Research Article

RP-HPLC Method Development and Validation for Simultaneous Estimation of Emtricitabine and Tenofovir in Pure and Pharmaceutical Dosage Form

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ABSTRACT

A simple, precise, accurate RP-HPLC method with PDA detector has been developed and subsequently validated for the simultaneous estimation of Emtricitabine and Tenofovir in pure and pharmaceutical dosage form. The estimation was carried out on Hyperclone C18 (250mm x 4.6mm i.d; particle size 5µm) column with a mixture of potassium di hydrogen phosphate buffer (pH-2.5 adjusted with orthophosphoric acid) and methanol in the ratio of 35:65 v/v as mobile phase at flow rate 1ml/min with UV detection performed at 254 nm. The retention times of Emtricitabine and Tenofovir were 2.580 min & 3.711 min respectively. The concentration range was found to be linear 30-70 µg/ml for Emtricitabine and 45-105 µg/ml for Tenofovir. The correlation coefficient (r2) was found to be 0.999 for both the drugs. The limit of detection was found to be 2.92 and 3.05 and the limit of quantification was found to be 3.05µg/ml and 10.07µg/ml for Emtricitabine and Tenofovir. The %RSD values were less than 2 for both the drugs. The assay of Emtricitabine and Tenofovir was found to be 99.77% and 100.12% respectively. The method was validated for linearity, accuracy, precision, robustness, LOD, LOQ as per ICH guidelines. The developed method was successfully used for the quantitative analysis of commercial available dosage form.

Keywords: Emtricitabine, Tenofovir, Method development, validation, RP-HPLC.

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INTRODUCTION

Emtricitabine (4-amino-5-flouro1-[(2S,5R)-2-(hydroxy methyl)-1,3-oxathiolan-5-yl]-1,2dihydro pyrimidin-2-one) marketed by Gilead Sciences with the brand name *Emtriva*.[1] Emtricitabine is the (-) enatiomer of a thio analog of cytidine. It has a molecular formula of $C_8H_{10}FN_3O_3$ and a molecular weight of 247.24. Emtricitabine is a nucleoside analogue used for the prevention of perinatal HIV-1 reverse transcriptase.[2] It also acts actively against Hepatitis B virus.[3,4] The drug works by inhibiting reverse transcriptase, the enzyme that copies HIV RNA into new viral DNA[5]. By interfering with this process, which is central to the replication of HIV, Emtricitabine can help to lower the amount of HIV, or "viral load", in a patient's body and can indirectly increase the number of immune system cells (called T cells or CD4+ T-cells). Both of these changes are associated with healthier immune systems and decreased likelihood of serious illness. Like Lamivudine, Emtricitabine, when used alone, it does not completely suppress viral replication. This allows drug resistant strains to emerge[6]. Emtricitabine is indicated in combination with other antiretroviral agents for the treatment of HIV infection in adults. Emtricitabine is commercially available and is approved by the FDA for treatment of HIV infection[7].

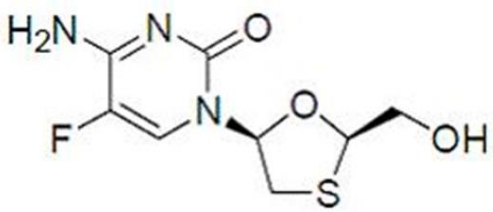

Figure 1: Structure of Emtricitabine

Tenofovir [8] ({[(2R) -1- (6-amino-9H-purin-9-yl) propan-2-yl] oxy} methyl) phosphoric acid) marketed by Gilead Sciences under the trade name Viread. It has a molecular formula of $C_{19}H_{30}N_5O_{10}P \bullet C_4H_4O_4$ and a molecular weight of 635.52. It belongs to a class of antiretroviral drugs known as nucleotide analogue reverse transcriptase inhibitors (NRTIs). It blocks reverse transcriptase, a crucial virus enzyme in human immunodeficiency virus 1 (HIV-1) and hepatitis B virus infections[9]. Tenofovir may be measured in plasma by liquid chromatography. Such testing is useful for monitoring therapy and to prevent drug accumulation and toxicity in patients with renal or hepatic impairment.[10-12] A review examined the use of Tenofovir as a pre-exposure prophylaxis against HIV infection. It is found that Tenofovir alone, as well as the Tenofovir/Emtricitabine combination, significantly decreased the risk of contracting HIV[13]. Tenofovir is

currently in late-stage clinical trials for the treatment of hepatitis B. Tenofovir is an acyclic nucleoside phosphonate di-ester analog of adenosine mono phosphate. Tenofovir requires initial di-ester hydrolysis for conversion to Tenofovir and subsequent phosphorylation by cellular enzymes to form Tenofovir diphosphate. Tenofovir diphosphate is a weak inhibitor of mammalian DNA polymerases α, β, and mitochondrial DNA polymerase γ. [14-17]

From the literature review it was found that various analytical methods like UV, HPLC and HPTLC have been reported for the estimation of Emtricitabine and Tenofovir. Present work is aimed to develop a new, simple, rapid, accurate RP-HPLC method for the simultaneous estimation of Emtricitabine and Tenofovir. The developed method was validated according to ICH guidelines.

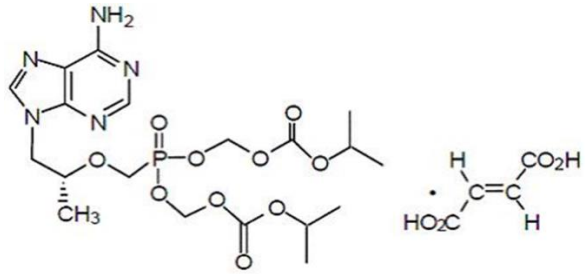

Figure 2: Structure of Tenofovir

MATERIALS AND METHODS

Chemicals and reagents

Tenofovir and Emtricitabine pure standard drugs were obtained from pharmatrain labs, Rajahmundry, India. Methanol and ortho phosphoric acid, Sodium Di hydrogen Ortho Phosphate, Milli-Q water were supplied by Merck chemical laboratories. Travuda, (200mg of Emtricitabine and 300mg of Tenofovir combination tablets) tablets was purchased from local market.

HPLC instrumentation and chromatographic condition

Chromatographic separation studies were carried out on a waters alliance 2695 with PDA detector in isocratic mode for the working standard solution of Emtricitabine and Tenofovir. Initially, trials were carried out using water: methanol, water: acetonitrile, Phosphate buffer: methanol in different ratios with different columns to obtain the desired system suitability parameters. After several trials Phosphate buffer: methanol (35:65 v/v), was selected as the mobile phase, which gave good resolution and sharp peaks. The same mobile phase was used as the diluents also. The method was optimized at 254 nm and the runtime was taken as 6min. Other chromatographic conditions like run length, sample application volume, sample application positions, distance between tracks, detection wavelength, were optimized to give reproducible R_f values and symmetrical peak shape.

Instrumentation

Column : Hyerclone C_{18} (250× 4.6 mm) 5µm

Mobile phase : Phosphate buffer (pH 2.5): Methanol (35:65v/v)

Flow rate : 1ml/min

Column temperature: 30°C

Injection volume : 20 µl

Wavelength : 254nm

Run time : 06 min

Retention times : 2.580(EMT), 3.711(TNF) min.

Preparation of standard stock solutions

Standard stock solution of Emtricitabine was prepared by dissolving 10 mg of drug in 10 ml of diluent to get the concentration of 1000µg/ml from which 0.5 ml was further diluted with the same solvent to get the final concentration of 50µg/ml.

Standard stock solution of Tenofovir was prepared by dissolving 15 mg of drug in 10 ml of diluent to get the concentration of 1500µg/ml from which 0.5 ml was further diluted with the same diluent to get the final concentration 75µg/ml.

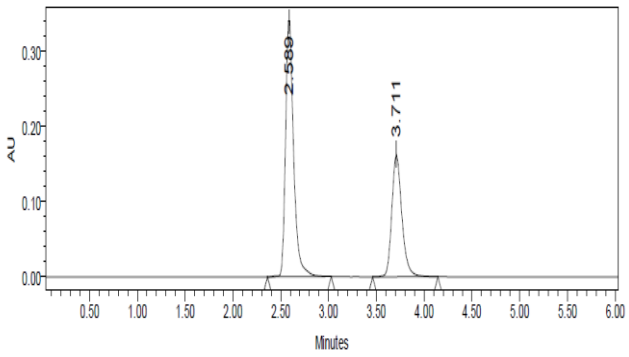

Figure 3: Chromatogram showing standard injection

Table 1: Chromatogram data of EMT &TNF

S.No	Peak Name	R_t	Area	Height	USP Plate Count	USP Tailing	USP Resolution
1	Emtricitabine	2.580	2233704	365596	4456	1.4	6.5
2	Tenofovir	3.711	1328106	174637	5823	1.3	

Assay of Emtricitabine and Tenofovir

20 tablets were weighed accurately and powdered in a mortar. An amount equivalent to 66.5 mg (tablet Containing 200 mg of ETB and 300 mg of TNF) was transferred to 50 ml volumetric flask and added 10ml of mobile phase and sonicated for 10 min and make up to 50 ml with mobile phase. The solution was filtered 0.45 µm pore size membrane filter. The filtered sample solution 5mL was diluted to 10 ml with mobile phase to get the solution containing ETB and TDF in 200 & 300 µg/ ml proportions respectively. The solution was injected in to HPLC and % assay was calculated. The results are depicted in **Table 2**.

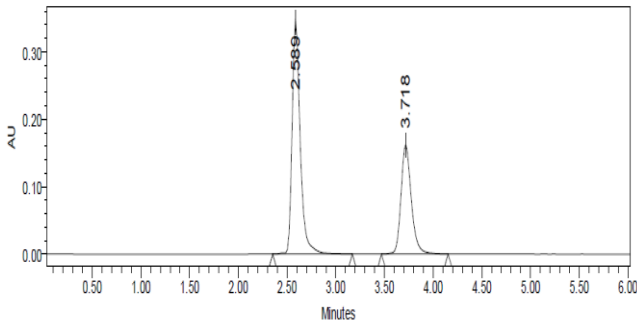

Figure 4: Chromatogram showing sample injection

Table 2: Analysis of marketed formulation data

Drug name	Label claim	Test conc. (µg/ml)	Mean amount found	% Drug content	% RSD
Emtricitabine	200 mg	198.6	99.3	0.14	198.6
Tenofovir	300 mg	299.8	99.9	0.18	299.8

Analytical method validation

Linearity

Accurately weighed and transferred 10 mg of Emtricitabine and 15mg of Tenofovir working standards into a 10ml clean dry volumetric flask added about 7mL of diluent and sonicated to dissolve it completely and made volume up to the mark with the same solvent (Stock solution).The linearity of the method was determined by pipetting out appropriate aliquots of the above stock solution into a series of 10 ml volumetric flasks and the volume was made up to the mark to obtain the concentration ranging from 30-70 µg/ml for Emtricitabine and 45-105 µg/ml for Tenofovir. Calibration curves were plotted with observed peak areas Vs concentration followed by the regression equations. The calibration curves for Emtricitabine and Tenofovir were shown in **Fig.No.5** and **Fig. No.**6 and their respective linearity parameters were given in **Table 3.**

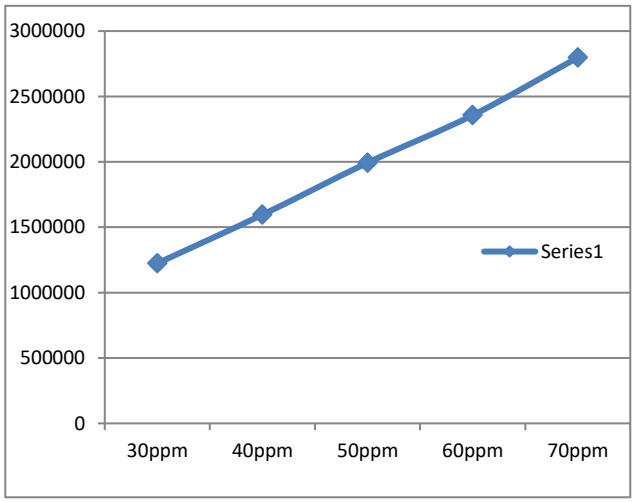

Figure 5: Linearity graph of Emtricitabine

Precision

The intra-day precision (%RSD) was carried out by analyzing standard drug solutions within the calibration range, six times on the same day. The Inter-day precision (%RSD) was carried out by analyzing drug solutions within the calibration range on three different days over a period of a week by different operators or by different instruments. The method precision was determined by injecting six working standard solutions and six sample injections. The peak areas of all the injections were taken and standard deviation, % relative standard deviation (RSD), was calculated. The precision parameters were given in **Table 4.**

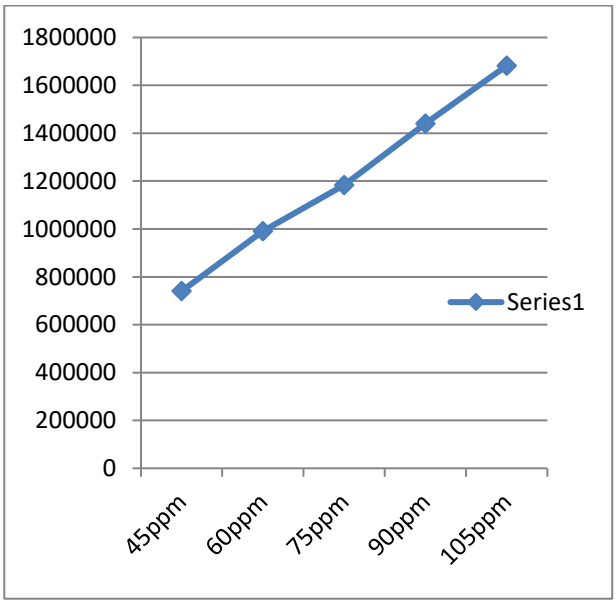

Figure 6: Linearity graph of Tenofovir

Table 3: Results of Linearity studies

Parameters	Emtricitabine	Tenofovir
Linearity range	30-70 µg	45-105 µg
Correlation coefficient (r^2)	0.999	0.999
Regression line equation	y = 39070x + 39803	y = 15561x + 39995
LOD	2.92	3.05
LOQ	3.05	10.07

Table 4: Precision results of Emtricitabine and Tenofovir

Sample No	Intra day		Inter day	
	% label claim		% label claim	
	ETB		TNF	
1	100.21	99.99	100.10	99.91
2	99.51	100.01	99.94	99.96
3	99.34	100.06	100.46	100.35
4	100.14	99.61	100.21	100.07
5	100.30	100.21	99.13	99.54
6	99.25	100.08	99.67	99.76
Mean	99.75	100.21	99.26	99.54
SD	0.2262	0.226247	0.3742	0.3742
% RSD	0.2264	0.2264	0.3742	0.3742

Accuracy (% Recovery)

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. The accuracy of the method was determined by recovery studies at three different concentration levels 50%, 100%, and150% by spiking known quantities of the drug analyte and % of recovery were calculated. The results were given in **Table 5.**

Specificity and Selectivity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. Two different samples were injected and studied with respective placebo. The HPLC chromatograms recorded for the drug matrix (mixture of the drug and placebo) showed almost no interfering peaks with in retention time ranges.

Table 5: % Recovery studies

Drug	Amount taken	Amount added	Total amount	% Recovery	S.D	%R.S.D
ETB	200	50	249.23	99.69	0.2371	0.2372
	200	100	300.45	100.15		
	200	150	350.10	100.02		
TNF	300	50	350.21	100.06	0.04618	0.04617
	300	100	400.25	100.06		
	300	150	449.92	99.98		

LOD and LOQ

LOD is the lowest amount of analyte in a sample that can be detected but not necessarily quantified as an exact value under the stated experimental conclusions. The quantification limit of an analytical procedure is the lowest amount of an analyte of a sample which can be quantitatively determined with suitable precision and accuracy. The LOD and LOQ were calculated from the slope(s) and the standard deviation (SD) of the peak areas using the formulae LOD = 3.3 σ/s and LOQ = 10 σ/s. The results were given in **Table 6.**

Table 6: LOD &LOQ for ETB& TNF

S.No	Drugs	LOD(µg/ml)	LOQ(µg/ml)
1	Emtricitabine	0.001	0.004
2	Tenofovir	0.005	0.015

Robustness

The robustness of the method was studied during the method development by varying the following parameters.

Flow rate: The standard solution of EMT and TNF were prepared and injected by varying the flow rate from 0.8ml/min to 1.2ml/min.

Mobile phase: Standard solution of ETB and TNF were prepared and injected by varying the mobile phase along with the optimized method. The results were tabulated in table 3. The results were tabulated in **Table 7.**

Table 7: Robustness study of Emtricitabine &Tenofovir

Chromatographic condition		Emtricitabine		Tenofovir	
		USP plate count	USP tailing	USP plate count	USP tailing
Flow rate	0.8	4327.2	1.3	6542.4	1.2
	1.0	5036.1	1.3	5839.5	1.2
	1.2	5674.5	1.4	6024.2	1.2
Mobile phase	5%less	6498.2	1.2	4577.3	1.3
	Actual	5026.5	1.3	6381.5	1.2
	5% more	6471.0	1.2	4476.1	1.3

CONCLUSION

A simple, accurate RP-HPLC method has been developed and validated for the estimation of ETB &TNF in combined dosage form. The proposed method is precise and it can be used for the simultaneous estimation of Emtricitabine & Tenofovir in pharmaceutical dosage form as well as routine quality control laboratories.

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