

A NEW AND PRECISE BIO-ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF EFAVIRENZ IN HUMAN PLASMA BY RP-HPLC

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ABSTRACT

A new, simple, rapid and accurate RP-HPLC method was developed for the estimation of Efavirenz (EFA) in human plasma and validated. The method was developed on SHISEIDO C18 column (250 x 4.6 mm i.d, 5 μ) using Acetonitrile : 20 mM phosphate buffer (pH 3.0). The wavelength used for detection of EFA is at 247 nm. The flow rate used for the elution is 1 ml/min. sample preparation for the extraction of drug was done using solid phase extraction method by Phenomenex STRATA[®] C18 sorbent, a 24 station SPE vacuum extraction assembly is used for the entire method. Methyl prednisolone was used as internal standard, the retention time of Efavirenz and internal standard were found to be 5.7 and 3.7 min respectively. Linearity was established in the range of 0.43 – 8.60 μ g/ml and the coefficient of regression (R²) value was found to be 0.995, having 0.037 as slope. The method was precise with %RSD < 2 for both intraday and interday precision. The accuracy of the method was performed over three levels of concentration and the recovery was in the range of 98-102%. The method can be successfully applied for quantifying the drug in human plasma for bioavailability and bioequivalence studies.

Keywords: RP-HPLC, Efavirenz, Phosphate buffer and Human plasma.

INTRODUCTION

Efavirenz^{1,2} (6-chloro- 4 - (2 - Cyclopropyl - 1 - ethynyl) - 4 - trifluoro methyl (4S) - 1, 4 - dihydro - 2H - benzo [D] [1,3] oxazin - 2-one) is a 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. For HIV infection that has not previously been treated, Efavirenz and Lamivudine in combination with Zidovudine or Tenofovir is the preferred NNRTI-based regimen. Efavirenz is also used in combination with other antiretroviral agents as part of an expanded post exposure prophylaxis regimen to prevent HIV transmission for those exposed to

materials associated with a high risk for HIV transmission^{3,4} (Fig. 1)

The present study is to develop a RP-HPLC method for the determination of EFA in human plasma. The Literature survey reports different analytical methods for EFA based on UV⁵⁻⁷, HPTLC⁸, HPLC⁹⁻¹⁵, stability indicating HPLC¹⁶⁻¹⁹, Bio-analytical^{20,21}, LC-MS^{22,23} were reported. However there were few methods reported for the determination of EFA in human plasma, the present aim is to develop a more precise, accurate, simple and RP-HPLC method for the estimation of EFA in human plasma and validate according to USFDA guidelines. The molar absorptivity of EFA was found maximum at 247 nm. The validated method will be used for the

quantification of EFA in bioavailability and bioequivalence studies.

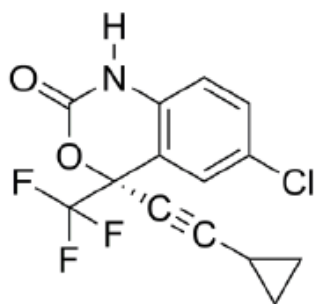


Fig. 1: Chemical structure of EFA

MATERIALS AND METHODS

Chemicals and reagents

Efavirenz (EFA) working standards was procured from Aurobindo pharma Ltd. Hyderabad. Tablets were purchased from the local Pharmacy of Tadepalligudem. HPLC grade water was purchased from Thermo Fisher Scientific Ltd., Mumbai. HPLC grade Acetonitrile, Methanol and Orthophosphoric acid were procured from Merck specialties Pvt. Ltd., Mumbai.

Instrumentation and analytical conditions

RP-HPLC method was performed on the HPLC system (Shimadzu) consisting of binary gradient pump with UV detector (LC-20AD). Rheodyne injector with 20 μ l fixed loop was used for injecting samples on SHISEIDO C18 column (250 x 4.6 mm i.d, 5 μ) in the present study.

Preparation of solutions

Preparation of Efavirenz (drug) Stock solution

Accurately weighed 10 mg of Efavirenz working standard and transferred to 10 ml volumetric flask. Added 5.0 ml of Methanol : Water (50 : 50) and sonicated to aid dissolution. Make up the volume up to the mark with Methanol : Water (50 : 50) stopper the flask and shaken gently to mix the contents.

Preparation of Methylprednisolone [IS] stock solution

Accurately weighed 10 mg of Methylprednisolone working standard and transferred to 10 ml volumetric flask. Add 5.0 ml of Methanol : Water (50 : 50) and sonicated to aid dissolution. Make up the volume to the mark with Methanol : Water (50 : 50) stopper the flask and shaken gently to mix.

Drug intermediate stock solution

Aliquoted 1 ml of drug stock solution into 10 ml volumetric flask to get the concentration 100 μ g/ml. Added required volume of diluent [50 : 50 v/v Methanol : Water] and sonicated to aid

dissolution. Make up the volume to the mark with diluent. Stopper the flask and shaken gently to mix.

Internal standard intermediate stock solution

Aliquoted 1 ml of internal standard stock solution into 10 ml volumetric flask to get the concentration 100 μ g/ml. Added required volume of diluent [50 : 50 v/v Methanol : Water] and sonicated to aid dissolution. Make up the volume to the mark with diluent. Stopper the flask and shaken gently to mix.

Preparation of Spiked Plasma Calibration Curve and Quality Control samples

Prepared the CC standards in the range of 0.43, 0.86, 2.15, 3.2, 4.3, 6.4, 7.5 and 8.6 μ g/ml by spiking CC spiking solutions and QC standards in the range of 0.43, 1.075, 4.3, 8.6 μ g/ml by spiking QC spiking solutions in to screened human plasma.

The calibration curve standards were prepared by aliquoting 43, 86, 215, 320, 430, 640, 750 and 860 μ l of drug intermediate stock solution into a 10 ml volumetric flask and make up the volume with the diluent to get the concentrations 0.43, 0.86, 2.15, 3.2, 4.3, 6.4, 7.5, and 8.6 μ g/ml. The QC standards were prepared by aliquoting 43, 86, 430 and 860 μ l of drug intermediate stock solution into a 10 ml volumetric flask and make up the volume with the diluent to get the concentrations 0.43 (LLOQ), 0.86 (LQC), 4.3 (MQC) and 8.6 (HQC) μ g/ml.

Aliquoted 20 μ l of each CC standards, QC sample, 20 μ l of internal standard solution (1 μ g/ml) into pre-labelled Eppendorf tubes containing 50 μ L of plasma and capped them tightly. Stored CC standard, QC samples, standard blank and standard zero samples in deep freezer [below - 80°C].

Sample Preparation

The plasma sample used for the developed method was K₂-EDTA. The plasma was taken out from the refrigerator and thawed. It is then spiked with required concentration of sample. For 50 μ l of plasma, 20 μ l of sample solution and 20 μ l internal standard were added and vortexed for 5 min.

All the samples were prepared by using solid phase extraction (SPE) method. The SPE cartridges were purchased from phenomenex India Pvt. LTD. The cartridges were contained with C₁₈ stationary phase. The cartridges must be clean and dry. They must be checked before working. The procedure for sample preparation was done in four steps.

Step 1

SPE cartridges (strata C18-E from Phenomenex) are conditioned with 0.5 ml of methanol. Care should be taken that adsorbent bed should not be dry, methanol should be present upto the level of adsorbent bed.

Step 2

Then the cartridges were loaded with 0.5 ml of vortexed plasma sample. By applying vacuum the plasma should be drained off. The drug will be attached to the adsorbent.

Step 3

The Cartridges are washed with HPLC water to liberate polar impurities.

Step 4

Finally the cartridges were loaded with 0.5 ml of mobile phase to elute the drug from the adsorbent. This eluent was collected in to the Eppendorf tubes by applying vacuum. After analysis all the plasma samples were stored in refrigerator at -70°C.

Preparation of the mobile phase

The elution was done using mobile phase consisting of Acetonitrile and 20 mM phosphate buffer pH-3 (30:70 v/v). The buffer was prepared by dissolving 6.8g of potassium dihydrogen orthophosphate in 1000 ml water adjusted the pH with 0.1 M ortho phosphoric acid up to 3.0 ± 0.1 . The buffer was filtered through a 0.5 μ nylon membrane filter before prior to use. Column was equilibrated for at least 30 min with mobile phase flowing through the system.

Optimized chromatographic conditions

Mobile phase used	:	Acetonitrile:
Phosphate buffer pH	3.0 ± 0.1	
Mobile phase ratio	:	30 : 70 v/v
Flow rate	:	1.0 ml/min
Column	:	SHISEIDO C18 (250 × 4.6 mm, 5 μ m)
Detector wavelength	:	247 nm using SPD 20-AD Dualwavelength detector
Data station	:	Lab solutions
Column temp	:	Ambient
Injection volume	:	20 μ L
Diluent	:	Methanol : Water
Ratio	:	50 : 50
Internal standard	:	Methyl prednisolone
Retention times	:	Methylprednisolone 3.7 min and Efavirenz 5.7 min
Tailing factor	:	Methylprednisolone 1.1 and Efavirenz 1.2
Theoretical plates	:	Methylprednisolone 4256 and Efavirenz 5964

METHOD VALIDATION²⁴

The objective of validation of a Bioanalytical procedure is to demonstrate that it is suitable for its intended purpose. Bio Analytical Method validation is the process of demonstrating that analytical procedures are suitable for their intended use" [US Food and Drug Administration Draft Guidance for Industry, 2000].

Performed the experiments involved in method validation as discussed below.

System Suitability

Processed aqueous standard at MQC level and ran the chromatographic device with injecting six consecutive from aqueous standard. Calculated the mean, standard deviation [SD] and coefficient of variation [CV] for response ratio and retention time of drug and IS.

Acceptance Criteria

Coefficient of variation [CV] for Response ratio should be $\leq 5\%$ and retention time of drug and IS should be $\leq 2\%$.

Selectivity and Specificity

Prepared and processed six blank matrix and six LOQ standards using six different lots of plasma from six individual healthy donors receiving no medication. Analyzed the samples on a chromatographic device for the assessment of potential interferences with endogenous substances. Evaluated the interference at the Retention time of analyte and internal standard [IS] by comparing the response in blank matrix against the mean response of the extracted LOQ standard.

Acceptance criteria

1. Response of the interfering peaks at the retention time of the analyte[s] should not be more than 20 % of the mean LOQ response.
2. Response of the interfering peaks at the retention time of internal standard should not be more than 5 % of mean of internal standard response.
3. At least 80 % of the screened matrix lots should meet the above-mentioned acceptance Criteria. If the criteria for selectivity are not met then repeat the evaluation or modify/change the method to eliminate interference.

Sensitivity**Limit of detection**

LOD is the lowest quantity of a substance that can be distinguished from the absence of that

substance (a blank value) with a stated confidence level (generally 99%).

Limit of quantification

LOQ is the lowest concentration at which the analyte can not only be reliably detected but at which some predefined goals for bias and imprecision are met. The LOQ may be equivalent to the LOD or it could be at a much higher concentration it cannot be lower than the LOD.

Acceptance Criteria

The analyte S/N should be 3:1 for LOD and 10:1 for LOQ.

Linearity

Evaluated at least three calibration curves generated using spiked samples to establish linearity. The matrix based standard curve consisted of minimum of eight standard points excluding blanks. The organization of calibration curve is given below

Calculated the mean concentrations, S.D, CV and % nominal for all calibrants of the three calibration curves using the chosen regression algorithm. Not included standard blank and standard zero in the calibration curve. However, included the accepted standard from calibration curve. Tabulated the results of calibration curve parameters e.g. slope, intercept and coefficient of correlation.

Acceptance criteria

1. Consider the first run of STD-1 and STD-8. If first run fails for the acceptance criteria, consider the second one. Never consider both the values of duplicate standards for calculation.
2. At least 75 % non-zero standards should meet the following criteria, including the LLOQ [lower limit of quantitation] & the ULOQ [upper limit of quantitation].
3. Deviation of the LOQ standard from nominal concentration should not be more than $\pm 20\%$.
4. Deviation of standards other than LOQ from nominal concentration should not be more than $\pm 15\%$.

Accuracy and Precision

Prepared calibration standards and quality control samples. Processed the batch of CC

Standards and QC samples as per the method. Determined the mean concentrations, standard deviation, accuracy and precision at each LQC, MQC and HQC concentration level. Assessed both within and between-run accuracy and precision by analysing three validation batches. Intra-day accuracy and precision were evaluated by analysis of quality control samples at four different levels [n = six at each level] on the same day. These levels were chosen to demonstrate the performance of the method and to determine the lower limit of quantification of the method. The upper limit of quantification was given by the highest level of the calibration curve. Samples with concentration above this upper limit of quantification should be diluted prior to reanalysis. To assure the inter-day accuracy and precision, the intra-day assays were repeated on three different days. The overall performance was calculated.

Acceptance Criteria

Acceptance criteria should be met for three validation batches including ruggedness batch for within and between batch accuracy and precision.

Stock Solution Stability

Prepared stock solution of analyte[s] and internal standard. Diluted this stock solution with reconstitution solution or any other suitable solvent to appropriate concentration at LQC, MQC and HQC level. Evaluated analyte[s] and internal standard separately as given below for short term and long-term stability conditions.

Short Term Stability

Withdrawn an aliquot from the stock [analyte & IS] and kept it on the bench and recorded the time and transferred the remaining stock into refrigerator. After completion of six hrs or as per the requirement again withdrawn the stock from the refrigerator [comparison sample] and recorded the time. Prepared injection dilutions from both the stocks i.e. stock withdrawn from the refrigerator and stock kept on the bench. Injected six replicates of both the dilutions. Calculated the mean response of stability sample and comparison sample. Calculated the % Stability as given below

$$\text{Stability} = \frac{\text{Mean Response of Stability Sample}}{\text{Mean Response of Comparison Sample}} \times 100$$

Long Term Stability

Stored the stock solutions of analyte[s] and internal standard in refrigerator [2-8°C] for at least 3 weeks. On the recommended period of storage, removed the stability samples i.e. Analyte[s] and IS and allowed them to reach room temperature and prepared their appropriate dilution [stability sample]. Recorded the time of withdrawal refrigerator. Consecutively prepared fresh stock of analyte and Internal Standard followed by their dilution

[comparison sample]. Acquired six consecutive injections for stability and comparison sample with the help of chromatographic device and recorded their peak responses.

Evaluated the long-term stability of stock by comparing the mean response of freshly prepared stock solution after applying correction factor of concentration of analyte[s] or IS [comparison sample] versus stored stock [stability sample].

$$\% \text{ Stability} = \frac{\text{Mean Area response of stability stock} \times \text{Conc. of comparison stock}}{\text{Area response of comparison stock} \times \text{Conc. of Stability stock}} \times 100$$

Acceptance criteria

Percent stability of drug and IS stocks should be within the range of 90 -110 % for both short term and long-term stock stability.

samples against mean of back calculated concentrations of freshly processed comparison samples.

Dry Extract Stability

Retrieved six replicates of each LQC and HQC from the deep freezer and allowed them to thaw at room temperature, processed and stored them in the refrigerator below 8°C for a period of 24 hrs or any other recommended period. Labelled them as dry extract stability samples with appropriate sample Labels. On the completion of recommended period, analyzed the dry extract samples using freshly processed calibration curve and QC samples. This stability experiment was carried out, whenever the sample processing involves evaporation before injecting on chromatographic device. Stability duration is calculated as difference between the storage time in refrigerator and reconstitution time of stability sample.

Acceptance criteria

The percent stability of the analyte[s] should be within 85-115%.

Evaluated the Dry extract stability by comparing the mean of back calculated concentration of stability sample against the mean of back calculated concentration of comparison sample.

Freeze Thaw Stability in Matrix

Conducted at least 3 cycles of freeze-thaw stability. Identified three sets, containing six replicates [FT-C1, FT-C2 & FT-C3] of each LQC, MQC and HQC samples for freeze-thaw cycles and labelled them for freeze-thaw cycles after bulk spiking and prior to storage. All sets of freeze-thaw stability samples were stored at recommended temperature in the deep freezer. After a minimum of 24 hrs freezing, retrieved all thawed three FT C-1, FT C-2 and FT C-3 sets from deep freezer, kept at room temperature up to complete thawing, vortexed all samples, After the completion of first freeze-thaw cycle the samples were restored at the same temperature. After a minimum of 12 hrs freezing, retrieved FT C-2 and FT C-3 sets from deep freezer, kept at room temperature up to complete thawing, vortexed all samples, on the completion of second freeze-thaw cycle and restore them in deep freezer at the same temperature. After a minimum of 12 hrs freezing, retrieved FT C-3 set from deep freezer, kept at room temperature up to complete thawing, vortexed all samples, on the completion of third freeze-thaw cycle. Processed FT-C3 samples as per method with a set of freshly spiked calibration curve standards and quality control samples Evaluated the freeze-thaw stability by comparing the mean of back calculated concentrations of stability samples against mean of back calculated concentrations of freshly spiked comparison samples.

Acceptance Criteria

The percent stability of the analyte[s] should be within 85-115 %.

Bench Top Stability in Matrix

Retrieved six samples each of LQC, MQC and HQC from freezer (-70°) and allowed them to thaw at room temperature or in water bath at room temperature for 6.0 to 8.0 hrs. After 6.0 to 8.0 hrs taken another set of QCs and CCs from deep freezer and thawed these samples. Processed all the sets of low and high QC samples and analysed using freshly processed calibration curve along with QC samples. Evaluated the stability by comparing the mean of back calculated concentrations of stability

Acceptance Criteria

The freeze-thaw stability should be within the range of 85- 115% at LQC & HQC levels.

Long Term Stability in Matrix

Performed the long-term stability at -80°C or as per protocol requirement for a required period. Following an appropriate storage period, removed six replicates of LQC & HQC samples from deep freezer and allowed the samples to thaw. Processed and analysed them against freshly spiked calibration standards and QC samples [comparison sample] as per method. Evaluated the long-term stability by comparing the mean of back calculated concentration for stability samples to the mean of back calculated concentration of comparison sample. The long term stability duration is calculated as the difference between the date of analysis of QC samples [stability samples] and the date of preparation of spiked stability QC samples i.e. date of spiking.

Acceptance criteria

Percent stability of analyte[s] in matrix should be within the range of 85 %-115 %.

$$\% \text{ Recovery} = \frac{\text{Mean Peak Response of Extracted Samples}}{\text{Mean Peak Response of Aqueous Samples}} \times 100$$

Calculated the mean % of recovery, standard deviation [SD] and coefficient of variation [CV] for each concentration of LQC, MQC and HQC. Also calculated the global mean recovery.

Ruggedness

Evaluate the Ruggedness of the method by analysing one batch of accuracy and precision using a different column. [Same type] with a different analyst employing the same instrument or another instrument.

Acceptance criteria

Same as mentioned in linearity [point-8.5.5.4], accuracy and precision [point-8.5.5.5].

Matrix Effect

Prepared and processed four different lots of biological matrices at a concentration equivalent LQC and HQC in triplicate. Analysed the samples on a chromatographic device along with one set of CC standards prepared from a different lot of screened plasma. Calculated the mean concentration of QC samples against CC curve for individual lots.

Recovery

Withdraw six aliquots of spiked quality control samples of three different concentrations corresponding to LQC, MQC and HQC [low, medium and high-quality control samples] from freezer. QC samples of P&A batches used for recovery experiments. Processed and analysed the QC samples as per the method and recorded their absolute chromatographic peak responses. Simultaneously prepared aqueous spiked standards of concentrations corresponding to LQC, MQC and HQC and analysed them as per method and recorded their absolute Chromatographic peak response. Calculated the mean response of each concentration level of the extracted standard and aqueous spiked standard. Compared mean response of spiked standards [LQC, MQC and HQC] with respect to the mean response obtained from each concentration level of the aqueous spiked standard. Determined the percentage recovery for each batch of LQC, MQC and HQC by using the formula

Acceptance criteria

The accuracy at LQC & HQC levels of at least three different lots [75% of screened Lots] should be $\pm 15\%$ of the nominal value and precision should be less than 15%. Matrix effect is not observed if it meets the acceptance criteria.

Anticoagulant Effect

Spiked HQC and LQC in plasma containing different anticoagulant other than used for validation. Aliquoted into different pre-labelled polypropylene tubes and stored them in deep freezer below -80°C. Processed and analysed six aliquots of LQC, MQC & HQC [in EDTA Plasma or any anticoagulant] and injected along with CC and QCs [CPDA Plasma]. Calculated mean % Nominal for LQC, MQC & HQC prepared with EDTA [or any anticoagulant] plasma.

Acceptance criteria

The effect of change in anticoagulant is deemed acceptable if the mean % nominal is within $\pm 15\%$ and CV is $\leq 15\%$.

RESULTS AND DISCUSSION

Selectivity/ Specificity

A method is said to be specific when it produces a response only for a single analyte. Method selectivity is the ability of the method to produce a response for the analyte in the presence of other interferences. In order to prove that the method chosen was specific and selective the following two sets of samples were processed and injected into the HPLC using the extraction procedure.

1. Blank sample of biological matrix (plasma containing sodium citrate (11%) as anticoagulant. (Fig.2: Showing chromatogram of blank).
2. Samples from the same biological matrix mentioned in step 1 spiked with the analyte at the lower limit of quantification (LLOQ) of the calibration curve and with the internal standard at the concentration level in the study. (Fig. 3: Showing chromatogram of optimised chromatographic trail).

System suitability

System suitability parameters such as asymmetric factor, resolution factor, theoretical plates, LOD and LOQ were evaluated from the chromatogram. (Table 1: Showing System suitability studies)

Sensitivity

The Limit of Detection for Efavirenz was 66 ng/ml.

The Limit of Quantification for Efavirenz was 346 ng/ml.

Linearity

Linearity and range of the method was analysed by preparing calibration curves using different concentrations of the standard solution containing the internal standard. The calibration curve was plotted using response factor and concentration of the standard solutions.

Linearity was established according to C_{max}^{19} of Efavirenz. It was 4.3 $\mu\text{g}/\text{ml}$ and the concentrations of Efavirenz were prepared as 10, 20, 50, 75, 100, 150, 175 and 200% of C_{max} . The approximate concentrations were 0.43, 0.86, 2.15, 3.2, 4.3, 6.4, 7.5, and 8.6 $\mu\text{g}/\text{ml}$. Linearity was established over the range of (0.43 to 8.6 $\mu\text{g}/\text{ml}$ for efavirenz) using the weighted least square regression analysis.

PRECISION AND ACCURACY

The precision and accuracy of the method was determined by analysing two batches each consisting of one set of calibration curve with six replicates of quality control samples at four concentration levels [Quality Control samples at

the lower limit of quantification (LLOQ), Low (LQC), Middle (MQC) and High(HQC)].

A. PRECISION

Precision is expressed as the percentage coefficient of variation (%CV) which is calculated as per the following expression: $CV = (\text{Standard Deviation} / \text{Mean}) \times 100$

Intra-run precision

Intra-run precision was determined by calculating the percentage coefficient of variation (% CV) of the results obtained in the same run. (Table 3: showing Precision study for Efavirenz (Intra run))

ACCURACY

Accuracy is reported as % nominal of the analysed concentration which is calculated as $\% \text{ Nominal} = (\text{Measured Concentration} / \text{Actual Concentration}) \times 100$

Accuracy was determined by calculating the percentage nominal of the calculated concentration from the actual values for quality control samples at each concentration level analyzed in a single run and the mean of percentage nominal at each level was reported (Table 4: showing Accuracy of Efavirenz).

Stock solution stability

Short term stock dilution stability at room temperature

Acceptance Criteria

% stability should be within 90 to 110% or the % change should be $\pm 10\%$.

$\% \text{Stability} = (\text{Mean response of stability samples} / \text{Mean response of comparison samples}) \times 100\%$.

$\% \text{Change} = 100 - (\text{Mean response of stability samples} / \text{Mean response of Comparison samples} \times 100)$

(Table 5: showing Short term stock solution study of Efavirenz).

Short term stock dilution stability in refrigerator

Acceptance Criteria: % change should be $\pm 10\%$.

$\% \text{Stability} = (\text{Mean response of stability samples} / \text{Mean response of comparison samples}) \times 100\%$.

$\% \text{Change} = 100 - (\text{Mean response of stability samples} / \text{Mean response of Comparison samples} \times 100)$

(Table 6: showing short term Stability study of Efavirenz).

Long term stock solution stability

Correlation factor (CF) = $\text{Concentration of comparison stock} / \text{Concentration of stability stock}$

$\%Stability = (\text{Mean response of stability samples} / \text{Mean response of comparison samples}) \times CF \times 100\%$

$\%Change = 100 - (\text{Mean response of stability samples} / \text{Mean response of comparison samples}) \times CF \times 100\%$. (Table 7: showing Long term stock solution stability study of Efavirenz).

Dry Extract Stability

Retrieved six replicates of each LQC, MQC and HQC from the deep freezer (-70°C) and allowed them to thaw at room temperature, processed and stored them in the refrigerator below 8°C for a period of 24 hrs. Labelled as dry extract stability samples and analyzed using freshly processed calibration curve and QC samples. (Table 8: showing dry extract stability study of Efavirenz).

Bench Top Stability in Matrix

Percentage stability of the QC samples were determined after 6 to 8 hours of thawing at room temperature. (Table 9: showing Bench top stability in matrix).

Freeze thaw stability

Six quality control samples each at LQC, MQC and HQC concentrations stored below -70°C for at least 24 hours were removed from the deep freezer and were allowed to thaw at least for three cycles. (Table 10: showing Stability study of Efavirenz)

Long-term (LT) stability

The stability of the analyte was evaluated by comparing each of the back calculated concentrations of stability Quality Control sample (QCs) with the Mean concentrations of the respective QCs freshly prepared. (Table 11: showing Stability study of Efavirenz).

Recovery

% Recovery of analyte at each level was calculated using the following expression

$\{[\text{Individual analyte peak area of extracted QCs} \times \text{Concentration of analyte added (unextracted sample)}] / [\text{Mean analyte peak area of aqueous QCs} \times \text{Concentration of analyte added (extracted sample)}]\} \times 100$

(Table 12: showing recovery (change in mobile phase) study for Efavirenz).

Robustness

Robustness of the method was studied by injecting the standard solutions with slight variations in the optimized conditions namely, $\pm 1\%$ in the ratio of acetonitrile in the mobile phase, varying pH range ± 1 and ± 0.1 ml of the flow rate. (Table 13: showing Ruggedness (change in flow rates) study for Efavirenz and Table 14: showing Ruggedness (change in mobile phase) study for Efavirenz)

Ruggedness

Ruggedness of the method was studied by changing the experimental conditions such as operators, instruments, source of reagents, solvents and column of similar type. (Table 15: showing ruggedness study for Efavirenz).

Matrix Effect

The drug was spiked in plasma, serum and urine matrices and injected into HPLC after SPE preparation in three replicates. The system suitability parameters were calculated accordingly. (Fig. 13: Spiked drug and internal standard in plasma and Fig. 14: Spiked drug and internal standard in serum). (Table 16: showing matrix effect of Zidovudine).

Observation

The chromatogram in serum showed no much difference when compared to plasma. So this method can be applied for the estimation of Zidovudine in serum samples. No peak was observed in urine.

Table 1: Showing System suitability studies

S.No	Parameters	Drug
1	Theoretical Plates	5964
2	Resolution factor	7.566
3	Asymmetric factor	1.203
4	Tailing factor	1.2
5	LOD(ng/ml)	66
6	LOQ(ng/ml)	346

Table 2: showing Concentration-Response factor for Efavirenz

Concentration (µg/ml)	Peak area of drug	Peak area of IS	Response factor
0.43	4947	104441	0.047366
0.86	9134	104461	0.107437
2.15	12046	104497	0.115276
3.20	16410	104481	0.157062
4.30	21438	104451	0.205245
6.40	33744	118689	0.284306
7.50	36410	114980	0.316664
8.60	36715	102871	0.356903

Table 3: showing Precision study for Efavirenz (Intra run)

S.NO	Nominal Concentration(µg/ml)			
	LLOQ	LQC	MQC	HQC
	0.43	0.86	4.3	8.6
1	0.4213	0.8561	4.3622	8.5812
2	0.4256	0.8593	4.3925	8.5775
3	0.4315	0.8652	4.3575	8.6291
4	0.4313	0.8691	4.3254	8.6383
5	0.4279	0.8546	4.3873	8.6042
6	0.4285	0.8578	4.3262	8.6152
Mean	0.4276	0.8603	4.3585	8.6075
S.D	0.0038	0.0056	0.0287	0.0248
C.V%	0.8961	0.6550	0.6601	0.2882
%Nominal	99.46	100.04	101.36	100.08
N	6	6	6	6

Table 4: showing Accuracy of Efavirenz

Level	Concentration of drug added(µg/ml)	Amount of drug recovered (µg/ml) in plasma sample	Recovery (%)	Amount of drug recovered(µg/ml) in Mobile phase	Relative Recovery (%)
LQC	0.86	0.85	Mean : 98.83, CV : 0.22, N:6	Mean : 98.96, CV :0.99, N :6	99.86
MQC	4.3	4.23	Mean : 98.37, CV: 0.034, N:6	Mean : 99.26, CV :0.98, N :6	99.10
HQC	8.6	8.35	Mean : 97.09, CV : 0.022, N : 6	Mean :99.17, CV :0.56, N :6	97.90

Table 5: showing Short term stock solution study of Efavirenz

Stock dilutions at room temperature	LQC	% Change	MQC	% Change	HQC	% Change
	0.43	--		4.3	--	8.6
After 1hr	0.43	0	4.3	0	8.6	0
After 2hr	0.429	0.232	4.289	0.255	8.582	0.209
After 3hr	0.428	0.465	4.276	0.303	8.578	0.255
N	3		3		3	

Table 6: showing short term Stability study of Efavirenz

Stock dilutions at 2-8°C temperature	LQC	% Change	MQC	% Change	HQC	% Change
	0.43	--		4.3	--	8.6
After 1hr	0.430	0	4.300	0	8.600	0
After 2hr	0.429	0.232	4.295	0.116	8.592	0.093
After 3hr	0.429	0.232	4.286	0.209	8.588	0.139
N	3		3		3	

Table 7: showing Long term stock solution stability study of Efavirenz

Stock dilutions at 2-8°C temperature	LQC	% Change	MQC	% Change	HQC	% Change
		0.43	--	4.3	--	8.6
After 1 st week	0.430	0	4.300	0	8.600	0
After 2 nd week	0.429	0.232	4.295	0.116	8.592	0.093
After 3 rd week	0.429	0.232	4.286	0.209	8.588	0.139
N	3		3		3	

Table 8: showing dry extract stability study of Efavirenz

Dry extract Stability	LQC	% Change	MQC	% Change	HQC	% Change
		0.43	--	4.3	--	8.6
After 24 hrs*	0.404	6.04	4.061	5.55	8.172	4.97

Table 9: showing Bench top stability in matrix

Bench Top Stability in Matrix	LQC	% Change	MQC	% Change	HQC	% Change
		0.43	--	4.3	--	8.6
After 6 hrs*	0.393	8.60	3.946	8.23	7.886	8.30

Table 10: showing Stability study of Efavirenz

Freeze and thaw	LQC	% Change	MQC	% Change	HQC	% Change
		0.43	--	4.3	--	8.6
Cycle 1	0.429	0.2325581	4.285	0.3488372	8.575	0.29069767
Cycle 2	0.427	0.6976744	4.274	0.6046512	8.552	0.55813953
Cycle 3	0.425	1.1627907	4.258	0.9767442	8.495	1.22093023
Mean	0.427		4.272		8.540	
S.D (+/-)	0.002		0.0135		0.0411	
C.V. (%)	0.4683		0.3177		0.4822	
%Nominal	99.30		99.35		99.31	
N	3	--	3	--	3	--

Table 11: showing Stability study of Efavirenz

Long Term plasma Sample at -70 ^o	LQC	% Change	MQC	% Change	HQC	% Change
		0.43	--	4.3	--	8.6
After 1 st week	0.425	1.1627907	4.258	0.9767442	8.494	1.23255814
After 2 nd week	0.421	2.0930233	4.234	1.5348837	8.445	1.80232558
After 4 th week	0.418	2.7906977	4.212	2.0465116	8.415	2.15116279
Mean	0.421		4.234		8.451	
S.D (+/-)	0.0035		0.0230		0.0398	
C.V. (%)	0.8335		0.5433		0.4718	
%Nominal	97.98		98.48		98.27	
N	3	--	3	--	3	--

Table 12: showing recovery (change in mobile phase) study for Efavirenz

Concentration Level	Concentration of Spiked sample (ng/ml) (extracted)	Concentration of aqueous sample (un extracted)	Peak area of extracted sample	Peak area of un extracted sample	Recovery
Level-I	0.43	0.43	4947	5241	94.39
Level-II	4.3	4.3	21438	22348	95.92
Level-III	8.6	8.6	36715	38926	94.31

**Table 13: showing Ruggedness
(change in flow rates) study for Efavirenz**

Sl. No	Flow rate (ml/ min)	Retention time (min)	Peak Area	RSD (%)	System suitability results	
					Plate count	Tailing factor
1	Less Flow (0.9)	5.8	21438	0.03	5974	1.23
			21444			
			21428			
2	Actual Flow (1.0)	5.7	21538	0.02	5964	1.13
			21437			
			21624			
3	More Flow (1.1)	5.6	21457	0.30	5944	1.13
			21463			
			21455			

**Table 14: showing Ruggedness
(change in mobile phase) study for Efavirenz**

Sl. No	Mobile Phase	Retention time (min)	Peak Area	RSD (%)	System suitability results	
					Plate count	Tailing factor
1	Less Organic (75:25)	5.8	21438	0.05	5969	1.24
			21508			
			21428			
2	Normal (65:35)	5.7	21424	0.89	5964	1.13
			21442			
			21417			
3	More Organic (60:40)	5.6	21526	0.09	5979	1.27
			21501			
			21441			

Table 15: showing ruggedness study for Efavirenz

Sl. No	Analyst	Retention time (min)	Peak Area	RSD (%)	System suitability results	
					Plate count	Tailing factor
1	Analyst 1	5.7	21438	0.03	5964	1.13
			21508			
			21428			
2	Analyst 2	5.6	21524	0.05	5974	1.24
			21442			
			21617			

Table 16: showing matrix effect of Zidovudine

Sl. No	Matrix	Retention time (min)	Peak Area	RSD (%)	System suitability results	
					Plate count	Tailing factor
1	Plasma	5.7	21525	0.6	5968	1.02
2	Serum	5.7	21438	0.8	6025	1.02
3	Urine	--	--	--	--	--

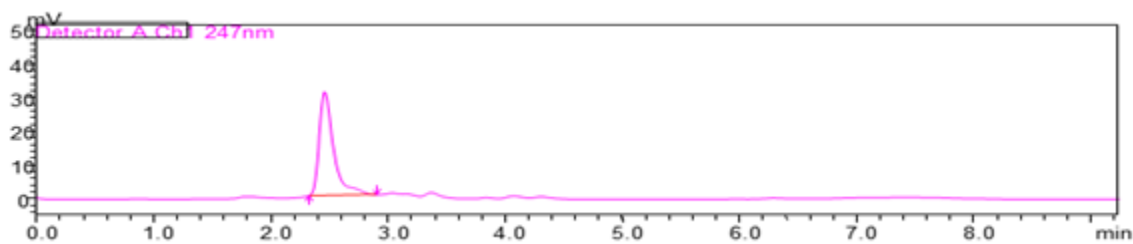


Fig. 2: Showing chromatogram of blank

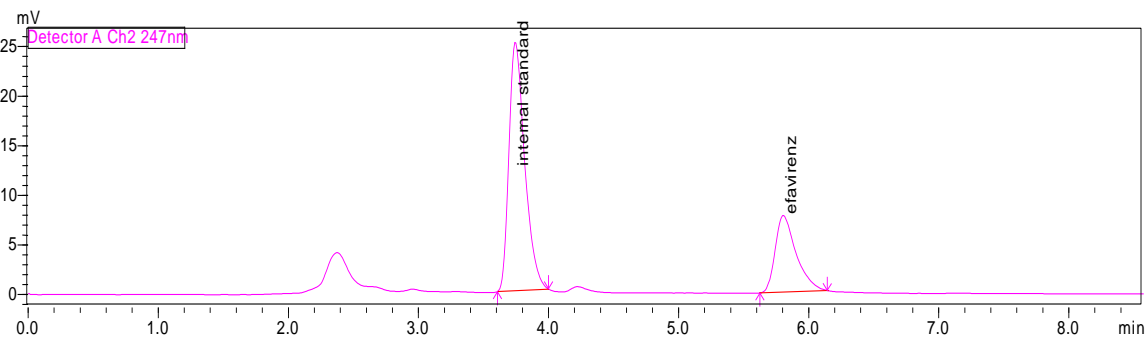


Fig. 3: Showing chromatogram of optimised chromatographic trail

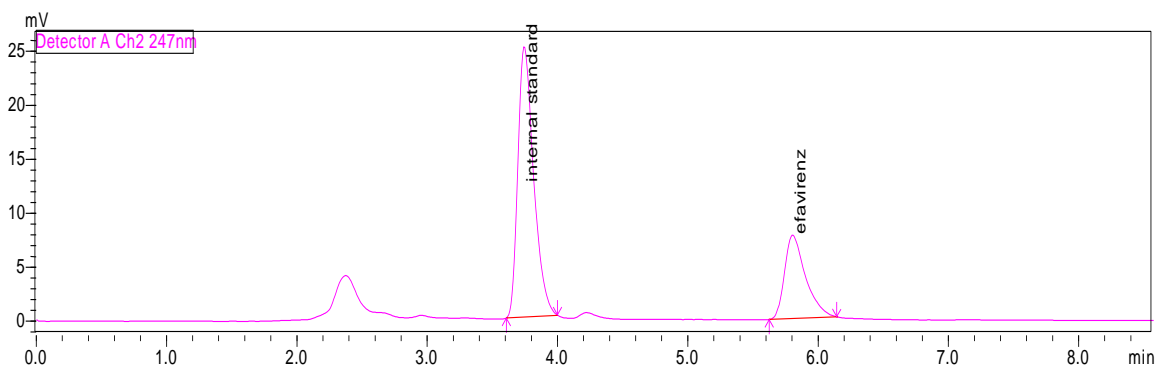


Fig. 4: showing Chromatogram for 0.43 µg/ml

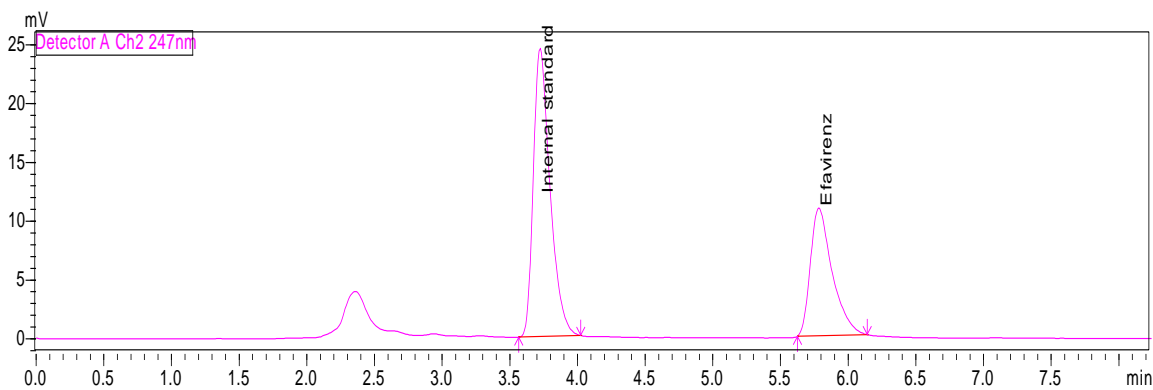


Fig. 5: showing Chromatogram for 0.86 µg/ml

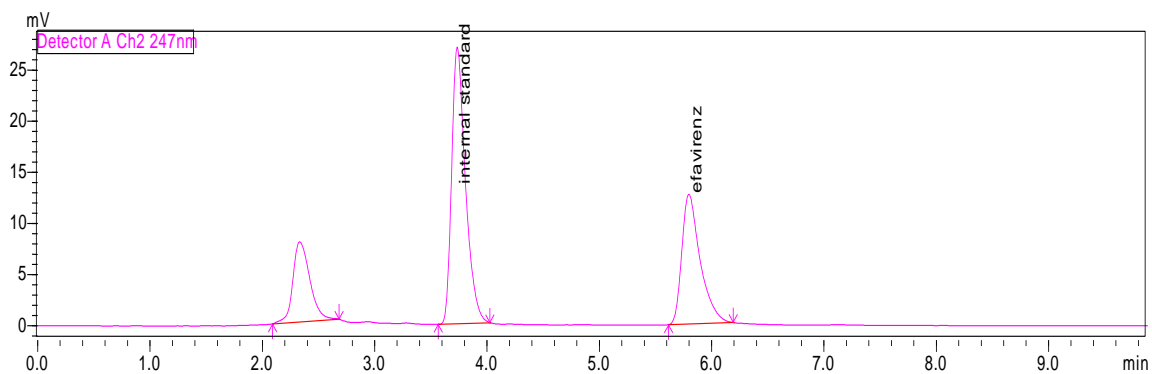


Fig. 6: showing Chromatogram for 2.15 µg/ml

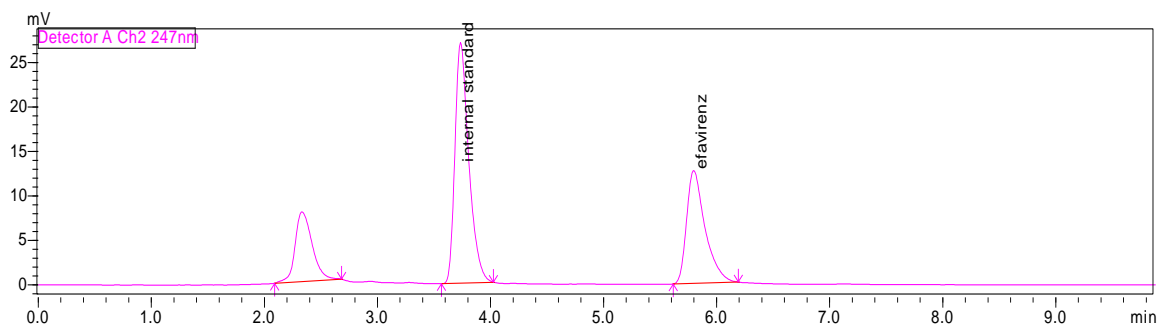


Fig. 7: showing Chromatogram for 3.2 µg/ml

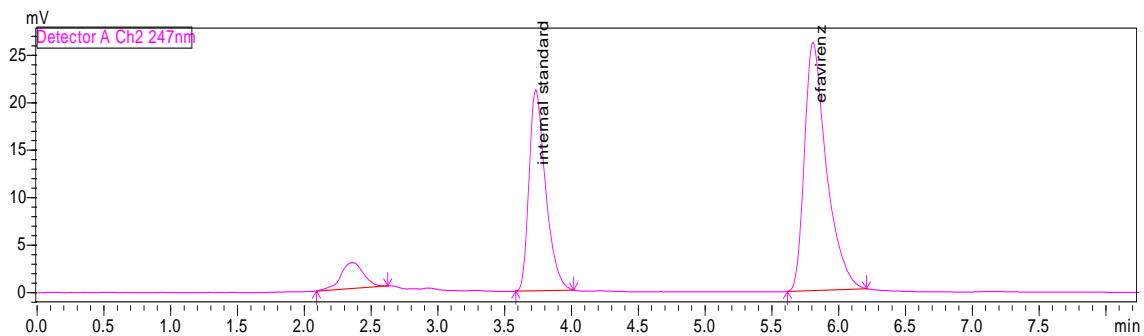


Fig. 8: showing Chromatogram for 4.3 µg/ml

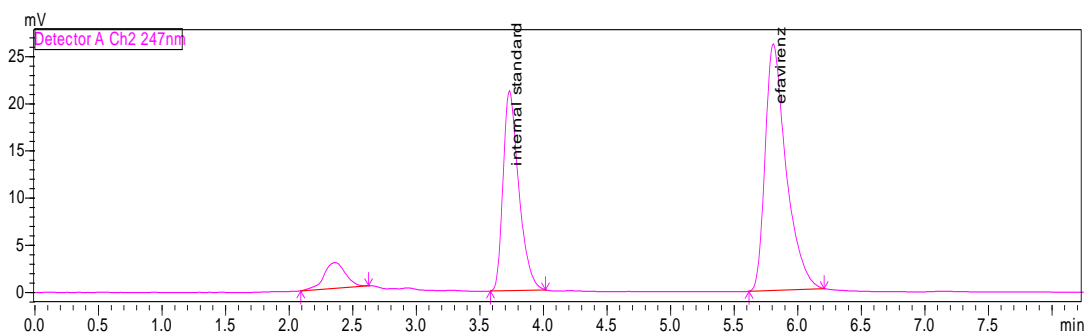


Fig. 9: showing Chromatogram for 6.4 µg/ml

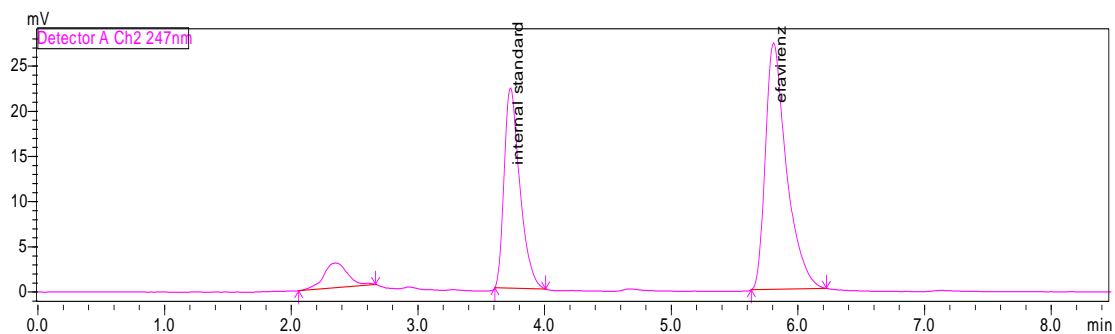


Fig. 10: showing Chromatogram for 7.5 µg/ml

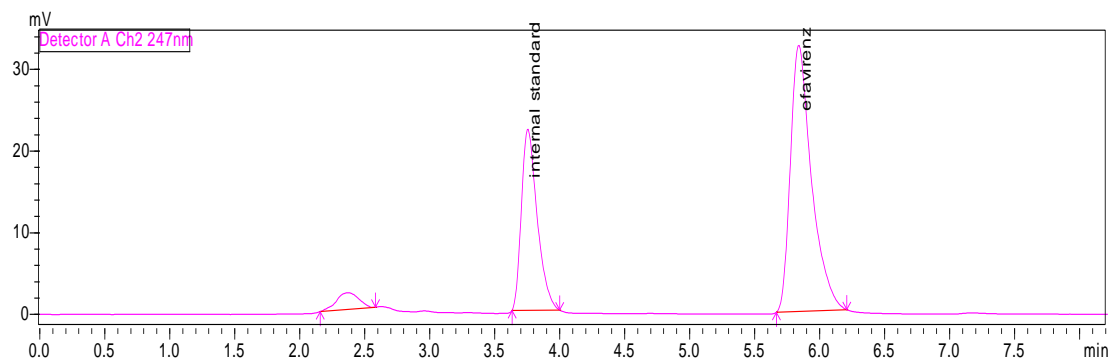


Fig. 11: showing Chromatogram for 8.6 µg/ml

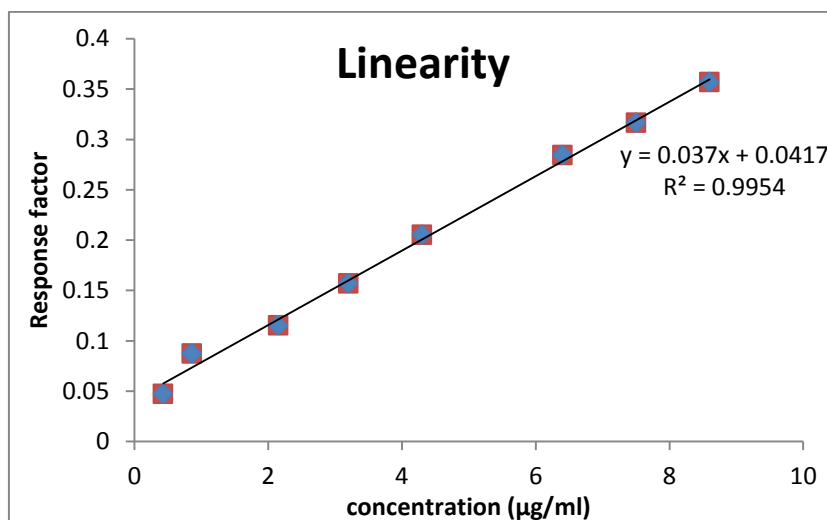


Fig. 12: showing Calibration curve of Efavirenz

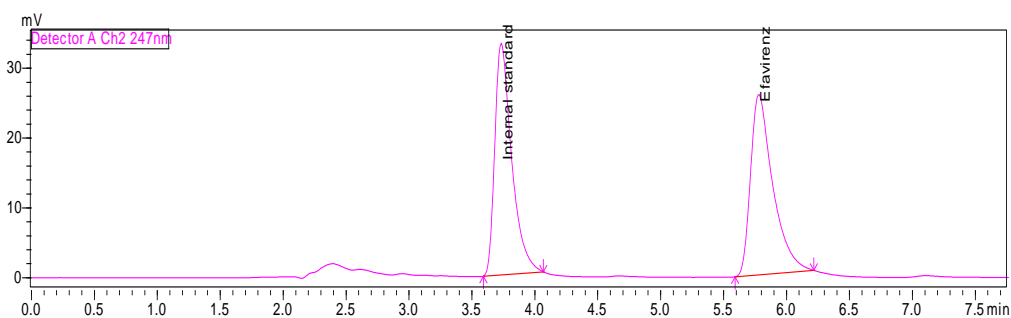


Fig. 13: Spiked drug and internal standard in plasma

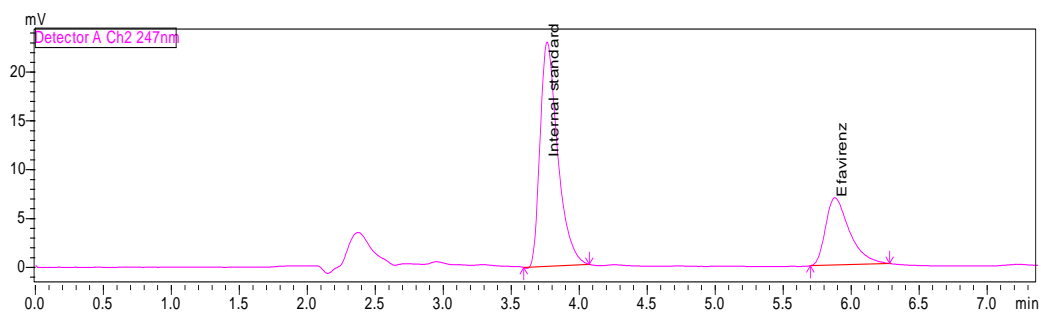


Fig. 14: Spiked drug and internal standard in serum

Anticoagulant Effect

Sl. No	Anticoagulant	Retention time (min)	Peak Area
1	K ₂ - EDTA	5.5	1603
2	K ₃ - EDTA	5.7	124

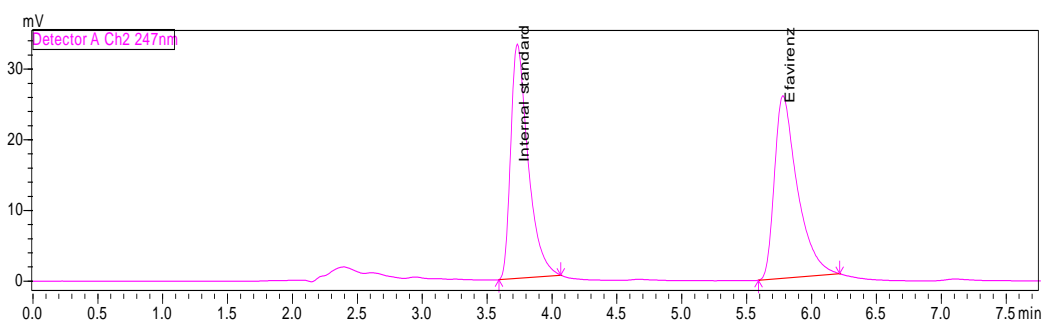


Fig. 15: Spiked drug and internal standard in plasma with K₃EDTA

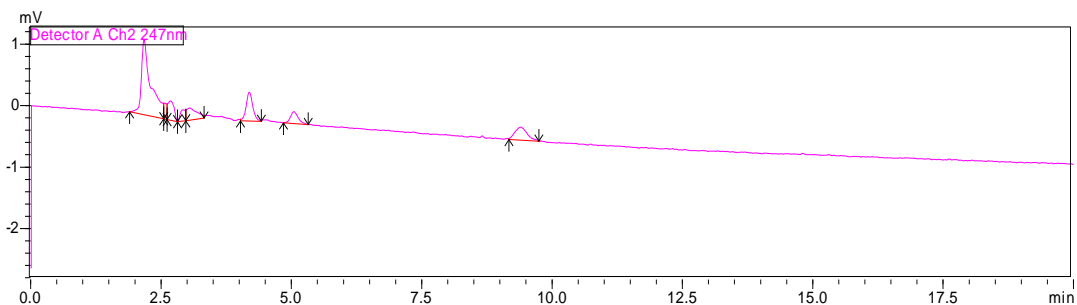


Fig. 16: Spiked drug and internal standard in plasma with K₄EDTA

Observation

The chromatogram with K₂EDTA is good when compared with K₃EDTA. K₃EDTA showed much interferences and negative peaks. The system suitability parameters were also poor for K₃EDTA.

CONCLUSION

The proposed method was found to be simple, precise, accurate and rapid for determination of Efavirenz in Human Plasma. The mobile phase is simple to prepare and economical. The method was validated as per USFDA guidelines, and validation acceptance criteria were met in all cases. In summary, the RP-HPLC method described in this work are reproducible, specific and sensitive enough for the selective and reliable determination of molecules in human plasma for routine bioequivalence and pharmacokinetic analysis and were validated in the specified ranges according to internationally accepted criteria. The sensitivity of the method was high as the limit of detection and limit of quantification are very low.

The selectivity and specificity of the method is good as there were no interferences or plasma peaks at the retention times of the drug. The linearity was done according to C_{max} with eight non-zero standards. Linearity was established with good regression coefficient and slope. The accuracy and precision data gives a result, which were within the acceptance limits. Consistent recoveries were observed. The method is specific in the presence of different anticoagulants [CPDA and EDTA] and matrices collected from different sources. The desired sensitivity was achieved with an LLOQ, which has within-batch and between-batch coefficients of variance [CVs], respectively.

Use of rapid extraction, chromatographic separation, makes them attractive procedures in high-throughput bioanalysis of analytes. The method is applied successfully to analysis and quantification of concentrations of analytes in plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies after therapeutic doses. However further studies needed in the pharmaceutical field to build up more sophisticated bioanalytical assay methods for the quantification of drug in human biological matrix. i.e., serum, tissue and urine.

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