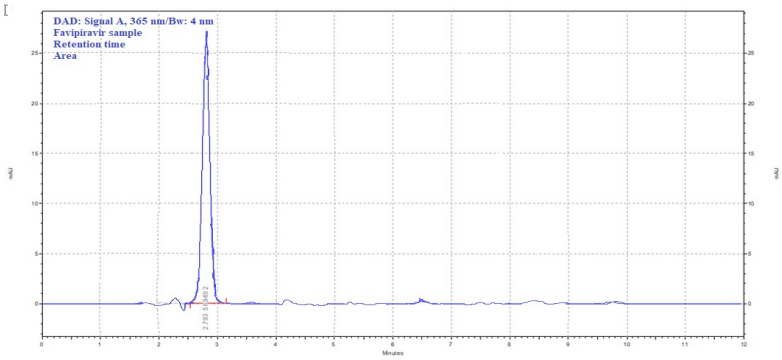

Fig 4. Sample Chromatogram of FAV

marketed formulations. The content of each component in the formulation was estimated by comparing the peak area of the test sample with that of the peak area of the standard which were found to be 99.85 % w/w for FAV sample chromatogram representing marketed formulation was shown in Fig 4. The development of an analytical method for the determination of drugs by Reverse Phase HPLC has received considerable attention in recent years because of their importance in quality control of drugs and drug products. Hence, the developed RP-HPLC methods can be adopted for the routine analysis of FAV in bulk and pharmaceutical dosage forms in quality control laboratories.

Conclusion

The present study demonstrated validated Reverse Phase High Performance Liquid Chromatography method for the determination of FAV available as tablet dosage form. The method was completely validated and showed satisfactory results. The method was free from interference of the other active ingredients and additives used in the formulation. The RP-HPLC method for the determination of FAV has various advantages like low solvent consumption, less retention time, excellent peak symmetry, highly sensitive, precise, accurate and robust. In fact, results of the study indicates that the developed methods were found to be accurate, precise, linear, sensitive, simple, economical, reproducible have short run time which makes the method rapid. Hence it can be concluded that this method may be employed for the routine quality control analysis of FAV in active pharmaceutical dosage forms.

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