World Inventia Publishers

Journal of Pharma Research
http://www.jprinfo.com/

Vol. 6, Issue 3, 2017 ISSN: 2319-5622 USA CODEN: JPROK3

Research Article

STRESS DEGRADATION STUDIES AND DEVELOPMENT OF HIGH RESOLUTION NEW VALIDATED STABILITY INDICATING ANALYTICAL METHOD FOR DETERMINATION OF EMTRICITABINE, TENOFOVIR AND EFAVIRENZ IN ITS BULK AND MULTICOMPONENT PHARMACEUTICAL DOSAGE FORMS IN THE PRESENCE OF DEGRADATION PRODUCTS AS PER ICH GUIDELINES

M. Madan Mohan Reddy [1, 2]*, D. Gowri Sankar [2], JVLN. Seshagiri Rao [3]

[1] Director, Quality & Regulatory Affairs, Eisai Pharmaceuticals India Pvt. Ltd, Ramky Pharma City (SEZ), Parawada, Visakhapatnam, Andhra Pradesh, Pin code: 531019, INDIA.

[2] Department of Pharmaceutical Analysis, College of Pharmaceutical Sciences, Visakhapatnam, Andhra University, Andhra Pradesh, Pin Code: 530003, INDIA.

[3] Department of Pharmaceutical Analysis, College of Pharmaceutical Sciences, Andhra University, Visakhapatnam, Andhra Pradesh, Pin Code: 530003, INDIA.

Received on: 09-03-2017; Revised and Accepted on: 13-03-2017

ABSTRACT

A Novel high resolution, sensitive,accurate, robust & Rugged stability indicating analytical method was developed for Trimultaneous determination of three active pharmaceutical ingredients for the Trimultaneous Determination of three active ingredients including Emtricitabine (ECB), Tenofovir (TNF) and Efavirenz (EFZ) in its bulk and pharmaceutical dosage forms by RP-HPLC-PDA and the analytical separation was carried out by reverse phase chromatography on inertsil ODS column. Thermosil C $_{18}$ (4.6 x 150mm, 5µm) column with the isocratic program. The mobile phase was 0.1 % Orthophosphoric acid buffer with (pH 3.0) Acetonitrile in proportion 40: 60 v/v respectively with isocratic programme the flow rate for the mobile phase elution is 1.0 ml per minute and the column oven temperature is maintained ambient, run time was 10 minutes. The quantification was achieved with PDA detector and the effluents were monitored at 260 nm for three drugs and their combination drug products were subjected to various stress conditions. The calibration curves for all four drugs was found to be linear and the correlation coefficient for all three drugs is not less than (r^2=0.999). The LOD Concentration for ECB, TNF, EFZ was found to be 0.23µg/mL, 0.24 µg/mL & 1.04 µg/mL respectively. Then the LOQ Concentration for 0.78µg/mL, 0.78µg/mL and 3.52µg/mL were found respectively. There was no interference observed with excepients and degradation products in the determination of API and FP thus providing the stability indicating superiority of the method.

Keywords: *High resolution, Forced degradation studies, RP-HPLC-PDA Detector, Emtricitabine (ECB), Tenofovir (TNF) and Efavirenz (EFZ), Stability-indicating Analytical method.*

INTRODUCTION

Emtricitabine (ECB) is a nucleoside reverse transcriptase inhibitor (NRTIs). Chemically it is 5-fluoro-1-(2R,5S)-[2-(hydroxymethyl)-1, 3-oxathiolan- 5-yl] cytosine (Fig 1). FTC is the (-) enantiomer of thio analogue of cytidine which differs from other cytidine analogues, in that it has fluorine in 5th position. FTC inhibits reverse transcriptase, the enzyme that copies HIV RNA into new viral DNA. FTC is used for the prevention of perinatal HIV-1 reverse transcriptase [1]. It is also active against Hepatitis B virus [2, 3].

Tenofovir disoproxil Fumarate (TNF), acyclic phosphonate nucleotide analogue, is a fumaric acid salt of the bis iso propoxy carbonyl oxy methyl ester derivative of tenofovir. Chemically it is [[(1R)-2-(6-Amino-9H-purin-9-yl)-1-methylethoxy]methyl] phosphonic acid (Fig 2) [4]. It is the first nucleotide reverse transcriptase inhibitor (NtRTIs) approved for use in combination with other antiretroviral agents in the treatment of HIV-1 infection in the United States. Unlike the nucleoside reverse transcriptase inhibitors, which must undergo three intracellular phosphorylation steps for activation, nucleotide analogues such as Tenofovir require only two such steps. This reduction in the phosphorylation requirement has the potential to produce more rapid and complete conversion of the drug to its pharmacologically active metabolite [5-7]. Efavirenz (EFZ) ((4S)-6-chloro-4-(2-cyclopropylethynyl)-4-(trifluoromethyl)-2,4- dihydro-1H-3,1-benzoxazin-2-one) is a non-nucleoside reverse transcriptase inhibitors. It is used in the treatment of HIV infection (Fig. 3.). It binds directly and reversibly to the catalytic site of the reverse transcriptase enzyme and therefore, interferes with viral RNA to DNA directed polymerase activities [8-10].

Different methods have been reported in the literature for monitoring plasma levels of ECB, TNF and EFZ individually. Rezk et al. have reported a simultaneous method for the estimation of TNF and EFZ in human plasma using a validated HPLC method and other methods with a rather long run time. Some other techniques used in individual analysis of TNF from plasma include HPLC with mass spectrometric methods. Further we felt the need for the development of a selective, fast and stability-indicating RP-HPLC method. To the best of our knowledge, no proved stability indicating with spectrum index based studies method has not been reported for the determination of ECB, TNF and EFZ drug substance and drug product for regular analysis and stability studies in quality control laboratory. The core-objective of this research work was to develop a fast, precise, sensitive and stability-indicating RP-HPLC method for the determination of process and degradation related impurities of ECB, TNF and EFZ .The developed method was successfully validated according to the USP 1225 Validation of Compendial Procedures and ICH guidelines [11-15].

***Corresponding author:**
M. Madan Mohan Reddy
Director, Quality & Regulatory Affairs,
Eisai Pharmaceuticals India Pvt. Ltd, Ramky Pharma City (SEZ),
Parawada, Visakhapatnam, Andhra Pradesh, Pin code: 531019, INDIA.
**E-Mail: mmadanreddy03@gmail.com*

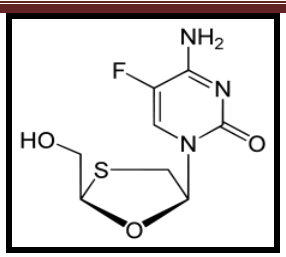

Fig. 1: Structure of Emtricitabine (ECB)

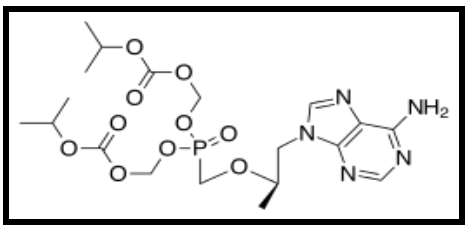

Fig. 2: Structure of Tenofovir (TNF)

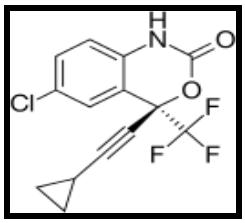

Fig. 3: Structure of Efavirenz (EFZ)

EXPERIMENTAL

1. Materials & Methods:

Pharmaceutical grade working standards Emtricitabine (ECB), Tenofovir (TNF) and Efavirenz (EFZ) were obtained from Eisai Pharmaceuticals as gratis samples. The HPLC-grade methanol was purchased from Merck. All other chemicals like acetonitrile, OPA, 0. 22µ membrane filter, 0.45µ filter paper and solvent used were of analytical grade (Merk). High purity water was prepared by using a Milli-Q RO system (Millipore). All the chemical and reagents were purchased from Merck chemicals.

2. Instrumentation:

The analysis was performed using waters-2695(Model alliance) High Performance liquid chromatography waters auto sampler–PDA detector 996 by using, Empower-software version-2, analytical balance (MettlerToledo) UV/Visible-Detector (Standard cell) and data handling system (Autochrome-3000), pH meter (lab India), Sonicator. The column used is Thermosil C $_{18}$ (4.6 x 150mm, 5µm) column with the Isocratic program.

3. Preparation of solutions:

Preparation of 0.1 % OPA buffer:

1 ml of Orthophosphoric acid was taken in a 1000ml volumetric flask and adjust the P^{H} with Diluted Triethylamine upto 3, finally the solution was filtered by using 0.45 Micron membrane filter, sonicate it for 10 mins.

4. Mobile phase (MP) Preparation:

Accurately measured 400 ml (40%) of above buffer and 600 ml of Acetonitrile HPLC (60%) were mixed and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

5. Preparation of blank solution:

Buffer and Acetonitrile 40:60 v/v this prepared solution was used as blank solution. This solution was also used for specificity blank solution

6. Preparation of Placebo Solution:

The placebo Solution was prepared by dissolving the Specified amount Excipients in diluent (in house made).

7. Preparation of STD stock solution:

Standard solution of ECB, TNF and EFZ were prepared by accurately weigh and transfer 20mg of Emtricitabine, 30mg of Tenofovir and 60mg of Efavirenz working standard into a 100 ml clean dry volumetric flask add about 70 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 1.5 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.(30ppm of EMT, 45ppm of TNF and 90ppm of EFA)

8. Preparation of STD solution:

From the Prepared individual Standard Stock Solution of ECB, TNF and EFZ take 0.3 ml of ECB, TNF and EFZ into a 10ml of standard flak to this add 10ml of diluent. Finally make up the solution upto the mark with diluent. The Final concentration of the individual was 30 µg/ml respectively.

9. Preparation of Test solution:

The test solution was prepared by taking an equivalent amount of ECB, TNF, EFZ first Accurately weigh 10 tablets crush in mortor and pestle and transfer equivalent to 20mg of Emtricitabine, 30mg of Tenofovir and 60mg of Efavirenz in (marketed formulation=126.53 mg of tablet Powder) sample into a 100mL clean dry volumetric flask add about 70 mL of Diluent and sonicate it up to 30 mins to dissolve it completely and make volume up to the mark with the same solvent. Then it is Filtered through 0.45 micron Injection filter. (Stock solution)

Further pipette 1.5 ml of Emtricitabine, Tenofovir and Efavirenz from the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. (30ppm of EMT, 45ppm of TENO and 90ppm of EFA)

Procedure:

Inject 10 µL of the standard, sample into the chromatographic system and measure the areas for Emtricitabine, Tenofovir and Efavirenz peaks and calculate the %Assay by using the formulae.

10. Optimization of HPLC Method:

The HPLC method was optimized and developed for Trimultaneous method for ECB, TNF, and EFZ. The mixed standard solution was injected in HPLC by the following chromatographic conditions.

The reverse phase chromatography was performed on inertsil ODS column Thermosil C $_{18}$ (4.6 x 150mm, 5µm) column with the isocratic program.The mobile phase was adjusted with 0.1 % Orthophosphoric acid buffer with (pH 3.0) Acetonitrile in proportion 40: 60 v/v respectively with isocratic programme the flow rate for the mobile phase elution is 1.0 ml per minute and the column oven temperature is maintained ambient, run time was 10 minutes. The quantification was achieved with PDA detector and the effluents were monitored at 260 nm for three drugs and their combination drug products were subjected to various stress conditions **(Table 1).**

11. Method validation:

The method validation was done according to the ICH guidelines. The following validation characteristic parameters are accuracy, precision, linearity, and specificity, LOD, LOQ, ruggedness and robustness.

11.1. Linearity and range:

Linearity of the method was studied by injecting the mixed standard solutions with the concentration ranges from of 10-50µg/mL for ECB, TNF and EFZ drug levels of increasing concentrations were prepared and injected six times into the HPLC system keeping the constant injection volume. The peak areas were plotted against the concentrations to obtain the linearity graphs.

11.2. Precision:

The precision of the optimized method was evaluated by carrying out six independent assays of test sample. %RSD of six assay values was calculated. Intermediate precision was carried out by the samples by using another instrument and with different analyst.

11.3. Limit of Detection and Quantification:

The LOD and LOQ procedures were performed on samples contain very lower concentrations of analytes under the ICH

guidelines. By applying the visual evaluation method, LOD was expressed by establishing the lowest concentration at which the analyte can be detected. LOQ was considered as the lowest concentration of analytes that can be detected and quantified, with acceptable accuracy and precision.

11.4. Robustness:

Robustness was studied by evaluating the effect of small variations in the chromatographic conditions. The conditions studied were flow rate altered by ±0.1ml/min, mobile phase composition. These chromatographic variations are evaluated for resolution between ECB, TNF,EFZ

11.5. System suitability:

The system suitability parameters with respect of tailing factor, theoretical plates, repeatability and resolution between ECB, TNF, EFZ peaks were defined.

11.6. Specificity:

The specificity of the analytical method is the ability of the method to estimate the analyte response in the presence of additional components such as impurities, degradation products and matrix [19]. The peak purity of ECB, TNF,EFZ were assessed by comparing the Retention time of standard ECB, TNF,EFZ good correlation was obtained between the Retention time of standard and sample of ECB, TNF and EFZ. The specificity method was also evaluated to ensure that there were no interference products resulting from forced degradation studies.

11.7. Forced degradation study:

Forced degradation or Stress testing of a drug substance will help to identify the degradation products, which can help to establish the intrinsic stability of the molecule .All stress decomposition studies were performed at an initial drug concentration 200µg/mL of ECB, TNF and EFZ.

The Stability indicating study of ECB, TNF and EFZ were undergoes acid, alkali and oxidation degradation, photolysis and heat condition. Placebo Interference: The placebo (in the present of excipients in tablet) sample were prepared as per the test method and analyzed in the HPLC. It expressed there is no additional peaks at the retention time of ECB, TNF and EFZ in the chromatograph it indicates that there is no placebo interference.

Acid Degradation:

Sample was treated with 3ml of 1N hydrochloric acid and kept for 10hrs. After 10hrs the solution was neutralized with 3ml of 1N sodium hydroxide, made the volume upto the mark with mobile phase and analyzed using HPLC.

Alkali Degradation:

Sample was treated with 3ml of 1N sodium hydroxide and kept for 10hr. After 10hr the solution was neutralized with 3ml of 1N hydrochloric acid, made the volume up to the mark with mobile phase and analyzed using HPLC.

Oxidative Degradation:

ECB, TNF and EFZ solutions of 200 and 20µg/ml were mixed with 3mL of 30%v/v aqueous hydrogen peroxide solution and kept for 10hrs. After 10hrs made the volume up to the mark with mobile phase and analyzed using HPLC.

Photolytic Degradation:

The ECB, TNF and EFZ samples were kept under UV light for different time intervals (15mins – 7days) and made the volume upto the mark with mobile phase and analyzed using HPLC. Thermal Degradation: Samples were heated at 800 C for 15mins -60mins and 2200 C for 2-5mins and analyzed.

11.8. Accuracy:

Accuracy was carried out by applying the method to drug sample (ECB, TNF and EFZ combination of tablets) to which known amounts of ECB, TNF and EFZ Standard powder corresponding to 50,100 and 150% of label claim was added, mixed and the powder was extracted and determined by the system in optimized mobile phase. The experiment was performed in triplicate and percentage recovery, % RSD was calculated.

11.9. Analysis of marketed formulation:

The marketed formulation was assayed by above description. The peak areas were monitored at 280nm and determination of sample concentrations were using by multilevel calibration developed on the same HPLC system under the same conditions using linear regression analyzed for ECB, TNF and EFZ in the same way as described above.

RESULTS AND DISCUSSIONS

The simultaneous HPLC method was optimized and developed for ECB, TNF and EFZ. The mixed standard solution was injected in HPLC by the following chromatographic conditions.The chromatographic separation was achived on inertsil ODS column. Thermosil C $_{18}$ (4.6 x 150mm, 5µm) column with the isocratic program.The mobile phase was optimized to 0.1 % Orthophosphoric acid buffer with (pH 3.0) Acetonitrile in proportion 40: 60 v/v respectively with isocratic programme the flow rate for the mobile phase elution is 1.0 ml per minute and the column oven temperature is maintained ambient, run time was 10 minutes. The quantification was achieved with PDA detector and the effluents were monitored at 260 nm for three drugs and their combination drug products were subjected to various stress conditions

1. Method Development and Optimization:

The HPLC procedure was optimized with a view to develop a suitable LC method for the analysis ECB, TNF and EFZ in fixed dose for bulk and combined dosage form. It was found that mobile phase consists of to 0.1 % Orthophosphoric acid buffer with (pH 3.0) Acetonitrile in proportion 40: 60 v/v respectively with isocratic program has given good resolution, theoretical plates, and for ECB, TNF and EFZ at the flow rate of 1.0 ml/min **(Table. 1; Fig. 4 & 5).**

Table No. 1: Optimized Chromatographic Conditions

Parameters	Method
Stationary phase (column)	Thermosil C $_{18}$ (4.6 x 150mm, 5µm)
Mobile Phase	0.1 % Orthophosphoric acid buffer with (pH 3.0) Acetonitrile in proportion 40: 60 v/v
pH	3.0
Flow rate (ml/min)	1.0ml/min
Run time (minutes)	10 mins
Column temperature (°C)	Ambient
Volume of injection loop (µl)	20µl
Detection wavelength (nm)	260 nm
Drugs RT (min)	2.291,2.826 & 5.525

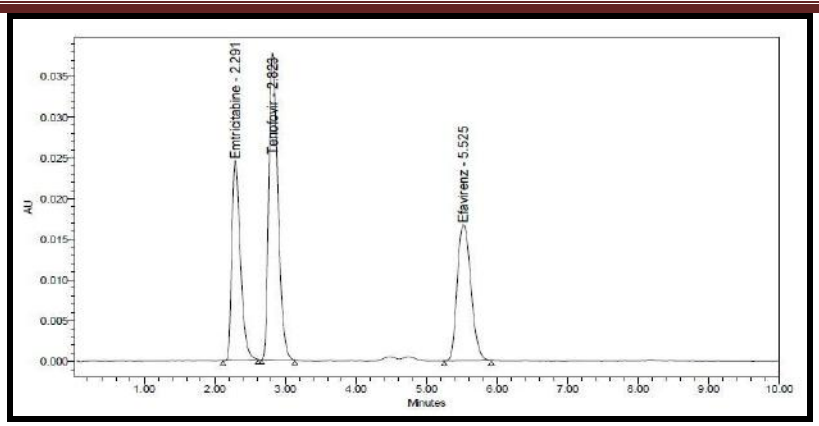

Fig. 4: Chromatogram of standard API MIXTURE (ECB, TNF and EFZ)

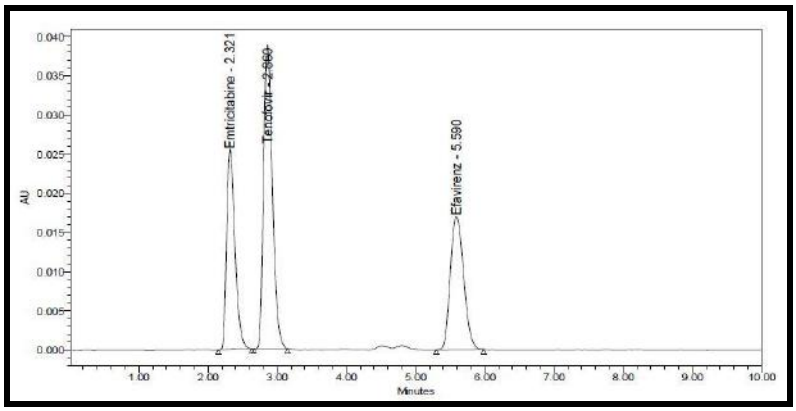

Fig. 5: Chromatogram of Sample DRUG PRODUCT MIXTURE (ECB, TNF and EFZ)

2. Validation of Developed Method:

The method validation was done according to the ICH guidelines. The following validation characteristic parameters accuracy, precision, linearity, and specificity, LOD, LOQ and robustness.

2.1. Linearity: The linearity five levels of concentrations with correlation regression curves are obtained the conc. range of 10-50µg/mL for ECB, 15-75µg/mL TNF and 30-150µg/mL EFZ. The reports of drug were found the linear in prepared conc. Where X was the conc of the drug in µg/ml & Y was area of the peak in the absorbance unit. The chromatograms were obtained during the linearity were shown in the (**Figure 6-9 & Table 2 &3.)**

Table No. 2: Linearity study of ECB and TNF

Linearity level	ECB		TNF	
	Conc. (µg/ml)	Mean Area	Conc. (µg/ml)	Mean Area
1	10	70032	15	111533
2	20	139904	30	224125
3	30	214202	45	344203
4	40	281523	60	451568
5	50	350367	75	562030
Correlation co-efficient		0.999		0.999
Slope		7022.x		7522.x
Intercept		518.9		160.7

Table No. 3: Linearity study of EFZ

Linearity level	EFZ	
	Conc. (µg/ml)	Mean Area
1	30	73968
2	60	149609
3	90	229201
4	120	301089
5	150	373690
Correlation co-efficient		0.999
Slope		2503.x
Intercept		234.2

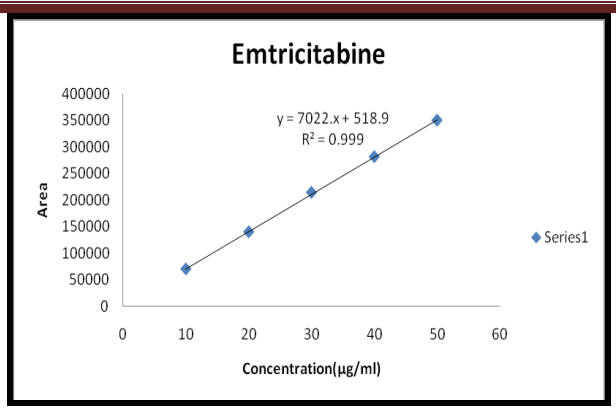

Fig. 6: Linearity curve for ECB

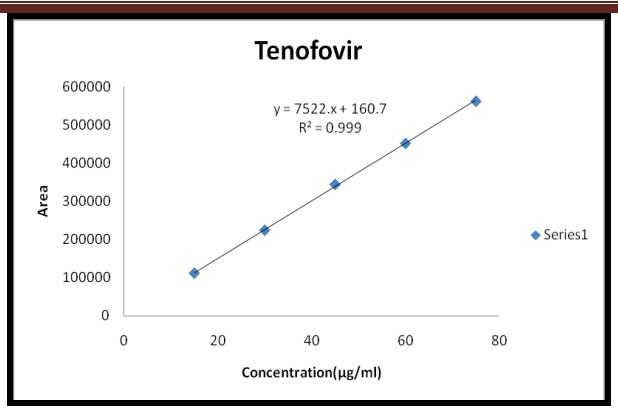

Fig. 7: Linearity curve for TNF

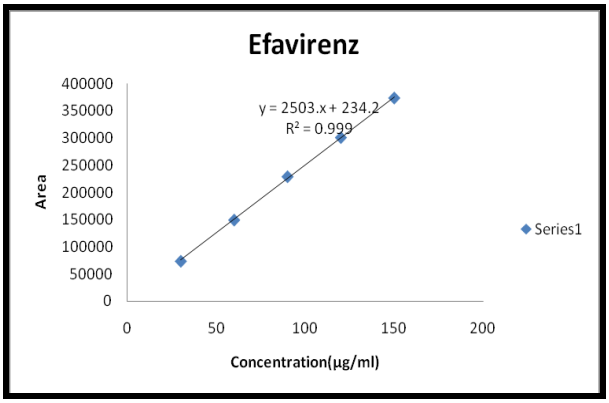

Fig. 8: Linearity curve for EFZ

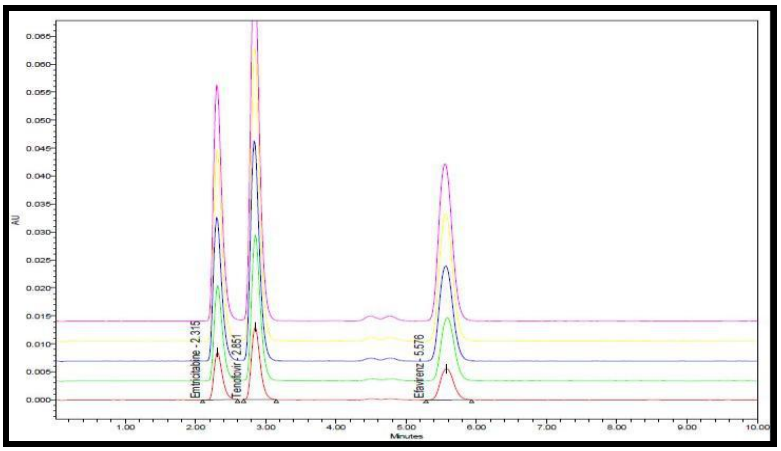

Fig. 9: Overlay linearity Chromatogram for linearity all Levels (1-5)

2.2. Precision: Precision of this analysis, as the intraday precision was evaluated by performing six individual test samples prepared & calculated the % RSD. Interday precision of this method was analyzed by the performing same the procedure with the various days by the person with the same developed environment. Resulting data of precision was given in the **Table 4 &5 (Fig.10)**. The % RSD values of the intra-day precision & interday precision study was < 2.0% for ECB,TNFand EFZ. This is confirmed that method was precise.

Table No. 4: Precision study of ECB and TNF.

Replicate	Area of ECB		Area of TNF	
	Intra-day precision	Inter-day precision	Intra-day precision	Inter-day precision
1	213869	211581	343021	337668
2	215322	212976	345443	339880
3	214393	212922	343710	338500
4	214047	213253	343686	337991
5	213566	211853	343663	336856
6	210975	213795	339480	340093
Mean	213695.1	212730.0	343167.1	338498.0
St. dev.	1463.2	847.9	1980.2	1272.5
% RSD	0.7	0.4	0.6	0.4

Table No. 5: Precision study of EFZ

Replicate	Area of EFZ	
	Intra-day precision	Inter-day precision
1	228951	226726
2	230317	225790
3	228344	225911
4	229939	228473
5	228902	228596
6	226840	229001
Mean	228882.1	227416.2
St. dev.	1236.1	1442.7
% RSD	0.5	0.6

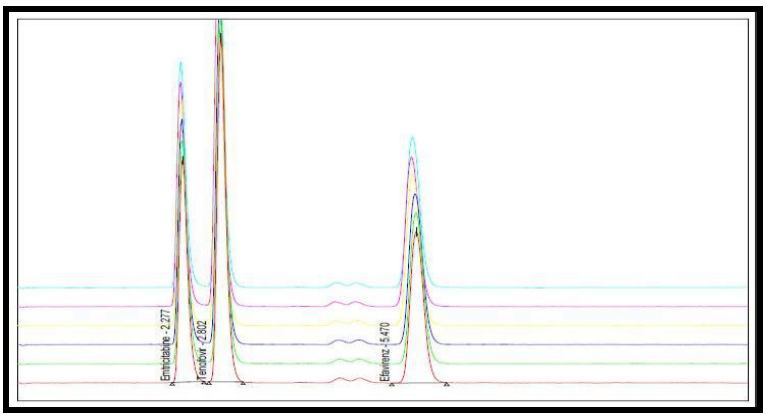

Fig. 10: Overlay precision Chromatogram for ECB, TNF and EFZ

2.3. LOD and LOQ: Limit of detection (LOD) & the limit of quantifications (LOQ) are evaluated by the serial dilutions of ECB, TNF and EFZ stock solutions in the ordered to be obtaining the signal to the noise ratio 3:1 for the LOD & 10:1 for the LOQ. The LOD Concentration for ECB, TNF,EFZ was found to be 0.23µg/mL,0.24 µg/mL,&1.04 µg/mL respectively. Then the LOQ Concentration for 0.78µg/mL, 0.78µg/mL and3.52µg/mL were found respectively. The chromatogram of the LOD and LOQ were shown in the **(Fig. 11 &12).**

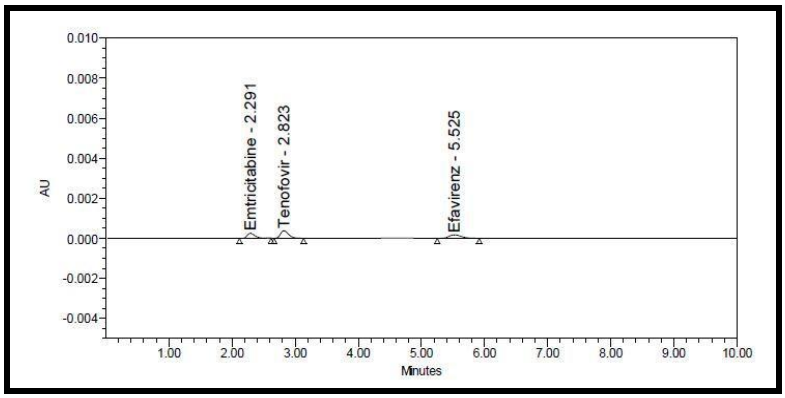

Fig. 11: Chromatogram of LOD study of ECB, TNF and EFZ

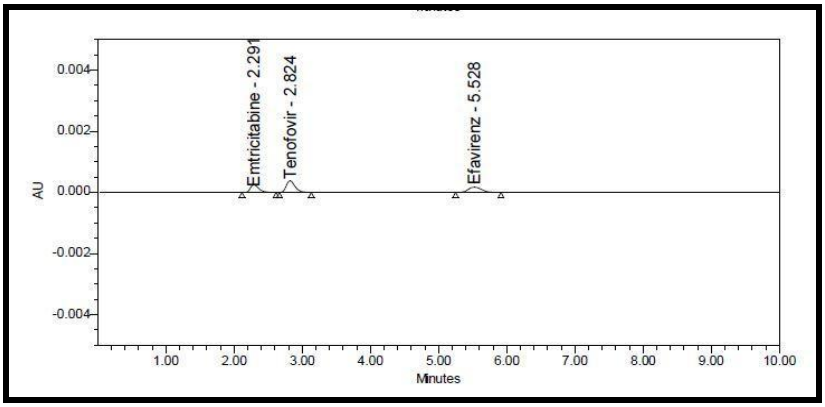

Fig. 12: Chromatogram of LOQ study of ECB, TNF and EFZ

2.4. Specificity: The specificity is a method for drug establishing by the verifying for the interferences with drug quantification from degradation products are formed during forced degradation study and peak purity for ECB, TNF and EFZ were found better under the various conditions. There were no other interferences of any other peaks and degradation products with the drug peaks.

2.5. System suitability: The system suitability parameters with respect of tailing factor, theoretical plates, repeatability and resolution between ECB, TNF and EFZ peaks were defined five replicate injections of the standard solution were injected and asymmetry, resolution, % RSD of peak area and theoretical plate were determined. For system suitability, asymmetry parameters should not more than 2.0, resolution should be more than 3.0 theoretical plate should not less than 3000 & % RSD for peak area should not be more than 2.0%, were full fill during all validation parameters all parameter are within the range of ICH prescribed Limits **(Table.6).**

Table No. 6: System suitability parameters for ECB, TNF and EFZ

System suitability parameters	ECB	TNF	EFZ
Retention time (min)	2.291	2.823	5.526
Repeatability of retention time; %R.S.D (n=5)	0.01	0.05	0.05
Repeatability of peak area; %R.S.D= (S.D./Mean)×100	0.7	0.6	0.5
Resolution (Rs)	-	2.38	9.84
Tailing factor (asymmetric factor)	1.40	1.35	1.24
USP plate count	2757.74	2479.32	4174.80
LOD (µg/mL)	0.23	0.24	01.04
LOQ (µg/mL)	0.78	0.78	3.52

2.6. Robustness: The robustness is studied by evaluating effects of small but the deliberate differences in method condition. The results of robustness for developed methods were started in the **Table 7.** The results are shown during all the different conditions of the test solution wasn't affective & in the accordance with an actual one. The suitability also found better; hence this method was conformed as robust. The chromatograms were Obtained during the robustness were shown in the **Figure 13-16.**

Table No. 7: Evaluation data of Robustness study of ECB, TNF and EFZ

	Parameters	Adjusted to	USP Plate Count	USP Tailing	USP Resolution
ECB	Flow Rate As per method 1.0ml/min	0.8 ml/min	2828.94	1.42	
		*1.0 ml/min	2768.97	1.41	
		1.2 ml/min	2773.51	1.43	
	Wavelength As per method 260nm	258 nm	2796.70	1.42	
		**260 nm	2768.97	1.41	
		262 nm	2811.61	1.43	
TNF	Flow Rate As per method 1.0ml/min	0.8 ml/min	2528.32	1.35	2.41
		*1.0 ml/min	2442.26	1.34	2.36
		1.2 ml/min	2442.59	1.35	2.37
	Wavelength As per method 260nm	258 nm	2466.2	1.34	2.38
		**260 nm	2442.26	1.34	2.36
		262 nm	2483.38	1.35	2.40
EFZ	Flow Rate As per method 1.0ml/min	0.8 ml/min	4312.75	1.21	9.61
		*1.0 ml/min	3976.31	1.2	9.26
		1.2 ml/min	4101.72	1.21	9.41
	Wavelength As per method 260nm	258 nm	4143.88	1.2	9.47
		**260 nm	3976.31	1.2	9.26
		262 nm	4196.02	1.22	9.52

**** Results for actual flow (1.0ml/min) have been considered from Assay standard.***
***** Results of actual optimized method wavelength has been considered from Accuracy standard.***

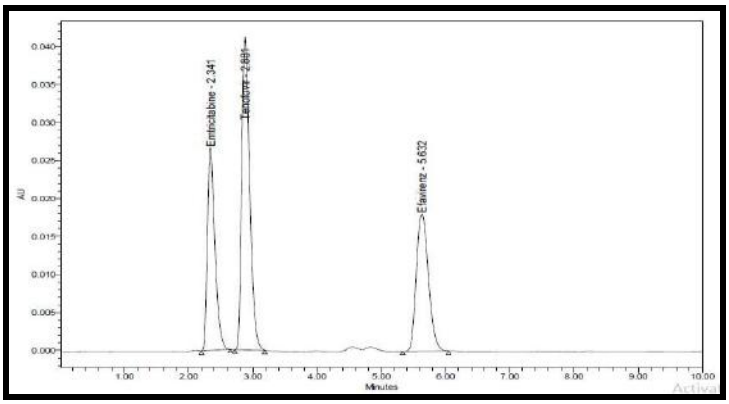

Fig. 13: Chromatogram of ECB, TNF and EFZ (0.8 ml/min flow rate)

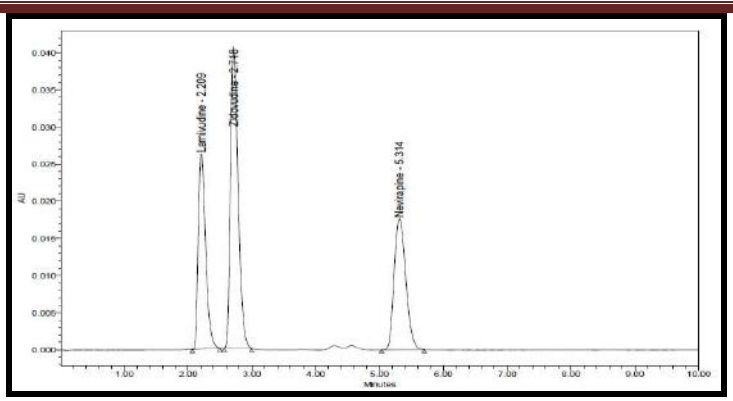

Fig. 14: Chromatogram of Chromatogram of ECB, TNF and EFZ (1.2 ml/min flow rate)

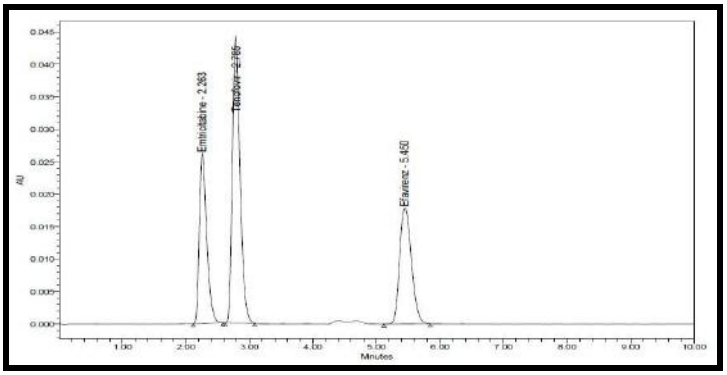

Fig. 15: Chromatogram of Chromatogram of ECB, TNF and EFZ (258 nm)

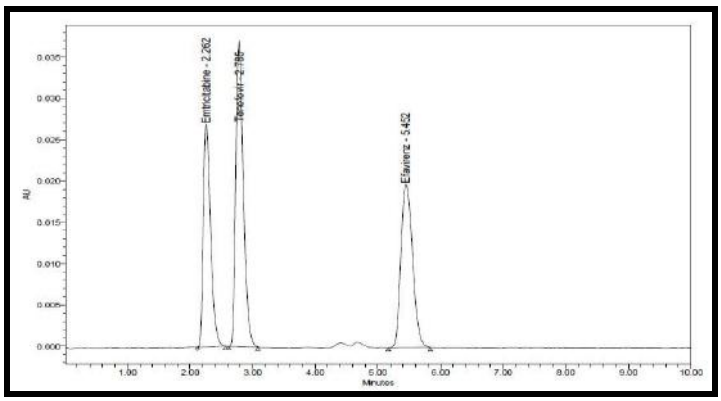

Fig. 16: Chromatogram of Chromatogram of ECB, TNF and EFZ (262 nm)

2.7. Solution stability study: ECB, TNF and EFZ Samples Stability was evaluated by shorting at the ambient temp & analysis was done in initial time, after 3hrs, 6 hrs, 12 hrs and 24 hrs. The analysis of the reports from all aged solutions was compared with those of from the freshly prepared solution (initial solution). **(Table 8-11)** shows results are obtained the stability of solution study at various intervals for a test preparations and it was conformed that the test solutions were stable upto the 24hrs at the ambient temp, because difference in the measured & the original values were < 2.0 %.

Table No. 8: Evaluation of solution stability for ECB, TNF and EFZ

S. No	Standard Area (Mean*6)	Standard area after 24hrs (Mean*3)	Sample area after 24hrs (Mean*3)	% Variation for standard & sample	% Assay
ECB					
1	213869	212867	212769	1.1 & 1.0	99.92
TNF					
1	343021	342019	342011	0.9 & 0.5	100.24
EFZ					
1	228951	227949	226849	1.1 & 0.4	100.48

2.8. Recovery studies (Accuracy): The recovery of ECB, TNF and EFZ was determined by the 3 various conc. levels. % recovery was found to be 98.87-101.04 % for ECB, 100.96-101.19% for TNF, 99.15-101.78% for EFZ **(Table 9).** The results are indicating that this method was accurate. Chromatograms obtained during the study of accuracy were shown in **Figure 17-19.**

Table No. 9: Accuracy study of ECB

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery
50%-1	105677	10	10.08	100.77%
50%-2	103685	10	9.87	98.87%
50%-3	103753	10	9.89	98.93%
100%-1	210997	20	20.12	100.60%
100%-2	20955 7	20	19.98	99.91%
100%-3	208850	20	19.91	99.57%
150%-1	316984	30	30.23	100.75%
150%-2	317885	30	30.31	101.04%
150%-3	318313	30	30.35	101.18%

Table No. 10: Accuracy study of TNF

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery
50%-1	169535	15	15.18	101.18%
50%-2	169523	15	15.18	101.17%
50%-3	169439	15	15.17	101.12%
100%-1	338335	30	30.29	100.96%
100%-2	338115	30	30.27	100.89%
100%-3	337931	30	30.25	100.84%
150%-1	508669	45	45.54	101.19%
150%-2	507269	45	45.41	100.91%
150%-3	507896	45	45.47	101.04%

Table No. 11: Accuracy study of EFZ

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery
50%-1	112964	30	29.87	99.55%
50%-2	114245	30	30.20	100.68%
50%-3	115489	30	30.53	101.78%
100%-1	228376	60	60.38	100.63%
100%-2	227414	60	60.12	100.21%
100%-3	227179	60	60.06	100.10%
150%-1	338676	90	89.54	99.49
150%-2	338096	90	89.39	99.32%
150%-3	337069	90	89.12	99.02%

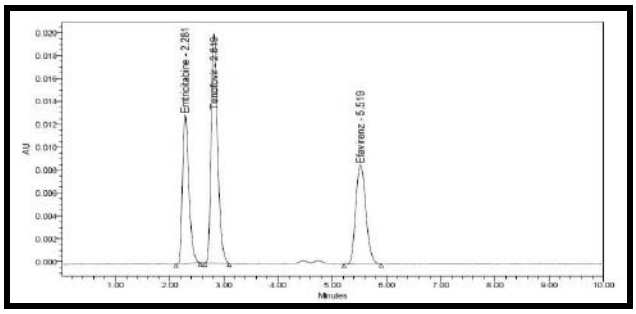

Fig. 17: Accuracy chromatogram for level-1 (50%)

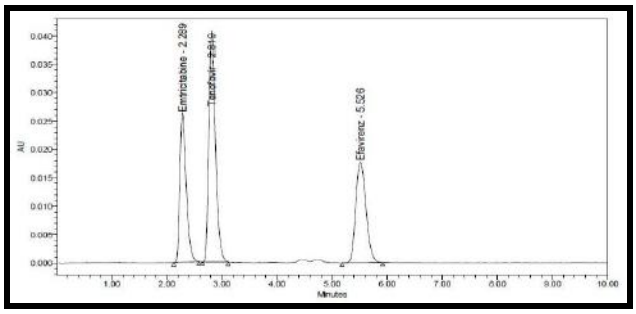

Fig. 18: Accuracy chromatogram for level-2 (100%)

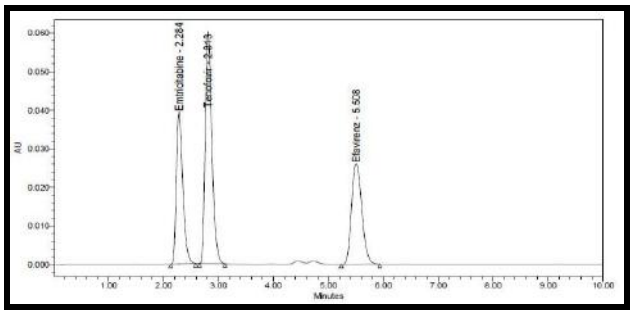

Fig. 19: Accuracy chromatogram for level-3 (150%)

2.9. Ruggedness: The ruggedness was studied by evaluating by different analysts but in the same chromatographic conditions. The results of ruggedness of developed method are started in the **Table 12.** The results are shown during by different analysts but in the

same chromatographic condition of the test solution wasn't affected & in the accordance with the actual. The suitability parameters are also been found good; hence this method was concluded as rugged. Chromatograms are obtained during ruggedness was shown in the **Fig: 20-25.**

Table No. 12: Evaluation data of Ruggedness study of ECB, TNF and EFZ

ID Precisions	No. of Injections	ECB		TNF		EFZ	
		Peak Area	RT	Peak Area	RT	Peak Area	RT
ID Precision - 1	1	210922	2.234	336500	2.750	227912	5.377
	2	210975	2.297	336560	2.802	227911	5.377
	3	2115311	2.287	335480	2.744	228840	5.470
ID Precision - 2	1	2115311	2.229	337668	2.748	225726	5.368
	2	212976	2.230	339880	2.748	225790	5.373
	3	212922	2.234	338560	2.750	225911	5.377
MEAN		21441.5	2.564	33788.6	8.99	226576	5.3876
STDEV		8742.7	0.0910	33452.2	0.0944	225672	5.3675
% RSD		0.7	1.3	0.7	1.05087	1.056	0.5645

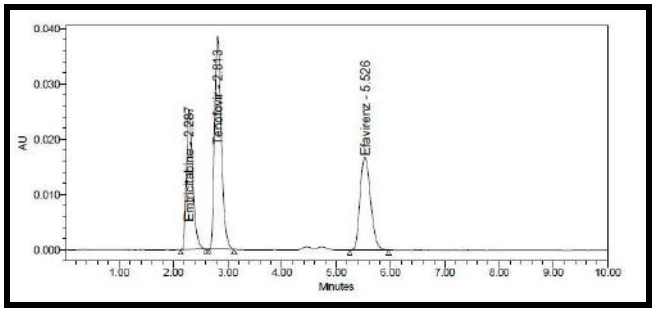

Fig. 20: Chromatogram of ECB, TNF and EFZ [ID Precision-1 (Injection-1)]

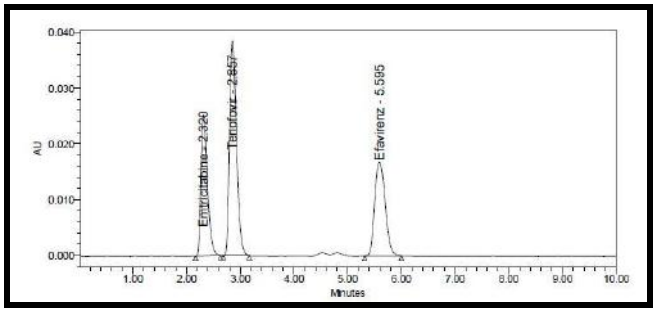

Fig. 21: Chromatogram of ECB, TNF and EFZ [ID Precision-1 (Injection-2)]

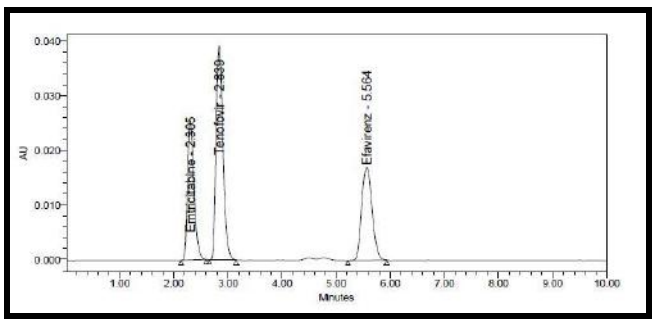

Fig. 22: Chromatogram of ECB, TNF and EFZ [ID Precision-1 (Injection-3)]

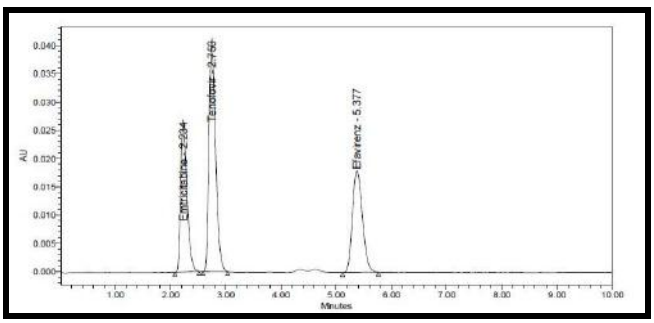

Fig. 23: Chromatogram of ECB, TNF and EFZ [ID Precision-2 (Injection-1)]

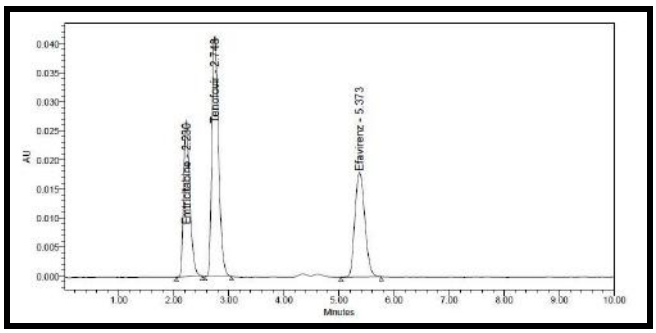

Fig. 24: Chromatogram of ECB, TNF and EFZ [ID Precision-2 (Injection-2)]

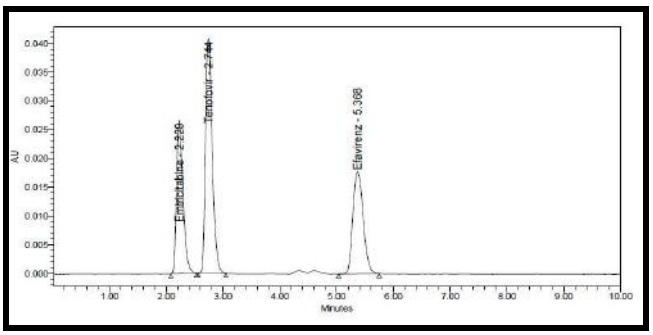

Fig. 25: Chromatogram of ECB, TNF and EFZ [ID Precision-2 (Injection-3)]

2.10. Analysis of a commercial formulation: Experimentally the results for the amount of ECB, TNF and EFZ in tablets, expressed as a percentage of label claims were in good agreement with the label claims there by suggesting that there is no interaction from the excipients which are commonly present in formulation of tablets.

2.11. Degradation study:

In a order to to establish the inherent stability and stability indicating assay method and to determine whether the analytical methods were stable ECB, TNF and EFZ dosage forms are stressed on the different conditions to applied degradation studies. The guidelines are expressed in ICH Q2A, Q3B, Q2B & FDA 21 CFR section of 211 all the required for development & for the validation of stability study.

Preparation of stock: Accurately weigh 10 tablets crush in mortor and pestle and transfer equivalent to 20mg of Emtricitabine, 30mg of Tenofovir and 60mg of Efavirenz in (marketed formulation=126.53

mg of tablet Powder) sample into a 100mL clean dry volumetric flask add about 70 mL of Diluent and sonicate it up to 30 mins to dissolve it completely and make volume up to the mark with the same solvent. Then it is filtered through 0.45 micron Injection filter. (Stock solution) The specific degradative conditions are described below.

Acid degradation: The Acid degradation was done by sample was treated with 3ml of 1N hydrochloric acid and kept for 10hrs at 60ºC. After 10hrs the solution was neutralized with 3ml of 1N sodium hydroxide, made the volume up to the mark with mobile phase and analyzed using HPLC. The degrading drug content was found up to 6.33% in the acidic condition **(Figure 26-30) & (Table 13 &14).**

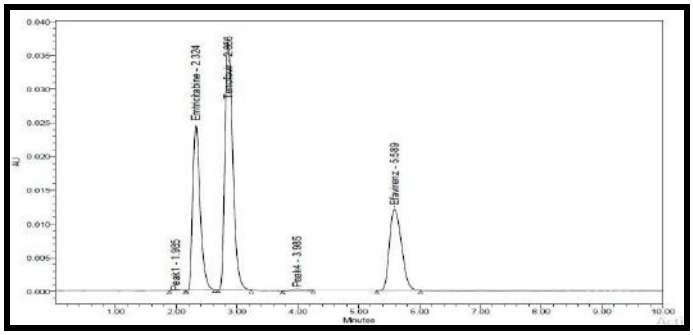

Fig. 26: Chromatogram of acidic forced degradation of ECB, TNF and EFZ

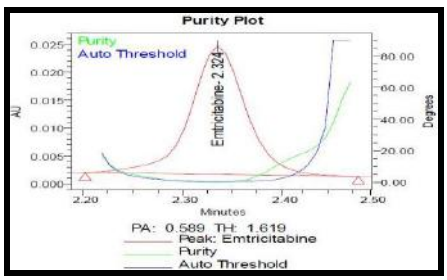

Fig. 27: Purity Plots for ECB in acidic forced degradation

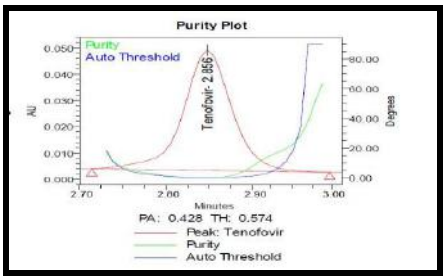

Fig. 28: Purity Plot for TNZ in acidic forced degradation

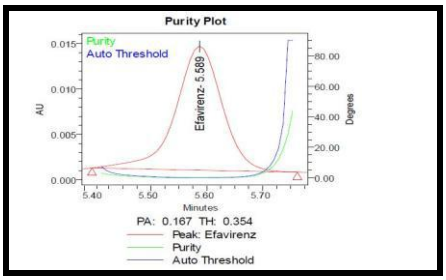

Fig. 29: Purity Plot for EFZ in acidic forced degradation

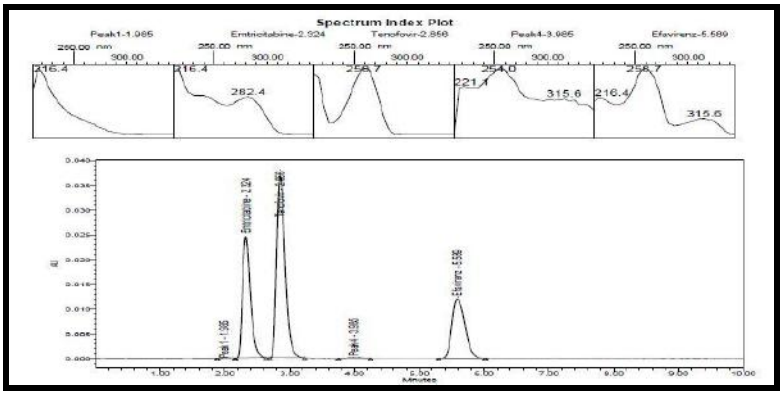

Fig. 30: Spectrum index for ECB, TNF and EFZ in acidic forced degradation

Alkaline degradation: The Alkaline degradation was done by sample was treated with 3ml of 1N sodium hydroxide and kept the sample for 10hr. After 10hr solution was neutralized to add 3ml of 1N hydrochloric acid, made the volume up to the mark with irrelevant media and analyzed using HPLC. In alkali degradation study, it was found to be 5.20% of the degraded drug **(Figure 31-35) & (Table 13 &14).**

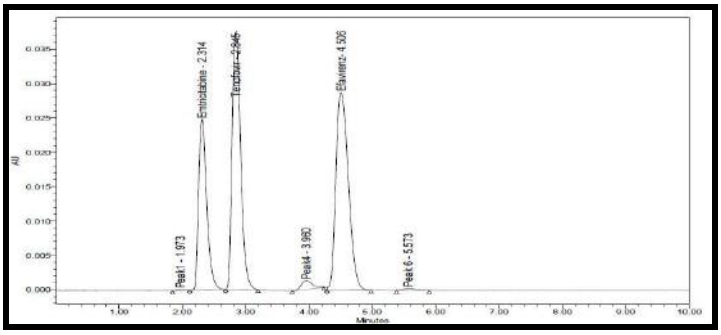

Fig. 31: Chromatogram of alkali forced degradation of ECB, TNF and EFZ

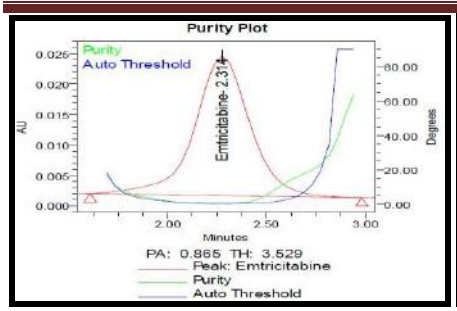

Fig. 32: Purity Plot for ECB in alkali forced degradation

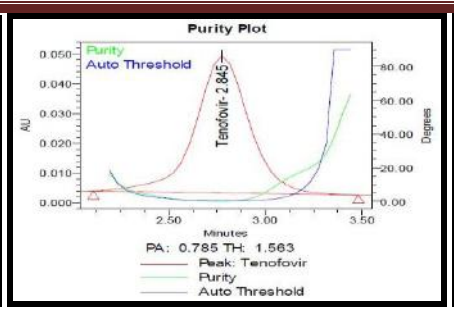

Fig. 33: Purity Plot for TNF in alkali forced degradation

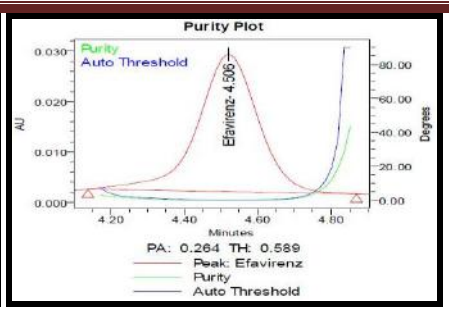

Fig. 34: Purity Plot for EFZ in alkali forced degradation

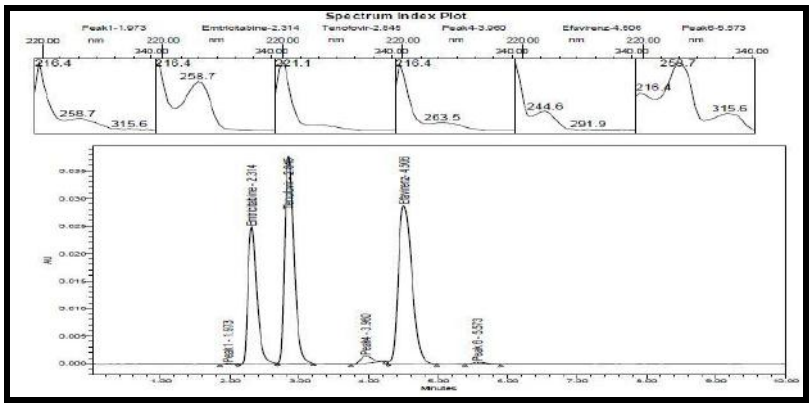

Fig. 35: Spectrum index for ECB, TNF and EFZ in Base (Alkali) forced degradation

Oxidative degradation: The oxidative degradation was done by sample was mixed with 3mL of 30%v/v aqueous hydrogen peroxide solution and kept for 10hrs. After 10hrs made the volume upto the mark with mobile phase and analyzed using HPLC. In oxidative degradation, it was found to be 6.36% of the degraded drug **(Figure 36-40)** & **(Table 13 &14).**

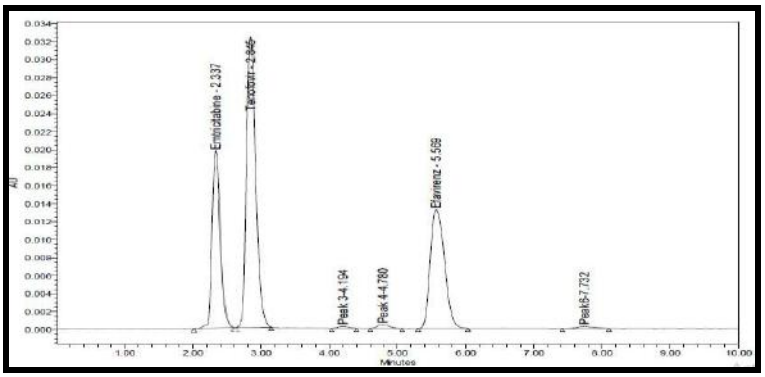

Fig. 36: Chromatogram of oxidative forced degradation of ECB, TNF and EFZ

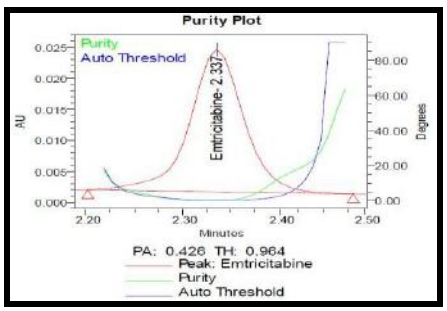

Fig. 37: Purity Plot for ECB in oxidative forced degradation

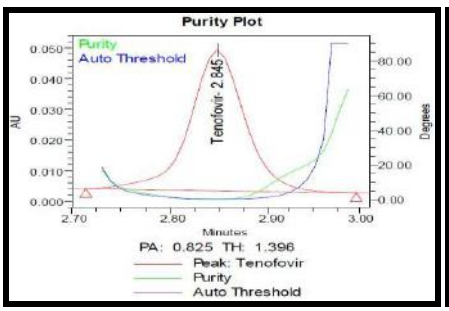

Fig. 38: Purity Plot for TNF in oxidative forced degradation

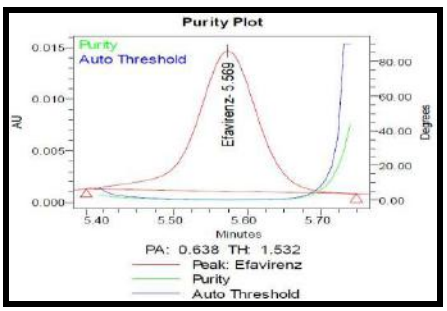

Fig. 39: Purity Plot for EFZ in oxidative forced degradation

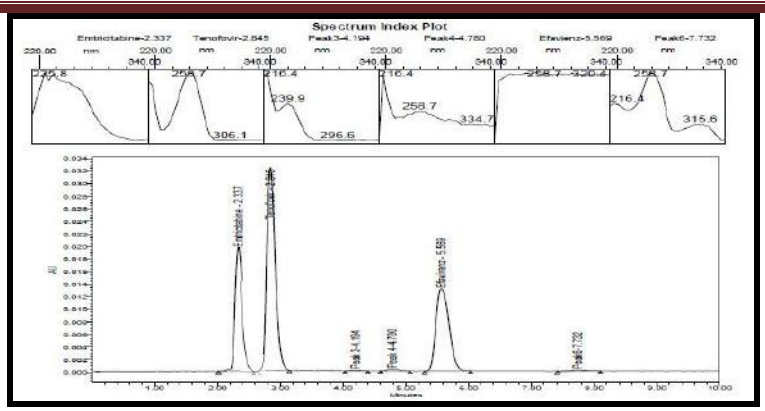

Fig. 40: Spectrum index for ECB, TNF and EFZ in oxidative forced degradation

Photolytic degradation: The photolytic degradation was done by exposing of drug content under the UV light for 15mins to 7days. There is 8.08% of the drug degradation observed in the above specific photolytic degradation condition **(Figure 41-45) & (Table 13 &14).**

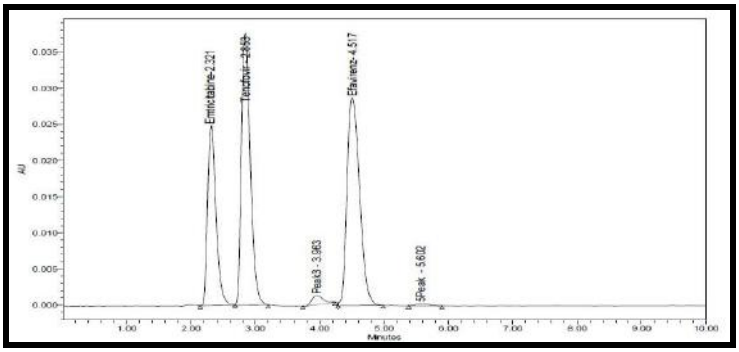

Fig. 41: Chromatogram of UV-light degradation of ECB, TNF and EFZ

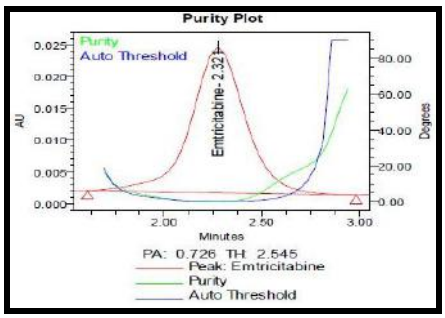

Fig. 42: Purity Plot for ECB in UV-light degradation

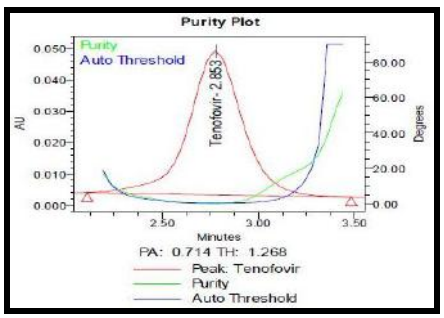

Fig. 43: Purity Plot for TNF in UV-light degradation

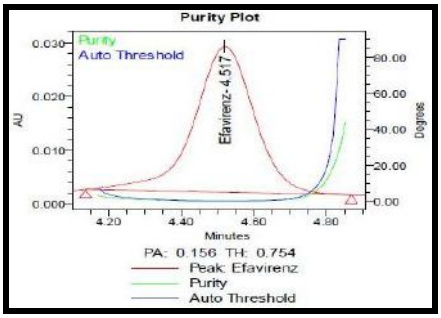

Fig. 44: Purity Plot for EFZ in UV-light degradation

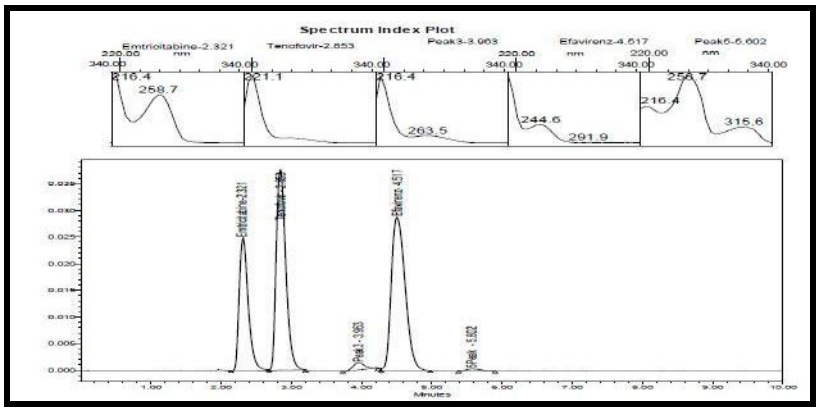

Fig. 45: Spectrum index for ECB, TNF and EFZ in Photolytic forced degradation

Thermal degradation: The Thermal degradation is to be performing by the exposing the solid drug at the 80°C for 15mins to 60mins and at 220°C for 2-5mins. Resultant chromatogram of thermal degradation study **(Figure 46-50) & (Table 13 &14).**was indicates that the drug was found to be slightly stable under thermal condition. It was only 3.20% of the drug content were degraded.

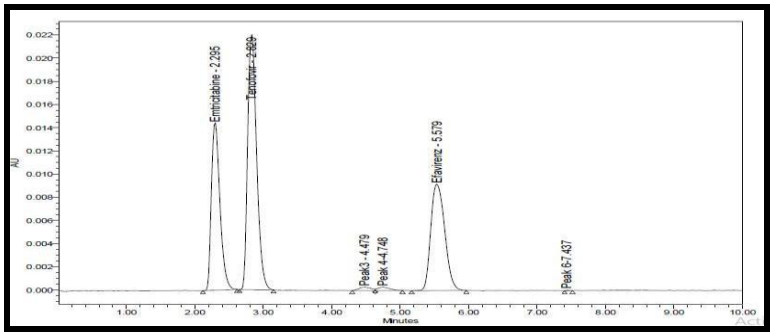

Fig. 46: Chromatogram of thermal degradation of ECB, TNF and EFZ

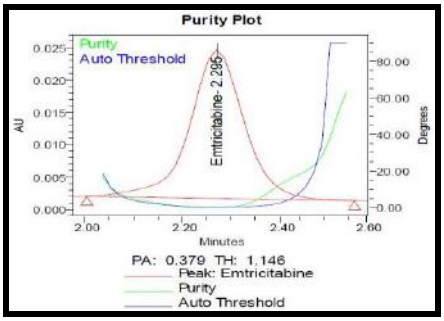

Fig. 47: Purity Plots for ECB in thermal degradation

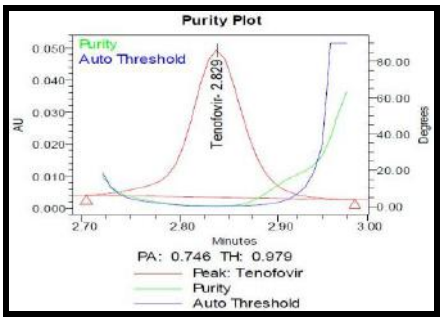

Fig. 48: Purity Plot for TNF in thermal degradation

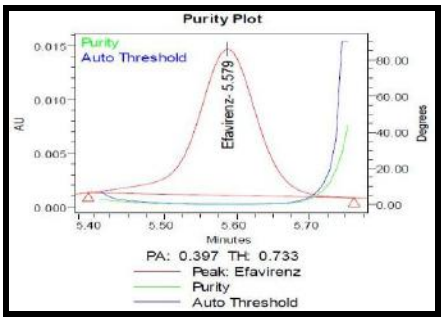

Fig. 49: Purity Plot for EFZ in thermal degradation

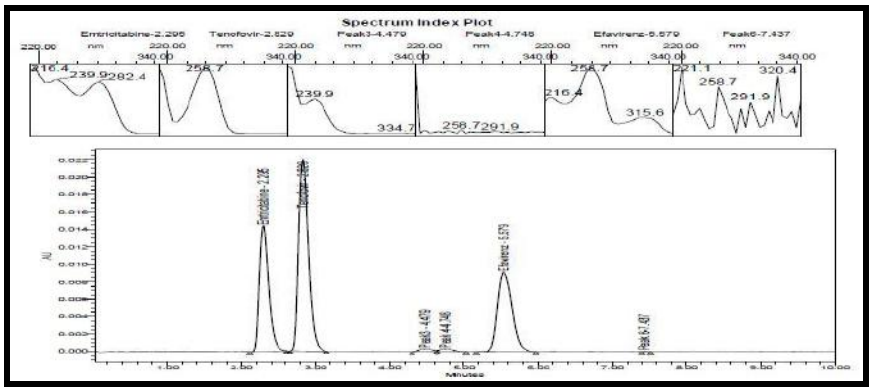

Fig. 50: Spectrum index for ECB, TNF and EFZ in Thermal forced degradation

Table No. 13: Peak purity results of ECB, TNF and EFZ

Stress Condition	*Purity Angle*			*Purity Threshold*			*Peak Purity*
	ECB	**TNF**	**EFZ**	**ECB**	**TNF**	**EFZ**	
Acid Degradation	0.589	0.428	0.167	1.619	0.574	0.354	Passes
Alkali Degradation	0.865	0.785	0.264	3.529	1.563	0.589	Passes
Oxidative Degradation	0.462	0.825	0.683	0.964	1.396	1.532	Passes
Thermal Degradation	0.379	0.746	0.397	1.146	0.979	0.733	Passes
Photolytic Degradation	0.726	0.714	0.154	2.545	1.268	0.754	Passes

Table No. 14: Percentage of degradation of ECB, TNF and EFZ

Drug Name		*Acid*	*Alkali*	*Oxidative*	*Photolytic*	*Thermal*
ECB	***Std Area***			207256		
	Sample Area	190116	197681	192023	195236	201805
	% of Degradation	8.27%	4.62%	7.35%	5.80%	2.63%
TNF	***Std Area***			331636		
	Sample Area	311008	322549	314556	312569	315584
	% of Degradation	6.22%	2.74%	5.15%	5.75%	4.84%
EFZ	***Std Area***			225979		
	Sample Area	215764	207313	211109	197313	221120
	% of Degradation	4.52%	8.26%	6.58%	12.69%	2.15%
Average of %Degradation		6.33%	5.20%	6.36%	8.08%	3.20%

CONCLUSION

A new RP-HPLC detailed stability indicating Analytical method was described in this manuscript provides a high resolution, simple, convenient and reproducible approach for the Trimultaneous estimation and quantification of Emtricitabine (ECB), Tenofovir (TNF) and Efavirenz (EFZ) in routine quality control analysis and also supporting method for advanced stability studies.

ACKNOWLEDGEMENT

The Corresponding author extends sincere thanks to Dr.D.Gowri Sankar, Dr.JVLN Seshagiri Rao for their valuable help, support and inspiration. I am also thankful and also acknowledge the immense help received from the scholars whose articles are cited and included in references of this manuscript. The author is also grateful to authors / editors / publishers of all those articles, journals and books from where the literature for this article has been reviewed and discussed.

REFERENCES:

1. Budawari S. The Merck Index, Merck and Co. Inc., New Jersey, **2001**;3568, 9151.
2. Sean C.S. Martindale. The Complete Drug Reference, Pharmaceutical Press, London, **2002**;620, 642.
3. Gish R.G, Trinh H, Leung N, Chan F.K.L, Fried M.L, Wright T.L, Wang C, Anderson J, Mondou E, Snow A, Sorbel J, Rousseau F. and Corey L. Safety and antiviral activity of emtricitabine (FTC) for the chronic hepatitis B infection: a two-year study. J Hepatol **2005**;43(1):60-66.
4. World Health Organization. Tenofovir Disoproxil Fumarate: Final Text for Addition to the International Pharmacopoeia. June, **2010**.
5. http://www.drugbank.ca/drugs/DB00300 accessed on 17/02/2014.
6. SD. Santhosham, VN. Bavani, M. Suresh. Int J Pharm Chem Res **2012**;1:1-5.
7. N. Devanaboyina, T. Satyanarayana, BG. Rao. Int J Res in Pharm and Bio **2012**;3(1):361-367.
8. Separation of efavirenz in human plasma by using reversed phase HPLC technique. J Chromatogr B **2008**;734(1):55-61.
9. Cespedes MS, Aberg JA. Neuropsychiatric complications of antiretroviral therapy. Drug safety: an international journal of medical toxicology and drug experience **2006**;29(10):865-74
10. Montgomery ER. Development and validation of a reverse phase HPLC method for analysis of efavirenz and its related substances in drug substance and capsule formulation. J Pharm Biomed Anal **2001**;2(5):267-284.
11. Wren, S.A.C. and Tchelitcheff, P. Use of Ultra-Performance Liquid Chromatography in Pharmaceutical Development. Journal of Chromatography A **2006**;1119:140-146.
12. ICH, Q2A Text on validation of analytical procedures, international conference on Harmonization, Oct. **1996**.
13. Chaibva FA, Walker RB. Development and validation of a stability-indicating analytical method for the quantitation of oxytocin in pharmaceutical dosage forms. J Pharm Biomed Anal **2007**;43:79–185.
14. International conference on harmonization (ICH), Q1AR2: stability testing of new drug substances, products IFPMA, Geneva, **2003**.
15. Singh S, Bakshi M. Guidance on conduct of stress tests to determine inherent stability of drugs. Pharm Technol **2000**;24:1-14.

How to cite this article:

M. Madan Mohan Reddy et al. STRESS DEGRADATION STUDIES AND DEVELOPMENT OF HIGH RESOLUTION NEW VALIDATED STABILITY INDICATING ANALYTICAL METHOD FOR DETERMINATION OF EMTRICITABINE, TENOFOVIR AND EFAVIRENZ IN ITS BULK AND MULTICOMPONENT PHARMACEUTICAL DOSAGE FORMS IN THE PRESENCE OF DEGRADATION PRODUCTS AS PER ICH GUIDELINES. J Pharm Res 2017;6(3):40-54.

Conflict of interest: The authors have declared that no conflict of interest exists.

Source of support: Nil