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R E S E A R C H A R T I C L E

A Novel Validated Stability Indacating Method for Simultaneous Estimation of Emtricitabine, Tenofovir and Efavirenz in Tablet Dosage Form by RP-HPLC

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A B S T R A C T

The objective of the current study was to develop and validate a rapid, precise, specific and stability-indicating reverse phase HPLC method for the quantitative determination the of three anti retroviral drugs emtricitabine (200mg), tenofovir (300mg), efavirenz (600 mg) used to treat HIV patients in its combined dosage form. The determination is done for the active pharmaceutical ingredient in its pharmaceutical dosage form in the presence of degradation products. The drug was subjected to stress conditions of acid, alkali, thermal, photolytic, humidity and peroxide. As per international conference on harmonization (ICH) prescribed stress conditions to show the stability-indicating power of the method. All the three drug solutions were scanned from 200-400 nm; it was observed that all the drugs show appreciable absorbance at 270nm. Hence detection was set at 270 nm for method development purpose. Attempts were made to get good separation between all the drugs by varying parameters like, flow rate, pH, buffer molarity, buffer components, type of organic modifier, gradient times, and buffer: organic modifier ratio but could not reduce the elution time of all the three in isocratic mode. To achieve this, experiments were conducted by changing the columns and mobile shares but unsuccessful in getting good peaks with less run time. Then method was optimized to separate all the three main peaks by changing to Gradient mode. The satisfactory chromatographic separation, with good peak shapes were achieved on Symmetry C18 (4.6 x 150mm, 3.5µm, Make: XTerra) or equivalent with mobile phase potassium di hydrogen sulphate : Methanol and linear gradient programming Time (min)/buffer% 0/30, 5/30, 6/70,12/70,13/30,14/30 with a flow rate of 1.0 ml/min. Several gradient conditions were tried before optimizing the final linear gradient programme. All the System Suitability parameters are within the acceptance limits. The calibration curves for emtricitabine, tenofovir and efavirenz were obtained by plotting the respective peak areas against their concentration. The graphs were found to be linear over the range 7.5-45.0µg/ml for emtricitabine,11.25-67.50µg/ml for tenofovir and 22.5-135.0µg/ml for efavirenz with the correlation coefficient 0.999,0.999 and 0.999 respectively for all the drugs which shows that the good correlation exists between peak areas and concentration of the drug. The low % RSD of intraday and inter day study show that the method is precise. The high % recovery values obtained for these drugs show that the method is accurate. The LOD values of emtricitabine, tenofovir and efavirenz were found to be 0.018µg/ml, 0.81µg/ml and 5.05µg/ml respectively. The LOQ was 0.060µg/ml, 0.252µg/ml and 0.162µg/ml for emtricitabine, tenofovir and efavirenz respectively. The low values of LOD and LOQ show that the method is sensitive and can estimate at micro gram level. The absence of additional peaks indicates the method is specific and the drugs were stable in the diluents for 8 hours which is sufficient to complete the work.

Keywords: Atripla (drugs emtricitabine (200mg), tenofovir (300mg), efavirenz (600 mg)), Forced degradation, Assay, Method Validation, RP-HPLC

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C O N T E N T S

1. Introduction

ATRIPLA is a fixed dose combination of three anti retroviral drugs emtricitabine (200mg), tenofovir (300mg), efavirenz (600 mg) used to treat HIV patients. These combinations are convenient to administer and may lead to better patient compliance. A tablet containing number of active ingredients is always a better dosage form which may have quick onset of action and extended therapeutic window. This type of dosage form might result in better patient compliance and also will be less in case of cost. Foreseeing the need of different analytical methods for the estimation of the ingredients of ATRIPLA, the ultimate goal of the work was to develop single HPLC method selective for three main components of ATRIPLA. Developing a single analytical method for the estimation of individual drugs in ATRIPLA is very challenging due to drug-drug and drug-excipients interaction. Extensive literature survey did not reveal any simple, sensitive analytical method for the simultaneous estimation and determination of all the three drugs in ATRIPLA. Here is an attempt to develop a new, sensitive, HPLC method for the simultaneous quantitative determination of emtricitabine, tenofovir and efavirenz. The list of marketed products is given in the table no 1.

ATRIPLA[1] is a combination of ANTI-HIV drugs. These oral administrative dosage forms are always convenient and lead to better compliance. This combination is beneficial and better compliance in terms of cost and therapeutic categorization. The CAS NUMBERS: 143491-57-0 , 202138-50-9 and 154598-52-4 respectively, chemically they are 4-amino-5-fluoro-1-[(2S,5R)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one, ({[(2R)-1-(6-amino-9*H*-purin-9-yl)propan-2-yl]oxy} methyl) phosphonic acid, (4S)-6-chloro-4-(2-cyclopropylethynyl)-4-(trifluoro methyl)-2, 4-dihydro-1H-3, 1-benzoxazin-2-one[2]. There are several research publications for determination of ATRIPLA. A reverse phase HPLC method was developed for simultaneous stability indicating estimation of emtricitabine, tenofovirdisoproxilfumerate and efavirenz in tablet dosage form[3]. Another novel rapid, sensitive and reproducible high performance liquid chromatographic method for quantitative determination of efavirenz,, lamivudine and tenofovirdisoproxil Fumerate in active pharmaceutical ingredients and its dosage forms[4]. A validated a simple, rapid reversed-phase high performance liquid chromatographic method for estimation of emtricitabine and tenofovirdisoproxilfumarate in tablet dosage form[5]is also available[5]. A validated a simple, accurate, precise and rapid high performance thin layer chromatographic method for the estimation of Tenofovir in tablet dosage forms[6]. Another interesting simple, rapid and precise method for the estimation of tenofovirdisoproxil fumarate (TDF) in pharmaceutical dosage form was developed[7].Development and validated a simple, precise, accurate and rapid high performance thin layer chromatographic method for the estimation of emtricitabine and tenofovir simultaneously in combined dosage form[8].Developmentof a new simple RP-HPLC method the for simultaneous estimation of emtricitabine, tenofovir and efavirenz[9].

2. Materials and Methods

Instrumentation Instrumention

Instrumentation:

Waters LC system equipped with 2695 pump and 2996 photodiode array detector was used. The output signals were monitored and integrated using waters Empower 2.0 software. Analytical balance (Model:AB 204S, Make: Mettle Toledo) and Micro balance(Model: XP 6, Make: Mettle Toledo) were used for weighing. Systronics digital pH meter 361 was used to adjust the pH of the buffer. Degassing of the mobile phase was done by sonication using Spinco Biotech Ultra Sonicator). Filtration was done by using Millipore vaccum filter**.**

Drugs and chemicals:

Pure standards of tenofovir, efavirenz, emtricitabine standards were kindly gifted from Hetero drugs Ltd., Hyderabad, India. The HPLC grade methanol, potassium di-hydrogen phosphate, ortho phosphoric acid were purchased from Merck.

Preparation of Solutions

Preparation of Mobile phase

Preparation of solutions:

Preparations of buffer:

Weighed about 7.0 gms of KH_2PO_4 into a 1000ml beaker and dissolved and diluted to 1000ml with milli-Q water. Adjusted the pH to 3.5 with Ortho phosphoric acid. And filtered through 0.45µm membrane filter.

Preparation of Mobile phase:

Mobile phase A: Methanol

Mobile phase B: pH 3.5 buffer

Preparation of diluent:

Methanol and buffer were mixed in the ratio 70:30 v/v and sonicated for 10 minutes.

Preparation of solutions for peak identification:

Preparation of Tenofovir standard solution for peak identification:

Weighed accurately 10 mg of tenofovir standard into a 25ml volumetric flask and added about 10ml of diluent, sonicated for 10 minutes to dissolve and diluted upto the mark with diluent.

Preparation of Emtricitabine standard solution for peak identification:

Weighed accurately 10mg of emtricitabine standard into a 25ml volumetric flask and added about 10ml of diluent, sonicated for 10 minutes to dissolve and diluted upto the mark with diluent.

Preparation of Efavirenz standard solution for peak identification: Weighed accurately 10mg of efavirenz standard into a 25ml volumetric flask and added about 10ml of diluent, sonicated for 10 minutes to dissolve and diluted upto the mark with diluent.

Preparation of standard solution:

Accurately weighed and transferred 200 mg of emtricitabine and 300mg of tenofovirand 600mg of efavirenz working standard into a 100ml clean dry volumetric flask, added about 70ml of diluent and sonicated to dissolve it completely and the volume is made up to the mark with the same diluent. Further 1.5ml of the above stock solution was transferred into a 100ml volumetric flask and dilute up to the mark with diluent.

Preparation of placebo solution:

Weighed accurately 362.9 mg of placebo powder into 100ml volumetric flask, added 30ml of the diluent and sonicated for 20min and diluted to the volume with diluents, filtered through 0.45µm. Further diluted 1.5 ml of this solution to 100ml with the diluent.

Test preparation:

Accurately weighed and finely powdered 20 tablets of ATRIPLA and transferred an amount of the powder equivalent to 200mg of emtricitabine into a 100ml of volumetric flask, added 30ml of the diluent and sonicated for 20min and diluted to the volume with diluent, filtered through 0.45µm filter. Further diluted 1.5ml of this solution to 100ml with diluent.

Optimized chromatographic conditions:

After systematic and detailed study of the various parameters involved in the method, the following conditions were employed.

Mobile phase: Gradient programmed was employed and given in the Table No.2

Column : Symmetry C18 (4.6 x 150mm, 3.5µm, Make: XTerra) or equivalent

Flow rate : 1.0 ml per min

Wavelength : 258 nm

Injection volume : 20 µL

Column oven Temperature: Ambient

Run time : 15min.

Procedure :

Column was equilibrated for at least 60 minutes with the mobile phase flowing through the system at a rate of 1.0ml/min. Detector was set at a wavelength of 258nm. Separately injected 20µL of diluent, placebo, peak identification solutions, standard solution, test solutions into the chromatograph and the chromatograms were recorded. The percent assay values of the tenofovir, efavirenz, and emtricitabine were calculated by using the following formulae. The representative model chromatograms of all the solutions are given below.

$$\%Assay = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{Avg.\ Wt}{Label\ Claim} \times 100$$

Where:

AT = Peak Area obtained with test preparation

AS = Peak Area obtained with standard preparation

WS = Weight of working standard taken in mg

WT = Weight of sample taken in mg

DS = Dilution of Standard solution

DT = Dilution of sample solution

P = Percentage purity of working standard.

Analytical Method Validation System suitability:

According to the USP 33 System suitability is the integral part of the chromatographic method. This test was conducted to verify that the reproducibility and effectiveness of the system is adequate for the analysis. To ascertain its effectiveness 20µL of freshly prepared standard solution containing 30µg/ml of emtricitabine, 45µg/ml of tenofovir and 90µg/ml of efavirenz was injected 6 times into the HPLC system by using optimized chromatographic conditions and System suitability results were calculated. The %RSD for the peak areas and retention times of the three drugs were found to be less than 2.0%. The theoretical plates were more than 2000 for all the three drugs. Tailing factor was found to be less than 2.0. The resolution between the adjacent peaks was found to be more than 6.0.All the results were tabulated in the tables 3,4 and 5

Specificity:

Blank and placebo interference:

A study to establish the interference of blank and placebo was conducted. Analysis was performed on placebo preparation described previously in triplicate equivalent to about the weight of placebo in portion of test preparation as per test method. Chromatograms of Blank and placebo solutions showed no peaks at the retention times of emtricitabine, tenofovir, efavirenz. This indicates that the

excipients used in the formulation did not interfere in the estimation. The chromatograms of blank and placebo using the proposed method were shown in figures 4.1.1 and 4.1.2.

Interference from degradation products:

Preparation of degradation samples:

Preparation of sample for Acid degradation:

ATRIPLA sample was refluxed with the 1M HCl at 60°C for 1hour and then neutralized with 1N NaOH. The sample was prepared as per the test method and then further diluted upto the required concentration with the diluent.

Preparation of sample for Alkaline degradation:

ATRIPLA sample was refluxed with the 1M NaOH. at 60°C for 1hour and then neutralized with 1M HCl The sample was prepared as per the test method and then further diluted upto the required concentration with the diluent.

Preparation of sample for Oxidative degradation:

ATRIPLA sample was refluxed with the 10%H_2O_2 by heating on water bath at 60°C for 1 hour. The sample was prepared as per the test method and then further diluted upto the required concentration with the diluent.

Preparation of sample for Photolytic degradation:

ATRIPLA sample was exposed to UV (200watt-hr/m^2) and visible (1.2 million lux hrs) The sample was prepared as per the test method and then further diluted upto the required concentration with the diluent.

Preparation of sample for Thermal degradation:

ATRIPLA sample was exposed to temperature at 105°c for 24hrs . The sample was prepared as per the test method and then further diluted upto the required concentration with the diluent.

Preparation of sample for Humidity degradation:

ATRIPLA sample was exposed to 85% humidity for 24hrs. The sample was prepared as per the test method and then further diluted up to the required concentration with the diluent. All the stressed samples were injected into the HPLC system by using optimized chromatographic conditions and the chromatographs were recorded. The chromatograms of the stressed samples were evaluated for peak purity of all the three drugs using PDA detector and Empower software. In all forced degradation samples all the three drugs passed the peak purity (purity angle is less than purity threshold). All the degradant peaks were well separated from the three drugs. Thus the method can be used for simultaneous estimation of tenofovir, efavirenz, and emtricitabine in bulk and pharmaceutical formulations and also the method is stability indicating. The results are given in the Table No's 6,7and 8The chromatograms are given in Figures 1 to 6

Method precision:

Precision of the method was conducted by performing the assay of ATRIPLA tablets 6 times. The samples were prepared six times according to the test preparation mentioned earlier and analyzed by using the test method. The % Assay values were calculated for all the three drugs and found to be in between 98.0% - 102.0%. The %RSD values were found to be less than 2.0%. The results were given in the table no 9

Limit of Detection and Limit of Quantification:

A study to establish the Limit of Detection and Limit of Quantification of tenofovir, efavirenz, emtricitabine was conducted. Limit of detection and Limit and quantification were established based on signal to noise ratio. A series of dilutions of the test solution were injected. Limit of detection was established by identifying the concentration which gives signal to noise ratio of about 3. Limit of Quantification was established by identifying the concentration which gives signal to noise ratio of about 10. The results of the LOQ and LOD are given in the table No. 10

Accuracy:

Accuracy for tenofovir, efavirenz, and emtricitabine was conducted by spiking these three drugs to the placebo powder at three different levels of the target concentration (i.e. 50%, 75%, 100%, 125% and 150%) and each level three times. The mean %Recovery and %RSD values were calculated. The %Recovery values for all the three drugs were found to be between 98.0% to102.0% and %RSD values were found to be less than 2.0%. The accuracy results were tabulated in the table No's 11,12, and 13.

Linearity and range:

Linearity of the detector response was established by plotting a graph of concentration versus peak area. A series of solutions of standard were prepared by appropriate dilutions of Linearity standard stock solution.

Preparation of Linearity stock solution:

Weighed accurately and transferred 25.0mg emtricitabine WS, 37.5mg tenofovir WS, 75.0mg of efavirenz WS into 100ml volumetric flask, added 30 ml diluent of the diluent and sonicated for 20min and diluted to the volume with diluent, filtered through 0.45µm filter.

Preparation of Linearity solutions:

Series of solutions in the range of 25% to 150% of target concentration were prepared by transferring 1.5mL, 3.0mL, 4.5mL, 6.0mL, 7.5mL, 15.0mL of Linearity stock solution into separate 50.0mL Volumetric flasks and making the volume up to the mark with the diluent. The detector response was found to be linear in the range of 7.5to 45.0µg/mL for emtricitabine, 11.25 to67.50µg/mL for tenofovir, 22.5to135.0µg/mL for efavirenz. The correlation coefficient values were found to be within the limits. The linearity and the regression data was tabulated in Tables No's 14,15,16 and 17.

Ruggedness:

A study to establish ruggedness of the method was conducted by preparing and analyzing the standard and test preparation on two different days by two different analysts on two different columns and two different HPLC systems. The system suitability parameters and the % Assay values of all the three drugs were calculated and the differences between the two analysts were evaluated and the method was found to rugged. The results were tabulated in the table no. 18,19& 20

Robustness:

A study to establish the effect of variation in flow rate, column temperature, pH of the buffer in the mobile phase was conducted. Standard and test solutions prepared as per the proposed method and were injected into the HPLC system. The system suitability parameters, and the %Assay values were evaluated and the method was found to be robust. All the results were tabulated in the table no 21

3. Results and Discussion

All the three drug solutions were scanned from 200-400 nm; it was observed that all the drugs show appreciable absorbance at 270nm. Hence detection was set at 270 nm for method development purpose. Attempts were made to get good separation between all the drugs by varying parameters like, flow rate, pH, buffer molarity, buffer components, type of organic modifier, gradient times, and buffer: organic modifier ratio but could not reduce the elution time of all the three in isocratic mode. To achieve this, experiments were conducted by changing the columns and mobile shares but unsuccessful in getting good peaks with less run time. Then method was optimized to separate all the three main peaks by changing to Gradient mode. The satisfactory chromatographic separation, with good peak shapes were achieved on Symmetry C18 (4.6 x 150mm, 3.5µm, Make: XTerra) or equivalent with mobile phase potassium di hydrogen sulphate : Methanol and linear gradient programming Time (min)/buffer% 0/30, 5/30, 6/70,12/70,13/30,14/30 with a flow rate of 1.0 ml/min. Several gradient conditions were tried before optimizing the final linear gradient programme.All the System Suitability parameters are within the acceptance limits. The calibration curves for emtricitabine, tenofovir and efavirenz were obtained by plotting the respective peak areas against their concentration. The graphs were found to be linear over the range 7.5-45.0µg/ml for emtricitabine,11.25-67.50µg/ml for tenofovir and 22.5-135.0µg/ml for efavirenz with the correlation coefficient 0.999,0.999 and 0.999 respectively for all the drugs which shows that the good correlation exists between peak areas and concentration of the drug.

The low % RSD of intraday and inter day study show that the method is precise. The high % recovery values obtained for these drugs show that the method is accurate. The LOD values of emtricitabine, tenofovir and efavirenz were found to be 0.018µg/ml, 0.81µg/ml and 5.05µg/ml respectively. The LOQ was 0.060µg/ml, 0.252µg/ml and 0.162µg/ml for emtricitabine, tenofovir and efavirenz respectively. The low values of LOD and LOQ show that the method is sensitive and can estimate at micro gram level. The absence of additional peaks indicates the method is specific and the drugs were stable in the diluents for 8 hours which is sufficient to complete the work.

Table: 1

Proprietary name	**Company**	**Formulation tablet**		
		Emtricitabine	Tenofovir	Efavirenz
ATRIPLA	Bristol-Myers Squibb	200mg	300mg	600mg

Table: 2 Gradient Program

Time (min)	**Mobile phase(A) %v/v**	**Mobile phase(B) %v/v**
0	70	30
5	70	30
6	30	70
12	30	70
13	70	30
14	70	30

Table: 3 System Suitability for Emtricitabine

S.no	**Retention time**	**Peak area**	**Theoretical plates**	**Tailing**
1	2.255	1759046	5124	1.1
2	2.258	1758962	5425	1.2
3	2.256	1759624	5325	1.1
4	2.261	1758656	6525	1.1
5	2.261	1745698	5264	1.2
6	2.256	1749685	5598	1.4
7	2.254	1746598	6884	1.1
8	2.255	1756321	5855	1.2
9	2.255	1754896	5894	1.1
10	2.254	1752146	5596	1.2
AVERAGE	2.2565	1754163		
SD	0.0026	5323.8		
%RSD	0.12	0.3		

Table: 4 System Suitability for Tenofovir

S.no	Retention time	Peak area	Theoretical plates	Tailing	Resolution
1	4.753	1198316	3124	1.2	6.2
2	4.756	1197891	3425	1.2	6.2
3	4.745	1197562	3325	1.1	6.3
4	4.756	1189562	4525	1.1	6.3
5	4.756	1187569	3264	1.1	6.1
6	4.749	1198562	3598	1.1	6.2
7	4.752	1179856	3884	1.1	6.4
8	4.756	1179995	3855	1.1	6.2
9	4.753	1179865	3894	1.1	6.4
10	4.753	1195625	4596	1.2	6.2
AVERAGE	4.753	1190480			
SD	0.004	8185.8			
%RSD	0.076	0.7			

Table: 5 System Suitability for Efavirenz

S.No	Retention time	Peak area	Theoretical plates	Tailing	Resolution
1	12.046	2550429	7124	1.1	15.1
2	12.043	2551456	7425	1.2	15.0
3	12.046	2545656	7325	1.2	15.1
4	12.051	2550123	8525	1.1	15.0
5	12.048	2549874	7264	1.1	15.0
6	12.046	2549865	7598	1.1	15.1
7	12.047	2547896	7884	1.2	15.1
8	12.048	2550123	7855	1.2	15.2
9	12.045	2511236	7894	1.2	15.0
10	12.049	2549687	8596	1.2	15.0
AVERAGE	12.047	2545635			
SD	0.002	12192			
%RSD	0.02	0.48			

Table: 6 Degradation Results of Emtricitabine

Stress Condition	Purity angle	Purity threshold	% Assay	% Degradation
Acid degradation	0.26	0.29	92.6	7.7
Alkali degradation	0.32	0.36	90.6	9.7
Thermal degradation	0.29	0.36	87.6	12.7
Humidity degradation	0.27	0.37	95.7	4.6
Photolytic degradation	0.24	0.25	97.9	2.4
Peroxide degradation	0.23	0.29	85.7	14.7

Table: 7 Degradation results of Tenofovir

Stress condition	Purity angle	Purity threshold	% assay	Degradation
Acid degradation	0.16	0.21	92.3	7.6
Alkali degradation	0.21	0.28	90.4	9.5
Thermal degradation	0.35	0.38	87.4	12.5
Humidity degradation	0.19	0.22	95.3	4.6
Photolytic degradation	0.18	0.29	97.7	2.2
Peroxide degradation	0.21	0.38	85.4	14.7

Table: 8 Degradation Results of Efavirenz

Stress condition	Purity angle	Purity threshold	% assay	Degradation
Acid degradation	0.53	0.59	92.8	7.7
Alkali degradation	0.41	0.59	90.8	9.7
Thermal degradation	0.36	0.41	87.8	12.7

Humidity degradation	0.40	0.55	95.1	5.4
Photolytic degradation	0.35	0.37	97.4	3.1
Peroxide degradation	0.19	0.23	85.8	14.7

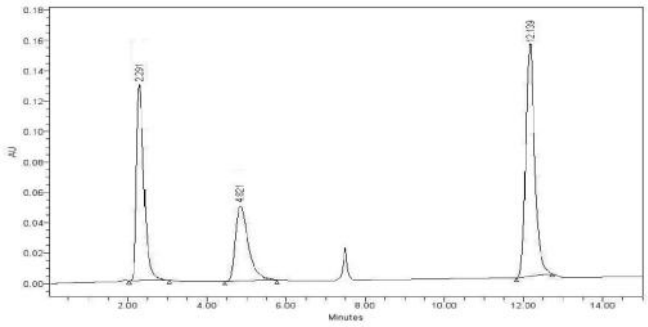

Fig.1 Representative Model chromatogram of Acid degradation

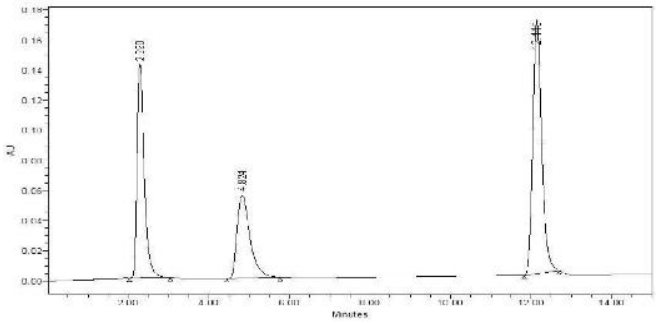

Fig.4 Representative Model Chromatogram of Humidity degradation

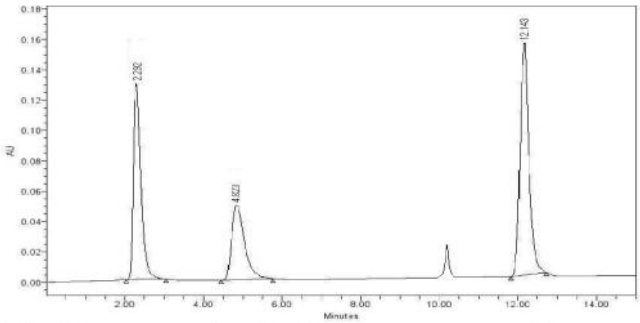

Fig. 2 Representative Model Chromatogram of Base degradation

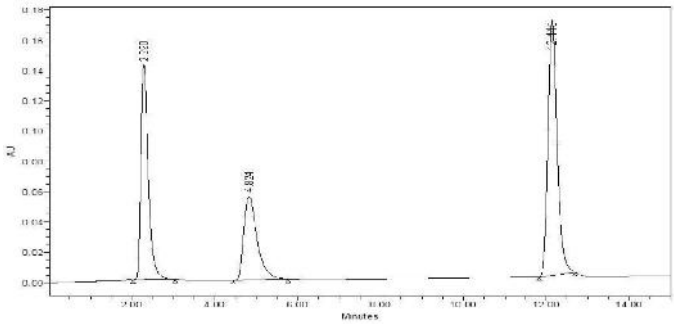

Fig.5 Representative Model chromatogram of Photolytic degradation

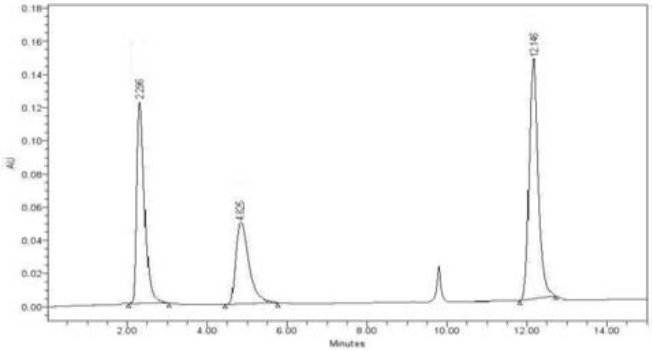

Fig.3 Representative Model Chromatogram of Thermal degradation

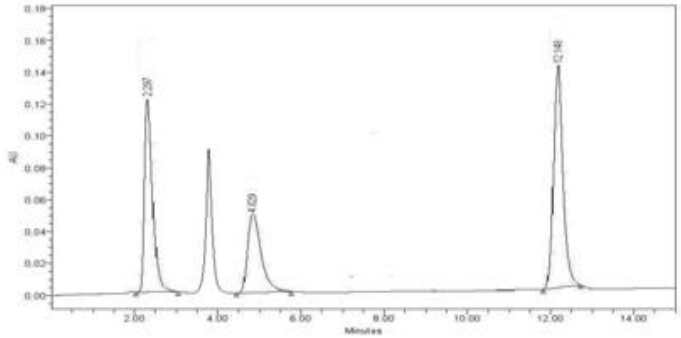

Fig.6 Representative Model Chromatogram of Peroxide degradation

Table: 9 Method Precision of Emtricitabine, Tenofovir & Efavirenz

S.No.	**%Assay**		
	Emtricitabine	**Tenofovir**	**Efavirenz**
1	99.4	99.6	98.4
2	100.2	98.7	99.8
3	99.2	99.9	100.5
4	99.8	100.3	100.7
5	100.1	99.6	100.6
6	99.1	100.8	101.1
AVERAGE	99.6	99.9	100.2
SD	0.5	0.8	1.0
% RSD	0.5	0.8	1.0

Table: 10 LOD and LOQ data

Component name	Limit of Detection	Limit of Quantification		
	Concentration (µg/ml)	Concentration (µg/ml)	% Mean recovery	% RSD
Emtricitabine	0.018	0.060	100.8	0.81
Tenofovir	0.081	0.252	99.9	0.92
Efavirenz	0.05	0.162	100.6	0.81

Table: 11 Accuracy for Emtricitabine

S.No.	%Spike level	Amount added(mg)	Amount found(mg)	%Recovery	Statistical parameters
1	**50%**	15.12	14.92	98.7	**Mean=98.8 SD=0.48 %RSD=0.49**
2		15.22	14.93	98.1	
3		15.16	15.09	99.5	
4		14.96	14.82	99.1	
5		15.31	15.13	98.8	
6		15.53	15.32	98.6	
7	**75%**	22.62	22.36	98.9	**Mean=98.9 SD=0.24 %RSD=0.25**
8		22.71	22.41	98.7	
9		22.61	22.42	99.2	
10	**100%**	31.23	31.16	99.8	**Mean=100.3 SD=0.50 %RSD=0.50**
11		30.91	31.12	100.7	
12		30.54	30.72	100.6	
13	**125%**	37.71	37.22	98.7	**Mean=99.1 SD=0.53 %RSD=0.54**
14		37.81	37.43	99.0	
15		37.62	37.52	99.7	
16	**150%**	45.81	45.91	100.2	**Mean=100.1 SD=0.57 %RSD=0.57**
17		45.63	45.81	100.4	
18		45.21	44.91	99.3	
19		45.93	46.32	100.8	
20		46.12	46.31	100.4	
21		45.52	45.33	99.6	

Table: 12 Accuracy for Tenofovir

S.No.	%Spike level	Amount added(mg)	Amount found(mg)	%Recovery	Statistical parameters
1	**50%**	22.51	22.41	99.6	**Mean=99.8 SD=0.28 %RSD=0.28**
2		22.49	22.51	100.1	
3		22.61	22.65	100.2	
4		22.45	22.38	99.7	
5		22.71	22.61	99.6	
6		22.52	22.44	99.6	
7	**75%**	33.72	33.12	98.2	**Mean=98.7 SD=0.44 %RSD=0.45**
8		33.81	33.41	98.8	
9		33.65	33.34	99.1	
10	**100%**	45.12	44.65	99.0	**Mean=99.0 SD=0.41 %RSD=0.41**
11		46.10	45.82	99.4	
12		45.86	45.2	98.6	
13	**125%**	56.26	55.82	99.2	**Mean=100.1 SD=0.84 %RSD=0.83**
14		56.28	56.32	100.1	
15		56.31	56.81	100.9	
16	**150%**	67.25	67.36	100.2	**Mean=100.3 SD=0.46 %RSD=0.46**
17		67.33	67.58	100.4	
18		67.25	66.93	99.5	
19		67.58	67.7	100.1	
20		67.59	68.21	100.9	
21		67.86	68.21	100.5	

Table: 13 Accuracy for Efavirenz

S.No.	%Spike level	Amount added(mg)	Amount found(mg)	%Recovery	Statistical parameters
1	**50%**	45.21	45.12	99.8	**Mean=99.5 SD=0.23 %RSD=0.23**
2		45.22	45.02	99.6	
3		45.36	45.11	99.4	
4		45.62	45.45	99.6	
5		45.56	45.21	99.2	
6		45.68	45.33	99.2	
7	**75%**	67.63	67.21	99.4	**Mean=100.3 SD=0.80 %RSD=0.80**
8		67.66	68.11	100.7	
9		67.58	68.16	100.9	
10	**100%**	91.12	91.22	100.1	**Mean=100.3 SD=0.85 %RSD=0.85**
11		90.51	91.68	101.3	
12		90.58	90.26	99.6	
13	**125%**	112.62	112.36	99.8	**Mean=99.9 SD=0.14 %RSD=0.14**
14		112.56	112.62	100.1	
15		112.71	112.64	99.9	
16	**150%**	135.51	135.48	100.0	**Mean=99.9 SD=0.42 %RSD=0.42**
17		135.21	135.55	100.3	
18		135.12	135.26	100.1	
19		135.26	135.61	100.3	
20		136.12	135.28	99.4	
21		136.56	135.66	99.3	

Table: 14 Linearity for Emtricitabine

S.NO.	Linearity level	Concentration (µg/ml)	Peak area
1	25	7.5	438762
2	50	15.0	879523
3	75	22.5	1309285
4	100	30.0	1759146
5	125	37.5	2168808
6	150	45.0	2598569

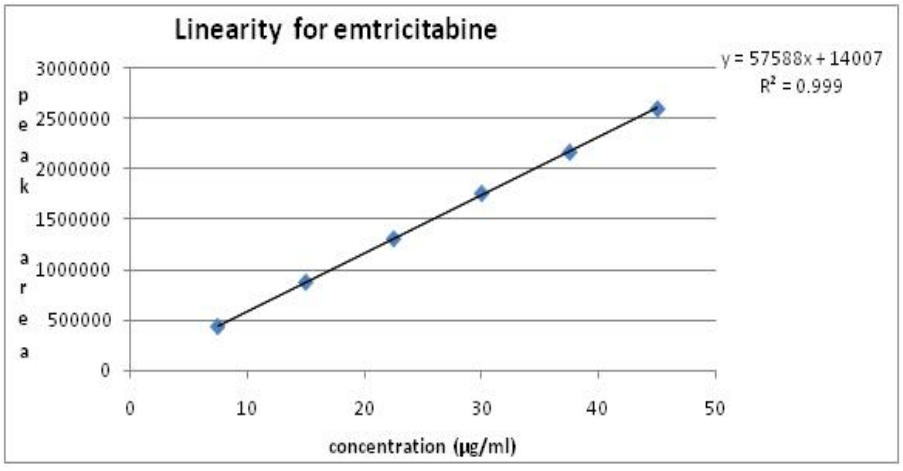

Fig. 7 Graph Representing Linearity of Emtricitabine

Table: 15 Linearity for Tenofovir

S.NO.	Linearity level	Concentration (µg/ml)	Peak area
1	25	11.25	298669
2	50	22.50	589154
3	75	33.75	888637
4	100	45.00	1197316

5	125	56.25	1477986
6	150	67.50	1779898

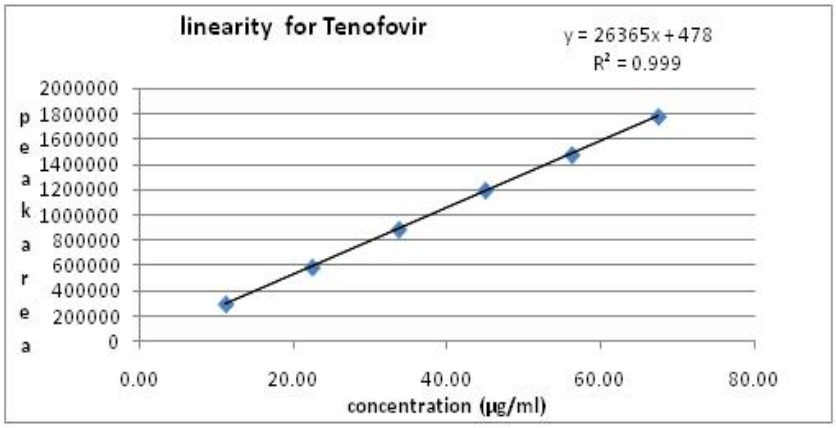

Fig .8 Graph representing linearity of Tenofovir

Table: 16 Linearity for Efavirenz

S.NO.	Linearity level	Concentration (µg/ml)	Peak area
1	25	22.5	638608
2	50	45.0	1195227
3	75	67.5	1889872
4	100	90.0	2540678
5	125	112.5	3087037
6	150	135.0	3727960

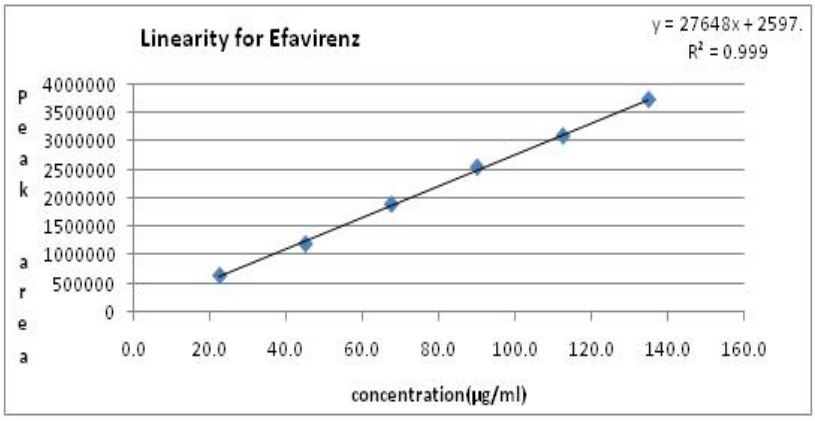

Fig. 9 Graph Representing Linearity of Efaverinz

Table: 17 Regression Data of the Proposed Method

Sno.	Parameters	Emtricitabine	Tenofovir	Efaverinz
1	Linearity (µg/ml)	7.5 – 45	11.25 - 67.50	22.5 – 135
2	Regression (mx+c)	57588x+1400	26365x+478	27648x+2597
3	Slope(m)	57588	26365	27648
4	Intercept(c)	1400	478	2597
5	Correlation coefficient (r^2)	0.999	0.999	0.999

Table: 18 Ruggedness of Emtricitabine

S.No	Emtricitabine		
	ANALYST-1	ANALYST-2	Overall results
1	98.5	98.7	**Mean** 99.8
2	99.4	99.5	**SD** 1.01
3	99.7	101.5	**%RSD** 1.01

4	100.5	100.8	
5	99.7	99.8	
6	98.4	101.1	
AVERAGE	99.4	100.2	
SD	0.8	1.07	
% RSD	0.8	1.07	

Table: 19 Ruggedness of Tenofovir

S.NO	**TENOFOVIR**		
	ANALYST-1	**ANALYST-2**	**OVERALL RESULTS**
1	99.4	99.2	
2	98.7	101.5	
3	100.5	98.7	
4	101.2	99.4	**Mean** 99.7
5	99.7	101.1	**SD** 1.11
6	98.2	98.8	**%RSD** 1.11
AVERAGE	99.6	99.8	
SD	1.1	1.21	
% RSD	1.1	1.21	

Table: 20 Ruggedness of Efavirenz

S.NO	**EFAVIRENZ**		
	ANALYST-1	**ANALYST-2**	**OVERALL RESULTS**
1	98.2	101.5	
2	98.9	99.4	
3	99.1	100.5	
4	99.3	99.8	
5	98.3	99.2	**Mean**: 99.6
6	99.7	101.5	**SD**: 1.08
AVERAGE	98.9	100.3	**%RSD**: 1.08
SD	0.6	1.02	
% RSD	0.6	1.02	

Table: 21A Robustness

Optimum conditions	**Modifications**	**Retention time**			**Asymmetric factor**		
		ET	TF	EF	ET	TF	EF
P^H	3.4	2.286	4.820	12.136	1.2	1.3	1.2
(3.5)	3.6	2.312	4.945	12.254	1.3	1.2	1.2
Column temperature	20	2.321	5.348	13.021	0.9	0.9	0.9
(25^oC)	30	2.161	4.516	11.988	1.1	1.1	1.1
Flow rate	0.9	2.432	5.237	13.362	1.3	1.2	1.3
(1.0 mL/min)	1.1	2.071	4.412	11.896	1.1	1.1	1.1
Wave length	260	2.287	4.827	12.143	1.1	1.2	1.1
(270nm)	256	2.288	4.829	12.141	1.1	1.1	1.1

Table: 21B Robustness

Optimum conditions	**Modifications**	**Theoretical plates**			**Resolution**	
		ET	TF	EF	TF	EF
P^H	3.4	2740	2169	15618	5.1	14.1
(3.5)	3.6	2736	2150	15610	6.1	15.1
Column temperature	20	2685	2168	15589	6.3	16.2
(25^oC)	30	2798	2214	15489	5.9	15.9
Flow rate	0.9	2645	2025	15365	6.5	16.8
(1.0 mL/min)	1.1	2865	2345	15798	5.9	15.8
Wave length	260	2752	2187	15635	6.0	15.0
(270nm)	256	2769	2197	15643	6.0	15.0

4. Conclusion

The proposed RP-HPLC method has been evaluated for the accuracy, precision and linearity. The method was found to be precise, accurate and linear over the linear concentration range. In this method, there was no interference from matrix sources. Moreover, the lower solvent consumption along with the short analytical run time of 14 minutes that allows the analysis of a large number of samples in a short period of time. Therefore, this RP-HPLC method can be used as a routine analysis of these drugs in bulk, pharmaceutical formulations and also for stability studies.

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