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A NEW RP-HPLC METHOD DEVELOPMENT AND VALIDATION, STRESS DEGRADATION STUDIES OF EFAVIRENZ IN BULK AND PHARMACEUTICAL DOSAGE FORM

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Abstract:
A new RP-HPLC method for the quantitative determination of Efavirenz was developed and validated as per ICH guidelines. The drug was injected into Hypersil Gold C_{18} column (150×4.6 mm i.d, 5μ) maintained at ambient temperature and effluent monitored at 252 nm. The mobile phase consisted of Acetonitrile [HPLC grade]: 25mM Ammonium acetate (50: 50V/V). The flow rate was maintained at 1.5 ml/min. The developed method shows high specificity for Efavirenz. The calibration curve for Efavirenz was linear from 0.066 and 3.024μg/ml respectively (r^2 for Efavirenz=0.999).The proposed method was adequate, sensitive, reproducible and specific for the determination of Efavirenz in bulk and pharmaceutical dosage forms.
Keywords: *Efavirenz, Hypersil Gold C_{18} column.*

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INTRODUCTION:

Efavirenz falls in the NNRTI class of antiretrovirals. Both nucleoside and non-nucleoside RTIs inhibit the same target, the reverse transcriptase enzyme, an essential viral enzyme which transcribes viral RNA into DNA. Unlike nucleoside RTIs, which bind at the enzyme's active site, it acts allosterically by binding to a distinct site away from the active site known as the NNRTI pocket. Efavirenz is not effective against HIV-2, as the pocket of HIV-2 reverse transcriptase has a different structure, which confers intrinsic resistance to the NNRTI class. As most NNRTIs bind within the same pocket, viral strains which are resistant to Efavirenz and are usually resistant to the other NNRTIs [1-3]. Various analytical methods have been reported for the estimation of Efavirenz, including spectrophotometric methods and HPLC. HPLC is the most widely used technique for the estimation of Efavirenz in human plasma, saliva, cerebrospinal fluid, and human blood cells, as well as for studying the drug metabolites in the urine. The suggested HPTLC and HPLC methods for assay of Efavirenz are expensive and need complex and sophisticated instrumentation. The titrimetric methods are reported to suffer from disadvantages like unstablity of the reagents, high cost of the chemicals, reduced sensitivity, etc. The present research work describes a UV spectrophotometric method for estimation of Efavirenz in API and its pharmaceutical preparation. The present method aims at developing a simple, accurate and precise RP-HPLC method for the estimation of Efavirenz in bulk and pharmaceutical dosage forms [4-11].
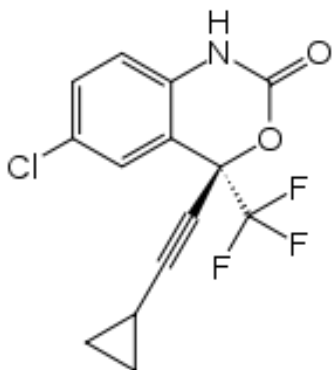

Fig.1: Chemical structure of Efavirenz

MATERIALS AND METHODS:

The reference sample of Efavirenz was obtained as a gift sample from Hetero laboratories, India. HPLC grade water (prepared by using 0.45 Millipore Milli – Q) was procured from Standard Reagents, Hyderabad. HPLC grade Acetonitrile and Methanol was bought from Merck, Mumbai. Buffers were prepared using Potassium dihydrogen ortho phosphate from Merck Chemicals, Mumbai respectively.

Instrumentation: A Waters HPLC system with Rheodyne 7725 injector ,dual wavelength UV-VIS absorbance and PDA detector was used throughout this study. An Hypersil gold,Sunfire C$_{18}$ (150×4.6, 5 □m) column was employed for the method development. The chromatographic system was monitored by Empower-2 software.

Analytes were monitored by UV detection at 252 nm using an isocratic mode with Acetonitrile: 25mM Ammonium acetate in the ratio 50:50 was used as mobile phase. The flow rate was set at 1.5 ml/min and effluent was monitored at 252 nm. The run time were maintained at 14 min. respectively. Solubility of the compounds was enhanced by sonication on an ultrasonicator (Power Sonic 510).

Selection of mobile phase: The objective of this experiment was to optimize the assay method for estimation of Efavirenz based on the literature survey. Various mobile phases were tested to select the best possible system. The various mobile phases used included 25mM Ammonium aceatate and Acetonitrile of pH 7.5 with 70:30 and 60:40 ratios. At 50:50 ratios of Acetonitrile and 25mM Ammonium acetate the peak was eluted at 10.1 min with Hypersil Gold. Better peak resolution and adequate retention time were obtained with the ratio of 50:50 Acetonitrile and 25mM Ammonium acetate .

Preparation of 25mM ammonium acetate buffer solution: 800mg of ammonium acetate was dissolved in sufficient water to produce 1000ml of solution and its pH was adjusted to 7.5.

Preparation of mobile phase: The mobile phase was prepared by mixing 500 ml of 25mM of ammonium acetate (pH 7.5) and 300 ml of Acetonitrile in a 1000 ml clean and dry flask. The mobile phase was then degassed using Ultra-Sonicator to remove dissolved gases and the resultant mobile phase was filtered through a 0.22 μm membrane filter under vacuum.

Preparation of Standard Stock Solution
Standard stock solution was prepared by accurately weighing 100 mg of Efavirenz and transferring them into a 100 ml clean dry volumetric flask containing mobile phase. The solution was sonicated for about 5 mins. and then made upto volume with the mobile

phase. The resultant mobile phase was filtered through a 0.22 μm membrane filter under vacuum. From this 5 ml of solution was taken & made upto 100 ml with mobile phase which has a concentration of 50μg/ml. The solution was sonicated for about 10 mins. and then made upto volume with the mobile phase. From the above solution 2 ml of the above solution was diluted to 100 ml in three different standard flasks with diluent to achieve a concentration of 1μg/ml for each solution. Different working standard solutions were prepared, as per respective concentrations of solutions needed by diluting with methanol.

Preparation of Sample solution
From sterile powder for injection, a quantity equivalent to label claim was weighed and transferred to a sintered glass crucible and extracted using diluent. The extract was filtered with whatmann filter paper and then the volume was made to 100 ml with diluent (Sample solution A). 5ml of sample solution A was pipette into a 100 ml standard flask, diluted and made up the volume up with diluent (Sample solution B). 2ml of sample solution B added to a 100 ml standard flask, diluted and made up the volume with diluent (Sample solution C).
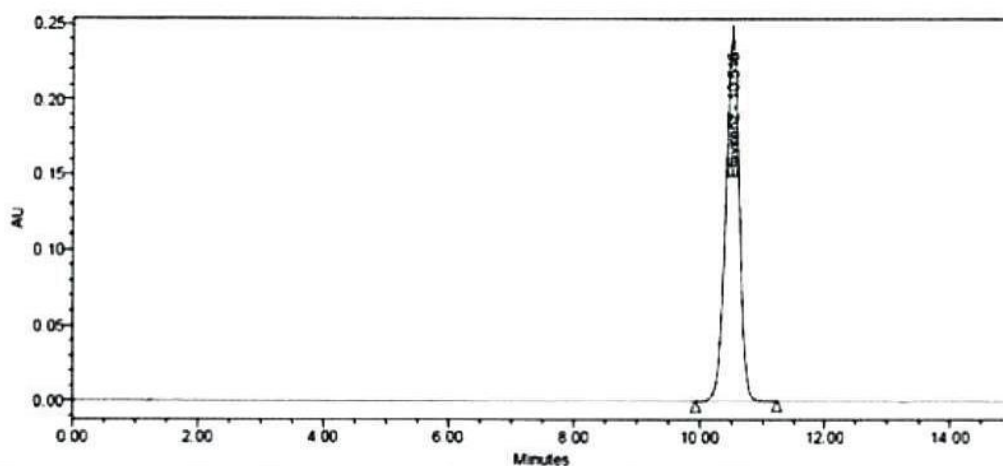

Fig 2: Chromatogram of Optimized trail.

RESULTS AND DISCUSSION:
Validation
Linearity: The linearity of the method was established by determining the absorbance of different concentrations of Efavirenz over a range of 0.066 and 3.024μg/ml respectively.

Table 1: Linearity table of Efavirenz

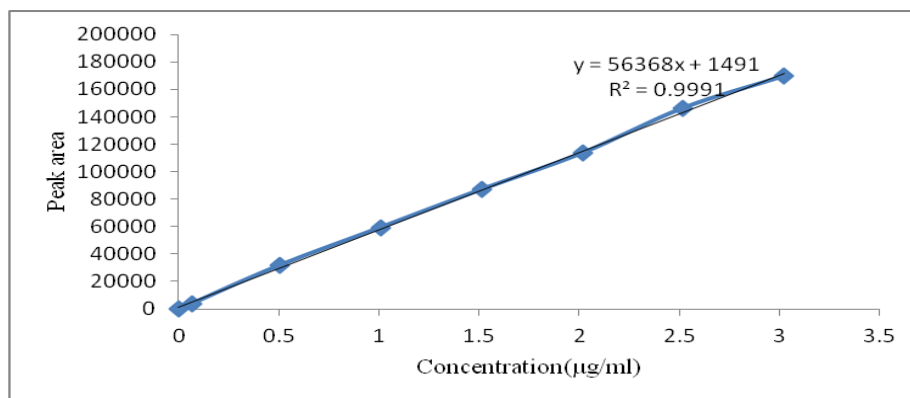| S. No.	Efavirenz Concentration (μg/ml)	Peak area
1	0.066	4053
2	0.504	31912
3	1.008	59428
4	1.512	87166
5	2.016	113633
6	2.520	146397
7	3.024	169654

Fig 3: Calibration curve of Efavirenz

Precision: Precision is one of the important factors which determine the reliability of an analytical method. The precision of the developed method was tested and was found to be suitable.

Table 2: Precision of Efavirenz

S. No.	Nominal Concentration (µg/ml)		
	LQC	MQC	HQC
	0.066	1.512	3.024
1	0.0652	1.511	3.022
2	0.066	1.512	3.023
3	0.0658	1.5115	3.0228
4	0.0648	1.512	3.0234
5	0.0656	1.511	3.023
Mean	0.065	1.511	3.023
S.D (+/-)	0.0005	0.0004	0.0005
%RSD	0.74	0.033	0.017

Accuracy: To determine the accuracy of the proposed method, recovery studies were carried out by analyzing the measured concentration and the added concentration of the drug. Each sample was injected thrice .The percent recoveries of the drugs were estimated.

Table 3: Accuracy level of Efavirenz.

Level	Concentration of drug added (µg/ml)	Amount of drug recovered (µg/ml) in placebo sample	Percentage Recovery in Placebo	Amount of drug recovered (µg/ml) in Mobile phase	Percentage Recovery in Mobile phase	Relative Recovery (%)
Level-I	0.067	0.066±0.001	Mean: 99.87	0.065±0.002	Mean: 99.24	99.54
Level-II	1.52	1.51± 0.01	Mean: 99.40	1.50±0.005	Mean : 99.52	99.46
Level-III	3.03	3.02±0.01	Mean : 99.56	3.02±0.005	Mean : 99.25	99.40

Robustness

The robustness of the proposed method was determined by analysis of aliquots from homogenous lots by differing physical parameters like volume of injection, wavelength which may differ but the responses were still within the limits of the assay.

Table 4: Robustness of Efavirenz.

Parameters		RT	Average Peak Area	Tailing factor	% RSD
Flow Rate	1.35 ml/min	11.853	55689	1.0	0.21
Flow Rate	1.65 ml/min	9.696	56015	1.0	0.14
Column Temperature	30˚C	10.278	55869	1.0	0.36
pH variation 1	pH 7.3	10.671	55986	1.0	0.32
pH variation 2	pH 7.7	10.572	55786	1.0	0.21
Mobile Phase Composition variation 1	Buffer: Acetonitrile (53:47)	8.846	56025	1.0	0.32
Mobile Phase Composition variation 2	Buffer: Acetonitrile (57:43)	13.299	55654	1.0	0.29

Ruggedness: Ruggedness is the degree of reproducibility of the results obtained under a variety of conditions. It was checked that the results were reproducible under different analysts.

Table. 5: Ruggedness of Efavirenz.

Sample	Analyst-1		Analyst-2	
	Mean peak area	%assay	Mean peak area	%assay
1	55236	98.86	55268	98.92
2	54593	97.7	55896	98.92
3	55613	99.53	55523	99.37
4	55231	98.85	55645	99.59
5	55836	99.93	55365	99.09
6	55024	98.48	55321	99.01
Mean		98.89		99.15
%RSD		0.79		0.27

STRESS DEGRADATION STUDIES

Blanks were prepared in the similar way for Acid/Base reagent and for Oxidation. Placebo was prepared in the similar way for Acid, Base, Peroxide, Heat and UV degradation.

Table 6: Stress Degradation studies of Efavirenz.

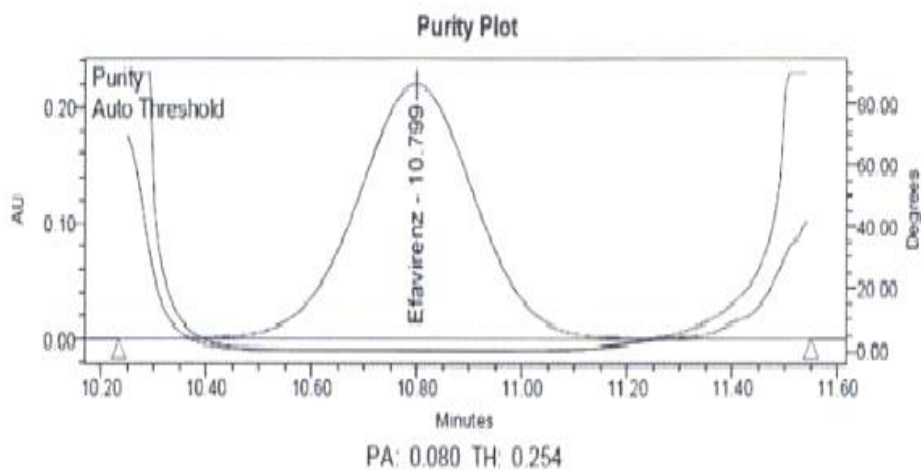| Sample name	Condition	% degradation	Purity angle	Purity threshold
Acid Stress sample	5ml 1N HCl, Heated on a water bath at 80˚C for 120 minutes	Nil	0.080	0.254
Base Stress sample	5 ml 1NaOH,kept at 80˚C for 120 minutes	10.06	0.083	0.259
Peroxide Stress sample	5ml 30% H_2O_2,heated on a water bath at 80˚C for 120 minutes	Nil	0.098	0.259
UV light Stress sample	Stressed under UV light for 72 hours	0.88	0.093	0.259
Heat Stress sample	Heated in a oven at 105˚Cfor 72 hours	0.78	0.084	0.261

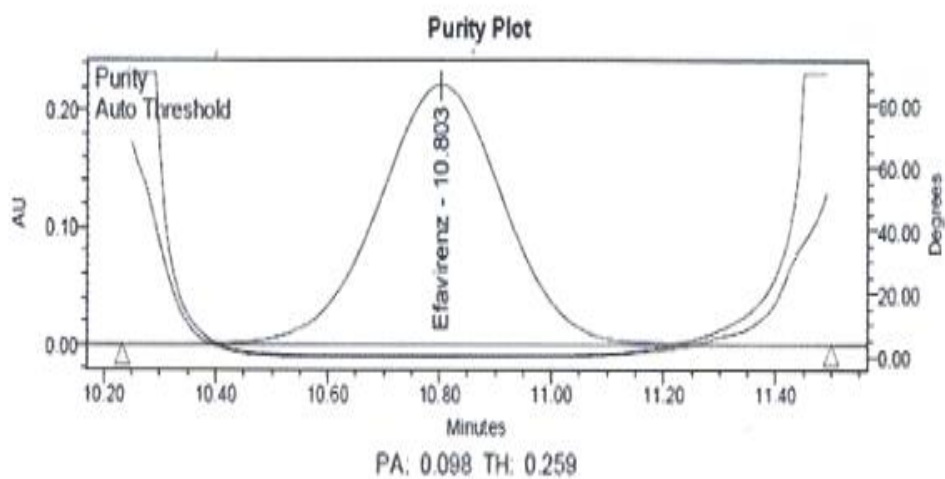**Fig 4: Acid stress sample :**

Fig 5: Base stress sample
:

Fig 6: Peroxide stress sample

Purity Plot

PA: 0.093 TH: 0.258
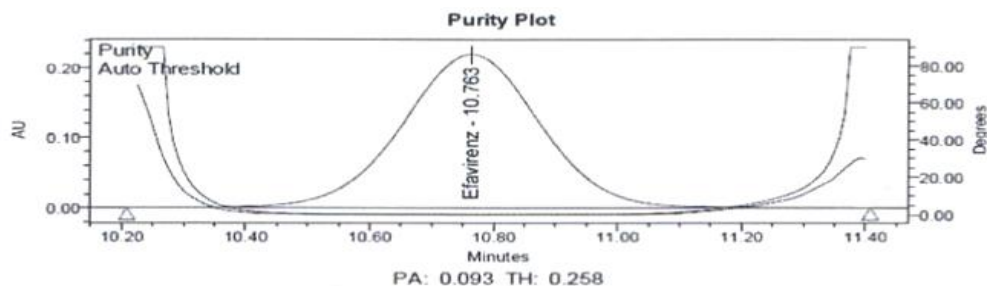
Fig 7: UV light sample :

Purity Plot
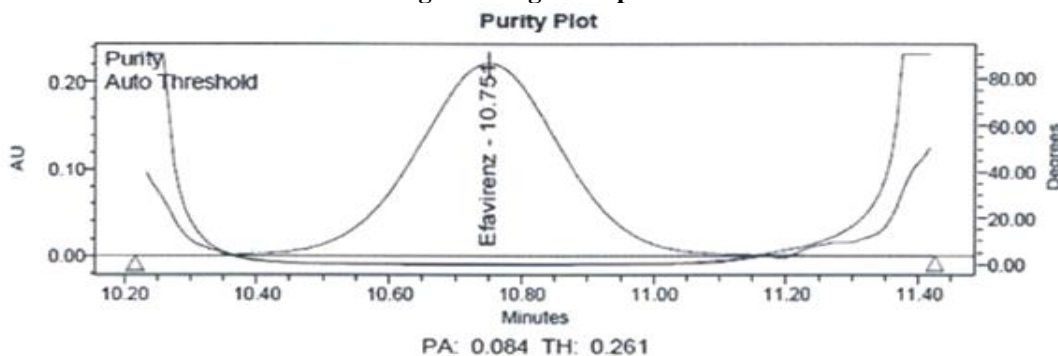

PA: 0.084 TH: 0.261

Fig 8: Heat stress sample:

Assay: Assay of different formulations available in the market was carried by injecting sample corresponding to equivalent weight into HPLC system and recovery studies were carried out.

Table. 7: Assay of Efavirenz.

Name	Labelled amount	Amount Found	% Label Claim ± % RSD	% Recovery ± % RSD
Efavirenz	100mg	98.5±1.0	98.5±1.0	98.4±0.8

CONCLUSION:

In the present work, an attempt was made to provide a newer, sensitive, simple, accurate and economical RP-HPLC method. It was successfully applied for the determination of Efavirenz in pharmaceutical dosage forms without the interferences of other constituents in the formulations.

Different mobile phase compositions were tried, to get good optimum results. Mobile phase and flow rate selection was done based on peak parameters (height, tailing, theoretical plates, capacity factor), run time etc. The system with Acetonitrile: 25mM Ammonium acetate in the ratio 50:50 was quite robust.

The optimum wavelength for detection was 252 nm at which better detector response for drug was obtained. The average retention time for Efavirenz was found to be 10.53 minutes.The calibrations was linear in concentration range of 0.066 and 3.024μg/ml for Efavirenz. The low values of % RSD indicate the method is precise and accurate. The mean recoveries were found in the range of 99.0 – 99.9 %. Sample to sample precision and accuracy were evaluated using, three samples of five and three different concentrations respectively, which were prepared and analyzed on same day. These results show the accuracy and reproducibility of the assay. Ruggedness of the proposed methods was determined by analysis of aliquots from homogeneous slot by different analysts, using similar operational and environmental conditions; the % RSD reported was found to be less than 2 %. Stress studies were also performed with acid

stress,base stress,uv light stress ,heat stress and peroxide stress that showed no difference even after introducing stress to the drug.There was no significant difference in the results achieved by the proposed method. The proposed method was validated in accordance with ICH parameters and the results of all methods were very close to each other as well as to the label value of commercial pharmaceutical formulation. It can be easily adopted for routine quality control for monitoring the assay in the API, in-process samples, and the finished formulations.

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