

Stability-Indicating RP-HPLC Method for the Simultaneous Estimation of Efavirenz, Tenofovir and Emtricitabine in Pharmaceutical Formulations

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Abstract: A simple, precise and rapid HPLC method has been developed for the simultaneous determination of Efavirenz, Tenofovir and Emtricitabine in pharmaceutical dosage form. The method was carried out using Zorbax C8 column (150 mm x 4.6 mm, 5 μ m) and mobile phase comprised of mixture of dilute orthophosphoric acid solution pH 2.4 \pm 0.02 as buffer and acetonitrile in the ratio of 70:30 v/v and degassed under ultrasonication. The flow rate was 1.0 mL/min and the effluent was monitored at 252 nm. The retention times of Efavirenz, Tenofovir and Emtricitabine were 1.81 min, 2.80 min and 7.30 min respectively. The method was validated in terms of linearity, precision, accuracy, specificity, limit of detection, limit of quantitation and by performing recovery study. Linearity was in the range of 600.13 to 1800.39 μ g/mL for Efavirenz, 300.54 to 900.44 μ g/mL for Tenofovir and 200.46 to 601.38 μ g/mL for Emtricitabine respectively. The percentage recoveries of all the three drugs were ranging from 98.2 to 101.9 for Efavirenz, 99.7 to 101.9 for Tenofovir and 98.7 to 101.6 for Emtricitabine respectively from the tablet formulation. The proposed method is suitable for the routine quality control analysis of simultaneous determination of Efavirenz, Tenofovir and Emtricitabine in bulk and pharmaceutical dosage form.

Keywords: Efavirenz, Tenofovir, Emtricitabine, RP-HPLC, Validation.

Introduction

Efavirenz (Fig.1) is a non-nucleosidase reverse transcriptase inhibitor used in the treatment of HIV infection. [1-2]. Chemically it is (S)-6-chloro-4-(cyclopropyl ethynyl)-1,4-dihydro-4-(trifluoromethyl)-2H-3,1-benzoxazin-2-one. Efavirenz inhibits the activity of viral RNA-directed DNA polymerase. Tenofovir disoproxil fumarate (Fig. 2) is an antiviral drug. Tenofovir disoproxil fumarate is an HIV-1, nucleotide reverse transcriptase inhibitor, which is a fumaric acid salt of bis-isopropoxycarbonyloxy methyl ester derivative of Tenofovir.

Chemically it is 9-[(R)-2-[[bis [[(isopropoxycarbonyl)oxy]methoxy] phosphinyl] methoxy] propyl]adenine fumarate (1:1) [3-4]. Emtricitabine (Fig. 3) is an antiviral drug. Emtricitabine is a synthetic nucleoside analog of cytidine with activity against human immunodeficiency virus type 1 (HIV-1) reverse transcriptase. Emtricitabine is a nucleoside reverse transcriptase inhibitor (NRTI) for the treatment of HIV infection in adults. Chemically it is 5-fluoro-1-(2R, 5S)-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine [5-6].

Literature survey reveals that few analytical methods have been reported for the determination of Efavirenz, Tenofovir and Emtricitabine individually in biological fluids and in pharmaceutical dosage forms. Few analytical methods using HPLC [7-10] have been reported for the simultaneous determination of Efavirenz, Tenofovir and Emtricitabine in combined dosage forms.

The objective of the present study was to develop and validate a simple, accurate and precise HPLC method for simultaneous determination of Efavirenz, Tenofovir and Emtricitabine.

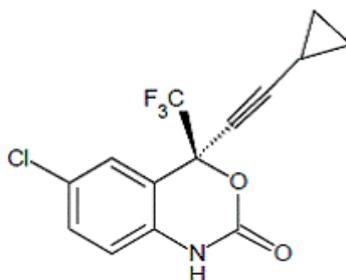


Fig. 1: Chemical structure of Efavirenz

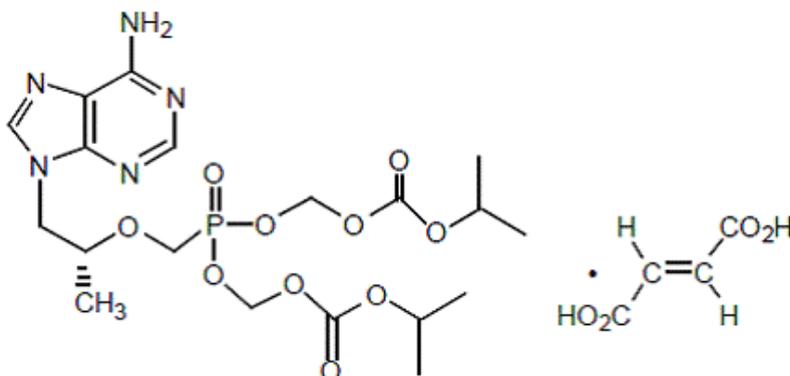


Fig. 2: Chemical structure of Tenofovir disoproxil fumarate

Materials and Methods

Instrumentation

The analysis of drugs was carried out on a Waters HPLC system on a Zorbax C8 column (150 mm x 4.6 mm, 5 μ m). The instrument is equipped with a 2695 pump with inbuilt degasser, 2998 photodiode array detector and a Rheodyne injector with 20 μ L sample loop. A 20 μ L Hamilton syringe was used for injecting the samples. Data was analysed by using Waters Empower 2 software. A double-beam Shimadzu UV-Visible 2450 spectrophotometer was used for spectral studies. Degassing of the mobile phase was done by using an ultrasonic bath sonicator. A Shimadzu balance was used for weighing the materials.

Chemicals and Solvents

The reference samples of Efavirenz (API), Tenofovir (API) and Emtricitabine (API) were obtained from Rainbow Pharma Labs, Hyderabad, India. The branded formulations (tablets) (TRUSTIVA tablets containing 600 mg of Efavirenz, 300 mg of Tenofovir and 200 mg of Emtricitabine) were procured from the local market. HPLC grade acetonitrile and analytical grade orthophosphoric acid was obtained from Qualigens Fine Chemicals Ltd, Mumbai, India. Hydrochloric acid, sodium hydroxide, hydrogen peroxide of analytical grade was obtained from Merck Chemicals Ltd, Mumbai, India. Milli-Q water was used throughout the experiment dispensed through 0.22 μ filter of the Milli-Q water purification system from Millipore, Merck KGaA, Darmstadt, Germany.

Chromatographic conditions

HPLC was connected with Zorbax C8 column (150 mm x 4.6 mm, 5 μ m) as stationary phase. A mixture of dilute orthophosphoric acid solution pH 2.4 \pm 0.02 as buffer and acetonitrile in the ratio of 70:30 v/v was prepared and used as mobile phase. The orthophosphoric acid buffer solution was prepared by transferring about 0.5 mL of orthophosphoric acid into 1000 mL standard flask, add 400 mL of Milli-Q water, mix and dilute to volume with Milli-Q water, sonicate for five minutes and cool to room temperature, measure the pH of above solution and finally adjusted the pH to 2.4 with orthophosphoric acid solution and filtered through 0.45 μ nylon filter. The 100% water was used as diluent. Injection volume was 10 μ L and flow rate was 1.0 mL/min and run time was 9.0 min. The column was maintained at ambient temperature and the fluent was monitored at 252 nm.

Preparation of standard solution

About 30 mg of Efavirenz, 15 mg of Tenofovir and 10 mg of Emtricitabine were accurately weighed and transferred into a 25 mL clean dry volumetric flask containing 15 mL of diluent and solicated for 30 min. The solution were cooled to room temperature and diluted to volume with diluent and used as standard stock solution. Standard stock solution was diluted to get a concentration of 600.13 to 1800.39 μ g/mL for Efavirenz, 300.54 to 900.44 μ g/mL for Tenofovir and 200.46 to 601.38 μ g/mL for Emtricitabine respectively.

Preparation of sample solution

Twenty tablets were weighed and finely powdered. An accurately weighed portion of powder sample equivalent to 30 mg of Efavirenz, 15 mg of Tenofovir and 10 mg of Emtricitabine was transferred to a 100 mL volumetric flask containing 60 mL of diluent. The contents of the flask were sonicated for about 30 min for complete solubility of the drug and volume made up with further quantity of diluent. Filter 20 mL of the above solution through 0.45 μ membrane filter, transfer 5 mL of the filtered solution into 25 mL volumetric flask and volume was made upto with mobile phase.

Method development

To develop a simple and robust method for the simultaneous determination of Efavirenz, Tenofovir and Emtricitabine in combined tablet dosage form using HPLC. The spectra of diluted solutions of the Efavirenz, Tenofovir and Emtricitabine in Milli-Q water were recorded separately on UV spectrophotometer. The peaks of maximum absorbance wavelengths were observed. The spectra of the all the three drugs Efavirenz, Tenofovir and Emtricitabine were showed that a balanced wavelength was found to be 252 nm. Preliminary development trials have performed with octyl and octadecyl columns with different types, configurations and from different manufacturers. Finally the expected separation and shapes of peak was succeeded in Zorbax C8 column.

To effect ideal separation of the drug under isocratic conditions, mixtures of solvents like water, methanol and acetonitrile with or without different buffers in different combinations were tested as mobile phases on a C8 stationary phase. A mixture of dilute orthophosphoric acid solution pH 2.4 \pm 0.02 as buffer and acetonitrile in proportion of 70:30 v/v was proved to be the most suitable of all the combinations since the chromatographic peaks obtained were better defined and resolved and almost free from tailing. Flow rates of the mobile phase were changed from 0.5-2.0 mL/min for optimum separation. A minimum flow rate as well as minimum run time gives the maximum saving on the usage of solvents. It was found from the experiments that 1.0 mL/min flow rate was ideal for the successful elution of the analyte. No interference in blank and placebo solutions for both drug peaks in the trail injections with a runtime of 9.0 min. The above optimized chromatographic conditions were followed for the simultaneous determination of Efavirenz, Tenofovir and Emtricitabine in bulk samples and its

combined tablet formulations. The chromatograms of standard and sample solutions of Efavirenz, Tenofovir and Emtricitabine were shown in Fig. 3 and Fig. 4.

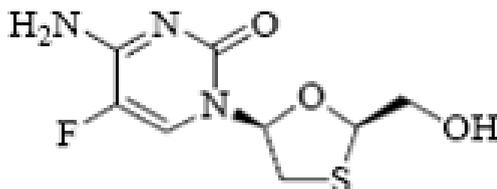


Fig. 3: Chemical structure of Emtricitabine

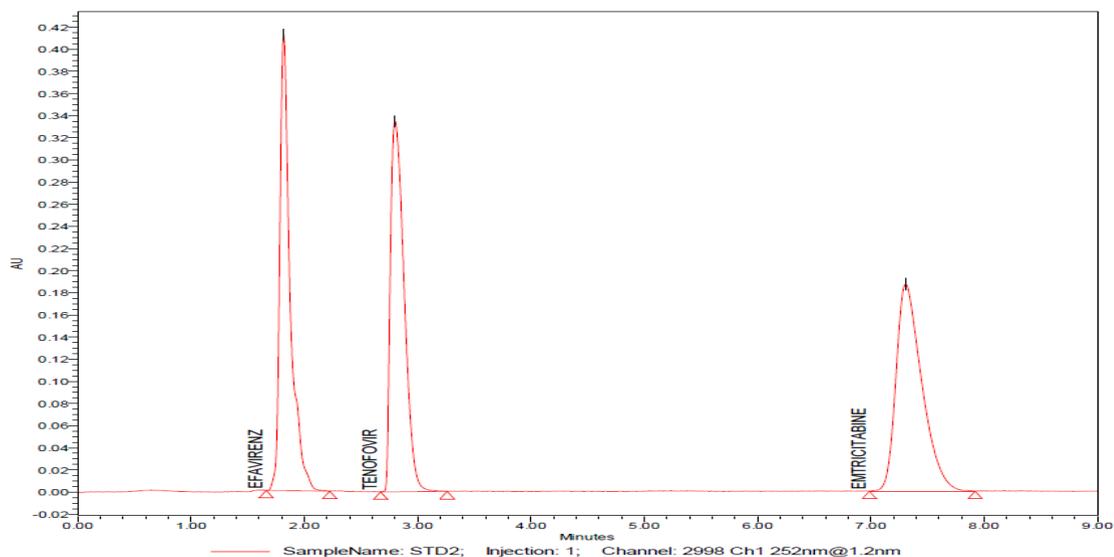


Fig. 4: Chromatogram of standard solution of Efavirenz, Tenofovir and Emtricitabine

Validation of the proposed method

The proposed method was validated as per ICH [11-12] guidelines. The parameters studied for validation were specificity, linearity, precision, accuracy, robustness, system suitability, limit of detection, limit of quantification, and solution stability.

Specificity

A study conducted to establish specificity of the proposed method involved injecting blank and placebo using the chromatographic conditions defined for the proposed method. It was found that there is no interference due to excipients in the tablet formulation and also found good correlation between the retention times of standard and sample. The chromatograms of blank and placebo for Efavirenz, Tenofovir and Emtricitabine were shown in Fig. 5 to 7.

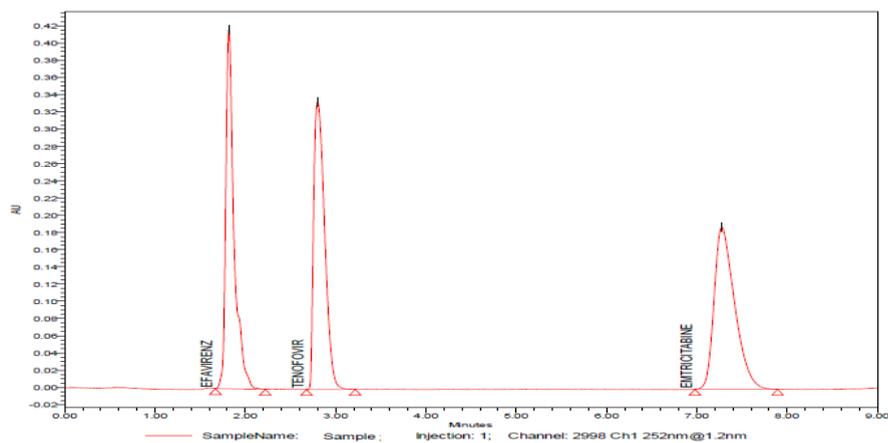


Fig. 5: Chromatogram of sample solution of Efavarinz, Tenofovir and Emtricitabine

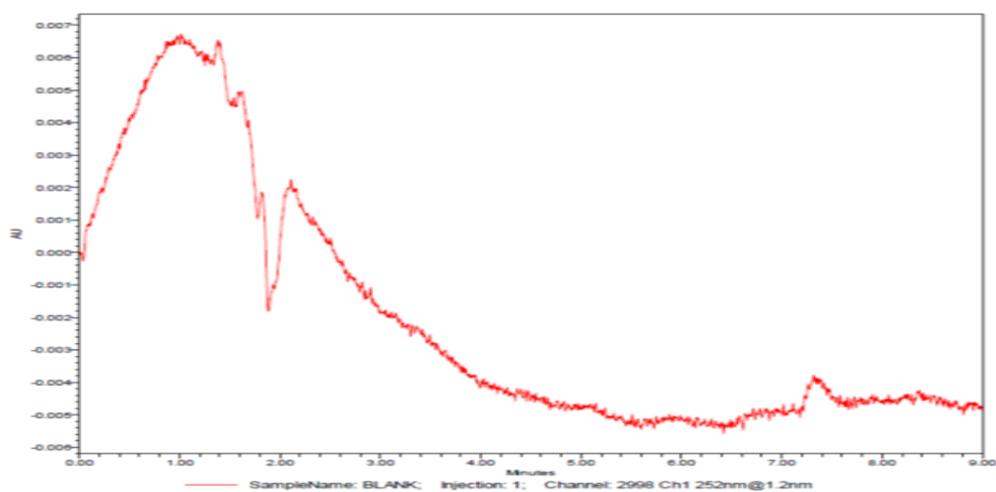


Fig. 6: Chromatogram showing no interference of blank for Efavarinz, Tenofovir and Emtricitabine

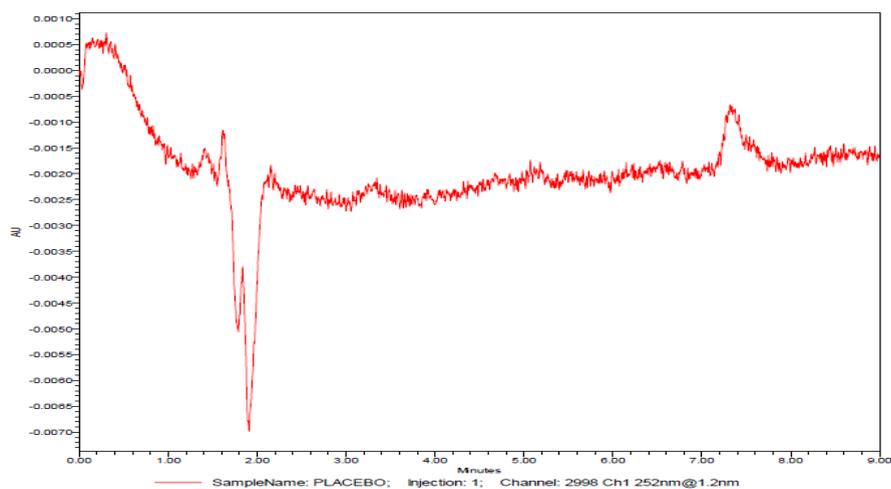


Fig. 7: Chromatogram showing no interference of placebo for Efavarinz, Tenofovir and Emtricitabine

Linearity

Linearity was performed by preparing mixed standard solutions of Efavirenz, Tenofovir and Emtricitabine at different concentration levels including working concentration mentioned in experimental condition i.e., 600.13 to 1800.39 $\mu\text{g/mL}$ for Efavirenz, 300.54 to 900.44 $\mu\text{g/mL}$ for Tenofovir and 200.46 to 601.38 $\mu\text{g/mL}$ for Emtricitabine respectively. Twenty microlitres of each concentration was injected in duplicate into the HPLC system. The response was read at 252 nm and the corresponding chromatograms were recorded. From these chromatograms, the mean peak areas were calculated and linearity plots of concentration over the mean peak areas were constructed individually. The regressions of the plots were computed by least square regression method. Linearity results were presented in Table 1 to 3 and linearity plots are shown in Fig. 8 to 10. In linearity, working concentration mentioned in experimental condition i.e., 12.0 mg/mL for Efavirenz, 6.0 mg/mL for Tenofovir and 4.0 mg/mL for Emtricitabine and respectively.

Table 1: Linearity study of Efavirenz

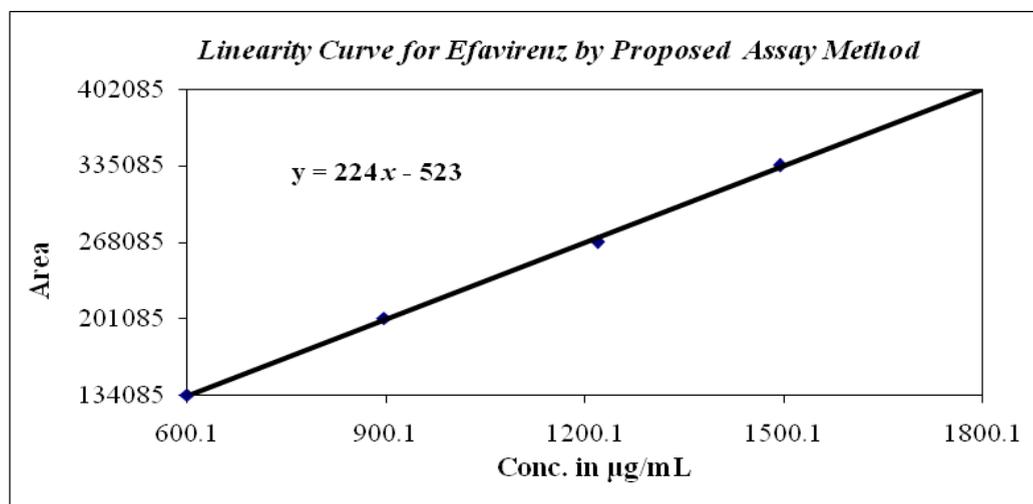
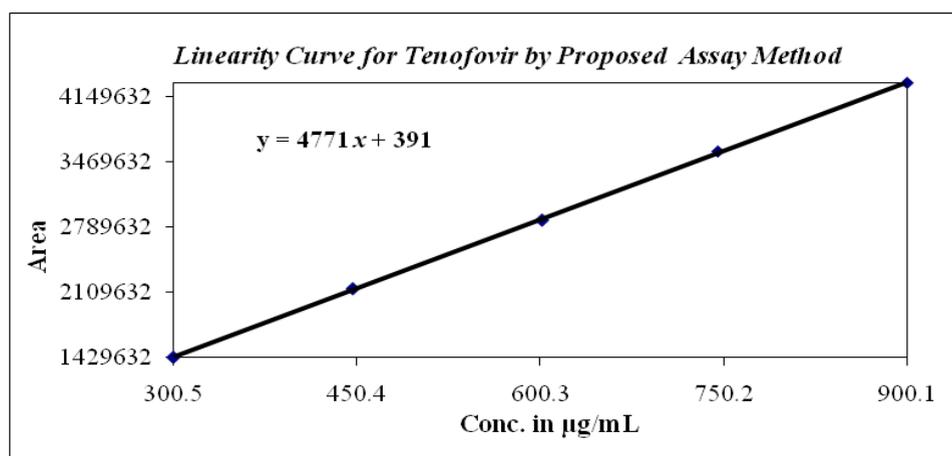
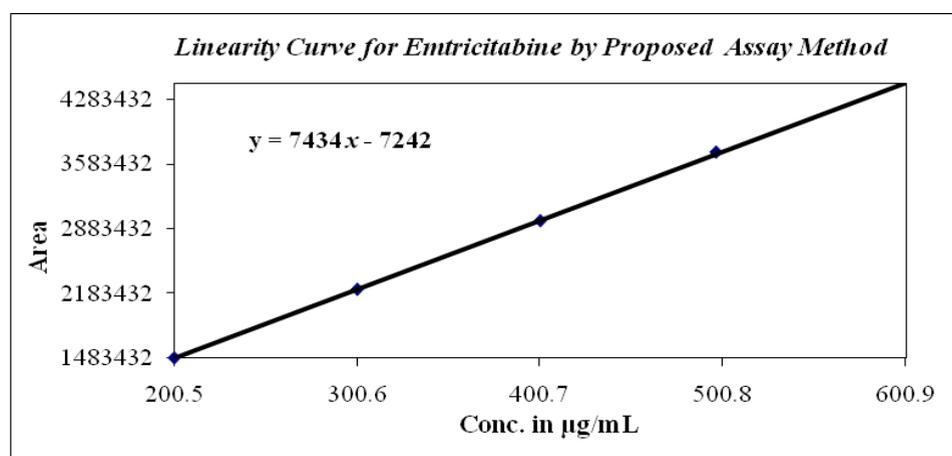
Level	Concentration of Efavirenz ($\mu\text{g/mL}$)	Mean peak area
Level-1	600.13	134085
Level-2	896.61	201127
Level-3	1219.39	268169
Level-4	1494.35	335211
Level-5	1800.39	402254
	Slope	224.0
	Intercept	-532.0
	Correlation Coefficient	0.9998
	Residual Sum of Squares	2567.0

Table 2: Linearity study of Tenofovir

Level	Concentration of Tenofovir ($\mu\text{g/mL}$)	Mean peak area
Level-1	300.54	1429632
Level-2	447.24	2144448
Level-3	602.28	2859264
Level-4	745.40	3574080
Level-5	900.44	4288896
	Slope	4771.0
	Intercept	391.0
	Correlation Coefficient	0.9999
	Residual Sum of Squares	15270.0

Table 3: Linearity study of Emtricitabine

Level	Concentration of Emtricitabine ($\mu\text{g/mL}$)	Mean peak area
Level-1	200.46	1483432
Level-2	300.69	2225147
Level-3	400.92	2966863
Level-4	497.14	3708579
Level-5	601.38	4452959
	Slope	7434.0
	Intercept	-7242.0
	Correlation Coefficient	0.9999
	Residual Sum of Squares	13678.0

**Fig. 8: Linearity plot of Efavirenz****Fig. 9: Linearity plot of Tenofovir****Fig. 10: Linearity plot of Emtricitabine**

Precision

Precision is the degree of repeatability of an analytical method under normal operational conditions. Precision of the method was performed as system precision, method precision and intermediate precision.

System precision

To study the system precision, five replicate mixed standard solutions of Efavirenz, Tenofovir and Emtricitabine were injected. The percent relative standard deviation (% RSD) was calculated and it was found to be 1.0 for Efavirenz, 0.9 for Tenofovir and 0.8 for Emtricitabine respectively, which are well within the acceptable criteria of not more than 2.0. Results of system precision studies are shown in Table 4.

Table 4: System precision

Injection number	Area of Efavirenz	Area of Tenofovir	Area of Emtricitabine
1	2652862	2818609	2954303
2	2638423	2818867	2975799
3	2601519	2775681	2922770
4	2614025	2782784	293410
5	2661929	2829090	2976218
Mean	2633752	2805006	2952638
%RSD	1.0	0.9	0.8

Method precision

The method precision study was carried out on six preparations from the same tablet samples of for Efavirenz, Tenofovir and Emtricitabine and percent amount of both were calculated. The %RSD of the assay result of six preparations in method precision study was found to be 0.8 for Efavirenz, 0.8 for Tenofovir and 0.7 for Emtricitabine respectively, which are well within the acceptance criteria of not more than 2.0. The results obtained for assay of Efavirenz, Tenofovir and Emtricitabine are presented in Table 5.

Table 5: Method precision

Sample number	% Assay		
	Efavirenz	Tenofovir	Emtricitabine
1	100.2	99.9	100.3
2	99.5	99.7	100.9
3	99.3	99.4	100.3
4	98.6	98.5	99.6
5	100.9	100.6	101.4
6	100.4	100.4	101.2
Mean	99.8	99.7	100.6
%RSD	0.8	0.8	0.7

Intermediate precision

The intermediate precision study was carried out by different analysts, different columns, different reagents using different HPLC systems from the same tablet of Efavirenz, Tenofovir and Emtricitabine and the percent amount for Efavirenz, Tenofovir and Emtricitabine

was calculated. The %RSD of the assay result of six preparations in intermediate precision study was 0.5 for Efavirenz, 0.4 for Tenofovir and 0.4 for Emtricitabine respectively, which are well within the acceptance criteria of not more than 2.0. The results of intermediate precision study are reported in Table 6 to 8.

Table 6: Intermediate precision study of Efavirenz

Preparation number	% Assay	Mean	%RSD
1	100.6	100.0	0.5
2	99.8		
3	99.6		
4	99.4		
5	100.4		
6	100.1		

Table 7: Intermediate precision study of Tenofovir

Preparation number	% Assay	Mean	%RSD
1	99.8	99.8	0.4
2	100.1		
3	99.6		
4	99.2		
5	100.1		
6	100.3		

Table 8: Intermediate precision study of Emtricitabine

Preparation number	% Assay	Mean	%RSD
1	100.4	100.8	0.4
2	101.2		
3	100.5		
4	100.3		
5	100.9		
6	101.3		

Accuracy

The accuracy of the method was determined by standard addition method. A known amount of standard drug was added to the fixed amount of pre-analyzed tablet solution. Percent recovery was calculated by comparing the area before and after the addition of the standard drug. The standard addition method was performed at three concentration levels of 50%, 100% and 150%. The solutions were analyzed in triplicate at each level as per the proposed method. The percent recovery and %RSD at each level was calculated and results are presented in Table 9 to 11. Satisfactory recoveries ranging from 98.2 to 101.9 for Efavirenz, 99.7 to 101.9 for Tenofovir and 98.7 to 101.6 for Emtricitabine respectively were obtained by the proposed method. This indicates that the proposed method was accurate.

Table 9: Recovery study for Efavirenz

Level	Amount of Efavirenz spiked (µg)	Amount of Efavirenz recovered (µg)	% Recovery	%RSD
50%	601.23	601.83	100.1	0.9
	600.85	590.03	98.2	
	598.65	600.68	100.3	
	602.36	593.58	98.5	
	597.66	594.80	99.5	
	600.01	601.72	100.3	
100%	1195.25	1195.68	100.0	0.9
	1196.69	1213.03	101.4	
	1189.49	1211.73	101.9	
150%	1781.77	1765.04	99.1	0.7
	1794.43	1763.60	98.3	
	1790.79	1796.22	100.3	
	1802.62	1777.92	98.6	
	1818.15	1799.01	98.9	
	1804.97	1792.25	99.3	
Mean % recovery				99.7
Overall %RSD				1.1

Table 10: Recovery study for Tenofovir

Level	Amount of Tenofovir spiked (µg)	Amount of Tenofovir recovered (µg)	% Recovery	%RSD
50%	300.31	304.83	101.5	1.0
	300.12	299.22	99.7	
	299.02	304.83	101.9	
	300.88	300.09	99.7	
	298.53	301.57	101.0	
	299.71	305.36	101.9	
100%	597.03	595.61	99.8	0.9
	597.75	605.70	101.3	
	594.15	592.69	99.8	
150%	889.99	896.79	100.8	0.3
	896.32	895.66	99.9	
	894.50	895.50	100.1	
	900.40	902.93	100.3	
	908.17	906.88	99.9	
	901.58	903.43	100.2	
Mean % recovery				100.5
Overall %RSD				0.8

Table 11: Recovery study for Emtricitabine

Level	Amount of Emtricitabine spiked (µg)	Amount of Emtricitabine Recovered (µg)	% Recovery	%RSD
50%	202.01	204.25	101.1	0.9
	201.89	200.83	99.5	
	201.15	204.27	101.6	
	202.40	202.40	100.0	
	200.82	204.11	101.6	
	201.61	203.04	100.7	
100%	401.61	401.62	100.0	0.6
	402.09	405.90	100.9	
	399.67	398.94	99.8	
150%	598.68	601.14	100.4	0.6
	602.94	600.84	99.7	
	601.72	599.59	99.6	
	605.69	600.16	99.1	
	610.91	602.79	98.7	
	606.48	601.93	99.2	
Mean % recovery				100.1
Overall %RSD				0.9

Robustness

The robustness study was performed by slight modification in flow rate of the mobile phase, pH of the buffer and composition of the mobile phase. The samples of Efavirenz at 1219.39 µg/mL, Tenofovir at 602.28 µg/mL and Emtricitabine at 400.92 µg/mL concentration were analyzed under these changed experimental conditions. It was observed that there were no marked changes in chromatograms, which demonstrated that the developed method was robust in nature.

Table 12: System suitability for Efavirenz

Parameter	Tailing factor	Theoretical plates
Specificity study	1.46	2689
Linearity study	1.52	2625
Precision study	1.55	2637
Robustness study		
Flow rate at 0.8 mL/min	1.45	2634
Flow rate at 1.2 mL/min	1.52	2680
pH of buffer 2.2	1.55	2647
pH of buffer 2.6	1.62	2689
Mobile phase:		
Buffer(68):Acetonitrile(32)	1.58	2724
Buffer(72):Acetonitrile(28)	1.63	2645

System suitability

System suitability was studied under each validation parameters by injecting six replicates of the standard solution. The system suitability parameters are given in Table 12 to 14.

Table 13: System suitability for Tenofovir

Parameter	Tailing factor	Theoretical plates
Specificity study	1.75	2565
Linearity study	1.74	2606
Precision study	1.77	2604
Robustness study		
Flow rate at 0.8 mL/min	1.75	2654
Flow rate at 1.2 mL/min	1.64	2561
pH of buffer 2.2	1.77	2559
pH of buffer 2.6	1.66	2520
Mobile phase:		
Buffer(68):Acetonitrile(32)	1.62	2678
Buffer(72):Acetonitrile(28)	1.72	2765

Table 14: System suitability for Emtricitabine

Parameter	Tailing factor	Theoretical plates
Specificity study	1.48	4712
Linearity study	1.49	4821
Precision study	1.49	4817
Robustness study		
Flow rate at 0.8 mL/min	1.52	4722
Flow rate at 1.2 mL/min	1.48	4716
pH of buffer 2.2	1.47	4765
pH of buffer 2.6	1.45	4742
Mobile phase:		
Buffer(68):Acetonitrile(32)	1.53	4857
Buffer(72):Acetonitrile(28)	1.52	4756

Limit of detection and Limit of quantification

Limit of detection (LOD) is defined as the lowest concentration of analyte that gives a detectable response. Limit of quantification (LOQ) is defined as the lowest concentration that can be quantified reliably with a specified level of accuracy and precision. For this study six replicates of the analyte at lowest concentration were measured and quantified. The LOD and LOQ of Efavirenz, Tenofovir and Emtricitabine are given in Table 15 to 17.

Table 15: LOD and LOQ of Efavirenz

Parameter	Measured value ($\mu\text{g/mL}$)
Limit of detection	37.82
Limit of quantification	114.60

Table 16: LOD and LOQ of Tenofovir

Parameter	Measured value ($\mu\text{g/mL}$)
Limit of detection	10.56
Limit of quantification	32.01

Table 17: LOD and LOQ of Emtricitabine

Parameter	Measured value ($\mu\text{g/mL}$)
Limit of detection	6.07
Limit of quantification	18.40

Stability studies

In order to demonstrate the stability of both standard and sample solutions during analysis, both solutions were analyzed over a period of 24 hours at room temperature. The results show that for both solutions, the retention time and peak area of Efavirenz, Tenofovir and Emtricitabine remained almost similar (%RSD less than 2.0) and no significant degradation within the indicated period, thus indicated that both solutions were stable for at least 24 hours, which was sufficient to complete the whole analytical process. Further forced degradation studies were conducted indicating the stability of proposed method. The results of the degradation studies are shown in the Table 18.

Table 18: Forced degradation study results for Efavirenz, Tenofovir and Emtricitabine

Stress Conditions	Degradation Time (Hrs)	Efavirenz		Tenofovir		Emtricitabine	
		%Assay	%Deg.	%Assay	%Deg.	%Assay	%Deg.
Control	--	99.8	--	99.7	--	100.6	--
Acid	1	76.6	-23.2	82.2	-17.5	90.7	-9.9
Base	1	78.0	-21.8	94.4	-5.3	94.5	-6.1
Peroxide	1	77.5	-22.3	77.0	-22.7	76.1	-24.5
Thermal	48	91.5	-8.3	96.3	-5.9	96.3	-4.3

Control sample

Twenty tablets were weighed and finely powdered. An accurately weighed portion of powder sample equivalent to 30 mg of Efavirenz, 15 mg of Tenofovir and 10 mg of Emtricitabine was transferred to a 100 mL volumetric flask containing 60 mL of diluent. The contents of the flask were sonicated for about 30 min for complete solubility of the drug and volume made up with further quantity of diluent. Filter 20 mL of the above solution through 0.45 μ membrane filter, transfer 5 mL of the filtered solution into 25 mL volumetric flask and volume was made upto with mobile phase.

Acid degradation sample

Twenty tablets were weighed and finely powdered. An accurately weighed portion of powder sample equivalent to 30 mg of Efavirenz, 15 mg of Tenofovir and 10 mg of Emtricitabine was transferred to a 100 mL volumetric flask containing 60 mL of diluent. The contents of the flask were sonicated for about 30 min for complete solubility of the drug with intermittent shaking at controlled temperature. Then 5 mL of 5N acid (Hydrochloric acid) was added, refluxed for 60 minutes at 60°C, then cooled to room temperature, neutralized with 5N base (Sodium hydroxide) and diluted to volume with diluent. About 25 mL of the above sample solution was filtered through 0.45 μ membrane filter. Pipetted 2 mL of the above filtered sample solution into a 10 mL volumetric flask and diluted to volume with diluent. Typical chromatogram of acid degradation for Efavirenz, Tenofovir and Emtricitabine is shown in Fig. 11.

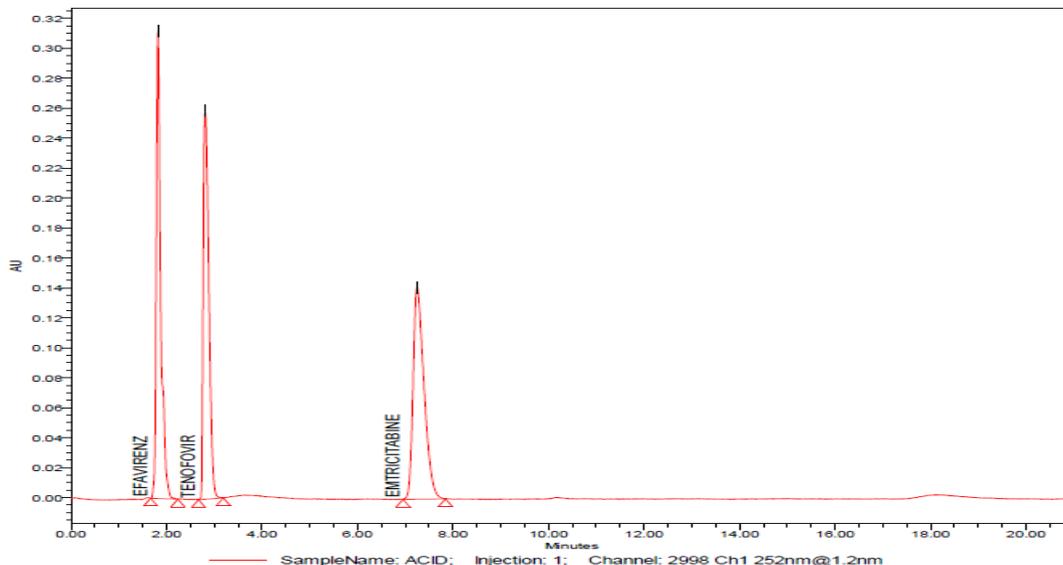


Figure 11: Chromatogram of acid degradation showing Efavirenz, Tenofovir and Emtricitabine

Base degradation sample

Twenty tablets were weighed and finely powdered. An accurately weighed portion of powder sample equivalent to 30 mg of Efavirenz, 15 mg of Tenofovir and 10 mg of Emtricitabine was transferred to a 100 mL volumetric flask containing 60 mL of diluent. The contents of the flask were sonicated for about 30 min for complete solubility of the drug with intermittent shaking at controlled temperature. Then 5 mL of 5N base (Sodium hydroxide) was added, refluxed for 60 minutes at 60°C, then cooled to room temperature, neutralized with 5N acid (Hydrochloric acid) and diluted to volume with diluent. About 25 mL of the above sample solution was filtered through 0.45 μ membrane filter. Pipetted 2 mL of the above filtered sample solution into a 10 mL volumetric flask and diluted to volume with diluent. Typical chromatogram of base degradation for Efavirenz, Tenofovir and Emtricitabine is shown in Fig. 12.

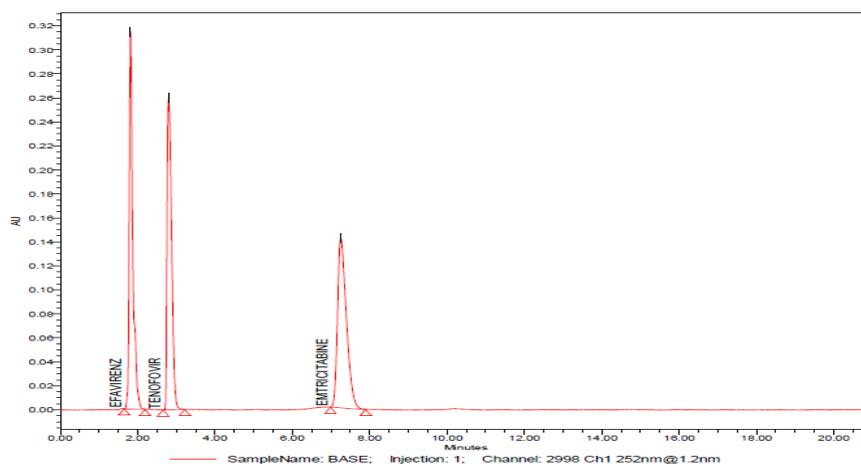


Figure 12: Chromatogram of base degradation showing Efavirenz, Tenofovir and Emtricitabine

Peroxide degradation sample

Twenty tablets were weighed and finely powdered. An accurately weighed portion of powder sample equivalent to 30 mg of Efavirenz, 15 mg of Tenofovir and 10 mg of Emtricitabine was transferred to a 100 mL volumetric flask containing 60 mL of diluent. The contents of the flask were sonicated for about 30 min for complete solubility of the drug with intermittent shaking at controlled temperature. Then 2 mL of 30% peroxide was added, refluxed for 60 minutes at 60°C, then cooled to room temperature and diluted to volume with diluent. About 25 mL of the above sample solution was filtered through 0.45 μ membrane filter. Pipetted 2 mL of the above filtered sample solution into a 10 mL volumetric flask and diluted to volume with diluent. Typical chromatogram of peroxide degradation for Efavirenz, Tenofovir and Emtricitabine is shown in Fig. 13.

Thermal degradation sample

Twenty tablets were weighed and finely powdered. The powder is exposed to heat at 105°C for about 2 days. An accurately weighed portion of powder sample equivalent to 30 mg of Efavirenz, 15 mg of Tenofovir and 10 mg of Emtricitabine was transferred to a 100 mL volumetric flask containing 60 mL of diluent.

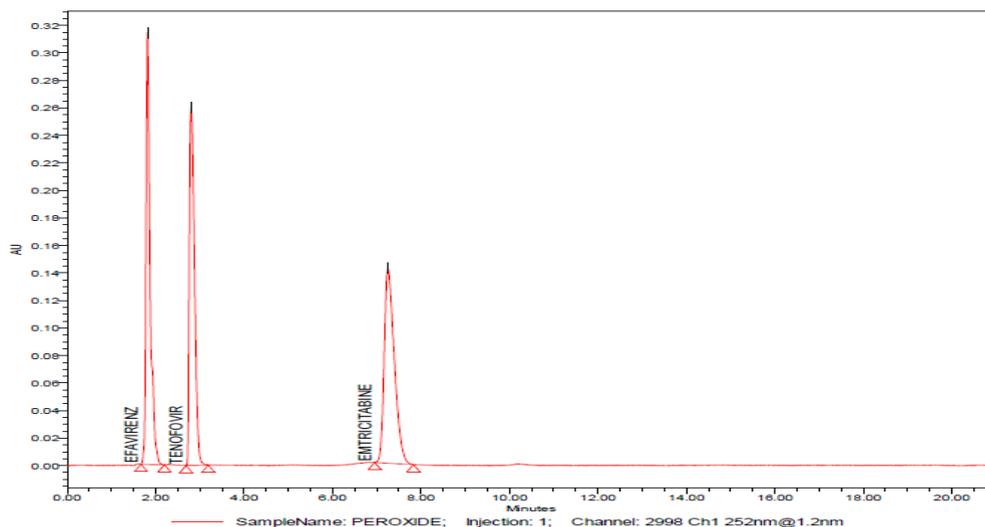


Figure 13: Chromatogram of oxidative degradation showing Efavirenz, Tenofovir and Emtricitabine

The contents of the flask were sonicated for about 30 min for complete solubility of the drug with intermittent shaking at controlled temperature and then cooled the solution to room temperature and volume made up with further quantity of diluent. Then this mixture was filtered through 0.45 μ membrane filter. 2.0 mL of this filtrate was further diluted to 10 mL with diluent. Typical chromatogram of thermal degradation for Efavirenz, Tenofovir and Emtricitabine is shown in Fig. 14.

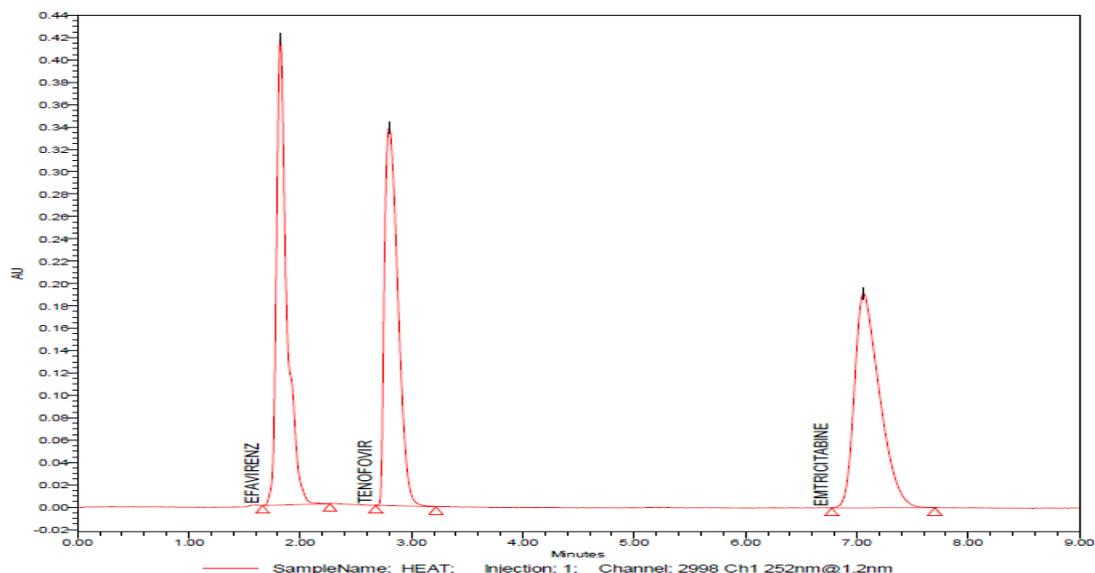


Figure 14: Chromatogram of thermal degradation showing Efavirenz, Tenofovir and Emtricitabine

Results and Discussion

The present study was aimed at developing a simple, sensitive, precise and accurate HPLC method for the simultaneous estimation of Efavirenz, Tenofovir and Emtricitabine from bulk samples and their tablet dosage forms. A non-polar C8 analytical chromatographic column was chosen as the stationary phase for the separation and simultaneous determination of Efavirenz, Tenofovir and Emtricitabine. Mixtures of commonly used solvents like water, methanol and acetonitrile with or without buffers in different combinations were tested as mobile phases. The choice of the optimum composition is based on the chromatographic response factor, a good peak shape with minimum tailing. A mixture of dilute orthophosphoric acid solution pH 2.4 ± 0.02 as buffer and acetonitrile in proportion of 70:30 v/v was proved to be the most suitable of all the combinations since the chromatographic peaks obtained were better defined and resolved and almost free from tailing. The retention times of the Efavirenz, Tenofovir and Emtricitabine were found to be 1.81 min for Efavirenz, 2.80 min for Tenofovir and 7.30 min for Emtricitabine respectively.

The linearity was found satisfactory for all the three drugs in the range of 600.13 to 1800.39 $\mu\text{g/mL}$ for Efavirenz, 300.54 to 900.44 $\mu\text{g/mL}$ for Tenofovir and 200.46 to 601.38 $\mu\text{g/mL}$ for Emtricitabine respectively. The regression equation of the linearity curve between concentrations of Efavirenz, Tenofovir and Emtricitabine over its peak areas were found to be $Y=224X-532.0$ (where Y is the peak area and X is the concentration of Efavirenz in $\mu\text{g/mL}$), $Y=4771X+391$ (where Y is the peak area and X is the concentration of Tenofovir in $\mu\text{g/mL}$) and $Y=7434X-7242$ (where Y is the peak area and X is the concentration of Emtricitabine in $\mu\text{g/mL}$) respectively. Precision of the method was studied by repeated injection of tablet solution and results showed lower %RSD values. This reveals that the method is quite precise. The percent recoveries of the drug solutions were studied at three different concentration levels. The percent individual recovery and the %RSD at each level were within the acceptable limits. This indicates that the method is accurate. The absence of additional peaks in the chromatogram indicates non-interference of the commonly used excipients in the tablets and hence the method is specific.

The deliberate changes in the method have not much affected the peak tailing, theoretical plates and the percent assay. This indicates that the present method is robust. The

system suitability studies were carried out to check various parameters such as theoretical plates and tailing factor. The lowest values of LOD and LOQ as obtained by the proposed method indicate that the method is sensitive. The solution stability studies indicate that both the drugs were stable up to 24 hours. The forced degradation studies indicate that all the three drugs Efavirenz, Tenofovir and Emtricitabine were stable in stability studies.

Conclusion

The proposed stability-indicating RP-HPLC method was simple, specific, sensitive, accurate and precise and can be used for simultaneous analysis of Efavirenz, Tenofovir and Emtricitabine in bulk samples and its tablet dosage forms.

References

1. Indian Pharmacopoeia, Govt. of India, Ministry of Health & Family Welfare, The Indian Pharmacopoeia Commission, Ghaziabad, 2010; 3: 2188.
2. The Merck Index, 14th Edn., Merck & Co., Inc., Whitehouse Station, NJ, USA, 2006; 598. Indian Pharmacopoeia, Govt. of India, Ministry of Health & Family Welfare, The Indian Pharmacopoeia Commission, Ghaziabad, 2010; 2: 1269.
3. The Merck Index, 14th Edn., Merck & Co., Inc., Whitehouse Station, NJ, USA, 2006; 1573.
4. The Merck Index, 14th Edn., Merck & Co., Inc., Whitehouse Station, NJ, USA, 2006; 607. Indian Pharmacopoeia, Govt. of India, Ministry of Health & Family Welfare, The Indian [1] Pharmacopoeia Commission, Ghaziabad, 2010; 2: 1272.
5. The Merck Index, 14th Edn., Merck & Co., Inc., Whitehouse Station, NJ, USA, 2006; 607. Raju NA, Rao JV, Prakash KV, Mukkanti K, Srinivasu K: Simultaneous estimation of Tenofovir disoproxil, Emtricitabine and Efavirenz in tablet dosage form by RP-HPLC: Oriental J. Chem. 2008; 24(2): 645-650.
6. The Merck Index, 14th Edn., Merck & Co., Inc., Whitehouse Station, NJ, USA, 2006; 607. Raju NA, Begum S: Simultaneous RP-HPLC method for the estimation of the Emtricitabine, Tenofovir disoproxil fumarate and Efavirenz in tablet dosage forms: Research J. Pharm. Tech. 2008; 1(4): 522-525.
7. The Merck Index, 14th Edn., Merck & Co., Inc., Whitehouse Station, NJ, USA, 2006; 607. Rao VPVSK, Kumar KK, Raveendranath TV, Madhuri PL, Sasikanth K: RP-HPLC method for the simultaneous estimation of Emtricitabine, Tenofovir and Efavirenz in pharmaceutical dosage form: J. Global Trends Pharm. Sci. 2011; 2(2):177-186.
8. The Merck Index, 14th Edn., Merck & Co., Inc., Whitehouse Station, NJ, USA, 2006; 607.
9. The Merck Index, 14th Edn., Merck & Co., Inc., Whitehouse Station, NJ, USA, 2006; 607. ICH Harmonised Tripartite Guideline. Validation of Analytical Procedures: Text and Methodology, Q2 (R1), USA, 2005; 1-13.
10. The Merck Index, 14th Edn., Merck & Co., Inc., Whitehouse Station, NJ, USA, 2006; 607. ICH Harmonised Tripartite Guideline. Stability Testing of New Drug Substances and Products, Q1A (R2), USA, 2003; 1-18.
11. Prashant SD, Roshan B, Nalini S, and Surendranath KV: Validated stability indicating RP-HPLC method for simultaneous determination of Tenofovir, Emtricitabine and Efavirenz and statistical approach to determine effect of variables: ISRN Chromatogr. 2013; 1-14.