Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2017, 9(5):45-52

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

RP-UPLC Method for the Simultaneous Quantification of Related Substances in Emtricitabine, Tenofovir Disoproxil Fumarate and Efavirenz Pharmaceutical Dosage Forms

Babu C[1*], L Narasimha Rao K[1], Devanna N[1] and Suresh Reddy KVN[2]

[1]*Department of Chemistry, Jawaharlal Nehru Technological University Anantapur, Anantapur, Andhra Pradesh, India*
[2]*Department of Chemistry, GITAM Institute of Technology, GITAM University, Visakhapatnam, Andhra Pradesh, India*

ABSTRACT

A stability-indicating reverse phase ultra-performance liquid chromatography (RP-UPLC) method, developed for the simultaneous quantification of eight related compounds in the dosage forms of Emtricitabine, Tenofovir Disoproxil Fumarate and Efavirenz. Efficient chromatographic separation was achieved on an Acquity UPLC BEH Phenyl (2.1×100 mm, 1.7 µm) column using mobile phase A (Buffer, 1.0 mL of TEA in 1000 mL of water pH adjusted to 4.0 ± 0.05 with diluted OPA) and mobile phase B (Buffer: Acetonitrile (20:80, v/v) in gradient mode with the flow rate of 0.5 mL/min and the peaks were monitored at 265 nm. In the developed method, the resolution of Emtricitabine, Tenofovir Disoproxil Fumarate, Efavirenz and related substances were found to be greater than 2.0. When the formulation samples are subjected to forced degradation the mass balance was found close to 99%. This method was validated in terms of limit of detection, limit of quantification, linearity, accuracy, precision and robustness as per ICH Q2R1. The test solutions were found to be stable in the diluent (Buffer: Methanol, 20:80 (v/v)) for 24 hours.

Keywords: Emtricitabine; Tenofovir disoproxil fumarate; Efavirenz; Related substances; Method validation; RP-UPLC

INTRODUCTION

Worldwide, there are about 40 million persons who have been suffering from human immunodeficiency virus-1 (HIV-1) or acquired immunodeficiency syndrome (AIDS) [1]. The goal of antiretroviral therapy for HIV-1 infection is to delay disease progression and increase the duration of survival by achieving maximal and prolonged suppression of HIV-1 replication. The treatment for AIDS involves the use of a combination of antiretroviral agents, typically a combination of at least three active substances, including a non-nucleoside reverse transcriptase inhibitor (NNRTI) or a protease inhibitor (PI) and two active substances from the nucleoside reverse transcriptase inhibitor/nucleotide reverse transcriptase inhibitor class (NRTI/NtRTI) [1]. Efavirenz [(4S)-6-Chloro-4-(2-cyclopropylethynyl)-4-(trifluoromethyl)-2,4-dihydro-1H-3,1-benzoxazin-2-one] is non-nucleoside reverse transcriptase inhibitor (NNRTI) and is used as part of highly active antiretroviral therapy (HAART) for treatment of human immunodeficiency virus (HIV) [2,3].

Emtricitabine is chemically known as 5-fluoro-1-(2R,5S)-[2-(hydroxymethyl)-1, 3 - oxathiolan - 5-yl) cytosine nucleoside reverse transcriptase inhibitor (NRTI)) [2-4]. This drug works by inhibiting reverse transcriptase, the enzyme that copies HIV RNA into new viral DNA. Tenofovir disoproxil fumarate is 9-[(R)-2 [[bis[[(isopropoxycarbonyl)oxy]-methoxy]phosphinyl]methoxy]propyl]adenine fumarate(1:1).

Tenofovir disoproxil is a nucleoside analog reverse transcriptase inhibitor (NRTI)) [2-4]. The combination of these three drugs are available in the market with the brand names of Viraday 30s tablets, Vonavir 30s tablets, Trustiva 30s tablets, Virotrenz 30s tablets, Teevir 30s tablets, Forstavir 30s tablets with 200 mg of Emtricitabine, 300 mg of Tenofovir Disoproxil and 600 mg of Efavirenz.

Literature survey showed that many analytical methods are available for the determination of these drugs either individually or in combination with other drugs through UV Spectrometry [5-8], HPLC [9-15], UPLC [16,17] and LC MS [18,19]. Mali et al. reported RP-HPLC method for the determination of these related substances in tablet dosage forms of Emtricitabine and Tenofovir with a run time of 45 min [15]. Madeesh et al. developed and validated a UPLC method for the simultaneous estimation of Lamivudine, Tenofovir and Efavurenz [16]. Stability Indicating RP-UPLC assay method was developed for Emtricitabine and TenofovirDisoproxil Fumarate [17]. But there is no RP-UPLC method reported for the simultaneous estimation of related substances in the combined dosage forms of Emtricitabine, Tenofovir Disoproxil Fumarate and Efavirenz. The objective of this study is to develop the stability indicating RP-UPLC method for determination of eight related substances in the combined dosage forms of Emtricitabine, Tenofovir Disoproxil and Efavirenz. The chemical names of all the analytes are presented in Table 1.

Table 1: Chemical names of Emtricitabine, Tenofovir and their impurities and Efavirenz

S. No	Name of the compounds	IUPAC Name
1	Emtricitabine	4-amino-5-fluoro-1-[(2*R*,5 *S*)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one.
2	Emtricitabine RC02	4-amino-1-[(2R, 3R, 5S)-2-(hydroxymethyl)-3-oxo-1,3λ4-oxathiolan-5-yl]pyrimidin-2(1H)-one.
3	Tenofovir Disoproxil	Bis{[(isopropoxycarbonyl)oxy]methyl} ({[(2R)-1-(6-amino-9H-purin-9-yl)-2-propanyl]oxy}methyl)phosphonate.
4	Mono POC PMPA	[2-(6-amino-purin-9-yl)-1-methyl ethoxymethyl]-phosphonic acid monoisopropoxycarbonyloxymethyl ester.
5	Isopropyl POC	Isopropyl ether (R)-9-(2-mono iso propoxy carbonyl oxy methyl phosphinomethyl) propyladenine.
6	n-POC PMPA	[2-(6-amino-purin-9-yl)-1-methyl-ethoxymethyl]-phosphonic acid isopropoxycarbonyloxymethyl ester-methoxycarbonyloxymethyl ester, Fumarate.
7	Tenofovir Mixed dimer	[2-[6-[[[9-[2-[6-[[[9-[2-(Bis-iso propoxycarbonyloxy methoxy phosphonyl-methoxy) propyl]-9H-purine-6-yl-amino] methyl] amino]-purine-9-yl]1-methyl-ethoxy methyl]-phosphonic acid mono iso propoxycarbonyloxy methyloxy methyl ester.
8	Dimer Impurity	[2-[6-[[[9-(Bis-isopropoxycarbonyloxy methoxy phosphonyl-methoxy)propyl]-9Hpurin-6-amino]methyl]amino]-purin-9-yl]-1-methyl-ethoxymethyl]-phosphonic acid diisopropoxycarbonyloxy methyl ester.
9	Efavirenz	(4*S*)-6-Chloro-4-(2-cyclopropylethynyl)-4-(trifluoromethyl)-2,4-dihydro-1*H*-3,1-benzoxazin-2-one
10	Efavirenz RC-A	(S)-2-(2-Amino-5-chlorophenyl)-4-cyclopropyl-1,1,1-trifluorobut-3-yn-2-ol.
11	Efavirenz RC-D	6-Chloro-2-cyclopropyl-4- (trifluoromethyl)quinoline.

MATERIALS AND METHODS

Chemicals and reagents

HPLC grade Acetonitrile was procured from Qualigens, India. Ortho-phosphoric acid is purchased from Merck, India. All other chemicals and solvents used were of analytical grade (Make: Rankem). Water used in the UPLC analysis was purified by the water purifier (Milli-Q Millipore). Reference standards of Efavirenz, Emtricitabine, Tenofovir disoproxil fumarate and all related substances are supplied by GSN Pharmaceuticals Private Limited, India as gift samples. Tablets of these drugs were purchased from local market. The mobile phase and all the solutions were filtered through a 0.22 µm nylon filter (Millipore).

Instrumentation

The Acquity UPLC H-Class system with PDA detector was used for the analysis. Analytical column used for this method is Acquity UPLC BEH phenyl 2.1 × 100 mm, 1.7 µm particle size.

Preparation of mobile phase A (buffer)

Mobile phase A was prepared by dissolving 1mL of triethylamine in 1000 mL of water and pH was adjusted to 4.0 ± 0.05 with diluted orthophosphoric acid and filtered through 0.22 µm nylon filter.

Preparation of mobile phase B
Mobile phase B was prepared by mixing of above buffer and Acetonitrile in 20:80 (% *v/v*).

Preparation of diluent
Diluent was prepared by mixing of above buffer and methanol in 80:20 (%*v/v*).

Standard solution preparation
10 mg of Emtricitabine, 15 mg of Tenofovir and 30 mg of Efavirenz are taken into a 50 mL volumetric flask and 35 mL of diluent is added and sonicated for 5 min to dissolve and made up to volume with diluent. Standard solution was prepared by taking 2 mL of the above solution into a 100 mL volumetric flask and made up to volume with diluent. The final concentrations of Emtricitabine, Tenofovir and Efavirenz are 4 µg/mL, 6 µg/mL and 12 µg/mL respectively.

Sample preparation
The amount equivalent to 20 mg of Emtricitabine (Finely powdered tablets) is weighed and transferred into a 10 mL volumetric flask added 7 mL of diluent and sonicated for 20 min to dissolve, made up to volume with diluent and filtered through 0.45 µm nylon filter. The final concentrations of Emtricitabine, Tenofovir disoproxil fumarate and Efavirenz are 2 mg/mL, 3 mg/mL and 6 mg/mL respectively.

Optimum chromatographic conditions
The analysis was carried out on Acquity UPLC BEH phenyl (2.1 × 100 mm, 1.7 µm) column maintained at 30°C using mobile phase A and mobile phase B in gradient mode (Table 2) with flow rate of 0.5 mL/min. Before delivering the mobile phase into the system, it was degassed and filtered through 0.45 µm nylon filter using vacuum. The injection volume was 3 µL and the detection was performed at 265 nm using a photo diode array (PDA) detector. The typical retention times of Emtricitabine, Tenofovir disoproxil and Efavirenz are 1.781, 6.365 and 11.722 minutes respectively. The counter ion fumaric acid is also found to be eluting at 0.654 minutes. The criticality of this method is to elute the entire active as well as their related impurities with optimum separation and symmetric peak shapes.

Table 2: Gradient program

Time (min)	Mobile phase A (*%v/v*)	Mobile phase B (*%v/v*)
0	90	10
2	70	30
4	70	30
7	65	45
11	15	85
12.4	15	85
13	90	10

RESULTS AND DISCUSSION

Optimum separation of Emtricitabine, Tenofovir disoproxil, Efavirenz and potential degradation impurities was achieved by the above optimized conditions. The aim of method validation is to confirm that the present method is suitable for its intended purpose as described in ICH guidelines Q2 (R1). The described method has been extensively validated in terms of specificity, forced degradation, limit of detection (LOD) and limit of quantification (LOQ), linearity, accuracy, precision, robustness and solution stability.

System suitability
To ensure that the system is working correctly during the analysis, the resolution, tailing factor, theoretical plates and %RSD were checked and the results of system suitability paratmeters are given in Table 3. The parameters such as tailing factor should be not more than 2.0, theoretical plate should be not less than 4,000 and %RSD for replicate injections of standard solution should not be more than 5.0. A typical chromatogram for system suitability is shown in Figure 1.

Table 3: System suitability results

S. No	Emtricitabine				Tenofovir				Efavirenz			
	RT (min)	Peak area	TF	TP	RT (min)	Peak area	TF	TP	RT (min)	Peak area	TF	TP
1	1.781	63068	0.9	4376	6.365	74957	1	207541	11.722	33812	1	767835
2	1.765	63178	1	4278	6.359	74892	1	204581	11.712	32587	1.1	758194
3	1.786	63092	0.9	4315	6.361	74912	1.1	203589	11.698	33058	0.9	745825
4	1.777	63142	1	4298	6.36	74792	1	201458	11.71	32978	1.1	764589
5	1.782	63012	1.1	4351	6.358	74058	1.1	203548	11.72	33789	1	745894
6	1.779	63251	1	4308	6.362	73992	1	208954	11.735	32589	1	765489
Mean		63124				74601				33136		
SD		84.939				449.52				550.46		
%RSD		0.13				0.6				1.66		

RT: Retention time, TF: Tailing factor, TP: Theoretical plates

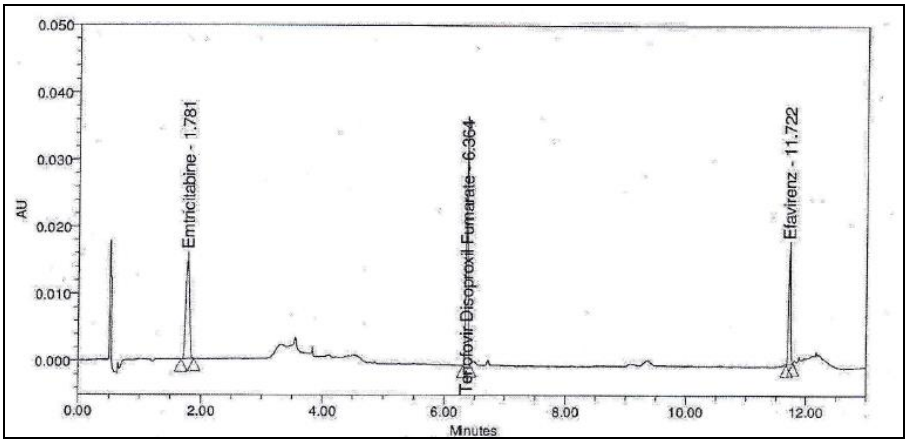

Figure 1: Typical chromatogram of standard solution

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. The specificity of the method is established by injecting blank and the impurity spiked sample and their corresponding chromatograms are shown in Figures 2 and 3. The results of specificity are presented in Table 4. The chromatograms in figures show that there was no interference of the blank and main drug substances with impurities and the developed method was successfully separated all the impurities with each other and with main drug. Hence, the RP-UPLC method used for the estimation of related substances in Emtricitabine, Tenofovir disoproxil fumarate and Efavirenz is very selective and specific.

Table 4: Specificity results of spiked sample

S. No	Name	RT	RT ratio	Resolution	Purity angle	Purity threshold
1	Fumaric acid	0.654	0.1	--	0.125	0.258
2	Emtricitabine RC 02	0.756	0.12	2.3	0.025	0.745
3	Emtricitabine	1.768	0.28	3.4	1.25	1.789
4	Mono POC PMPA	3.829	0.6	5.3	0.369	1.036
5	Isopropyl POC	5.265	0.83	3.3	0.036	0.521
6	Tenofovir Disoproxil	6.334	1	3.8	0.426	2.135
7	n- POC PMPA	6.531	1.03	5.2	0.751	0.902
8	Tenofovir mixed dimer	7.053	1.11	4.6	0.209	0.534
9	Efavirenz RC-A	11.321	1.79	4.3	0.011	0.201
10	Efavirenz	11.703	1.85	5.2	0.925	3.103
11	Tenofovir Dimer	11.809	1.86	3.1	3.659	4.021
12	Efavirenz RC-D	12.314	1.94	4.2	5.912	6.385

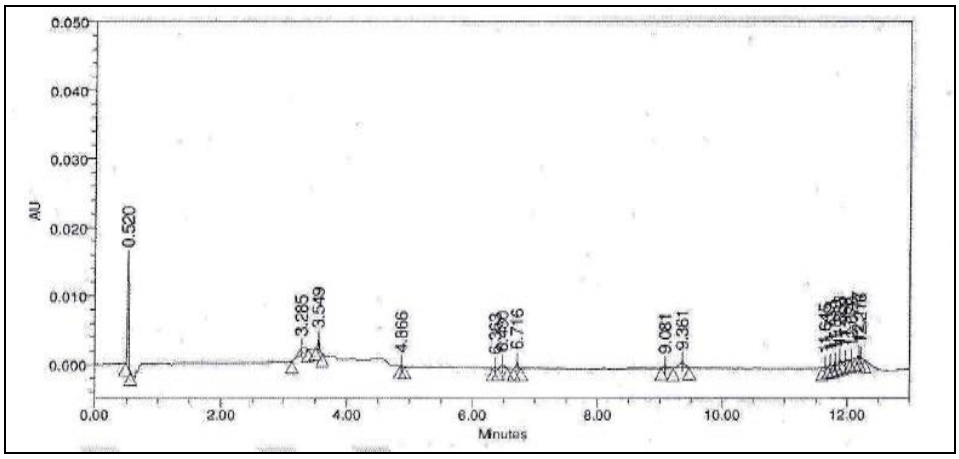

Figure 2: Typical chromatogram of blank solution

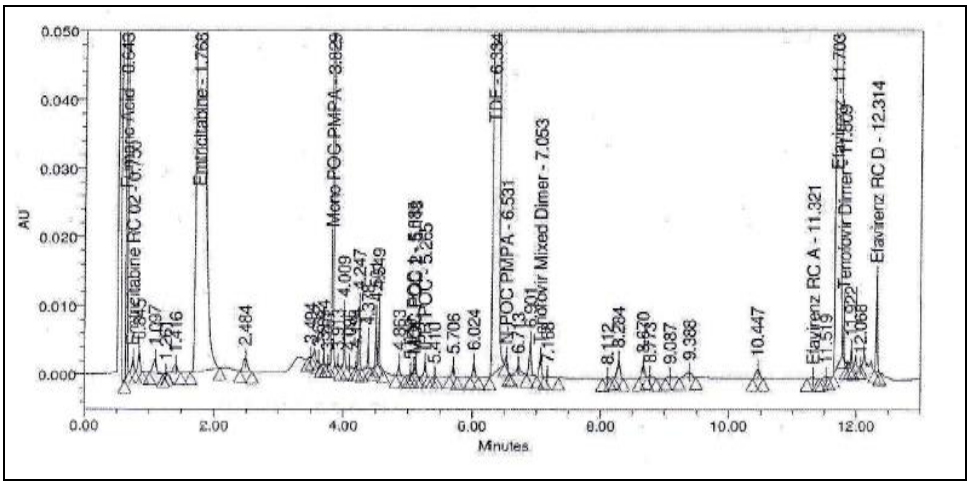

Figure 3: Typical chromatogram of spiked sample solution

Forced degradation studies

Forced degradation studies were performed to provide an indication of the stability indicating property and specificity of the proposed method. Intentional degradation was attempted to stress the samples under conditions like thermal at 60°C for 7 days, humidity (90% relative humidity) for 7 days, photolytic sample (1.2 million lux hrs), acid hydrolysis (using 0.5 N HCl at room temperature for 2 hours), base hydrolysis (using 0.5N NaOH at room temperature for 2 hours) and oxidative degradation (using 3.0% H_2O_2 at room temperature for 2 hours) to evaluate the ability of the proposed method to separate degradation products from each other and active ingredients as well. To check and ensure the homogeneity (peak purity) of all peaks in the stressed sample solutions, a wide range of wavelength was applied using photo diode array detector and the corresponding results are tabulated in Table 5. In forced degradation, it is observed that Emtricitabine is susceptible for degradation in oxidation stress condition, whereas Tenofovir susceptible for base stress condition and found to be stable in all other stress conditions.

Table 5: Forced degradation results

Sample details	Emtricitabine			Tenofovir Disoproxil			Efavirenz			
	% Degrad.	% assay	Mass balance	% Degrad.	% assay	Mass balance	% Degrad.	% assay	Mass balance	Peak purity
As such sample	0.12	99.5	-	0.36	100.6	-	0.1	101.2	-	Pass
Thermal	0.63	97.3	98.3	0.52	99.8	99.4	0.25	100.5	99.5	Pass
Photolytic	0.92	97.5	98.8	0.65	98.4	98.1	0.36	99.6	98.7	Pass
Humidity	0.83	98.6	99.8	0.81	97.6	97.5	0.51	97.9	97.1	Pass
Acid	1.12	97.5	99	0.93	96.8	96.8	0.3	98.6	97.6	Pass
Base	0.93	100.5	101.8	9.3	91.6	99.9	0.12	99.1	97.9	Pass
Oxidative	7.8	89.6	97.8	1.1	97.9	98.1	0.29	100.2	99.2	Pass

Limit of detection and quantification

Limit of detection (LOD) and limit of quantification (LOQ) for all impurities are determined by injecting a series of solutions of known concentration till the signal-to-noise ratio became as 3:1 and 10:1, respectively and the corresponding values are given in Table 6. The found LOQ values are sufficient to quantify these impurities below 0.2% of the drug concentration as per the limits defined by pharma regulating agencies.

Table 6: % LOD and LOQ values of analytes along with S/N ratios

S. No	Name	% LOD	s/n ratio	% LOQ	s/n ratio
1	Emtricitabine RC 02	0.03	2	0.1	11
2	Emtricitabine	0.02	3	0.07	12
3	Mono POC PMPA	0.01	2	0.03	13
4	Isopropyl POC	0.02	3	0.07	11
5	Tenofovir Disoproxil	0.03	3	0.1	10
6	n- POC PMPA	0.02	2	0.07	12
7	Tenofovir mixed dimer	0.03	3	0.1	11
8	Efavirenz RC-A	0.01	2	0.03	10
9	Efavirenz	0.02	3	0.07	11
10	Tenofovir Dimer	0.03	2	0.1	10
11	Efavirenz RC-D	0.03	3	0.1	11

Linearity and relative response factor

The series of solutions were prepared by diluting the impurity stock solution to different concentrations from LOQ to 150% (i.e. LOQ to 0.4% with respect to Emtricitabine (2 mg/mL), Tenofovir disoproxil fumarate (3 mg/mL) and Efavirenz (6 mg/mL) except monoester (1.0% and isopropyl POC 0.3%). The correlation coefficients (r), y-intercepts and relative retention factor (RRF) values are given in Table 7, which shows that there is an excellent correlation between peak areas and concentration of all analytes.

Table 7: Linearity results for Emtricitabine, Tenofovir, Efavirenz and its all impurities

S. No	Name	Correlation coefficient (r^2)	Y-intercept at 100% level	RRF
1	Emtricitabine RC 02	0.991	-1.5	1.21
2	Emtricitabine	0.997	1.3	1
3	Mono POC PMPA	0.999	0.9	1.32
4	Isopropyl POC	0.998	-0.3	1.2
5	Tenofovir Disoproxil	0.996	-2.5	1
6	n- POC PMPA	0.998	-1.7	0.85
7	Tenofovir mixed dimer	0.999	-2.1	1.1
8	Efavirenz RC-A	0.993	1.2	1.12
9	Efavirenz	0.991	-0.9	1
10	Tenofovir Dimer	0.998	-1	1.08
11	Efavirenz RC-D	0.995	-1.1	1.41

Accuracy

The accuracy was determined in triplicate by spiking respective impurities in sample at LOQ, 100% and 150% specification level with respect to analyte concentration of Emtricitabine (2.0 mg/mL), Tenofovir disoproxil fumarate (3 mg/mL) and Efavirenz (6 mg/mL). The recovery values are found within the range of 97.5% to 106.2% with the %RSD of less than 3.3 which indicate that the method is more reliable and accurate. The results of accuracy study are summarized in Table 8**.**

Table 8: Accuracy results

S. No	Sample name	LOQ level	100% level	150% level	%RSD
1	Emtricitabine RC 02	103.5	101.2	99.8	1.8
2	Emtricitabine	101.5	99.9	100.6	0.8
3	Mono POC PMPA	105.3	101.8	103.2	1.7
4	Isopropyl POC	97.5	100.3	102.1	2.3
5	Tenofovir	98.7	99.3	101.5	1.5
6	n-POC PMPA	98.3	100.5	103.1	2.4
7	Tenofovir mixed dimer	99.5	102.9	104.6	2.5
8	Efavirenz RC-A	106.2	101.3	99.8	3.3
9	Efavirenz	104.2	101.6	100.9	1.7
10	Tenofovir Dimer	105.2	102.6	104	1.3
11	Efavirenz RC -D	104.6	102.3	101.2	1.7

Precision

The precision of the method was verified by injecting six individual preparations, spiked with all impurities at 0.2% level except monoester and isopropyl POC with respect to the target concentration of Emtricitabine (2.0 mg/mL), disoproxil fumarate (3 mg/mL) and Efavirenz (6 mg/mL). Monoester and isopropyl POC were spiked at 1% and 3% of the drug concentrations, respectively. Results of method precision are given in Table 9. Results in Table 9 indicate that the % RSD for all impurities was found below 10.0%. Results of intermediate precision at different days with different lots of analytical columns are included in Table 10, which shows that the %RSD for all impurities was below 10%.

Table 9: Method precision results

S. No	Name	Spl-1	Spl-2	Spl-3	Spl-4	Spl-5	Spl-6	% mean	% RSD
1	Emtricitabine RC 02	0.21	0.19	0.21	0.2	0.23	0.18	0.2	8.61
2	Mono POC PMPA	1.02	0.99	1.08	0.98	1.03	1.05	1.03	3.64
3	Isopropyl POC	0.31	0.32	0.29	0.28	0.31	0.32	0.31	5.39
4	n- POC PMPA	0.2	0.19	0.18	0.21	0.21	0.18	0.2	7.07
5	Tenofovir mixed dimer	0.19	0.2	0.23	0.21	0.19	0.22	0.21	7.9
6	Efavirenz RC-A	0.23	0.21	0.19	0.2	0.21	0.22	0.21	6.73
7	Tenofovir Dimer	0.21	0.23	0.21	0.22	0.23	0.23	0.22	4.44
8	Efavirenz RC -D	0.19	0.21	0.22	0.2	0.21	0.22	0.21	5.61

Table 10: Intermediate precision results

S. No	Name	Spl-1	Spl-2	Spl-3	Spl-4	Spl-5	Spl-6	% mean	% RSD
1	Emtricitabine RC 02	0.2	0.18	0.19	0.21	0.18	0.2	0.19	6.26
2	Mono POC PMPA	1.1	1.1	1.09	1.02	1.08	1.03	1.07	3.34
3	Isopropyl POC	0.29	0.3	0.28	0.35	0.32	0.33	0.31	8.47
4	n- POC PMPA	0.18	0.2	0.21	0.23	0.19	0.21	0.2	8.61
5	Tenofovir mixed dimer	0.22	0.19	0.23	0.2	0.24	0.22	0.22	8.59
6	Efavirenz RC-A	0.21	0.23	0.24	0.19	0.22	0.21	0.22	8.08
7	Tenofovir Dimer	0.22	0.19	0.23	0.19	0.21	0.23	0.21	8.67
8	Efavirenz RC -D	0.21	0.22	0.19	0.18	0.19	0.18	0.2	8.43

Robustness

The robustness of the method was checked by intentional changes in flow rate, column temperature and pH of the mobile phase. The flow rate of the mobile phase was changed to 0.45 mL/min and 0.55 mL/min. The effect of pH was studied at pH 3.9 and 4.1. The effect of column temperature was studied at 25°C and 35°C. The resolution between adjacent peaks was evaluated and the resolution was found greater than 2.0.

Solution stability

To check the stability, both standard and impurity spiked samples were kept at refrigerator condition (5°C) and room temperature (25°C). Much change was not observed in the area of the respective impurities. The results of solution stability studies confirmed that both standard and test solutions were stable up to 24 hrs.

CONCLUSION

A specific, linear, precise and accurate stability indicating ultra-performance liquid chromatographic method has been developed for the quantification of eight impurities in the combined dosage forms of Emtricitabine, Tenofovir disproxil fumarate and Efavirenz. The method has been validated for specificity, linearity, accuracy, precision, robustness and stability. The method is linear in the range of LOQ to 150% of the specification concentration for all the impurities with a correlation coefficient not less than 0.991. The accuracy of the method is in the range of 97.5 to 106.2% for all impurities. As the method is validated according to international council of harmonization (ICH) guidelines, it could be adopted for the analysis of all the related substances in the dosage forms of Emtricitabine, Tenofovir and Efavirenz both in quality control and routine analysis of pharmaceutical industries and research laboratories.

REFERENCES

[1] http://aidsinfo.nih.gov/guidelines
[2] S Budavari, MJ O'Neil, A Smith, PE Heckelman, JF Kinneary. The Merck Index, Whitehouse Station, NJ: Merck Research Laboratories Division of Merck & Co, **2001**.
[3] SC Sweetman. Martindale: the complete drug reference. Pharmaceutical press, **2009**.
[4] www.rxlist.com
[5] R Sharma; K Mehta. *Indian J Pharm Sci.* **2010**, 72(4), 527-530.
[6] KA Kumar; K Kannan; T Vetrichelvan. *Dig J Nanomater Bios.* **2011**, 6(3), 1085-1090.
[7] AS Atul; HB Charashila; JS Sanjay. *Pak J Pharm Sci.* **2009**, 22(1), 27-29.
[8] SV Sutar; SS Patil; SA Pishvikar; HN More. *Int J Pharm Sci Res.* **2012**, 3(11), 4363-4366.
[9] T Sharma; N Mishra; KM Swapna; CSI Sudam; DG Sankar. *Asian J Pharm Clin Res.* **2012**, 5(3), 108-110.
[10] CHV Reddiah; PR Devi; K Mukkanti; P Srinivasu. *Int J Pharm Phytopharmacol Res.* **2012**, 2(1), 40-45.
[11] LAR Prasad; JVLNS Rao; P Srinivasu; J Varasprasad; J Hemalatha. *Int J Pharm.* **2013**, 3(1), 136-144
[12] A Srinath; B Sneha; A Akhila; R Ahmed; RG Kulkarni. *Int J Pharm Sci Res.* **2014**, 5(12), 5491-5497.
[13] P Vanaja, N Anusha, VSG Prasad. *Int J Pharm Pharm Sci.* **2013**, 5(3), 116-121.
[14] A Dunge; C Varalakshmi; VV Daisy; D Sanjana; H Jos; A Erwin. *J Sep Sci.* **2010**, 33(12), 1708-1716.
[15] AD Mali; UB More. *Int J Pharma Sci Res.* **2016**, 7(4), 1662-1669.
[16] SK Madeesh; Y Ismail; V Gunasekaran. *Int J Pharm.* **2012**, 2(3), 656-660.
[17] BV Purnima; TVB Reddy; YS Rao; G Ramu; D Ramachandran. *Am J Analyt Chem.* **2015**, 6, 807-821.
[18] MM Krishna; B Laxminarayana; PN Rao, IJ Kumar. *Biomed Chromatogr.* **2012**, 26 (10), 1202-1209.
[19] MM Krishna; PN Rao; IJ Kumar; B Laxminarayana. *Acta Pharma Sinica B.* **2012**, 2(5), 472-480.