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January-March 2016

International Journal of CHEMICAL AND PHARMACEUTICAL ANALYSIS

eISSN: 2348-0726 ; pISSN : 2395-2466

Research Article | Volume-3 | Issue-2 | Article ID: 934

STABILITY INDICATING HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OFEFAVIRENZ, EMTRICITABINE AND TENOFOVIR IN COMBINED PHARMACEUTICAL DOSAGE FORM

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Received: 17 January 2016 / Revised: 8 March 2016 / Accepted: 9 March 2016 / Available online : 31 March 2016

ABSTRACT

A very accurate and precise simultaneous estimation by stability indicating RP-HPLC gradient method was developed for Assay test of Efavirenz, Emtricitabin and Tenofovir disoproxil tablet dosage form. The experiment was carried out on Hypersil BDS C18, (150mm x 4.6 mm), 5µm column using the gradient composition of phosphate buffer pH 3.5 as mobile phase A and mixture of methanol, acetonitrile and water 500:350:150 v/v. degas as mobile phase B at flow rate 1.5ml/min and detection wavelength 265 nm. The retention time of Efavirenz was about 13.8 min, Emtricitabin was about 2.9 min and Tenofovir disoproxil was about 8.0 min. The detector response was linear from in the range of 50 % to 150 % test concentration i.e. 120.00 ppm to 360.00 ppm for Efavirenz, 40.00 ppm to 120.00 ppm for Emtricitabine and 60.00 ppm to 180.00 ppm for Tenofovirdisoproxil fumarate.

Keywords *–RP-HPLC , gradient method, Assay , Efavirengz, Emtricitabine ,Tenofovirdisoproxil*

1. INTRODUCTION

The new proposed method was simple, accurate, precise, linear and rugged. Method was validated as per ICH guidelines[1,2,3,4] for simultaneous estimation of Efavirenz, Emtricitabine, and Tenofovirdisoproxil in tablet dosage form hence can be use for routine analysis. Efavirenz[5] (S)-6chloro(cyclopropylethylethynyl-1,4-(trifluoromethyl)-2H-1-benzoxazin-2-one) non-nucleoside reverse transcriptase inhibitor (NNRTI) and is used as part of highly active antiretroviral therapy (HAART) for treatment of human immunodeficiency virus (HIV) . Emtricitabine[5] is 4-amino-5-fluoro-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-2-(1H)-pyrimidon. Emtricitabine is a nucleoside reverse transcriptase inhibitor(NRTI). The drug works by inhibiting reverse transcriptase, the enzyme that copies HIV RNA into new viral DNA. Tenofovir[5] is [{1R)-2-(6-amino-9Hupurin-9-yl-1-methylethoxy}methyl]phosphonic acid. Tenofovir is a nucleoside analog reverse transcriptase inhibitor (NRTI). Literature survey reveals few chromatographic method were reported along with other antiretroviral reveals drugs like Rilpivirin, Emtricitabine, Lamivudine and Tenofovir.[6,7] The objextive of the present study was to develop the stability indicating method for combination drug dosage form of Efavirenz, Emtricitabine and Tenofovir disoproxil tablet.

2. EXPERIMENTAL

2.1 Chemical and Reagents

Working standard of Efavirenz, Emtricitabine and Tenofovirdisoproxil fumarate of Macleods Pharmaceutical Ltd. Mumbai, India were used with purity 99.1%, 99.5% and 98.7% respectively. The combination drug dosage form of Efavirenz, Emtricitabine and Tenofovirdisoproxil and in (600 mg / 200 mg / 300 mg) tablets of Macleods Pharmaceutical Ltd. Mumbai, India were used. Water(Milli-Q), Sodium dihydrogen phosphate monohydrate (Merck), Orthophosphoric acid (Rankem), Methanol(Merck, HPLC grade), Acetonitrile(Merck, HPLC), Hydrochloric acid (35.4% w/v) (Merck), Sodium hydroxide(Thomas Baker), Hydrogen peroxide 30% w/w(Thomas Baker) were used. Filter paper : GF/C (Glass Micro fibre, 25 mm, Whatman), 0.45 µm Nylon (25 mm, Advanced Micro devices Pvt Ltd (Mdi)), 0.45 µm PVDF(25 mm, Advanced Micro devices Pvt Ltd (Mdi)) were used.

2.2 Instruments / Equipments

HPLC (with UV and PDA detector) (Shimadzu LC-2010 CHT), Analytical Balance(Sartorius), pH meter(Lab India), Hot air oven(Expo hi-tech), Photostability Chamber(Atlas (Suntest CPS$^+$)), Column used Hypersil BDS C18 (150X4.6mm), 5µ.

2.3 Methodology

The separation of drug was achieved with gradient method on a reverse phase Hypersil BDS C18, (150 mm x 4.6 mm), 5µm column at wavelength 265nm, injection volume 10µl and column oven temperature was25°C. The gradient program is of 20 minutes and is as follow:

Tabel 1: Gradient program

Time (min.)	Mobile phase A (%)	Mobile phase B (%)	Comment
0	90	10	Linear gradient
4	50	50	Linear gradient
5	40	60	Linear gradient
9	30	70	Linear gradient
14	10	90	Linear gradient
17	90	10	Re-equilibration
20	90	10	Re-equilibration

Buffer solution: Buffer solution was prepared by dissolving 2.75 g of sodium dihydrogen phosphate monohydrate in 1000ml water, mixed. Adjusted the pH to 3.5± 0.05 with 10% v/v orthophosphoric acid.Filtered the solution through 0.45µm nylon filter.

Diluent: Mixture of methanol and water 85:15 v/v, degassed.

Mobile phase A : Buffer pH 3.5

Mobile phase B : Mixture of methanol, acetonitrile and water (500:350:150 v/v), degassed.

Standard Preparation:

Stock Solution A: 40 mg Emtricitabine working standard and 60 mg Tenofovirdisoproxil fumarate working standard were taken in 50ml volumetric flask added the 30ml diluent then it was sonicated, and made up volume up to mark with diluent, mixed.

Stock Solution B: 60 mg Efavirenz working standard were taken in 50ml volumetric flask added the 30ml diluent then it was sonicated and made up volume up to mark with diluent, mixed.

Standard solution: Diluted the 5 ml stock solution A and 10ml of stock solution B to 50ml with diluent and mixed. The concentration of standard solution was 240, 80 and120 µm/ml of EfavirenzEmtricitabine and Tenofovirdisoproxil fumarate respectively.

Sample solution: weighed 10 tablets for average weight and crushed them to a fine powder. Weighed accurately and transfer tablets powder equivalent to about 200 mg of Emtricitabine to a 250 mL volumetric flask. Added 150 mL of diluent and shacked mechanically for 5 minutes and sonicated for 20 minutes with intermittent shaking. Allowed to equilibrate to room temperature and diluted to volume

with diluent, mixed. Centrifuged the solution at 4000 rpm for 5 minutes. Diluted 5 mL of the supernatant solution to 50 mL with diluent, mixed. Filtered the solution through 0.45 µm PVDF (25mm) filter discarding first few mL of the filtrate.Used the filtrate. The concentration of solution was 240, 80 and120 µm/ml of EfavirenzEmtricitabine and Tenofovirdisoproxil fumarate respectively.

2.3 Method Validation

A. Specificity

To exclude the possibility of interference with excipients in the region of elution of EfavirenzEmtricitabine and Tenofovirdisoproxil fumarate. The blank, placebo solution, impurity solutions, standard solution and sample solution were prepared and injected as described in the methodology. There was no interference observed due to blank, placebo and impuritiesat the same retention time as the peaks of Efavirenz,Emtricitabine and Tenofovirdisoproxil fumarate in standard solution and sample solution chromatograms.

1.1 Forced degradation study

Stress testing (forced degradation study can help to identify the likely degradation products, stability of the molecules and also validate the stability and specificity of the analytical procedure. Study was performed for following parameter.

- Forced degradation with Heated the powder at 80°C for 24 hours,
- Forced degradation with Photolytic degradation, Powder covered with aluminum foil exposed. Also powder was directly exposed (without aluminum foil) in the photo stability chamber, as per ICH guidelines[8],
- Forced degradation with Thermal and Humidity at 40^{o}C/75%RH for 24 hours exposed,
- Forced degradation with Acid media, powder was kept in 0.2M HCl for 10 min at room temperature,
- Forced degradation with Base media, powder was kept in 0.1M NaOH for 60 min. at 80^{o}C on water bath.
- Forced degradation with Oxidative degradation media, powder was kept in 10 ml of 3% H_2O_2 for 2.5 hrs. at 80^{o}C on water bath.

Summary of forced degradation results

The summary of degradationgivenwithTabel 2. The maximum degradationwasobserved in acid media and chromatograms of samewereshown in Figure E

Force Degradation condition	% Degradation			Peak Purity		
	Efa	Emtri	Teno DF	Efa	Emtri	Teno DF
Initial	----	----	----	1.000	1.000	1.000
Heat at 80°C in oven for 24 hours (Thermal Degradation)	0.1	X	X	1.000	1.000	1.000
Photolytic degradation, as per ICH guidelines (Control)	----	----	----	1.000	1.000	1.000
Photolytic degradation, as per ICH guidelines (Exposed)	0.2	X	X	1.000	1.000	1.000
Thermal and Humidity at 40°C/75% RH for 24 hours	1.9	X	X	1.000	1.000	1.000
10 mL of 0.2 M HCl kept at room temperature for 10 mins	X	4.1	14.2	1.000	1.000	1.000
10 mL of 0.1 M NaOH kept at 80°C for 60 min on water bath	X	1.6	12.7	1.000	1.000	1.000
10 mL of 3 % H_2O_2 - kept at 80°C for 2.5 hours on water bath	2.7	1.8	X	1.000	1.000	1.000

Note: X indicates No degradation, EfaindicatesEfavirenz; EmtriindicatesEmtricitabine and Teno DF indicatesTenofovirdisoproxil fumarate

Conclusion: Forced degradation study

The peaks due to Efavirenz, Emtricitabine and Tenofovirdisoproxil fumarate were found to the spectrally pure in all the degradation conditions, indicating that there was no co-elution with main peaks.

Based on the above results it was concluded that the method for assay of Efavirenz, Emtricitabine and Tenofovirdisoproxil fumarate estimation in Efavirenz, Emtricitabine and Tenofovirdisoproxil fumarate (600/200/300 mg) tablets was specific and stability indicating.

B. Solution Stability

Considering the decomposition of analytes and standards over a time period the method development should investigate the stability of analytes and standards. It is measure of bias in assay result generated during preselected time interval.

To demonstrate the stability of both standard and sample solutions during analysis, both solutions were analyzed over a period at room temperature and at 2-8°C. The standard and sample solutions were prepared as described in methodology and stored at controlled room temperature (20°C – 25°C) and at 2-8°C. The stored solutions were injected at initial, 2 hours, 12 hours, 24 hours and 48 hours. The Absolute difference in assay of peaks due to Efavirenz, Emtricitabine and Tenofovirdisoproxil fumarate peaks at each time interval, with respect to initial assay was calculated for standard solution and sample solution. The obtained results are presented in Table 3 for standard solution and Table 4 for sample solution.

Tabel 3: Solution stability result for standard solution of Efavirenz, Emtricitabine and Tenofovir disoproxil fumarate

Time (hours)	Efavirenz in standard solution					
	CRT (20°C – 25°C)			At 2-8°C		
	Mean Area	%Assay	Absolute difference	Mean Area	%Assay	Absolute difference
Initial	724268	99.5	-	-	-	-
2 hours	718123	98.6	0.9	721431	99.1	0.4
12 hours	718611	98.7	0.8	718953	98.7	0.8
24 hours	731570	100.4	0.9	727794	99.9	0.4
48 hours	724146	99.8	0.3	724761	99.9	0.4
	Emtricitabine in standard solution					
Initial	875444	99.9	-	-	-	-
2 hours	876211	100.0	0.1	875532	100.0	0.1
12 hours	874026	99.8	0.1	876327	100.0	0.1
24 hours	877494	99.9	0.0	872327	99.4	0.5
48 hours	876358	99.3	0.6	876371	99.3	0.6
	Tenofovir disoproxil fumarate in standard solution					
Initial	1111473	99.0	-	-	-	-
2 hours	1109389	98.8	0.2	1111213	99.0	0.0
12 hours	1105044	98.4	0.6	1107171	98.6	0.4
24 hours	1114931	99.8	0.8	1107601	99.1	0.1
48 hours	1106281	98.8	0.2	1109288	99.1	0.1

Tabel 4: Solution stability result for sample solution of Efavirenz, Emtricitabine and Tenofovir disoproxil fumarate

Conclusion : Based on the above data it had been concluded that the standard solution and sample solution can be used up to 48 hours after preparation when stored at controlled room temperature (20°C – 25°C) and at 2°C – 8°C.

C. Filter Compatibility

Sample solution was prepared as described in the methodology. The following variations were carried out at the sample filtration stage, the sample solution was centrifuged and diluted as per methodology. The sample solution were centrifuged and filtered through Whatman GF/C (25 mm) filter, 0.45 µm nylon (25 mm) filter and 0.45µm PVDF (25 mm) filter

The obtained solutions were analysed and the assay results were determined. The absolute difference between the results obtained with the centrifuged solution and filtered solution were calculated. The results are presented in result table 5.

Tabel 5: Filter Compatibility result for Assay of Efavirenz, Emtricitabine and Tenofovir disoproxil fumarate

Filter Type	Efavirenz			Emtricitabine			Tenofovir disoproxil fumarate		
	Mean	%	Adso Diff	Mean	%	Adso Diff	Mean	%	Adso Diff
Centrifuged	602417	100.3	-	809698	98.5	-	998338	98.9	-
Whatman GF/C	605094	100.7	0.4	809646	98.5	0.0	998461	98.9	0.0
0.45µm nylon	606971	101.0	0.7	811891	98.7	0.2	1001953	99.2	0.3
0.45µm PVDF	603354	100.4	0.1	807347	98.2	0.3	995324	98.6	0.3

Conclusion: The obtained results using the Whatman GF/C (25 mm), 0.45µm nylon filter (25 mm) and 0.45µm PVDF filter (25 mm) in sample are well within the acceptance criteria i.e. absolute difference of not more than 2.0.

However, 0.45µm PVDF filter was selected as the filter of choice

D. Filter Saturation

The saturation of 0.45µm PVDF (25 mm) filter was optimised by filtering and discarding 1.0 mL, 3.0 mL and 5.0 mL sample solution using separate filters, followed by filtration of further 10 mL aliquots and collection of the filtrates in separate test tubes.

Each sample was analysed and the results were calculated. The absolute difference in the results obtained between two consecutively filtered aliquots was calculated and the minimum volume of solution required to saturate the filter was determined. The results are presented in table 6.

Tabel 6:Filter Saturation results for Assay of Efavirenz, Emtricitabine and Tenofovir disoproxil fumarate

Volume discarded	Efavirenz			Emtricitabine			Tenofovir disoproxil fumarate		
	Mean area	% Assay	Abs Diff	Mean area	% Assay	Abs Diff	Mean area	% Assay	Abs Diff
1.0 mL	604397	101.0	-	822870	99.5	-	1010983	98.5	-
3.0 mL	608489	101.7	0.7	827850	100.1	0.6	1017868	99.2	0.7
5.0 mL	607379	101.5	0.2	826825	100.0	0.1	1016809	99.1	0.1

Conclusion: From the above results, it was concluded that the volume of 3.0 mL is sufficient to saturate the filters.

E. Linearity and Range

To determine Linearity, a series of solutions were prepared by quantitative dilutions of the stock solution of Efavirenz, Emtricitabine and Tenofovirdisoproxil fumarate standards to obtain solutions at 50 %, 80 %, 100 %, 120 % and 150 % of the working concentration of (0.24 mg/mL (240 ppm)) Efavirenz, (0.08 mg/mL (80 ppm)) Emtricitabine and (0.12 mg/mL (120 ppm)) Tenofovirdisoproxil fumarate. This corresponded to a concentration range of 120.00 ppm to 360.00 ppm for Efavirenz, concentration range of 40.00 ppm to 120.00 ppm for Emtricitabine and concentration range of 60.00 ppm to 180.00 ppm for Tenofovirdisoproxil fumarate.

Each solution was injected in duplicate and the peak areas were recorded. Slope, intercept, correlation coefficient of the regression line and residual sum of squares were calculated.

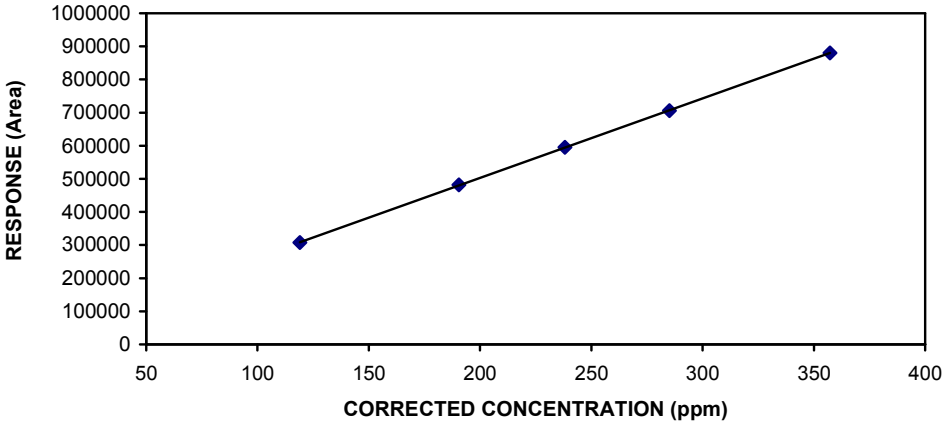
The values of concentration, corrected concentration and mean peak area are presented in table 7 for Efavirenz, table 8 for Emtricitabine and table 9 for Tenofovirdisoproxil fumarate. A graph of mean peak area vs. corrected concentration (ppm) were plotted in figure (a) for Efavirenz, (b) for Emtricitabine and (c) Tenofovirdisoproxil fumarate.

The plot of peak area of each sample against respective concentration was found to be linear in the range of 120 – 360, 40 - 120, and 60 - 180 ppm with correlation coefficient of 0.99997, 0.99993 and 0.99995 and linear regression equation Being Y=2399.01785x+22789.00820 Y=10443.90443x+2357.09259, and Y=8586.12019x+5775.49522 for EfavirenzEmtricitabine and Tenofovirdisoproxilfumerate respectively. Linear regression least square fit, slope (m), intercept (b), standard deviation, residual sum of squares and correlation coefficient data obtained from the measurements

Tabel 7: Linearity results for assay of Efavirenz.

Level	Theoretical Conc. (ppm)	Corrected Conc. (ppm)	Mean area
50 %	120.00	119.08	307381
80 %	192.00	190.53	481709
100 %	240.00	238.16	595067
120 %	288.00	285.79	706187
150 %	360.00	357.24	880352
SLOPE			2399.01785
INTERCEPT			22789.00820
CORRELATION COEFFICIENT			0.99997
RESIDUAL SUM OF SQUARES			10606540.00158
RANGE: 50 % to 150 % of target concentration (i.e. 120.00 ppm to 360.00 ppm)			

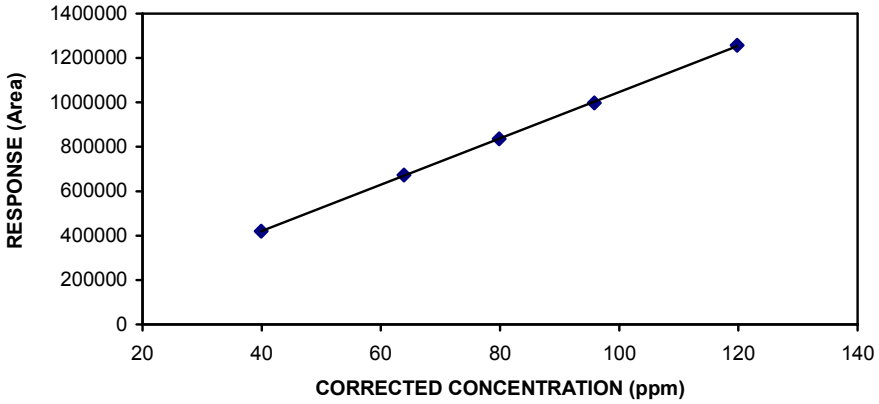
Figure (a) - LINEARITY OF EFAVIRENZ



Tabel 8: Linearity results for Assay of Emtricitabine.

Level	Theoretical Conc. (ppm)	Corrected Conc. (ppm)	Mean area
50 %	40.00	39.93	419411
80 %	64.00	63.89	672612
100 %	80.00	79.86	835518
120 %	96.00	95.83	997599
150 %	120.00	119.79	1256898
SLOPE			10443.90443
INTERCEPT			2357.09259
CORRELATION COEFFICIENT			0.99993
RESIDUAL SUM OF SQUARES			53099711.17000
RANGE: 50 % to 150 % of target concentration (i.e. 40.00 ppm to 120.00 ppm)			

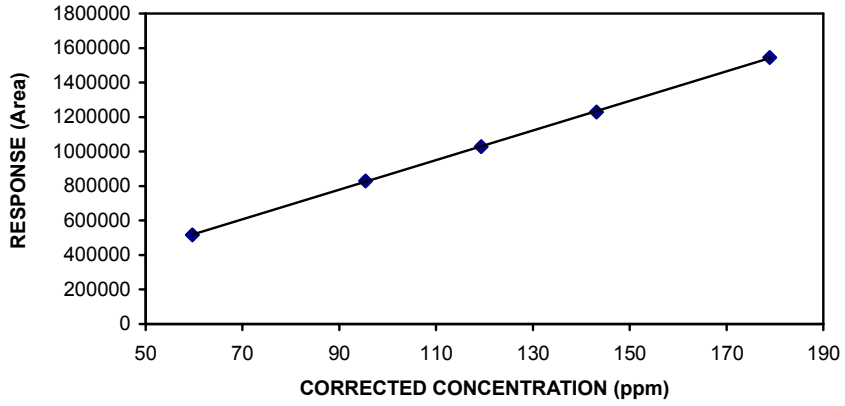
Figure (b) - LINEARITY OF EMTRICITABINE



Tabel 9: Linearity results for Assay of Tenofovirdisoproxil fumarate.

Level	Theoretical Conc. (ppm)	Corrected Conc. (ppm)	Mean area
50 %	60.00	59.64	517294
80 %	96.00	95.43	829110
100 %	120.00	119.29	1029061
120 %	144.00	143.15	1229083
150 %	180.00	178.93	1545436
SLOPE			8586.12019
INTERCEPT			5775.49522
CORRELATION COEFFICIENT			0.99995
RESIDUAL SUM OF SQUARES			61691201.75295
RANGE: 50 % to 150 % of target concentration (i.e. 60.00 ppm to 180.00 ppm)			

Figure (c) - LINEARITY OF TENOFOVIR DISOPROXIL FUMARATE



Conclusion: The correlation co-efficient was found to be 0.99997 for Efavirenz, 0.99993 for Emtricitabine and 0.99995 for Tenofovirdisoproxil fumarate, which are well within the acceptance criteria of not less than 0.999. Hence it had been concluded that the method was linear in the range of 50 % to 150 % i.e. 120.00 ppm to 360.00 ppm for Efavirenz, 40.00 ppm to 120.00 ppm for Emtricitabine and 60.00 ppm to 180.00 ppm for Tenofovirdisoproxil fumarate.

F. Precision

i. System Precision

To check system precision Efavirenz, Emtricitabine and Tenofovirdisoproxil fumarate standard solution was prepared as per methodology and peak response were measured in five replicates. The mean and relative standard deviations were calculated. The results are presented in the following result table 10.

Tabel 10: System presicion for Assay of Efavirenz, Emtricitabine and Tenofovir disoproxil fumarate

Injection No.	Peak Area (Efavirenz)	Peak Area (Emtricitabine)	Peak Area (Tenofovirdisoproxil fumarate)
1	558279	859918	1035348
2	558097	861901	1037830
3	558399	863434	1039892
4	559025	864985	1042928
5	558087	862761	1040995
Mean	558377	862600	1039399
% RSD	0.07	0.22	0.28

Conclusion: The relative standard deviations for areas of peaks due to Efavirenz, Emtricitabine and Tenofovir disoproxil fumarate in five replicate injections of standard solution are 0.07 %, 0.22 % and 0.28 % respectively, which are well within the acceptance criteria of not more than 2.0 %.

ii. Repeatability

The assay was carried out as described in the methodology on six samples The % assay of Efavirenz, Emtricitabine and Tenofovirdisoproxil fumarate were calculated. The mean, relative standard deviation and 95 % confidence interval of the results were calculated. The results obtained for assay of Efavirenz, Emtricitabine and Tenofovir disoproxil fumarate are presented as below.

Table 11: Repeatability results for Assay of EfavirenzEmtricitabine and Tenofovir disoproxil fumarate

Sample No	Spl. wt (mg)	Peak Area (Mean)			% Assay		
		Efavi	Emtri	Teno DF	Efavi	Emtri	Teno DF
1	1612.82	564235	850247	1022967	100.4	98.2	97.8
2	1616.15	559684	847315	1020033	99.3	97.6	97.3
3	1611.65	564690	848341	1020313	100.5	98.0	97.6
4	1610.92	564664	849372	1020202	100.6	98.2	97.7
5	1610.08	559172	847245	1018031	99.6	98.0	97.5
6	1611.63	557300	845495	1015275	99.2	97.7	97.1
MEAN					99.9	98.0	97.5
% RSD					0.64	0.26	0.27
95 % CONFIDENCE INTERVAL					0.51	0.20	0.21

Conclusion: The relative standard deviation of the assay results for six individual sample preparations in repeatability for Efavirenz was 0.64 %, Emtricitabine was 0.26 % and Tenofovirdisoproxil fumarate was 0.27 % for Efavirenz, Emtricitabine and Tenofovirdisoproxil fumarate tablets (600 mg / 200 mg / 300 mg) which was well within the acceptance criteria of not more than 2.0 %.

G. Accuracy / Recovery

Recovery solutions were prepared by spiking Efavirenz, Emtricitabine and Tenofovirdisoproxil fumarate to placebo powder to obtain solutions in the range 50 % to 150 % (i.e. at 50 %, 100 % and 150 %) of the target concentration (0.24 mg/mL (240 ppm)) Efavirenz, (0.08 mg/mL (80 ppm)) Emtricitabine and (0.12 mg/mL (120 ppm)) Tenofovirdisoproxil fumarate in triplicate.

The % recovery of Efavirenz, Emtricitabine and Tenofovirdisoproxil fumarate were calculated for each of the recovery solution and the mean recovery was determined. The results are presented in the result table 12,13 and 14 respectively

Table 12: Recovery study for Assay test of Efavirenz

Level	Efavirenz spiked (mg)	Wt. of placebo (mg)	Mean Area	Efavirenz recovered (mg)	% Recovery	Mean
50 %	310.76	511.08	295951	314.45	101.2	101.5
	316.03	511.10	302003	320.88	101.5	
	317.20	511.40	304247	323.26	101.9	
100 %	610.98	510.08	587896	624.64	102.2	101.7
	606.10	510.94	577584	613.68	101.3	
	608.72	511.10	581477	617.82	101.5	
150 %	900.07	511.08	847583	900.55	100.1	100.8
	898.81	511.59	851802	905.03	100.7	
	901.18	512.02	862051	915.92	101.6	
Mean % Recovery					101.3	
% RSD					0.62	

Table 13: Recovery study for Assay test of Emtricitabine

Level	Emtricitabine spiked (mg)	Wt. of placebo (mg)	Mean Area	Emtricitabine recovered (mg)	% Recovery	Mean
50 %	101.59	511.08	444828	102.76	101.2	100.4
	102.80	511.10	444627	102.72	99.9	
	102.30	511.40	443247	102.40	100.1	
100 %	199.68	510.08	873232	201.73	101.0	100.6
	200.13	510.94	864750	199.77	99.8	
	200.29	511.10	875543	202.26	101.0	
150 %	300.83	511.08	1304937	301.46	100.2	100.3
	302.41	511.59	1307969	302.16	99.9	
	303.30	512.02	1322344	305.48	100.7	
Mean % Recovery					100.4	
% RSD					0.55	

Table 14: Recovery study for Assay test of Tenofovirdisoproxil fumarate

Level	Tenofovirdisoproxil fumarate spiked (mg)	Wt. of placebo (mg)	Mean Area	Tenofovirdisoproxil fumarate recovered (mg)	% Recovery	Mean
50 %	150.81	511.08	529287	152.75	101.3	101.1
	151.73	511.10	531912	153.51	101.2	
	152.10	511.40	531823	153.48	100.9	
100 %	299.47	510.08	1046661	302.06	100.9	100.6
	299.15	510.94	1034102	298.44	99.8	
	298.41	511.10	1045363	301.69	101.1	
150 %	450.05	511.08	1556160	449.10	99.8	100.4
	449.45	511.59	1560996	450.49	100.2	
	450.39	512.02	1581114	456.30	101.3	
Mean % Recovery					100.7	
% RSD					0.62	

Conclusion: The % recovery for Efavirenz, Emtricitabine and Tenofovirdisoproxil fumarate at 50 %, 100 % and 150 % of target concentration (0.24 mg/mL (240 ppm)) Efavirenz, (0.08 mg/mL (80 ppm)) Emtricitabine and (0.12 mg/mL (120 ppm)) Tenofovirdisoproxil fumarate ranged from 100.1 % to 102.2 % for Efavirenz, 99.8 % to 101.2 % for Emtricitabine and 99.8 % to 101.3 % for Tenofovirdisoproxil fumarate respectively, which are well within the acceptance criteria of 97.0% to 103.0 %.

The mean recoveries are 101.3 % for Efavirenz, 100.4 % for Emtricitabine and 100.7 % for Tenofovirdisoproxil fumarate, which were also within the acceptance criteria of 98.0 % to 102.0 %.

Based on the above obtained recovery results, it was concluded that method for assay of Efavirenz, Emtricitabine and Tenofovirdisoproxil fumarate in Efavirenz, Emtricitabine and Tenofovirdisoproxil fumarate tablets (600 mg / 200 mg / 300 mg) was accurate

H. Robustness

Experimental and result: The Assay method was carried out as described in the methodology and by making the following alterations in the chromatographic conditions

- Changing the flow rate 1.5±0.2 ml/min (1.3 mL / min, 1.7 mL / min)
- Changing Column oven temperature 25±5^{o}C(20°C, 30°C)
- Changing the pH of mobile phase Buffer3.5±0.2 (pH = 3.3, pH = 3.7)

The observed values were presented in table 15.

Tabel 15: Robustness study results for Assay of Efavirenz, Emtricitabine and Tenofovir disoproxil fumarate

Altered condition	Efavirenz			Emtricitabine			Tenofovir disoproxil fumarate		
	Mean area	% Assay	Abs Diff	Mean area	% Assay	Abs Diff	Mean area	% Assay	Abs Diff
Unaltered	---	99.9	-	---	98.0	-	---	97.5	-
Flow 1.3	732104	101.1	1.2	952134	98.9	0.9	1164065	97.4	0.1
Flow1.7	571334	100.7	0.8	727610	98.7	0.7	885577	96.8	0.7
Temp 20^{0}C	633252	101.0	1.1	827249	99.4	1.4	1017339	97.2	0.3
Temp 30^{0}C	638774	101.2	1.3	824163	98.8	0.8	1012065	96.7	0.8
pH 3.3	546816	101.7	1.8	813002	97.2	0.8	978721	97.1	0.4
pH 3.7	546955	101.6	1.7	819106	97.2	0.8	979862	97.1	0.4

Conclusion : By making above alteration no significant change in result observed hence method siRoubst for routine analysis.

I. System suitability

To check the system suitability, Efavirenz, Emtricitabine and Tenofovirdisoproxil fumarate standard solution was prepared as described in the methodology. The standard solution was injected into the HPLC system at the start of each validation parameter and peak responses were measured. The system suitability parameters of mean and relative standard deviation of areas, and the theoretical plates and tailing factor for the peaks due to Efavirenz, Emtricitabine and Tenofovirdisoproxil fumarate were calculated. The comparative results are presented in the following result tables 16,17,18 respectively.

Tabel 16: System suitability data for Efavirenz

Sr. No	Validation parameters		Retention time (min)	Tailing Factor	Theoretical plates	Mean area	% RSD (Five replicate injections)
1	Specificity	1	13.9	1.0	91487	598398	0.15
		2	13.8	1.0	82318	469971	0.06
		3	13.8	1.0	79124	469157	0.19
		4	13.8	1.0	70589	474439	0.13
2	Solution Stability	Initial	13.7	0.9	49386	727819	0.21
		24 hours	13.6	1.0	49117	726080	0.47
		48 hours	13.7	0.9	42848	723214	0.23
3	Filter Compatibility		13.9	1.0	97569	602025	0.48
4	Filter Saturation		13.9	1.0	93973	594408	0.07
5	Linearity and Range		13.8	1.0	97352	599645	0.29
6	Repeatability		13.7	1.0	87488	558377	0.07
7	Intermediate Precision		13.3	1.0	89487	629589	0.02
8	Accuracy		13.7	1.0	85661	550113	0.13

Tabel 17: System suitability data for Emtricitabine

Sr. No	Validation parameters		Retention time (min)	Tailing Factor	Theoretical plates	Mean area	% RSD (Five replicate injections)
1	Specificity	1	2.9	1.1	6786	841629	0.23
		2	2.9	1.2	6264	883524	0.07
		3	2.9	1.2	6168	884751	0.07
		4	2.9	1.2	5999	890783	0.14
2	Solution Stability	Initial	2.7	1.1	4958	877868	0.25
		24 hours	2.7	1.1	5050	876885	0.08
		48 hours	2.7	1.1	4817	878126	0.08
3	Filter Compatibility		2.9	1.1	6823	841431	0.09
4	Filter Saturation		2.9	1.1	6705	834321	0.11
5	Linearity and Range		2.9	1.1	6613	838504	0.08
6	Repeatability		2.8	1.1	6244	862600	0.22
7	Intermediate Precision		2.5	1.2	6305	835506	0.38
8	Accuracy		2.8	1.1	6223	860988	0.04

Tabel 18: System suitability data for Tenofovir disoproxil fumarate

Sr. No	Validation parameters		Retention time (min)	Tailing Factor	Theoretical plates	Mean area	% RSD (Five replicate injections)
1	Specificity	1	8.0	1.1	39133	1031737	0.19
		2	8.0	1.1	35493	989133	0.10
		3	8.0	1.1	33169	985670	0.12
		4	8.0	1.1	30462	980948	0.26
2	Solution Stability	Initial	7.8	1.0	21697	1117270	0.38
		24 hours	7.7	1.0	21764	1112415	0.06
		48 hours	7.8	1.0	20088	1108906	0.04
3	Filter Compatibility		8.0	1.1	41529	1011286	0.07
4	Filter Saturation		8.0	1.1	39918	1026276	0.14
5	Linearity and Range		8.0	1.1	42171	1012114	0.05
6	Repeatability		8.0	1.1	38174	1039399	0.28
7	Intermediate Precision		7.5	1.1	36803	1042288	0.30
8	Accuracy		7.9	1.1	37504	1026864	0.06

Based on the overall results of system suitability, the following acceptance criteria are recommended:

1. The column efficiency should not be less than 2000, 8000 and 20000 theoretical plates for Emtricitabine, Tenofovirdisoproxil and Efavirenz peaks respectively.
2. Relative standard deviation for area due to Efavirenz, Emtricitabine and Tenofovirdisoproxil fumarate peak in five replicate injections of standard solution should be not more than 2.0 %.
3. Tailing factor for Efavirenz, Emtricitabine and Tenofovirdisoproxil fumarate peak should be not more than 2.0.

Figure D: Typical elution pattern for Assay of Efavirenz, Emtricitabine and Tenofovir disoproxil fumarate

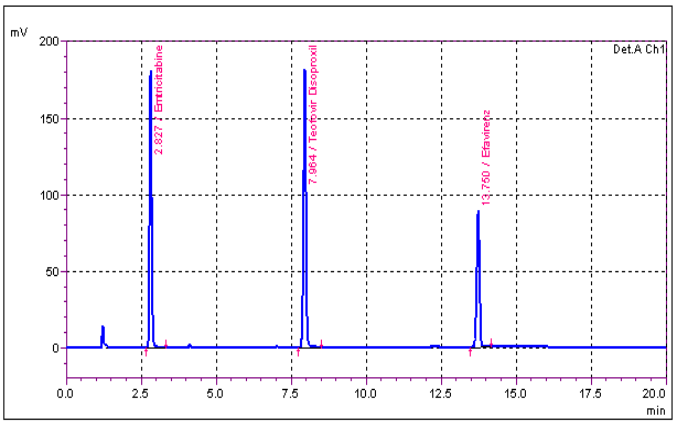
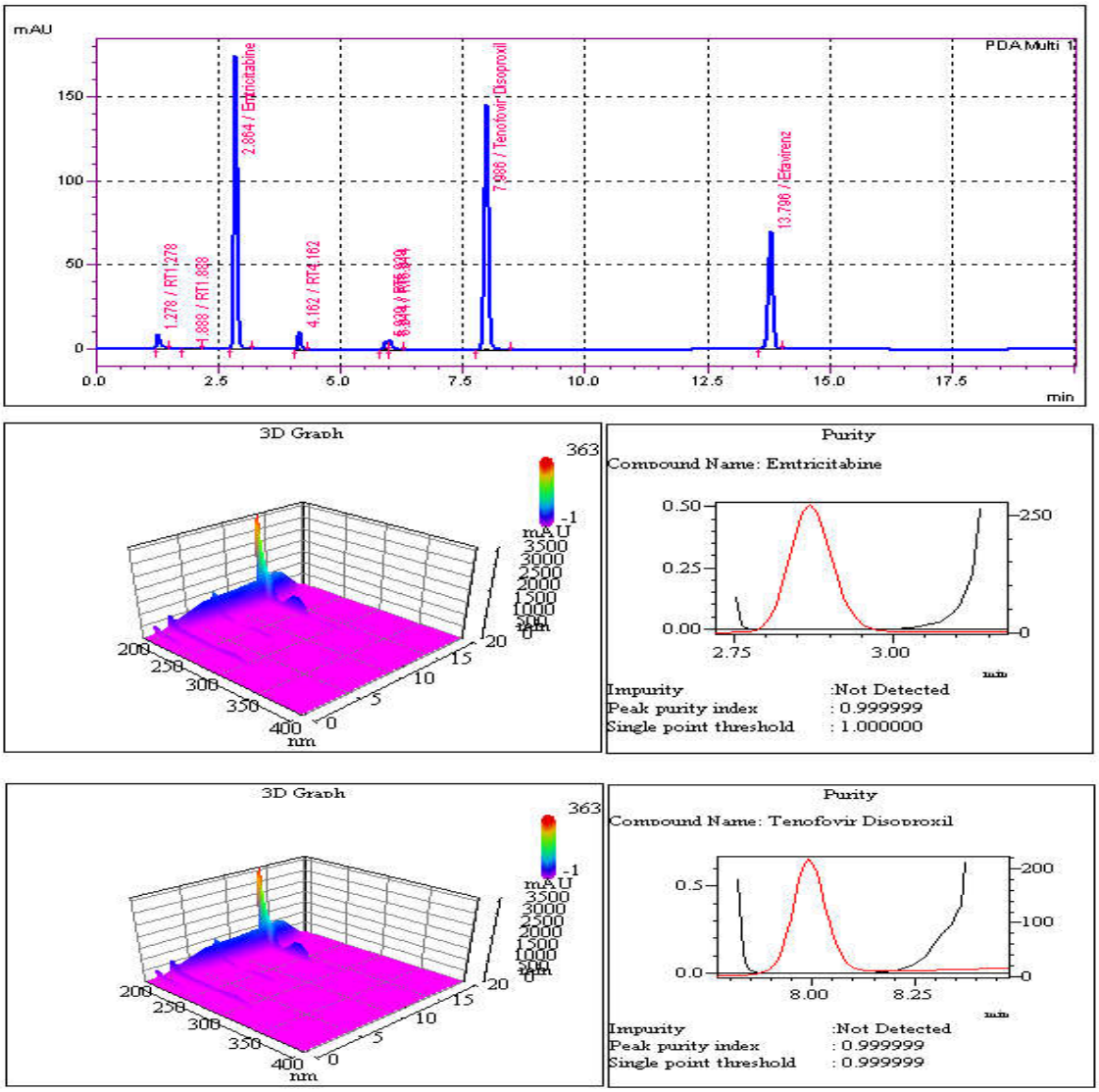
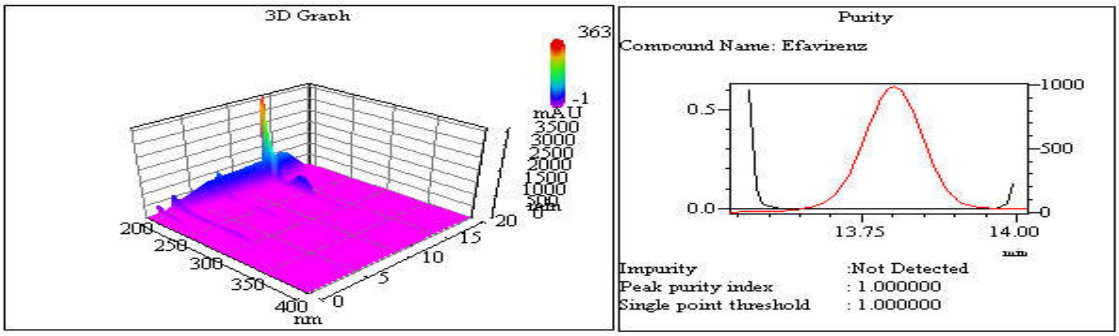


Figure E: The Maximum degradation (0.2MHCl) of Efavirenz, Emtricitabine and Tenofovirdisoproxil fumarate..





4. CONCLUSION

The method for the determination of assay of Efavirenz, Emtricitabine and Tenofovirdisoproxil fumarate in Efavirenz, Emtricitabine and Tenofovirdisoproxil fumarate (600 mg /200 mg / 300 mg) tablets was validated. The method was evaluated for its specificity, precision, solution stability, accuracy, linearity and range, and robustness. The method meets all the acceptance criteria

Hence it can be concluded that the method has been suitable for its intended use, i.e. to determine the assay of Efavirenz, Emtricitabine and Tenofovirdisoproxil fumarate in Efavirenz, Emtricitabine and Tenofovirdisoproxil fumarate (600 mg /200 mg / 300 mg) tablets.

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