Original Article

A Validated Rp-Hplc Method For Simultaneous Determination Of Lamivudine, Tenofovir Disoproxil Fumarate And Efavirenz In Tablet Formulation

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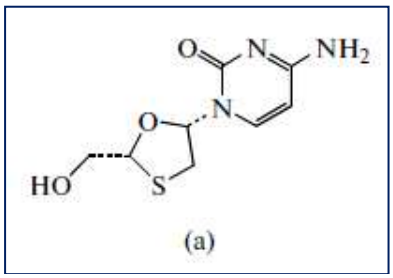
Abstract

A simple, rapid, specific, accurate, robust and reproducible Reverse phase HPLC method was developed and validated for the estimation of Emtrictabine, Tenofovir and Efavirenz in pure and pharmaceutical dosage forms. The quantification was carried out using Inertsil ODS-3V column C_{18} (250×4.6mm, 5µm) with mobile phase composing of Acetonitrile: 1%IPA in 80:20%v/v at flow rate 1ml/min, detection was carried out at 256nm using PDA detector with injection volume 20µl, the retention time was found to be 2.4, 2.8 and 5.2. The proposed method was validated as per ICH Q2B guidelines. The method produced linear response in the concentration ranges of 5-25µg/ml (R^2= 0.999), 7.5-37.5µg/ml (R^2= 0.999) and 15-45µg/ml (R^2= 0.999). The recovery studies were carried out and found to be within 98-102%. The %RSD was found to be within limit. LOD &LOQ of method was found to be within limit. The proposed method was statistically evaluated and can be applied for the routine analysis and quality control of raw materials, formulation for Emtricitabine, Tenofovir and Efavirenz.

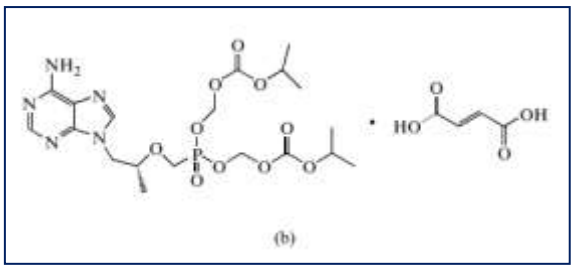
Keywords: Emtricitabine, Tenofovir and Efavirenz, RP-HPLC, ICH Q2B Guidelines.

INTRODUCTION

Lamivudine (LAM), chemically (2R,5S)4amino 1[2(hydroxylmethyl)1,3oxathiolan5yl]2(1H) pyrimidinone [1] is converted intercellularly in stages to the triphosphate. This triphosphate halts the DNA synthesis of retroviruses, including HIV, through competitive inhibition of reverse transcriptase and incorporation into viral DNA[2]. Tenofovir disoproxil fu marate (TDF) is converted inter cellular to the diphosphate that halts the DNA synthesis of HIV through the same mechanism. Chemically it is a bis (iso propyloxy) carbonyloxymethyl ester of (R)9 (2 phosphono-methoxypropyl) adenine with fumaric acid. Efavirenz (EFV) is chemically designated as (4S) 6 chloro4 (cyclopropylethylnyl) 1,4dihydro 4 (trifluoro methyl) 2H3, 1benzoxazin2one [1]. HIV Protease inhibitors are antiretroviral that act by binding reversibly to HIV Protease thereby preventing the cleavage of the viral precursor polyproteins. This results in the formation of immature viral particles incapable of infection other cells. The combination of LAM, TDF and EFV is used in pharmaceutical preparations for the treatment of AIDS. The chemical structures of LAM, TEN and EFV are shown in Fig. 1.



(a)



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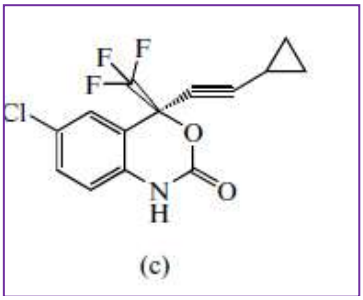

Fig.1. Chemical structure of a) Lamuvudine b) Tenofovir c) Efavirinz.

Several methods were reported for the estimation of LAM, TDF and EFV [3–8] individually and in combination with other antireteroviral drugs [9–15]. Simultaneous estimation of LAM, TDF and EFV by UV methods also reported [16]. Because of no chromatographic method for the simultaneous determination of LAM, TDF and EFV in a combined dosage form has yet been reported, it was essential to develop such a method for bulk and tablet formulations. The method described is rapid, economical, precise, and accurate and can be effec tively used for routine quality control analysis of tab lets. The developed method was validated as per ICH norms [17–19].

EXPERIMENTAL

The instrument and chromatographic conditions. Shimadzu HPLC system (Shimadzu corporation Ky oto, Japan) consisted of a pump (LC10 ATvp solvent deliver module, SPD10 Avp UVVisible detector) run under Winchrome software, with manual injecting fa cility programmed at 20 µL capacity per injection was used. The column used was Phenomenex Luna C18 (150 mm × 4.6 mm, 5.0 µm particle size). Different mobile phases were tested in order to find the best con ditions for separation of LAM, TDF and EFV. The mobile phase contained acetonitrile : methanol : water (30 : 45 : 25, v/v/v) and the flow rate was maintained at 0.5 mL/min. UV detection was carried out at 258 nm. The mobile phase and samples were filtered through a 0.45 µm membrane filter. Mobile phase was degassed by Sonica ultrasonic cleaner (model 2200 MH) prior to use. All determinations were performed at ambient temperature.

MATERIALS AND REAGENTS

Pharmaceutical grade working standards LAM, TDF and EFV were obtained from Strides arco labs, Bangalore, India. All chemicals and reagents were of HPLC grade and were purchased from Qualigens India Pvt. Ltd., Mumbai, India.

AIM AND PLAN OF WORK:

Aim:

Emtricitabine, Tenofovir and Efavirenz drugs are selected for the present study. According to the literature survey it was found that few analytical methods like UVspectrophotometric, RP-HPLC, LC-MS, HPLC DAD and UPLC were reported for estimation of Emtricitabine, Tenofovir and Efavirenz individually or different combinations. Hence it was felt that there is a need for new method development and validation in RP-HPLC for estimation of Emtricitabine, Tenofovir and Efavirenz in pure drug and pharmaceutical dosage form.

The main aim of my present study is to develop a new, simple, precise, accurate and rapid RP-HLC method and to validated as per ICH Q2B guidelines which should be followed for routine analysis of drugs.

Plan of Work:

- Literature Survey
- Acquirement of Standard drug
- Selection of Chromatographic mode
- Standard and Sample preparation
- Selection of Column, Detector and Mobile phase
- Preliminary Run
- Optimization of the method
- Method developed and validated according to ICH guidelines

RESULTS & DISCUSSION

Selection of Detector Wave length by UV/ VISIIBLE Spectroscopy:

A standard solutions of Emtricitabine, Tenofovir and Efavirenz concentration 10µg/ml were prepared and scanned in the UV region i.e., 200 to 400 nm to detect the maximum wavelength (Fig.2).

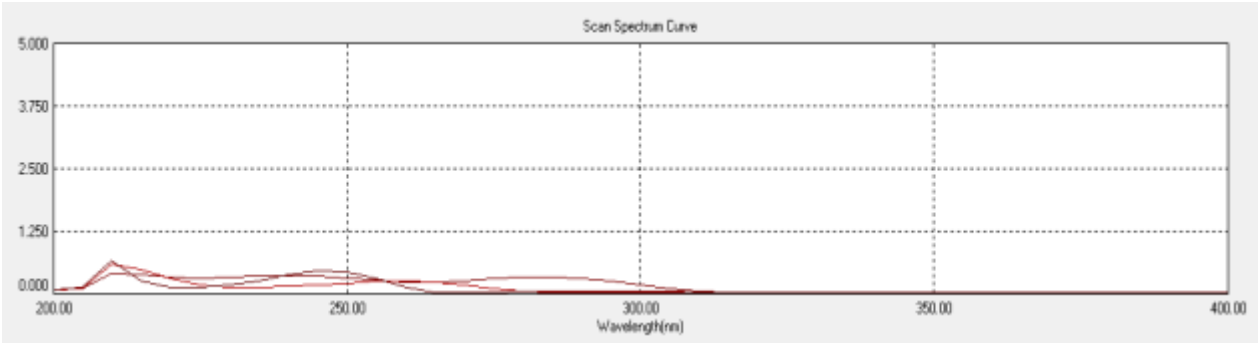

Fig 2: Absorbance spectrum of emtricitabine, tenofovir and efavirenz

DISCUSSION:

The resulting solution was scanned in the range of 200-400nm. From spectrum 256nm was selected based on the isobestic point.

Chromatographic Conditions:

The Mobile phase consisted of Acetonitrile: 1% IPA in the ratio of 80:20. Contents of mobile phase were filtered before use through a 0.22 µm membrane filter and sonicated for 15min. The mobile phase was pumped with 1.0 ml/min flow rate from the solvent reservoir to the column. The injection volume was 10 µl.The column oven temperature was maintained at 30^0c. The eluents were detected at 251nm.

OPTIMIZED METHOD

Chromatographic conditions:

Stationary phase: Inertsil ODS-3V (4.6×250mm×5µm)
Mobiles phase : Acetonitrile : 1% IPA (80:20)
Flow rate: 1ml/min
Detector wavelength: 256nm
Injector volume: 20µl
Temperature: 30°c

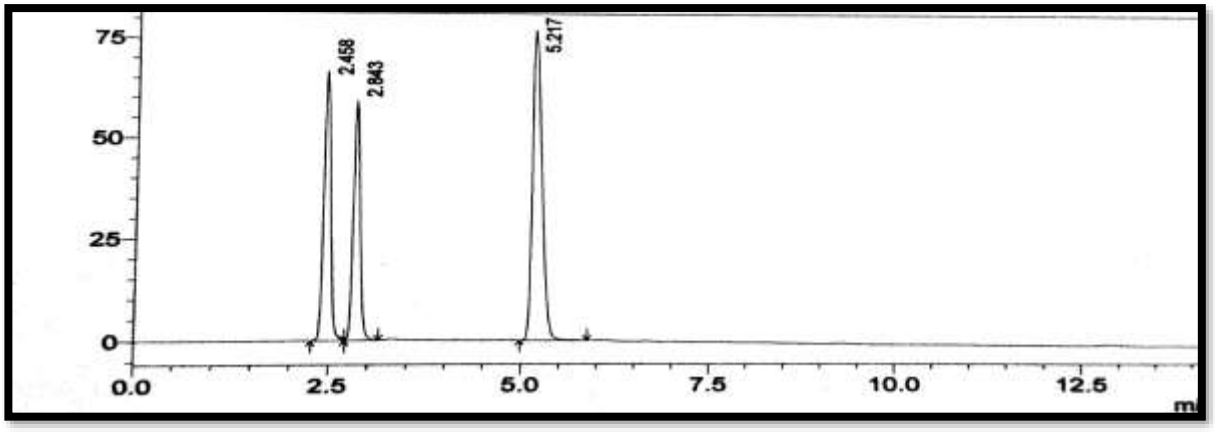

Fig. 3. Chromatogram of optimized method

Name of the drug	Retention time	Tailing factor	Peak area	Theoretical plates	Resolution
Emtricitabine	2.458	1.265	414083	3012	0.000
Tenofovir	2.843	1.287	360195	4031	2.149
Efavirenz	5.217	1.225	678413	7295	11.213

DISCUSSION:

The resolution between two anlytes was good. All the results were found to be within the acceptance criteria. Hence the method was considered to be optimized.

METHOD VALIDATION LINEARITY:

Preparation of solutions

From the standard stock 0.05, 0.1, 0.15, 0.2, 0.25 ml and 0.075, 0.15, 0.22, 0.30, 0.375 ml and 0.15, 0.30, 0.45, 0.60, 0.75 ml of Emtricitabine, Tenofovir and Efavirenz solutions were pipetted out and transferred into 10 ml volumetric flask and make up the volume with diluent. The solutions were degassed and passed through 0.45µm membrane filter for filtration. The concentrations of the Emtricitabine 5-25 µg/ml and Tenofovir 7.5-37.5 µg/ml and Efavirenz 15-75 µg/ml was prepared and injected.

Table 1: Linearity study of Emtricitabine

Linearity Level	Concentration (µg/ml)	Area		Mean
		Set 1	Set 2	
1	5	200821	200564	200692.5
2	10	390085	396605	397345
3	15	603238	602785	603011.5
4	20	826185	827510	826847.5
5	25	1053717	1055144	1054430
Y= 42740x-24628				R^2= 0.999

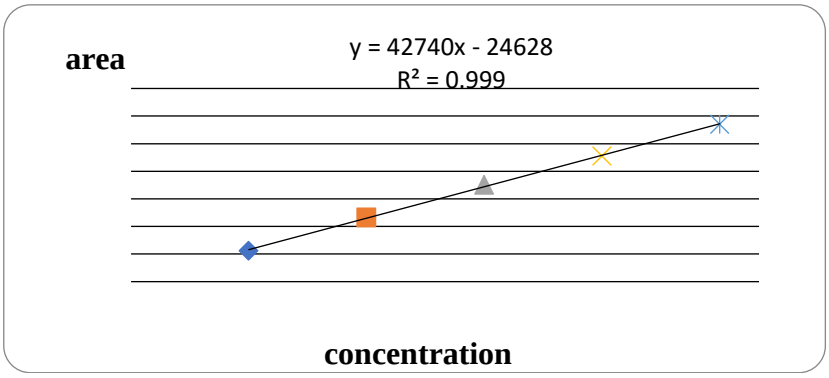

Fig 4. Linearity plot of Emtricitrabine

Table 2: Linearity study of Tenofovir

Linearity Level	Concentration (µg/ml)	Area		Mean
		Set 1	Set 2	
1	7.5	260864	256253	258558.5
2	15	506593	496736	501614.5
3	22.5	783279	781370	782324.5
4	30	1018964	1019169	1019066.5
5	37.5	1291860	1291723	1291791.5
Y= 34452x-4504				R^2= 0.999

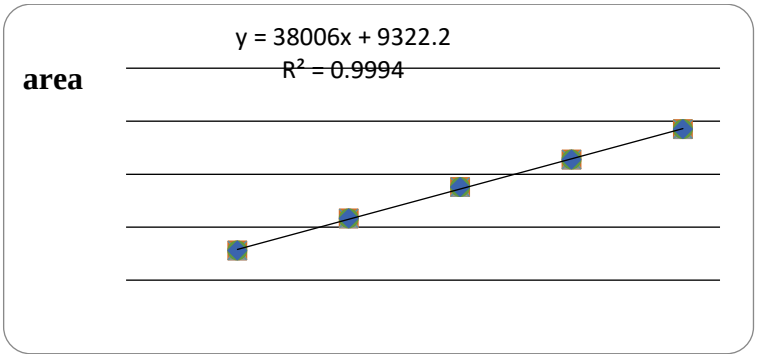

Fig 5. Linearity plot of Tenofovir

Table 3: Linearity study of Efavirenz

Linearity Level	Concentration (µg/ml)	Area		Mean
		Set 1	Set 2	
1	15	558428	556156	557292
2	30	1161150	1160928	1161039

3	45	1753051	1753085	1753068
4	60	2274457	2279002	2276729.5
5	75	2855850	2844008	2849929
Y = 38006x + 9322				R^2= 0.999

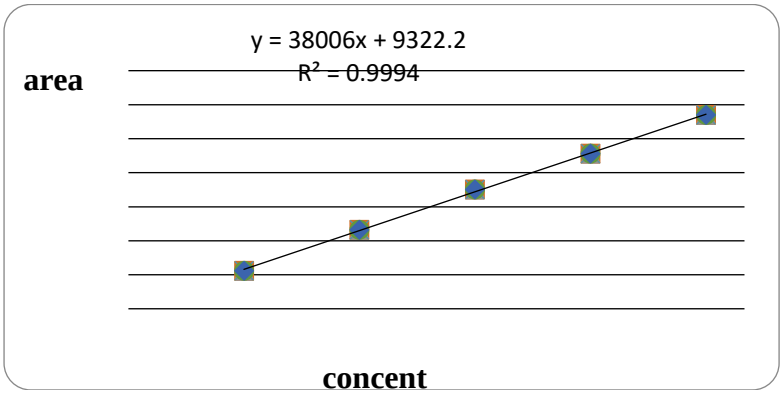

Fig.6. Linearity plot of Efavirenz

PRECISION:

Preparation of solutions

From the standard stock solution 0.15 ml of Emtricitabine and 0.225 ml of Tenofovir and 0.45 ml of Efavirenz were pipette out and transferd into six 10 ml volumetric flasks and make up the volume with the diluent. The solutions were degassed and passed through 0.45µm membrane filter for filtration. The concentration of Emtricitabine 15 µg/ml and Tenofovir 30 µg/ml and Efavirenz 45 µg/ml solutions were injected for six times into the HPLC in the same day and three conjugative days the area for all six injections were measured.

Table 4: Intraday Precision study of Emtricitrabine

S.No	Concentration (µg/ml)	Concentration Found (µg/ml)	Percentage %	Average %	S.D	%RSD
1	15	14.84	98.93	98.27	0.045	0.30
2		14.75	98.33			
3		14.67	97.8			
4		14.87	99.1			
5		14.57	97.1			
6		14.76	98.4			

Table 5: Intraday Precision study of Tenofovir

S.No	Concentration (µg/ml)	Concentration Found (µg/ml)	Percentage %	Average %	S.D	%RSD
1	22.5	23.95	106.4	104.8	0.123	0.52
2		23.52	104.5			
3		23.38	103.9			
4		23.82	105.9			
5		23.13	102.8			
6		23.70	105.3			

Table 6: Intraday Precision study of Efavirenz

S.No	Concentration (µg/ml)	Concentration Found (µg/ml)	Percentage %	Average %	S.D	%RSD
1	45	45.97	102.1	101.6	0.108	0.23
2		46.02	102.2			
3		45.68	101.5			
4		45.55	101.1			
5		45.55	100.7			
6		45.91	102			

INTERMEDIATE PRECISION:

Table 7: Intermediate Precision study of Emtricitrabine

S.No	Concentration (µg/ml)	Concentartion (µg/ml)	Percentage %	Average %	S.D	%RSD
1	15	14.75	98.36	98.42	0.035	0.23
2		14.88	99.24			
3		14.69	97.95			
4		14.72	98.15			
5		14.85	99.03			
6		14.67	97.82			

Table 8: Intermediate Precision study of Tenofovir

S.No	Concentration (µg/ml)	Concentration Found (µg/ml)	Percentage %	Average %	S.D	%RSD
1	22.5	23.60	104.8	105.06	0.072	0.30
2		23.91	106.2			
3		23.53	104.6			
4		23.51	104.5			
5		23.82	105.9			
6		23.49	104.4			

Table 9: Intraday Precision study of Efavirenz

S.No	Concentration (µg/ml)	Concentration Found (µg/ml)	Percentage %	Average %	S.D	%RSD
1	45	45.5	101.2	101.6	0.11	0.24
2		46.08	102.4			
3		45.55	101.2			
4		45.93	102			
5		45.80	101.7			
6		45.56	101.2			

DISCUSSION:

The %RSD of six standard injection results was within range.

ACCURACY:

Preparation of Emtricitabine, Tenofovir and Efavirenz combined Tablet dosage form:

An equivalent weight of 10 mg of Emtricitabine, 15 mg of Tenofovir and 30 mg of Efavirenz were weighed. All other excipients like MCC, SLS, Povidine, crospovidine were weighed as per requirement. All the ingredients except magnesium stearate were blended in the mortar and passed through the sieve. To the above mixture magnesium stearate was added and blended well for uniform dispersion and tablets of desired weight were punched using rotary tablet punching machine.

Preparation of Sample Solution:

The average weight of tablets were determined by weighing 20 tablets and powdered. Tablet powder was equivalent to 10 mg of Emtricitabine, 15 mg of Tenofovir and 30 mg of Efavirenz were weighed and transferred into 10 ml volumetric flask about 4 ml of diluents was added and degassed for 15 min for the complete dissolution of the drug, volume was made upto the mark with diluentand mixed. Above solution was filtered through whatᵓman filter paper number 41 i.e; sample stock solution.

Preparation of Solutions:

Accuracy was performed in 3 different levels for Emtricitabine, Tenofovir and Efavirenz, spiked known quantity of Emtricitabine, Tenofovir and Efavirenz yet 50%, 100%, 150% level into the analyzed samples in triplicate for each level. From the results % recovery was calculated.

Preparation of 50% Solution:

From the sample solution, pipetted out 0.15 ml and transferred into three separate 10 ml volumetric flasks; to the same volumetric flask 0.075, 0.1125 and 0.225 ml of Emtricitabine, Tenofovir and Efavirenz standard solutions were added

and make up to the mark with diluent and degassed. The solution was filtered through 0.45microns membrane filter and injected into the system.

Preparation of 100 % Solution:

From the sample solution, pipetted out 0.15 ml and transferred into three separate 10 ml volumetric flasks; to the same volumetric flask 0.15, 0.22 and 0.45 ml of Emtricitabine, Tenofovir and Efavirenz standard solutions were added and make up to the mark with diluent and degassed. The solution was filtered through 0.45microns membrane filter and injected into the system.

Preparation of 150% Solution:

From the sample solution, pipetted out 0.15 ml and transferred into three separate 10 ml volumetric flasks; to the same volumetric flask 0.225, 0.3375 and 0.675 ml of Emtricitabine, Tenofovir and Efavirenz standard solutions were added and make up to the mark with diluent and degassed. The solution was filtered through 0.45microns membrane filter and injected into the system.

Table 10: Accuracy study of Emtricitabine

S. no	% Level	Concentration Present (µg/ml)	Concentration Added (µg/ml)	Concentration Found (µg/ml)	Concentration Recovery (µg/ml)	% Recovery	Average %	SD	%RSD
1	50%	15	7.5	22.78	7.78	103.7	101.6	0.07	0.32
2				22.53	7.53	100.4			
3				22.57	7.57	100.9			
4	100%		15	30.02	15.02	100.1	99.9	0.05	0.18
5				30.08	15.08	100.5			
6				29.87	14.87	99.1			
7	150%		22.5	37.9	22.9	1017	99.96	0.23	0.61
8				37.5	22.5	100			
9				37.1	22.1	98.2			

Table 11: Accuracy study of Tenoovir

S. no	% Level	Concentration Present (µg/ml)	Concentration Added (µg/ml)	Concentration Found (µg/ml)	Concentration Recovery (µg/ml)	% Recovery	Average %	SD	%RSD
1	50%	22.5	11.25	33.51	11.01	97.8	99.9	0.13	0.38
2				33.97	11.47	101.9			
3				33.75	11.25	100			
4	100%		22.5	45.27	22.77	101.2	100.5	0.20	0.44
5				44.72	22.22	98.7			
6				45.37	22.87	101.6			
7	150%		33.75	56.02	33.52	99.3	100.7	0.26	0.46
8				56.57	34.07	100.9			
9				56.92	34.42	101.9			

Table 12: Accuracy study of Efavirenz

S. no	% Level	Concentration Present (µg/ml)	Concentration Added (µg/ml)	Concentration Found (µg/ml)	Concentration Recovery (µg/ml)	% Recovery	Average %	SD	%RSD
1	50%	45	22.5	68.11	23.11	102.7	100.83	0.26	0.38
2				67.77	22.77	101.2			
3				67.20	22.2	98.6			
4	100%		45	89.80	44.8	99.5	101.13	0.84	0.92
5				91.73	46.73	103.8			
6				90.05	45.05	100.1			
7	150%		67.5	112.65	67.75	100.2	99.8	0.16	0.14
8				112.54	67.54	100			
9				112.12	67.12	99.4			

ROBUSTNESS:

Deliberate variations were made to the optimized HPLC conditions, to evaluate robustness were:

1. Wave length variations.
2. Column oven temperature variation
3. Flow rate variations

1. Wave length varied by ±5nm:

Standard preparation of 15 µg/ml solution of Emtricitabine, 22.5 µg/ml of Tenofovir and 45 µg/ml of Efavirenz solutions were prepared and injected into HPLC system with variation in wave length, varied by ±5nm, i.e., 251nm and 261nm. System suitability parameters were evaluated.

2. Column over temperature varied by ±5^{0}c:
Standard preparation of 15 µg/ml solution of Emtricitabine, 22.5 µg/ml of Tenofovr and 45 µg/ml of Efavirenz solutions were prepared and injected into HPLC system with variation in flow rate, varied by ±5^{0}c, i.e.,25 ^{0}Cand 35^{0}C. System suitability parameters were evaluated.

3. Flow rate varied by ±0.2 ml /minute:
Standard preparation of 15 µg/ml solution of Emtricitabine, 22.5 µg/ml of Tenofovir and 45 µg/ml of Efavirenz solutions were prepared and injected into HPLC system with variation in flow rate i.e., 0.8 ml/min and 1.2 ml/min. System suitability parameters were evaluated.

Acceptance criteria: % RSD should be not more than 2%.

Table 13: Robustness study of Emtricitabine , Tenofovir and Efavirenz

SL.NO	parameters	Normal	Variation	%RSD		
				Emtricitabine	Tenofovir	Efavirenz
1	Wavelength	256nm	251	0.17	0.155	0.133
			261	0.48	0.14	1.2
2	Temperature	30^{0}c	25	0.198	0.285	0.148
			35	0.438	0.266	0.043
3	Flow rate	1ml/min	0.8	0.089	0.034	0.048
			1.2	0.02	0.15	0.20

LOD & LOQ:
Preparation of calibration curve from the serial dilutions of standard was repeated for three times .the limit of detection and limit of quantification was calculated by using average value of slope and standard deviation of intercept.

Table 14: LOD&LOQ Study of Emtricitabine, Tenofovir and Efavirenz

Name of the Drug	LOD(µg/ml)	LOQ(µg/ml)
Emtricitabine	0.065	0.198
Tenoovir	0.22	0.66
Efavirenz	0.09	0.29

Preparation of sample solution:
The average weight of tablets were determined by weighing 20 tablets and powdered. 1.7 g of equivalent weight of tablet power was weighed and transferred into 10 ml volumetric flask and about 4 ml of diluent was added and degassed for 15 minutes for the complete dissolution of drug, volume was made up to the mark with diluent and mixed. Above solution was filtered through What'sman filter paper i.e; primary stock. From the above solution 1 ml was pipetted out and transferred into 10 ml volumetric flask and made up to the mark with diluent. This solution was used for assay studies.

Table 15: Assay studies of emtricitabine, tenofovir and efavirenz

Name of the drug	Label claim (mg)	Amount found (mg)	Assay %	%RSD
Emtricitabine	10	10.18	101.83	0.76
Tenofovir	15	14.74	98.26	0.05
Efavirenz	30	30.06	100.17	0.42

SUMMARY & CONCLUSION

Validation Parameters	Acceptance criteria	Results		
		Emtricitabine	Tenofovir	Efavirenz
Linearity	The correlation coefficient should be NLT 0.999.	0.999	0.999	0.999
Intraday precision	The % RSD of peaks obtained from the 6 replicate injection should be NMT 2.0%.	0.30	0.52	0.23
Intermediate precision	The % RSD of peaks obtained from the 6 replicate injection should be NMT 2.0%.	0.23	0.30	0.24
Accuracy	The % recovery at each level should be NLT 98.0% and NMT 102% of the amount added.	100.4	100.36	100.58
LOD (µg/ml)	_	0.065	0.22	0.66

LOQ (µg/ml)	_		0.198	0.09	0.29
Assay	The amount of assay % in the formulation should be NLT 98.0% and NMT 102.0%.		101.83	98.26	100.17
Robustness	Variation in Flow rate (0.8ml/min, 1.2ml/min)	% RSD should be NMT 2 %	% RSD 0.8ml/min=0.089 1.2ml/min=0.02	% RSD 0.8ml/min=0.034 1.2ml/min=0.15	% RSD 0.8ml/min=0.048 1.2ml/min=0.20
	Variation in Wave length (251nm, 261nm)		% RSD 251nm= 0.17 261nm= 0.48	% RSD 251nm= 0.155 261nm= 0.14	% RSD 251nm= 0.133 261nm= 1.2
	Variation in temperature (25°c,35°c)		% RSD 25°c= 0.198 35°c= 0.438	% RSD 25°c= 0.285 35°c=0.266	% RSD 25°c= 0.148 35°c=0.043

CONCLUSION

Here by concluded that the method shown was sensitive, reproducible, accurate and precise. This is proved by the low percentage relative standard deviation. RP-HPLC method can be effectively applied for the routine analysis of emtricitabine, tenofovir and efavirenz in quality control analysis.

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