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VALIDATED RP-HPLC METHOD FOR THE ASSAY OF TENOFOVIR DISOPROXIL FUMARATE AND EMTRICITABINE IN TABLETS AND BULK DRUG

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ABSTRACT

The pharmaceutical combined dosage forms of Emtricitabine and Tenofovir disoproxil fumarate are used for the treatment to help stop the spread of HIV in your body. A reverse-phase high-performance liquid chromatography method has been developed for simultaneous estimation of Emtricitabine and Tenofovir disoproxil fumarate available in combined tablet dosage form. The method was developed using Discovery 150x4.6mm, 5µ**,** and mobile phase composition of potassium dihydrogen phosphate Buffer: Acetonitrile (60:40A). The flow rate was adjusted at 1 ml/min for isocratic elution and detection was performed at 280 nm with UV detector. The retention time for Tenofovir disoproxil fumarate and Emtricitabine was found to be 3.039 min and 2.185 min, respectively. The method was validated as per ICH guidelines. The linearity range was found to be 3.75 to 22.5 µg/ml for Tenofovir disoproxil fumarate and 2.5 to 15 µg/ml for Emtricitabine. The percent recovery was found to 99.68 % for Tenofovir disoproxil fumarate, and 99.61 % for Emtricitabine, which indicate that method is accurate. The %RSD was found to be less than 2%, which indicates the developed method is precise. The proposed methods were successfully applied for the quantification of Tenofovir disoproxil fumarate and Emtricitabine in pharmaceutical formulations without any interference from excipients.

KEYWORDS: Tenofovir Disoproxil fumarate (TDF), Emtricitabine, RP-HPLC, Robustness and Accuracy.

1. INTRODUCTION

Emtricitabine (EMT) is chemically 4-amino-5-fluoro-1-[(2R, 5S)-2-(hydroxymethyl)- 1, 3-oxathiolan-5-yl]-1, 2-dihydropyrimidin-2-one. Emtricitabine is a nucleoside reverse transcriptase inhibitor (NRTI) for the treatment of HIV infection in adults. Emtricitabine is an analogue of cytidine. The drug works by inhibiting reverse transcriptase, the enzyme that copies HIV RNA into new viral DNA.[1]

Tenofovir disoproxil fumarate (TSF) is chemically 9-[(R)-2[[bis [[(isopropoxycarbonyl) oxy] methoxy] phosphinyl] methoxy] propyl] adenine fumarate (1:1). Tenofovir disoproxil fumarate is a prodrug of tenofovir, belongs to a class of antiretroviral drugs known as nucleotide analogue reverse transcriptase inhibitors (nRTIs), which block reverse transcriptase, an enzyme crucial to viral production in HIV-infected people.[2-5]

Emtricitabine and Tenofovir disoproxil fumarate in combination are available in tablet dosage forms in the ratio of 2:3 (200 mg of Emtricitabine and 300 mg of Tenofovir disoproxil fumarate). Literature survey reveals good numbers of analytical methods are available for the estimation of these drugs. Various analytical methods including HPTLC[6], and HPLC methods[7-17] are available for the simultaneous estimation of Emtricitabine and Tenofovir disoproxil fumarate in pharmaceutical dosage forms. We tried to develop a simple method for the estimation of Emtricitabine and Tenofovir disoproxil fumarate by RP-HPLC in pharmaceutical dosage forms. The proposed method was optimized and validated as per the International Conference on Harmonization (ICH) guidelines.[18]

2. MATERIALS AND METHODS

2.1. INSTRUMENTATION AND CHROMATOGRAPHIC CONDITIONS

To develop a high pressure liquid chromatographic method for simultaneous estimation of Emtricitabine and Tenofovir disoproxil fumarate using WATERS HPLC 2965 SYSTEM. The instrument is BDS C8-150 mm x 4.6mm, 5µ. A 10µl with Auto Injector and PDA Detector was used.

2.2 CHEMICALS AND SOLVENTS

The working standard of Emtricitabine and Tinofovir Disoproxil fumatare were obtained as gift samples from Hetero labs, Hyderabad, India. The market formulation Tenof EM TABLETS tablets ((200 mg of Emtricitabine and 300 mg of Tenofovir disoproxil fumarate)) were procured from local market. HPLC grade water was purchased from Qualigens Ltd, Mumbai, India. Acetonitrile (HPLC Grade), potassium dihydrogen phosphate, Methanol and Ortho-phosphoric acid were obtained from E.Merck (India) Ltd, Mumbai, India.

2.3 PREPARATION OF BUFFERS

Preparation of 0.1% Ortho-Phosphoric Acid Buffer

Accurately 1ml of OPA in a 1000ml of Volumetric flask add about 900ml of milli-Q water added and degas to sonicate and finally make up the volume with water.

Preparation of 0.01N KH2PO4 Buffer

Accurately weighed 1.36gm of Potassium dihydrogen Ortho phosphate in a 1000ml of Volumetric flask add about 900ml of milli-Q water added and degas to sonicate and finally make up the volume with water then PH adjusted to 5.4 with dil. Orthophosphoric acid solution. Preparation of standard stock solutions: Accurately weighed 5 mg of Emtricitabine, 7.5mg of Tenofovir Disoproxil Fumarate and transferred to 50ml volumetric flasks and 3/4 th of diluents was added to these flasks and sonicated for 10 minutes. Flask was made up with diluents and the solution was filtered through 0.45µm membrane filter and labeled as Standard stock solution. 100µg/ml of Emtricitabine 150µg/ml Tenofovir Disoproxil Fumarate.

Preparation of standard working solutions (100% solution)

1ml from each stock solution was pipette out and taken into a 10ml volumetric flask and made up with diluents. The solution was filtered through 0.45µm membrane filter (10µg/ml of Emtricitabine 15µg/ml of Tenofovir Disoproxil Fumarate).

Inject 10μl of the standard solution into the chromatographic system and measure the area for the emtricitabine and Tenofovir disoproxil fumarate peaks and calculate the % Assay by using the assay formula.

Preparation of sample stock solutions

1 vial equivalent to 200 mg Emtricitabine & 300 mg Tenofovir Disoproxil Fumarate was transferred into a 500ml volumetric flask, 400ml of diluents was added and sonicated for 25 min, further the volume was made up with diluent and the solution was filtered through 0.45µm membrane filter (400µg/ml of Emtricitabine 600µg/ml of Tenofovir Disoproxil Fumarate).

Preparation of sample working solutions (100% solution)

0.5ml of filtered sample stock solution was transferred to 20ml volumetric flask and made up with diluent. The solution was filtered through 0.45µm membrane filter (10µg/ml of Emtricitabine 15µg/ml of Tenofovir Disoproxil Fumarate).

Inject 10μl of the sample solution into the chromatographic system and measure the area for the emtricitabine and Tenofovir disproxil fumarate peaks and calculate the % Assay by using the assay formula.

Preparation of Tablet Samples

Twenty tablets were weighed and powdered. An amount of tablet powder equivalent to 50 mg of TDF, and 15 mg of Emtricitabine was accurately weighed and transferred to 100 mL volumetric flask containing 70 mL of diluent. This mixture was subjected to sonication for 30 min to ensure complete extraction of drugs, made up to 100 mL with diluents, and filtered. From this solution, 1mL was taken and diluted to 10 mL with diluent to get final concentration of 50 g/mL TDF, and 15 g/mL Emtricitabine. Optimized chromatographic conditions are listed in Table 1.The chromatogram of TDF, and Emtricitabine standard is presented in Fig. 1.

Table 1: Optimized Chromatographic conditions.

Column Used	Discovery 150x4.6mm, 5µ
Buffer used	**(0.01N KH2PO4)**
Mobile phase	Buffer: Acetonitrile (60:40A)
Flow rate	1ml/min
Diluent	Water: ACN (50:50)
Wavelength	280nm
Temperature	30°C

Injection Volume	10µl

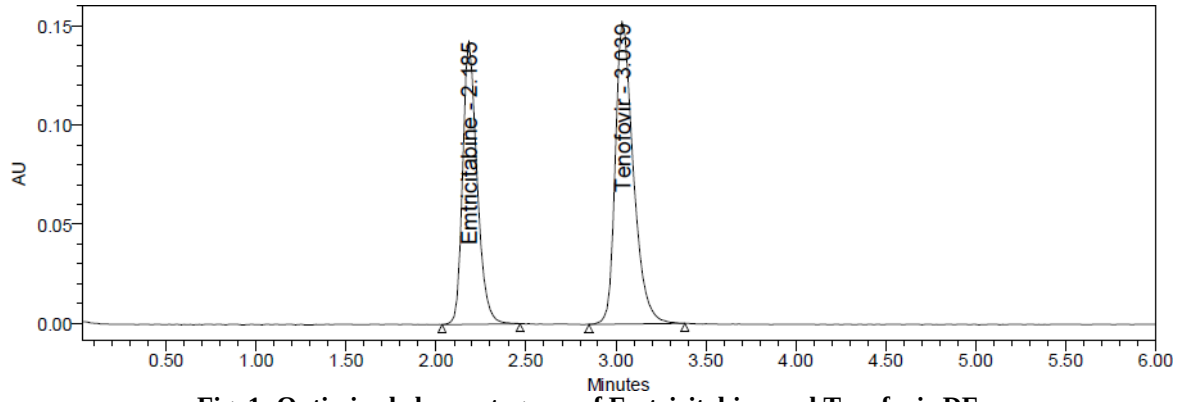

Fig. 1: Optimized chromatogram of Emtricitabine and Tenofovir DF.

3. METHOD VALIDATION

The method was validated according to ICH guidelines in terms of: Linearity, Accuracy, Precision, Limit of detection, Limit of quantification, Robustness and Stability indicating capability. Different trail runs were performed and selected the optimized trail for evaluation.

3.1. System suitability parameters

System suitability testing is performed to check the instrument performance, whether it is giving accurate results or not. In this test we check the Tailing factor and Plate count of analyte. System suitability was performed at the start of the method validation and on each day as a first experiment.

3.2. Linearity

The linearity of the method is determined by preparing three individual series of solutions in the range of Emtricitabine (2.5 – 15 µg/ml), and Tenofovir disproxil fumarate (3.75 – 22.5 µg/ml). The obtained peak areas are plotted against concentration.

3.2.1. Preparation of linearity solutions

25% Standard solution: 0.25ml each from two standard stock solutions was pipetted out and made up to 10ml. (2.5µg/ml of Emtricitabine 3.75µg/ml of Tenofovir Disoproxil Fumarate)

50% Standard solution: 0.5ml each from two standard stock solutions was pipetted out and made up to 10ml. (5µg/ml of Emtricitabine 7.5µg/ml of Tenofovir Disoproxil Fumarate)

75% Standard solution: 0.75ml each from two standard stock solutions was pipetted out and made up to 10ml. (7.5µg/ml of Emtricitabine 11.25µg/ml of Tenofovir Disoproxil Fumarate)

100% Standard solution: 1.0ml each from two standard stock solutions was pipetted out and made up to 10ml. (10µg/ml of Emtricitabine 15µg/ml of Tenofovir Disoproxil Fumarate)

125% Standard solution: 1.25ml each from two standard stock solutions was pipetted out and made up to 10ml. (12.5µg/ml of Emtricitabine 18.75µg/ml of Tenofovir Disoproxil Fumarate)

150% Standard solution: 1.5ml each from two standard stock solutions was pipetted out and made up to 10ml (15µg/ml of Emtricitabine 22.5µg/ml of Tenofovir Disoproxil Fumarate)

3.3. Precision

Precision of an analytical method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogenous sample. Precision of analytical method is usually expressed as the standard deviation and relative standard deviation.

3.3.1 Intraday precision (Repeatability)

The Relative standard deviation calculated for data obtained within one day is often called intraday precision (within one day). Intraday Precision was performed and % RSD for Emtricitabine and Tenofovir Disoproxil Fumarate were found to be 0.2% and 0.5% respectively.

3.3.2 Intermediate precision

Intermediate precision was performed with 24 hrs time lag and the %RSD Obtained for Emtricitabine and Tenofovir Disoproxil Fumarate was found to be 0.2% and 0.3%.

3.4. Accuracy

The accuracy of the analytical method was determined by applying the method to the analyzed samples, to which known amounts of analyte had been added. The accuracy was calculated from the test results as the percentage of analyte recovered by the assay.

3.4.1 Preparation of 50% spiked Solution: 0.5ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

3.4.2 Preparation of 100% spiked Solution: 1.0ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

3.4.3 Preparation of 150% spiked Solution: 1.5ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Three concentrations 50%, 100%, 150%, were injected in a triplicate manner and amount Recovered and % Recovery were displayed in Table No 5.

Acceptance Criteria

The % Recovery for each level should be between 98.0 to 102 .0%

3.5. Limit of detection and limit of quantification

Limit of detection (LOD) and limit of quantification (LOQ) of Emtricitabine, and Tenofovir disproxil fumarate were determined by calibration curve method. Solutions of Emtricitabine, and Tenofovir disproxil fumarate were prepared in linearity range and injected in triplicate. Average peak area of three analyses was plotted against concentration.

3.6. Method robustness

The Robustness of the developed method was determined by making small deliberate changes in flow rate (± 1 ml/min), column temperature (± 5%), organic mobile phase ratio (± 10%), along with the optimized method.

4. RESULTS

In order to develop and validate a chromatographic method for simultaneous quantification of TDF, and Emtricitabine by RP-HPLC, several trials were undertaken. Initially the drug solution was analyzed using a mixture of Water: Methanol (50:50A), Water: Acetonitrile (50:50A), OPA: Acetonitrile (50:50A), OPA: Acetonitrile (55:45A), at a flow rate of 1 mL/min, in which case the peak resolution and symmetry were not satisfactory. Several mobile phase compositions were tried, but the peak asymmetry and tailing were observed. Then, the buffer composition was changed and a mixture of Potassium diphosphate buffer: Acetonitrile (50:50A) at 50:50 v/v provided good peaks at a flow rate of 1 mL/min with detection at 280 nm. The retention times of TDF, and Emtricitabine were found to be 3.039, and 2.185 min respectively. The proposed method obeyed linearity for Emtricitabine in the range of 2.5 -15 µg/mL (r2 = 0.999) and for TDF within 3.75 – 22.5 µg/mL (r2 = 0.999). The LOD and LOQ value were found to be 0.05 and 0.5 g/mL for Emtricitabine and TFD, which indicate proper sensitivity of the proposed method.

The proposed method was also found to be specific, as the chromatogram (Fig. 2) obtained by injecting tablet solution showed no peaks close to the individual retention times of TFD, and Emtricitabine.

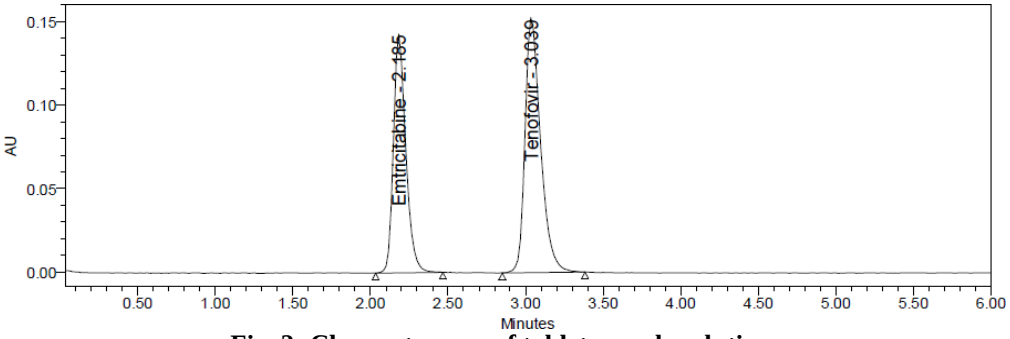

Fig. 2: Chromatogram of tablet sample solution.

The proposed method gave consistent results indicating its precision as observed from the %RSD data as well as the intermediate precision (Table 2 & Fig.3). Accuracy of the method was analyzed using recovery studies for the commercially available formulations. The percentage recovery values (Table 3 & Fig.4) were in the range of 99.26 to 99.91 % for TDF, and 99.25 – 99.91 % for Emtricitabine, which indicate that there are no interferences from excipients in formulation.

Table 2: Results of precision of Emtricitabine and TDF.

Parameter	Intraday Precision		Intermediate Precision	
	Emtricitabine	Tenofovir	Emtricitabine	Tenofovir
Area	791439	1189645	758990	1063132
	792185	1188902	759006	1070542
	791689	1176501	758185	1069492
	790401	1177475	759053	1067004
	793967	1179384	756701	1069208
	791152	1179420	756508	1061494
Mean	791806	1181888	758074	1066812

Std. Dev.	1213.6	5834.8	1184.6	3706.7
%RSD	0.2	0.5	0.2	0.3

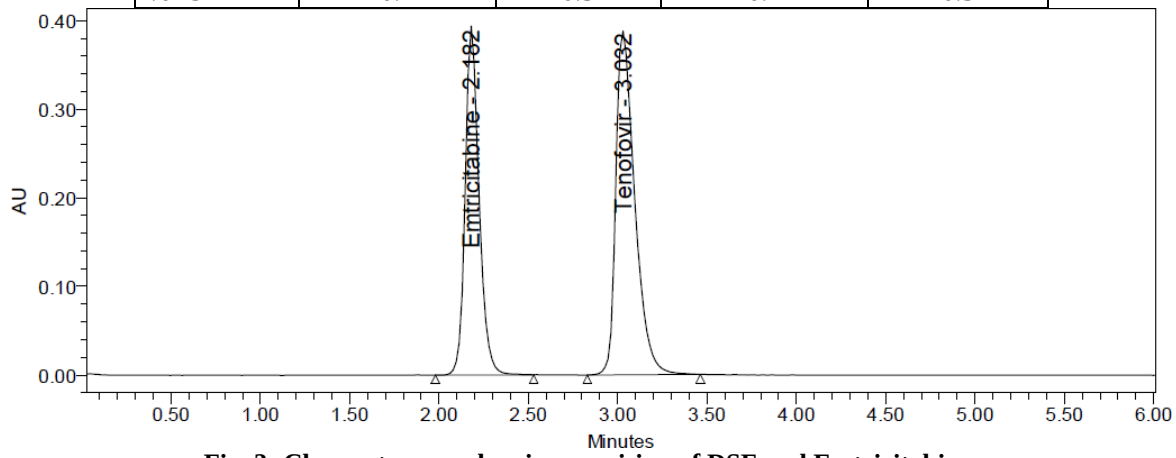

Fig. 3: Chromatogram showing precision of DSF and Emtricitabine.

Table 3: Accuracy of Emtricitabine and Tenofovir Disproxil fumarate.

	Emtricitabine			TDF		
% Level	**Amount Spiked (µg/mL)**	**Amount recovered (µg/mL)**	**% Recovery**	**Amount Spiked (µg/mL)**	**Amount recovered (µg/mL)**	**% Recovery**
50%	5	4.96258547	99.25	7.5	7.45623579	99.42
	5	4.97420978	99.48	7.5	7.45362262	99.38
	5	4.98672429	99.73	7.5	7.4442881	99.26
100%	10	9.95133531	99.51	15	14.9940073	99.96
	10	9.98247968	99.82	15	14.981331	99.88
	10	9.97244227	99.72	15	14.9803133	99.87
150%	15	14.8933944	99.29	22.5	22.4805206	99.91
	15	14.9603535	99.74	22.5	22.4513738	99.78
	15	14.9857567	99.91	22.5	22.4307198	99.69
Mean % Recovery	99.61			99.68 %		

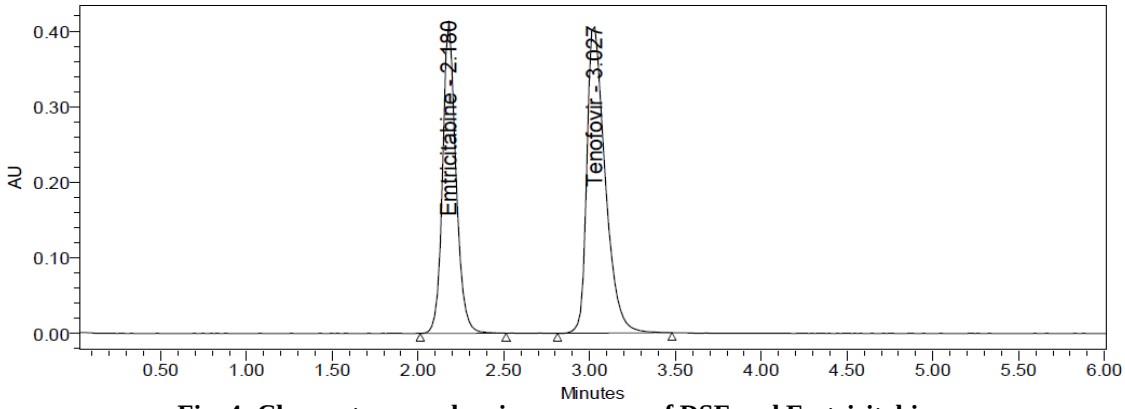

Fig. 4: Chromatogram showing accuracy of DSF and Emtricitabine.

Small deliberate variations in the chromatographic conditions such as flow rate and mobile phase composition did not produce significant effect on the parameters like tailing factor (<1.2) and number of theoretical plates (>2000). These data was presented in table 4 and Fig.5.

Table 4: Result of System Suitability study.

S.No	Emtricitabine			Tenofovir disproxil fumarate			
Inj.	**RT (min)**	**USP Plate Count**	**Tailing**	**RT (min)**	**USP Plate Count**	**Tailing**	**USP Resolution**
1	2.183	3423	1.23	3.038	4403	1.35	5.1
2	2.183	3446	1.22	3.039	4362	1.34	5.1
3	2.184	3440	1.23	3.039	4420	1.36	5.1
4	2.185	3449	1.23	3.041	4359	1.34	5.1

5	2.192	3418	1.23	3.042	4390	1.37	5.1
6	2.185	3418	1.24	3.043	4422	1.37	5.1

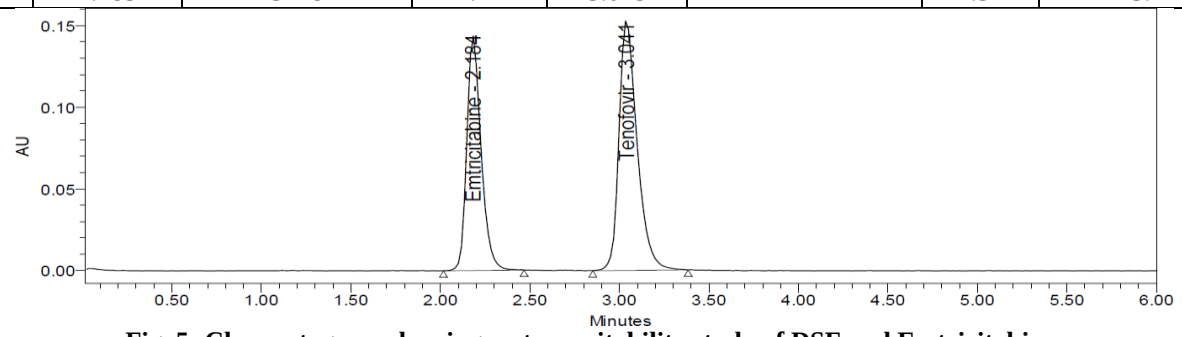

Fig. 5: Chromatogram showing system suitability study of DSF and Emtricitabine.

Analysis of the marketed formulation by the proposed method

TDF (300 mg) and Emtricitabine (200mg) in marketed tablets were analyzed using assays, and the results showed good agreement with the contents as stated. The marketed formulation contained metformin hydrochloride. Since there was no evidence of excipient interaction with the peaks of interest, and the suggested method might be effectively used to estimate TDF and Emtricitabine simultaneously in a combination tablet dose form. Table 5 and Fig. 6 presents the assay findings.

Table 6: Estimation of TDF & Emtricitabine in marketed formulation.

Drug	Label Claim	Amount found	% Assay
DSF	300 mg	299.16 mg	99.72
Emtricitabine	200 mg	198.26 mg	99.13

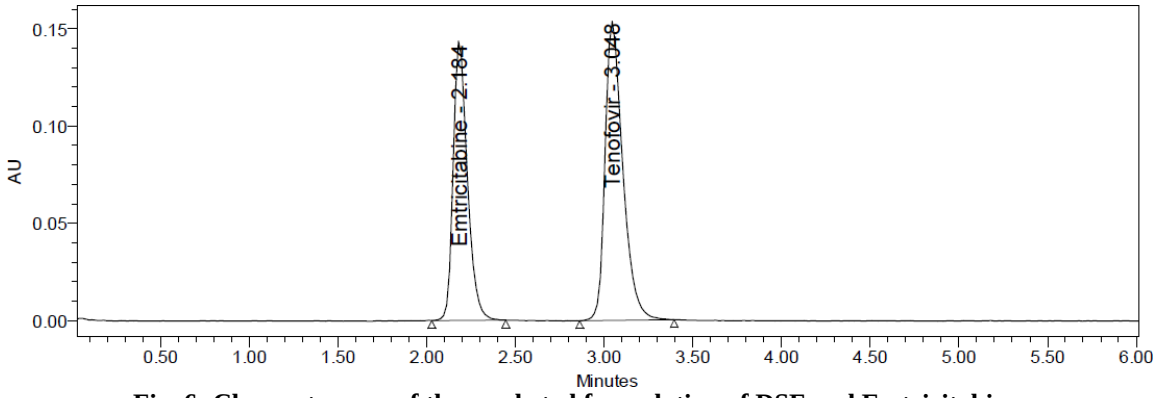

Fig. 6: Chromatogram of the marketed formulation of DSF and Emtricitabine.

6. DISCUSSION

As there is a developing interest for anti-HIV drugs, it is also necessary to develop a rapid, sensitive and robust analytical method. The statistical investigation of obtained information demonstrated that the proposed RP-HPLC method was accurate, linear, robust and economical. The optimized method is appropriate for determining of pharmaceutical drugs in the marketed formulation with virtually no interferences of excipients. Forced degradation data also confirmed that the degradation of three drugs was within the acceptable range. Hence, the method can be effectively applied to the quality control analysis.

In conclusion, this study was aimed at the quantification of TDF, and Emtricitabine in bulk and tablets. The developed method was found to be linear, accurate, robust and reliable. An evident advantage is the simplicity of sample preparation and speed of analysis, since the three compounds were eluted within 10 minutes. The RP-HPLC method also indicated the stability of drugs under various stress conditions, so it can also be employed as stability indicating method. Hence, the developed method can be used for routine analysis in quality control laboratories and for further research.

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