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Analytical Method Development and Validation for the Estimation of Efavirenz by RP-HPLC Method in Bulk and Pharmaceutical Dosage Form

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A B S T R A C T

The chromatographic conditions were successfully developed for the separation of Efavirenz by using Kromosil Column (100-5 C18.60x4.6mm), flow rate was 1ml/min, mobile phase ratio was Acetonitril: Phosphate buffer P^H 5.0: Methanol (40:50:10 v/v), detection wavelength was 247 nm. The Spectroscopic method was done in solvent using methanol and the instrument lab India 3000+ with UV win software. The instrument used was WATERS HPLC Auto Sampler, Separation module 2675, UV detector, Empower-software version 2. The retention time were found to be 3.221 min. The system suitability parameters for Efavirenz such as theoretical plates and tailing factor were found to be 3015. The analytical method was validated according to ICH guidelines (ICH, Q2 (R1)). The linearity study of Efavirenz was found in the concentration range of 29.69 µg/ml to 237.52 µg/ml and correlation coefficient (r^2) was found to be 0.999 respectively, % recovery was found to be 99.4% respectively. %RSD for repeatability and precision was found to be <2. LOD values were 0.024 µg/ml and LOQ value were 0.066 respectively for Efavirenz.

Keywords: Efavirenz, HPLC, LOQ, ICH guidelines

A R T I C L E I N F O

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1. Introduction

Efavirenz belongs to the NNRTI class of anti retrovirals [1]. It acts allosterically by binding to a distinct site known as the NNRTI pocket, away from the active site [2].

Analytical methods

Methods are developed for new products when no official methods are available. Alternate methods for existing (non-pharmacopoeial) products are developed to reduce the cost and time for better precision and ruggedness [3]. Trial runs

are conducted, method is optimized and validated. When alternate method proposed is intended to replace the existing procedure comparative laboratory data including merit/demerits are made available [4].

Description of the Various Analytical Methods

Titrimetric and gravimetric method of analysis is suitable when the sample is present in pure form or when no interference is observed in the mixture with other materials. Ultraviolet and visible spectrometric method is suitable when no Interference is observed in the mixture. HPLC and GC methods are more advantageous than the above due to their capability in separating organic mixtures and quantitative estimations [5].

AAS is used mainly for quantitative estimation in ppm and ppb levels of elements. Infra-red spectroscopy though mainly used for qualitative analysis can be used for quantitative estimation also [6]. Out of all the above methods, thin layer chromatography plays a very important role in analysis due to its adaptability, flexibility, and cost and time. It can be used both for qualitative and quantitative determination [7]. After separation spots can be scanned with the help of a scanner and quantitative measurement can be made [8].

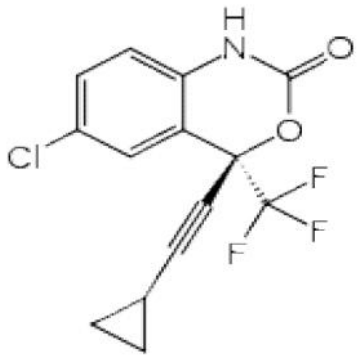

Figure 1: Efavirenz

Chromatography: Chromatography is a technique used in analytical chemistry to separate and identify components of mixtures. The name comes from the Greek term for "color writing" because this method was originally used to separate colored samples. The advent of high-performance liquid chromatography (HPLC)[9].in this system pressure is applied to the column, forcing the mobile phase through at much higher rate. The pressure is applied using a pumping system. The action of the pump is critical, since it must not pulsate and mix up the sample being separated in the solvent, causing it to lose resolution [10]. Development of pumps has proceeded quite quickly over the last several years, and now it is possible to achieve good resolution under the conditions required for HPLC.

2. Experimental

Apparatus: The instrument used for the study was Waters HPLC Auto Sampler, Separation module 2675, UV detector with Empower-software version-2 [11].

Reagents and Materials

The solvents used were Methanol, Water, Acetonitrile, Sodium hydroxide, Potassium dihydrogen ortho phosphate

Selection of detection wavelength:

By appropriate dilution of standard stock solution (1200µg\ml) with diluent, various concentrations like 80µg\ml, 100 µg\ml, and 120 µg\ml of Efavirenz were prepared separately [12]. The solutions were scanned the double beam spectrophotometer in the spectrum in the spectrum mode between the wave length ranges of 400nm-190nm. The λ_{max} of Efavirenz was found to be 247nm which was selected as the analytical wavelength for further analysis [13].

Selection of mobile phase

Pure drug of Efavirenz was subjected into the HPLC system and run in different solvent system. Different mobile phase systems like mixed phosphate buffer, Acetonitrile and phosphate buffer and acetonitrile, methanol and phosphate buffer were tried in order to determine the best conditions for the separation of Efavirenz. It was found that acetonitrile; methanol and pH 5.0 phosphate buffer gives satisfactory result compared to other mobile phases, Finally, the optimal composition of the mobile phase employed was acetonitrile, methanol and pH 5.0 phosphate buffer in the ratio (40:10:50 v\v\v). The mobile phase was ultra-sonicated for 20minutes and then filtered through a 0.45µ membrane filter [14].

Chromatographic trials for Estimation of Efavirenz by RP- HPLC.

Trial-1 Chromatographic conditions

Column	:	Inertsil ODS 100x4.6, 5micron
Mobile phase ratio:		Acetonitrile: pH 5.0 phosphate Buffer: methanol in the ratio of 50:50v/v
Detection wavelength	:	247nm
Flow rate	:	0.1ml/min
Injection volume	:	10µl
Retention time	:	9 .45 min

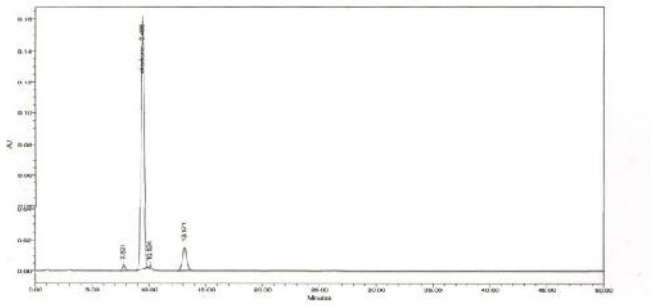

Figure 2: Chromatogram of Trial-1

Observation: The retention time of Efavirenz was found to be 9.4 min and the resolution from the degradation peak was found to be 1.12. In order to have better resolution it was decided to use methanol along with the existing mobile phase

Trial-2 Chromatographic condition

Column	:	Inertsil ODS 100x4.6, 5micron
Mobile phase ratio	:	Acetonitrile: pH 5.0 phosphate Buffer: methanol in the ratio of 30:60:10v/v

Detection wavelength : PDA (200- 400nm)
Flow rate : 0.1ml/min
Injection volume : 0µl
Retention time : 37.293 min

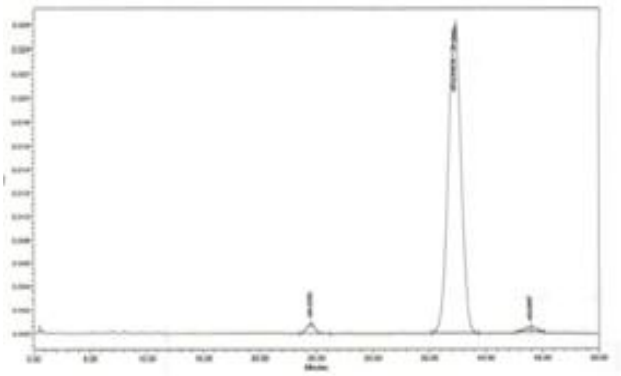

Figure 3: Chromatogram of trial-2

Observation: The retention time of the Efavirenz is found to be 37.25 min, and the resolution from the degradation peak was found to be 2.76. But retention time of Efavirenz is more in order to reduce the retention time; it was decided to change the mobile phase composition.

Trial-3 Chromatographic condition

Column : Inertsil ODS 100x4.6, 5micron
Mobile phase ratio : Acetonitrile: pH 5.0 phosphate Buffer: methanol in the ratio of 40:50:10 v/v
Detection wavelength : PDA (200- 400 nm)
Flow rate : 0.1 ml/min
Injection volume : 10 µl
Retention time : 1.056 min

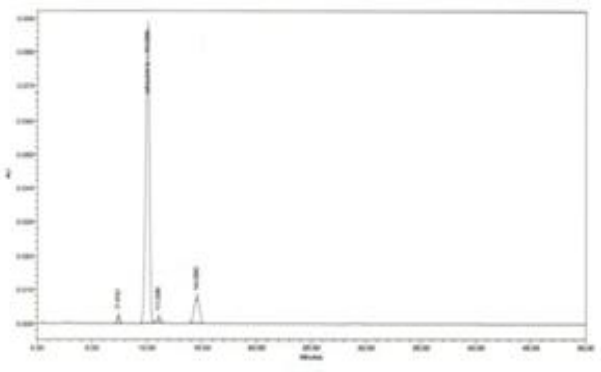

Figure 4: Chromatogram of trial-3

Observation: The retention time of the Efavirenz is found to be 10.056min, and the resolution from the degradation peak was found to be 1.85. But retention time of Efavirenz is more in order to reduce the retention time, it was decided to change the column and flow rate.

Trial-4 Chromatographic conditions (Optimized Method):

Column : Kromosil 100-5 C18.60×4.6mm
Mobile phase ratio : Acetonitrile: pH 5.0 phosphate Buffer : Methanol in the ratio of 40:50:10v/v
Detection wavelength: PDA (200- 400nm)
Flow rate : 1.5ml/min
Injection volume : 10µl
Retention time : 3.221 mins

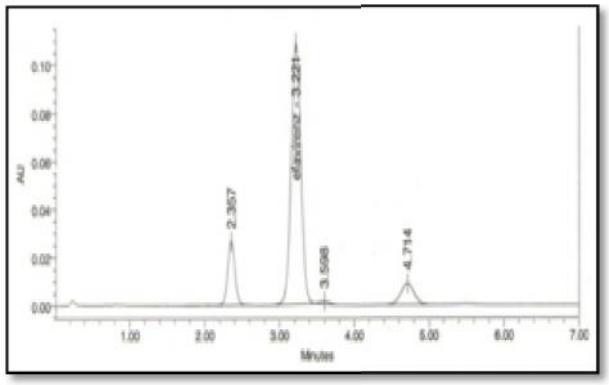

Figure 5: Chromatogram of trial-4 (Optimized Method)

Observation: The chromatographic conditions of the Trail-4 have been finalized, which meets the criteria of minimum RT, better resolution from degradation peak, minimal consumption of organic solvent, good peak symmetry and less run time.

Procedure

Preparation of phosphate buffer

2.95 grams of KH_2PO_4 and 5.45 grams of K_2HPO_4 was weighed and taken into a 1000 ml beaker, dissolved and diluted to 1000 ml with HPLC water and pH was adjusted to 5 with Orthophosphoric acid. The resulting solution was sonicated and filtered [15].

Preparation of mobile phase

Pure drug of Efavirenz was subjected into the HPLC system and run in different solvent system. Different mobile phase systems like mixed phosphate buffer, Acetonitrile and phosphate buffer and acetonitrile, methanol and phosphate buffer were tried in order to determine the best conditions for the separation of Efavirenz[16]. It was found that acetonitrile; methanol and pH 5.0 phosphate buffer gives satisfactory result compared to other mobile phases, Finally, the optimal composition of the mobile phase employed was acetonitrile, methanol and pH 5.0 phosphate buffer in the ratio (40:10:50 v\v\v)[17]. The mobile phase was ultra-sonicated for 20 minutes and then filtered through a 0.45µ membrane filter [18].

Efavirenz standard preparations: About 60 mg of Efavirenz was accurately weighed and transferred into 50 ml volumetric flask and dissolved in 30 ml of diluent. It was sonicated for 10 minutes [19]. The above solution was diluted to 1200 µg\ml with the diluents [20]. The standard solution was filtered with 0.45 µm membrane filter [21].

3. Results and Discussion

Method Validation Parameters

Specificity: The system suitability for specificity was carried out to determine whether there is any interference of any impurities in retention time of analytical peak. The specificity was performed by Injecting blank.

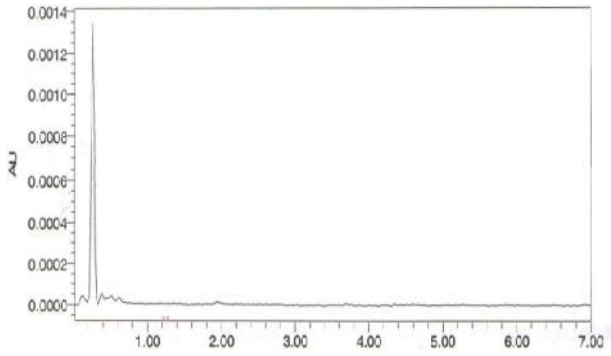

Figure 6: Chromatogram of Blank

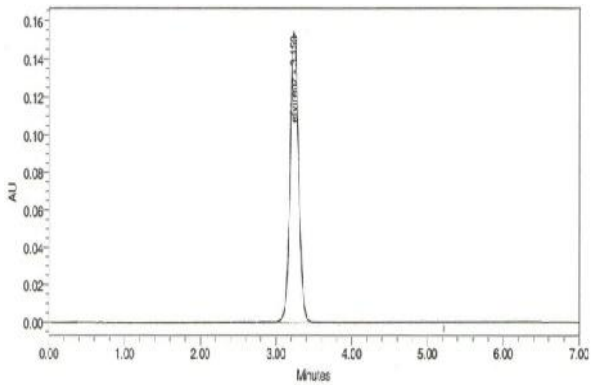

Figure 7: Chromatogram of Sample

Linearity

Standard solutions of different concentrations were injected separately and the chromatograms were recorded. Peak areas were recorded for each injected concentration of drug and the calibration curve was constructed for Efavirenz. A set of solutions for Efavirenz of concentration 25, 50, 75, 100, 150 and 200% µg\ml were choosen. Triplicate of each concentration of the drug were prepared separately. From these triplicate solutions, 10 µL injections of each concentration of the drug were injected.

Acceptance criteria: Correlation coefficient should be not less than 0.999.

Range

Based on precision, linearity and accuracy data it can be concluded that the assay method is precise, linear and accurate in the range of 25-200 ppm for Efavirenz respectively.

Accuracy

Five replicate injection of the standard solution and three replicate injections of each sample solution were injected separately and chromatograms were recorded. The concentration of each drug was estimated by comparing sample peak with standard.

Acceptance criteria: As the results were found to be within the acceptance limits (98% to 102%), the method was accurate.

Precision

Five replicate injections of the standard solution and each of six sample solutions were made separately and chromatograms were recorded. The chromatogram of the sample solution of Efavirenz and the observation results of six separate sample solutions are as follows.

Acceptance criteria

- As the results were found within acceptable limits, the method was precise.

Selection of solvent

Solutions of Efavirenz were prepared by dissolving in mobile phase and UV spectrum of each was recorded by scanning between 200-400 nm.

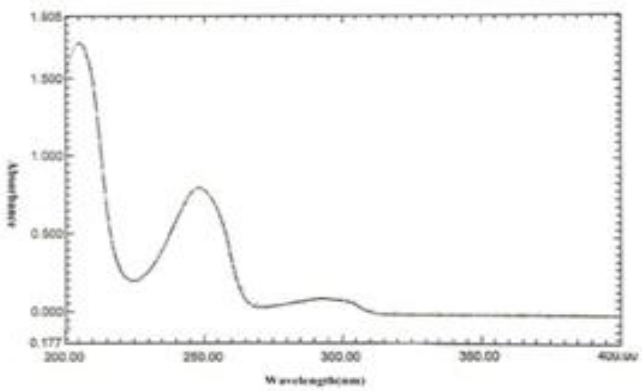

Figure 8: Overlain Spectra of Efavirenz

Validation of the Method

Linearity

Efavirenz: A set of solutions for Efavirenz of concentration 25, 50, 75, 100, 150 and 200% µg\ml were choosen. Triplicate of each concentration of the drug were prepared separately. From these triplicate solutions, 10 µL injections of each concentration of the drug were injected.

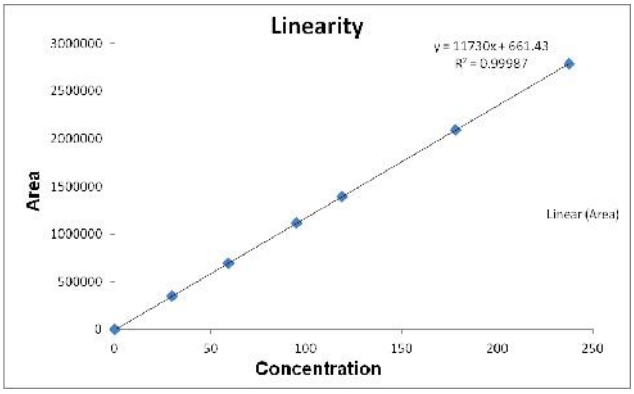

Figure 9: Calibration graph of Efavirenz

Table 1: Calibration data of Efavirenz

S. No	%Level	Conc in ppm	Area
1	25	29.69	349283
2	50	59.38	697455
3	75	95.01	1115273
4	100	118.76	1393823
5	150	178.14	2090178
6	200	237.52	2786545
Correlation		**0.99987**	
Slope		**11726.9**	
Intercept		**1100.27**	
Residual Sum of Square		**1446877884**	

Recovery studies

Five replicate injection of the standard solution and three replicate injections of each sample solution were injected separately and chromatograms were recorded. The concentration of each drug was estimated by comparing sample peak with standard.

Table 2: Showing accuracy results for Efavirenz

Average weight	1220.3	1220.3	1220.3
Test Weight	65.6(50%)	121.9(100%)	179.7(150%)
Test area 1	776901	1434091	2108028
Test area 2	775042	1445633	2106014
Average area	775972	1439862	2107021
Results in mg	596.49	595.64	591.27
%label claim	99.4	99.3	98.3

Robustness:

Table 3: System Suitability Results for Efavirenz

	Flow	
	Flow (1.4ml/min)	**Flow (1.6ml/min)**
Working Standard Area	1447758	1331471
	1448149	1332351
	1443095	1335332
	1443990	1334467
	1446591	1334707
Overall Statistical Analysis		
Mean	1445917	1333366
SD	7288	2653
%RSD	0.16	0.12

Precision:

Table 4: Peak name: Efavirenz

Observation/ Results				
Acceptance Criteria : RSD should be not more than 2.0%				
Concentration	**Sample**	**Assay**	**Statistical Analysis**	
40ppm	Sample 1	98.2	Mean	98.9
	Sample 2	99.5		
	Sample 3	99.1	SD	0.479
	Sample 4	99.3		
	Sample 5	98.8	%RSD	0.5
	Sample 6	98.6		

Table 5: LOD and LOQ

Drug	**LOD (µg/ml)**	**LOQ (µg/ml)**	**SN Ratio for LOD**	**SN Ratio for LOQ**
Efavirenz	0.024	0.066	3.88	10.79

4. Conclusion

Analysts are forever in search of rapid, sensitive and accurate methods of analysis, that are viable quantitative study. In this study, a suitable analytical method was developed for the determination of Efavirenz in pharmaceutical dosage forms and bulk drug by RP-HPLC After doing my trails, the analytical method used for determination of Efavirenz in pharmaceutical dosage forms and bulk drug had good sensitivity and simplicity to measure the drug concentration in pharmaceutical dosage forms. The Reverse Phase High Performance Liquid Chromatography method was developed a stability indicating assay method. Pure drug chromatogram was run in different mobile phases containing methanol, water, Acetonitrile, Phosphate Buffer. And different columns like C_8 & C_{18} were used. The retention time and tailing factor were calculated. Finally the mobile phase with Acetonitrile: Phosphate buffer: Methanol in the ratio of 40: 50: 10v/v was found to be good. A linear response (r^2 – 0.999) was observed in range of 29.69 µg/ml to 237.52µg/ml. The limit of Detection (LOD) was 0.02µg/ml. and the limit of Quantitation (LOQ) was 0.06µg/ml. The method was validated for accuracy and precision. The proposed method can be used for the stability studies as the method separates EFA from its degradation products and excipients. The proposed RP-HPLC method was found to be simple, specific, precise, accurate, rapid and economical for simultaneous estimation of Efavirenz in dosage form and bulk. This method was validated as per ICH guidelines. The sample recoveries in formulation were in good agreement with their respective label claim and showed non-interference from formulation excipients in the estimation hence, this method can be easily and conveniently adopted for routine and stability of Efavirenz tablet dosage form.

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