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Development and Validation of Ultra Performance Liquid Chromatographic and Mass Spectrometric Method for Quantitation of Efavirenz from Active Pharmaceutical Ingredient

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ABSTRACT

A novel, simple, rapid and stability-indicating reversed-phase ultra performance liquid chromatographic and mass spectrometric method was developed and subsequently validated for quantitation of Efavirenz (EFV) from drug substance matrix. The separation was achieved in 2.5 minutes on Waters ACQUITY UPLC BEH C_{18} (50 x 2.1) mm, 1.7µm column in isocratic mode with flow rate 0.4 mL/min. Mobile phase used was 0.01 M ammonium acetate buffer pH 7.5 and acetonitrile in ratio 50:50 v/v. Detection was carried out at the maximum wavelength of 247 nm using a photodiode array detector. The retention time of Efavirenz was found 1.8 minutes. Specificity of the method was established on drug substance by hydrolytic and oxidative stress conditions. Validation of analytical method was carried out as per the current ICH guidelines for linearity, recovery, precision, limit of detection, limit of quantification and robustness parameters.

Keywords: Efavirenz (EFV), UPLC, Antiretroviral, Stability indicating, ICH

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INTRODUCTION

Efavirenz is chemically (*S*)-6-Chloro-4-(cyclopropylethynyl)-1,4-dihydro-4-(trifluoromethyl)-2*H* -3,1-benzoxazin-2-one with molecular mass 315.67 g/mol[1]. It is used as non-nucleoside reverse transcriptase inhibitor[2] in human immunodeficiency virus infection (HIV) theropy in combination with other antiretroviral drugs[3]. Literature survey revealed that several analytical methods have been published for the determination of EFZ by RP-HPLC[4-8] and by UPLC[9-11]. Those published methods were having minimum 5 minutes run time in gradient mode of elution. However the exhaustive literature survey revealed that none of the most recognized pharmacopoeias or any journals published the method with less than 5 minutes run time in isocratic mode of elution. So we developed ultra performance liquid chromatographic isocratic procedure with less than 2.5 minutes run time which will serve as rapid, reliable, accurate, sensitive and stability indicating for estimation of EFV. Also this method can be directly used for mass spectrometry analysis. A chemical structure of EFV is presented in figure 1.

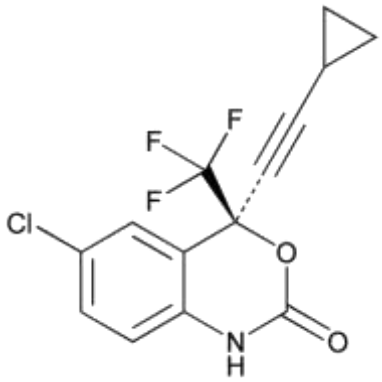


Figure 1: Chemical structure of Efavirenz(EFV)

MATERIALS AND METHODS

Chemicals and Reagents

Ammonium acetate (GR grade), Ammonia solution 30% v/v (GR grade) and Acetonitrile (HPLC grade) were purchased from Merck Fine Chemicals (Mumbai, India). The 0.2µm nylon filters were purchased from Advanced Micro Devices Pvt Ltd., India. High purity Efavirenz (EFV) was purchased from LGC Promochem India Ltd. Double distilled water was used throughout the experiment. Other chemicals used were of AR or GR grade.

Chromatograpic conditions

The Waters ACQUITY UPLC ultra performance liquid chromatographic system was used comprised of degasser, quaternary pump, auto injector, column compartment with heater & chiller facility, photodiode array detector and system control. Data collection and data processing were

accomplished by using Waters Empower chromatography data software. The separation of EFV was achieved on Waters ACQUITY BEH C_{18} (50 x 2.1) mm, 1.7µm column in isocratic mode. Mobile phase consisted of 0.01M ammonium acetate buffer pH 7.5 and acetonitrile in ratio of 50:50 v/v. The mobile phase pumped through column with flow rate of 0.4 mL/min in isocratic mode. Injection volume was 1.0 µL throughout the study. Based on the response of EFV peak the optimum wavelength 247 nm was selected.

Blank Preparation

Mobile phase was used as a blank solution throughout the study. Blank chromatogram is presented in figure 2.

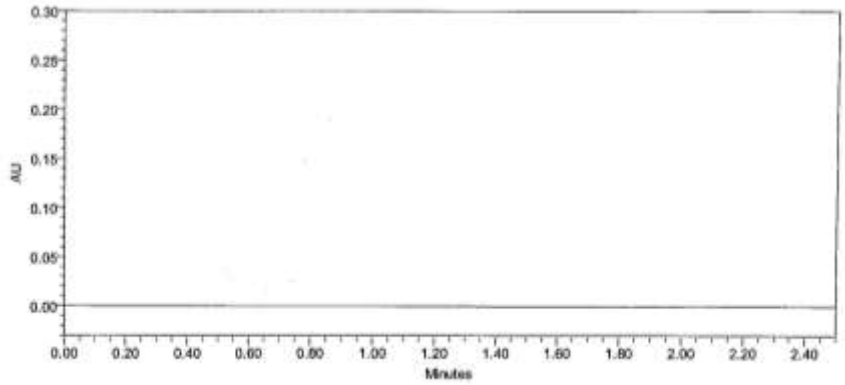
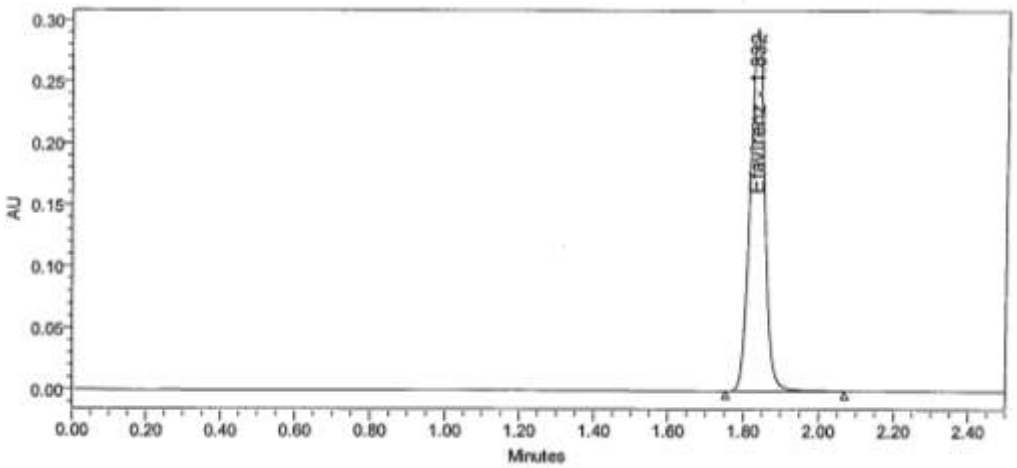


Figure 2: Typical UPLC chromatogram of Blank

Standard solution preparation

Dissolved 20 mg of EFV standard into 20 mL mobile phase and mixed. 2 mL of this solution was further diluted to 20 mL with mobile phase and mixed to achieve the concentration of EFV 100 µg/mL. Standard chromatogram is presented in figure 3.



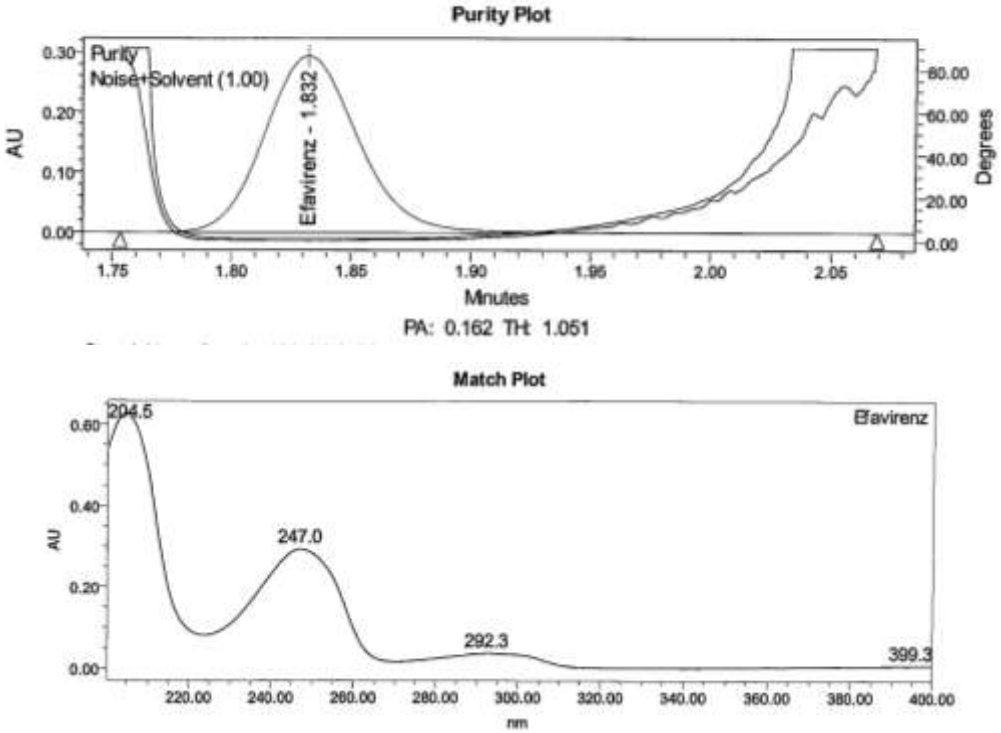
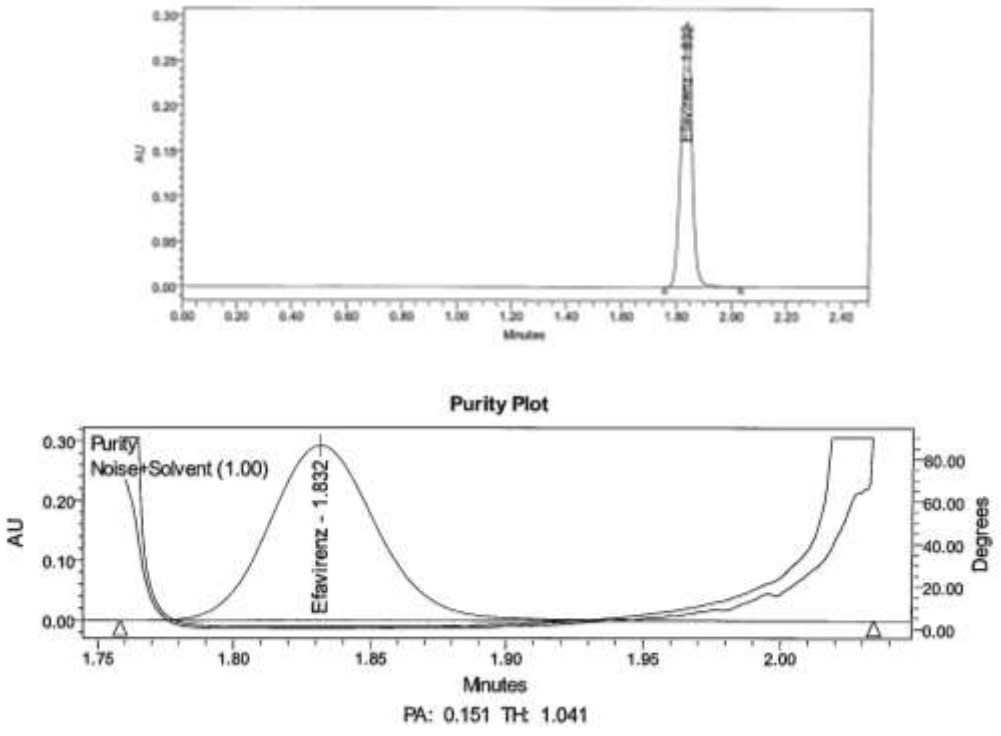


Figure 3: Typical UPLC chromatogram, Purity profile and UV spectra of EFV Standard

Sample solution preparation

Dissolved 20 mg of EFV standard into 20 mL mobile phase and mixed. 2 mL of this solution was further diluted to 20 mL with mobile phase and mixed to achieve the concentration of EFV 100 µg/mL. Sample chromatogram is presented in figure 4.



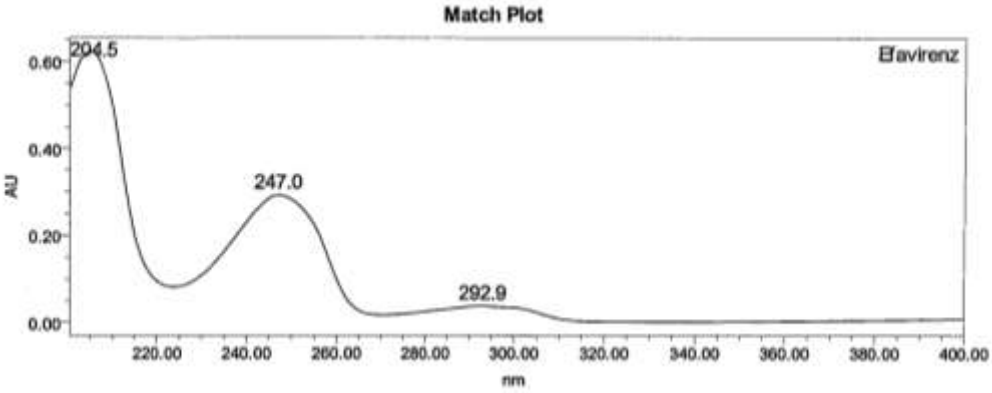


Figure 4: Typical UPLC chromatogram, Purity profile and UV spectra of EFV Sample

Method Validation

The optimized RP- UPLC method was validated according to ICH guidelines[12], with respect to specificity, accuracy, precision, linearity, limit of detection, limit of quantification and robustness.

Forced degradation studies

For hydrolysis degradation the sample solutions containing EFV 100µg/mL were treated with 5 mL 0.1N hydrochloric acid and 5 mL 0.1N sodium hydroxide solutions for 1 hr on water bath at 80°C. All the solutions prepared were quenched to their original pH before injection. For oxidation degradation the sample solution was treated with 5 mL of 5% v/v Hydrogen Peroxide and exposed to 80°C on water bath for 1 hr. The data obtained evaluated for the peak purity indices for the analyte peaks in stressed sample solutions by using PDA detector.

System suitability test

The system suitability test evaluated according to United States Pharmacopoeia by calculating % relative standard deviation, theoretical plates and tailing factor.

Precision

The precision of the analytical method was established by method precision and intermediate precision. Method precision was evaluated by assaying six sample solutions of EFV of same batch where as intermediate precision was carried out on next day by using different instrument and different column by using the same batch used under method precision study.

Accuracy

Accuracy of the method was evaluated by determining percent recovery of the EFV spiked in triplicate at three concentration levels 50, 100 and 150% respectively of test conc. in mobile phase.

Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD and LOQ for EFV was determined by signal to noise ratio of 3:1 and 10:1 respectively by injecting a series of dilute solutions with known concentrations.

Linearity

The linearity of EFV was established in the range of 50 to 150% of test concentration (50.68 µg/ml to 152.03 µg/ml) using serial dilutions of standard solutions.

Robustness

The robustness is the capacity of method to remain unaffected by small but deliberate changes in chromatographic conditions. Robustness was established by testing the influence of small changes in column temperature ($\pm 5^0$C), change in flow rate ($\pm$10%) and changes in organic composition of mobile phase ($\pm$ 2 %).

Solution stability

To demonstrate the stability of standard and sample solution, the solutions were analyzed over a period of 16 hrs stored at room temperature and the peak area counts observed were studied for all time points.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

The mechanism of retention in the reverse phase packing is due to the partitioning of the molecule into the lipophylic stationary phase, which primarily depends upon the lipophylicity of the compound. The other factor that influences the degree of retention is the nature of the mobile phase. The reversed-phase chromatography allows efficient separation of substances with different polarities by altering the composition/polarity of the mobile phase[13]. Retention, selectivity and peak symmetry of compounds are strongly influenced by the sorbents/stationary phases. Strongly distorted peaks of the compounds are often observed when unsuitable RP sorbents are used, due to the interaction of the compounds with free silanol groups on the sorbent matrix. EFV is weak acid compound with dissolution constant ranging 9 to 11[14]. The main objective of this investigation was to develop a rapid and reliable method on reverse phase stationary phase which will be helpful to analyze the samples in very short time. We initiated the development by using the Waters ACQUITY BEH C_{18} column on UPLC by using the ammonium acetate as a buffer in mobile phase and acetonitrile as organic modifier. Due to low percentage of organic modifier used, the retention of EFV observed higher on the stationary phase with peak symmetry more than 1.5. The higher retention observed due to the low polarity of the mobile phase. Hence we optimized the acetonitrile composition in mobile phase to achieve the retention time 1.8 with symmetry near to 1.0. The optimum performance of chromatography was achieved in 0.01M ammonium acetate buffer with pH 7.5 and acetonitrile in ration 50:50 v/v as a mobile phase with 0.4 mL/min flow are

in isocratic mode. The 247 nm wavelength of analysis was selected based on the spectral analysis of EFV peak by using the PDA.

METHOD VALIDATION RESULTS

Forced degradation studies

All stressed sample solutions were studied for the purity angle and purity threshold values. In all the above stressed solution chromatograms the purity angle found less than the purity threshold which proved the homogeneity of the analyte peaks. The data interpretation confirmed that there were no co-eluting peaks with the analytes which proved the ability of the method to assess unequivocally the analyte of interest in the presence of potential interference. Major degradation observed in oxidative stressed condition at 5 mL 5 % v/v hydrogen peroxide. The % degradation, purity angle and purity threshold values of all stressed conditions are tabulated in Table 1. The stressed sample chromatograms of EFV along with peak purity profile and mass spectra are presented in figure 5,6,7,8,9 & 10.

Table 1: Forced degradation data of EFV

Parameters	% Assay	% Deg[a]	PA[b]	PT[c]
Control sample	100.17	NA	0.151	1.041
Acid Deg. Sample (5ml 0.1NHCl/1hr)	99.28	0.89	0.167	1.049
Base Deg. sample (5ml 0.1NHCl/1hr)	99.53	0.64	0.156	1.039
Peroxide Deg. sample (5ml 5% H_2O_2/1hr)	97.13	3.04	0.203	1.069

[a] Percentage Degradation. [b] Purity angle. [c] Purity Threshold

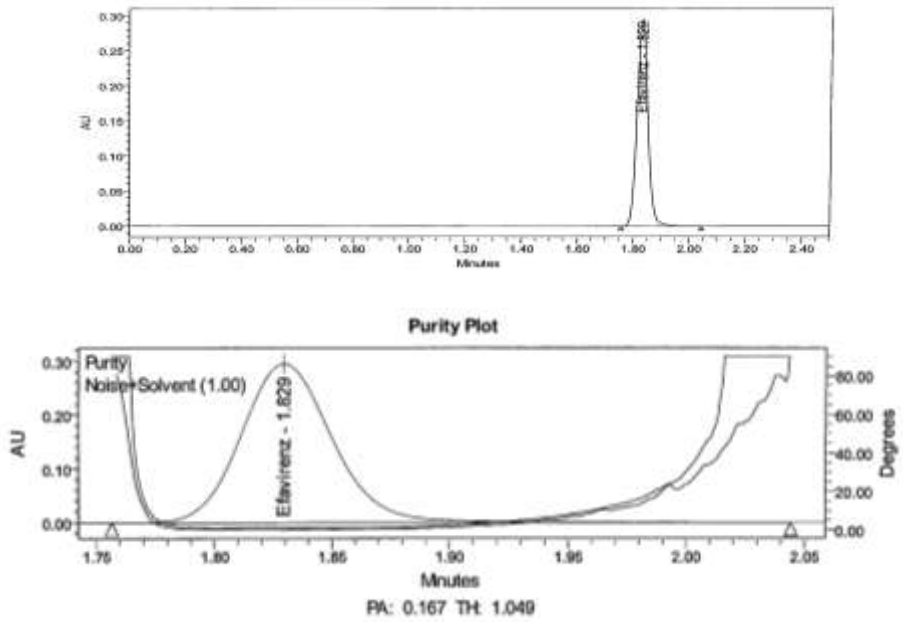


Figure 5: Typical UPLC chromatogram and Purity profile of EFV acid Stressed Sample

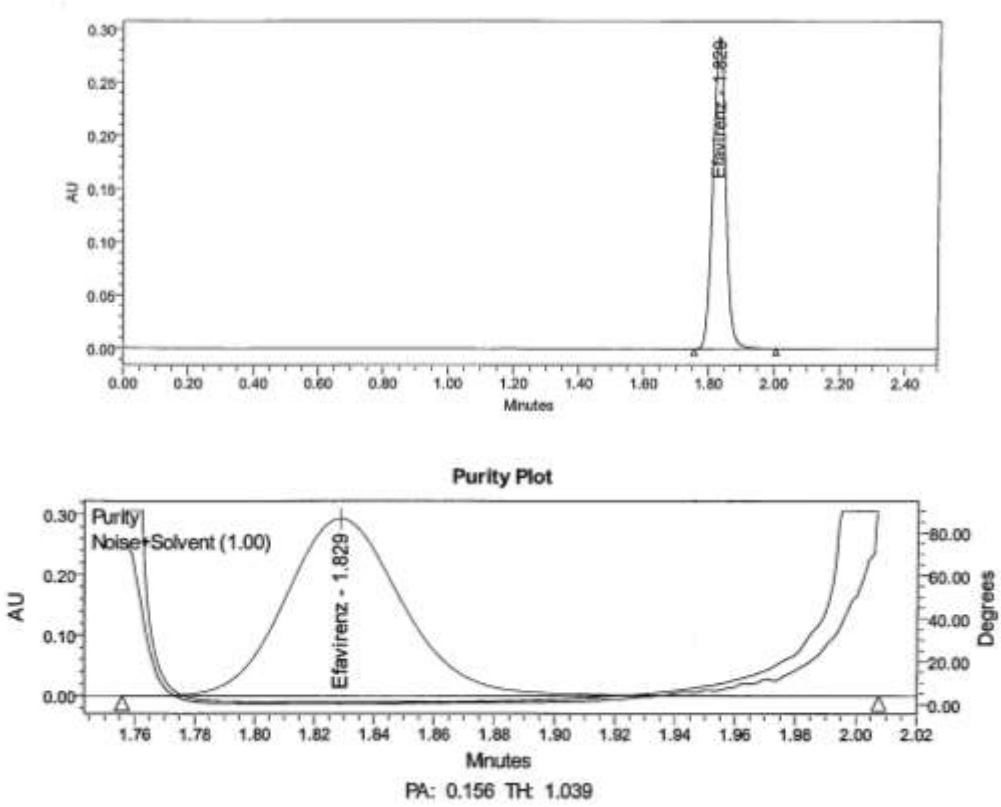


Figure 6: Typical UPLC chromatogram and Purity profile of EFV base Stressed Sample

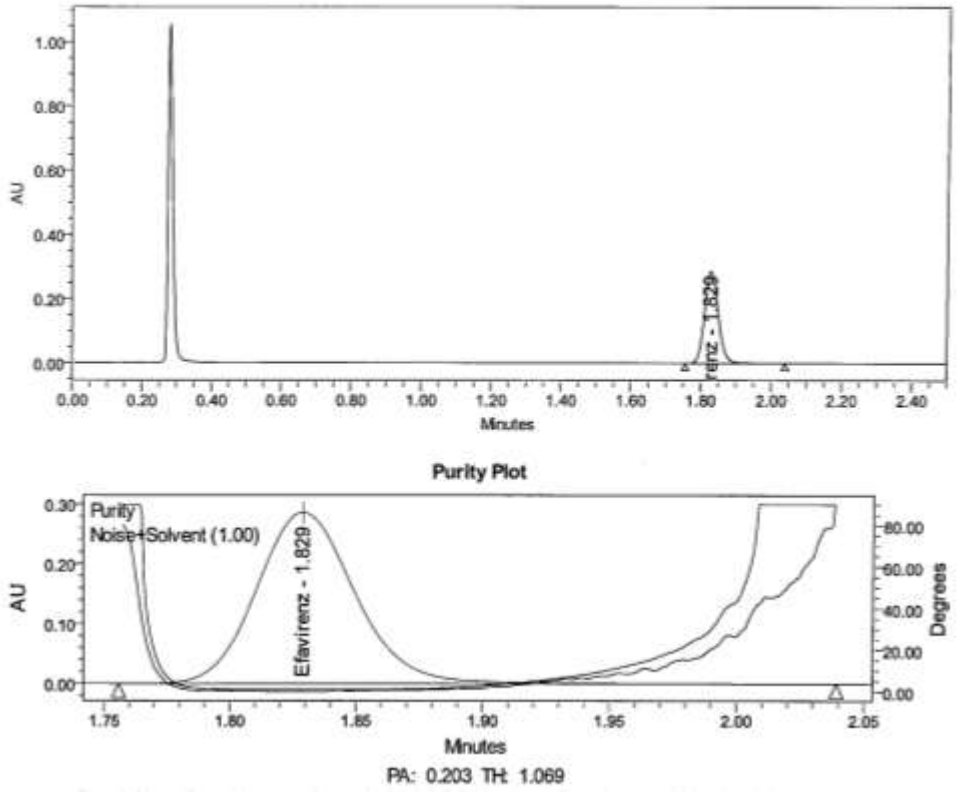


Figure 7: Typical UPLC chromatogram and purity profile of EFV Oxidation Stressed sample

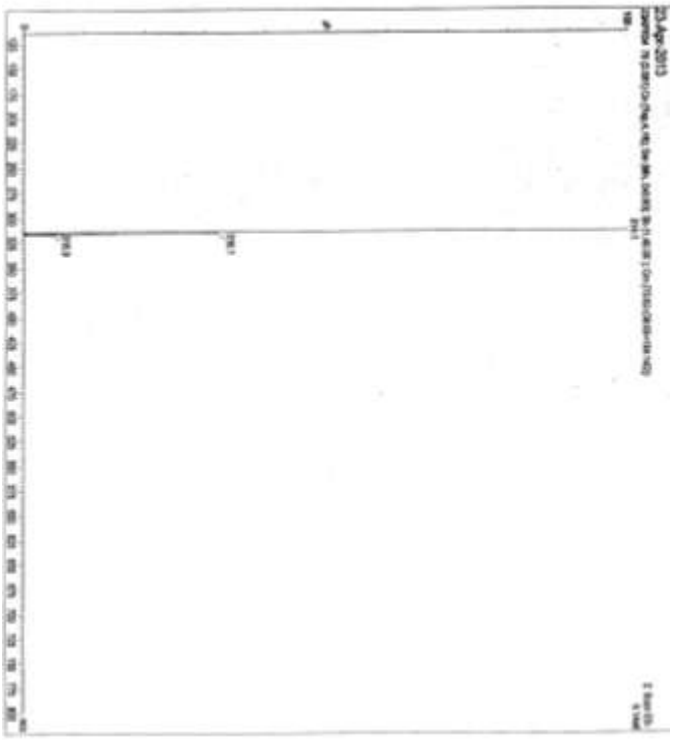


Figure 8: Typical mass spectrometric chromatogram of acid degradation of EFV Sample

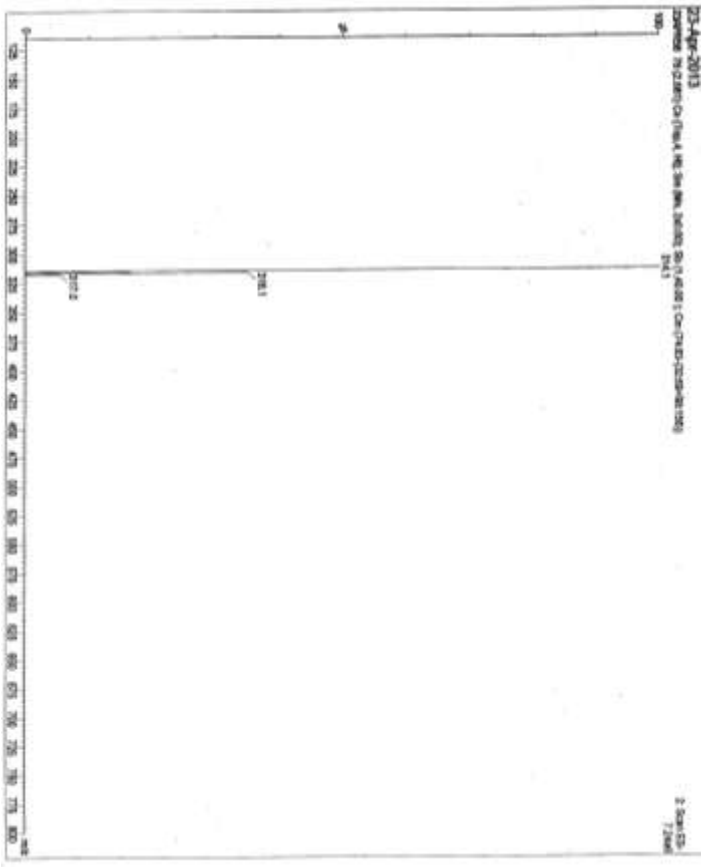


Figure 9: Typical mass spectrometric chromatogram of alkali degradation of EFV Sample

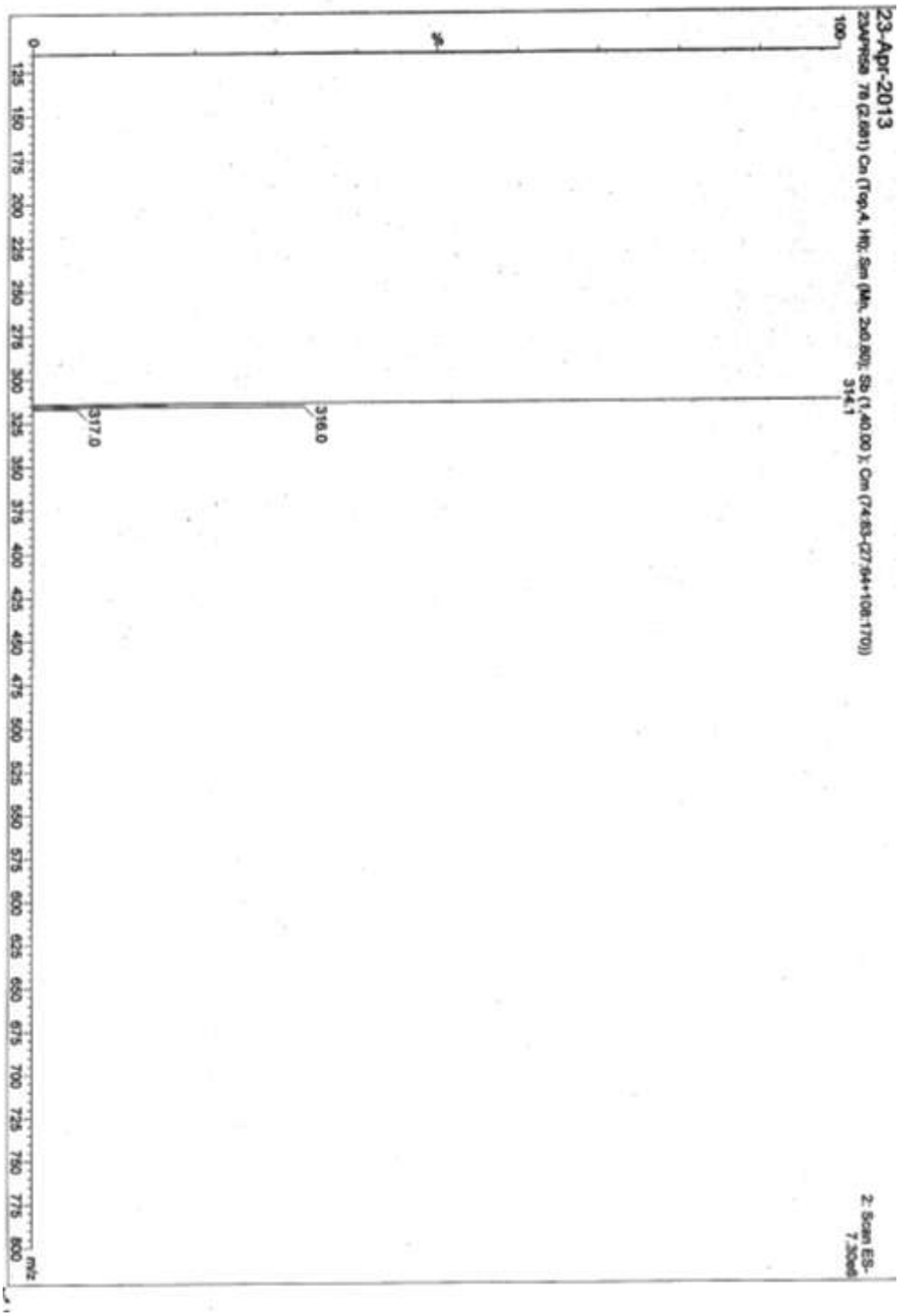


Figure 10: Typical mass spectrometric chromatogram of peroxide degradation of EFV Sample

System suitability test

The system suitability test was evaluated according to United States Pharmacopoeia and the results are tabulated in Table 2.

Table 2: Method validation results of EFV

Parameters	EFV
System precision (% R.S.D.)	0.25
Tailing factor (NMT 2)	1.12
Theorotical plates (NLT 2000)	10740
Method Precision[a]	100.17
Method Precision (% R.S.D.)	0.44
Intermediate Precision[a]	99.85
Intermediate Precision (% R.S.D.)	0.21
Accuracy (% Recovery at 50%)[b]	99.58
Accuracy (% Recovery at 100%)[b]	100.34
Accuracy (% Recovery at 150%)[b]	100.36
LOD (µg/mL)[a]	0.2027
LOQ (µg/mL)[a]	0.5068

[a]Average of six determinations, [b]Average of three determinations

Precision

The RSD of six replicate method precision results and intermediate precision results was studied and found less than 1 %. Percentage RSD of method precision and intermediate precision are tabulated in Table 2.

Accuracy

Accuracy of the method was evaluated at three different levels in triplicate on spiked samples. The average % recovery results are tabulated in Table 2.

Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD and LOQ for EFV were established from the RSD of six replicate injections and the data is tabulated in Table 2. The representative chromatograms of EFV LOD and LOQ are presented in figure 11 & 12.

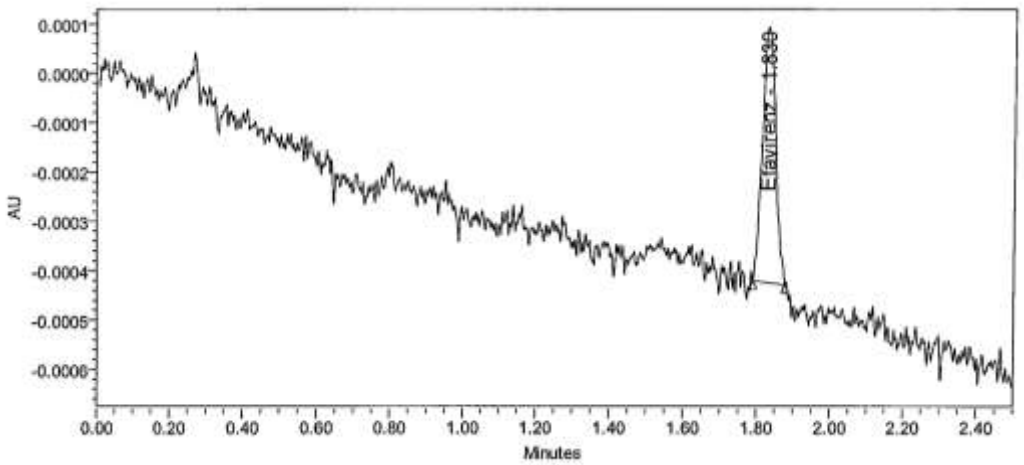


Figure 11: Typical LOD chromatogram of EFV

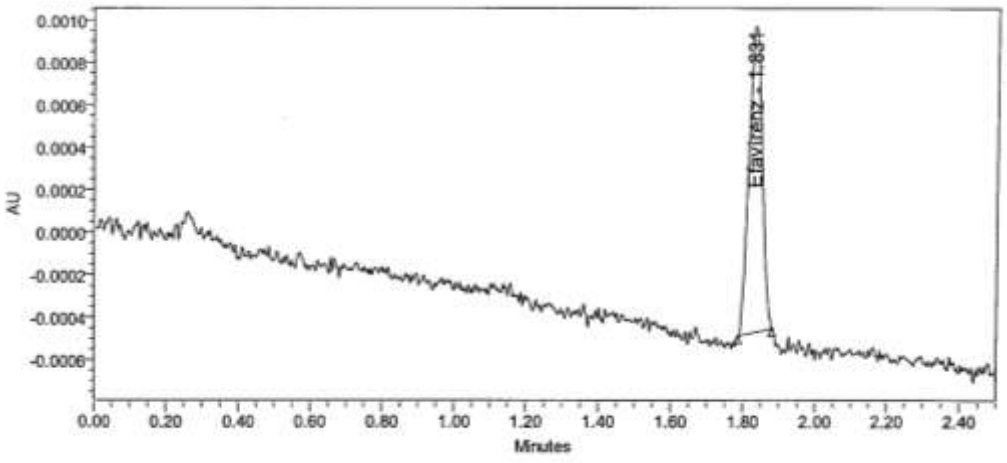


Figure 12: Typical LOQ chromatogram of EFV

Linearity

The linearity plot of EFV was studied and the following regression equation was established.

$y = 7969x - 7475$ ($R^2 = 0.99997$)

Robustness

The robustness data obtained was evaluated for the system suitability criteria as per the United States pharmacopoeia. The data obtained confirmed that the method is robust for the above parameters.

Solution Stability

The results confirmed that the peak areas of the EFV remained within acceptable range and no significant degradation was observed during this period. Thus the study concluded that the solutions can be used for the analysis after the preparation of 16 hrs.

CONCLUSION

A new rapid RP-UPLC/MS method with less than 2.5 minutes run time was successfully developed for the quantitation of EFV from drug substance matrix. The validation of proposed method was carried out as per current ICH guidelines. The method validation results confirmed that the method is selective, precise, accurate, robust and stability indicating. The proposed method provides optimum selectivity between the analyte peak and the degradants formed under degradation study. As the run time is very short we can analyze more number of samples in a very short time. In the proposed method the EFV elutes at 1.8 minutes and the analysis can be completed in less than 2.5 minutes. Hence this method can be used for the release and stability testing in quality control laboratories. Moreover, this method can also be used for the dosage forms after establishment of the specificity studies. Under validation study sample solution found stable

for 16 hrs at room temperature which can be helpful to cater the multiple unattended sample analysis or any breakdown of the instrument during analysis.

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