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METHOD DEVELOPMENT AND VALIDATION OF RP- HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF LAMIVUDINE AND TENOFOVIR DISOPROXIL FUMARATE IN PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

The analysis of Lamivudine and Tenofovir by improved High Performance liquid chromatography method with PDA detector is investigated. Lamivudine (LAM) and Tenofovir Disoproxil Fumarate (TDF) belongs to a class of antiretroviral drugs known as nucleotide analogue reverse transcriptase inhibitors (NRTIs), which block reverse transcriptase, an enzyme crucial to viral production. In the present study an RP-HPLC method was developed and validated for the simultaneous estimation of Lamivudine and Tenofovir in pharmaceutical dosage forms. The chromatographic system used in this study is equipped with Symmetry C8 column and PDA detector set at 260nm, with a mobile phase of phosphate buffer (pH 3, adjusted with orthophosphoric acid) and Methanol in the ratio of 40:60 at a flow rate of 0.8 ml/min and the injection volume set at 20 µl with 10 minutes of run time. The described method was linear over a concentration range of 25 –125 µl for the simultaneous estimation of Lamivudine and Tenofovir with a good linearity response of 0.9990 and 0.9996 respectively. The retention time of Lamivudine was 2.44 min and of Tenofovir was 3.97min. The results of analysis were validated. The results of the study showed that the proposed RP-HPLC method was simple, rapid, precise and accurate, which is useful for the routine determination of Lamivudine and Tenofovir in bulk drug and pharmaceutical dosage form.

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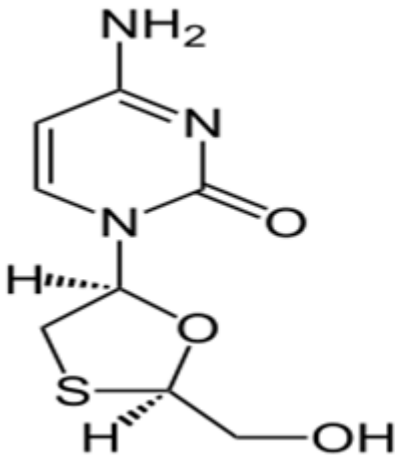
KEYWORDS : *Lamivudine, Tenofovir, RP-HPLC, NRTI's.*

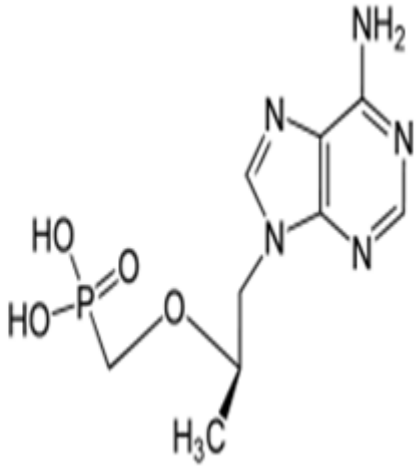
INTRODUCTION

Lamivudine[15] (2',3'-dideoxy-3'-thiacytidine, commonly called 3TC) is a potent nucleoside analog reverse transcriptase inhibitor (NRTI). It is chemically known as 4-amino-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one. Lamivudine is an analogue of cytidine. It can inhibit both types (1

and 2) of HIV reverse transcriptase and also the reverse transcriptase of hepatitis B. Lamivudine has been used for treatment of chronic hepatitis B at a lower dose than for treatment of HIV. It improves the seroconversion of e-antigen positive hepatitis B and also improves histology staging of the liver. Tenofovir Disoproxil Fumarate[16] is a prodrug form of Tenofovir. Tenofovir belongs to a class of antiretroviral drugs known as nucleotide analogue reverse transcriptase inhibitors (NRTIs), which block reverse transcriptase, an enzyme crucial to viral production. It is chemically know as ({[(2R)-1-(6-amino-9H-purin-9-yl) propan-2-yl] oxy} methyl) phosphoric acid. Both the drugs act by inhibiting the HIV reverse transcriptase enzyme competitively and act as a chain terminator of DNA synthesis. Literature survey reveals that Tenofovir Disoproxil Fumarate and Lamivudine are estimated individually by UV, derivative – HPLC, Plasma RP-HPLC and Plasma LC/MS/MS methods. RP-HPLC, LC-MS/MS and HPTLC methods were reported for the simultaneous estimation of Emtricitabine and Tenofovir Disoproxil Fumarate in human plasma and in formulations. To the best of our knowledge, there is no reported RP-HPLC method for Simultaneous Estimation of Lamivudine and Tenofovir Disoproxil Fumarate in pharmaceutical formulations, previous to our work. The wave length selection is made at 260 nm since all the two compounds maximum absorbance in UV spectrum as reported in the literature is in 260 nm. As the drug was polar in nature, RP-HPLC method was preferred. Thus, efforts were made to develop fast, selective and sensitive analytical method for Simultaneous estimation of Lamivudine and Tenofovir Disoproxil Fumarate in their combined dosage form.



LAMIVUDINE



TENOFOVIR DISOPROXIL FUMARATE

MATERIALS AND METHODS:

Chemical and Reagents:

Lamivudine & Tenofovir working standard, Potassium dihydrogen orthophosphate-HPLC grade (MERCK), Methanol-HPLC grade (MERCK), Acetonitrile-HPLC grade (MERCK), O-phosphoric acid, triethylamine and Milli Q water.

APPARATUS:

Waters HPLC (Alliance-2695) with water's PDA detector (2996), ACE Symmetry C8 (4.6 x 150mm, 5 µm) column, Digital pH meter (Digisun Electronics-7007), Electronic balance (Shimadzu-AY220), Photo stability chamber (Thermolab-943/03/0607), Centrifuge (Thermolab-R_8C), Volumetric flask and Pipettes (Borosilicate).

Preparation of Buffer:

Weigh accurately 7.8gm of Potassium dihydrogen orthophosphate into a beak containing 1000 ml of Milli-Q water, and Sonicate to dissolve completely. Add 1ml of triethylamine and adjusted the pH of the solution to 3.0±0.05 with dilute orthophosphoric acid. Filter through 0.45µm membrane filter.

Preparation of Mobile phase:

Mix a mixture of above Buffer 400ml (40%) and 600ml of Methanol HPLC (60%) and degas in ultrasonic water bath for 5 minutes. Filter through 0.45µm membrane filter under vacuum filtration.

Preparation of Standard Solution:

Accurately 10mg of Lamivudine & Tenofovir were weighed and transferred into 10ml volumetric flask, about 7ml of diluent was added and sonicated for 5 minutes to dissolve it. The volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter (Stock solution). From this 0.75ml of solution was pipette out and transferred into 10ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter.

Preparation of Sample Solution:

20 Lamivudine & Tenofovir tablets were weighed and the average weight was calculated. Accurately the sample equivalent to 10mg of Lamivudine & Tenofovir was weighed & transferred into 10ml volumetric flask about 7ml of diluent was added and sonicated for 5 minutes to dissolve it content. The volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter. 0.75ml of sample solution was pipetted out and transferred into 10ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter.

METHOD DEVELOPMENT:

Procedure:

In order to separate the three components different chromatographic conditions were tried. Columns like C8, C18 of different lengths, different particles size were tried. The C8 (symmetry) of 4.6 x 150mm and 5 µm particle size shown results. The mobile phase was optimized by trying different mobile phase's methanol/water, acetonitrile/water, buffer (pH 3)/acetonitrile/methanol, buffer (pH 4)/methanol and buffer (pH3)/methanol in different proportions at different flow rates. The mobile phase containing phosphate buffer and methanol has shown some promising results. For further optimization phosphate buffer of different pH in different proportions were tried. The mobile phase containing potassium dihydrogen orthophosphate buffer (pH 3): methanol in the proportion of 40:60%v/v gave symmetric peaks with good resolution and theoretical plates. Two drugs showed good signals at 260 nm which was used as detections wave length throughout the analysis. With this optimized mobile phase system suitability study was performed.

Inject 20µl of the standard and sample solution into the chromatographic system and measure the area for the Lamivudine & Tenofovir peaks and calculate the %Assay by using the assay formula.

Optimized Chromatographic Conditions:

Equipment: High performance liquid chromatography equipped with Auto Sampler Waters Empower software, PDA-2996 detector.

Column : Symmetry C18 (4.6 x 150mm, 5 µm, Make: ACE) column or equivalent.
Mobile Phase : Buffer (pH=3): Methanol in the ratio of 40:60%v/v.
Flow rate : 0.8 ml / min
Wavelength : 260 nm
Injection volume : 20 µl
Run time : 10 min
Column temperature: Ambient
Technique : Isocratic

METHOD VALIDATION:

The validation of the current method has been performed in accordance with USP requirements for assay determination (Category-I: analytical methods for quantitation of active ingredients in finished pharmaceutical products) which include accuracy, precision, selectivity, linearity, range, robustness and ruggedness.

SYSTEM SUITABILITY:

The standard stock solution was injected five times into HPLC system as per test procedure. The system suitability parameters were evaluated from standard Chromatograms obtained, by calculating the % RSD of retention times, tailing factor, theoretical plates and peak areas from five replicate injections.

PRECISION:

a) Reproducibility/ System Precision:

Stock solution was injected six times into HPLC system as per test procedure. The system precision parameters were evaluated from standard Chromatograms obtained, by calculating the % RSD of peak areas from six replicate injections.

b) Repeatability/ Method precision:

The method precision was determined by preparing the sample from the tablet formulation for five times and six successive injections of 20µl of working sample solution were injected and the chromatograms were recorded.

ACCURACY:

Accuracy was determined in terms of percent recovery. Sample solution spiked with the analytes at the three different concentration levels 50, 100, 150 µg/ml of both Lamivudine and Tenofovir Disoproxil Fumarate. Another set of standard mixtures at the same concentration levels was also prepared with the diluents. Sample and standard solutions are injected into HPLC system in triplicate. Percentage recoveries of Lamivudine and Tenofovir Disoproxil Fumarate were calculated by using formula

Formula:

$$\%\text{Recovery} = \frac{\text{(Amount recovered)}}{\text{(Actual amount added)}} \times 100$$

LINEARITY: The linearity of the method was established by spiking a series of standard solutions and working samples at concentrations of 25, 50, 75,100,125,150 µg/ml of Lamivudine and Tenofovir Disoproxil Fumarate were prepared and injected into the chromatographic system. Construct the calibration curves for standard solutions by plotting their response ratios (ratios of the peak area of the analyte) against their respective slope, intercept, and correlation coefficient were determined.

SPECIFICITY:

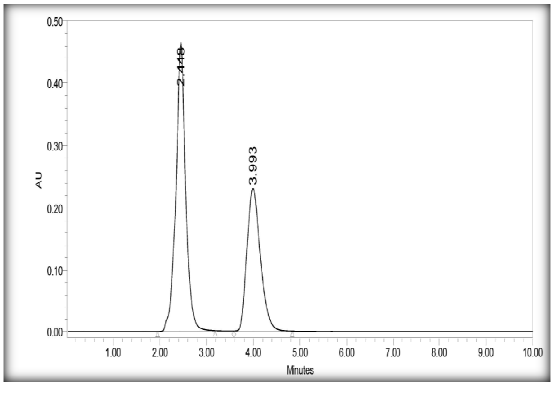
Specificity was performed to exclude the possibility of interference with excipients in the region of elution on Tenofovir Disoproxil Fumarate. The specificity and selectivity of the method was tested under normal conditions and the results of the tests produced that the components other than the drug did not produce a detectable signal at retention time of Lamivudine and Tenofovir Disoproxil Fumarate.

ROBUSTNESS:

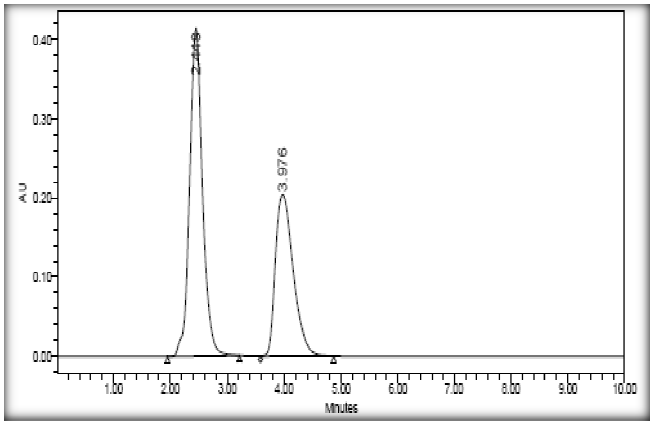
The robustness of the developed method was investigated by evaluating the influence of small deliberate variations in procedure variables like flow rate (±0.1 ml/min) and the change in mobile phase ratio (±5%).

RUGGEDNESS:

The ruggedness of the method was investigated by evaluating the influence of different analyst, different labs, different analyst, and different lots of reagents, different elapsed assay times, different assay temperature, different days etc.



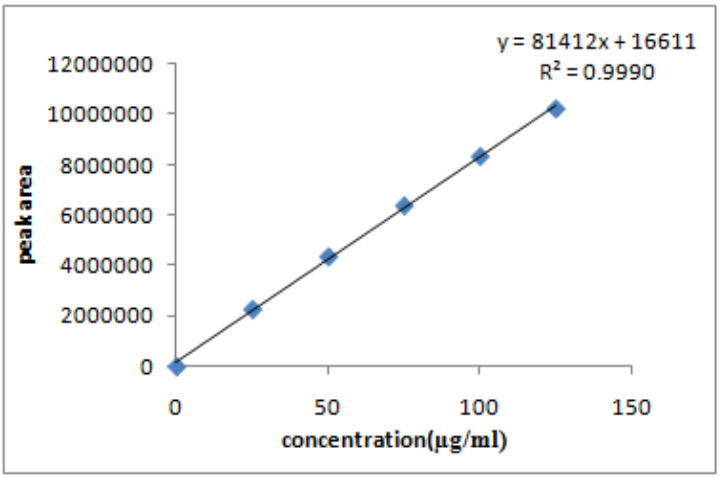
Standard chromatogram



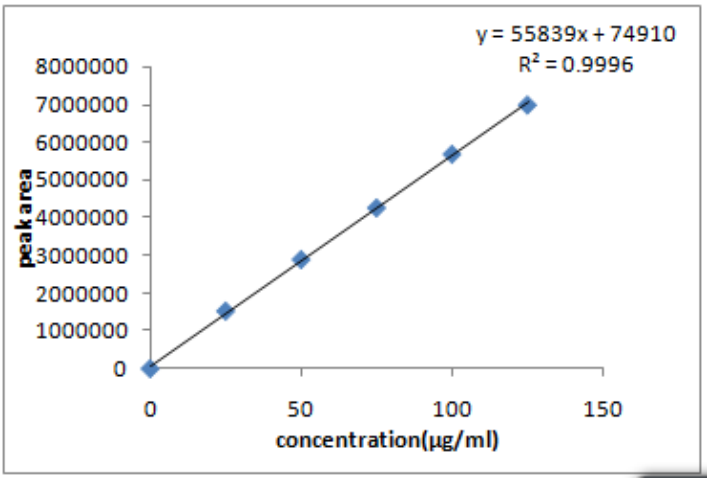
Sample chromatogram

	Name	Retention Time (min)	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Lamivudine	2.45	6739592	463588	2736.06	1.0
2	Tenofovir	3.99	4637107	231647	2853.19	1.3

	Name	Retention Time (min)	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Lamivudine	2.448	6709473	454296	2796.42	1.1
2	Tenofovir	3.976	4598839	230126	2895.13	1.4



Linearity of Lamivudine



Linearity of Tenofovir

Table No.1 Showing List of System suitability parameters of Lamivudine & Tenofovir

Parameter	Area		Retention Time		Theoretical Plates		Tailing Factor	
	LAM	TDF	LAM	TDF	LAM	TDF	LAM	TDF
	6673432	4458842	2.445	3.976	2766.2	2853.2	1.1	1.3
	6573180	4496725	2.443	3.968				
	6769056	4619732	2.446	3.986				
	6684315	4396428	2.445	3.967				
	6769112	4458678	2.447	3.974				
MEAN	12.226	19.871	12.226	19.871				
SD	81224	82923	0.0015	0.0076				
%RSD	0.24	0.36	0.012	0.038				

Table No.2 Showing Results of Precision for Lamivudine & Tenofovir

Parameter	System Precision		Method Precision	
	Lamivudine	Tenofovir	Lamivudine	Tenofovir
Area	6852536	4576258	6859856	4396432
	6926931	4657326	6926578	4457356
	6795873	4491437	6795357	4491874
	6678317	4713814	6678336	4513756
	6784739	4713814	6784868	4513647
	6807672	4624293	6807468	4624667
MEAN	6807678	4629290	6808744	4499622
SD	82011	85924	82770	75534
%RSD	1.20	1.74	1.21	1.67

Table No.3 Showing Results of % Recovery studies for Lamivudine & Tenofovir

Inj. Sample	Spike level	Amount present	Amount recovered	% Recovered	Mean recovery	Acceptance Criteria
Lamivudine	50 %	5mg	4.94mg	98.8%	98.96%	98-102%
	100 %	10mg	9.96mg	99.6%		
	150 %	15mg	14.77mg	98.4%		
Tenofovir	50 %	5mg	4.92mg	98.45%	98.84%	98-102%
	100 %	10mg	9.91mg	99.1%		
	150 %	15mg	14.87mg	98.99%		

Table No.4 Showing results of Linearity for Lamivudine and Tenofovir

Linearity Level	Concentration	Area
I	25µg/ml	2266976
II	50µg/ml	4954694
III	75µg/ml	6730592
IV	100µg/ml	8384491
V	125µg/ml	10209389
Correlation Coefficient	0.9990	

Linearity Level	Concentration	Area
I	25µg/ml	1529418
II	50µg/ml	2898127
III	75µg/ml	4568836
IV	100µg/ml	5693545
V	125µg/ml	6999247
Correlation Coefficient	0.9996	

Table No.5 Showing Robustness results for change in flow rate and mobile phase of Lamivudine & Tenofovir

Flow rate	Inj. Sample	Area	Plate count	Tailing	RT	%RSD
0.7ml/min	**Lamivudine**	8344338	2671.86	1.1	2.79	**LAM-1.37**
	Tenofovir	5675524	2876.79	1.4	4.54	
0.9ml/min	**Lamivudine**	6590717	2575.82	1.1	2.58	
	Tenofovir	4500085	2800.83	1.5	3.54	
Mobile phase variation						
35:65	**Lamivudine**	6329717	2652.12	1.2	2.44	**TFV-1.56**
	Tenofovir	3990067	2795.62	1.4	3.97	
45:55	**Lamivudine**	7408362	2580.74	1.0	2.44	
	Tenofovir	5077859	2785.37	1.4	3.34	

Table No.6 showing results of Robustness

S. No	Column Code	Instrument Code	Analyst	Result Obtained (%)	
				Lamivudine	Tenofovir
1.	C-01	W-29	I	99.5	99.25
2.	C-02	W-30	II	99.85	99

Parameter	Result observed		Acceptance Criteria
	Lamivudine	Tenofovir	
Percentage Content	99.67	99.12	98 – 102%
%RSD	0.24	0.17	NMT 2%

Table No.7 showing validation parameters

S. No	Parameter	Requirement	Results		Acceptance criteria
			Lamivudine	Tenofovir	
1.	System Suitability	RT	2.445	3.974	
		Tailing factor	1.1	1.3	NMT 2
		Plate count	2766.24	2853.19	NLT 2000
		Assay value	99.56%	99.1%	98-102%
2.	Accuracy	% Recovery	98.96%	98.84%	98-102%
3.	Precision	%RSD	1.20	1.74	NMT 2%
4.	Specificity	No interference	Pass	Pass	No interference
5.	Linearity	Correlation coefficient	0.9990	0.9996	NLT 0.999
6.	Range	Concentration	25-125µg/ml	25-125µg/ml	Nil
7.	Robustness	%RSD	1.37	1.56	NMT 2%
8.	Ruggedness	%RSD	0.24	0.17	NMT 2%

RESULTS AND DISCUSSION:

The present study was carried out in developing a simple, precise, accurate, and rapid Reverse Phase High Performance Liquid Chromatography method for simultaneous estimation of Lamivudine and Tenofovir with different chromatographic conditions were tried. The symmetry C8 column, mobile phases containing potassium dihydrogen orthophosphate buffer (pH 3): methanol at a ratio of 40:60%v/v, and the flow rate of 0.8 ml/min found to resolve two components with good peak symmetry and theoretical plates. The retention times for Lamivudine and Tenofovir were found to be 2.4 min and 3.97 min respectively. System suitability parameters were studied with the five replicates standard solution of the drugs and the calculated parameters are within the acceptance criteria. The tailing factor, the number of theoretical plates and HETP or all in the acceptable limits. The specificity of the method was assessed by comparing the retention time of standard Lamivudine, Tenofovir and sample, good correlation was correlation was obtained between the retention time of standard and sample. Placebo and blank were injected and there were no peaks. There are no interferences hence method is specific. The linearity range for both Lamivudine and Tenofovir were found to be 25-125 ppm respectively. The regression equation for Lamivudine and Tenofovir were found to be as y = 81412x + 16611(R^2 = 0.9990) for Lamivudine and that of Tenofovir was y = 55839x +74910 (R^2 = 0.9996) and relative standard deviation (%RSD) was found to be less than 2% for sample analysis that

proves method is precise. The recovery studies shown recovery of the sample is between 98-102% that proves methods accuracy. The analysis of sample by second analyst did not shown any effect on its performance. The small deliberate changes in mobile phase composition, pH of the buffer and flow rate did not show any impact on retention time, peak symmetry, resolution and theoretical count.

CONCLUSION:

A new method is developed for Simultaneous Estimation of Lamivudine and Tenofovir by RP-HPLC method. The sample preparation is simple and the analysis time is short. The analytical procedure is validated as per ICH guidelines and shown to be accurate, precise and specific. This method represents a fast analytical procedure for the simultaneous quantitation of Lamivudine and Tenofovir. The method is amenable to the routine analysis of large numbers of samples with good precision and accuracy. Hence it can be concluded that the proposed HPLC method is sensitive and reproducible for the analysis of Lamivudine and Tenofovir in quality control laboratories with short analysis time.

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