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DEVELOPMENT AND VALIDATION OF STABILITY INDICATING METHOD FOR THE SIMULTANEOUS ESTIMATION OF EMTRICITABINE, TENOFOVIR DISOPROXIL FUMARATE AND EFAVIRENZ IN PHARMACEUTICAL DOSAGE FORMS BY RP-HPLC

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ABSTRACT

A stability indicating method was developed for the simultaneous estimation of Emtricitabine, Tenofovir and Efavirenz in pharmaceutical dosage form by reverse phase high performance liquid chromatography (RP-HPLC) and validated. The chromatographic separation was performed using the Kromasil C_{18} (250mm × 4.6mm, 5µ) column run in an isocratic mode with a flow rate of 1mL/min at ambient temperature. The mobile phase consists of 0.01N Ammonium acetate and Acetonitrile in the ratio 65:35 (v/v/) and detected at the wave length 260nm. The retention times for Emtricitabine, Tenofovir and Efavirenz were found to be 2.28min, 4.03min and 2.79min respectively. The drugs obeyed Beer's law in the concentration range of 50µg/mL to 300µg/mL, 150µg/mL to 900µg/mL and 75µg/mL to 450µg/ml respectively. The method was validated as per ICH guidelines for accuracy, precision, specificity, ruggedness, robustness and stability. The standard solution was subjected to stress conditions such as acidic, basic, oxidative, neutral, photolytic and thermal conditions. The net degradation was found to be within the limits.

Keywords: Emtricitabine, Tenofovir, Efavirenz, Stability indicating, Method development, Validation, RP-HPLC.

INTRODUCTION

Emtricitabine (EMT)[1] (Fig.1A), 4-amino-5-fluoro-1-[(2*R*,5*S*)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one, is a white to off-white powder, soluble in water and methanol and practically insoluble in methylene chloride with pKa value of 2.65. It is used as antiretroviral drug in the treatment of HIV and AIDS. Tenofovir Disoproxil Fumarate (TDF)[2] (Fig.1B), [[(2R)-1-(6-aminopurin-9-yl) propan-2-yl]oxymethyl-(propan-2-yloxycarbonyloxymethoxy)phosphoryl]oxymethyl propan-2-ylcarbonate;(E)-but-2-enedioic acid, is a white to off-white crystalline powder, soluble in methanol and dimethyl Formamide, sparingly soluble in water with pKa 3.75. It is used as antiretroviral drug in the treatment of HIV and AIDS. Efavirenz (EFA)[3] (Fig.1C), (4S)-6-Chloro-4-(2-cyclopropylethynyl)-4-

(trifluoromethyl)-2,4-dihydro-1H-3,1-benzoxazin-2-one, is a white to slightly pink powder, soluble in methanol with pKa value of 12.52. It acts as antiretroviral agent and used in the treatment of HIV infection and AIDS. According to the literature survey[4-11], very few methods were developed for the simultaneous estimation of Emtricitabine, Tenofovir and Efavirenz in pharmaceutical dosage forms. The present study aimed to develop and validate the stability indicating method for the simultaneous estimation of Emtricitabine, Tenofovir and Efavirenz in pharmaceutical dosage form using RP-HPLC.

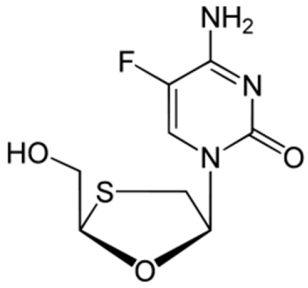

Fig.1A: Chemical Structure of Emtricitabine

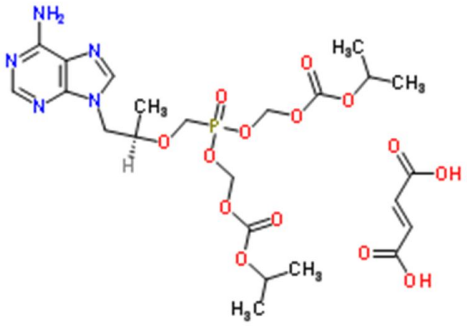

Fig.1B: Chemical Structure of Tenofovir Disoproxil Fumarate

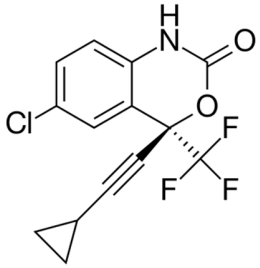

Fig.1C: Chemical Structure of Efavirenz

MATERIALS AND METHOD

Emtricitabine, Tenofovir and Efavirenz working standards were supplied by Hetero Drugs Pvt. Ltd., Hyderabad, India as gift samples. The tablets were purchased from local pharmacy. All the chemicals used in the method were of AR grade. All the solvents used were of HPLC grade.

The HPLC analysis was performed using Waters 2998 model equipped with an autosampler, Photo Diode Array detector and done on empower software. Column used was Kromasil C18 (250mm × 4.6mm, 5µ).

Preparation of Buffer (0.01N Ammonium Acetate): Transfer 0.77g of ammonium acetate in to a 1000mL volumetric flask; add about 100ml of milli-Q water and mix. Finally make volume up to the mark with milli-Q water.

Preparation of Mobile Phase: Mixture of Buffer and Acetonitrile in the ratio 65:35 (v/v) respectively.

Preparation of Diluent: Mixture of water and acetonitrile in the ratio 50:50 (%v/v) respectively

Preparation of Standard solution:

(200µg/mL Emtricitabine, 600µg/mL Efavirenz & 300µg/mL Tenofovir Disoproxil Fumarate)

20mg of Emtricitabine, 60mg of Efavirenz and 30mg of Tenofovir Disoproxil Fumarate working standards were accurately weighed and transferred into a 10mL volumetric flask. 7mL of diluent was added, sonicated to dissolve and make up to final volume with diluent. From the above stock solution, 1mL was pipetted into a 10mL volumetric flask and the volume was made up to mark with diluent.

The standard solution was injected into the HPLC system and chromatogram was recorded (Fig.2A).

Preparation of Sample solution:

20 tablets (Atripla) were weighed accurately and the average weight was calculated. Then the tablets were crushed and fine powder was collected. An amount equivalent to 20mg of Emtricitabine was weighed and transferred into 10mL volumetric flask. 7mL of diluent was added and sonicated for 30min with intermediate shaking. Volume was made up with diluent. The above solution was filtered using HPLC filters. 1mL of the above solution was pipette into 10mL volumetric flask and made up with diluent.

The sample solution was injected into the HPLC and chromatogram was recorded (Fig.2B). A blank solution was also injected and chromatogram was recorded (Fig.2C).

Method validation[12]:

The standard solution was injected into the HPLC system six times and system suitability parameters were noted in the table 1.

The specificity study was conducted using placebo solution. The placebo interference with the peaks of drugs is to be noted (Fig.3).

Precision (%RSD) was determined by injecting the six samples of solution.

To determine the accuracy of the test method, samples were prepared by spiking drug materials with the equivalent amount of placebo at 50%, 100% and 150% of the target concentration. The average % recoveries were determined.

Linearity was determined by preparing the series of standard solutions and injecting into the HPLC system. A graph is plotted to concentration versus peak area, results and graphs were summarized in table 2 and figures 4A, 4B and 4C. LOD and LOQ were determined using the formula mentioned in ICH guidelines, based on calibration curves.

Ruggedness (%RSD) was determined by analyzing the samples on different days. Robustness was determined by varying the optimum conditions such as ±5% of organic phase, ±0.2mL/min flow rate and ±5ºC column oven temperature with respect to test method.

The stability of drugs in solution was determined by repeated analysis of samples during the course of experimentation on the same day and also after storage of drug solution for 24h under laboratory conditions.

Forced degradation studies[13] were conducted by exposing the standard solution to the stress conditions like acidic (hydrochloric acid), basic (sodium hydroxide), oxidative (hydrogen peroxide), neutral (water), photolytic (UV light) and thermal (heat) conditions. The chromatograms were recorded (Fig.5) and results were summarized in table 3.

RESULTS

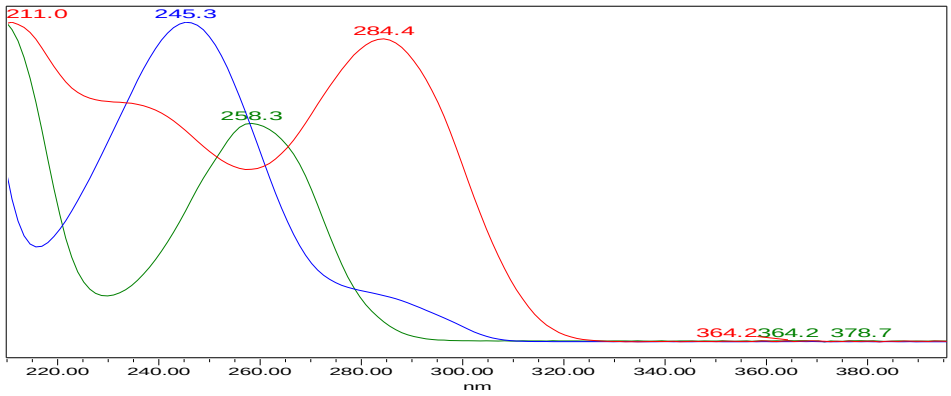

Fig. 6: Overlay UV Spectrum of EMT, TDF and EFA

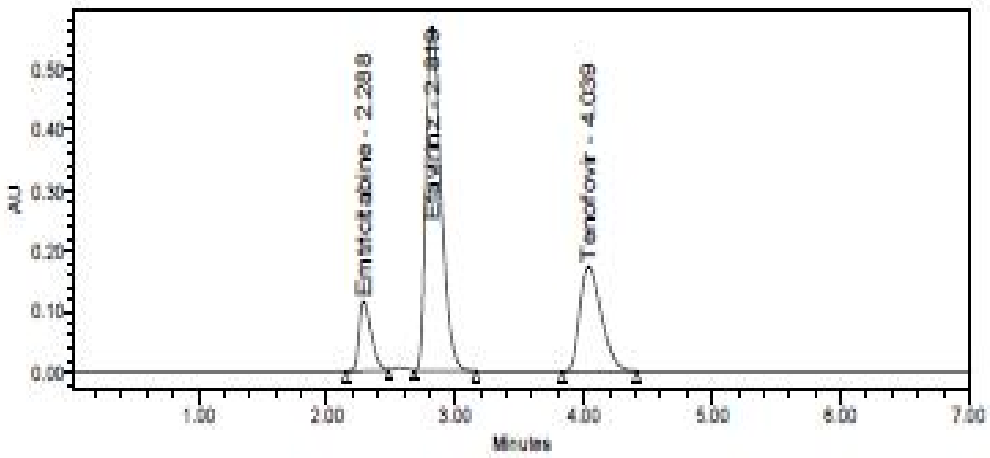

Fig.2A: Standard chromatogram

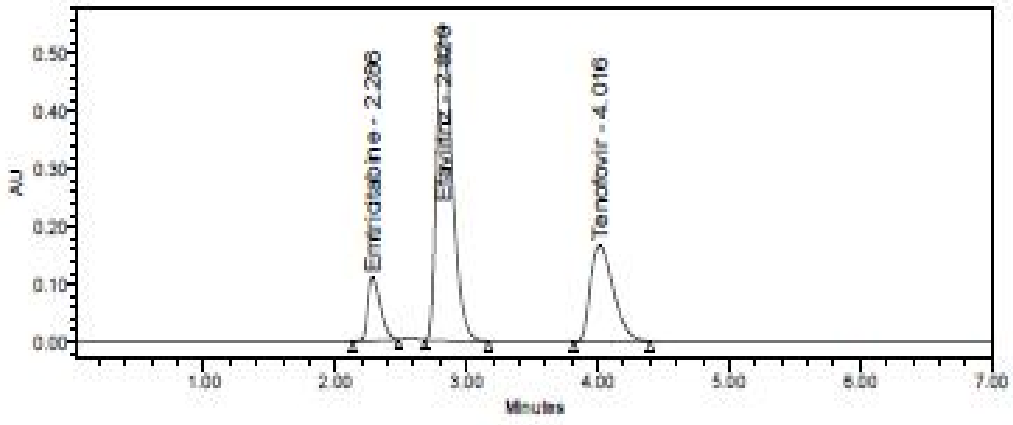

Fig.2B: Sample chromatogram

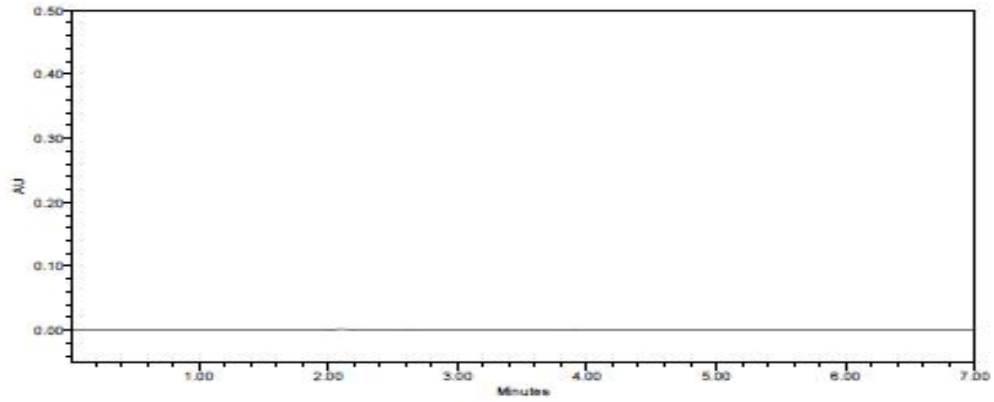

Fig.2C: Blank chromatogram

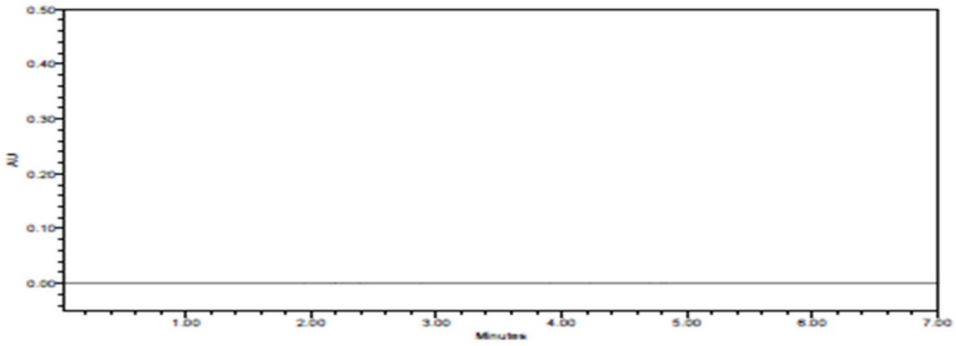

Fig.3: Placebo chromatogram

Parameter	**Emtricitabine**	**Efavirenz**	**Tenofovir**
Specificity	Specific	Specific	Specific
Precision (%RSD)	0.5	0.5	0.6
Accuracy (% Recovery)	99.60%-100.07%	99.02%-99.60%	99.60%-100.14%
Linearity range (µg/ml)	50-300	75-450	150-900
Correlation coefficient, r	0.9998	0.9996	0.9996
Limit of Detection (µg/ml)	0.32	1.23	0.15
Limit of Quantitation (µg/ml)	0.96	3.73	0.46
Ruggedness (%RSD)	0.9	0.7	0.6
Robustness	Robust	Robust	Robust

Stability	Stable	Stable	Stable
USP Plate Count	3331	2979	2947
USP Tailing factor	1.41	1.44	1.21
USP Resolution		2.7	5.2

Table 2: LINEARITY RESULTS

Parameter (Unit)	**Emtricitabine**	**Efavirenz**	**Tenofovir**
Linearity range (µg/mL)	50-300	75-450	150-900
Regression equation, y=mx+c	y=3685.1x+9178.7	y=7917.5x+45083	y=7102.4x+15511
Slope, m	3685	7917	7102
Regression coefficient, r^2	0.9996	0.9993	0.9992
Correlation coefficient, r	0.9998	0.9996	0.9996

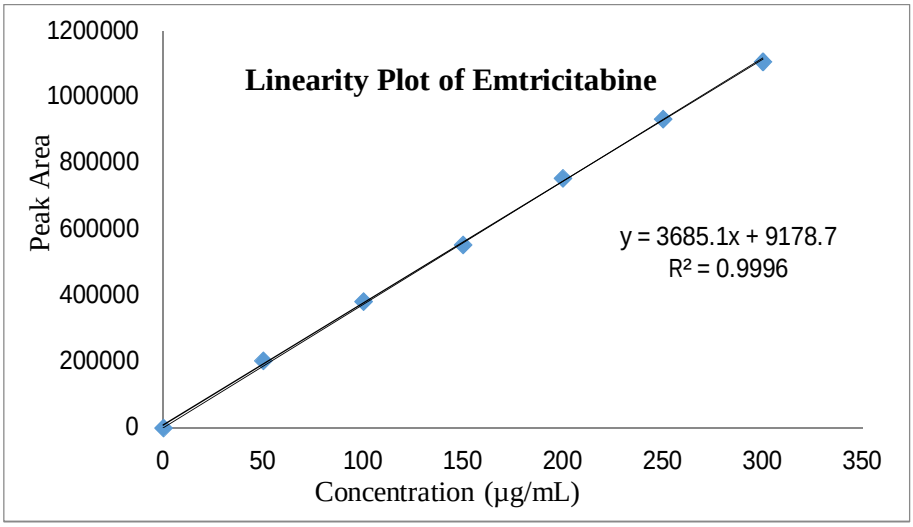

Fig.4A: Linearity plot of Emtricitabine

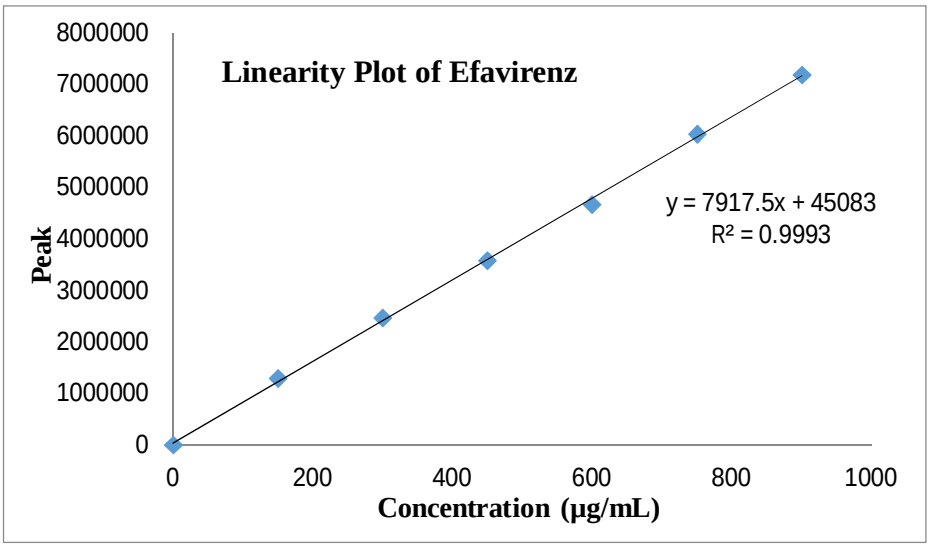

Fig.4B: Linearity plot of Efavirenz

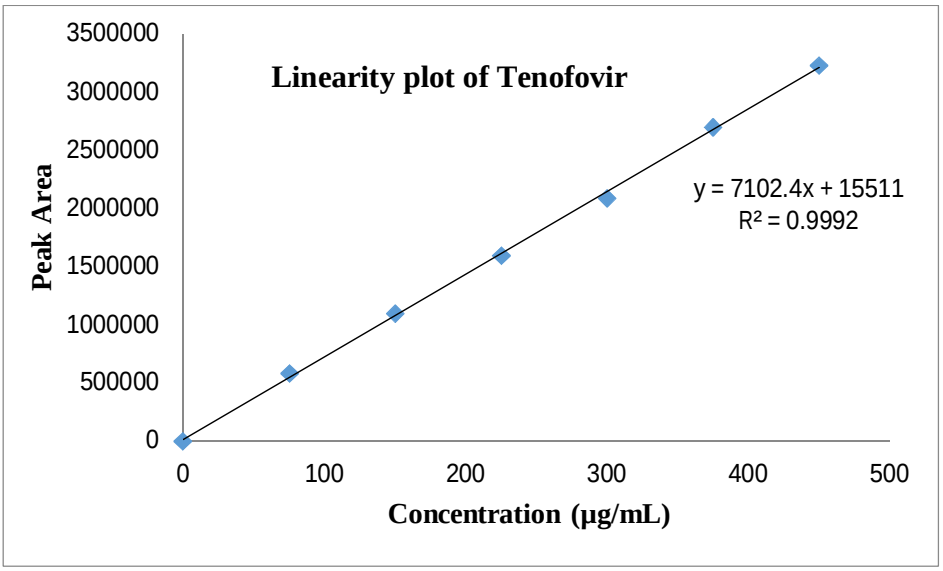

Fig.4C: Linearity plot of Tenofovir

Table 3: FORCED DEGRADATION STUDIES RESULTS.

Drug	Parameters	Stress Condition					
		Acidic heat	Basic	Oxidative	Photolytic	Neutral	Dry
Emtricitabine	% Assay	95.49	97.04	98.06	99.44	99.32	99.18
	Purity Angle	3.073	2.972	1.863	1.571	0.073	7.726
	Purity Threshold	4.273	3.268	2.280	2.280	0.273	8.27
Efavirenz	% Assay	95.21	97.28	98.45	99.40	99.29	99.18
	Purity Angle	0.520	0.720	0.742	0.563	0.120	0.806
	Purity Threshold	1.284	1.283	0.995	0.785	0.284	1.295
Tenofovir Disoproxil Fumarate	% Assay	95.30	97.22	98.53	99.31	99.20	99.14
	Purity Angle	0.097	0.090	0.131	0.105	0.097	0.121
	Purity Threshold	1.309	1.303	0.312	0.318	0.309	1.306
% Area of	degradation Peak	0.51	-	-	-	-	-

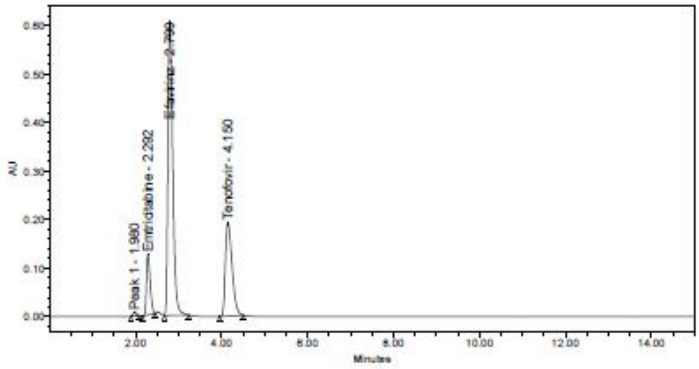

Fig.5A: Acid Degradation study chromatogram

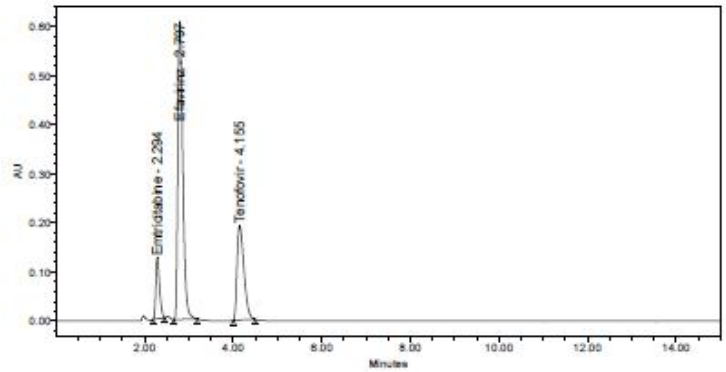

Fig.5B: Base Degradation study chromatogram

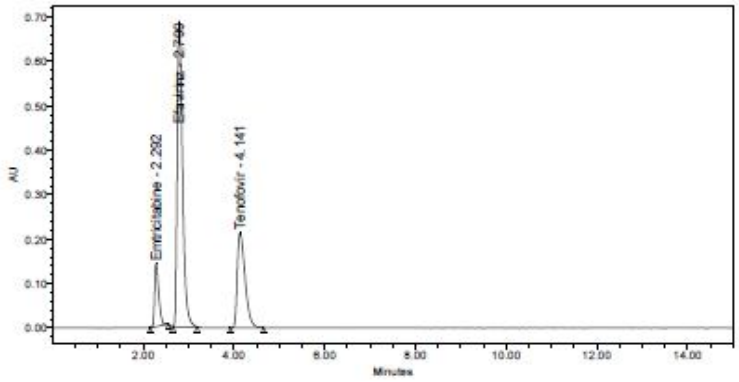

Fig.5C: Oxidative Degradation study chromatogram

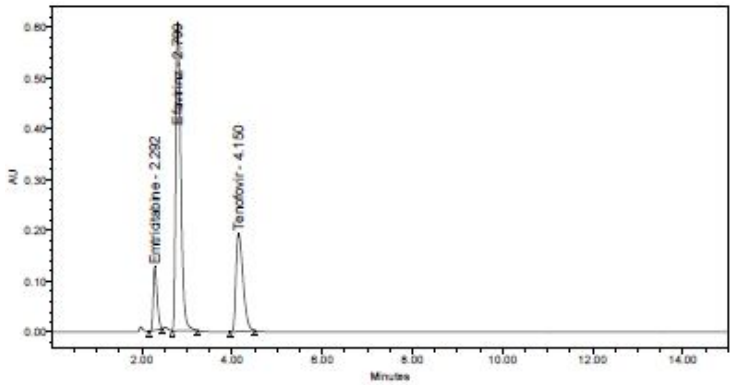

Fig.5D: Neutral Degradation study chromatogram

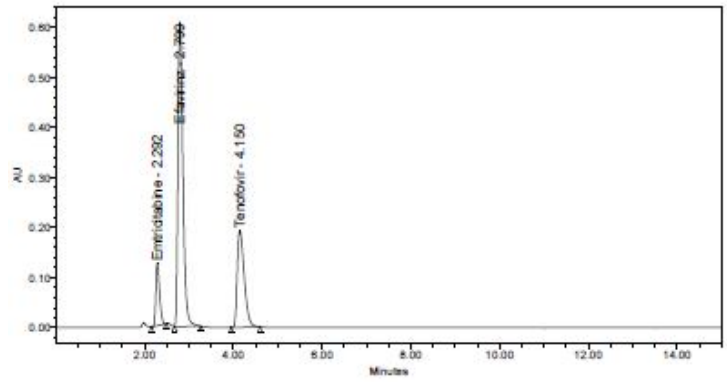

Fig.5E: Photolytic Degradation study chromatogram

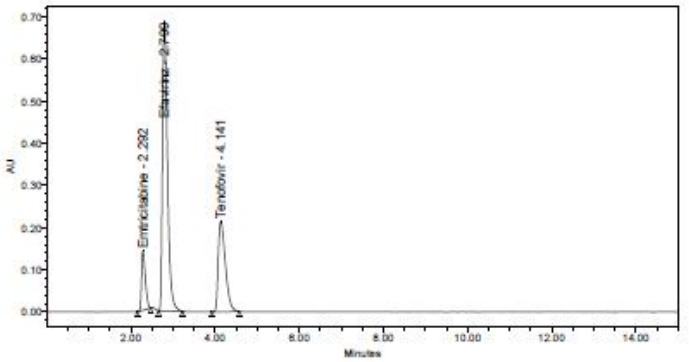

Fig.5F: Thermal Degradation study chromatogram

DISCUSSION

At the starting, various mobile phase ratios were tried to separate the drugs. Based on their peak parameters, run time and resolution, optimized conditions were determined. The standard solution of 10µg/mL was prepared and scanned in the range of 200-400nm. 260nm was selected as detection wavelength based on the overlay UV spectrum (Figure 6). The chromatographic separation was performed using Kromasil C18, 250mm × 4.6mm, 5µ column. 0.01N Ammonium acetate: acetonitrile (65:35) run in isocratic mode and flow rate 1.0ml/min was selected. Emtricitabine, Efavirenz and Tenofovir Disoproxil Fumarate were found to be 2.28min, 2.79min and 4.03min respectively

A linear response was observed in the concentration range of 50µg/mL – 300µg/mL for Emtricitabine, 150µg/mL – 900µg/mL for Efavirenz and 75µg/mL – 450µg/mL for Tenofovir with correlation coefficient of 0.999.

The %RSD for Emtricitabine, Efavirenz and Tenofovir Disoproxil Fumarate were found to be 0.5, 0.5 and 0.6 respectively. The % recoveries were found to be 99.90% - 100.07% for Emtricitabine, 99.02%-99.60% for Efavirenz and 99.60%-100.14% for Tenofovir Disoproxil Fumarate.

The results of ruggedness, robustness and stability confirmed that the developed method is rugged, robust and stable up to 24h.

The forced degradation studies confirmed that the drugs were stable under stress conditions such as acidic, basic, oxidative, neutral, photolytic and thermal conditions. The net degradation was found to be within the limits. The peak purity angle is less than the peak purity threshold.

CONCLUSION

A stability indicating RP-HPLC method was developed for the simultaneous estimation of Emtricitabine, Efavirenz and Tenofovir in bulk drug and pharmaceutical dosage form. The method was validated according to ICH guidelines. The method was found to accurate, precise, specific, stable, rugged and robust. From the degradation studies, it is concluded that the drugs were stable in stress conditions. The proposed method is used for the simultaneous estimation of Emtricitabine, Efavirenz and Tenofovir in routine and quality control analysis of tablet formulations.

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