Development and Validation of RP-HPLC Method for Estimation of Efavirenz in Bulk and Tablet Formulation

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ABSTRACT

Efavirenz is a non-nucleoside reversetranscriptase inhibitor widely used in the management of Human Immunodeficiency Virus (HIV) infection. The present study aimed to develop and validate simple, accurate, precise, and economical UV-Spectrophotometric and reverse phase high-performance liquid chromatographic (RP-HPLC) methods for the estimation of Efavirenz in bulk drug and pharmaceutical dosage forms. The spectrophotometric method was developed using acetonirile as solvent, and the absorbance was measured at a wavelength of 248 nm. The RP-HPLC method was optimized using a Hyperclone C18 analytical column (250×4.6 mm, 5 µm particle size) with a mobile phase consisting of Phosphate buffer and Methanol in the ratio of 35:65 % v/v ratio, delivered at a flow rate of 1.0 mL/min and uv detected at 248 nm. The developed methods were validated in accordance with the International Council for Harmonisation (ICH) guidelines for linearity, accuracy, precision, specificity, robustness, limit of detection (LOD), and limit of quantification (LOQ). The RP-HPLC method showed a retention time of 2.25 minutes and linearity over the concentration range of 5-25 µg/mL with an R^2 value of 0.999. Recovery studies yielded values ranging from 99.2 % to 102.0 %, while precision studies showed standard deviation (% RSD) values below 2.00. The LOD and LOQ were found to be 2.186 µg/mL and 6.626 µg/mL respectively. System suitability tests further verified robust chromatographic performance, with a tailing factor 1.12 and theoretical plates 3854. The method demonstrated high sensitivity and selectivity, effectively separating the analyte from excipients, making it ideal for routine quality control and stability testing in pharmaceutical manufacturing.

Keywords: Efavirenz, Spectrophotometric Method, RP-HPLC, Method Development, Validation, ICH Guidelines, Pharmaceutical Dosage Forms.

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1. INTRODUCTION

Human Immunodeficiency Virus (HIV) remains one of the world's most serious public health challenges, requiring continuous therapeutic intervention [1]. Efavirenz is a highly effective, first-line antiretroviral medication extensively utilized globally in the management and prophylaxis of HIV-1 infections [2]. Pharmacologically, it is classified as a non-nucleoside reverse transcriptase inhibitor (NNRTI). It exerts its therapeutic action by directly targeting and non-competitively binding to the HIV-1 reverse transcriptase enzyme. This binding induces a conformational change that disrupts the enzyme's ability to transcribe single-stranded viral RNA into double-stranded DNA, a pivotal step in the viral life cycle [3].

Efavirenz works by specifically targeting the HIV-1 reverse transcriptase enzyme, an essential enzyme required by the virus to convert its single-stranded viral RNA into double-stranded DNA, a critical step in the viral life cycle. By binding non-competitively to this enzyme, Efavirenz alters its structure and function, thereby preventing the transcription process and inhibiting the production of new viral particles. As a result, the viral load in the bloodstream is significantly reduced, helping to slow disease progression, preserve immune function, and decrease the risk of HIV transmission. Efavirenz is commonly administered as an oral tablet or capsule, usually taken once daily, which enhances patient adherence to therapy. It is frequently used as part of combination antiretroviral therapy (cART) and is available in fixed-dose combination formulations such as Atripla, which combines Efavirenz with other antiretroviral agents to improve treatment effectiveness and reduce pill burden. Marketed under brand Sustiva and Stocrin, Efavirenz has been extensively utilized worldwide as a cornerstone therapy for HIV/AIDS.

By halting this replication process, Efavirenz significantly suppresses the viral load, preserves the patient's immune function, and slows disease progression. Clinically, it is administered once daily, maximizing patient adherence, and forms a critical cornerstone of combination antiretroviral therapy (cART) [4]. Ensuring the precise quantification of Efavirenz in commercial pharmaceutical formulations is mandatory to maintain its therapeutic efficacy, prevent sub-therapeutic dosing or toxicity, and comply with stringent regulatory quality standards [5].

Various analytical techniques, including UV-Visible spectrophotometry and High-Performance Liquid Chromatography (HPLC), are fundamental tools in pharmaceutical quality control [6, 7]. While UV-Vis spectroscopy offers a rapid and economical approach for routine preliminary analysis [8], Reverse Phase HPLC (RP-HPLC) remains the universal "gold standard" for drug estimation. RP-HPLC provides superior specificity, high resolution, and ruggedness, making it indispensable for evaluating complex formulations and conducting stability-indicating regulatory testing [9, 10].

A thorough literature survey reveals that several analytical methods have been reported for the estimation of Efavirenz. However, the modern pharmaceutical industry is currently experiencing a paradigm shift towards "Green Analytical Chemistry" and high-throughput methodologies [11-13]. Many existing chromatographic methods for antiretroviral drugs rely on complex, hazardous mobile phases or suffer from prolonged chromatographic run times. This not only increases the consumption of toxic organic solvents but also elevates operational costs and environmental hazards [14, 15]. Modern analytical research strongly emphasizes minimizing toxic solvent usage, reducing analysis time, and ensuring eco-friendly quality control without compromising on analytical precision or sensitivity [16-18].

To address these existing literature gaps, the present investigation aims to systematically develop, optimize, and comprehensively validate a novel, rapid, cost-effective, and highly precise RP-HPLC method for the quantitative estimation of Efavirenz in both bulk drug substance and marketed tablet dosage forms.

The proposed method utilizes an optimized, simple isocratic mobile phase designed for rapid elution, thereby ensuring high laboratory throughput. Furthermore, complete method validation was performed in strict accordance with the International Council for Harmonisation (ICH) Q2(R1) regulatory guidelines, comprehensively evaluating parameters such as linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), and system robustness [19, 20]. This streamlined approach is intended to provide enhanced reliability and suitability for routine, sustainable quality evaluation in modern pharmaceutical enterprises.

HIV is still one of the world's most serious public health issues. A weakened immune system is exploited by these opportunistic infections or malignancies, which indicate the presence of AIDS, the last stage of HIV infection. Tens of millions of people have contracted HIV since the start of the epidemic. But since the development of efficient antiretroviral medication (ART), HIV has changed from a deadly illness to a chronic illness that can be controlled.

2. Drug Information

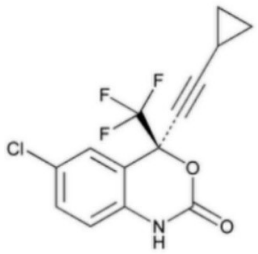

Fig. No. 01 – Structure of Efavirenz

Generic Name: Efavirenz
Brand Names: Sustiva, Stocrin (often found in combination pills like Atripla).
Chemical Class: Benzoxazinone derivative.
Therapeutic Category: Antiretroviral agent (NNRTI).
Molecular Formula: C14H9ClF3NO2
Molecular Weight: 315.68 g/mol
Chirality: The drug contains one chiral center and is usedas the (S)-enantiomer.
Physical Properties: Appearance: White to slightly pink crystalline powder.
Melting Point: 139°C – 141°C.
Dissociation Constant (pKa): 10.2 (Weakly acidic due to the carbamate nitrogen).
Chemical Name: (S)-6-Chloro-4-(cyclopropylethynyl)-4-(trifluoromethyl)-1,4-dihydro-2H-3,1-benzoxazin-2-one
Category: Non-Nucleoside Reverse Transcriptase Inhibitor (NNRTI). [21]

3. MATERIALS AND METHODS

Chemicals and reagents

The active pharmaceutical ingredients (API) standard of Efavirenz was generously provided by Pharmatrain Labs, Rajahmundry, India, ensuring analytical grade purity. HPLC grade reagents Methanol, ortthophosphoric acid, sodium

dihydrogen orthophosphate and milli-Q double distilled water were supplied by Merck Chemical Laboratories. Commercial formulated Efavirenz tablets (Sustiva 600 mg) were purchesed from a local pharmacy for assay evaluation.

Apparatus and Instrumentation

Ultraviolet-Visible (UV-Vis) Spectroscopy

A popular analytical method for the qualitative and quantitative examination of pharmaceutical substances is UV-Vis spectroscopy. The technique relies on molecules in the 200–800 nm wavelength range absorbing ultraviolet and visible light. Because UV-Vis spectroscopy is easy, quick, accurate, economical, and requires little sample preparation, it is the method of choice for pharmaceutical analysis. It is widely used in stability testing, dissolution studies, assay determination, and drug identification. In research and industrial laboratories, the methodology is also crucial for the development and validation of methods for the quality control of pharmaceutical formulations and bulk medicinal substances.

HPLC instrumentation and chromatographic condition

All chromatographic separations and quantitative evaluations were conducted using the Waters Alliance 2695 HPLC system equipped with photodiode array (PDA) detector. Data were taken by using of standard chromatography software.

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Instrumentation

Column : Hyperclone C18 Coloumn (250 × 4.6 mm internal diameter, 5 µm particle size

Mobile phase : Phosphate buffer (pH 2.5) : Methanol (35:65 v/v)

Flow rate : 1.0 ml/min

Column temperature: 30^0**C**

Injection volume: 20 µl

Wavelength : 248 nm

Run time : 10 min

Preparation of standard stock solutions

The primary standard stock solution was prepared by accurately weighing and dissolving 50 mg of pure Efavirenz in 50 ml of the diluents (mobile phase) to yield concentration of 1000 µg/ml. Intermediate stock solution of 100 µg/ml was prepared by transferring 5 ml of the primary stock solution into 50 ml volumetric flask and diluting up to the mark. Further serial dilutions continue to ranging from 5 to 25 µg/ml for calibration.

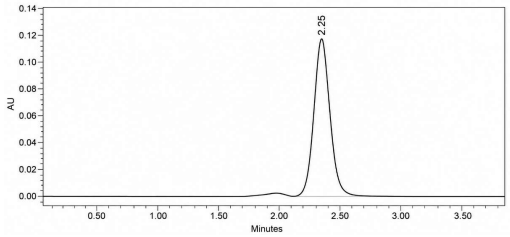

Fig. No. 01 – Chromatogram of Efavirenz Optimization of Mobile Phase

Observation: Efavirenz peak was eluted at 2.25min, each with high levels of resolution. Due tovery good plate count and tailing factor, thisapproach was optimized and validated.

Table No 1: Chromatogram data of Efavirenz

Drug	RT	Area	Height	Theoretical plates
Efavirenz	2.25	2846766	265596	3854

Calibration Curve Development

Standard solutions of efavirenz were prepared at concentrations of 5, 10, 15, 20 and 25 ppm for the calibration curve. After pipetting each standard solution into a 100 mL volumetric flask, acetonitrile was used to dilute them to the appropriate volume. To ensure consistency, duplicate dilutions were independently prepared for each concentration.

A 20 µL volume of each prepared solution was injected into the RP-HPLC system. Chromatographic analysis was performed under the specified conditions, with a UV detector set at 248 nm to measure the retention time of efavirenz.

Method Validation

The newly developed RP-HPLC method was validated based on essential performance criteria, including linearity, accuracy, precision, selectivity, range, forced degradation tests, robustness, and system adaptability.

Development and Validation of RP-HPLC Method

The analysis of efavirenz was successfully carried out through the development and validation of a reliable RP-HPLC method. The chemical was effectively separated using a Hyperclone C18 column and a mobile phase consisting of phosphate buffer and methanol in a 35:65 v/v ratio. Detection was performed at a wavelength of 248 nm, with a constant flow rate of 1.0 mL/min. The chromatographic data obtained demonstrated the high selectivity of the method, confirming that no

interference from excipients was observed during the analysis. The effectiveness of the new method was proven by the complete separation of analytes in under 10 minutes.

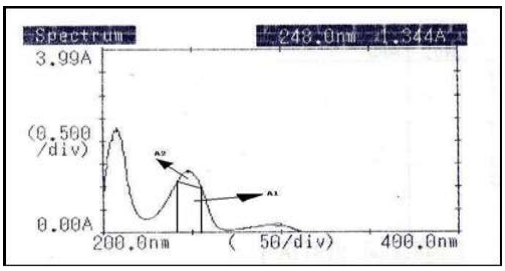

Figure No.02 – UV overlay spectra of Efavirenz

Linearity of the Method

For efavirenz, the technique showed a robust linear correlation over the concentration range of 5-25 ppm. Plotting peak area against concentration allowed for the creation of a calibration curve, and linear regression analysis was performed on the data. **Figure 02** shows the regression equation, y = 1000000x+378716, with a correlation coefficient (r2) of 0.999, or almost unity. These findings support the method's superior linearity, which makes it ideal for quantitative analysis.

Optimization and Development of an HPLC Method

To obtain unique, well-resolved peaks with the ideal retention period, the chromatographic configurations were optimised. A mobile phase consisting of phosphate buffer and methanol (35:65 v/v) at a flow rate of 1.0 mL/min was used for the initial attempts. The chromatographic peaks that were produced under these circumstances showed good symmetry and sharpness. Because of its superior chromatographic responsiveness and peak resolution, the phosphate buffer: methanol (35:65 v/v) mobile phase was chosen as the most appropriate for the entire investigation based on these observations.

System Suitability Studies

To verify the method's dependability and performance, system suitability assessments were performed. This thorough analysis confirmed the method's appropriateness for its designated purpose. Multiple chromatographic factors were evaluated, such as: Retention time: Efavirenz elueted efficiently retention time 2.25 minutes. Peak area variability: Determined to be within permissible ranges, showcasing method reliability. Tailing factor: Recorded as below 2, suggesting excellent peak symmetry. Theoretical plates: Over 3854 plates were noted, indicating superior column performance. The method's high sensitivity facilitated precise peak identification, and in every instance, efavirenz was effectively isolated from excipients. This validates the method's applicability for standard analytical use.

Specificity of the Method

The elution time of efavirenz was analyzed to evaluate the precision of the RP-HPLC technique. As the compound was consistently detected at the expected retention time, this method is deemed reliable for research and pharmaceutical purposes.

Accuracy

Accuracy was determined by performing recovery studies by spiking specific concentrations of pure drug in a pre-analyzed sample solution of 50 µg/ml of efavirenz. To pre-analyze the sample solution, a known amount of standard stock solution was added which was at different levels 50, 100, and 150%. The solutions were analyzed by the proposed method. The mean % recovery was calculated.

Repeatability

It was determined by analyzing the same solution of 10 µg/ml of efavirenz standard solution repeatedly.

Robustness

In the robustness study, the following parameters have been changed one by one and observed their effect on system suitability test and assay.

i. Change Wavelength ± 1nm
ii. Change flow rate ±0.1mL/min.

5. Result and discussion

5.1 Optimization by HPLC Method

5.1.1 Linearity

The linearity has been performed by determining a standard calibration curve using a concentration range of 5-25 µg/ml. Least squares linear regression analysis of the average peak area versus concentration data were used to evaluate the linearity of the method. The values obtained were linear correlation co-efficient for calibration curve was found to be 0.999 in Fig. 03 and Table 02.

Table No. 02: Linearity data of Eavirenz standard

S. No	Conc. in ppm	Replications	AUC	Mean
1	5	R1	1542258	1542526
		R2	1542794	
2	10	R1	2852445	2846763
		R2	2841082	
3	15	R1	4123187	4132971
		R2	4142755	
4	20	R1	5299246	5302263
		R2	5305280	

5	25	R1	6436865	6437635
		R2	6438405	

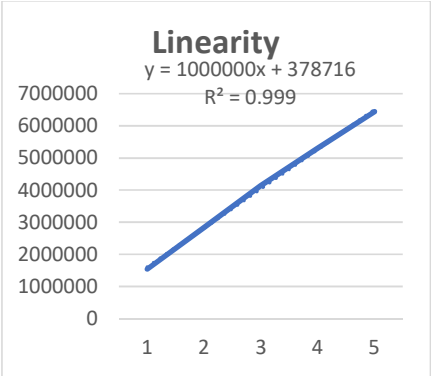

Fig. No. 03 - Calibration curve of Efavirenz

5.1.2 Precision

Precision was analyzed by calculating variations of the method in intraday (repeatability performed by analyzing standard solution on the same day) and inter-day (repeatability carried out by analyzing standard solution on three different days). The precision study showed the very closeness of the results in Table no. 04.

Table No. 04 – Inter-day and intra-day data of Efavirenz

S. No	Conc.	AUC			Mean	SD	% RSD
		I	II	III			
Inter-day	5	1542519	1542523	1542528	1542519	4.5092497 5	0.00029
	10	2846756	2846764	2846767	2846756	5.6862407	0.00019
	15	4132965	4132971	4132969	4132965	3.0550504 6	0.00007
Intra-day	5	1542533	1542530	1542527	1542527	3.0	0.00019
	10	2846770	2846763	2846768	2846763	3.6055512 8	0.00012
	15	4132991	4132983	4132990	4132983	4.3588989 4	0.00010

5.1.3 Accuracy

Accuracy was determined by performing recovery studies by spiking specificconcentrations of pure drug in a pre-analyzed sample solution of 50µg/ml of Efavirenz. To pre-analyze the sample solution, a known amount of standard stock solution was added which was at different levels 50, 100, and 150%. The % recovery was found to be between 99-102% [Table No. 05], which is the acceptable limit as per the ICH guidelines.

Table No. 05 – Accuracy data of Efavirenz

% level	Amount spiked	Amount recovered	% recovery	Mean recovery
50%	5	4.96	99.2	100.3
	5	5.05	101	
	5	5.10	102	
100 %	10	10.09	100.9	
	10	10.11	101.1	
	10	9.92	99.2	
150 %	15	14.85	99	
	15	15.30	102	
	15	15.02	100.1	

5.1.4 Repeatability

The low values of % RSD are indicative of the accuracy and reproducibility of the method. The repeatability of the method was determined by analyzing the same solution of 10 µg/ml of Efavirenz standard solution repeatedly in Table No. O6.

Table No. 06 – Repeatability study of Efavirenz

S No	Conc.	AUC
1	10	2846755
2	10	2846752
3	10	2846767
4	10	2846787
5	10	2846765
6	10	2846760
Mean		2846764
SD		12.48
%RSD		0.00043%

5.1.5 Limit of Detection (LOD) and Limit of Quantification (LOQ)

Limit of Detection (LOD) and Limit of Quantification (LOQ) of the developed method has been studied. It was found in the permissible range i.e. LOD, 2.186 µg/ml and LOQ, 6.626 µg/ml tabulated in Table No. 07.

LOD

The LOD was estimated from the set of five calibration curves used to determine method linearity. The calibration curve was repeated for 6 times and the SD of the intercept was calculated then LOD was calculated as follow:

LOD= (3.3*SD)/slope.

Where,

SD= the standard deviation of the y-intercept of 5 calibration curves.

Slope= the mean slope of the 5 calibration curves

LOQ

The LOQ was estimated from the set of five calibration curves used to determine method linearity.

The LOQ may be calculated as:

LOQ = 10 × (σ/S).

Table No. 07 - LOD & LOQ data of Efavirenz

S. No	Drug	LOD (µg/ml)	LOQ (µg/ml)
1	Efavirenz	2.186	6.626

Robustness: The robustness of the method was studied during the method development by varying the following parameters.

Flow rate: The standard solution of efavirenz were prepared and injected by varying the flow rate from 0.8ml/min to 1.2ml/min.

Change in wavelength: Standard solution of efavirenzwas prepared and injected by varying the wavelength along with the optimized method. The results were tabulated in table No. 08.

Table No. 08 – Robustness of Efavirenz

Chromatographic condition		AUC	RT
Change in Flow rate	0.8ml/mn	2846761	2.25
	1.0ml/mn	2846768	2.24
	1.2ml/mn	2846770	2.25
Change in wavelength	246 nm	2846771	2.25
	248 nm	2846764	2.23
	250 nm	2846760	2.25

Assay of marketed formulation

The validated RP HPLC technique was successfully employed to estimate drug content is commercially available Sustiva tablets.

Table No. 09 – Formulation Assay of Efavirenz

S. N.	Formulation	Label Claim	Amount Found	% Drug Recovered	% RSD
01	Sustiva Tablets	600 mg	598.8 mg	99.8 ±0.5%	0.40

Summary of validation parameter

Table No. 10 - Summary of validation parameters

Parameters	Observation
Linearity Range(µg/ml)	5-25
Regression Equation (y=mx+c)	y=1000000x+378716
Correlation Coefficient (R^2)	0.999
LOD (µg/ml)	2.186
LOQ(µg/ml)	6.626
Repeatability (%RSD)	0.00043
Inter-day precision (%RSD)	0.00007-0.00029
Intra-day precision (%RSD)	0.00010-0.00019
Accuracy (%RSD)	99.20-102.00
Assay	99.9%

6. CONCLUSION

A number of different chromatographic settings were applied, and the discoveries that were found are explored in the chapters that came before this one. The goal was to develop an RP-HPLC technique for measuring efavirenz that was accurate, linear, specific, and acceptable in terms of its stability indicating capabilities.One pump and a flat baseline separation are all that are required for isocratic elution, which is a basic method that produces results that are simple and repeatable. Because of this, it was suggested that gradient elution be used instead for the current investigation. Furthermore, the medication did not dissolve in water. In the appropriate composition, these solvents may be used for the purpose of developing and validating the more recent processes. Following the scanning of the reference drug solution across a range of 200 to 400 nm, the detection wavelength was chosen. Since the majority of HPLC work can be done in the wavelength range of 240-320 nm, the ultraviolet spectrum of efavirenz indicated that this is the most convenient time for it to be done. In addition, it was discovered that the optimal flow rate for analysis was 1 milliliter per minute, and the optimal injection volume was 20 microliters. Based on the findings, it seems that the methodology that was created is yet another method that is suited for assaying, and it has the potential to assist in the study of efavirenz in a variety of formulations.

When it comes to the estimate of EFZ in pharmaceutical and bulk dosage forms, the two approaches that have been developed have been proven to be quick, sensitive, exact, and repeatable. The validation of the techniques is carried out in accordance with the guidelines established by the International Conference on Harmonization (ICH). It has been determined that this approach is not only innovative but also quick, and that it has the potential to be used by both academic institutions and enterprises for the purpose of estimating combination medications.

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AUTHOR CONTRIBUTIONS: Hasim Khan conceptualized the review, structured the content, and wrote the initial draft. Nilesh mandloi contributed to literature collection, critical analysis, and manuscript revision. Mohini Patidar assisted in compiling data,preparing tables and figures, and literature synthesis.Prabhat Kumar Das, Prachi Nagde and Nitin Deshmukh were provided critical feedback, edited the manuscript, and ensured clarity and coherence. All authors read andapproved the final manuscript.

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