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ORIGINAL ARTICLE

Development and validation of analytical method for quantitation of Emtricitabine, Tenofovir, Efavirenz based on HPLC

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Abstract This paper describes the development and validation of a HPLC method for the quantitation of Emtricitabine, Tenofovir, and Efavirenz in pure form and pharmaceutical formulations. The Zorbax SB CN, (250 × 4.6 mm, 5 μm) column was used. UV detection was performed at 260 nm. The mobile phase consisted of methanol (A) and buffer at pH 4.5(B) using the gradient: 0–10 min (90% B), 10–22 min (35% B), and 22–25 min (90% B). The flow rate was 1.5 ml/min in ambient temperature. The injection volume of sample was 20 μl. The method showed to be linear ($r^2 > 0.999$), precise (RSD < 0.76%), accurate (recovery of 100.09% for Emtricitabine, 99.88% for Tenofovir and 100.04% for Efavirenz), specific and robust. Three batches of Emtricitabine, Tenofovir, and Efavirenz tablets were assayed by the validated method. The Emtricitabine contents in the tablet samples varied from 99.94 to 101.60%. The Tenofovir content in the tablet samples varied from 99.13 to 101.81% while Efavirenz content varied from 100.01 to 101.67%.

1. Introduction

Tenofovir Disoproxil Fumarate (TDF) is a fumaric acid salt of the bisisopropoxycarbonyloxymethyl ester derivative of tenofovir. Chemically it is described as 9-[(R)-2[[bis[[(isopropoxycarbonyl)oxy]methoxy]phosphinyl] methoxy]propyl] adenine fumarate (Budawari, 2001). Fig. 1a shows the nucleotide reverse transcriptase inhibitor (NtRTI) used in combination with other antiretrovirals for the treatment of HIV infection (Martindale, 2002). Emtricitabine (FTC) is a nucleoside reverse transcriptase inhibitor (NRTI). Chemically it is described as 5-fluoro-1-(2R,5S)-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl] cytosine (Fig. 1b). FTC is the (−) enantiomer of thio analog of cytidine which differs from other cytidine analogs, in that it has fluorine in the 5th position. FTC is an antiviral agent used for the prevention of perinatal HIV-1 reverse transcriptase (Budawari, 2001). It is also active against Hepatitis B virus (Martindale, 2002; Gish et al., 2005).

Efavirenz is a human immunodeficiency virus type-I (HIV-I) specific non nucleoside reverse transcriptase inhibitor (NNRTI). Efavirenz is chemically described as (S)-6-chloro-4-(cyclopropylethynyl)-1, 4-dihydro-4-(trifluoromethyl)-2H-3,

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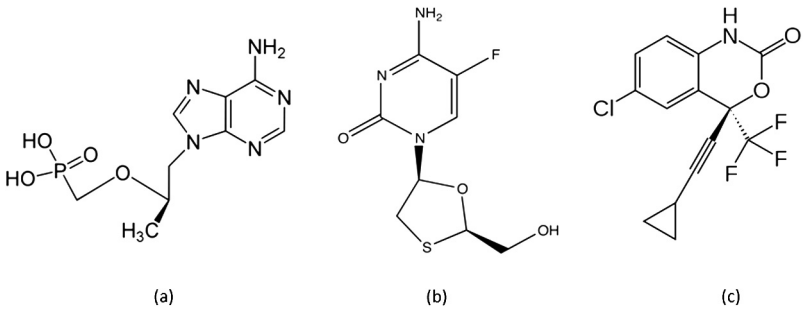

(a) (b) (c)

Figure 1 Chemical structure of (a) Tenofovir, (b) Emtricitabine and (c) Efavirenz.

1-benzoxazin-2-one (Fig. 1c) Physician's Desk Reference, 2008. A literature survey reveals that analytical methods based on HPLC (Malipatil and Nandedkar, 2009; Sharma and Pooja Gupta, 2009; Appala et al., 2008; Rolim-Neto, 2011; Montgomery et al., 2001), HPTLC (Joshi et al., 2009; Laxman et al., 2010), and UV spectrometry (Sockalingam et al., 2005; Shirkhedkar Atul et al., 2009) are available for the determination of these drugs individually and in combination with other drugs in different dosage forms. Some papers have described the analysis of combination with other drugs in plasma, based on HPLC (Kandagal et al., 2008; Deirdre Fox et al., 2011; Rezk et al., 2005), stability indicating (Sagar et al., 2009; Rao and Nikalje, 2009). However, there is no method reported regarding the quantitation of Emtricitabine, Tenofovir and Efavirenz.

Hence, the aim of this study was to develop a simple, precise, accurate, and validated HPLC method, using UV detection to quantify Emtricitabine, Tenofovir, and Efavirenz in pure form for pharmaceutical formulations. The molar absorptivity of Emtricitabine, Tenofovir and Efavirenz in the UV region was found to be at 260 nm. The validated method was applied to the analysis of tablets containing Emtricitabine, Tenofovir and Efavirenz (200 + 300 + 600 mg).

2. Experimental

2.1. Reagents and materials

Emtricitabine, Tenofovir, and Efavirenz reference standards were procured from Cipla Laboratories, Mumbai. Tablets were purchased from local pharmacy which were manufactured by Viraday, Cipla, Mumbai, India. Ultra-pure water was obtained from a Millipore system (Bedford, MA, USA). Methanol (HPLC grade) was obtained from E-Merck (India) Ltd, Mumbai, India. All other chemicals used in the analysis were of AR grade.

2.2. Instrumental and analytical conditions

The HPLC analyses were carried out on Waters 2695 separation module (Waters Corporation, USA) equipped with auto sampler and Waters 2998 PDA detector, Zorbax SB CN, (250 × 4.6 mm, 5 μm) column. UV detection was performed at 260 nm. UV spectra scanning from 190 to 400 nm was recorded online for peak identification. The mobile phase consisted of methanol (A) and buffer at pH 4.5(B). The best separation was obtained between Emtricitabine, Tenofovir and Efavirenz using the gradient: 0–10 min (90% B), 10–22 min (35% B), and 22–25 min (90% B). The flow rate was 1.5 ml/min. The injection volume of sample was 20 μl. The separation of Emtricitabine, Tenofovir and Efavirenz was evaluated in different proportions of these solvents and for each condition, retention factor (k) and resolution (R) were calculated and data are presented in Table 1.

2.3. Preparation of buffer (pH 4.5)

Two grams of ammonium acetate was weighed and dissolved in 1000 ml of water. The pH of the solution was adjusted to 4.5 with orthophosphoric acid. The solution was filtered through 0.45 μm membrane filter.

2.4. Preparation of standard solution

About 100 mg of Emtricitabine, 150 mg of Tenofovir, and 300 mg of Efavirenz reference standards were accurately weighed and transferred into a 100 ml volumetric flask. 20 ml of methanol was added to ensure complete solubilization and the volume was adjusted with the mobile phase. Further dilutions were made to get a final concentration of 0.08 mg/ml of Emtricitabine, 0.12 mg/ml of Tenofovir and 0.24 mg/ml of Efavirenz.

Table 1 Retention factor (k) and Resolution (R) for Emtricitabine, Tenofovir and Efavirenz.

Emtricitabine Retention factor (K)	Tenofovir Retention factor (K)	Efavirenz Retention factor (K)	Resolution(R) for Tenofovir	Resolution(R) for Efavirenz
0.61	2.34	4.90	2.43	1.19
1.66	3.18	4.93	11.25	4.11
3.63	4.17	6.90	10.56	6.12
3.45	8.65	12.43	17.41	7.47
3.63	13.31	16.75	29.51	9.46

2.5. Analysis of fixed dose combination tablets

Three different batches (A: X31006; B: X31079; C: X31025) of tablets were analyzed using the validated method. For the analysis, six replicates of each batch were assayed. The average weight of the tablets was determined. Tablets were finely powdered. A portion of the powder, equivalent to about 200 mg of Emtricitabine, 300 mg of Tenofovir and 600 mg of Efavirenz was accurately weighed and transferred into a 500 ml volumetric flask followed by the addition of 100 ml of methanol. The solution was sonicated for 30 min and diluted with mobile phase to volume. Further dilutions were made to get the final concentration equivalent to 0.08 mg/ml of Emtricitabine, 0.12 mg/ml of Tenofovir and 0.24 mg/ml of Efavirenz.

2.6. Validation (ICH Q2A, 1994; ICH Q2B, 1996)

2.6.1. Linearity

Standard stock solutions containing 1 mg/ml of Emtricitabine, 1.5 mg/ml of Tenofovir and 3 mg of Efavirenz were prepared, in triplicate. Aliquots of these solutions were diluted with the mobile phase to five different concentrations, corresponding to 40–120 μg/ml of Emtricitabine, 80–160 μg/ml of Tenofovir and 200–280 μg/ml of Efavirenz. Calibration curves for the different concentrations versus peak area were plotted for Emtricitabine, Tenofovir and Efavirenz and the obtained data were subjected to regression analysis using the least squares method with a weighting factor of $1/x$.

2.6.2. Precision

The intra-day precision was evaluated by analyzing six sample solutions ($n = 6$), at the final concentration of analyses of 80 μg/ml of Emtricitabine, 120 μg/ml of Tenofovir and 240 μg/ml of Efavirenz. Similarly the inter-day precision was evaluated in three consecutive days ($n = 18$). Emtricitabine, Tenofovir, and Efavirenz concentrations were determined and the relative standard deviations (RSD) were calculated.

2.6.3. Accuracy

Emtricitabine, Tenofovir, and Efavirenz reference standards were accurately weighed and added to a commercial formulation of tablet powder, at three different concentration levels (80, 100 and 120 μg/ml of Emtricitabine, Tenofovir and Efavirenz respectively). At each level, samples were prepared in triplicate and the recovery percentage was determined.

2.6.4. Specificity

Spectral purities of Emtricitabine, Tenofovir, and Efavirenz chromatographic peaks were evaluated using the UV spectra recorded by a UV detector. In addition, a solution containing a mixture of the tablet excipients was prepared using the sample preparation procedure and injected onto the chromatograph, to evaluate possible interfering peaks.

2.6.5. Robustness

Six sample solutions were prepared and analyzed under the established conditions and by variation of the following analytical parameters: flow rate of the mobile phase (1.35 and 1.65 ml/min), methanol and buffer as the mobile phase (±2% of organic solvent) and mobile phase pH (4.3, 4.7). Emtricitabine, Tenofovir, and Efavirenz contents were determined for each condition and the obtained data were submitted for statistical analysis.

2.6.6. Detection and quantitation limits

Limit of detection (LOD) (signal-to-noise ratio of 3) and limit of quantification (LOQ) (signal-to-noise ratio of 10) were measured based on the signal-to-noise ratio. Determination of the signal-to-noise ratio is performed by comparing measured signal samples with known low concentration of analyte with those of blank samples establishing the minimum concentration at which the analyte can be reliably detected and quantified.

3. Results and discussion

The chromatographic parameters were initially evaluated using a Zorbax SB CN (250 × 4.6 mm, 5 μm) column and a mobile phase composed of methanol and buffer gradient. The conditions for method development using the above said column, with different proportions of mobile phase were performed to obtain a good peak (Table 2). Under these conditions the retention factors obtained for Emtricitabine, Tenofovir and Efavirenz were 4.6, 14.3 and 17.7 and a short

Table 2 Chromatographic parameters for Emtricitabine, Tenofovir and Efavirenz at different mobile phase compositions using a Zorbax SB CN,(250 × 4.6 mm, 5 μm) column.

Trial	Mobile phase composition	Injection volume	Flow ml/min	pH (Buffer)	Defect
1	Isocratic Acetonitrile:Buffer (30:70)	20 μl	1	3.5	Split peeks; less number of theoretical plates were present
2	Isocratic Methanol:Buffer (40:60)	20 μl	1	3.5	Split peeks
3	Isocratic Methanol:Buffer (30:70)	20 μl	1.2	4	More retention time, resolution was not satisfactory
4	Gradient Acetonitrile:Buffer	20 μl	1.5	4.5	Resolution was not good and more tailing
5	Gradient Methanol:Buffer	20 μl	1.5	4.5	Less retention time, More theoretical plates, less tailing, peek shape and resolution was found Good

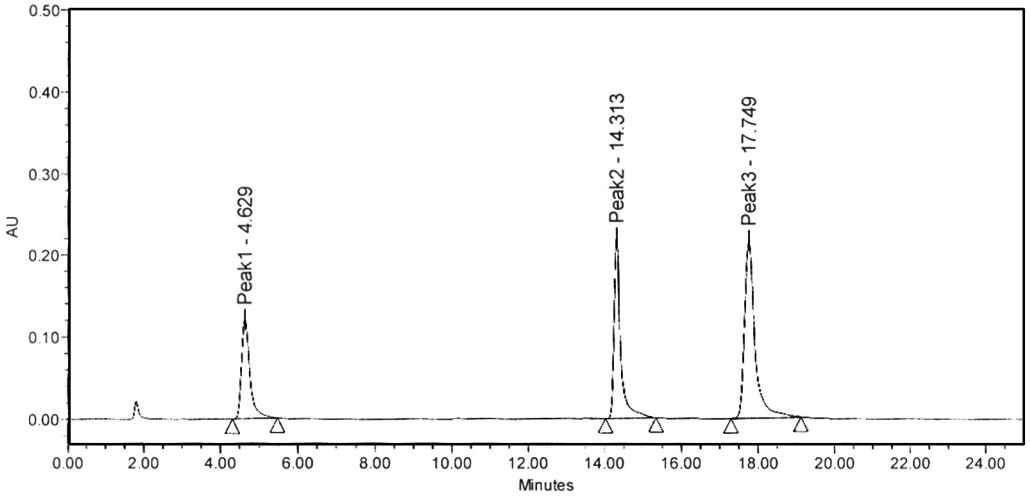

Figure 2 Schematic representation of chromatogram for Emtricitabine, Tenofovir and Efavirenz.

Table 3 Calibration curve data for Emtricitabine, Tenofovir and Efavirenz.

Regression parameters	Emtricitabine	Tenofovir	Efavirenz
Regression coefficient (r^2)	0.999	0.999	0.999
Slope ± standard error	18275 ± 0.034	18754 ± 0.04	19719 ± 0.02
Intercept ± standard error (%)	−1.03 ± 0.21	−5.73 ± 0.01	37.56 ± 0.02
Concentration range(μg/ml)	40–120	80–160	200–280
Number of points	5	5	5

run rime (21 min), and so, this condition was adopted in subsequent analysis (Fig. 2).

After the evaluation of Emtricitabine, Tenofovir, and Efavirenz UV spectrum in the range of 200–400 nm, the wavelength of 260 nm was selected for detection due to the adequate molar absorptivity of Emtricitabine, Tenofovir, and Efavirenz in this region and the higher selectivity of this wavelength regarding possible interfering compounds or solvents in the sample.

3.1. Validation

3.1.1. Linearity

A linear correlation was found between the peak areas and the concentrations of Emtricitabine, Tenofovir and Efavirenz in the assayed range. The regression analysis data are presented in Table 3. The regression coefficients (r^2) obtained were higher than 0.999 for both compounds (Fig. 3) which attest the linearity of the method.

3.1.2. Analysis of fixed dose combination tablets

Samples of fixed dose combination of commercial tablets containing 200 mg of Emtricitabine, 300 mg of Tenofovir and 600 mg of Efavirenz were analyzed using the validated method. The results obtained are presented in Table 5. All the analyzed batches presented Emtricitabine, Tenofovir and Efavirenz contents very close to the labeled amount. The Emtricitabine content in the tablet samples varied from 99.77 to 101.53%. The Tenofovir content in the tablet samples varied from 99.27 to 101.65% while Efavirenz content varied from 100.01 to 101.67%.

3.1.3. Precision

Mean contents of Emtricitabine, Tenofovir, and Efavirenz in the intra-day precision analysis ($n = 6$) were 80 μg/ml (RSD = 0.76%), 120 μg/ml (RSD = 1.06%) and 240 μg/ml (RSD = 0.68%) respectively. For the intra-day precision $n = 18$ the mean contents obtained were 80 μg/ml (RSD = 0.77%), 120 μg/ml (RSD = 1.05%) and 240 μg/ml (RSD = 0.73%) for Emtricitabine, Tenofovir, and Efavirenz respectively. RSD values lower than 2% assure the precision of the method. The results obtained are presented in Table 4.

3.1.4. Accuracy

Accuracy was investigated by means of addition of Emtricitabine, Tenofovir, and Efavirenz reference standards to a mixture of the tablet excipients. The mean recovery ($n = 9$) was found to be 100.09%, (RSD = 0.51%), 99.88% (RSD = 0.30%) and 100.04% (RSD = 0.19%) for the drugs Emtricitabine, Tenofovir and Efavirenz respectively for demonstrating the accuracy of the method. The results obtained are presented in Table 6.

3.1.5. Specificity

Peak purities higher than 99.3% were obtained for Emtricitabine, Tenofovir and Efavirenz in the chromatograms of sample solutions, demonstrating that other compounds did not co-elute with the main peaks. The chromatogram obtained

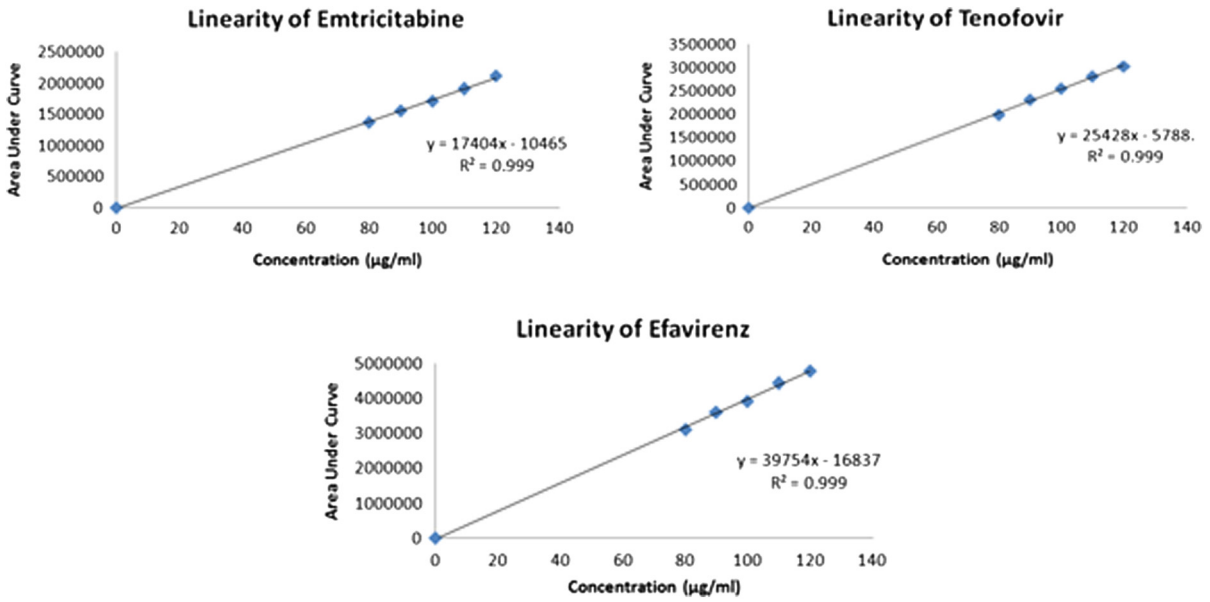

Figure 3 Linearity profile of Emtricitabine, Tenofovir and Efavirenz.

Table 4 Assay of Emtricitabine, Tenofovir and Efavirenz in the fixed dose combination tablets.

Sample	Batch	Content (%) + S.D.		
		Emtricitabine	**Tenofovir**	**Efavirenz**
Viraday (Cipla)	A	100.03 ± 0.42	101.65 ± 0.44	100.01 ± 0.21
	B	101.53 ± 0.51	99.27 ± 0.32	101.09 ± 0.27
	C	100.31 ± 0.32	99.59 ± 0.37	101.48 ± 0.32

S.D = Standard Deviation; (n = 6).

Table 5 Precision studies for Emtricitabine, Tenofovir and Efavirenz.

Compound	% Assay (Day-1, Analyst-1, Instrument-1)	% RSD of Assay (N = 6)	% Assay (Day-2, Analyst-2, Instrument-2)	% RSD of Assay (N = 6)
Emtricitabine	100.52	0.76	99.31	0.77
Tenofovir	101.07	1.06	100.64	1.01
Efavirenz	99.39	0.68	99.09	0.73

Table 6 Recovery studies for Emtricitabine, Tenofovir and Efavirenz by standard added commercial formulations.

Drug	Levels (%)	Amount recovered (mg)	% Recovery	Mean % recovery	% RSD
Emtricitabine	80	364.06	100.26	100.09	0.55
	100	402.64	099.68		0.48
	120	444.17	100.34		0.31
Tenofovir	80	542.85	099.97	099.88	0.45
	100	603.03	099.82		0.48
	120	661.71	099.84		0.63
Efavirenz	80	1088.66	100.02	100.04	0.21
	100	1206.13	100.09		0.27
	120	1328.31	100.02		0.18

Table 7 Robustness studies for Emtricitabine, Tenofovir and Efavirenz.

S.No	Condition	% RSD		
		Emtricitabine	Tenofovir	Efavirenz
1	Flow (+10%)	0.37	0.28	0.52
2	Flow (−10%)	0.31	0.18	0.24
3	Temperature (23 °C)	0.3	0.21	0.44
4	Temperature (27 °C)	0.41	0.43	0.32
5	Wave length (+2 nm)	0.29	0.67	0.28
6	Wave length (−2 nm)	0.42	0.21	0.18
7	pH of buffer (2.8)	1.02	0.81	0.25
8	pH of buffer (3.2)	0.64	0.2	0.23
9	Organic (+2%)	0.18	0.27	0.09
10	Organic (−2%)	0.24	0.2	0.12

with the mixture of the capsule excipients showed no interfering peaks in the same retention time of Emtricitabine, Tenofovir and Efavirenz.

3.1.6. Robustness

Statistical analysis showed no significant difference between results obtained employing the analytical conditions established for the method and those obtained in the experiments in which variations of some parameters were introduced. Thus, the method showed to be robust for changes in mobile phase flow rate 1.35 and 1.65 ml/min, methanol: buffer proportion (±2% of organic solvent), mobile phase pH (4.3 and 4.7). The results obtained are presented in Table 7.

3.1.7. Detection and quantitation limits

According to the determined signal-to-noise ratio, Emtricitabine, Tenofovir, and Efavirenz presented limits of detection of 0.38, 0.76, 0.94 μg/ml and limits of quantitation of 1.25, 2.5, 3.10 μg/ml, respectively where the compound proportion was found in the sample solutions injected onto the chromatograph. However, the objective of the method is the quantitation of Emtricitabine, Tenofovir, and Efavirenz, so the values obtained for Emtricitabine, Tenofovir, and Efavirenz should be considered as the limit of method sensitivity.

The development of a simple and reliable method is essential to assure the identification and quantitative determination of antiretroviral drugs since the problem of counterfeit or substandard antiretrovirals is well established all over the world. The quality control of the antiretroviral pharmaceutical preparations marketed nowadays may help to assure the treatment efficacy and avoid the development of resistance to antiretrovirals drugs.

4. Conclusion

This study was the first report of development and validation of Emtricitabine, Tenofovir, and Efavirenz in pure form for pharmaceutical formulations. The developed method showed to be a simple, precise, accurate and suitable technique to quantify the antiretrovirals and might be employed for quality control analysis as well as in further studies in other matrices, such as plasma. Emtricitabine, Tenofovir, and Efavirenz tablets analyzed by the validated method showed adequate quality and drug contents in concordance with the labeled amount.

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