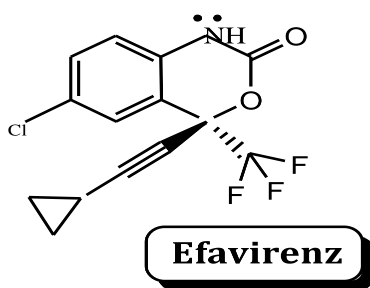
# CHAPTER - V

## ANALYSIS OF EFIVARIENZ IN PURE AND PHARMACEUTICAL FORMULATIONS BY VISIBLE SPECTROPHOTOMETRY AND RP-HPLC

## PART–A: NEW VISIBLE SPECTROPHOTOMETRIC METHODS FOR THE QUANTIFICATION OF EFAVIRENZ [EFZ] IN PURE AND TABLET FORMS

### 5.01-A: INTRODUCTION

Efavirenz [(S)-6-chloro-4-(cyclopropylethynyl)1,4-dihydro-4(trifluoromethyl) -2H-3,1-benzoxazin-2-one belongs to the class of non-nucleoside reverse transcriptase inhibitors and is used in the treatment of HIV infection1-4. Efavirenz bind directly to heterodimeric HIV-1 reverse transcriptase and appear to inhibit viral RNA- and DNA-dependent DNA polymerase activities by disrupting the catalytic site of the enzyme. **It** is available in the brand names such as **Sustiva** and **Stocrin [Label claim]**. It is never used alone and is always given in combination with other drugs.




Literature survey reveals that only HPLC methods are available for the estimation of efavirenz in combination with other drugs, in its dosage form and in plasma [5-10]. But only one UV spectrophotometric [11] and visible spectrophotometric [12] method has been reported in literature. As the analytically useful functional groups present in EFZ have not been fully exploited for designing suitable visible spectrophotometric methods and therefore still offer a scope to develop more number of new visible spectrophotometric methods with better sensitivity, selectivity, precision and accuracy. The author has made some attempts in this direction and succeeded in developing eight new visible spectrophotometric methods for the quantification of Efavirenz [EFZ] in pure and tablet dosage forms.

**5.02-A: EXPERIMENTAL**

**i)UV-Visible Spectrophotometer:** An Elico SL-159 model,2nm high resolution, double beam, 1cm length quartz coated optics; Wavelength range190-1100nm ; High stability, linearity, precision instrument was used for all the spectral measurements.

**ii) Preparation of reagents:** Analytical grade chemicals and reagents were used in the preparation of solutions and all the solutions were prepared in triply distilled water.

**Method $M_{1a}$:** Solutions of various reagents such as **MBTH solution** (Fluka; 0.2%, $8.56x10^{-3}M$), **IBDA solution** (Qualigens,0.2%; $6.21x10^{-3}M$and **Acetic acid solution** (Qualigens; 2.3M) were prepared in the same way as described under SVD in chapter – II (p.82).

**Method $M_{1b}$:** Solutions of various reagents such as MBTH Solution (Fluka; 0.2%, $8.56 \times 10^{-3}M$ and Ce(IV)solution (Merck); 1%, $9.35 \times 10^{-3}M$ were prepared in the same way as described under SVD in chapter – II (p.82).

**Method $M_{1c}$:** Solution of **MBTH solution** (Fluka; 0.2%, $8.56x10^{-3}M$)is prepared by dissolving 200mg of 3-Methyl-2-Benza Thiazolinone Hydrazone (MBTH) with distilled water in 100mL volumetric flask and **$NaIO_4$ solution**(BDH; 0.2%, $9.35 \times 10^{-3}M$) is prepared by dissolving 200mg of sodium meta periodate in 100mL of distilled water and standardized iodometrically.

**Method $M_5$**

| | | |
|---|---|---|
| **AMV Solution** (Loba; 0.3%, $1.80x10^{-1}$ M) | : | Prepared by dissolving 300mg of ammonium meta vanadate in 100mL of distilled water |
| **$H_2SO_4$ (18M)** | : | AR Conc (18M)Sulphuric acid was used directly. |

**Method $M_6$**

| | | |
|---|---|---|
| **PFC Solution** (BDH,0.1%,) | : | 100mgs of Potassium Ferricynide was accurately weighed and transferred into 100mL volumetric flask ,dissolved in triple distilled water and made up to the mark. |
| **Fe(III) Solution** (Wilsonlabs,0.054%) | : | 54mgs of Ferric chloride was accurately weighed and transferred into 100mL volumetric flask ,dissolved in triple distilled water and made up to the mark. |

**Method $M_9$:** Solutions of various reagents such as **NQS solution** (Loba, 0.5%,$1.92 \times 10^{-2}$M) and **NaOH solution** (Loba, 20%, 5.0M)were prepared in the same way as described under SVD in chapter – II .

**Methods $M_{11a}$ and $M_{11b}$:** Solutions of various reagents such as **Tpooo solution** (Fluka; 0.2%, $5.70 \times 10^{-3}$M) and **ARS solution** (0.2%,$5.84 \times 10^{-3}$M) and HCl (Qualigens; 0.1M) were prepared in the same way as described in **Chapter – II**. Chloroform of analytical grade is used as it is.

**c) Preparation of standard drug solution**

0.1% stock solution of efivarienz was freshly prepared by transferring accurately weighed 100mg of efivarienz into 100mL volumetric flask and dissolved in double distilled water, and then made up to the mark. Then working standard solutions, 100μg $mL^{-1}$ and 200μg $mL^{-1}$ are prepared by transferring 10.0mL , and 20.0mL of the stock solution into two 100mL standard flasks respectively and made up to the mark. Working standard concentration of 100μg.$mL^{-1}$ for the methods $M_{11a}$ and $M_{11b}$ and 200μg.$mL^{-1}$ was used for the following

methods $M_{1a}$, $M_{1c}$, $M_3$, $M_5$, $M_6$ and $M_9$. An aliquot of this solution was used for the determination of each zidovudine drug as per the procedures described earlier.

**d)Pharmaceutical preparations**

Twenty tablets of each efivarienz drug was finely powdered in a small dish. Fifty mg of this powder was dissolved in about 10.0mL of ethanol and filtered through a Whatman No. 42 filter paper. The filtrate was made up to mark with distilled water in a 100mL volumetric flask. A suitable volume of the filtrate was accurately diluted with water to get a sample concentration of 100µg.mL$^{-1}$ for the methods $M_{11a}$ and $M_{11b}$ and 200µg.mL$^{-1}$ working standard solution was used for the following methods $M_{1a}$, $M_{1c}$, $M_3$, $M_5$, $M_6$ and $M_9$. An aliquot of this solution was used for the determination of each efivarienz drug as per the procedures described earlier.

## 5.03-A: PROPOSED PROCEDURES

### Method $M_{1a}$

Aliquots of standard efavirenz solution (0.5-3.0mL) were transferred into a series of 20.0mL calibrated tubes. Then 1.0mL (9.35 x $10^{-3}$M) of IBDA solution,1.0mL of acetic acid solution were and the total volume was adjusted to 10.0 mL and kept in a water bath for 45min. The solutions were cooled suddenly. After that 1.0mL (8.56 x $10^{-3}$M) of MBTH solution was added and kept aside for 10min. The volume was made up to the mark with distilled water. The absorbance was measured at 640nm against a similar reagent blank. The amount of efavirenz was computed from its calibration graph. (**Fig.5.09, P.208)**

**Method $M_{1b}$**

Aliquots of standard efavirenz solution (0.5-3.0mL) were transferred into a series of 20.0mL calibrated tubes. Then 1.0mL (8.56 x $10^{-3}$M) of MBTH solution was added and kept aside for 5min. After that 1.0mL (1.58 x $10^{-2}$ M) of cerric ammonium sulphate was added and kept aside for 10min. The volume was made upto the mark with distilled water. The absorbance was measured at 630nm against a similar reagent blank. The amount of efavirenz was computed from its calibration graph. (**Fig.5.10, P.208**).

**Method $M_{1c}$**

Into a series of 20.0mL calibrated tubes standard efavirenz solution (0.5-3.0mL) were transferred. Then 1.0mL (9.35 x $10^{-3}$M) of $NaIO_4$ solution,1.0mL of acetic acid solution were and the total volume was adjusted to 10.0 mL and kept in a water bath for 45min. The solutions were cooled suddenly. After that 1.0mL (8.56 x $10^{-3}$M) of MBTH solution was added and kept aside for 10min. The volume was made up to the mark with distilled water. The absorbance was measured at 650nm against a similar reagent blank. The amount of efavirenz was computed from its calibration graph(**Fig.5.11, P.208**).

**Method-$M_5$**

Aliquots of standard efavirenz drug solution (0.5mL-30.0mL) were delivered in to a series of 20mL calibrated tube. To each tube 2.0mL of AMV (4.27x $10^{-1}$ M) reagent and 3.0mL of 18 M $H_2SO_4$ were added to each tube and the contents were heated for 20min in boiling water bath. After cooling the volume was made up to 20mL with distilled water. The resulting absorbance of the green color was measured at 770nm against a reagent blank. The amount of drug was computed from to appropriate calibration graph (**Fig.5.12, P.208**).

**Method $M_6$**

Different aliquots of (0.5-3.0mL) of standard efavirenz solution are transferred into a series of 20.0mL calibrated tubes and then a solution of 1.0mL of Fe (III) is added. The tubes are stoppered and shaken well for 5min. Then 1.0mL of PFC solution was added into each tube and is closed with lids .After five minutes 1mL of 1N HCl is added and the final volume was made up to 20mL with distilled water. The absorbance of the solution in each tube is measured immediately at 790nm against a similar reagent blank. The amount of the drug is calculated from its calibration graph(**Fig.5.13, P.209**).

**Method – $M_9$**

Varying aliquots of standard efavirenz solution (0.5 – 3.0mL; 100μg.mL$^{-1}$) were transferred into a series of 25mL calibrated tubes containing 2mL of 5.0N NaOH and 0.5mL (1.92 x $10^{-2}$M) of NQS reagent solution was added in each tube and the contents were heated at $50^0$c for 5min and cooled for 2min in ice water. This operation was performed in the dark. After cooling the contents in the tube were rinsed with 1.0mL of water. Then 0.5mL of con $H_2SO_4$ was added slowly, mixed and the absorbance were measured after 5min at 464nm against a reagent blank prepared similarly. The amount of efavirenz was calculated from its calibration graph (**Fig.5.14, P.209**).

**Method – $M_{11a}$ &$M_{11b}$**

Into a series of 125mL separating funnels containing aliquots of standard efavirenz solution (0.5-3.0mL for the methods $M_{11a}$, & $M_{11b}$ respectively), 6.0mL of 0.1M HCI solution and 2.0mL 0.2% TPooo dye (for Method$M_{11a}$), 1.0mL of 0.2% ARS dye solution (for Method$M_{11b}$)were added successfully. The total volume of aqueous phase in each separating funnel was adjusted to 15.0mL with distilled water. To each separating funnel 10mL of chloroform was added and the contents were shaken for 2min. The two phases were allowed

to separate and the absorbance of the separated chloroform layer was measured at λmax (TPooo 430nm;ARS 650nm) against a similar reagent blank. In both spectrophotometric methods **[$M_{11a}$ & $M_{11b}$]** a standard graph was prepared by plotting the increasing absorbance values versus concentration of efavirenz (μg/mL). The concentration of the unknown was read from the standard graph or computed from the respective regression equation derived using the Beer's law data(**Fig.5.15&5.16, P.209**).

### 5.04-A: RESULTS AND DISCUSSIONS

#### i. Spectral Characteristics

In order to ascertain the optimum wavelength of maximum absorption ($\lambda_{max}$) of the colored species formed in the above methods, specified amounts of efavirenz were taken and colors were developed separately by following the above procedures. The absorption spectra were scanned on a spectrophotometer in the wave length region of 340 to 900nm against similar reagent blank or distilled water. The reagent blank absorption spectrum of each method was also recorded against distilled water. The results were graphically represented in **Fig.5.01** to **5.08, P.206-207**. The absorption curves of the colored species in each method show characteristics absorption maxima where as the blank in each method has low or no absorption in this region.

#### ii. Parameters fixation [Optimization of variables]

The experimental variables for the formation of the stable and sensitive colored products by the proposed methods were optimized. The optimum conditions for the color development of methods (**$M_{1a}$, $M_{1b}$, $M_{1c}$, $M_5$, $M_6$, $M_9$, $M_{11a}$** and **$M_{11b}$**) were established by varying the parameters one at a time, keeping the others fixed and observing the effect

produced on the absorbance of the colored species. Several experiments were carried out for this purpose and the conditions so obtained were incorporated in recommended procedures.

**For Method – $M_6$ [AMV]**

The method involves the reaction of the drug **EFZ** with AV in acid medium. The effect of various parameters, such as conc and volume of AV, nature and strength of acid, order of addition of reagents, solvent for final dilution were studied and the optimum conditions developed and recorded in **Table.5.01, P. 212**.

**For Method – $M_5$ [Fe(III) – $K_3Fe(CN)_6$]**

The optimum conditions in this method were fixed basing on the study of the effects of various parameters such as volumes of $3.32 \times 10^{-3}$ M ferric chloride solution, $3.02 \times 10^{-3}$ M potassium ferricyanide solution and 1N HCl, time and temperature necessary for complete color development, the stability and intensity of the colored species after final dilution were established by measuring absorbance's at 700nm and results were incorporated in **Table.5.02, P.213.**

**iii. Optical Characteristics**

In order to test whether the colored species formed in the above methods, adhere to Beer's law the absorbance's at appropriate wave lengths of a set of solutions containing varying amounts of EFZ and specified amounts of reagents (as given in the proposed procedures for each method) were recorded against the corresponding reagent blanks. The Beer's law plots of these systems are recorded against the corresponding reagent blanks are recorded graphically (**Figs.5.09** to **5.16,P.208-209**). The optimum photometric range (Ringbom plot) of these systems is recorded graphically (**Figs.5.17** to **5.24**, **P. 210-211**).

**a) Linearity and sensitivity**

Under optimum conditions, a linear relation was obtained between absorbance and concentration of efavirenz in various ranges 0.5-10 µg/mL that are represented in table. The regression analysis of the plot using the method of least squares was made to evaluate the intercept (a), slope (b), regression coefficient (r) and standard deviations of slope and intercept (**Table.5.05a & 5.05b.P.216-217**). The high value of the regression coefficient (close to unity) of the regression equation and the negligible value of the intercept corroborate the linearity of the calibration plot .

The sensitivity of the proposed methods was indicated by the fairly high values of molar absorptivities and low values of sandell's sensitivity values. Sandell's sensitivity (S) represents the number of micrograms of the determinant per milliliter of a solution having an absorbance (A) of 0.001 for a path length (l) of 1.0cm. Thus, S [µg $cm^{-2}$] = $10^{-3}$/a, where a = (b/Molecular weight of efavirenz)×1000, where b, molar absorptivity, equals A/cl, where c is the molar con centration of the determinant and l = 1 cm is the path length.

**b)Limits of detection (LOD) and quantification (LOQ)**

The limits of detection (LOD) and quantification (LOQ) were calculated according to the same guidelines using the formulae: LOD = $3.3S_a/b$ and LOQ = $10S_a/b$ where $\mathbf{S_a}$ is the standard deviation of five determinations and **s** is the slope of the calibration curve. The limits of detection (LOD) and quantitation (LOQ) for the propsed methods were calculated and are presented in (**Table.5.05a & 5.05b, P. 216-217**).

### iv) Precision

Precision refers to the reproducibility of measurements within a set of measurements. One of the most common statistical terms employed is the standard deviation. The precision of each proposed method was ascertained from the absorbance values obtained by actual determination of six replicates of a fixed amount of efavirenz in total solution. The percent relative standard deviation and percent were calculated for the proposed methods (**Table.5.03, P. 214**).

### v) Accuracy(recovery study)

The accuracy and validity of the proposed methods were further ascertained by performing recovery studies. Pre-analyzed tablet powder was spiked with pure efavirenz at one concentration levels and the total was found by the proposed methods. The results of this study given in Table 4 indicated that the recovery was good, and that the co formulated substances did not interfere in the determination (**Table.5.04, P.215**).

### vi) Application to tablets analysis

The validity of the proposed methods was checked by applying them to assay in one brand of tablets. **Table.5.06a &5.06b,P.218-219** gives the results of assay and reveal that there is close agreement between the results obtained by the proposed methods and the label claim. These results were also compared statistically with those obtained by a UV reference method by applying Student's t-test for accuracy and F-test for precision. At the 95% confidence level, the calculated t- and F-values did not exceed the tabulated values (t = 2.327 and F = 5.05) suggesting that the proposed methods are as accurate and precise as the reference method

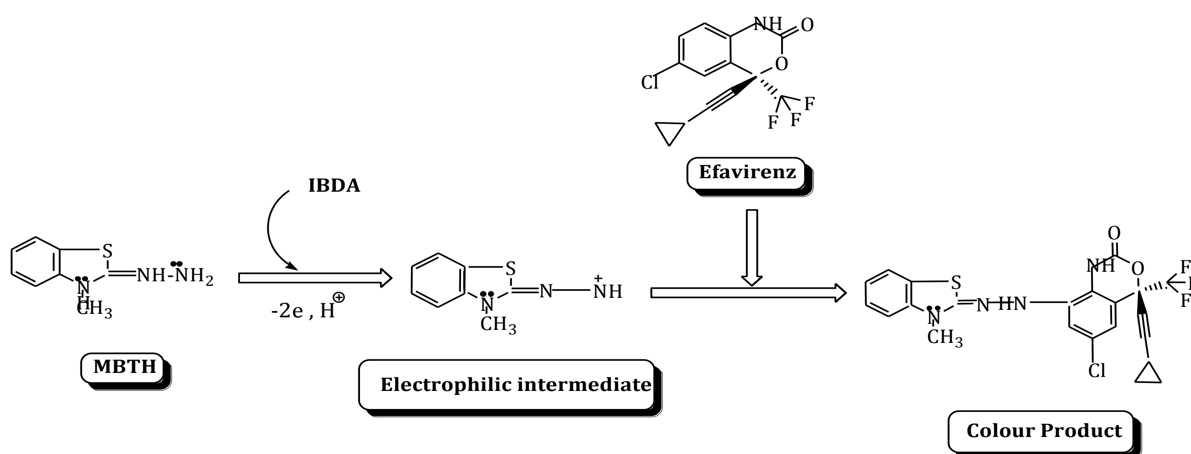
### vii)Reaction mechanisms of the proposed methods

It is difficult to predict the exact nature of colored species formed in the proposed methods. The reviews concerning the reagents used for the development of color by exploiting appropriate functional moieties in Efavirenz, an attempt has been made to indicate the nature of colored species in each of the proposed methods.

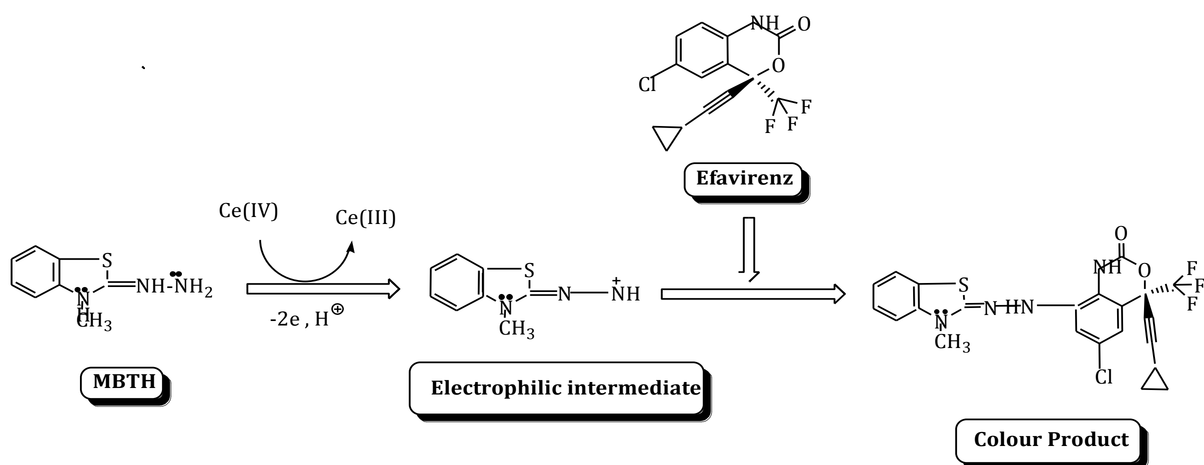
**Method – $M_{1a}$, $M_{1b}$** and **$M_{1c}$**

Under the optimized reaction conditions MBTH looses two electrons and one proton during oxidation with oxidants such as IBDA, Ce(IV), $NaIO_4$ forming an electrophilic intermediate, which is the active coupling species. These active species reacts with the coupler (i.e.) stavudine by electrophilic attack on the most nucleophilic site in the benzene ring of the coupler giving oxidative coupled products.

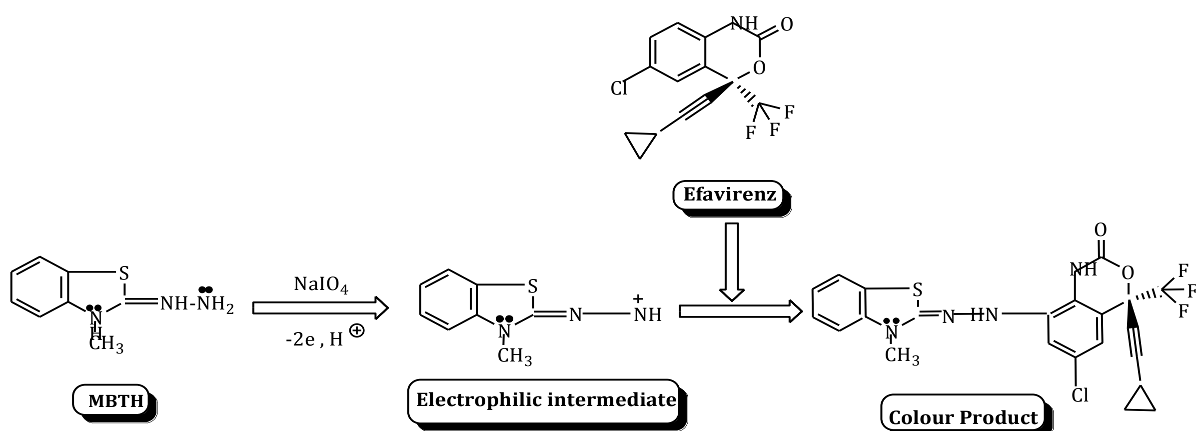
**Method – $M_{1a}$,**




**Method – $M_{1b}$**




## Method – $M_{1c}$

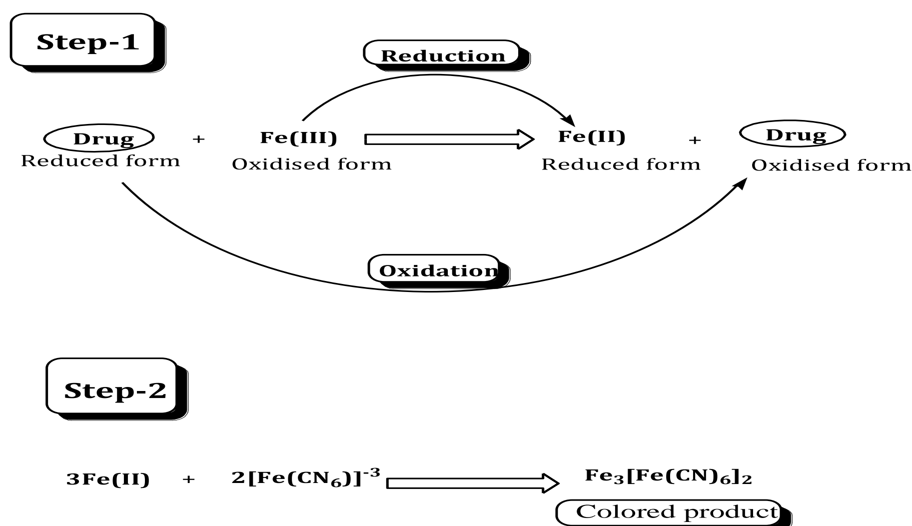



## Method - $M_5$

Reducible groups present in the EFZ probably effects the reduction of 1,2 or 3 oxygen atoms from exemplified vanadate, thereby producing one or two more of possible reducing species which have a characteristic intense blue color.

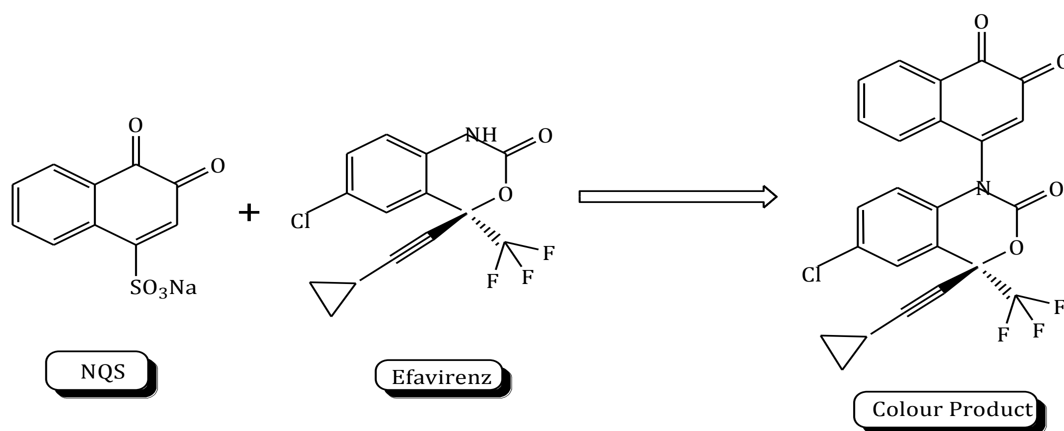
## Method – $M_6$

The reduced form of Fe (III) (i.e. Fe (II) has a tendency to give a colored complex on treatment with $[Fe\ (CN)_6]^{3-}$.




**Method - $M_{10}$**

In this method, the presence of imino group of EFZ permits the development of new spectrophotomertrc method for its determination through the nucleophilic substitution reaction with NQS.

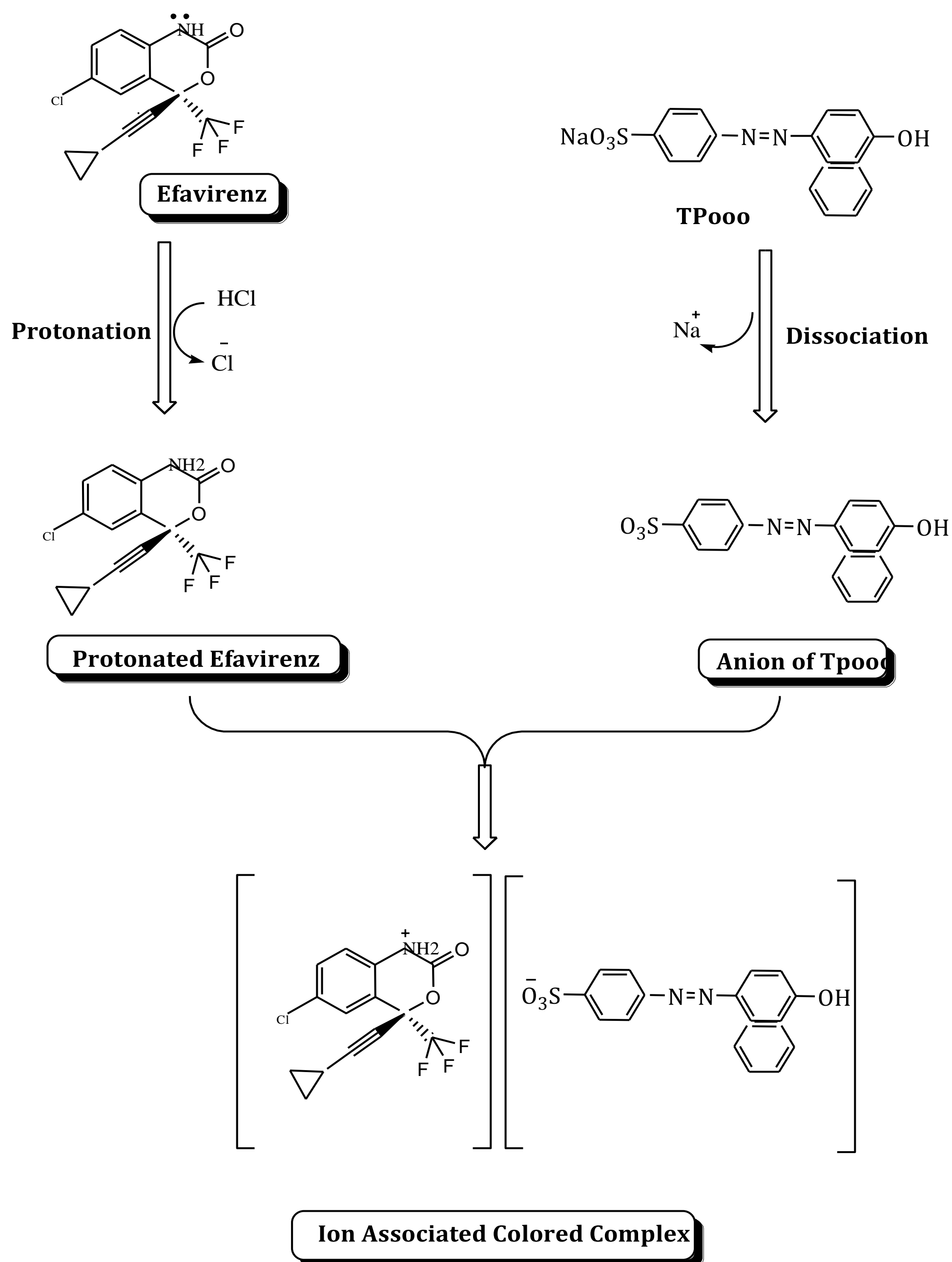



**Method –$M_{11a}$ & $M_{11b}$**

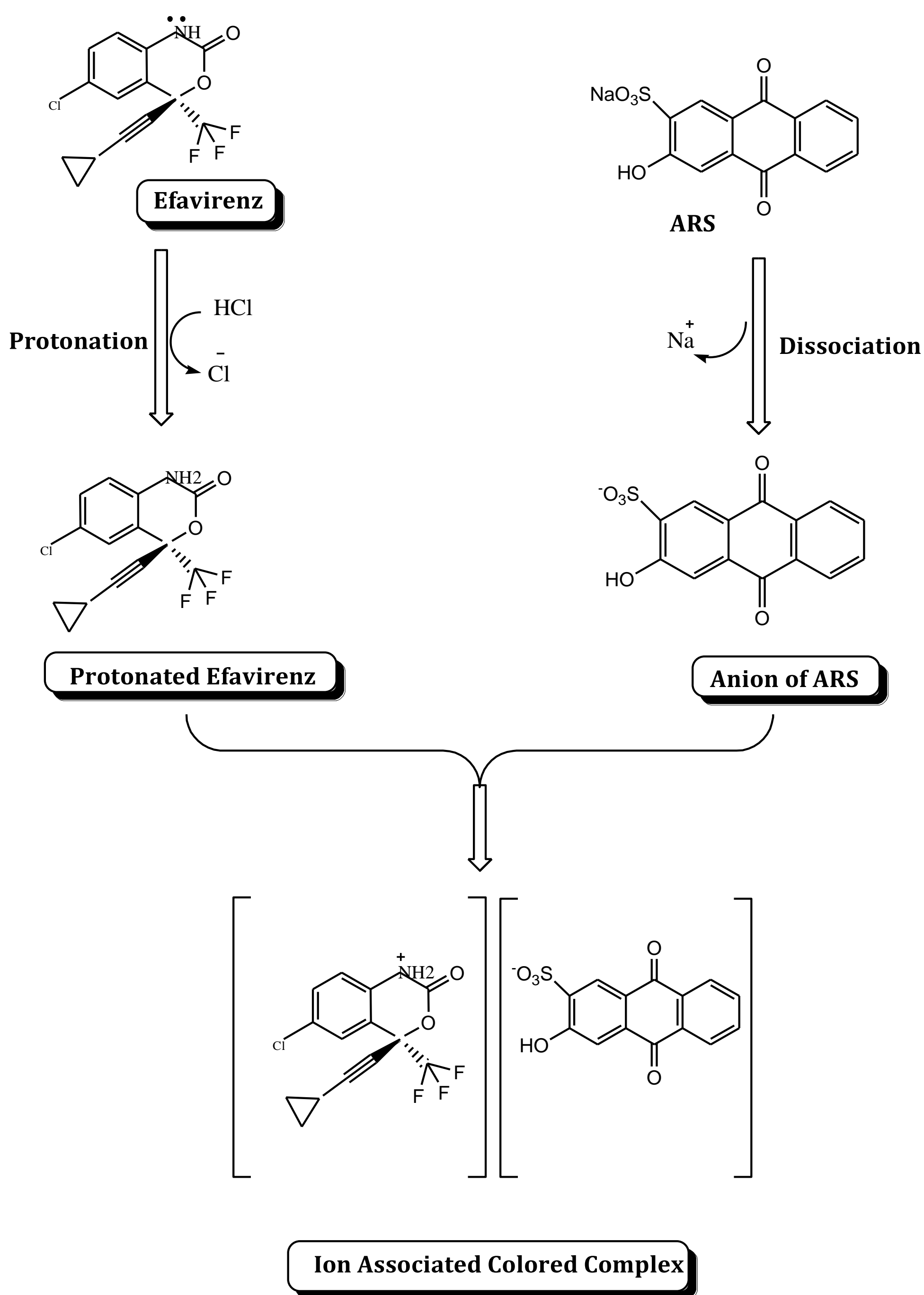
EFZ being a base, forms an ion association complex with an acidic dye (TPooo, ARS) which is extractable into chloroform from the aqueous phase. The protonated nitrogen (positive charge) of the drug molecule in acid medium is expected to attract the oppositely

charged part (negative charge) of the dye and behave as a single unit being held together by electrostatic attraction. Based on the analogy, the structures of ion association complexes are shown in scheme.

**Method –$M_{11a}$**



**Method –$M_{11b}$**



## 5.06-A: CONCLUSIONS

The visible Spectrophotometric methods thus developed for the estimation of efavirenz were found to be simple and useful with high accuracy, precision, repeatability. Sample recoveries in formulations using the above proposed methods were in good agreement with their respective label claim or theoretical drug content, thus suggesting the validity of the proposed methods and non interference of formulation excipients in its estimation.

**Fig.5.01. Absorption spectrum ofEFZ MBTH,IBDA,AcOH($M_{1a}$)**

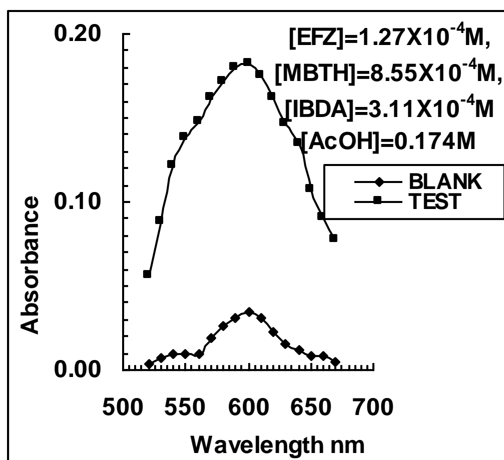



**Fig.5.02. Absorption spectrum of EFZ-MBTH, Ce(IV)($M_{1b}$)**

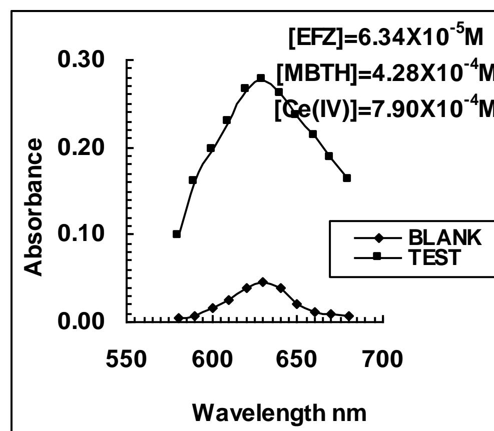



**Fig.5.03. Absorption spectrum of EFZ-MBTH,$NaIO_4$($M_{1c}$)**

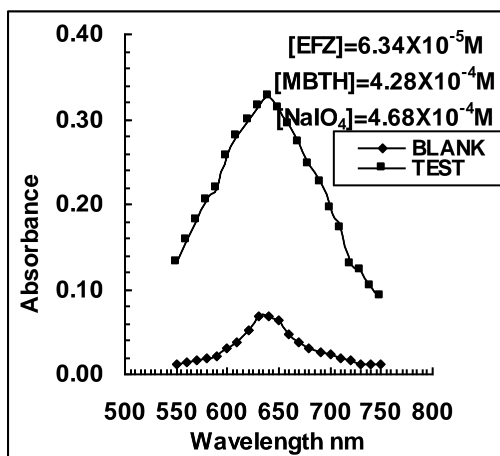



**Fig.5.04: Absorption spectrum of EFZ with AMV ($M_5$)**

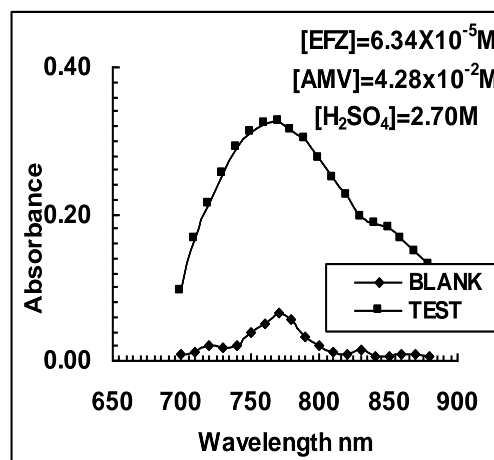


**Fig.5.05: Absorption spectrum of EFZ with Fe(III)- PFC ($M_6$)**

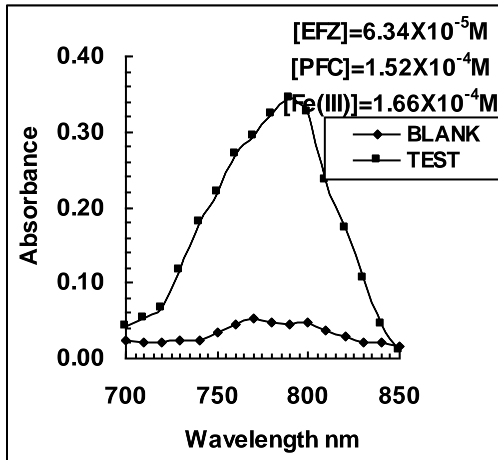



**Fig.5.06: Absorption spectrum of EFZ with NQS ($M_9$)**

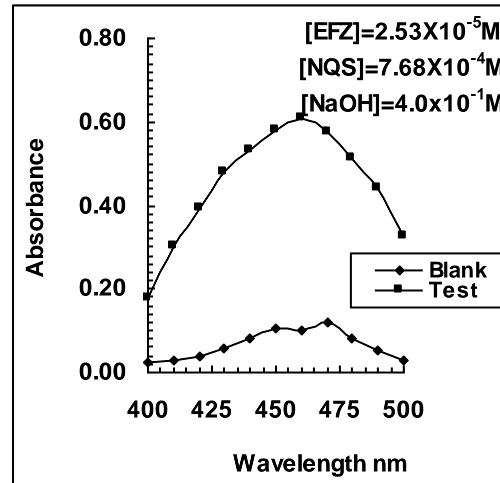



**Fig.5.07: Absorption spectrum of EFZ with TPooo ($M_{11a}$)**

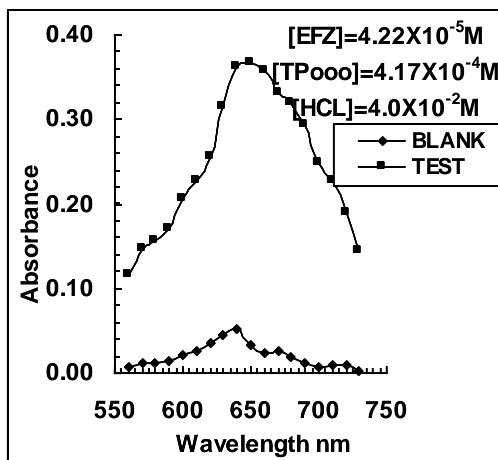



**Fig .5.08: Absorption spectrum of EFZ with ARS ($M_{11b}$)**

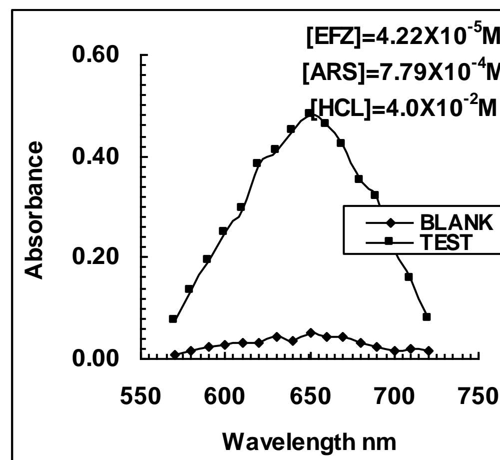


**Fig. 5.09: Beer's law plot of EFZ with with MBTH – IBDA( $M_{1a}$)**

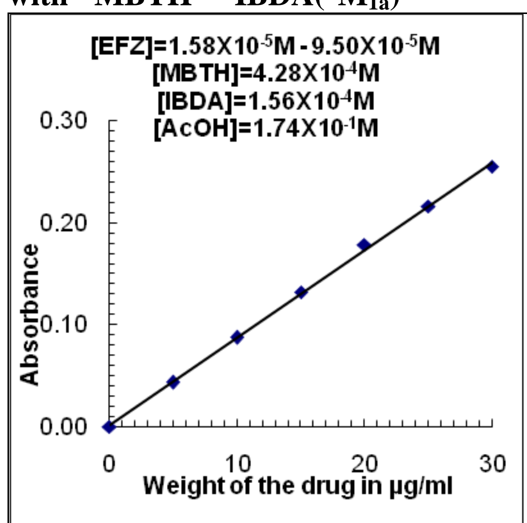



**Fig. 5.10: Beer's law plot of EFZ MBTH - Ce(IV)( $M_{1b}$)**

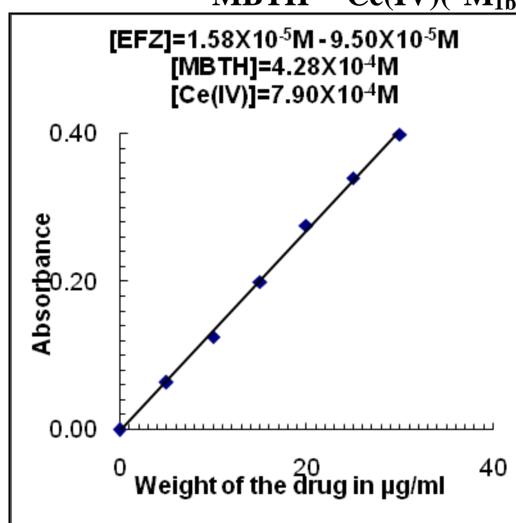



**Fig.5.11: Beer's law plot of EFZ with MBTH – $NaIO_4$( $M_{1C}$)**

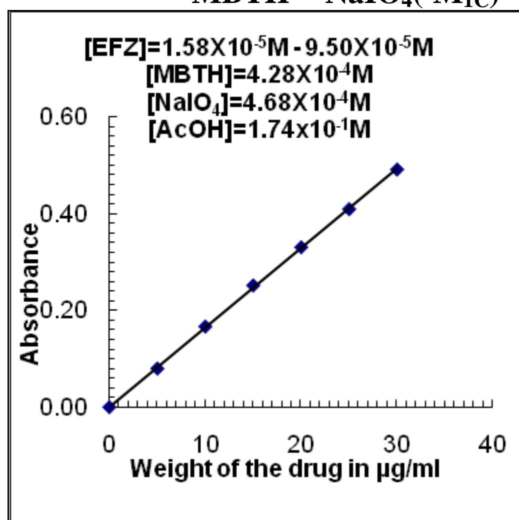



**Fig. 5.12: Beer's law plot of EFZ with AMV( $M_5$**

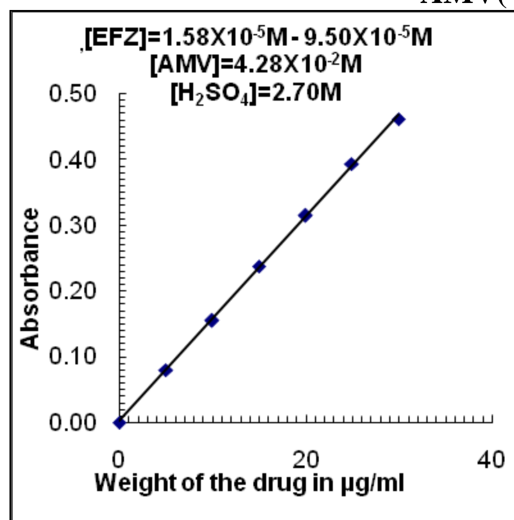


**Fig. 5.13: Beer's law plot of EFZ with Fe(III)- PFC( $M_6$)**

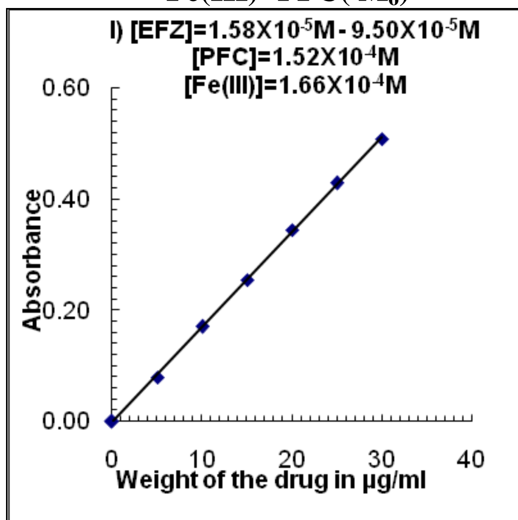



**Fig. 5.14: Beer's law plot of EFZ with NQS($M_9$)**

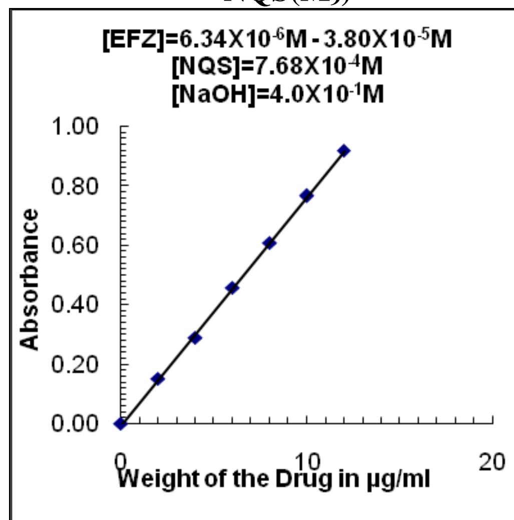



**Fig. 5.15: Beer's law plot of EFZ with TPooo( $M_{11a}$)**

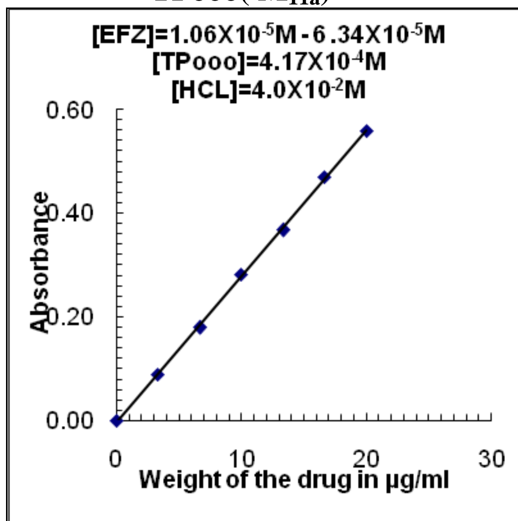



**Fig. 5.16: Beer's law plot of EFZ with ARS( $M_{11b}$)**

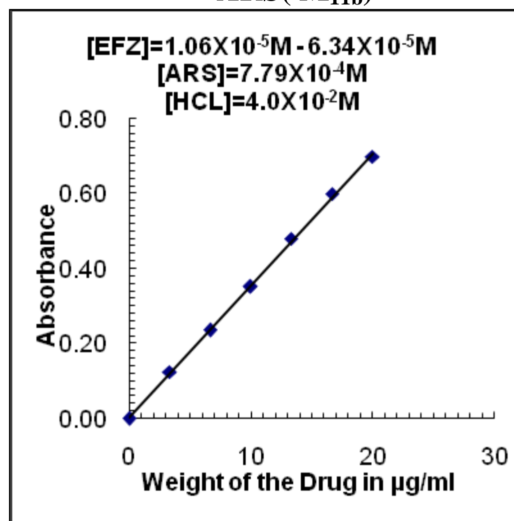
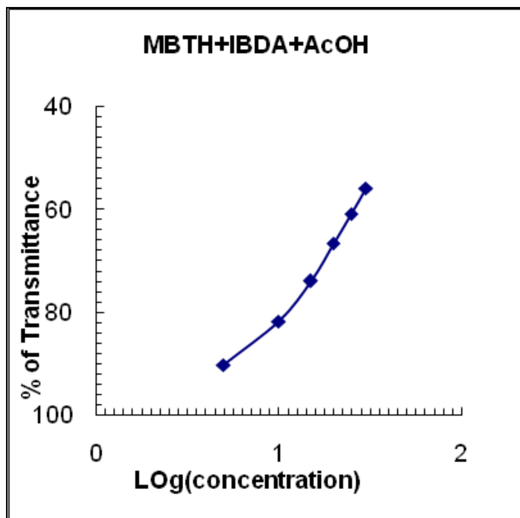


**Fig. 5.17: Ringbom plot of EFZ with MBTH – $NaIO_4$( $M_{1a}$)**

**Fig. 5.18: Ringbom plot of EFZ with MBTH - Ce(IV)( $M_{1b}$)**



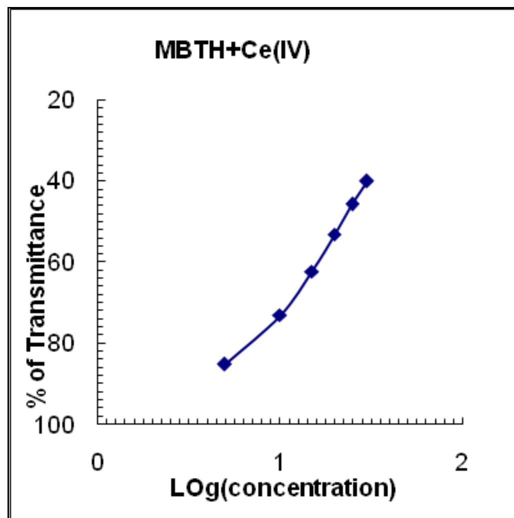
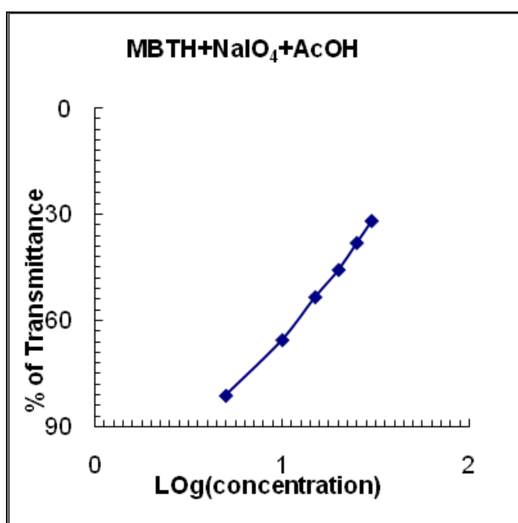




**Fig.5.19: Ringbom plot of EFZ with MBTH – $NaIO_4$( $M_{1C}$)**

**Fig. 5.20: Ringbom plot of EFZ with AMV( $M_5$)**



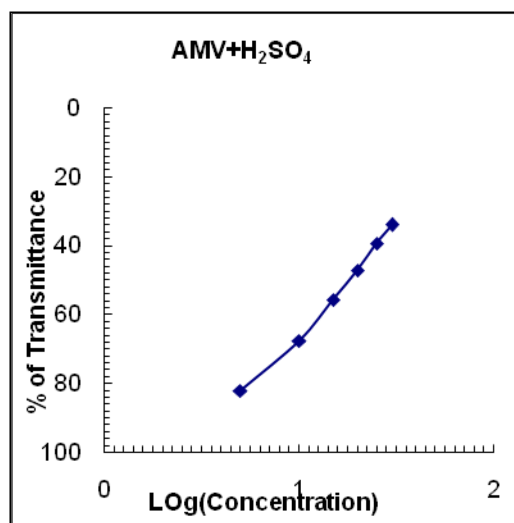



**Fig. 5.21: Ringbom plotof EFZ with Fe(III)- PFC( $M_6$)**

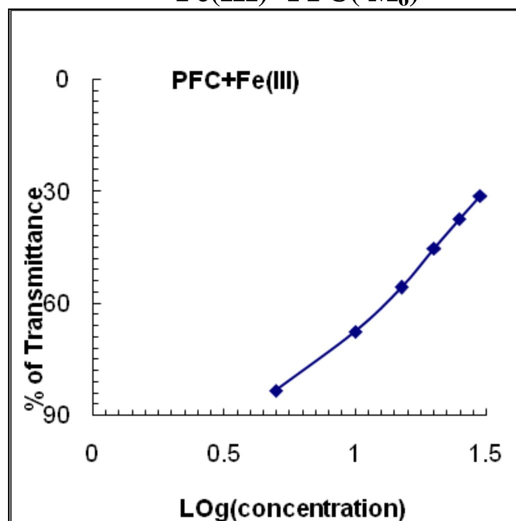



**Fig. 5.22: Ringbom plotof EFZ with NQS($M_9$)**

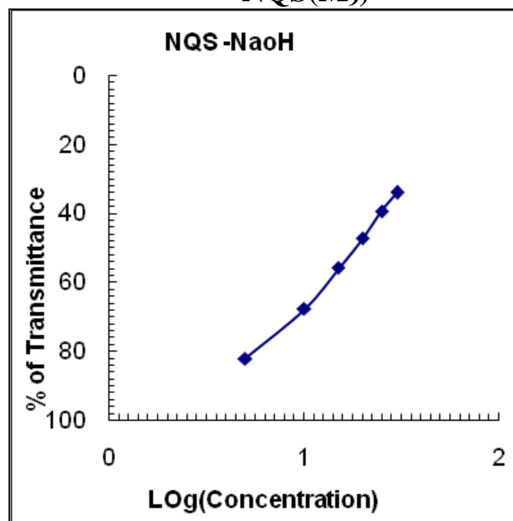



**Fig. 5.23: Ringbom plotof EFZwith TPooo( $M_{11a}$)**

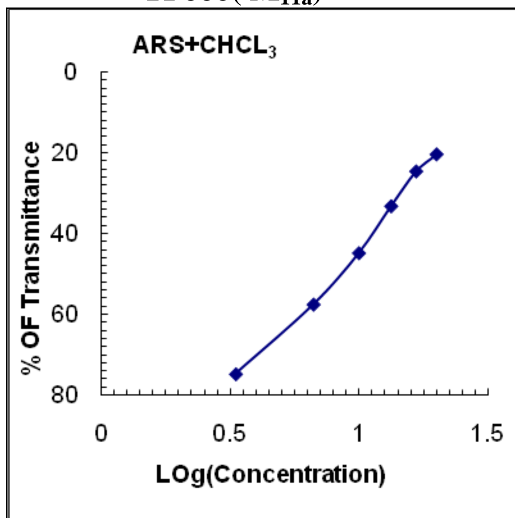



**Fig. 5.24: Ringbom plotof EFZS with ARS( $M_{11b}$)**

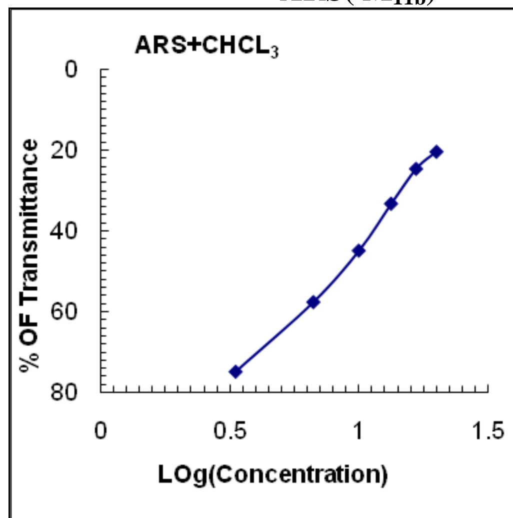


**Table.5.01**
**Optimum conditions established in method $M_5$ for EFZ**

| Parameter | Optimum range | Conditions in procedure | Remarks |
|---|---|---|---|
| $\lambda_{max}$ (nm) | 680 - 730 | 700 | - |
| Volume of ferric chloride ($3.32x10^{-3}$M) required for oxidation. | 0.8 - 1.5mL | 1.0mL | Optimum conditions furnished in column were preferred for broad coverage of Beer's Law limits and stability of colored species formed. |
| Vol of Potassium ferri cyanide ($3.02x10^{-3}$M) for formation of ferrous ferricyanide. | 0.4 - 0.7mL | 0.5mL | - |
| HCl (1.0N) necessary for maintenance of acidity prior to dilution. | 0.8 - 1.5mL | 1.0mL | - |
| Temperature and time necessary for complete development of color. | 5 - 15min.<br>Room temp. | 10min.<br>Room temp. | - |
| Stability period | Immediate -1hr. | Immediate-1hr. | - |

**TABLE.5.02**
**Optimum conditions established in method $M_6$ for EFZ**

| Parameter | Optimum range | Conditions in procedure | Remarks |
|---|---|---|---|
| $\lambda_{max}$ (nm) | 740 – 780 | 760 | |
| Effect of volume of $2.61x10^{-2}$M of AV solution. | 0.7 - 1.3mL | 1.0mL | 1.0mL of $2.61x10^{-2}$M of AV solution was necessary for covering broad range of Beer's law limits. |
| Effect of volume of Conc. $H_2SO_4$ on color development | 3.0-5.0mL | 4.0mL | <3.0mL of conc. $H_2SO_4$ results in low absorbance values and >5.0mL has no additional value. |
| Effect of the order of addition of reagent on color development | EVD,AV solution, Conc. $H_2SO_4$ | EVD, AV solution, Conc. $H_2SO_4$ | The change in the order of addition has no effect. |
| Effect of temperature and time | Boiling water bath 20-30min. | Boiling water bath 20min. | It was found that boiling water bath was necessary for uniform temperature and maximum color development. Heating on a boiling water bath for 20min. is necessary for maximum color development |
| Solvent for final dilution | Ethanol | Ethanol | The absorbance of the test solution decreased when water was used instead of ethanol for final dilution. |
| Stability period after final dilution | 5min-24 hours | 5min. | |

**Table.5.03**
**Intra-day precision and accuracy results**

| Proposed methods | EVD taken $\mu g.mL^{-1}$ | Intra-day[a] | | |
|---|---|---|---|---|
| | | EVD found[c], $\mu g.mL^{-1}$ | Precision[d] | Accuracy[e] |
| **Method $M_{1a}$** | 20.0 | 19.92 | 0.208 | -0.40 |
| **Method $M_{1c}$** | 20.0 | 19.90 | 0.260 | -0.50 |
| **Method $M_{1c}$** | 20.0 | 19.94 | 0.163 | -0.30 |
| **Method $M_5$** | 20.0 | 19.89 | 0.255 | -0.55 |
| **Method $M_6$** | 20.0 | 19.96 | 0.370 | -0.20 |
| **Method $M_9$** | 10.0 | 9.93 | 0.303 | -0.70 |
| **Method $M_{11a}$** | 15.0 | 14.95 | 0.468 | -0.33 |
| **Method $M_{11b}$** | 15.0 | 14.96 | 0.543 | -0.26 |

**a = 6 replicates;c= mean±standard error; d=relative standard deviation, %; e= bias %**

**Table.5.04**

**The results of percentage recovery values of Efavirenz [EFZ] by the proposed spectrophotometric methods**

| Proposed methods | SVD in tablet $\mu g.mL^{-1}$ | Pure SVD added $\mu g.mL^{-1}$ | Total found $\mu g.mL^{-1}$ | Pure ABS recovered %±SD* |
|---|---|---|---|---|
| **Method $M_{1a}$** | 20.0 | 10.0 | 30.05± 0.42 | 100.16 |
| **Method $M_{1c}$** | 20.0 | 10.0 | 29.88± 0.42 | 99.60 |
| **Method $M_{1c}$** | 20.0 | 10.0 | 30.11± 0.42 | 100.36 |
| **Method $M_5$** | 20.0 | 10.0 | 29.99± 0.42 | 99.96 |
| **Method $M_6$** | 20.0 | 10.0 | 29.85± 0.42 | 99.85 |
| **Method $M_9$** | 10.0 | 10.0 | 19.98± 0.42 | 99.90 |
| **Method $M_{11a}$** | 15.0 | 10.0 | 24.85± 0.42 | 99.40 |

**Table.5.05a**
**Optical and regression characteristics of the proposed methods for Efavirenz [EFZ]**

| Name of the Parameter | Method $M_{1a}$ | Method $M_{1b}$ | Method $M_{1c}$ | Method $M_5$ | Method $M_6$ |
|---|---|---|---|---|---|
| Maximum Wavelength $\lambda_{max}$ | 640 | 630 | 640 | 770 | 790 |
| Beer's Law Limits µg/mL | 5.0-30.0 | 5.0-30.0 | 5.0-30.0 | 5.0-30.0 | 5.0-30.0 |
| Optimum Photometric Range µg/mL | 10.0-25.0 | 15.0-30.0 | 10.0-25.0 | 10.0-20.0 | 10.0-25.0 |
| Sandell's Sencitivity(µg/cm$^2$ / 0.001 Absorbance) | 1.16E-01 | 7.81E-02 | 6.25E-02 | 6.25E-02 | 6.25E-02 |
| Molar Absorptivity lt/mole/cm | 2.69E+03 | 4.31E+03 | 5.17E+03 | 4.88E+03 | 5.43E+03 |
| Slope (b) | 8.53E-03 | 1.37E-02 | 1.64E-02 | 1.55E-02 | 1.72E-02 |
| Intercept(a) | 2.53E-03 | -5.27E-03 | 1.87E-03 | 3.33E-03 | -2.73E-03 |
| Standard Deviation on Slope($S_b$) | 1.29E-04 | 2.53E-04 | 1.23E-04 | 1.57E-04 | 1.38E-04 |
| Standard Deviation on Intercept($S_a$) | 2.52E-03 | 4.93E-03 | 2.39E-03 | 3.05E-03 | 2.68E-03 |
| Standard Error on Estimation($S_e$) | 3.52E-03 | 6.91E-03 | 3.35E-03 | 4.27E-03 | 3.75E-03 |
| Correlation Coefficient ( r ) | 0.9993 | 0.9990 | 0.9998 | 0.9997 | 0.9998 |
| Limit of Detection (LOD) µg/mL | 0.8850 | 1.0829 | 0.4372 | 0.5921 | 0.4673 |
| Limit of Quantification (LOQ) µg/mL | 2.9499 | 3.6097 | 1.4574 | 1.9736 | 1.5578 |

**Table.5.05b**
**Optical and regression characteristics of the proposed methods for Efavirenz [EFZ]**

| Name of the Parameter | Method $M_9$ | Method $M_{11a}$ | Method $M_{11b}$ |
|---|---|---|---|
| Maximum Wavelength $\lambda_{max}$ | 460 | 430 | 650 |
| Beer's Law Limits µg/mL | 2.0-12.0 | 3.33-20.0 | 3.33-20.0 |
| Optimum Photometric Range µg/mL | 4.0-8.0 | 10.0-20.0 | 6.66-16.66 |
| Sandell's Sencitivity(µg/cm$^2$ / 0.001 Absorbance) | 1.32E-02 | 3.70E-02 | 0.0265 |
| Molar Absorptivity lt/mole/cm | 2.45E+04 | 8.95E+03 | 1.11E+04 |
| Slope (b) | 7.75E-02 | 2.83E-02 | 3.50E-02 |
| Intercept(a) | -9.40E-03 | -5.60E-03 | 7.40E-03 |
| Standard Deviation on Slope($S_b$) | 6.86E-04 | 2.63E-04 | 4.49E-04 |
| Standard Deviation on Intercept($S_a$) | 5.34E-03 | 3.42E-03 | 5.83E-03 |
| Standard Error on Estimation($S_e$) | 7.48E-03 | 4.79E-03 | 8.17E-03 |
| Correlation Coefficient ( r ) | 0.9998 | 0.9998 | 0.9996 |
| Limit of Detection (LOD) µg/mL | 0.2068 | 0.3619 | 0.4998 |
| Limit of Quantification (LOQ) µg/mL | 0.6893 | 1.2064 | 1.6659 |

**Table.5.06a**

**Assay of Efavirenz [EFZ] in Pharmaceutical Formulations**

| Sample | Amount taken | | Amount found in proposed methods | | | | Reference Method | Recovery in proposed methods | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | $M_{1a}$ | $M_{1b}$ | $M_{1c}$ | $M_5$ | | | $M_{1a}$ | $M_{1b}$ | $M_{1c}$ | $M_5$ |
| **Sustiva[a] (tablet)** | **100mg** | **AVG** | 98.97 | 98.99 | 98.86 | 98.74 | 98.89 | **%REC** | 98.97 | 98.99 | 98.86 | 98.74 |
| | | **SD** | ±0.152 | ±0.158 | ±0.155 | ±0.156 | ± 0.153 | **%RSD** | ±0.153 | ±0.159 | ±0.156 | ±0.157 |
| | | **F** | 1.013 | 1.066 | 1.026 | 1.039 | | | | | | |
| | | **t** | 0.862 | 1.078 | 0.323 | 1.617 | | | | | | |
| **Sustiva[b] (tablet)** | **150mg** | **AVG** | 593.52 | 594.78 | 593.94 | 593.4 | 589.98 | **%REC** | 98.92 | 99.13 | 98.99 | 98.9 |
| | | **SD** | ± 4.45 | ±4.39 | ±4.27 | ± 4.65 | ± 4.36 | **%RSD** | ±0.749 | ±0.738 | ±0.718 | ±0.783 |
| | | **F** | 1.041 | 1.013 | 1.042 | 1.137 | | | | | | |
| | | t | 1.33 | 1.816 | 1.498 | 1.294 | | | | | | |

**a & b Tablets from two different pharmaceutical companies.**

***Average of six determinations are considered, AVG=Average, SD=Standard deviation, F=F-test value, t=t-test value; Theoretical values at 0.05 level of confidence limit F=5.05, t=1.812.**

****%REC=% of Recovery, %RSD=%of Relative standard deviation; Recovery of 10.0mg added to the preanalyzed formulations (Average of six determinations)**

## Table.5.06b

## Assay of Efavirenz [EFZ] in Pharmaceutical Formulations

| Sample | Amount taken | | Amount found in proposed methods | | | | Reference Method | Recovery in proposed methods | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | $M_6$ | $M_9$ | $M_{11a}$ | $M_{11b}$ | | | $M_6$ | $M_9$ | $M_{11a}$ | $M_{11b}$ |
| **Sustiva[a] (tablet)** | **100mg** | **AVG** | 99.01 | 98.99 | 98.94 | 98.95 | 98.89 | **%REC** | 99.01 | 98.99 | 98.94 | 98.95 |
| | | **SD** | ±0.149 | ±0.148 | ±0.159 | ±0.150 | ± 0.153 | **%RSD** | ±0.150 | ±0.149 | ±0.160 | ±0.151 |
| | | **F** | 1.054 | 1.068 | 1.079 | 1.040 | | | | | | |
| | | **t** | 1.294 | 1.078 | 0.539 | 0.647 | | | | | | |
| **Sustiva[b] (tablet)** | **150mg** | **AVG** | 594.12 | 593.28 | 591.66 | 593.7 | 589.98 | **%REC** | 99.02 | 98.88 | 98.61 | 98.96 |
| | | **SD** | ±4.54 | ±4.54 | ±4.28 | ±4.58 | ± 4.36 | **%RSD** | ±0.764 | ±0.756 | ±0.723 | ±0.771 |
| | | **F** | 1.084 | 1.084 | 1.037 | 1.103 | | | | | | 98.95 |
| | | t | 1.566 | 1.248 | 0.635 | 1.407 | | | | | | |

**a & b Tablets from two different pharmaceutical companies.**

***Average of six determinations are considered, AVG=Average, SD=Standard deviation, F=F-test value, t=t-test value; Theoretical values at 0.05 level of confidence limit F=5.05, t=1.812.**

****%REC=% of Recovery, %RSD=%of Relative standard deviation; Recovery of 10.0mg added to the preanalyzed formulations (Average of six determinations)**

# PART-B: RP- HPLC METHOD FOR THE ESTIMATION OF EFAVIRENZ IN BULK AND IN PHARMACEUTICAL FORMULATIONS

## 5.01-A: INTRODUCTION

Literature survey reveals that many chromatographic methods [5-10] for the determination of efavirenz in biological fluids. So far, very few assays have been reported for the estimation of efavirenz in pharmaceutical formulations. The availability of an HPLC method with high sensitivity and selectivity will be very useful for the determination of efavirenz in pharmaceutical formulations. This made the author to develop to a simple, precise and accurate with high sensitivity and selectivity reversed-phase HPLC method for the estimation of efavirenz in bulk and in pharmaceutical formulations.

## 5.02-A: EXPERIMENTAL

**a) Materials and Methods: E**favirenz was obtained as a gift sample from Hetero Pharma Ltd, Hyderabad. Acetonitrile and water used were of HPLC grade (Qualigens). Commercially available efavirenz tablets (Levroxa 250mg, Ranbaxy) were procured from local market.

**b)Instrumentation**

Quantitative HPLC was performed on liquid Chromatograph, Water separation 2996, PDA detector module equipped with automatic injector with injection volume 20µL, and 2693 pump. A RP C-18 Gemini NX $C_{18}$ column (250 x 4.6 mm; particle size 5µm) was used. The HPLC system was equipped with Empower Software.

**c) HPLC conditions**

The contents of the mobile phase were acetonitrile [Orthophosphoric acid] and waterin the ratio of 70:30 v/v. They were filtered before use through a 0.45μm membrane filter, and pumped from the respective solvent reservoirs to the column at a flow rate of 1.0 mL/min. The run time was set at 30.0min and the column temperature was ambient. Prior to the injection of the drug solution, the column was equilibrated for at least 30min with the mobile phase flowing through the system. The eluents were monitored at 240 nm.

**d) Preparation of standard stock solution:** A standard stock solution of the drug was prepared by dissolving 50mg of efavirenz in 50mL volumetric flask containing 30mL of mobile phase, sonicated for about 15 min and then made up to 50mL with diluent to get 1mg/mL standard stock solution.

**e) Working standard solution:** 1.0mL of stock solution was taken in 100mL volumetric flask and thereafter made up to 50mL with mobile phase to get a concentration of 10μg/mL.

**f) Preparation of sample solution:** Twenty tablets (Levroxa 250mg, Ranbaxy) were weighed, and then powdered. A sample of the powdered tablets, equivalent to 50mg of the active ingredient, was mixed with 25mL of diluent. The mixture was allowed to stand for 1hr with intermittent sonication to ensure complete solubility of the drug, and then filtered through a 0.45μm membrane filter, followed by adding mobile phase to obtain a stock solution of 1.0 mg/mL. 1.0mL of this solution was transferred to a 100mL volumetric flask and made up to sufficient volume with mobile phase to give a concentration of 10μg/mL.

### 5.03-B: ASSAY METHOD

Aliquots of standard efavirenz stock solution were taken in different 10mL volumetric flasks and diluted up to the mark with the mobile phase such that the final concentrations of efavirenz are in the range of 10.0-60.0μg.$mL^{-1}$. Each of these drug solutions (20μL) was injected three times into the column, and the peak areas and retention times were recorded. Evaluation was performed with PDA detector at 210nm and a calibration graph was obtained by plotting peak area *versus* concentration of efavirenz (**Figure.5.26,P.223**). The plot of peak area of each sample against respective concentration of efavirenz was found to be linear in the range of 10.0–60.0 μg.$mL^{-1}$ with correlation coefficient of 0.999.The respective linear regression equation being **Y=22119.684x+6829.3428**. The regression characteristics, such as slope, intercept, and % RSD were calculated for this method and given in **Table.5.07,P.223.**

**Fig.5.25. Typical chromatogram of efavirenz by HPLC**

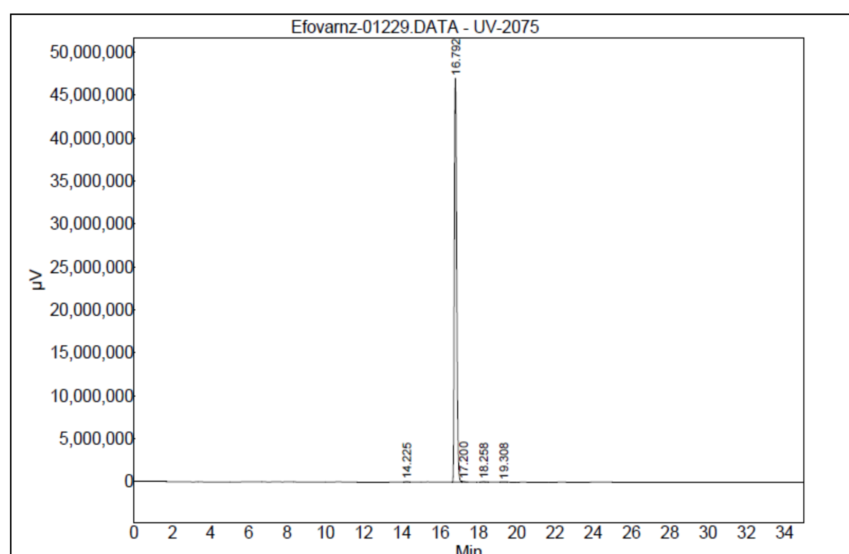


**Fig.5.26. Calibration curve of efavirenz by HPLC**

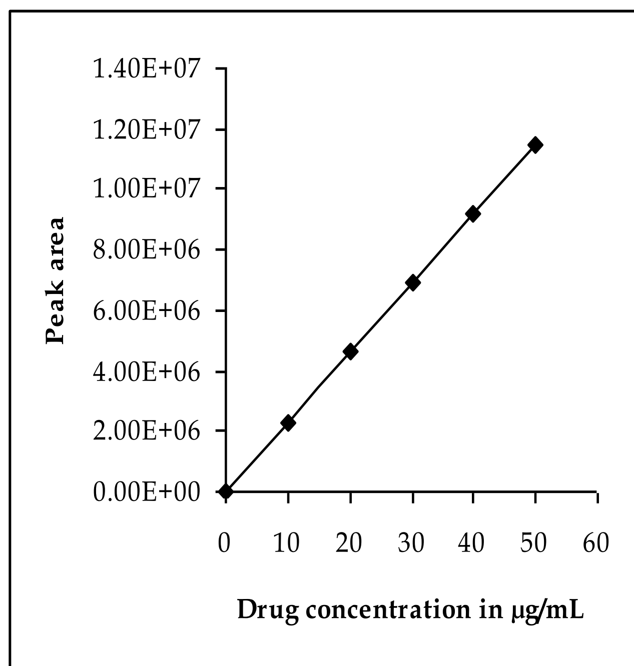



**Table.5.07**

**Linear regression data for calibration curves.**

| Parameters | Linear regression data |
|---|---|
| **Concentration range, µg $mL^{-1}$** | 10.0 - 60.0 |
| **Slope, m** | 22119.684 |
| **Intercept, b** | 6829.3428 |
| **Correlation coefficient** | 0.9999 |

## 5.03-B: RESULTS & DISCUSSION

The system suitability tests were carried out on freshly prepared standard stock solution of efavirenz. From the typical chromatogram of efavirenz as shown in (**Fig.5.25,P.222**), it was found that the retention time was 16.792min. A mixture of acetonitrile and water in the ratio of 70:30 v/v was found to be most suitable to obtain

a peak well defined and free from tailing. In the present developed RP-HPLC method, the standard and sample preparation required less time and no tedious extraction were involved. A good linear relationship (r=0.9999) was observed between the concentration range of 10.0-60.0μg/mL. The assay of efavirenz tablets was found to be 101.50%. From the recovery studies (**Table.5.08,P.224)** it was found that about 99.26% of efavirenz was recovered which indicates high accuracy of the method. The absence of additional peaks in the chromatogram indicates non-interference of the common excipients used in the tablets. This demonstrates that the developed HPLC method is simple, linear, accurate, sensitive and reproducible. Thus, the developed method can be easily used for the routine quality control of bulk and tablet dosage forms of efavirenz.

**Table. 5.08**

**Results of HPLC assay and recovery studies**

| Sample | Amount claim, mg/tablet | %found by the proposed method | % Recovery* |
|---|---|---|---|
| **Sustiva** | 100.0 | 99.98 | 99.98 |

***Average of three different concentration levels.**

## 5.04-B: CONCLUSIONS

The HPLC method developed in this study has the sensitivity, selectivity and reproducibility which make it versatile and valuable, specifically in determination of EFZ in pharmaceutical dosage form. The method can also be readily adapted to routine quality control analysis of EFZ in bulk and in pharmaceutical formulations within a short analysis time.