A study on the development and validation of RP-HPLC method for the simultaneous estimation of Emtricitabine, Tenofovir Disoproxil Fumarate, and Efavirenz in both bulk and pharmaceutical formulations.

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ABSTRACT

An RP-HPLC method was developed and validated for the simultaneous quantification of Emtricitabine, Tenofovir Disoproxil Fumarate, and Efavirenz in both bulk and pharmaceutical formulations. The chromatographic separation of Emtricitabine, Tenofovir Disoproxil Fumarate and Efavirenz was achieved on a YMC PACK ODS AQ (150 mm x 4.6 mm ID, 5 µm), Waters Alliance HPLC system equipped with 2998 Photo Diode Array (PDA) detector and Empower 2 software. The optimized mobile phase was consisted of potassium dihydrogen phosphate buffer (pH adjusted to 4.5 with OPA) and Acetonitrile (40:60, v/v). The flow rate was 1mL / min and effluents were monitored at 257nm. Emtricitabine, Tenofovir Disoproxil Fumarate, and Efavirenz were found to have retention times of 2.185 min, 4.848 min, and 15.682 min, respectively. The resolution values for the two adjacent peaks were determined to be 4.15 and 12.08. The linearity was found in the concentration range of 35-65ppm with correlation coefficient was 0.999. The regression equation of Emtricitabine, Tenofovir Disoproxil Fumarate and Efavirenz was found to be Y = 31987x + 2256, Y = 17161x + 4100 and Y = 54966x + 9250 respectively. LOD values were found to be 0.24ppm for Emtricitabine, 0.78ppm for Tenofovir Disoproxil Fumarate and 0.56ppm for Efavirenz and LOQ values were found to be 0.72ppm for Emtricitabine, 2.37ppm for Tenofovir Disoproxil Fumarate and 1.68ppm for Efavirenz. The percentage recoveries were between 99.64 %-99.97 % for Emtricitabine, 100.04 %-100.08 % for Tenofovir Disoproxil Fumarate and 99.92 %-100.05 % for Efavirenz respectively. The proposed method was effectively employed to quantitatively assess Emtricitabine, Tenofovir Disoproxil Fumarate, and Efavirenz in bulk and pharmaceutical formulations in accordance with the ICH guidelines.

KEY WORDS: Emtricitabine, Tenofovir Disoproxil Fumarate and Efavirenz, RP-HPLC, PDA detector, ICH guidelines.

1. INTRODUCTION

Human Immunodeficiency Virus type-1 (HIV-1) cripples the immune system, making the host vulnerable to pathogenic agents, culminating in acquired immunodeficiency syndrome (AIDS), a life-threatening, chronic ailment. HIV-1 targets vital components of the immune response, such as macrophages, dendritic cells, and T-lymphocytes, particularly the CD4+ T cells. Emtricitabine (EMT), Tenofovir Disoproxil Fumarate (TDF), and Efavirenz (EFV) were developed to combat HIV and manage AIDS by addressing these issues. EMT is a powerful antiviral drug that is a chemically synthesized fluoro analogue of thiacytidine. TDF is a derivative of AMP with antiviral properties [1, 2]. An NNRTI drug, EFV is prescribed in the HAART protocol for treating HIV-1. EMT, TDF, and EFV combination drug is safe, effective, and provides quick and intense action against HIV, according to clinical studies. Therefore, this combination has been given clinical approval for managing AIDS and HIV-1. In the present investigation, a unique RP-HPLC technique for the concurrent quantification of EMT, TDF, and EFV in drug formulations has been developed and validated [3]. The above-mentioned drug IUPAC names, structures, physico-chemical characteristics, and pharmacological classification are listed in Tables 1 and 2.

Table 1: Chemical structures of EMT, TDF and EFV [4, 5, 6]

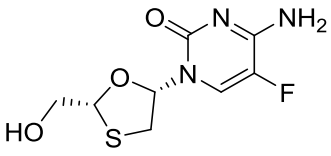
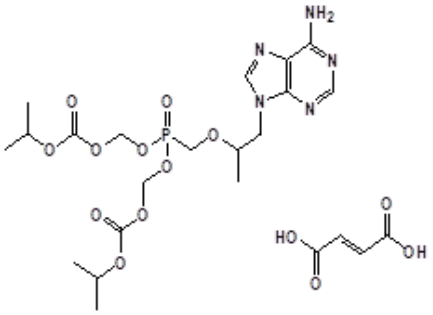
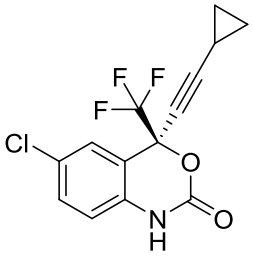
Drug	IUPAC Name	Structure
EMT	4-amino-5-fluoro-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl] pyrimidin-2-one	
TDF	[[(2R)-1-(6-aminopurin-9-yl) propan-2-yl]oxymethyl-(propan-2-yloxycarbonyloxymethoxy) phosphoryl]oxymethylpropan-2-yl carbonate; (E)-but-2-enedioic acid	
EFV	**(**4S)-6-Chloro-4-(2-cyclopropylethynyl)-4-(trifluoromethyl)-2,4-dihydro-1H-3,1-benzoxazin-2-one	

Table 2: Physico-chemical properties of EMT, TDF and EFV[7]

Properties		EMT	TDF	EFV
Chemical formula		$C_8H_{10}FN_3O_3S$	$C_{23}H_{34}N_5O_{14}P$	$C_{14}H_9ClF_3NO_2$
Molecular mass		247.24g/mol	635.52g/mol	315.675 g/mol
Appearance		White to off-white crystalline powder		
Smell		No odor		
Taste		Bitter		
State		Crystalline form		
Solubility	Soluble in	H_2O, MeOH	MeOH and dimethyl formamide	MeOH, ACN, dimethyl sulfoxide, chloroform,
	Insoluble in	methylene chloride	H_2O	H_2O

2. MATERIALS AND METHODS

2.1. Reagents

Emtricitabine, tenofovir disoproxil fumarate, and efavirenz were bought from Hetero Drugs Ltd., Hyderabad, India. ACN and methanol of HPLC quality were purchased from SD Fine Chem. Ltd., India. The source of the water was Milli-Q. Ortho-phosphoric acid (OPA), potassium dihydrogen ortho-phosphate (PDP), and ammonium acetate were purchased from Merck Ltd., India.

2.2. Experimental procedure

Using a HPLC system (Waters Alliance) coupled with a 2998 PDA detector and Empower 2 software for data collection and processing, the separation technique was carried out. As the stationary phase, YMC PACK ODS AQ (150 mm x 4.6 mm ID, 5 µm) was employed. The investigation makes use of a semi-micro weighing scale (India), Whatman no. 42 filter paper, and an ultrasonic bath sonicator (Frontline FS 4, Mumbai, India).

2.3. Preparation of mobile phase

3.4g of PDP was carefully weighed and poured into 1000 ml reagent bottle containing 900 ml of Milli Q grade water [8, 9]. The contents were shaken and volume made with water. pH of the buffer was brought to 4.5 with OPA. The buffer was then filtered using 0.45µm pore size filter paper before use and mixed with HPLC grade Acetonitrile with 40:60, v/v[10].

2.4. Diluent

Diluent consisted of equal concentrations of methanol and water (50:50, v/v).

2.5. Standard solution preparation

50 mg each of EMT, TDF, and EFVwas taken in a 100ml flask & dissolved in diluent (MeOH: H_2O=1:1). Sonicate for 5 min and volume was adjusted to 100ml. Then 5ml of this solution is transfer to 50 ml flask and volume was brought up with a dilutant. The mixture so obtained was filtered using 0.45µm pore size filter units before use [11].

2.6. Preparation of linearity solutions

For assay methods, preparing standard solutions at seven-concentration level from 70 to 130% of the target analyte concentrations generally performs this study and was evaluated by linear regression analysis [12, 13].

Procedure: 6 repeated injections of above-prepared standard solution containing 50 ppm were administered to initially test the system suitability. Subsequently, injections of reference standard solutions at various concentrations—70 to 130% of the drug substance—were loaded (35ppm, 40ppm, 50ppm, 60ppm, 65ppm). To verify the linearity of the sample in the aforementioned concentration levels, the correlation coefficient (r^2) was computed after each solution was administered in duplicate.

2.7. Preparation of sample solution

20 Trustiva® tablets were each weighed separately, and their average weight was determined. Using this calculation, each tablet's weight was established. The required concentration was prepared, filtered using a 0.45 µm membrane filter, and the quantity was then brought up to the desired level using the mobile phase[14,15].

3. RESULTS AND DISCUSSIONS

Chromatograms for the four trials are depicted in Figures 1-4.

3.1.1. TRAIL-1

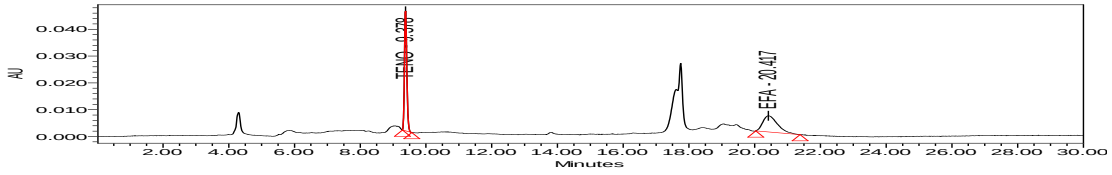


Figure 1: Chromatogram for Trial-1

Stationary Phase	: Kromacil (250*4.6) mm
Mobile phase	: Phosphate Buffer (pH3.0): MeOH-40:60, v/v
Detection	: 250nm
Flow rate	: 1.0 ml/min
Sample size	: 20µl
Column Temp.	: 40ºC
Dilutant	: MeOH: H_2O (1:1)

3.1.2. TRAIL-2

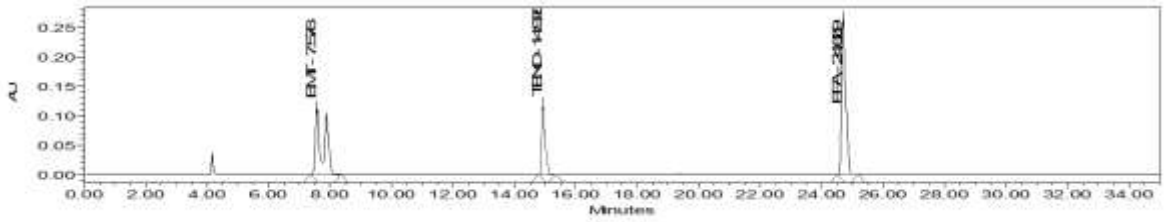


Figure 2: Chromatogram for Trial-2

Stationary Phase	: Kromacil (250*4.6) mm
Mobile phase	: Phosphate Buffer (pH3.0): ACN-60:40, v/v
Detection	: 250nm
Flow rate	: 1.0 ml/min
Sample size	: 20µl
Column Temp.	: 40ºC
Dilutant	: MeOH: H_2O (1:1)

3.1.3.TRAIL-3

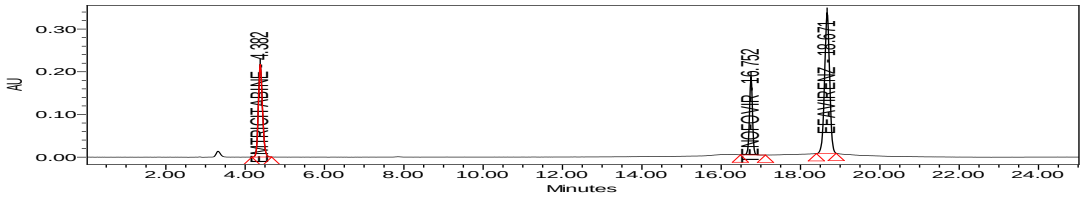


Figure 3: Chromatogram for Trial-3

Stationary Phase	: Kromacil (250*4.6) mm
Mobile phase	: Phosphate Buffer (pH 3.0): MeOH-30:70, v/v
Detection	: 250nm
Flow rate	: 1.0 ml/min
Sample size	: 20µl
Column Temp.	: 40ºC
Dilutant	: MeOH: H_2O (1:1)

3.1.4. TRAIL-4 (OPTIMIZED)

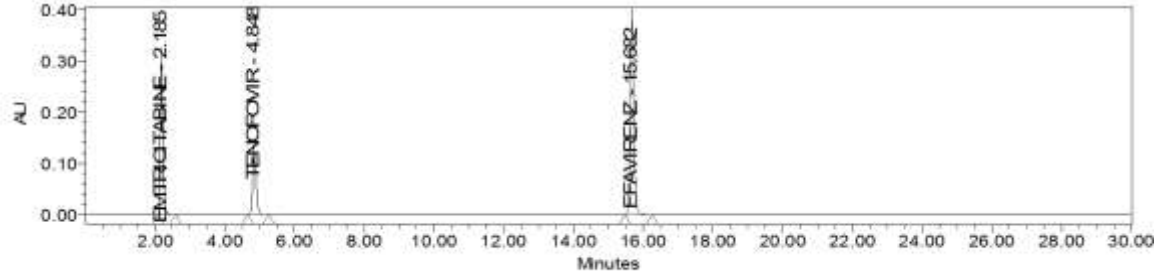


Figure 4: Chromatogram for Trial-4

Stationary Phase : YMC PACK ODS AQ (150 mm x 4.6 mm ID, 5 µm)
Mobile phase : PDP: ACN (40:60, v/v).
Detection : 257nm
Flow rate : 1.0 ml/min
Sample size : 20µl
Column Temp. : 40ºC
Dilutant : MeOH: H_2O (1:1)

3.2. Assay of standard and sample solution

Assay % was determined by analyzing the peak areas of the sample and standard chromatograms, and results are presented in Table 3 for EMT, TDF, and EFV. A dilution of 20 µL of sample and standard solution containing 50 ppm of each drug was pumped into the HPLC column for 6 times.

Table 3: Assay studies findings

Drug	Trustiva® Label claim (mg/tablet)	Amount detected* (mg/tablet)	Label claim %	RSD %
EMT	200	200.36	100.18	0.2
TDF	300	299.45	99.82	0.2
EFV	600	599.95	99.99	0.3

* Mean of six determinations

3.3. Method optimization

Numerous variables and mobile phase ratios were tested to optimize the proposed technique. Using YMC PACK ODS AQ (150 mm x 4.6 mm ID, 5 µm), an acceptable separation and better peak symmetry for EMT, TDF, and EFV were achieved. To improve reliability and validity, a mobile phase comprising a blend of PDP: ACN (40:60, v/v) was supplied at

1mL/min. Using a PDA detector, the drugs were examined in the 200–400 nm wavelength range. Figure 5 depicts the findings obtained using a PDA detector at 257 nm. EMT, TDF, and EFV all had retention times (RT) that were discovered to be 2.185, 4.848, and 15.682 minutes, correspondingly. Figure 6 depicts the chromatograms of EMT, TDF, and EFV sample and standard solutions.

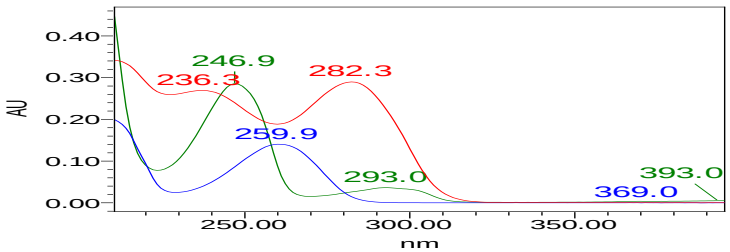


Figure 5: UV spectra of EMT, TDF, and EFV

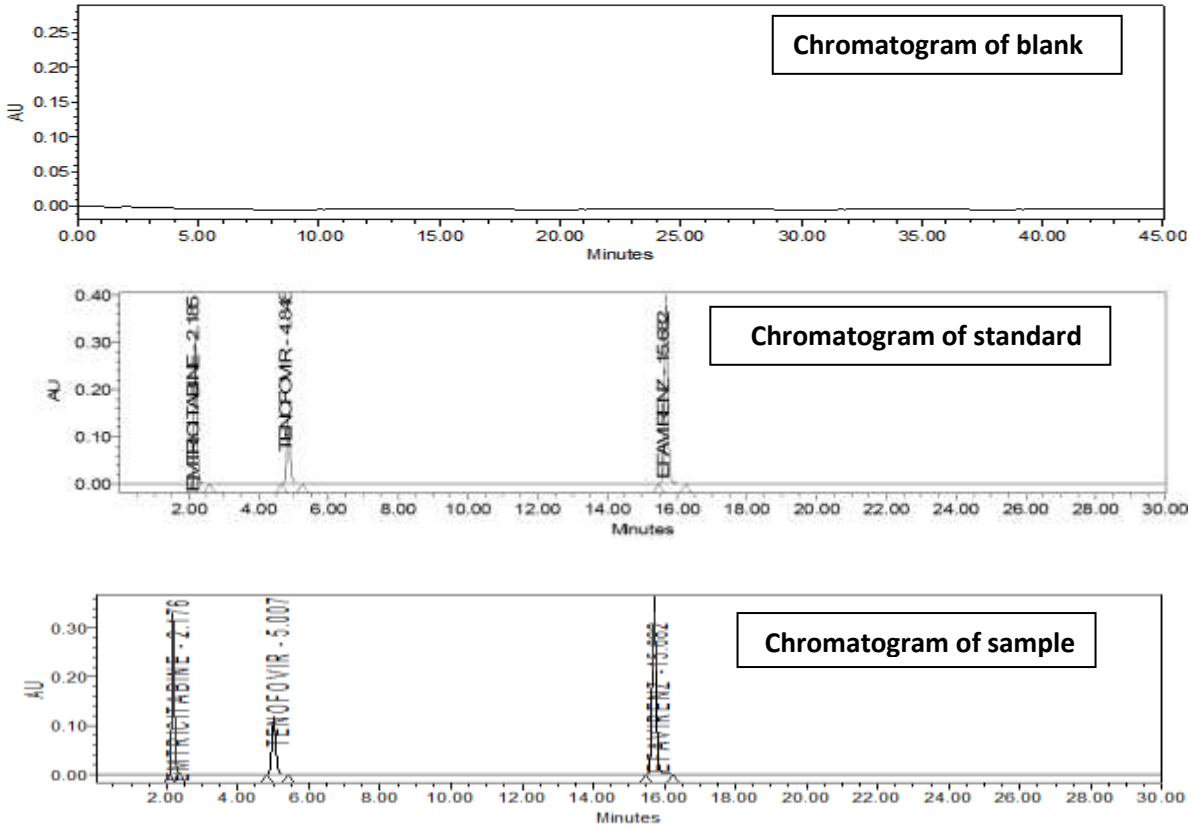


Figure 6: Chromatogram of blank, standard and sample solutions of EMT, TDF, and EFV

3.4. Method validation

To ensure that the proposed RP-HPLC technique is reliable for effective quality control screening in laboratories, it was validated in accordance with ICH guideline Q2 (R1).

3.4.1. System suitability

The findings for the system suitability (Table 4) of the devised technique for the concurrent quantification of emtricitabine, tenofovir disoproxil fumarate, and efavirenz are acceptable, and all outcomes were compliant within the allowable levels, therefore verifies the system suitability of the developed technique.

3.4.2. Specificity

Investigation on the impact of adjuvants often found in the mixed dose form of EMT, TDF, and EFV on the analysis at ideal circumstances has revealed no influence. Loading of the placebo solution into the apparatus proved the specificity of the developed procedure. Figure 7 displayed the typical chromatogram of the placebo.

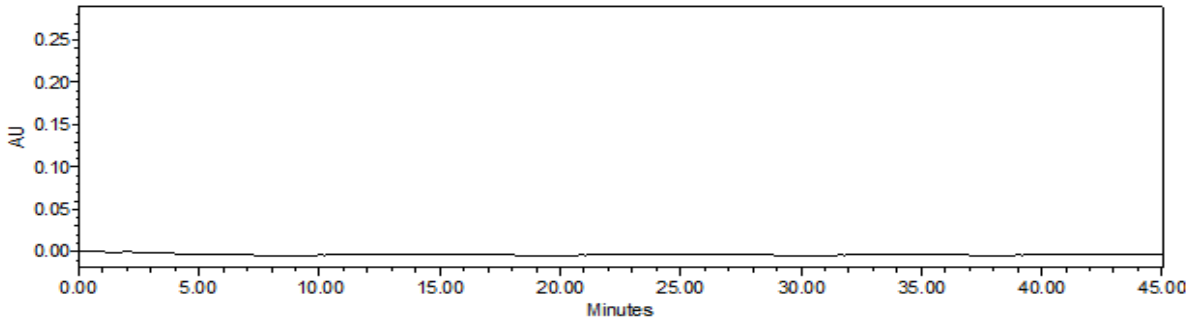


Figure 7: Chromatogram of placebo for EMT, TDF, and EFV

Table 4: System suitability parameters of EMT, TDF, and EFV

Parameters	EMT	TDF	EFV	Allowable limit
RT (min)	2.185	4.848	15.682	-----
Theoretical plates (N)	2457	3348	5973	Above 2000
Asymmetry factor	1.01	1.02	1.01	Below 2
Resolution		4.15	12.08	Above 2

3.4.3. Linearity

6 repeated injections of above-prepared standard solution containing 50 ppm were administered to initially test the system suitability. Subsequently, injections of reference standard solutions at various concentrations—70 to 130% of the drug substance—were loaded (35ppm, 40ppm, 50ppm, 60ppm, 65ppm). To verify the linearity of the sample in the aforementioned concentration levels, the pearson correlation (r^2) was determined, as can be seen in Figure 8, and the regression analysis was performed. The findings are reported in Tables 5 and 6.

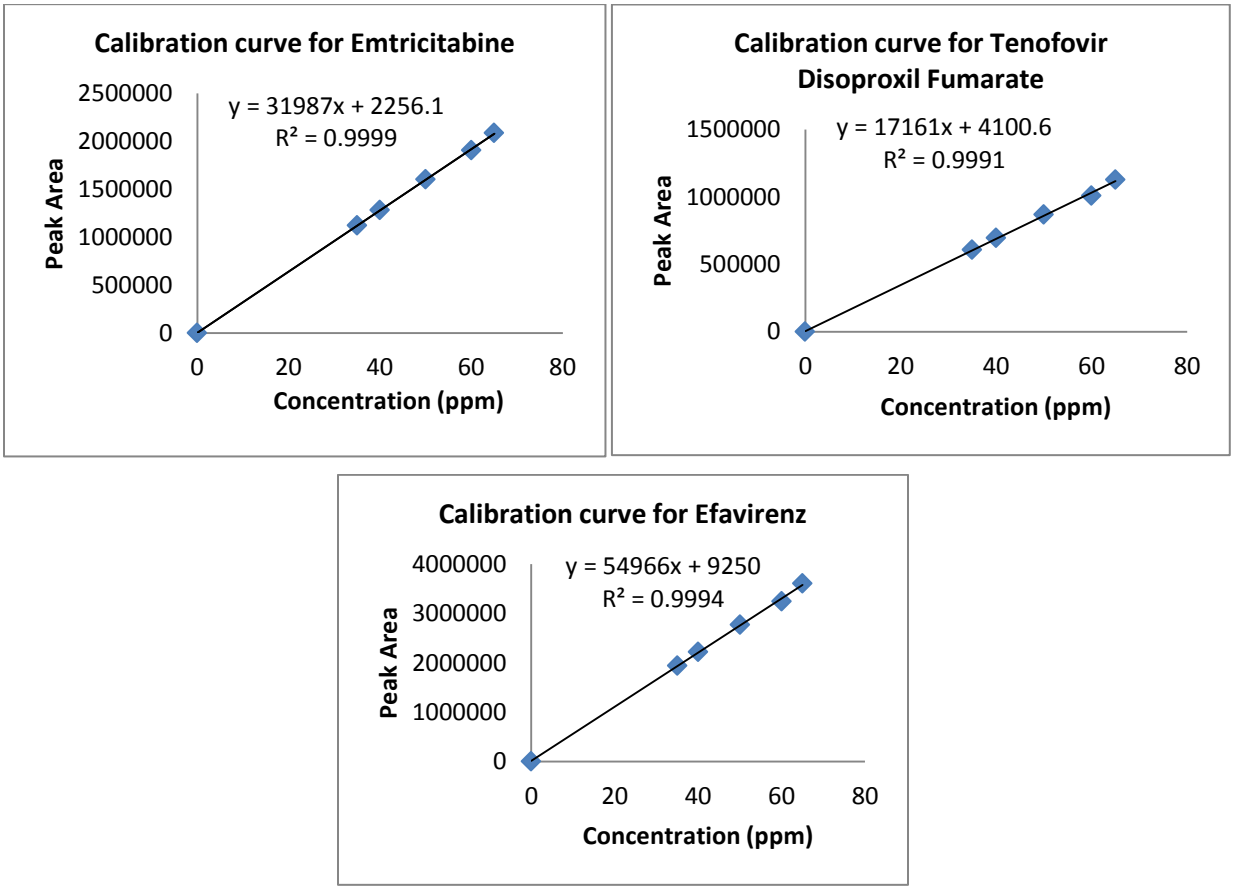


Figure 8: Standard calibration curves of EMT, TDF, and EFV

Table 5: Linearity of Emtricitabine, Tenofovir Disoproxil Fumarate and Efavirenz

EMT Conc. (ppm)	Peak Area	TDF Conc. (ppm)	Peak Area	EFV Conc. (ppm)	Peak Area
35	1124250	35	608964	35	1943050
40	1284857	40	695959	40	2220629
50	1606071	50	869949	50	2775786
60	1907285	60	1010939	60	3248943
65	2087892	65	1128934	65	3608522

Table 6: Optical and regression parameters of EMT, TDF, and EFV

Optical and regression parameters	Emtricitabine	Tenofovir Disoproxil Fumarate	Efavirenz
Detection wavelength (nm)	257		

Linearity range (ppm)	35-65		
Regression Equation (y=mx+C)	31987x + 2256	17161x + 4100	54966x + 9250
Slope (m)	31987	17161	54966
Intercept (C)	2256	4100	9250
r^2	0.999	0.999	0.999
LOD (ppm)	0.24	0.78	0.56
LOQ (ppm)	0.72	2.37	1.68

3.4.4. Accuracy

By using the standard addition approach, the respective recoveries of emtricitabine, tenofovir disoproxil fumarate, and efavirenz were calculated in order to assess the suggested method's accuracy. Recovery trials were conducted by mixing standard solutions of emtricitabine, tenofovir disoproxil fumarate, and efavirenz at concentrations of 50%, 100%, and 150% to the pre-analyzed solution of Trustiva® powder. The findings for accuracy of the suggested approach are shown in Table 7.

Table 7: Results of accuracy studies of EMT, TDF, and EFV

Accuracy findings of Emtricitabine						
Conc. (%)		**Quantity added (ppm)**	**Amount recovered (ppm)**	**% Recovery**	**% Mean Recovery**	**RSD %**
50%	S_1	25	24.87	99.88	99.87	0.18
	S_2	25	24.62	99.68		
	S_3	25	25.51	99.04		
100%	S_4	50	50.22	99.04	99.97	0.10
	S_5	50	49.23	99.86		
	S_6	50	50.71	99.02		
150%	S_7	75	74.67	100.69	99.64	0.12
	S_8	75	74.83	100.51		
	S_9	75	74.39	100.72		
Accuracy findings of TDF						
Conc (%)		**Quantity added (ppm)**	**Amount recovered (ppm)**	**% Recovery**	**% Mean Recovery**	**RSD %**
50%	S_1	25	25.25	100.20	100.04	0.28
	S_2	25	24.43	100.72		
	S_3	25	25.55	99.20		
100 %	S_4	50	50.63	99.26	100.08	0.17
	S_5	50	49.86	100.92		
	S_6	50	50.83	100.06		

150 %	S_7	75	75.24	99.05	100.05	0.03
	S_8	75	75.56	100.08		
	S_9	75	75.41	100.01		
Accuracy findings of Efavirenz						
Conc (%)		**Quantity added (ppm)**	**Quantity recovered (ppm)**	**% Recovery**	**% Mean Recovery**	**RSD %**
50%	S_1	25	25.15	100.20	100.05	0.14
	S_2	25	24.88	100.92		
	S_3	25	25.11	99.04		
100 %	S_4	50	50.37	99.14	99.92	0.20
	S_5	50	49.77	100.74		
	S_6	50	49.84	100.88		
150 %	S_7	75	75.26	99.08	99.96	0.17
	S_8	75	75.32	99.03		
	S_9	75	74.62	100.76		

3.4.5. Precision

Using sample solution of Trustiva® powder from one batch, method precision was conducted by pumping into the column a homogeneous sample preparation containing 50 ppm of EMT, TDf, and EFV for 6 times. This was done to check that the proposed technique was operating correctly. Table 8 presents the method precision findings. The technique's riggedness was determined by injecting six times of 50 ppm of EMT, TDF, and EFV into the HPLC system. This procedure was done to conduct precision on within-laboratory changes, including various days and separate researchers. Table 9 presents the findings of ruggedness.

Table 8: Method precision of EMT, TDF, and EFV

Concentration (ppm)	**EMT % Assay**	**TDF % Assay**	**EFV % Assay**
50	99.18	99.89	100.01
50	99.62	99.87	99.98
50	100.01	100.01	100.01
50	100.01	100.06	100.04
50	99.96	99.97	99.84
50	100.01	100.02	99.98
Average	99.80	99.97	99.98
SD	0.34	0.08	0.07
RSD %	0.34	0.08	0.07

Table 9: Ruggedness of EMT, TDF, EFV

Conc. (ppm)	Peak Area		
	EMT	**TDF**	**EFV**
50	1607834	869883	2774527
50	1606622	869867	2777381
50	1606071	869453	2778377
50	1608942	869923	2778283
50	1608933	869361	2773847
50	1605692	869672	2778465
Average	1607349	869693	2776813
SD	1427.54	239.81	2082.39
RSD %	0.09	0.03	0.07

3.4.6. LOD & LOQ

The analyte's least concentration in a given sample which is noticeable and not measurable is known as the limit of detection (LOD). LOD is referred to as a quantity at a given signal-to-noise ratio (SNR), which is commonly 3:1. Limit of quantitation (LOQ) is analyte's least concentration in a given sample that may be detected with appropriate precision and accuracy utilizing the technique's specified active parameters. The ICH approved an SNR of 10:1 for LOQ. According to the calculations, detection and quantitation can alternatively be computed using the following equations and the findings for LOD and LOQ of EMT, TDF, and EFV were tabulated in Table 6.

$$LOQ = 3.3 \times \frac{\sigma}{S}$$

$$LOQ = 10 \times \frac{\sigma}{S}$$

Where σ is S.D. of the response
S is the curve's slope

3.4.7. Robustness

To test the technique's robustness, the mobile phase's makeup was purposefully changed, with the amount of organic phase being increased by 10% and the flow rate decreased by 0.1 mL. The outcomes of robustness were noted in Tables 10, 11, and 12, which guarantee that the established technique would not be influenced by relatively minor or intentional changes in technique's parameters and provides evidence of its performance under regular usage. There were no significant disparities in the system suitability findings.

Table 10: Robustness findings for EMT

Variations in parameters		RT (min)	Avg. peak area*	RSD %	System suitability findings	
					Theoretical Plates	Asymmetry
Buffer: ACN	34:66,	2.375	1610746	0.02	2847	1.04

	v/v					
	46:54, v/v	2.174	1605763	0.06	2783	1.02
Flow Rate (mL/min)	0.9	2.891	1582258	0.01	2954	1.06
	1.1	2.176	1577969	0.05	2645	1.03

* Average of 6 values

Table 11: Robustness data of TDF

Variations in parameters		**RT (min)**	**Avg. peak area***	**RSD %**	**System suitability findings**	
					Theoretical Plates	**Asymmetry**
Buffer: ACN	34:66, v/v	4.926	967508	0.09	3772	1.02
	46:54, v/v	4.382	967584	0.08	3429	1.04
Flow rate (mL/min)	0.9	5.007	868235	0.05	3253	1.04
	1.1	4.699	848512	0.02	3227	1.05

* Average of 6 values

Table 12: Robustness data of Efavirenz

Variations in parameters		**RT (mins)**	**Avg. peak area***	**RSD %**	**System suitability findings**	
					Theoretical Plates	**Asymmetry**
Buffer: ACN	34:66, v/v	14.417	2778110	0.15	5468	1.01
	46:54, v/v	15.007	2794379	0.09	5298	1.02
Flow rate (mL/min)	0.9	16.752	2782502	0.10	5842	1.04
	1.1	14.875	2861014	0.06	5739	1.06

* Average of 6 values

4. SUMMARY AND CONCLUSION

According to ICH recommendations, the current RP-HPLC technique was developed and validated for the quantitative detection of EMT, TDF, and EFV in bulk as well as therapeutic dose forms. This approach was designed for quick and precise quantification with strong chromatographic peak separation without any detectable interference peaks. The establishment of the RP-HPLC technique for the concurrent quantification of EMT, TDF, and EFV utilized various marketed columns and several mobile phase ratios. The YMC PACK

ODS AQ (150 mm x 4.6 mm ID, 5 µm) provided the optimum separation; the mobile phase entailing PDP buffer (pH set to 4.5 using OPA): ACN (40:60, v/v), was supplied at a 1 mL/min flow rate on a HPLC system (Waters Alliance) coupled with a 2998 PDA detector. Depending on the peak area, separation was achieved at 257 nm. EMT, TDF, and EFV had retention times of 2.185 min, 4.848 min, and 15.682 min, correspondingly, with resolutions of 4.15 min and 12.08 min.EMT, TDF, EFV all showed linearity in the 35-65 ppm range having r^2 value of 0.999, and their corresponding % recoveries ranged from 99.64% to 99.97%, 100.04% to 100.08%, and 99.92% to 100.05%. EMT, TDF, EFV had RSD% estimates below 2%, indicating accuracy of the suggested approach. EMT, TDF, EFV, correspondingly, had RSD% readings of 0.34%, 0.08%, and 0.07% for method precision. The suggested approach is accurate, as shown by the RSD% estimates of ruggedness that were discovered to be 0.09% for EMT, 0.03% for TDF, and 0.07% for EFV, correspondingly. LOD findings for the drugs were reported to be 0.24ppm for Emtricitabine, 0.78ppm for Tenofovir Disoproxil Fumarate and 0.56ppm for Efavirenz and LOQ findings for the drugs were reported to be 0.72ppm for Emtricitabine, 2.37ppm for Tenofovir Disoproxil Fumarate and 1.68ppm for Efavirenz.The RSD% estimates of the robustness analyses were determined to be below 2%, indicating that the presented approach is robust. The results demonstrate that the presented technique was precise and reliable for estimating EMT, TDF, and EFV simultaneously in bulk and therapeutic dosage forms. The established approach is uncomplicated, sensitive, efficient, linear, specific, precise, rugged, reliable, and unique. The monitoring and evaluation of these drugs in commercial and pharmaceutical forms may thus be done using the RP-HPLC technique.

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