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DEVELOPMENT AND VALIDATION OF SIMULTANEOUS RP-HPLC METHOD FOR ESTIMATION OF TENOFOVIR AND LAMIVUDINE IN BULK AND PHARMACEUTICAL FORMULATIONS

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ABSTRACT

A simple, specific, accurate and precise RP-HPLC method has been developed for the simultaneous determination of Tenofovir and Lamivudine from combined dosage form by reverse phase C18 column (*Younglin (S.K) Gradient System* UV (250mm x 4.6mm) 5µ). The sample was analysed using Acetonitrile 80 ml and water 20ml(pH 6.2 ,0.05 % OPA) as a mobile phase at a flow rate of 1.0ml/min and detection at 260nm. The retention time for tenofovir and lamivudine was found to be 2.933 min and 6.966 min respectively. The stability assay was performed for this combination and was validated for accuracy, precision, linearity, specificity and sensitivity in accordance with ICH guidelines. Validation revealed the method is specific, rapid, accurate, precise, reliable, and reproducible. Calibration plots were linear over the5-25 µg/mL for tenofovir and 5-25 µg/mL for lamivudine, respectively, and recoveries from combined dosage form were between 98 and 102%. The method can be used for estimation of combination of these drugs in combined dosage form.

KEYWORDS: Tenofovir , Lamivudine , RP-HPLC.

INTRODUCTION

Tenofovir[Figure 1] is chemically known as({[(2*R*)-1-(6-amino-9*H*-purin-9-yl)propan-2-yl]oxy}methyl)phosphonic acid. It inhibit the activity of HIV reverse transcriptase by competing with natural substrate deoxyadenosine 5'-triphosphate and ,after incorporation into DNA ,by DNA chain termination. Lamivudine [Figure 2] is chemically known as 4-amino-1-

[(2*R*,5*S*)-2-(hydroxymethyl) -1,3-oxathiolan-5-yl] -1,2-dihydropyrimidin-2-one. It is nucleoside reverse transcriptase inhibitor with activity against HIV-1 and hepatitis- B.

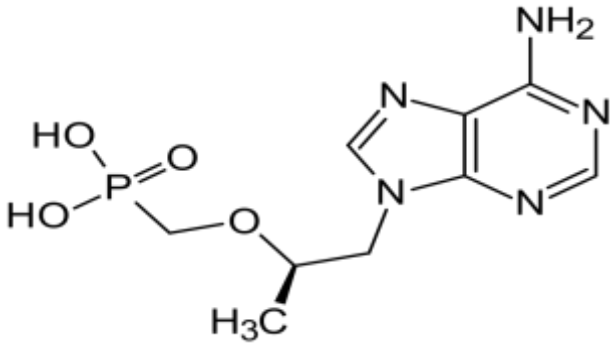

Figure 1: ({[(2*R*)-1-(6-amino-9*H*-purin-9-yl)propan-2-yl]oxy}methyl)phosphonic acid

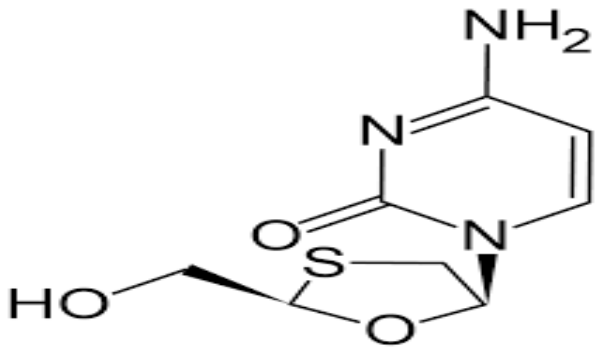

Figure2: 4-amino- 1- [(2*R*,5*S*)- 2-(hydroxymethyl) -1,3 -oxathiolan-5-yl]-1,2- dihy dropyrimidin- 2- one

Experimentals

Instrument

A High Performance Liquid Chromatographsystem, the purity determination performed on a stainles steel column 250mm long, 4.6mm internal diameter filled with Octadecyl silane chemically bonded to porous silica particles of 5µm diameter reverse phase C18 column (*Younglin (S.K) Gradient System UV* (250mm x 4.6mm) 5µ). Optimized chromatographic conditions are listed in Table 1.

MATERIALS AND CHEMICALS

Tenofovir & Lamivudine supplied as a gift sample by Mylan Laboratories Ltd, Sinnar, Nashik, India. All the chemicals used of HPLC Grade (Merk Ltd., Mumbai) and double distilled water was used for mobile phase preparation.

Preparation of Standard Stock Solution Solution A:.Weigh accurately about 10 mg of tenofovir working standard in a 10.0 ml volumetric flask. Dissolve and dilute up to mark with diluent. Solution B: Weigh accurately about 10 mg of lamivudine working standard in a 10.0 ml volumetric flask. Dissolve and dilute up to mark with diluent and that give concentration 1000 and 1000 µg/mL for tenofovir and lamivudine respectively.

Mixtured Standard Preparation From the standard stock solution, the mixed standard solutions were prepared using acetonitrile to contain 10µg/mL of tenofovir and 10µg/mL of lamivudine.

Preparation of test sample from Sample Stock Preparation : Take 10 µgm/ml+ 10 µgm/ml sample for assay (0.2 ml from tab stock and makeup 10 ml with 0.2 ml from tab stock and makeup 10 ml with mobile phase.

Selection of analytical wavelength

Each solution was scanned using double beam UV visible spectrophotometer in the spectrum mode between the wavelength range of 400 nm to 200 nm and their spectra was overlaid. The wavelength selected was 260 nm**.**

Selection of detection wavelength: UV detector was selected, as it is reliable and easy to set at constant wavelength. A fix concentration of analyte were analysed at different wavelengths. As per the response of analyte, 260 nm was selected.

Linearity Study: From the standard stock solution of Tenofovir and Lamivudine 0.25 mL were taken in 10 mL volumetric flask diluted up to the with Acetonitrile such that final concentration of Tenofovir and Lamivudine in the range 5-25 µg/mL of Lamivudine and 5-25 µg/mL of Lamivudine respectively. Volume of 20µl of each sample was injected with the help of Hamilton Syringe. All measurements were repeated five times for each concentration and calibration curve was constructed by plotting the peak area versus the drug concentration.

Method Validation: The proposed method was validated as per ICH guidelines. The solutions of the drugs were prepared as per the earlier adopted procedure given in the experiment.

Accuracy: It was done by recovery study using standard addition method at 80%, 100% and 120% level; known amount of tenofovir and lamivudine standard was added to preanalysed sample and subjected to the proposed HPLC method.

Precision: Precision of the method was studied as intra-day and inter- day variation and also repeatability of sample injections. Intra- day precision was determined by analyzing, the three different concentration 10 µg/mL,15 µg/mL and 20µg/mL of tenofovir and 10µg/mL, 15 µg/mL and 20µg/mL of lamivudine respectively, for three times in the same day. Inter day variability was assessed using above mentioned three concentration analysed on two different days, over a period of one week.

Repeatability: It was performed by injecting sample 10µg/mL of tenofovir and 10 µg/mL of lamivudine into the system and measuring the peak area. It was repeated for six times.

Ruggedness: Ruggedness of the method was studied by two different analyst using same operational and environmental condition. An appropriate concentration 10µg/mL of tenofovir and 10 µg/mL of lamivudine was analysed and concentration were determined. The procedure was repeated for six times.

Robustness: Robustness of the method was studied by making deliberate variation in parameters such as flow rate (± 0.1mL), % of Acetonitrile in the mobile phase composition (± 10%), and change in detection wavelength (±2 nm) and the effect on the results were examined. It was performed using 10µg/mL and 10 µg/mL solution of tenofovir and lamivudine in triplicate.

Limit of Detection and Limit of Quantification

The limit of detection (LOD) and the limit of quantification (LOQ) for tenofovir and lamivudine were determined from standard deviation of the response and the slope.

LOD= σ/S X 3.3;

LOQ= σ/S X 10

RESULTS AND DISCUSSION

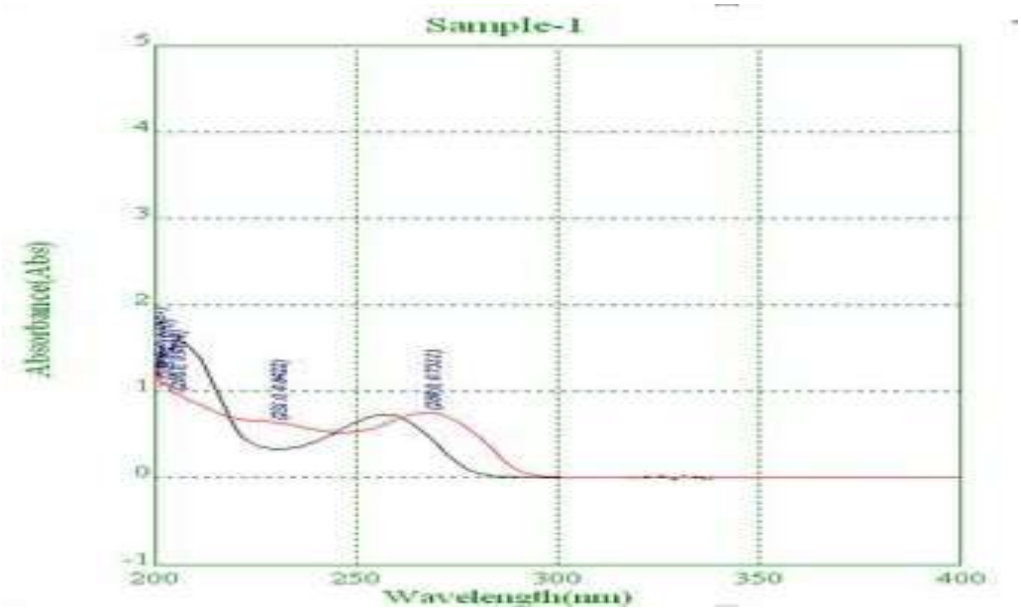

Figure 3: Overlain spectra of Tenofovir and Lamivudine

HPLC Method Development and Optimization: The finally optimized chromatographic conditions are.

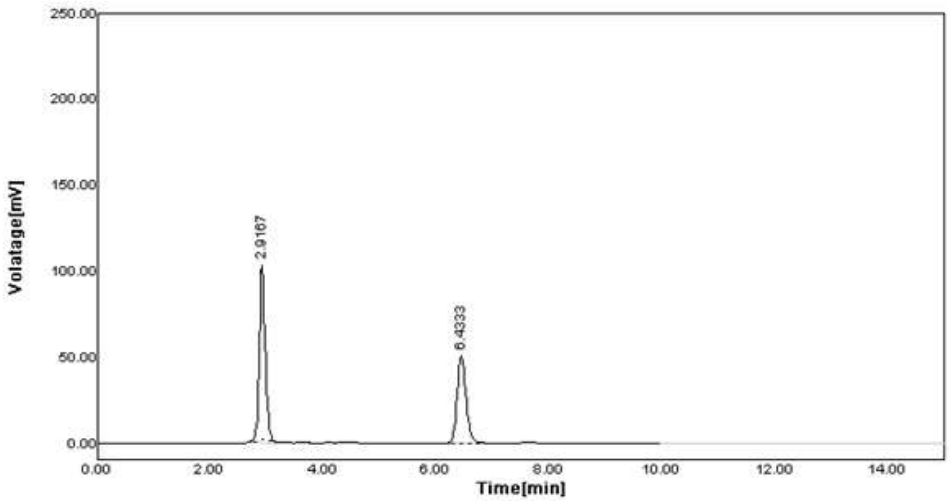

Figure 4:optimized chromatogram of tenofovir and lamivudine.

Sr. No.	Parameter	Description
1	Stationary Phase	C_{18} column with 250 mm x 4.6 mm i.d. and 5 µm particle size
2	Mobile Phase	Acetonitrile : Water (80:20) pH 6.2 with OPA
3	Flow Rate	0.7 ml/min
4	Detection wavelength	260 nm

5	Detector	UV detector
6	Injector	Rheodyne Injection
7	Injection volume	20µl
8	Column Temperature	Ambient

2.Linearity

Table No 1. Linearity of Lamivudine

Sr. No.	Concentration µg/ml	Area	RSD
1	5	143.21	1.60
2	10	282.64	1.49
3	15	418.94	1.09
4	20	551.48	1.14
5	25	661.56	0.76

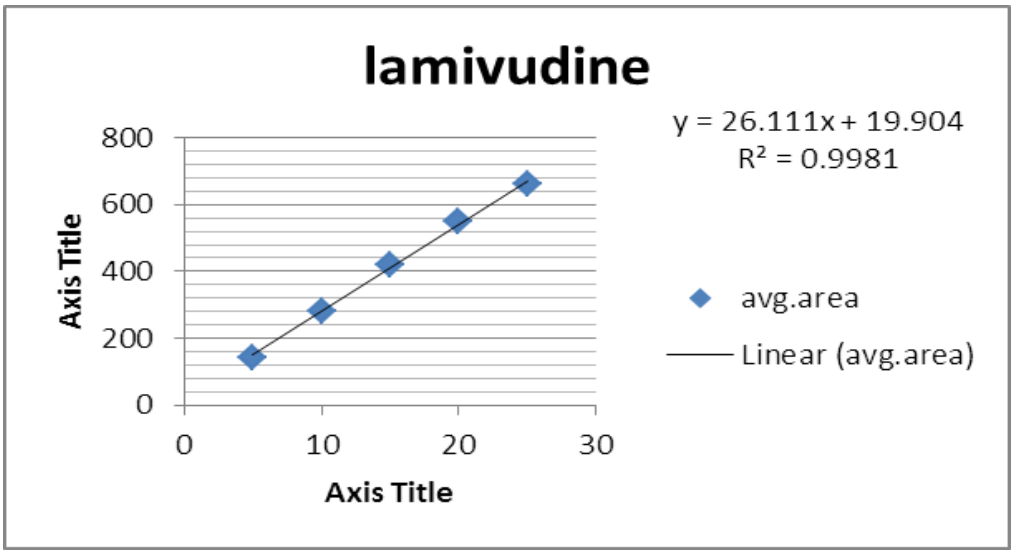

Figure 5:Linearity studies of lamivudine

Table 2: Linearity studies of tenofovir

Sr. No.	Concentration µg/ml	Area	RSD
1	5	145.26	0.98
2	10	306.25	1.49
3	15	477.12	0.44
4	20	620.84	1.03
5	25	766.66	0.99

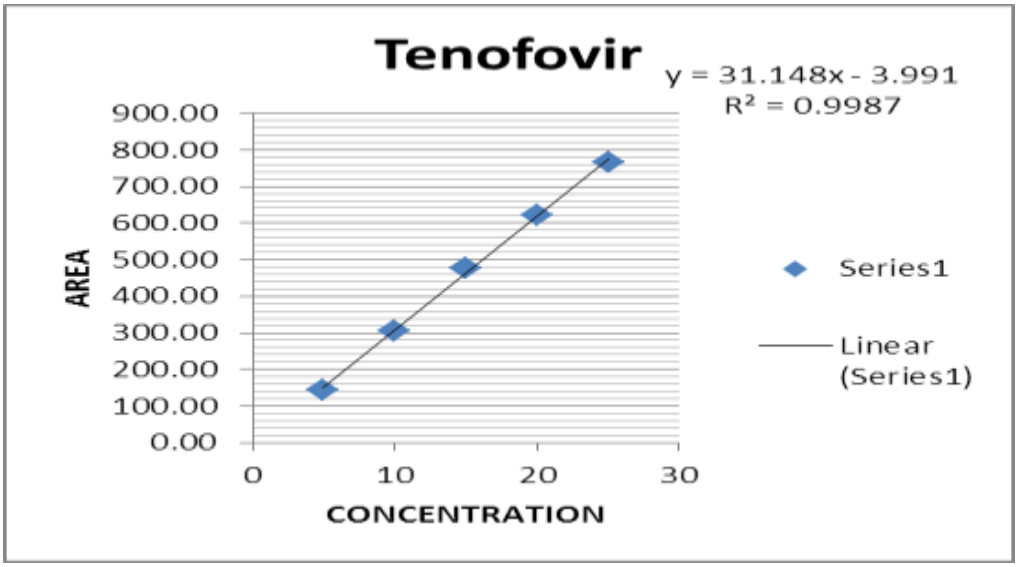

Figure 6:Linearity studies of tenofovir

Analysis of marketed formulation

Brand name:Lamivudine and Tenofovir Disoproxil Fumarate Tablets IP 300mg/300mg

Table 3:Analysis of marketed formulation

Sr.no	Amount present in mg		Amount found in mg		% Label claim	
	Tenofovir	Lamivudne	Tenofovir	Lamivudne	Tenofovir	Lamivudne
1	20	20	19.81	20.64	99.05	103.20
2	20	20	19.95	20.82	99.75	104.10
Mean	_	_	19.56	20.43	99.40	103.5
SD	_	_	3.16	0.13	4.41	3.32
%RSD	_	_	0.51	0.61	1.01	0.13

Method Validation

Accuracy: Recovery studies were performed to validate the accuracy of developed method. To pre analysed tablet solution, a definite concentration of standard drug (80%, 100%, and 120%) was added and then its recovery was analyzed (Table No.4). Statistical validation of recovery studies shown in (Table No. 5).

Table 4: Recovery studies of Tenofovir

Conc.	Amount added (µg/mL)	Amount found (µg/mL)	%Recovery	Mean recovery	Mean added (µg/mL)	Mean found	% RSD
80%	8	18.02	100.29	101.10	8	18.09	0.51
	8	18.15	101.90				
100%	10	20.10	101.00	103.21	10	20.32	1.93
	10	20.54	105.41				
120%	12	21.77	98.14	99.36	12	21.92	0.97
	12	22.07	100.58				

Table 5: Recovery studies of Lamivudine

Conc.	Amount added (µg/mL)	Amount found (µg/mL)	%Recovery	Mean recovery	Mean added (µg/mL)	Mean found	% RSD
80%	8	18.40	105.06	103.95	8	18.31	0.70
	8	18.22	102.84				
100%	10	20.10	99.48	100.04	10	20.08	0.14
	10	20.06	100.60				
120%	12	21.76	98.05	99.08	12	21.89	0.81
	12	22.01	100.11				

Precision: The method was established by analyzing various replicates standards of Cefexime and Linezolid. All the solution were analyzed thrice in order to record any intra-day & inter-day variation in the result. The result obtained for intraday are shown in Table No 5 & 6, the result obtained for interday variation are shown in the Table No .6& 7 respectively.

Table 6: Intra-day precision study of Tenofovir

Conc µg/ml	Peak area		Mean area	SD	% RSD
	Trial 1	Trial 2			
10	309.84	302.78	306.31	4.99	1.63
15	479.83	480.84	480.33	0.73	0.15
20	621.05	621.29	621.17	3.07	0.49

Table 7 : Inter-day precision study of Tenofovir

Conc µg/ml	Peak area		Mean area	SD	% RSD
	Day 1	Day 2			
15	306.27	308.21	307.24	0.62	0.61
30	498.21	489.14	498.19	0.42	0.22
45	605.96	610.96	608.46	1.32	0.51

Table 8: Intra-day precision study of Lamivudine.

Conc µg/ml	Peak area		Mean area	SD	% RSD
	Trial 1	Trial 2			
10	288.72	284.51	286.62	2.98	1.04
15	422.45	427.46	424.96	3.54	0.83
20	558.89	566.69	562.79	5.52	0.98

Table 9: Inter-day precision study of Lamivudine.

Conc µg/ml	Peak area		Mean area	SD	% RSD
	Day 1	Day 2			
15	279.24	276.58	277.91	1.32	1.29
30	418.98	421.96	420.47	1.02	0.10
45	547.79	551.69	549.74	2.36	0.15

Repeatability

Table 10: Repeatability studies on Tenofovir

Concentration of Tenofovir(mg/ml)	Peak Area	Amount found	%Amount found
15	470	14.96	99.76
15	470.23	14.97	99.80
15	470.34	15.26	101.73
	Mean	15.11	100.74
	SD	0.15	1.02
	%RSD	1.02	1.01

Table 11: Repeatability studies on Lamivudine

Concentration Of Lamivudine(mg/ml)	Peak Area	Amount found	%Amount found
15	421.41	15.37	102.47
15	411.57	15.00	100.00
15	420.91	15.35	102.33
	Mean	15.27	101.80
	SD	0.26	1.72
	%RSD	1.02	1.69

Ruggedness: Ruggedness of the method was studied by two different analyst using same operational and environmental condition. An appropriate concentration 10.0µg/mL of tenofovir and 10.0µg/mL of lamivudine was analysed and concentration were determined.

Table 12: Ruggedness studies on Tenofovir

Condition	Mean	± SD n=3	%RSD
Analyst1	306.31	4.99	1.63
Analyst2	479.83	0.73	0.15

Table 13: Ruggedness studies on Lamivudine

Condition	Mean	± SD n=3	%RSD
Analyst1	286.62	2.98	1.04
Analyst2	424.96	3.54	0.83

Robustness: The Robustness of a method is its ability to remain unaffected by small deliberate changes in parameters. To evaluate the robustness of the proposed method, small but deliberate variations in the optimized method parameters were done. The effect of changes in mobile phase composition and flow rate on retention time and tailing factor of drug peak was studied. The mobile phase composition was changed in ± 1 ml proportion and the flow rate was varied by ± 0.1 ml min^{-1}, of optimized chromatographic condition.

Table 14: Robustness studies on Tenofovir

Conc µg/ml	Peak area		Mean area	SD	% RSD
	Trial 1	Trial 2			
10	309.84	302.78	306.31	4.99	1.63
15	479.83	480.84	480.33	0.73	0.15
20	621.05	621.29	621.17	3.07	0.49

Table 15: Robustness studies on lamivudine

Conc µg/ml	Peak area		Mean area	SD	% RSD
	Trial 1	Trial 2			
10	288.72	284.51	286.62	2.98	1.04
15	422.45	427.46	424.96	3.54	0.83
20	558.89	566.69	562.79	5.52	0.98

Limit of detection (LOD)

LOD is calculated from the formula = 3.3 σ /S

σ = Standard deviation of the response, S= slope of the calibration curve

Tenofovir = 0.31

Lamivudine = 0.50

Limit of quantification (LOQ)

LOQ is calculated from the formula = 10 σ / S

σ = Standard deviation of the response, S= slope of the calibration curve

Tenofovir = 0.94

Lamivdine = 1.53

CONCLUSION

A simple rapid method accurate and précised method was developed for simultaneous RP-HPLC determination of Tenofovir and Lamivudine. The method is validated and demonstrated a wide Linerity Accuracy,Precision,System suitability,Rubustness. The proposed method is a simple and rapid procedure. In RP-HPLC method, the analyte were resolved using Acetonitrile : (0.1%OP,A 80:20 %v/v) at pH 6.2, flow rate 1.0ml/min, on HPLC pump Spectra System 600E with Phenomenex C18 150 × 4.6mm 5 µm column autochro 3000 Software detector was used for the study. The detection was carried out at 260 nm. The method gave the good resolution and suitable retention time. The results of analysis in all the method were validated in terms of accuracy, precision, robustness, linearity and range**.**

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