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Development and Validation of RP-HPLC Method for the Simultaneous Estimation of Tenofovir Alafenamide Fumarate and Emtricitabine in Bulk and Tablet Dosage Form

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ABSTRACT

New Analytical method was developed for the estimation of Emtricitabine and Tenofovir Alafenamide Fumarate in drug product by liquid chromatography. The chromatographic separation was achieved on Cosmosil C18 column (250mm×4.6ID, 5µm) at ambient temperature. The separation achieved employing a mobile phase consists of Methanol:water(80:20v/v). The flow rate was 0.8ml/ minute and ultra violet detector at 252nm. The average retention time for Emtricitabine and Tenofovir Alafenamide Fumarate found to be 4.277min and 5.293min. The proposed method was validated for selectivity, precision, linearity and accuracy. All validation parameters were within the acceptable range. The assay methods were found to be linear from 10-50µg/ml for Emtricitabine and 15-75µg/ml for Tenofovir Alafenamide Fumarate.

Keywords: Emtricitabine and Tenofovir Alafenamide Fumarate, HPLC, Methanol and validation.

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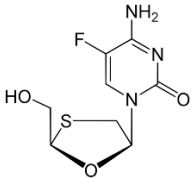
INTRODUCTION

Drug Profile

Emtricitabine: Emtricitabine (2'-deoxy-5-fluoro3'thiacytidine, FTC), with trade name Emtriva is a nucleoside reverse transcriptase inhibitor (NRTI) for the prevention and treatment of HIV infection in adult



Structure of emtricitabine

IUPAC Name: 4-amino-5-fluoro-1-[(2R, 5S)-2(hydroxymethyl)-1, 3-oxathiolan-5-yl]-1, 2dihydropyrimidin-2-one

Molecular formula: C8H10FN3O3S

Molecular Weight: 247.248 g/mol.

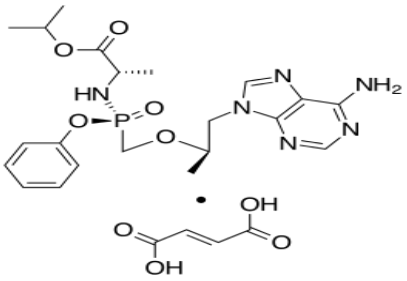
Solubility: Soluble in ACN, Water, and Methanol.

Pka: 14.29.

Mechanism of action: Emtricitabine works by inhibiting reverse transcriptase, the enzyme that copies HIV RNA into new viral DNA. Emtricitabine is a synthetic nucleoside analogue of cytidine. It is phosphorylated by cellular enzymes to form emtricitabine 5'-triphosphate, which is responsible for the inhibition of HIV-1 reverse transcriptase. It competes with the natural substrate deoxycytidine 5'triphosphate and incorporates into nascent viral DNA, resulting in early chain termination. Therefore emtricitabine inhibits the activity of HIV-1 reverse transcriptase (RT) both by competing with the natural substrate deoxycytidine 5'-triphosphate and by its incorporation into viral DNA. By inhibiting HIV-1 reverse transcriptase, 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

Tenofovir Alafenamide Fumarate: Tenofovir alafenamide fumarate (INN/USAN; trade name Vemlidy) is a nucleotide

reverse transcriptase inhibitor and a prodrug of tenofovir. It was developed by Gilead Sciences for use in the treatment of HIV infection and chronic hepatitis B, and is applied in the form of Tenofovir alafenamide fumarate (TAF). Closely related to the commonly used reverse transcriptase inhibitor Tenofovir disoproxil fumarate (TDF), TAF has greater antiviral activity and better distribution into lymphoid tissues than that agent.



Structure of Tenofovir alafenamide fumarate

IUPAC Name: Isopropyl (2S)-2-[[[(1R)-2-(6aminopurin-9-yl)-1-methyl-ethoxy] methyl-phenoxyphosphoryl] amino] propanoate**.**

Molecular formula: C21H29N6O5P**.**

Molecular Weight: 476.466 g/mol**.**

Solubility: Soluble in methanol, Acetonitrile and water

Pka: 11.36

Mechanism of action: Tenofovir alafenamide fumarate is a nucleotide reverse transcriptase inhibitor (NRTI) and a novel ester prodrug of the antiretroviral tenofovir. Following oral administration, TAF is converted in vivo to tenofovir, an acyclic nucleoside phosphonate (nucleotide) analog of adenosine 5′-monophosphate. Tenofovir mimics normal DNA building blocks but is lacking a 3'-OH molecule required for phosphodiester bond linkage. By competing with regular nucleotides for incorporation into proviral DNA and prevention of the formation of the 5' to 3' phosphodiester linkage required for DNA elongation, Tenofovir causes early chain termination and prevents proviral DNA transcription.

EXPERIMENTAL

Equipments: The chromatographic technique performed on a HPLC workstation software, Cosmosil C18 (250mm×4.6ID,5µ), Wenser ultra sonicator, Wenser high precision balance , Vaccum micro filtration unit with 0.45µ membrane filter was used in the study.

Materials: Pharmaceutically pure sample of Emtricitabine/Tenofovir Alafenamide fumarate were obtained as gift samples from Mecleod pharmaceutical, Gujarat, India. HPLC-grade Methanol and water

Chromatographic conditions:

Column: C18 column (250 x 4.6 mm,5µ)
Mobile phase: Methanol: Distill water in proportion of 80:20 v/v
Detector: 252nm
Injection volume: 20 µl
Flow rate: 0.8 ml/min
Temperature: Ambient
Run time: 10 min
Diluents: Mobile Phase

Selection of Mobile Phase

Standard solution of EMT and TA were injected into the HPLC system and run in different solvent systems. Mixture of different solvents were tried in order to determine optimum chromatographic conditions for effective separation of EMT and TA. After several permutation and combination, it was found that mixture of methanol: water gives satisfactory results as compared to other mobile phases. Finally, the optimal composition of the mobile phase methanol: water in the ratio of 80:20v/v was selected, as it gave high resolution of EMT and TA with minimal tailing.

Preparation of mobile phase: Mobile phase was prepared by mixing 80ml of methanol with 20ml of distilled water.

Preparation of standard stock solution:About 45 mg of TA and 30 mg of EMT were accurately weighed & transferred to 10 ml volumetric flasks. Both the drugs were dissolved in 5 ml of mobile phase with shaking and then volume was made up to the mark with the mobile phase to get 4500µg/ml of TA and 3000µg/ml of EMT of standard stock solution of each drug. Then it was ultrasonicated for 10 minutes and filtered through 0.20µ membrane filter..

Preparation of sample solution: Accurately weighed twenty tablets were ground to obtain fine powder equivalent to 200mg of Emtricitabine and 25 mg of Tenofovir Alafenamide fumarate sample were weighed and transferred to 100 ml of volumetric flask and dissolved in diluent. The flask was shaken and volume was made up to mark with diluents to give a primary stock solution. From the above solution 1 ml of solution is pipetted out into a 10 ml volumetric flask and volume was made up to mark with diluents to give a solution containing 30µg/ml of Emtricitabine and 45µg/ml Tenofovir Alafenamide fumarate.

RESULTS AND DISCUSSIONS

Determination Of Working Wavelength (λ max): 10 mg of the Emtricitabine and Tenofovir Alafenamide fumarate standard drug is taken in a 10 ml volumetric flask and dissolved in Diluent and volume made up to the mark, from this solution 0.1ml is pipette into 10 ml volumetric flask and made upto the mark with the Water to give a concentration 10 µg/ml. The above prepared solution is scanned in uv between 200-400 nm using Water as blank. The λmax was found to be 252nm.

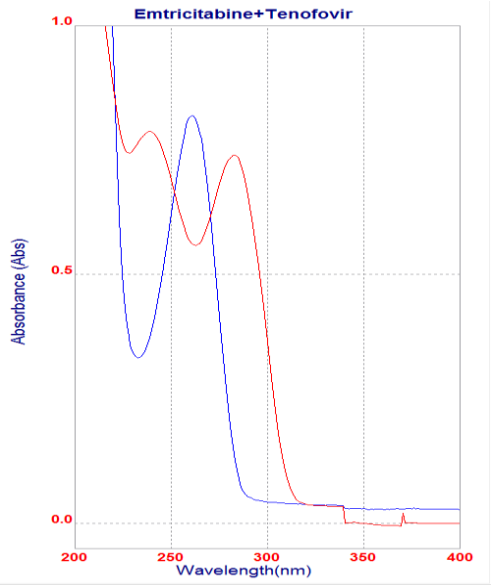


Figure: 1 Chromatogram of Emtricitabine and Tenofovir Alafenamide fumarate

Method Validation

Linearity: Linearity was studied by analyzing five standard solutions covering the range of 10-50g/mL for Emtricitabine and 15-75µg/ml of Tenofovir Alafenamide fumarate. From the primary stock solution 1.0ml, 1.5ml, 2.0ml, 2.5ml, 3.0ml and 1.5ml,3.0ml,45ml,60ml,75ml,of aliquots are pipetted into 10 ml volumetric flasks and made up to the mark with the mobile phase to give a concentrations of 10µg/mL, 20µg/ml, 30µg/mL, 40µg/mL, 50µg/mL of Emtricitabine and 15g/mL, 30µg/mL, 45µg/mL, 60µg/mL and 75µg/mL of Tenofovir Alafenamide fumarate.

Calibration curve with concentration verses peak areas was plotted by injecting the above prepared solutions and the obtained data were subjected to regression analysis using the least squares method.

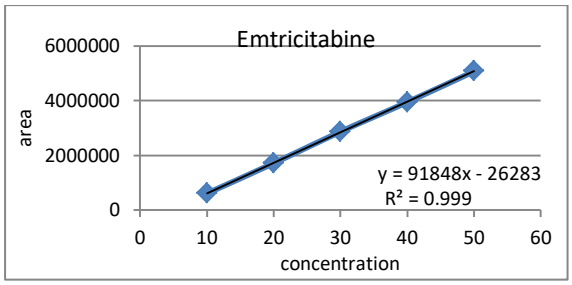


Figure 2: Linearity (calibration) curve Emtricitabine

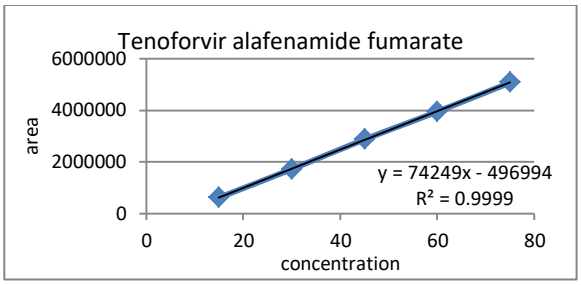


Figure 3: Linearity (calibration) curve of Tenofovir alafenamide fumarate

Result: A linear relationship between peak areas versus concentrations was observed for Emtricitabine and Tenofovir Alafenamide fumarate in the range of 10% to 50% and 15% to75% of nominal concentration. Correlation coefficient was 0.9993 and 0.9991 for both Emtricitabine and Tenofovir Alafenamide fumarate which prove that the method is linear in the range of 10% to 50% and 15% to75%.

Limit of detection and limit of quantification: The limit of detection (LOD) and limit of quantification (LOQ) were separately determined based on standard deviation of the y-intercept and the slope of the calibration curve by using the equations (1) and (2), respectively.

LOD = 3.3 σ /S ..... (1)

LOQ =10 σ /S ..... (2)

Where, σ = the standard deviation of the response (STEYX) S = the slope of the calibration curve. Slope S may be estimated from the calibration curve of the analyte.

Table 1: LOD and LOQ values Calculated from calibration curve .

	Emtricitabine(mg)	Tenofovir alafenamide fumarate
LOD	0.079%	0.047%
LOQ	0.24%	0.14%

Precision

Repeatability: The precision of the method was checked by repeated preparation (n=6) of 30µg/ml of Emtricitabine and 45µg/ml of Tenofovir Alafenamide fumarate without changing the parameter of the proposed chromatographic method. And measure the peak areas and retention times.

Table 2: Result of repeatability (intra-day)

Drug	Conc (µg/ml)	Morning	Evening	Mean	%RSD
Emtricitabine	30	2532980	2533416	2533059	0.06%
		2531529	2535640		
		2532579	2532208		
TAF	45	2870144	2871352	2871167	0.09%
		2867512	2874794		
		2869554	2873645		

Intermediate precision: The Intra and Inter-day precision was determined by analysis of the marketed formulation on the same day at different time intervals and on different days respectively

Table 3: Result of repeatability (intra-day)

Drug	Conc (µg/ml)	Day1	Day2	Mean	%RSD
Emtricitabine	30	2532980	2529331	2533059	0.06%
		2531529	2534937		
		2532579	2535014		
TAF	45	2870144	2872721	2871167	0.09%
		2867512	2872706		
		2869554	2871935		

Result: The relative standard deviation values for repeatability precision were found less than 2%. %RSD of repeatability was 0.06% for Emtricitabine and 0.09% for tenofovir alafeamide fumarate and %RSD of intermediate precision was 0.08% for Emtricitabine and 0.07% for Tenofovir alafenamide fumarate.

4.**3 Accuracy**

The accuracy of the method was determined by calculating the recoveries of Emtricitabine and Tenofovir Alafenamide fumarate by analyzing solutions containing approximately 50%, 100% and 150% of the working strength of Emtricitabine and Tenofovir Alafenamide fumarate. The percentage recovery results obtained are listed in Table 4 &5

Table 4: The accuracy results for Emtricitabine

%Concentration	Area	Amount added (mg)	Amount found (mg)	% Recovery	Mean % recovery
50%	2528334	10	29.94	99.8	99.80
100%	3405037	20	39.93	99.82	
150%	4301062	30	49.90	99.8	

Table 5: The accuracy results for Tenofovir alafenamide fumarate

%Concentration	Area	Amount added(mg)	Amount found (mg)	% Recovery	Mean % recovery
50%	2869077	15	44.98	99.95	99.94
100%	3944458	30	59.98	99.96	
150%	5067816	45	74.95	99.93	

Result: The accuracy study was performed for % recovery of Emtricitabine and Tenofovir alafenamide fumarate. The % recovery was found to be 99.80% and 99.94% respectively (NLT 98% and NMT 102%)

Robustness: Robustness is the measure of a method remain unaffected by small, deliberate changes in method parameters like flow rate and detection wavelength on assay of the analyte of interest. Here the detection wavelength varied ±2nm and flow rate was varied ±0.2 ml/min. The results were shown in (Table no. 6&7).

Table 6: Results of Emtricitabine

Parameter		Area	Mean	SD	%RSD
Flowrate	0.9	1572158	1573945	1550.66	0.0985204
	0.7	1574736			
	0.8	1574940			
Wavelenght	250	1575147	1574796	441	0.028003
	254	1574301			
	252	1574940			

Table 7: Results of of Tenofovir alafenamide fumarate

Parameter		Area	Mean	SD	%RSD
Flowrate	0.9	1719803	1720171	1964.57	0.1142178
	0.7	1722294			
	0.8	1718417			
Wavelenght	250	1721703	1719281	2125.66	0.123636
	254	1717724			
	252	1718417			

Result

The results of Robustness of the present method had shown that changes are not significant we can say that the method is Robust.

Table 8: Summary for results of validation parameters

Validation Parameter		Acceptance criteria	Results	
			Emtricitabine	Tenofovir alafenamide fumarate
Linearity		Correlation coefficient NLT 0.9990	0.9991	0.9992
Accuracy (%Recovery)		98 – 102 %	99.80 %	99.94%
Precision	Repeatability	%RSD NMT 2.0	0.06 %	0.09 %
	Intermediate	%RSD NMT 2.0	0.08 %	0.07 %
Robustness	Change in wavelength	%RSD NMT 2.0	0.028 %	0.12 %
	Change in flow rate	%RSD NMT 2.0	0.09%	0.11 %
Limit of detection (µg/ml)			0.079 %	0.047 %
Limit of quantitation (µg/ml)			0.24%	0.14%

CONCLUSION

From the above experimental results it was concluded that, this newly developed method for the simultaneous estimation of Emtricitabine and Tenofovir Alafenamide fumarate was found to be simple, precise, accurate and high resolution and shorter retention time makes this method more acceptable and cost effective and it can be effectively applied for routine analysis in research institutions, quality control department in meant in industries, approved testing laboratories.

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