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SIMULTANEOUS ESTIMATION OF LAMIVUDINE AND TENOFOVIR ALAFENAMIDE FUMARATE IN BULK DRUG PRODUCT BY RP-HPLC METHOD

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ABSTRACT
New analytical method was developed for the estimation of Lamivudine and Tenofovir alafenamide fumarate in drug product by liquid chromatography. The chromatographic separation was achieved on C18 column (Eclipse XDB-Phenyl 250*4.6mm) at ambient temperature. The separation achieved employing a mobile phase consists of 0.1%v/v Trifluoro acetic acid in water: Methanol (300:700). The flow rate was 1.0ml/ minute and ultra violet detector at 260nm. The average retention time for Lamivudine and Tenofovir alafenamide fumarate found to be 2.412 min and 4.669 min. 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 300.0 – 900.00µg/ml for Lamivudine and 6.3 –18.8µg/ml of Tenofovir alafenamide fumarate.

KEYWORDS: Lamivudine, Tenofovir alafenamide fumarate, Isocratic, HPLC, Eclipse XDB-Phenyl, Trifluoro acetic acid, Acetonitrile, Methanol and validation.

1. INTRODUCTION

LAMIVUDINE

Lamivudine[1-2] commonly called 3TC, is an antiretroviral medication used to prevent and treat HIV/AIDS. It is also used to treat chronic hepatitis B when other options are not possible. It is effective against both HIV-1 and HIV-2. It is typically used in combination with other antiretrovirals such as zidovudine and abacavir. Lamivudine may be included as part of post-exposure prevention in those who have been potentially exposed to HIV. Lamivudine is taken by mouth as a liquid or tablet. Lamivudine is chemically designated as 4-Amino-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin2-one. Its molecular formula is C8H11N3O3S and its molecular weight is 229.26 g/mol.

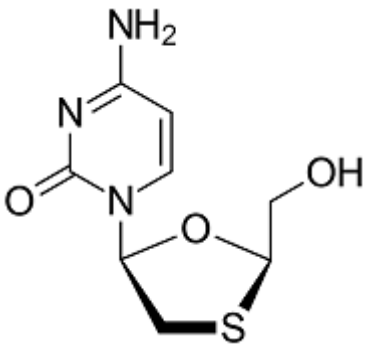


Fig. 1: Chemical structure: Lamivudine.

Tenofovir alafenamide fumarate

Tenofovir alafenamide (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.

Tenofovir alafenamide fumarate is chemically designated as Isopropyl (E)-but-2-enedioic acid; propan-2-yl (2S)-2-[[[(2R)-1-(6-aminopurin-9-yl)propan-2-yl]oxymethyl-phenoxyphosphoryl]amino]propanoate. Its molecular formula is C46H62N12O14P2, and its molecular weight is 1069.02 g/molg/mol.

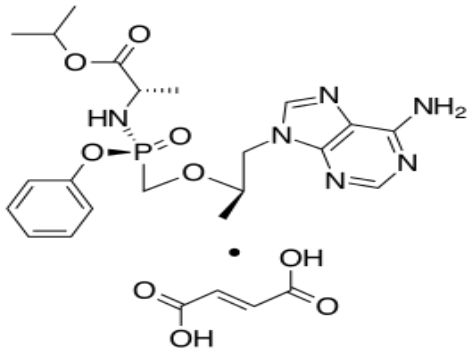


Fig. 2: Chemical structure: Tenofovir alafenamide fumarate.

2. MATERIALS AND METHODS

Equipments: The chromatographic technique performed on a waters 2695 with 2487 detector and Empower2 software, reversed phase Phenyl column (Eclipse XDB-Phenyl 250*4.6, 5µm) as stationary phase, Ultrasonic cleaner, Scaletech analytical balance and Vacuum micro filtration unit with 0.45µ membrane filter was used in the study.

Materials: Pharmaceutically pure sample of Lamivudine/Tenofovir alafenamide fumarate were obtained as gift samples from Fortune pharma training institute, Sri Sai nagar colony, KPHB, Hyderabad, India.

HPLC-grade Methanol and Acetonitrile were obtained from qualigens reagents pvt ltd. Trifluoro acetic acid (AR grade) was from sd fine chem.

Chromatographic conditions The sample separation was achieved on a (Eclipse XDB-Phenyl 250*4.6mm) Phenyl column, aided by mobile phase mixture of 0.1%v/v Trifluoro acetic acid in water: Methanol (30:70). The flow rate was 1.0ml/ minute and ultra violet detector at 260nm that was filtered and degassed prior to use, Injection volume is 5µl and ambient temperatures.

Preparation of mobile phase

Buffer Preparation: Taken accurately 1ml of Trifluoro acetic acid in 1000ml of water.

Mobile phase: Then added 30 volumes of buffer and 70 volumes of Methanol mixed well and sonicated for 5 min.

Diluents: Water: Acetonitrile : 50:50 v\v.

Preparation of standard stock solution

A 150 mg of pure Lamivudine 40 mg of and 5 mg of Tenofovir alafenamide fumarate were weighed and transferred to 10 ml of volumetric flask and dissolved in diluent. The flask was shaken and volume was made up to mark with diluent to give a primary stock solution. From the above solution 0.4ml of solution is pipette out into a 10 ml volumetric flask and volume was made up to mark with water to give a solution containing 600.0µg/ml of Lamivudine and 12.5µg/ml Tenofovir alafenamide fumarate.

Preparation of sample solution: Accurately weighed twenty tablets were ground to obtain fine powder equivalent to 150 mg of Lamivudine and 5mg of Tenofovir alafenamide fumarate sample and transferred to 25 ml of volumetric flask and dissolved in diluent. The flask was shaken and volume was made up to mark with diluent to give a primary stock solution. From the above solution 0.4 ml of solution is pipette out into a 10 ml volumetric flask and volume was made up to mark with diluents to give a solution containing 600.0µg/ml of Lamivudine and 12.5µg/ml Tenofovir alafenamide fumarate.

Method validation

System suitability

The typical values for evaluating system suitability of a chromatographic procedure are RSD <2%, tailing factor <1.5 and theoretical plates >3000. The retention time, peak area, theoretical plates and tailing factor were evaluated for system.

Linearity

Linearity was studied by analyzing five standard solutions covering the range of 50.0 -150.0µg/ml for Lamivudine and 6.3-18.8µg/ml Tenofovir alafenamide fumarate. From the primary stock solution 0.125ml, 0.187ml, 0.25ml, 0.312ml, 0.375 ml of aliquots are pipette into 10 ml volumetric flasks and made up to the mark with the water to give a concentrations of 50.0 µg /mL, 75.0µg/mL, 100.0µg/mL,125.0µg/mL and 150.0µg/mL of Lamivudine and 6.3g/mL, 9.4µg/mL, 12.5µg/mL, 15.6µg/mL and 18.8 µg/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.

2.5.3. 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.

$LOD = 3.3\ \delta/S$

$LOQ = 10\ \delta/S$

Where,

δ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte.

2.5.4. Method precision

The precision of the method was checked by repeated preparation(n=6) of 100.0µg/ml of Lamivudine and 12.5µg/ml Tenofovir alafenamide fumarate without changing the parameter of the proposed chromatographic method. And measured the peak areas and retention times.

2.5.5. Accuracy

The accuracy of the method was determined by calculating the recoveries of Lamivudine and Tenofovir alafenamide fumarate by analyzing solutions containing approximately 50%, 100% and 150% of the working strength of Lamivudine and Tenofovir alafenamide fumarate.

2.5.6. 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.

3. RESULTS AND DISCUSSIONS

Determination of Working Wavelength (λ max): 10 mg of the Lamivudine 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 of 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 260nm.

After several initial trails with mixtures of methanol, water, Acetonitrile and buffer in various combinations and proportions, a trail with a mobile phase mixture of 0.1%v/v Trifluoro acetic acid in water: Methanol (30:70). At flow rate was 0.8mL/ minute brought sharp peaks. The chromatogram was shown in Fig 3.

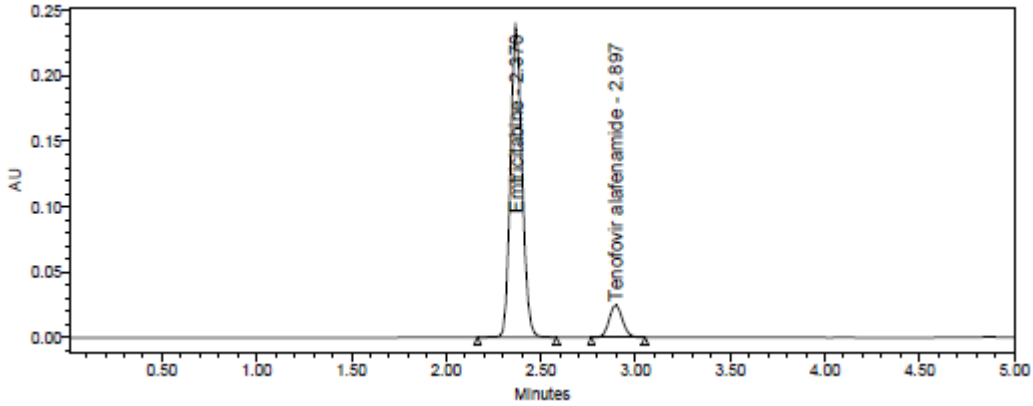


Fig 3: Chromatogram of Lamivudine and Tenofovir alafenamide fumarate.

System suitability

The system suitability of the method was checked by repeated preparations for Tenofovir alafenamide fumarate and Lamivudine. The typical values for evaluating system suitability of a chromatographic procedure are RSD <2%, tailing factor <1.5 and theoretical plates >3000. The retention time, peak area, theoretical plates and tailing factor were evaluated for system, System suitability data of Tenofovir alafenamide fumarate and Lamivudine are shown in Table 1.

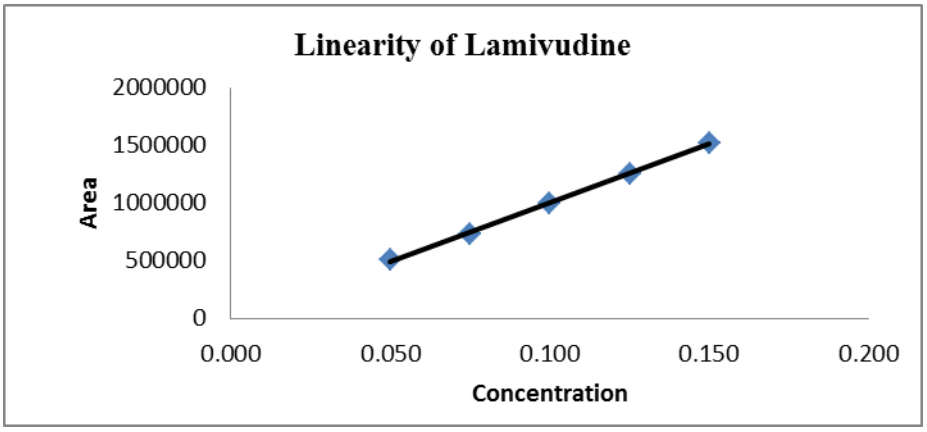
Table 1: System suitability data of Lamivudine and Tenofovir alafenamide fumarate.

Parameter	Lamivudine	Tenofovir alafenamide fumarate	Acceptance criteria
Retention time	3.596	4.671	+-10
Theoretical plates	9708	9841	>3000
Tailing factor	1.18	1.13	<1.50
% RSD	0.21	0.19	<2.00

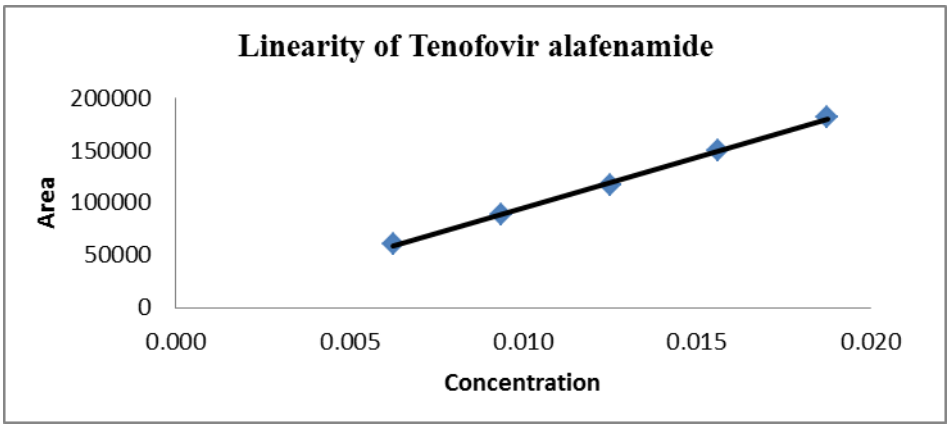
Linearity

Linearity was studied by analyzing five standard solutions covering the range of 50.0 -150.0µg/ml for Lamivudine and and 6.3 -18.8µg/ml Tenofovir alafenamide fumarate. From the primary stock solution 0.125ml, 0.187ml, 0.25ml, 0.312ml, 0.375 ml of aliquots are pipette into 10 ml volumetric flasks and made up to the mark with the water to give a concentrations of 50.0 µg /mL, 75.0µg/mL, 100.0µg/mL, 125.0µg/mL and 150.0µg/mL of Lamivudine and 6.3g/mL, 9.4µg/mL, 12.5µg/mL, 15.6µg/mL and 18.8 µg/mL of Tenofovir alafenamide fumarate in Table 2 and Table 3.

A linear relationship between peak areas versus concentrations was observed for Lamivudine and Tenofovir alafenamide fumarate in the range of 50% to 150% of nominal concentration. Correlation coefficient was 1.000 and 0.9999 for Lamivudine and Tenofovir alafenamide fumarate.



Calibration curve: Lamivudine:



Calibration curve: Tenofovir alafenamide fumarate

Table 2: Linearity data of Lamivudine.

Level	Concentration (mg/mL)	Peak area
50%	0.300	10005696
75%	0.450	1431355
100%	0.600	1851581
125%	0.750	2194208
150%	0.900	2607788
Correlation		1.0000

Table 3: Linearity data of Tenofovir alafenamide fumarate.

Level	Concentration (mg/mL)	Peak area
50%	0.006	89373
75%	0.009	134333
100%	0.013	181107
125%	0.016	228803
150%	0.019	276512
Correlation		0.9999

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.

The slope S may be estimated from the calibration curve of the analyte.

Table 4: LOD and LOQ values Calculated from calibration curve.

	Lamivudine mg	Tenofovir alafenamide fumarate mg
LOD	0.034	0.0002
LOQ	0.103	0.001

Method precision (repeatability)

The precision of the method was checked by repeated preparation(n=6) of 50.0µg/ml of Lamivudine and 12.5µg/ml Tenofovir alafenamide fumarate without changing the parameter of the proposed chromatographic method. And measure the peak areas and retention times. The precision of the method (% RSD) was found to be <1% showing good repeatability. The values of percentage RSD for Lamivudine and Tenofovir alafenamide fumarate are shown in Table 5 and Table 6.

Table 5: Summary of peak areas for method precision of Lamivudine.

	Retention time	Peak area	% Assay
1	2.412	1821825	99.7
2	2.411	1816004	99.6
3	2.412	1820231	99.4
4	2.408	11826610	99.5
5	2.412	1839151	100.6
6	2.411	1879371	100
Mean	2.411	1833865	99.8
%RSD	**0.06**	**1.29**	**0.46**

Table 6: Summary of peak areas for method precision of Tenofovir alafenamide fumarate.

Sample No	Retention time	Peak area	% Assay
1	4.673	182071	99.1
2	4.672	182342	99.8
3	4.677	181025	99.3
4	4.678	180623	98.7
5	4.678	182125	99.5
6	4.677	182444	100.3
Mean	4.676	181772	99.5
%RSD	**0.06**	**0.42**	**0.56**

Accuracy (recovery study)

The accuracy of the method was determined by calculating the recoveries of Lamivudine and Tenofovir alafenamide fumarate by analyzing solutions containing approximately 50%, 100% and 150% of the working strength of Lamivudine and Tenofovir alafenamide fumarate. The percentage recovery results obtained are listed in Table 7 & 8.

Table 7: Recovery data of Lamivudine.

LEVEL	S.NO	%Recovery of Lamivudine	Average
50	1	99.4	99.3%
	2	99.8	
	3	98.8	
100	1	99.7	99.5%
	2	99.6	
	3	99.4	
150	1	99.3	99.6%
	2	99.7	
	3	99.8	

Table 8: Recovery data of Tenofovir alafenamide fumarate.

LEVEL	S.NO	%Recovery of Tenofovir alafenamide fumarate	Average
50	1	99.6	99.1%
	2	99.2	
	3	98.5	
100	1	99.1	99.4%
	2	99.8	
	3	99.3	
150	1	99.7	99.9%
	2	99.6	
	3	99.3	

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 9&10) the results of Robustness of the present method had shown that changes are not significant was found to be the method is Robust.

Table 9: Results of Lamivudine.

parameter	Rt of Lamivudine	Theoretical plates	Asymmetry
Decreased flow rate (0.7ml/min)	2.998	9738	1.12
Increased flow rate (0.9ml/min)	2.016	7368	1.06
Wave Length 258nm	2.413	8267	1.07
262nm	2.414	8342	1.07

Table 10: Results of Tenofovir alafenamide fumarate.

parameter	Rt of Tenofovir alafenamide fumarate	Theoretical plates	Asymmetry
Decreased flow rate (0.7ml/min)	5.364	10256	1.14
Increased flow rate (0.9ml/min)	4.201	9213	1.11
Wave Length 258nm	4.669	9872	1.12
262nm	4.672	9815	1.13

Ruggedness: The ruggedness of the method was studied by analyzing the sample and standard preparations by two analysts. The results were shown in Table 11&12. The %RSD assay values between two analysts was calculated, this indicates the method was rugged.

Table 11: Ruggedness data for Lamivudine.

		%Assay	%RSD
Analyst-1	Lamivudine	99.7	0.07%
Analyst-2		99.6	

Table 12: Ruggedness data for Tenofovir alafenamide fumarate.

		%Assay	%RSD
Analyst-1	**TENOFOVIR ALAFENAMIDE FUMARATE**	99.1	0.50%
Analyst-2		99.8	

CONCLUSION

From the above experimental results it was concluded that, newly developed method for the simultaneous estimation of LAMIVUDINE 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 pharmaceutical industries, approved testing laboratories.

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