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Novel analytical method development and validation for the quantitative analysis of Efavirenz in bulk and pharmaceutical dosage forms by RP-HPLC

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Abstract
A convenient, simple, specific, accurate, precise, rapid, inexpensive isocratic Reversed Phase-High Performance Liquid Chromatography (RP-HPLC) method was developed and validated for the quantitative determination of Efavirenz in pharmaceutical tablet dosage forms. RP-HPLC method was developed by using Welchrom C_{18} Column (4.6 X 250 mm, 5µm), Shimadzu LC-20AT Prominence Liquid Chromatograph. The mobile phase composed of 10 mM Phosphate buffer (pH-3.0, adjusted with triethylamine): acetonitrile (50:50 v/v). The flow rate was set to 1.2 $mL.min^{-1}$ with the responses measured at 246 nm using Shimadzu SPD-20A Prominence UV-Vis detector. The retention time of Efavirenz was found to be 9.563 minutes. Linearity was established for Efavirenz in the range of 2-10 $\mu g.mL^{-1}$ with correlation coefficient 0.9999. The LOD and the LOQ were found to be 0.0183 $\mu g.mL^{-1}$ and 0.0555 $\mu g.mL^{-1}$ respectively. The amount of Efavirenz present in the formulation was found to be 99.82%. The validation of the developed method was carried out for specificity, linearity, precision, accuracy, robustness, limit of detection, limit of quantitation. The developed method can be used for routine quality control analysis of Efavirenz in pharmaceutical tablet dosage form.

Keywords: Efavirenz, Isocratic RP-HPLC, Method Validation, Quality control.

1. Introduction
Efavirenz (EFA) is chemically (4S)-6-chloro-4-(2-cyclopropylethynyl)-4-(trifluoromethyl)-1H-3, 1-benzoxazin-2-one (Fig. 1). It is a white powder form and used as antiretroviral agent, for the treatment of HIV infection. It has an empirical formula of $C_{14}H_9ClF_3NO_2$ and molecular weight of 315.6750. Efavirenz belongs to a class of antiretroviral drugs known as non-nucleoside reverse transcriptase inhibitor (NNRTI) and is used as part of highly active antiretroviral therapy (HAART) for the treatment of a human immunodeficiency virus (HIV) type-1. It is practically insoluble in water and soluble in methanol, acetonitrile, NaOH, HCl. The drug is also used in combination with other anti-retroviral agents for the treatment of HIV-1 infection in children and adults. The trade name of EFA is Efavir (CIPLA), and the usual dose of EFA in film coated tablet is 600 mg/day and a capsule is 200 mg. Some example for combinations of EFA is lazid-E (EMCURE), Zidovudine 300 mg, Lamivudine 150 mg, Efavirenz 600 mg. Both nucleoside and non-nucleoside RTIs inhibit the same target, the reverse transcriptase enzyme, an essential viral enzyme which transcribes viral RNA into DNA. Unlike nucleoside RTIs, which bind at the enzyme's active site, NNRTIs act allosterically by binding to a distinct site away from the active site known as the NNRTI pocket. EFA is not effective against HIV-2, as the pocket of the HIV-2 reverse transcriptase has a different structure, which confers intrinsic resistance to the NNRTI class. However, more than 50% of patients starting EFA treatment experience its related neuropsychiatric adverse events (NPAEs), such as dizziness, feeling of drunkenness and sleep disorders and even severe psychiatric symptoms have been reported by EFA.
The literature survey reveals that there are some analytical methods of EFA were reported, including UV spectrophotometry [1-5], HPLC [5-10]. However, the requirement of very simple, quick, economical and reliable analytical method for quality control purpose always necessities to see a novel and better methods. In addition to that the reported RP-HPLC methods suffer from the low sensitivity. Thus the present study has aimed to develop simple, sensitive, and economical, efficient method that could estimate EFA in pure drug and in tablet formulations. Chemical structure of EFZ is shown in Figure 1.

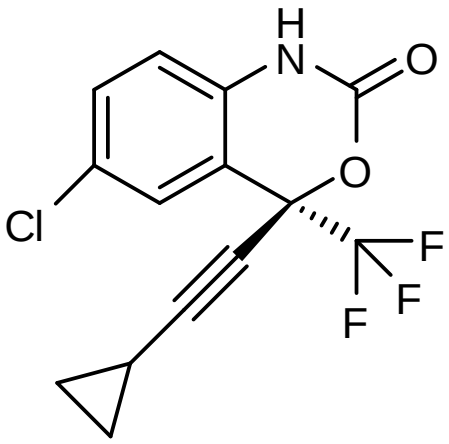

Fig 1: Chemical structure of Efavirenz

2. Materials and methods

2.1. Chemicals and Reagents

The reference sample of EFA standard was kindly supplied as gift sample by Hetero Drugs Ltd., Hyderabad, Andhra Pradesh, India. All the chemicals were analytical grade. Potassium dihydrogen orthophosphate and phosphoric acid from Rankem Ltd., Mumbai, India, while acetonitrile (HPLC grade) and triethylamine (HPLC grade) from Merck Pharmaceuticals Private Ltd., Mumbai, India. Ortho phosphoric acid used to be of HPLC grade and purchased from Merck Specialties Private Ltd., Mumbai, India. Commercial tablets of EFA formulation were procured from local market.

2.2. Instrumentation

Quantitative HPLC was performed on in isocratic high performance liquid chromatography (Shimadzu LC-20AT Prominence Liquid Chromatograph) with a LC-20AT VP pump, manual injector with loop volume of 20 µL (Rheodyne), programmable variable wavelength Shimadzu SPD-20A Prominence UV-Vis detector and Welchrom C_{18} Column (4.6 X 250 mm, 5µm particle size). The HPLC system was equipped with "Spinchrome" software. In addition an electronic balance (Shimadzu TX223L), digital pH meter (Systronics model 802), a sonicator (spectra lab, model UCB 40), UV-Visible Spectrophotometer (Systronics model-2203) were used in this study. To attain accuracy and reliability of the results calibrated glassware (Borosil, India) was used.

2.3. Chromatographic conditions

EFA was analyzed by various reversed phase columns like C_8 and C_{18} columns. Among C_8 and C_{18} columns, C_{18} (250 mm X 4.6 mm, 5 µm) column was selected. Various combinations of acetonitrile, phosphate buffer and methanol with triethylamine as column modifier were tested. The mixture of 10 mM Phosphate buffer (pH adjusted to 3.0 using triethylamine) and Acetonitrile in ratio of 50:50 v/v was selected as mobile phase and UV detection wavelength was 246 nm with a flow rate of 1.2 $mL.min^{-1}$. Injection volume was 20 µL, with ambient temperature, run time was 12 minutes and retention time was 9.563 minutes.

2.4. Preparation of mobile phase

A 10mM Phosphate buffer was prepared by dissolving 6.056 g of potassium dihydrogen orthophosphate in 445 mL of HPLC grade water. To this 55 mL of 0.1M phosphoric acid was added and pH was adjusted to 3.0 with triethylamine. The above prepared buffer and acetonitrile were mixed in the proportion of 50:50 v/v and was filtered through 0.45 µm nylon membrane filter and degassed by sonication.

2.5. Preparation of Standard solution

About 10 mg of pure EFA was accurately weighed and dissolved in 10 mL of mobile phase to get 1 $mg.mL^{-1}$ stock solution. Working standard solution of EFA was prepared with the mobile phase. The final volume was made with the mobile phase. The standard solution was filtered through 0.45 µm nylon membrane filter and degassed by sonication.

2.6. Preparation of test Sample solution

The content of 10 tablets of (Efavir) was accurately weighed and transferred into a mortar and finely powdered. Then average weight was determined. From this, tablet powder which is equivalent to 100 mg of EFA was taken and transferred in to 100 ml of volumetric flask and the drug was extracted in 70 mL of mobile phase. After filling the volume to the mark with the mobile phase and resulting solution was filtered through 0.45µm nylon filter and degassed by sonication. Eventually this solution was further suitably diluted to the desired concentrations for chromatography.

2.7. Selection of analytical wavelength

For the selection of analytical wavelength, 10 µg/mL EFA standard solution was prepared with the mobile phase and UV spectrum was recorded by scanning the in the UV range of 200 to 400 nm using diluents as a blank. The results showed that the absorption maximum from the spectrum (λmax) of EFA was found to be 246 nm and the detector was selected at this wavelength for monitoring the eluents for analysis.

2.8. Calibration curve for Efavirenz

Replicates of each calibration standard solutions (2, 4, 6, 8, 10 $\mu g.mL^{-1}$) were injected using a 20 µL fixed loop system and the chromatograms were recorded. Calibration curves were constructed by plotting concentration of EFA on X-axis and peak areas of standard EFA on Y-axis and regression equations were computed for EFA.

2.9. Optimization and method development

In order to produce an optimized method for separation utilizing RP-HPLC several trials were carried out by varying the commonly used solvents, their compositions and flow rate and stationary phases. Mixtures of commonly utilized solvents like water, methanol and acetonitrile with or without different buffers in different combinations were tested as mobile phases on a C_{18} stationary phase. This was undertaken by varying one parameter at a time and keeping all other conditions constant which results better resolution, short run time, minimal peak tailing and good reproducibility of the results. Eventually an analytical column of Welchrome C_{18} (250 mm X 4.6 mm, 5µm) column and a mobile phase containing of a mixture of 10 mM Phosphate buffer (pH adjusted to 3.0) and acetonitrile in ratio of 50:50 v/v were found to be most suitable for analysis of EFA. A mobile phase flow rate and detection wavelength adjusted to 1.2 mL/minute, 246 nm, respectively. Injection volume was 20 µL and with ambient temperature were also proved to be suitable in the present study. Optimized chromatographic conditions for the development method are shown in Table 1.

Table 1: Optimized chromatographic conditions for proposed method

Parameter	Chromatographic conditions
Instrument	SHIMADZU LC-20AT prominence liquid chromatograph
Column	WELCHROM C_{18} Column (4.6 X 250 mm, 5 µm)
Detector	SHIMADZU SPD-20A prominence UV-Vis detector
Diluents	10 mM Phosphate Buffer(pH-3): Acetonitrile (50:50 v/v)
Mobile phase	10 mM Phosphate Buffer(pH-3): Acetonitrile (50 : 50 v/v)
Flow rate	1.2 $mL.min^{-1}$.
Detection wave length	By UV at 246 nm.
Run time	12 minutes
Temperature	Ambient temperature (25ºC)
Volume of injection loop	20 µL

3. Validation of a proposed method

The developed method of analysis was validated as per the ICH Q2 (R1) guide lines[11] for the parameters like system suitability, specificity, linearity, precision, accuracy, robustness, and system suitability, limit of detection (LOD) and limit of quantitation (LOQ).

3.1. System suitability

System suitability tests are an integral part of chromatographic method which was used to verify reproducibility of the chromatographic system. To ascertain its effectiveness, certain system suitability test parameters were checked by repetitively injecting the drug solution at the concentration level 10 µg mL^{-1} for EFA to check the reproducibility of the system.

At first the HPLC system was stabilized for 40 minutes. One blank followed by six replicates of a single calibration standard solution of EFA was injected to check the system suitability. To ascertain the systems suitability for the proposed method, the parameters such as theoretical plates, peak asymmetry, retention time and parameters were taken and results are presented in Table 2.

Table 2: System suitability test parameters for Efavirenz

Parameters	Result
Retention time (R_t)	9.563
Theoretical plates [th.pl] (Efficiency)	22869
Theoretical plates per meter [t.p/m]	457389
Tailing factor (asymmetry factor)	1.025

3.2. Specificity

The method specificity was assessed by comparing the chromatograms obtained from the synthetic drug mixture and blank solution. In fact the blank solution was prepared by mixing the most commonly used excipients such as lactose anhydrous, microcrystalline cellulose and magnesium stearate have been added to the placebo solution. The effect of wide range of excipients and other additives usually present in the formulations of EFA in the determinations under optimum conditions was investigated. The drug to excipients ratio used was similar to that in the commercial formulations. The specificity of the RP-HPLC method was established by injecting the mobile phase and placebo solution in triplicate and recording the chromatograms. The representative chromatogram of placebo is shown in Fig. 2. The specificity results are presented in Table 3.

Table 3: Specificity study

Name of the solution	Retention time (R_t) min.
Mobile phase	No peaks
Placebo	No peaks
Efavirenz 10 $\mu g.mL^{-1}$	9.563 min.

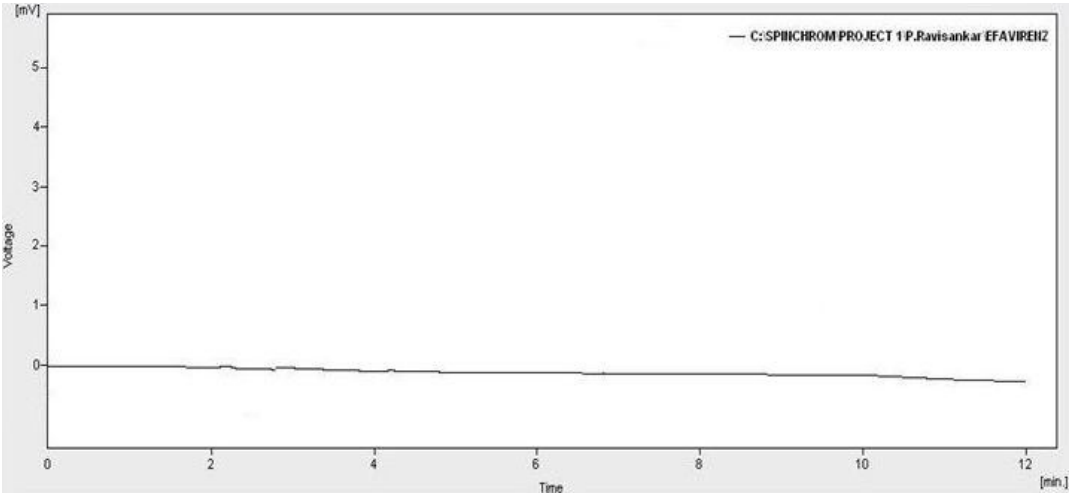

Fig 2: Chromatogram of placebo.

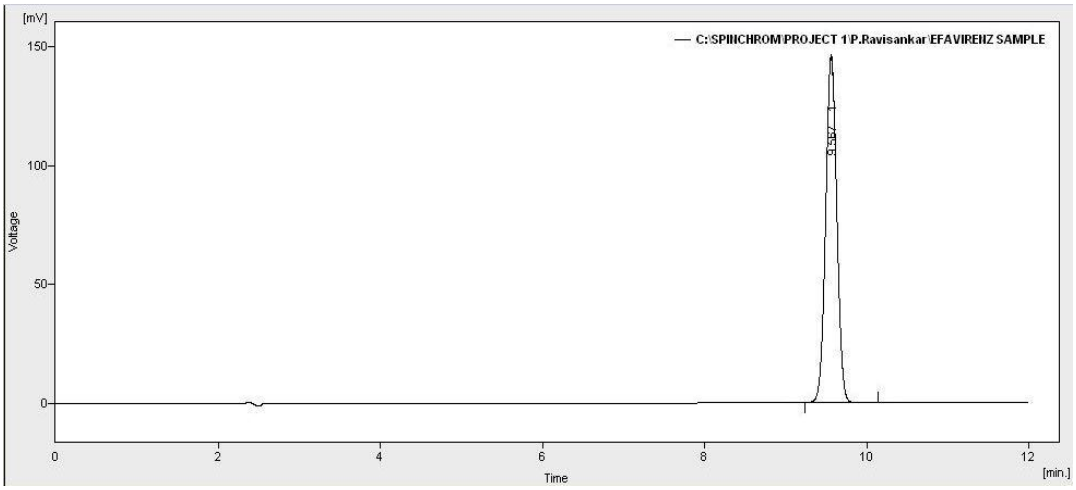

Fig 3: A typical chromatogram of Synthetic drug

3.3. Linearity

The linearity graphs for the proposed assay methods were obtained over the concentration range of 2-10 µg.mL^{-1} of EFA. Method of least square analysis was carried out for getting the slope, intercept and correlation coefficient, regression data and linearity data values and the results are presented in Table 4 and Table 5. The representative chromatograms indicating the EFA are shown in Fig. 4 to 8. A calibration curve was plotted between concentration and area response and statistical analysis of the calibration curve is shown in Fig. 9.

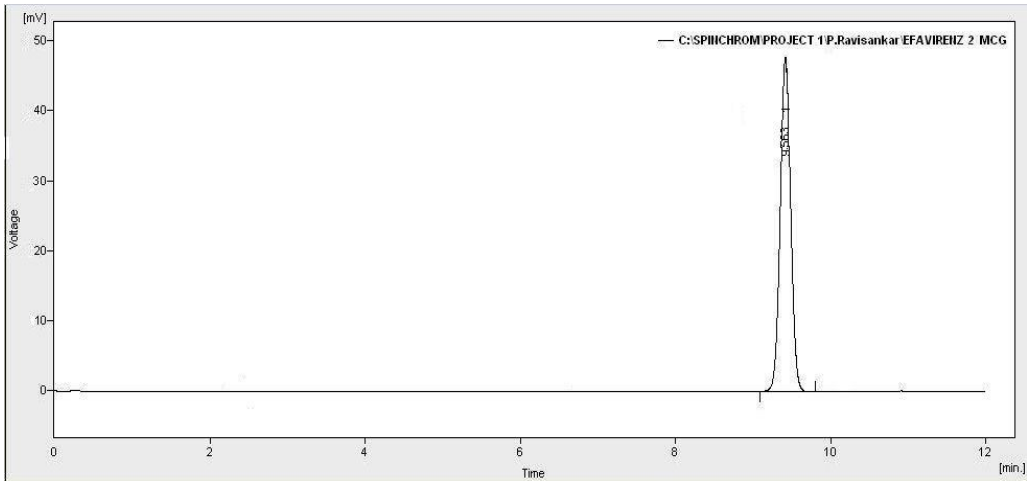

Fig 4: Standard chromatogram of Efavirenz (2 µg.mL^{-1}).

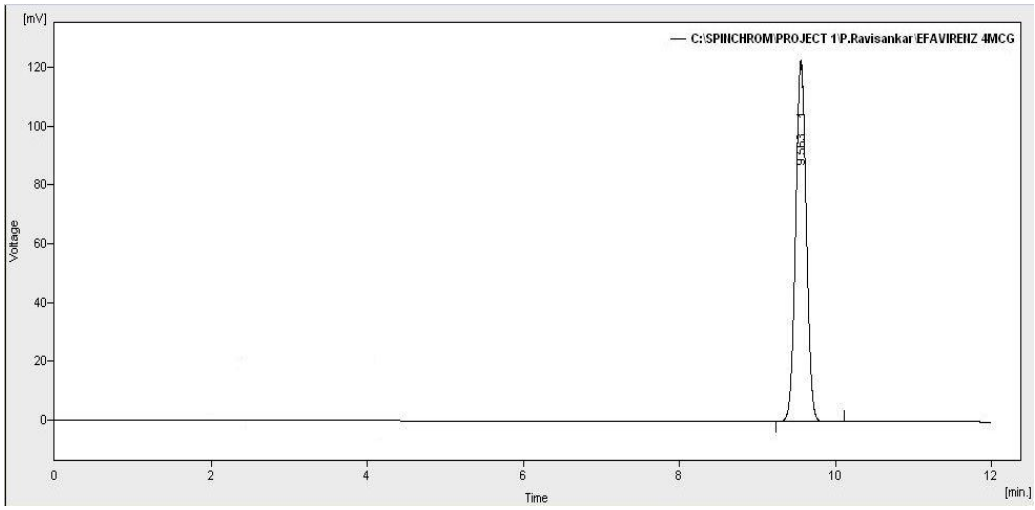

Fig 5: Standard chromatogram of Efavirenz (4 µg.mL^{-1}).

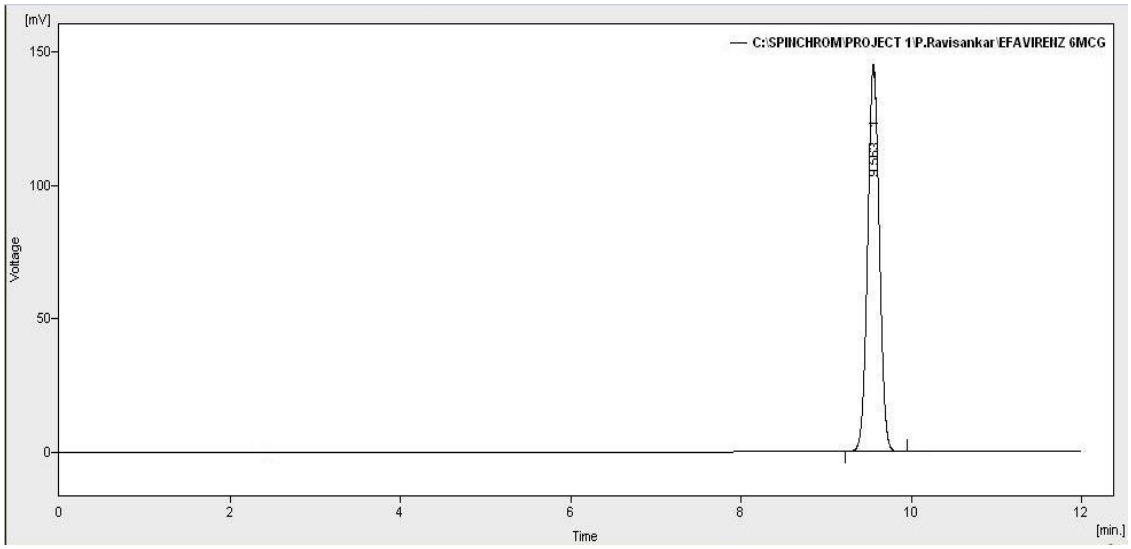

Fig 6: Standard chromatogram of Efavirenz (6 µg.mL^{-1}).

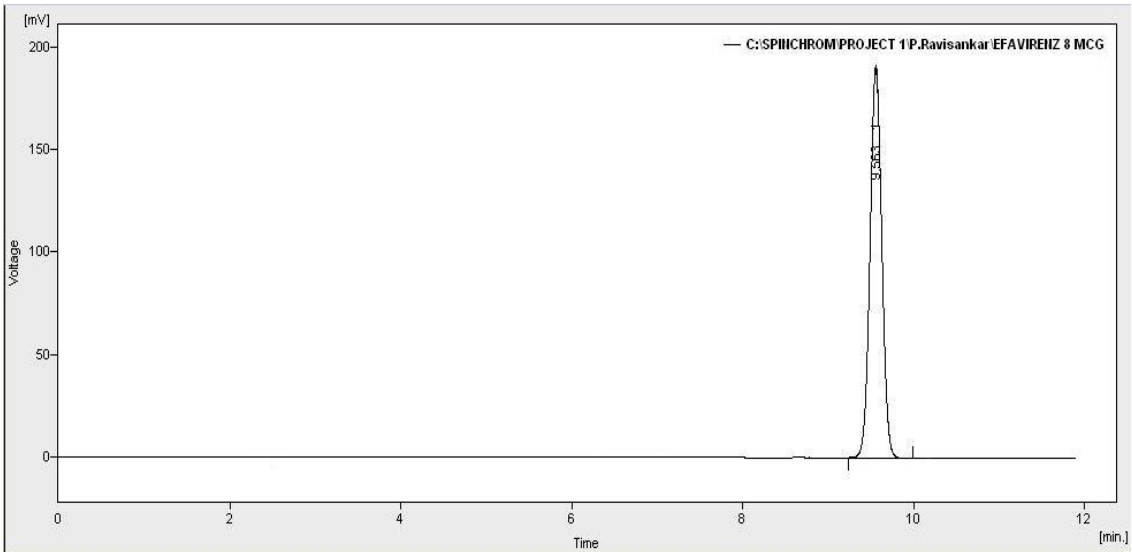

Fig 7: Standard chromatogram of Efavirenz (8 µg.mL^{-1}).

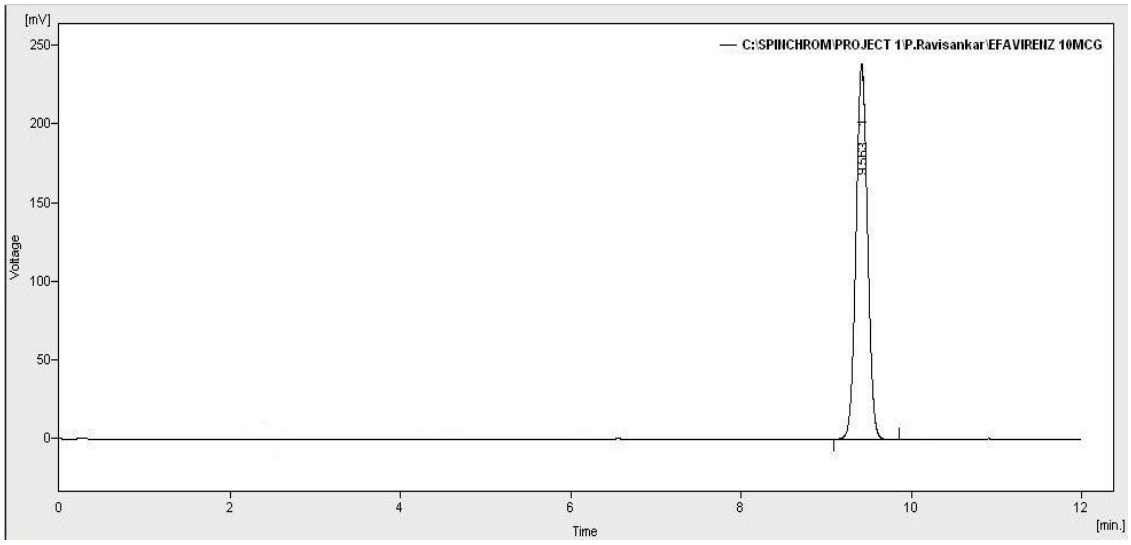

Fig 8: Standard chromatogram of Efavirenz (10 µg.mL^{-1}).

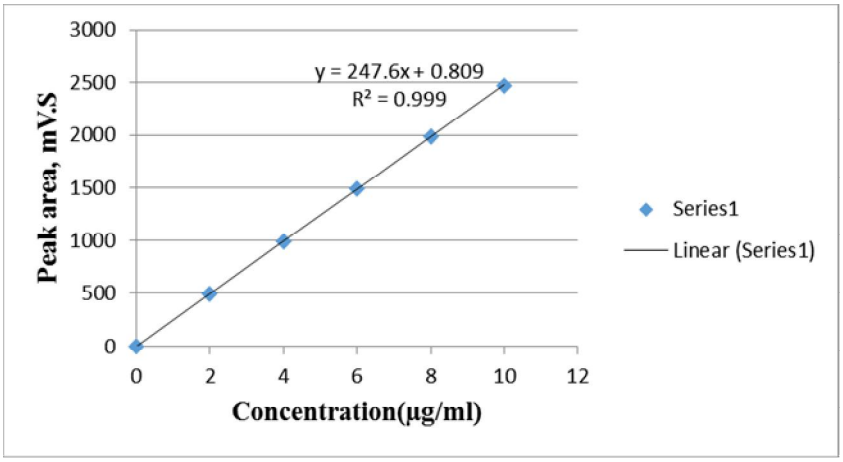

Fig 9: Calibration plot of Efaviranz.

Table 4: Linear regression data of the proposed HPLC method of Efavirenz

Parameter	Method
Detection wavelength(λ max)	By UV at 246nm
Linearity range (µg/ml)	2-10 $\mu g.mL^{-1}$
Regression equation (Y=a+bx)	Y= 247.6 x + 0.809
Slope(b)	247.6
Intercept(a)	0.809
Standard deviation of slope (S_b)	0.2273
Standard deviation of intercept (S_a)	1.3768
Standard error of estimation (Se)	1.9023
Correlation coefficient (r^2)	0.9999
% Relative standard deviation* i.e., Coefficient of variation(CV)	0.98
Percentage range of errors* (Confidence limits) 0.05 significance level 0.01 significance level	0.2745 0.3608

*Average of 6 determinations; Acceptance criteria < 2.0.**3.4.**

Table 5: Linearity data of Efavirenz

S.No	Concentration, $\mu g.mL^{-1}$.	Retention time, (R_t) min.	Peak area, mV.s.
1	0	-	0
2	2	9.563	496.183
3	4	9.563	991.366
4	6	9.563	1487.549
5	8	9.563	1984.732
6	10	9.563	2480.619

Precision

Intra-day and inter-day precision study of EFA was carried out by estimating corresponding responses 3 times on the same day and on 3 different days in the concentration of 6 µg/mL. The percent relative standard deviation (% RSD) was calculated which is within the acceptable criteria of not more than 2.0. The results for intra-day and inter-day precision are presented in Table 6 and Table 7 respectively.

Table 6: Results of Precision study (Intra-day)

Sample	Concentration ($\mu g.mL^{-1}$)	Injection no.	Peak area	% RSD*
Efavirenz	6	1	1487.549	0.1067
		2	1483.542	
		3	1486.889	
		4	1487.264	
		5	1486.438	
		6	1484.742	

*acceptance criteria < 2.0.

Table 7: Results of Precision study (Inter-day)

Sample	Concentration ($\mu g.mL^{-1}$)	Injection no.	Peak area	%RSD*
Efavirenz	6	1	1487.549	0.1445
		2	1486.583	
		3	1482.481	
		4	1485.471	
		5	1484.952	
		6	1482.253	

*acceptance criteria < 2.0.

3.5. Accuracy (Recovery studies)

The accuracy of the method was determined by calculating recovery of EFA by the method of addition. Known amount of EFA at 80 %, 100 % and 120 % was added to a pre analyzed sample solutions. The recovery studies were carried out in the tablet in triplicate each in the presence of placebo. The mean percentage recovery of EFA at each level was not less than 99 % and not more than 101 %. The results are presented in Table 8.

Table 8: Recovery data of the Efavirenz by RP-HPLC method

Recovery level	Amount taken (mg)	amount added (mg)	Total Amount (mg)	% recovery (mg)	Mean % Recovery	%RSD (n=3)
80 %	8	5	13	12.94	99.22	0.576
100 %	10	5	15	14.87	98.63	0.507
120 %	12	5	17	16.91	99.52	0.344

3.6. Robustness

The robustness of the developed method were determined by analyzing the samples under a variety of conditions of the method parameters such as a variation of the pH of the buffer, flow rate (± 0.2 mL/min), detection wavelength (± 5 nm) and mobile phase composition(± 0.2 mL/min). It was observed that there was no significant effect on chromatographic parameters which demonstrated that the developed method was robust in nature. The results are presented in Table 9.

Table 9: Robustness results of Efavirenz

S. No	Parameter	Optimized	Used	Retention time (R_t), min	Plate count[$]	Peak asymmetry[#]	Remark
1.	Flow rate (±0.2mL.min^{-1})	1.2 mL.min^{-1}	1.0 mL.min^{-1}	9.567	22874	1.027	*Robust
			1.2 mL.min^{-1}	9.563	22869	1.025	*Robust
			1.4 mL.min^{-1}	9.559	22862	1.024	*Robust
2.	Detection wavelength (±5nm)	246 nm	240 nm	9.563	22872	1.025	Robust
			246 nm	9.563	22869	1.025	Robust
			250 nm	9.563	22865	1.025	Robust
3.	Mobile phase composition (±5%)	50:50 v/v	55:45 v/v	9.566	22875	1.023	*Robust
			50:50 v/v	9.563	22869	1.025	*Robust
			45:55v/v	9.561	13853	1.026	*Robust

Acceptance criteria (Limits)

[#]Peak Asymmetry < 1.5, [$] Plate count > 3000, *significant change in Retention time.

3.7. LOD and LOQ

Limit of Detection is the lowest concentration in a sample that can be detected, but not necessarily quantified under the stated experimental conditions. The limit of quantitation is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy. Limit of Detection and Limit of Quantitation was calculated using following formula LOD = 3.3(SD)/S and LOQ= 10 (SD)/S, where SD = the standard deviation of response (peak area) and S = the slope of the calibration curve. The LOD and LOQ values are presented in Table 10.

Table 10: Limit of Detection (LOD) and Limit of Quantitation (LOQ)

Parameter	%RSD
Limit of Detection (LOD)	0.0183 µg.mL^{-1}
Limit of Quantitation (LOQ)	0.0555 µg.mL^{-1}

3.8. Solution Stability

Stability of the sample solution evaluated by keeping the Sample solutions for 72 hours at room temperature and analyzed each sample by the end of the prescribed storage hour. The percent RSD and % assay difference from the freshly analyzed sample were computed. The percentage changes in the assay results after 72 hours storage were compared with freshly analyzed sample and % RSD values were also estimated. The % RSD values found to be 0.253. The stock solutions showed no significant change in analyte composition: retention time and peak areas of EFA. Similarly, the standard solutions of EFA solutions were also found to be stable at room temperature over a 72 hours period, which was sufficient for the whole analytical process.

3.9. Analysis of marketed formulation

The proposed validated method was successfully applied to determine the EFA in their tablet dosage form. A sample solution was prepared tablet containing 600 mg Efavir as described in the experimental procedure. After filtration through 0.45 µm nylon filter the solution was diluted to required concentration and injected into Liquid Chromatograph. The assay was repeated for six times and the amount of the drug present per tablet was estimated from calibration equation. The amount found was determined. The results are represented in Table 11.

Table 11: Assay results of Efavirenz formulation

S. No	Formulation	Labelled amount	Amount found* (mean±SD)
1	Efavir	600 mg	598.97 mg ± 0.135

***** Average of 6 determinations.

4. Results and discussion

The mobile phase consisting of 10 mM phosphate buffer (pH-3.0): acetonitrile (50:50 % v/v at 1.2 mL.min^{-1} flow rate was optimized which gave sharp peak, minimum tailing factor. The retention time for EFA was 9.563 minutes. UV spectra of EFA showed that the drug absorbed maximum at 241 nm, so this wavelength was selected as the analytical wavelength. Optimized chromatographic conditions and system suitability parameters are shown in Table 1 and Table 2. The specificity was determined to test the interference of commonly used excipients. The comparison of standard and blank chromatograms indicates no co-eluting peak in the chromatograms as well shaped peaks also indicates the specificity of the method. Therefore, it was concluded that the method is specific. The specificity results are summarized in Table 3.The chromatograms of placebo and sample peak are shown in Fig 2 and Fig 3. The calibration curve for EFA was found to be linear over the range of 2-10 µg.mL^{-1}. The data of regression analysis and the calibration data are shown in Table 4 and Table 5. The regression equation was found to be Y= 247.6 x + 0.809 with correlation coefficient is r^2=0.9999 which indicates this method has good linearity. The representative chromatograms indicating the EFA are shown in Fig. 4 to 8. The linearity of the graph is shown in Fig. 9. Precision was studied to find out intra and inter day variations in the test methods of EFA for the three times on the same day and different day. The intra-day and inter-day precision obtained was % RSD (< 2.0) indicates that the proposed method is quite

precise and reproducible and results are shown in Tables 6 and 7. Recovery studies of the drug were carried out for the accuracy parameter at three different concentrations levels i.e., multiple level recovery studies. A known amount of EFA standard was added into pre-analyzed sample and subjected them to the proposed HPLC method. The % recovery was found to be within the limits as listed in Table 8. Generally the mean percentage recovery of EFA at each level was not less than 99 % and not more than 100 %. In this case the percentage recovery of EFA was found to be in the range of 99.22 % to 99.52 %. The method precision was done and the low % RSD values indicate that the proposed method which was in good agreement with precision. Robustness were done by small changes in the chromatographic conditions like mobile phase flow rate, temperature, mobile phase composition etc., It was observed that there were no marked changes in the chromatograms. In fact the parameters are within the limit which indicates that the method has robustness and suitable for routine use. The Robustness results are presented in Table 9. The limit of detection (LOD) and limit of quantitation (LOQ) was calculated based on the standard deviation (SD) of the response and the slope (S) of the calibration curve at levels approximating the LOD and LOQ. The limit of detection (LOD) was 0.0183µg/mL and the limit of quantitation (LOQ) was 0.0555 µg/mL which shows that this method is very sensitive. The results are presented in Table 10.Eventually the proposed validated method was eventually applied for quantitative determination of the marketed formulation EFAVIR tablets. 20 µL of sample solution was injected into liquid chromatograph and chromatogram was recorded and the mean assay value was found to be 99.82. Satisfactory results were achieved. The mean % found for the drug was in good agreement with the label claim and results are presented in Table 11.

5. Conclusion

A New validated RP-HPLC method has been developed for the quantitative determination of EFA in bulk and pharmaceutical tablet dosage forms. Statistical analysis of the results shows that the proposed procedure has good precision and accuracy. The method was completely validated shows satisfactory results for all the method validation parameters tested and the method was free from interference of the other active ingredients and additives used in the formulation. In fact results of the study indicate that the developed method was found to be simple, reliable, accurate, linear, sensitive, economical, and reproducible and 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 EFA in active pharmaceutical ingredient (API) and pharmaceutical preparations.

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