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METHOD DEVELOPMENT AND VALIDATION OF SIMULTANEOUS ESTIMATION OF EMTRICITABINE AND TENOFOVIR ALAFENEMIDE IN BULK AND TABLET DOSAGE FORM BY RP-HPLC

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Abstract

A simple, rapid, accurate and economical method has been developed for the simultaneous estimation of Emtricitabine and Tenofovir Alafenamide in tablet dosage form by RP-HPLC Technique. The linearity of the method was found to be in the range of 40µg/ml- 120µg/ml for Tenofovir Alafenamide and 320µg/ml-960µg/ml for Emtricitabine. The percentage purity of the drugs was found as 98 and 97%w/w for Tenofovir Alafenamide and Emtricitabine respectively. The method was also found to be accurate, precise, robust and rugged. The limit of detection and the limit of quantification were found to be 10.53µg/ml and 31.91µg/ml for Tenofovir Alafenamide and 87.35µg/ml and 364.71µg/ml for Emtricitabine respectively.

Keywords: Tenofovir Alafenamide (TEN), Emtricitabine (EMT), UV-Visible spectroscopy.

Introduction

Emtricitabine [EMT] is a nucleoside reverse transcriptase inhibitor (NRTIs) with chemicals it's 5-fluoro-1-(2R, 5S)-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl) cytosine. EMT is the (-) enantiomer of thio analog of cytidine which differ from other cytidine analogs, in that it has fluorine in the 5th position. EMT is an antiviral agents used for the prevention of perinatal HIV-1 reverse transcriptase.

It is also active against Hepatitis B virus. Tenofovir Alafenamide (a pro drug of tenofovir) is a nucleotide analogue reverse transcriptase inhibitor (nRTIs), which blocks reverse transcriptase, an enzyme crucial for the viral production in HIV-infected individuals. Chemically it is (9[(*R*)2 [[bis[[(isopropoxycarbonyl)oxy]

methoxy] phosphinyl]methoxy]propyl]. The present study was aimed to develop a simple, rapid and accurate method for the simultaneous estimation of Emtricitabine and Tenofovir Alafenamide in bulk and tablet dosage form using RP-HPLC method and to validate it using ICH guidelines.

Materials and Methods

Chemicals and Reagents

Pure drug sample of Emtricitabine and Tenofovir alafenamide was kindly supplied as a gift sample by Mylan Laboratories Ltd., Hyderabad. The Tablets used for the analysis was TAFERO-EM manufactured by Hetero Labs Ltd, Himachal Pradesh, India, containing Emtricitabine 200 mg and Tenofovir Alafenamide 25 mg per tablet.

Other chemicals used are Water HPLC Grade (Thermo Fisher Scientific India Pvt. Ltd), Methanol HPLC Grade (s d fine-chem Limited) and Acetic acid HPLC Grade (HiMedia Laboratories Pvt. Ltd).

Instrumentation

Instruments used in the work are Shimadzu LC-20 AT HPLC, A SHIMADZU model PHARMASPEC-1800 UV-Visible double beam spectrophotometer with 1cm matched quartz cell, Shimadzu electronic balance AY 220. Elico pH meter LI 127, Ultra sonicator – Enertech, Hamilton 702 NR 25µL Syringe (22s/51/3), Cuvettes – quartz cells.

Selection of Detection Wavelength

Ultra Violet spectrum of 10 µg / ml Emtricitabine and Tenofovir AF in diluents (mobile phase composition) has been recorded by scan in the range of 200nm to 400nm.

By using the UV spectrum, wavelength has been selected as 272nm. Hence wavelength of both drugs show good absorbance.

Selection of Mobile phase

Solvent selectivity (solvent type), solvent strength (percentage of organic solvent in the mobile phase), strength and pH of buffer, flow rate etc. were varied to determine the chromatographic conditions that gave the best separation (Table 1).

Table 1: Selection of mobile phase.

Mobile phase	Observation
Methanol : Water	Good Separation with increased retention time
Methanol : Water (pH 3.26)	Good Separation with increased retention time
Methanol : Water (pH 2.56)	Good Separation with increased retention time
Methanol : Water (pH 2.72)	Good separation with symmetric peaks And less retention time
Methanol : Water (pH 2.57)	Good Separation with increased retention time
Methanol : Water (pH 2.94)	Good Separation with increased retention time

Preparation of Mobile phase

Mobile phase solution A and B are prepared separately by taking HPLC grade Methanol as Mobile phase A, Mobile phase B was prepared using HPLC Grade water and pH was adjusted to 2.72 using HPLC Grade Acetic acid. It was then sonicated for 15min and filtered through 0.45µ membrane filter.

Selection of Ratio of Mobile phase

In a mobile phase system consisting Methanol : Water (pH 2.72) in different ratios like 50:50, 60:40, 70:30, 80:20, 90:10, 40:60, 30:70, 20:80 %v/v, a mixture of Emtricitabine and Tenofovir Alafenamide were injected. Symmetrical peaks with good resolution was obtained with a ratio of 80:20 %v/v and hence selected for further studies.

Selection of Flow rate

Keeping all the parameters of mobile phase system constant, the chromatograms were recorded with different flow rates like 0.8, 1 and 1.2ml/ min. With flow rate 0.8 and 1 ml/ min, peaks were not symmetrical. But a flow rate of 1.2ml/min gave good symmetrical peaks and hence selected for further studies.

Table 2: Optimized Chromatographic Condition.

Parameters	Conditions
Stationary phase	Phenomenex C18 Column (250mm × 4.6 i.d 5µ)
Mobile phase	Solvent A: Methanol, Solvent B: Water
pH	2.72 adjusted with Acetic acid
Solvent ratio	80:20
Detection wavelength	272nm
Flow rate	1.2 ml/min
Temperature	25^0C

Preparation of standard stock solution

5mg of Tenofovir Alafenamide and 40mg of Emtricitabine was taken separately on a 25ml standard flask. Then the drug was dissolved in 10ml of methanol and then final volume was made up with methanol. 4ml from this solution was further transferred to separate 10ml standard flask and the volume was made up with diluent and sonicated for 5 minutes. Then 20µl of each single and mixed drug solutions were injected into the chromatographic systems and the chromatograms were recorded.

Analysis of Tablet Formulation

Twenty tablets were weighed accurately and the average weight was calculated. The tablets were then grounded to fine powder. An accurately weighed tablet powder equivalent to 5mg of Tenofovir Alafenamide and 40mg of Emtricitabine was transferred into 25ml standard flask. Dissolved the content in little amount of methanol and the volume is again made up with methanol. The solution was then sonicated using ultrasonicator for 15 minutes and was filtered using Membrane filter. 4ml of the above prepared solution was transferred into a 10ml standard flask and the volume was made up to 10ml with diluent. 20µl of this solution was injected into the RP-HPLC system and the chromatogram was recorded.

Method Validation

The method was validated using ICH guidelines by determining the following parameters: Linearity, Accuracy, Precision, Robustness, Ruggedness, Precision, Detection limit and Quantification limit.

Linearity

Five different concentrations of standard Tenofovir Alafenamide (40, 60, 80, 100, 120 µg/ml) and Emtricitabine (320, 480, 640, 800, 960 µg/ml) were prepared and the linearity was evaluated using Linear regression analysis.

Accuracy

The accuracy of the method was determined using recovery analysis. A known quantity of mixed pure drug was added to the pre analyzed tablet formulation at 50%, 75%, and 100% levels. The recovery studies were carried out three times and the percentage recovery and percentage relative standard deviation was calculated.

Precision

In order to determine the precision of the proposed method tablet solution at a particular concentration level (within the working range) were prepared and analyzed in three replicates during the same day (intra-day) and on three consecutive days (inter-day). And the percentage relative standard deviation was also calculated.

Robustness

Robustness of the method was estimated by introducing small changes in the mobile phase ratio and flow rate, and the effect in the results was recorded.

Ruggedness

Ruggedness was determined by performing analysis of the drug following the recommended procedures by three different analysts.

Detection and Quantification Limit

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated based on the intercept standard deviation and the curve slope.

$$\text{LOD} = \frac{3.3\sigma}{S} \qquad \text{LOQ} = \frac{10\sigma}{S}$$

Results and Discussions

Selection of Detection Wavelength

Ultra Violet spectrum of 10 µg / ml Emtricitabine and Tenofovir AF in diluents (mobile phase composition) has been recorded by scan in the range of 200nm to 400nm. By using the UV spectrum, wavelength has been selected as 272nm. Fig. 1

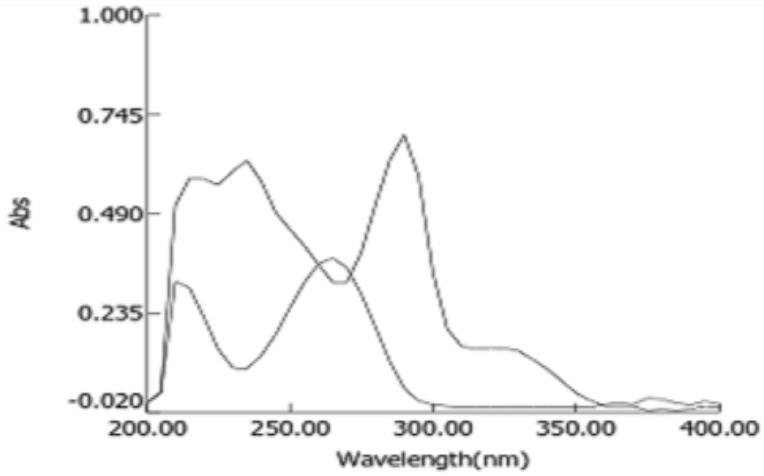

Figure 1: UV Spectrum of Emtricitabine and Tenofovir alafenamide.

Analysis of Tablet formulation

Simultaneous estimation of Emtricitabine and Tenofovir Alafenamide in combined dosage forms by High Performance Liquid Chromatography was carried out using optimized chromatographic conditions. The standard and sample solutions were prepared and chromatograms were recorded. The recorded chromatogram of Blank, Emtricitabine, Tenofovir Alafenamide, standard and formulation chromatograms are given. The assay procedure was repeated for three times and mean peak area, mean weight of standard drugs and sample were taken and calculated. The percentages of individual drugs found in formulations, mean and relative standard deviation in formulations were calculated and presented in Table 3.

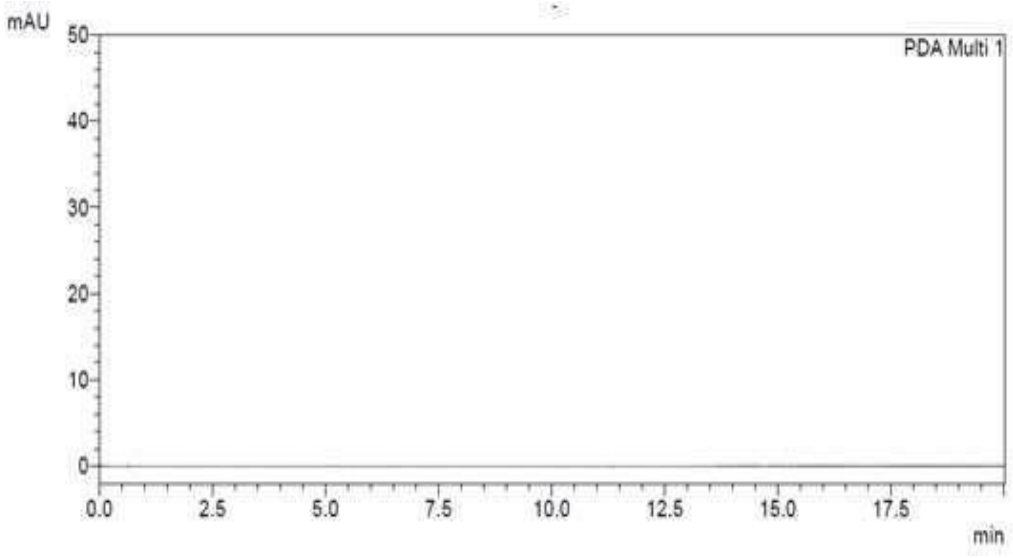

Figure 2: Chromatogram of Blank.

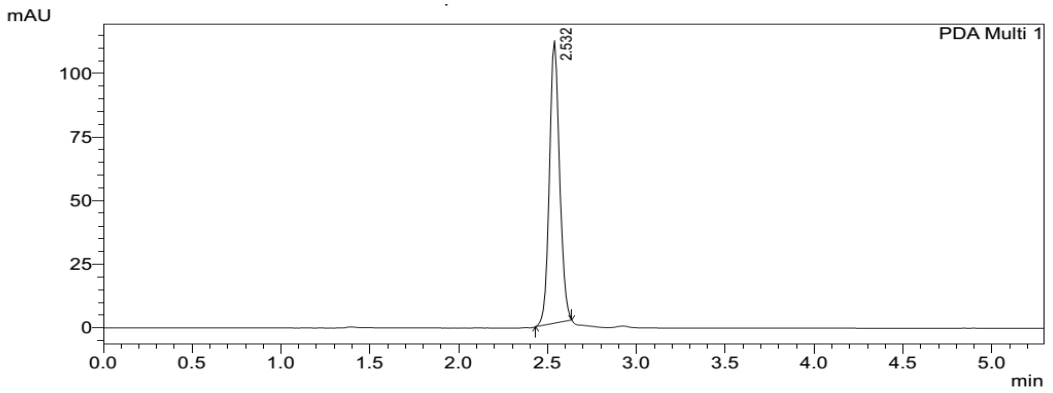

Figure 3: Chromatogram of Emtricitabine standard drug.

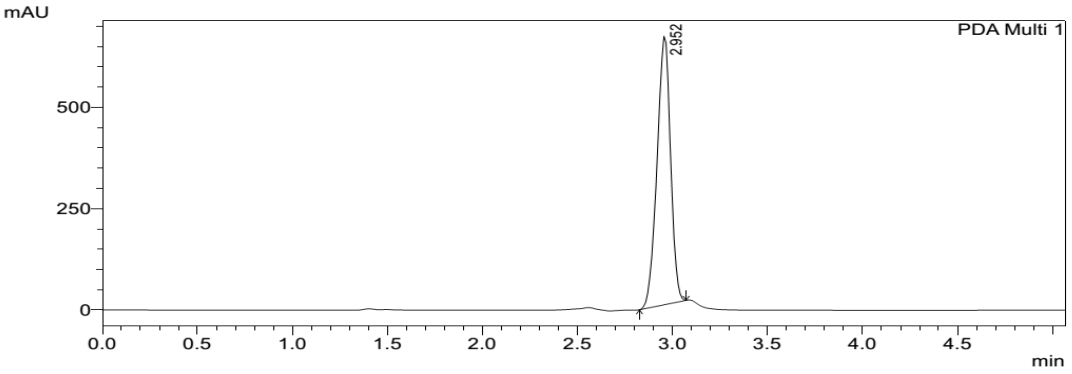

Figure 4: Chromatogram of Tenofovir Alafenamide standard drug.

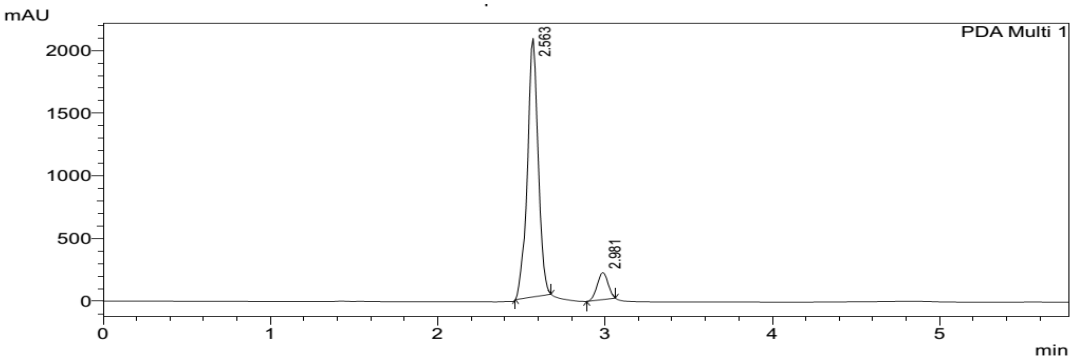

Figure 5: Chromatogram of standard drug in combination.

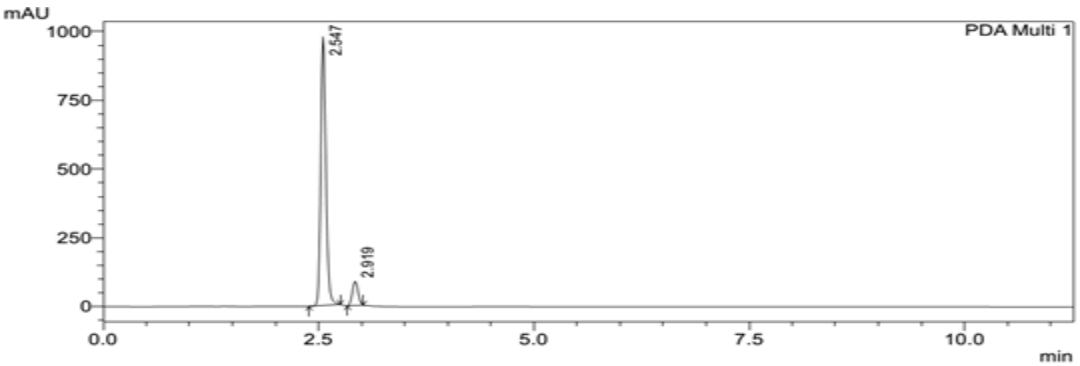

Figure 6: chromatogram of Formulation.

Table 3: Results of assay of Tablet formulation.

Marketed formulation	Drug	Peak Area	Estimated amount (mg)	% purity	%RSD
TAFERO-EM (25:200)	TEN (80µg/ml)	1808385 1808412 1808396	24.3 24.5 24.4	97.2 98 97.6	0.4
	EMT (640µg/ml)	17770083 17770114 17770098	193.3 194 193.8	96.5 97 96.9	0.25

Method validation

Linearity

The linearity of the both the drugs were determined and the results are shown in Figure.7, Figure.8 and Table 4.

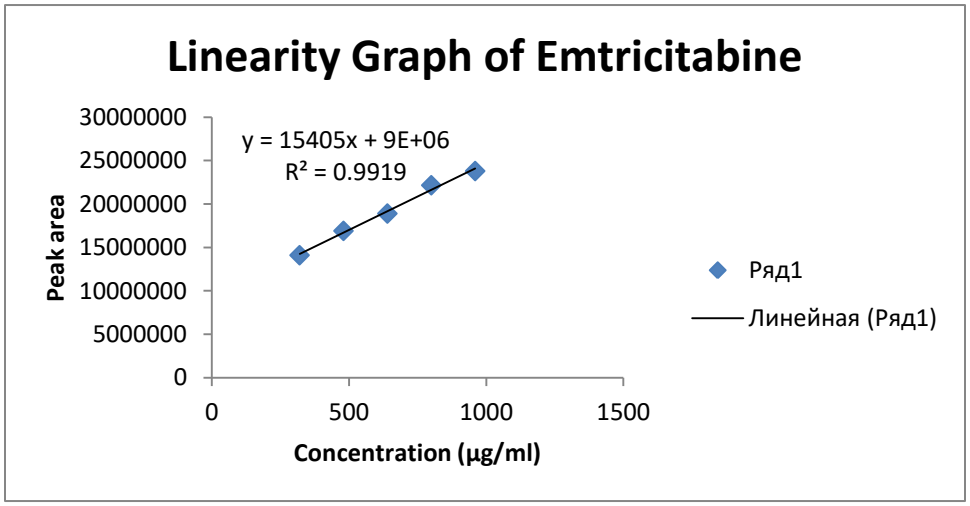

Figure 7: Linearity graph of Emtricitabine.

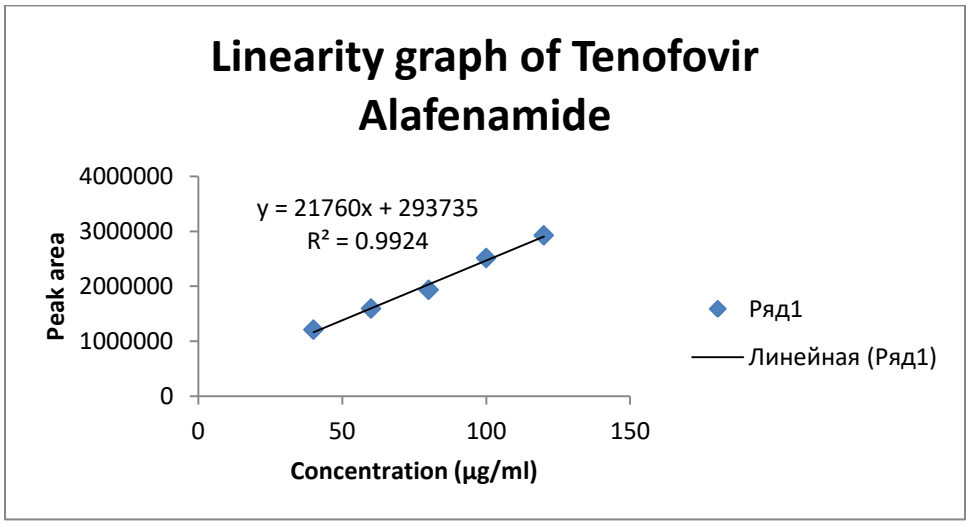

Figure 8: Linearity graph of Tenofovir Alafenamide.

Table 4: Linearity results.

TENOFOVIR ALAFENAMIDE		EMTRICITABINE	
Concentration (µg/ml)	Peak area	Concentration (µg/ml)	Peak area
40	1209062	320	14103800
60	1592582	480	16910155
80	1933588	640	18907508
100	2512155	800	22164392
120	2925274	960	23801092

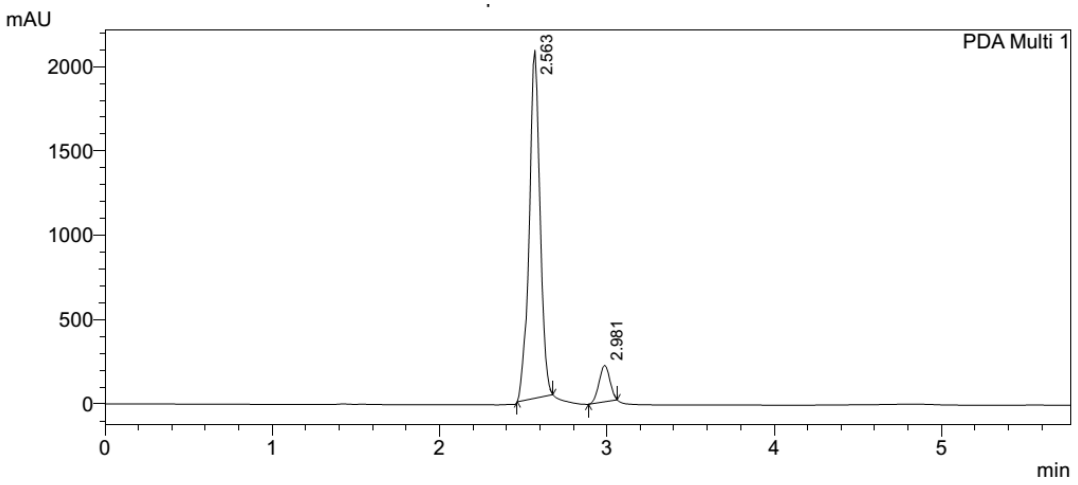

Figure 9: Chromatogram of standard solution of TEN at 40µg/ml and EMT 320µg/ml.

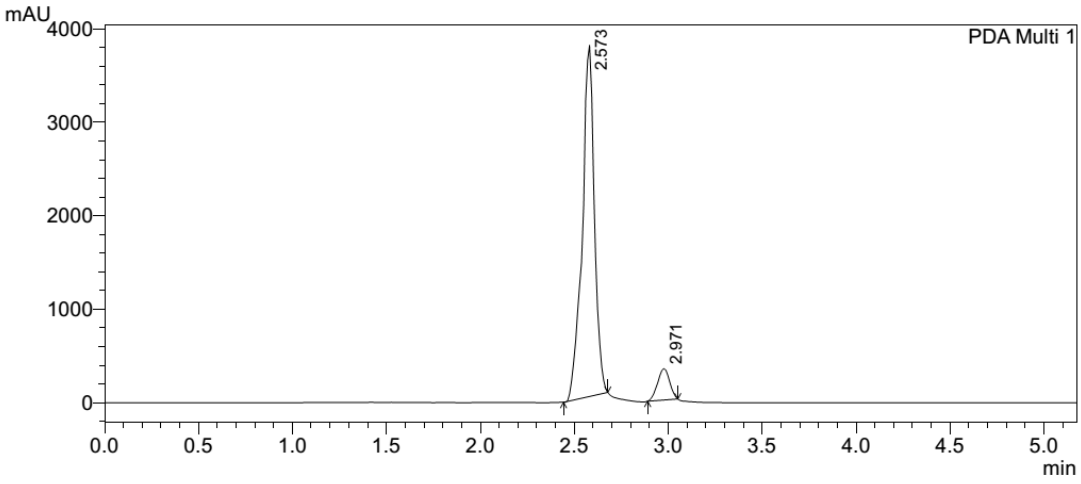

Figure 10: Chromatogram of standard solution of TEN at 60µg/ml and EMT 480µg/ml.

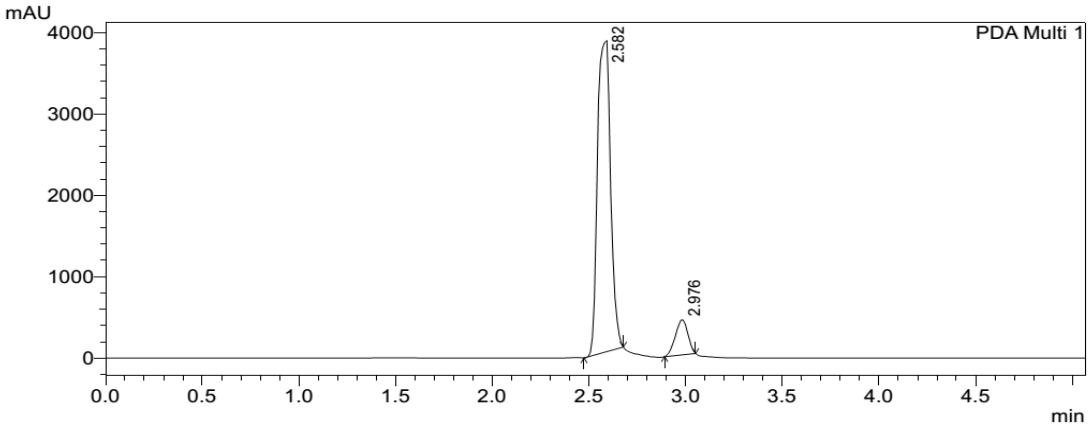

Figure 11: Chromatogram of standard solution of TEN at 80µg/ml and EMT 640µg/ml.

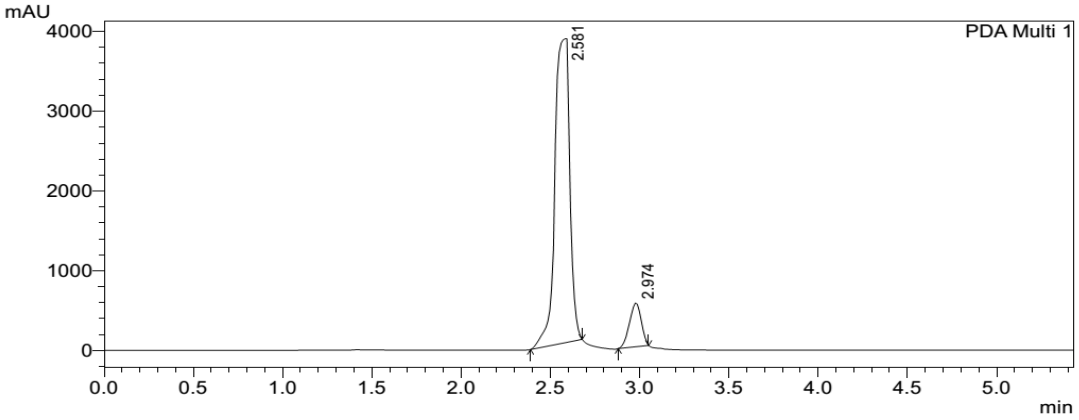

Figure 12: Chromatogram of standard solution of TEN at 100µg/ml and EMT 800µg/ml.

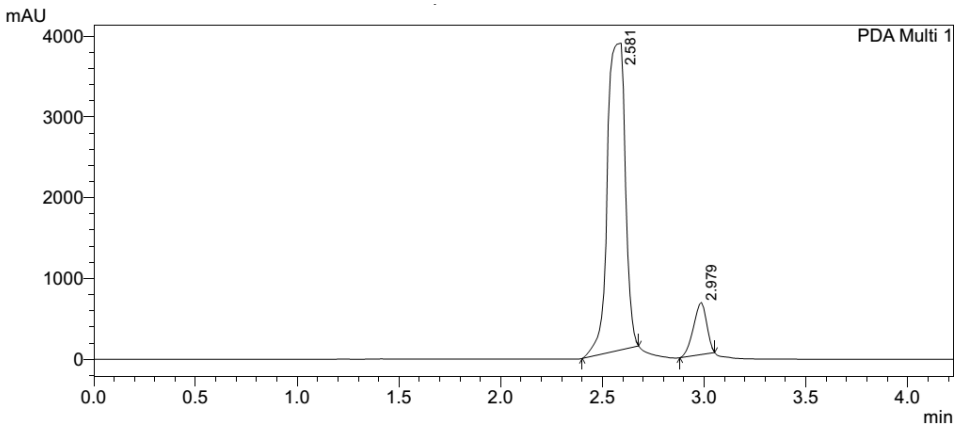

Figure 13: Chromatogram of standard solution of TEN at 120µg/ml and EMT 960µg/ml.

Accuracy

The accuracy of the method was determined at three percentage levels 50%, 75% and 100%. The recovery studies were carried out three times and the percentage recovery and percentage relative standard deviation was found to be less than 2 and the results are given in Table 5.

Table 5: Results of accuracy studies.

Drug	Theoretical % target level	Amount added (mg)	Amount recovered(mg)	% Recovery	% RSD
TEN	50	40	24.9 25.1 24.7	99.6 100.4 98.8	0.8
	75	60	25.0 24.8 24.7	100 99.2 98.8	0.61
	100	80	25.1 24.7 24.6	100.4 98.8 98.4	1.05
EMT	50	320	199.1 198.7 198.9	99.5 99.3 99.4	0.1
	75	480	195.3 196.1 195.4	97.6 98.0 97.7	0.20
	100	640	194.3 195.1 194.9	97.1 97.5 97.4	0.20

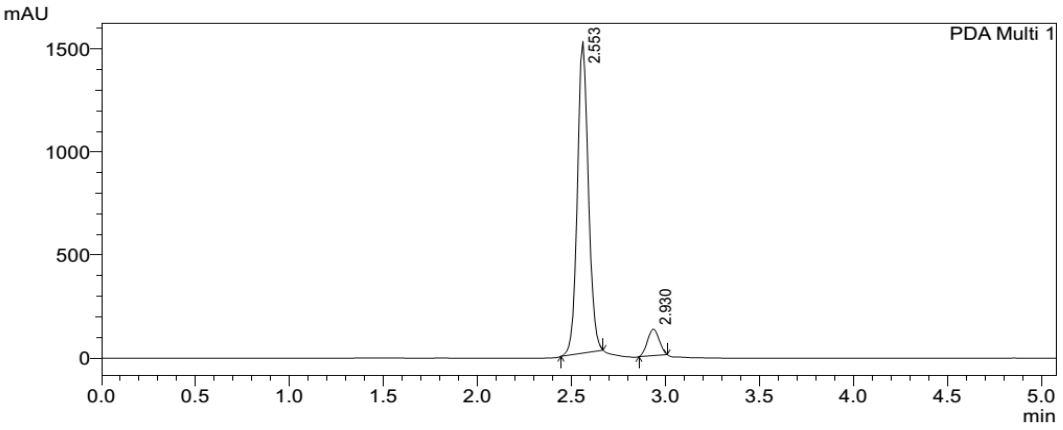

Figure 14: Result of accuracy at 50%.

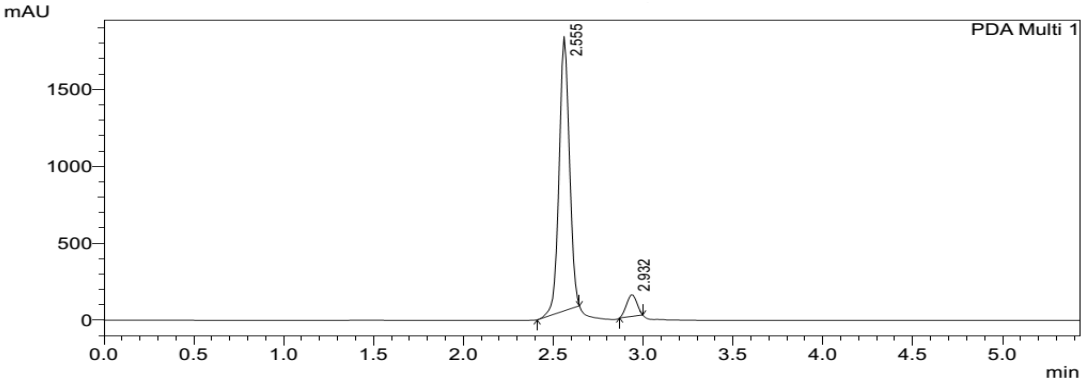

Figure 15: Result of accuracy at 75%.

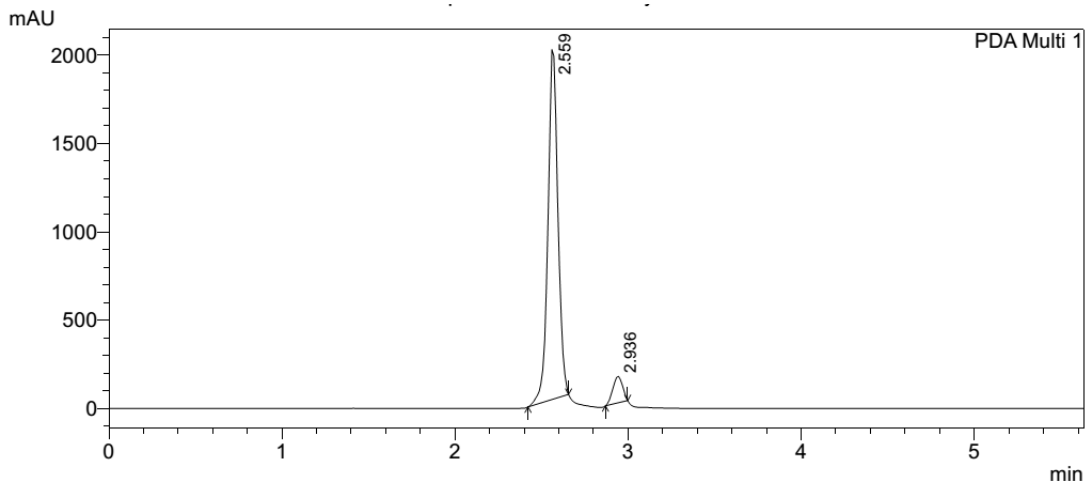

Figure 16 : Result of accuracy at 100%.

Precision

Tablet solution at a particular concentration level (80µg/ml of TEN and 640µg/ml of EMT) were prepared and analyzed in three replicates during the same day (intra-day) and on three consecutive days (inter-day). And the percentage relative standard deviation was also calculated and the results are shown in Table 6 and 7.

Table 6: Results of Interday Precision.

Day	Peak area of TEN	Mean	% RSD	Peak area of EMT	Mean	% RSD
Day 1 Day 2 Day 3	1808412 1808391 1808389	1808397	0.05	17770089 17770081 17770098	17770089	0.06

Table 7: Results of Intraday Precision.

Time	Peak area of TEN	Mean	% RSD	Peak area of EMT	Mean	% RSD
0^{th} Hour 3^{rd} Hour 6^{th} Hour	1808411 1808392 1808391	1808398	0.04	17770114 17770085 17770089	17770096	0.06

Robustness

Robustness of the method was estimated by introducing small changes in the mobile phase ratio and flow rate, and the effect in the results was shown in the Table 8.

Table 8: Robustness results.

Parameter altered	Values	Tenofovir Alafenamide		Emtricitabine	
		Theoretical plate	Tailing factor	Theoretical plate	Tailing factor
Mobile phase ratio	90:10	7898 7891 7874	1.075 1.067 1.071	6929 6936 6928	1.183 1.191 1.187
	70:30	8906 8941 8916	1.063 1.065 1.069	6936 6931 6929	1.259 1.254 1.252
Flow rate	1.1 ml/min	9333 9349 9321	0.961 0.964 0.963	7065 7159 7043	1.062 1.043 1.063
	1.3 ml/min	9364 9316 9359	1.007 1.012 1.019	6315 6359 6312	1.299 1.219 1.291

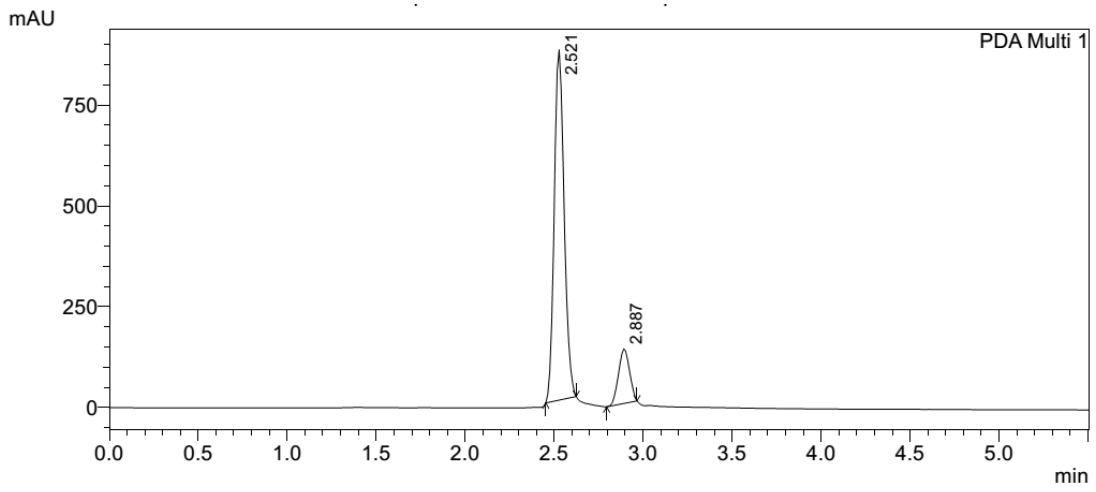

Figure 17: Robustness at mobile phase ratio 90:10.

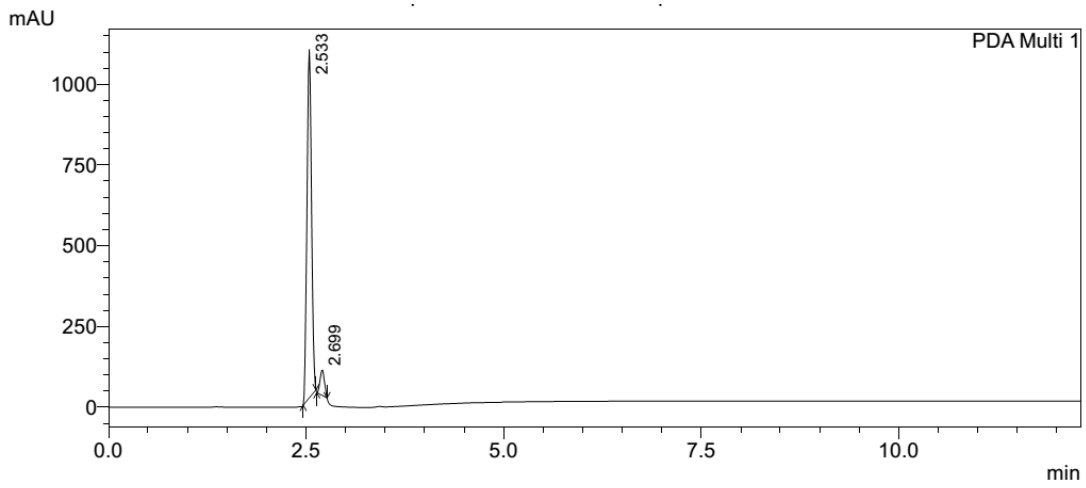

Figure 18: Robustness at mobile phase ratio 70:30.

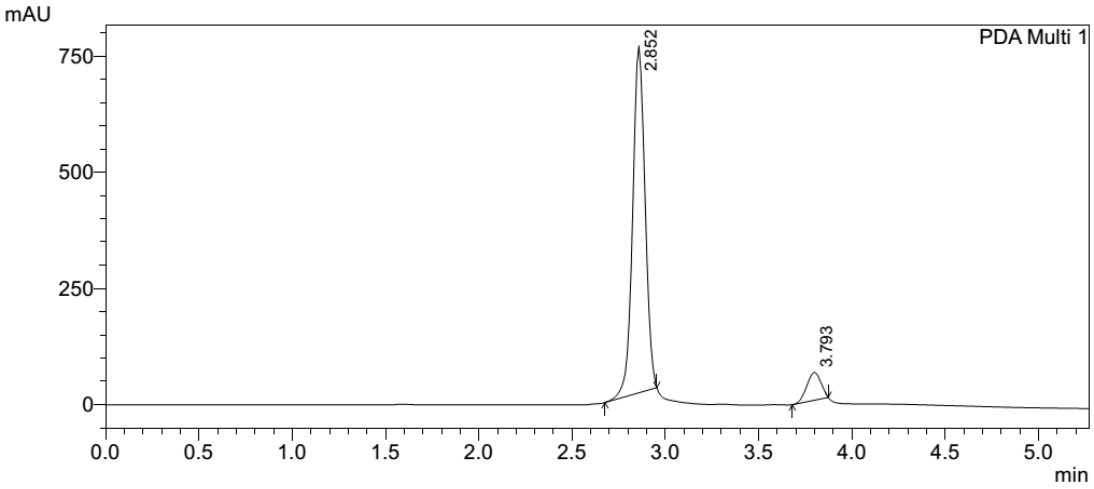

Figure 19: Robustness at Flow rate 1.1 ml/min.

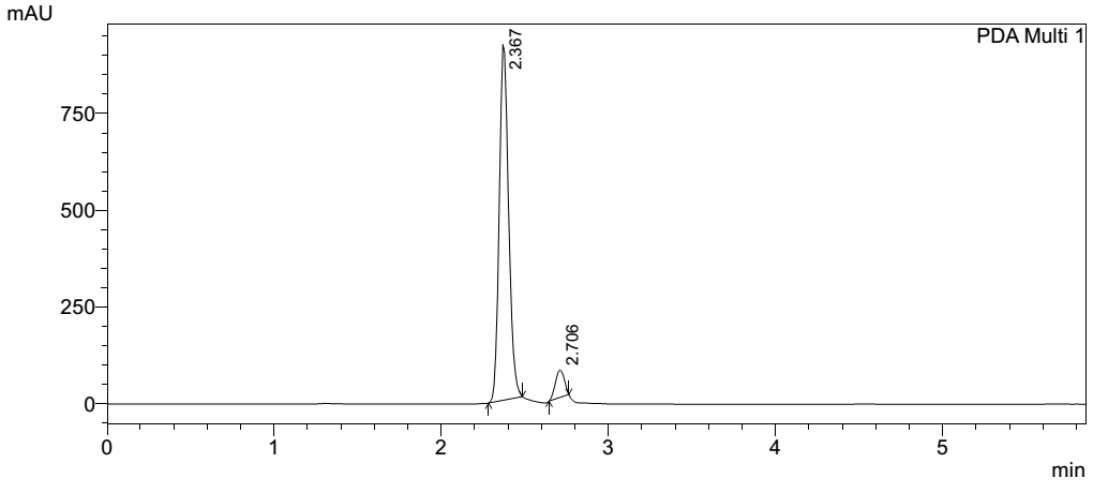

Figure 20: Robustness at Flow rate 1.3 ml/min.

Ruggedness

Ruggedness was determined by performing analysis of the drug following the recommended procedures by three different analysts and the results are shown in Table 9 and the proposed method was found to be rugged.

Table 9: Ruggedness results.

Drug	Analyst	Amount taken (µg/ml)	Amount recovered (mg)	% Content	% RSD
TEN	Analyst I	80	24.4	97.6	0.61
	Analyst II		24.7	98.8	
	Analyst III		24.5	98	
EMT	Analyst I	640	194.2	97.1	0.1
	Analyst II		193.9	96.9	
	Analyst III		194.1	97	

LOD and LOQ

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated based on the intercept standard deviation and curve slope and the results were shown in Table 10.

Table 10: Results of LOD and LOQ.

TENOFOVIR ALAFENAMIDE		EMTRICITABINE	
LOD (µg/ml)	LOQ (µg/ml)	LOD (µg/ml)	LOQ (µg/ml)
10.53	31.91	87.35	264.71

Conclusion

The developed RP-HPLC method was found to be rapid as it has a very less retention time, and also the method was found to be simple, economical and also the method uses the water in acetic acid as one of the buffer system, though the acetic acid acts as a volatile buffer it helps the column from degenerating also will not affect the stability of the column. So as compared to the already developed methods the present method has more advantages in all the aspects of less time consumption, economical and maintenance of column stability. So the

newly developed method can be used for the routine analysis of Tenofovir Alafenamide and Emtricitabine in combined dosage form.

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