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

G. Kamal Yadav*, P Vivek Sagar, P
Vaagdevi Pharmacy College, Warangal

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Abstract:

A simple, Accurate, precise method was developed for the simultaneous estimation of the Lamivudine and Tenofovir in Tablet dosage form. Chromatogram was run through C_{18} Inertsil 5µ, 250mm×4.6mm column using phosphate buffer:acetonitrile:methanol(40:20:40) as mobile phase was pumped through column at a flow rate of 1.0 ml/min. Temperature was maintained at 30°C. Optimized wavelength selected was 257.0 nm. Retention time of Lamivudine and Tenofovir were found to be 3.4min and 4.5min. % RSD of the Lamivudine and Tenofovir were and found to be 0.42 and 0.21 respectively. The method is linear over a concentration range of 3.75 – 22.50 µg/ml for Lamivudine and 50 to 300 µg/ml for Tenofovir. The method was validated for system suitability, accuracy, precision, linearity and ruggedness. The system suitability parameters were within limit, hence it was concluded that the method was suitable to perform the assay. It was also used for determining lower concentration of drug in its solid dosage forms. Therefore it was concluded that the proposed method can be used for analysis of Lamivudine and Tenofovir Disoproxil Fumerate in Pharmaceutical dosage forms.

Key words: *Tenofovir, Lamivudine, stability indicating, RP- HPLC*

Corresponding author:
Dr. G.Kamal Yadav,
Vaagdevi Pharmacy College, Warangal
Email: gaddak70@gmail.com

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1. INTRODUCTION:

Lamivudine
4-amino-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one is a reverse transcriptase inhibitor used to treat HIV and hepatitis B infections. Lamivudine is a nucleoside reverse transcriptase inhibitor (NRTI) with activity against Human Immunodeficiency Virus Type 1 (HIV-1) and hepatitis B (HBV) to disrupt viral DNA synthesis. Tenofovir ({[(2R) -1 -(6 –amino -9H – purin -9 -yl) propan -2-yl] oxy} methyl) phosphonic acid is a nucleotide analog indicated in the treatment of HIV infections. tenofovir is activated by a bi-phosphorylation it acts as an antiviral acyclic nucleoside phosphonate. It is a potent inhibitor of the viral reverse transcriptase with an inhibitory constant. The literature review reveals that few RP-HPLC methods for the estimation of Lamivudine and Tenofovir are available alone and in combination with other drugs. Few methods are also reported for estimation of both drugs from formulation[1-5]. we intend to develop a Stability indicating RP-HPLC method by simultaneous determination with simple, rapid, greater sensitivity and faster elution.

2. MATERIALS AND METHODS:

Lamivudine and Tenofovir pure drugs (API) received as gift sample from Aurobindo pharma Ltd. Combined form of Lamivudine and Tenofovir tablets was purchasd from the local market. Acetonitrile, Phosphate buffer, Methanol, Potassium dihydrogen ortho phosphate buffer, perchloric acid Ortho-phosphoric acid. All the above chemicals and solvents are purchased from Merck.

2.1. Preparation Solutions:

2.1.1.Preparation of Standard solutions:

Transfer an accurately weighed quantity of about 3mg of Lamivudine working standard and 40mg of Tenofovir Disoproxil Fumerate working standard in to 100ml volumetric flask add 75ml of Mobile phase and sonicate to dissolve the content, and make up to the volume with mobile phase and further dilute 10ml in to 100 ml with diluents, mix.

2.1.2. Samples Preparation

10 Tablets of contents were weighed and triturated in glass mortar. The quantity of powder equivalent to 100 mg of active ingredient present in Lamivudine and Tenofovir was transferred into a 100 ml clean dry volumetric flask, 7 ml of diluent was added to it and was shaken by mechanical stirrer and sonicated for about 30 minutes by shaking at intervals of five minutes each and was diluted up to the mark with diluent to give a concentration of 1000 µg/ml and allowed to stand until the residue settles before taking an aliquot for further dilution (stock solution). 3 ml of upper clear solution was transferred to a 10 ml volumetric flask and diluted with diluent up to the mark to give the respective concentrations as per with standard solution. The solution was filtered through 0.45 µm filter before injecting into HPLC system.

2.1.3. Cc standards;

Calibration curve standards were prepared by pipetting suitable aliquots from stock solution into separate 10 ml volumetric flasks and the volume was made up to the mark with diluent to obtain the CC standards in the range of 3.75 - 22.5 µg/ml and 50 - 300 µg/ml concentrations for Lamivudine and Tenofovir respectively.

2.2. Diluent: Mobile phase is used as diluent.

2.3. Chromatographic conditions:

The new HPLC method for estimation of Lamivudine and Tenofovir was developed and validated using C_{18} Develosil ODS HG-5RP 150mm×4.6mm column. 65 volumes of HPLC grade 40 volumes of 0.01M Phosphate buffer adjusted to pH 5.0 20 Volumes of Acetonitrile and 40 volumes of Methanol and (40:20:40 % v/v) as mobile phase. Separation was achieved through isocratic elution mode at 0.8 mL/ min flow rate and the effluent was monitored at 257nm.

2.4. System suitability:

- The system suitability parameters were determined by preparing standard solutions of Lamivudine and Tenofovir. The solutions were injected six times and the parameters like peak tailing, resolution and USP plate count were determined. The RSD for the peak area of Lamivudine and Tenofovir Disoproxil Fumerate for 5 replicate injections should not be more than 2%. Tailing Factor for Tenofovir Disoproxil Fumerate and Lamivudine should be not more than 2.

2.5. Method validation

The method validation was performed in accordance with ICH guidelines

2.5.1. Linearity

Inject each level into the chromatographic system and measure the peak area. Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.

2.5.2. Accuracy

Accuracy was determined by the recovery studies of the analyte. It is determined by standard addition method where the test solution of known quantity is spiked with standard solutions at three levels i.e., 50%, 100% & 150% in triplicate. Mean percentage recoveries at all the levels were calculated.

2.5.3. Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurement obtained from multiple sampling of the same homogenous sample under the prescribed conditions. Precision of an analytical procedure is usually expressed the variance, standard deviation of

coefficient of variation of a series of measurement. Precision of the method is determined in terms of System precision and Method precision

2.5.4. Robustness

Small deliberate changes in method like Flow rate and mobile phase ratio, are made. The actual flow rate is 1.0 ml/min. Change the flow rate ± 0.2 ml/min and observed USP tailing and USP plate count. Actual mobile phase ratio was buffer: ACN is 60:40 .It was changed to 65:35 and 55:45 and observed USP tailing and USP plate count. Standard solutions were injected in sextet. System suitability parameters are evaluated by making the deliberate changes.

2.5.5. Specificity:

The specificity was studied by establishing the interference of placebo with the drug. A sample of placebo was injected into the HPLC system as per the test procedure. Chromatogram of placebo should not show any peak at the retention time of analyte peak.

3. RESULTS AND DISCUSSION:

3.1 Assay of formulation:

Assay of the formulation is performed as per the givem procedure. This was done in triplicate. The amount of drug present in the formulation was calculated from standard graph. The % assay of Lamivudine and Tenofovir obtained was 99. 63 and 98.48 % respectively. Representative chromatograms for standard, test and blank was given in figures 3,4 &5. Peak areas were given in table no. 1.

3.2 System suitability

System suitability parameters were determined according to ICH guidelines. Plate count was more than 2000, tailing factor was less than 2 and resolution was more than 2. All the system suitable parameters were passed and were within the limits. The results showing system suiability parameters were given in table no. 2

3.3 Validation

3.3.1. Linearity

The linearity was determined at six concentration in the range of 3.75 – 22.50 µg/ ml for Lamivudine and 50 - 300 µg/ ml for Tenofovir. The Peak areas against concentration were plotted and the calibration curve was constructed. The calibration curve was illustrated in Figure 3. The Correlation coefficient (r^2) was greater than 0.99 within the concentration range for both the drugs. The results for linearity were given in the table 3.

3.3.2. Accuracy

Accuracy of the method wsa established at three levels of concentrations by standard addition method. Triplicate injections were given at each level of accuracy and percentage recoveries were calculated. The mean % Recovery was obtained was 99.32 % and 100.22 % for Lamivudine and Tenofovir respectively. The results for accuracy was given in the table 4.

3.3.3.Precision:

The precision of the method was studied by considering system precision and method precision. System precision was studied by taking six replicate injections from same homogenous standard solution and peak areas were determined. Average area, standard deviation and % RSD were calculated for two drugs. Method precision was studied by taking six replicate injections from test solution and peak areas were determined. Average area, standard deviation and % RSD were calculated for two drugs. The % RSD of Lamivudine for System precision was found to be 0.31 and 0.35 and for Tenofovir it was found to be 0.01 and 0.02. The results for precision were given in the table 5 & 6

3.3.4. Robustness:

Robustness of the method was studied by making deliberate changes in flow rate, column oven temperature and mobile phase ratio. After making each change in the conditions, chromatograms were recorded by injecting the standard solutions in six replicates. System suitability parameters were checked at each level. System suitability parameters were not much affected and all the parameters were passed. % RSD was within the limit. Results were given in the table 7.

3.3.5. Specificity

The Chromatograms of Standard and Sample are identical with nearly same Retention time. No interference due to Placebo and Sample at the retention time of analyte which shows that the method was specific.

4. CONCLUSION:

In the present study a new RP-HPLC method was developed for Stability indicating and simultaneous estimation of Lamivudine and Tenofovir Disoproxil Fumerate in Pharmaceutical dosage forms and Bulk drugs. The analysis is resolved by using a on C_{18} Inertsil 5µ, 250mm×4.6mm column using phosphate buffer:acetonitrile:methanol(40:20:40) as mobile phase the flow was quite satisfactory. The flow rate was 0.8ml/min and the analyte was monitored at 257nm at which better detector response for drugs were obtained. The retention time for Lamivudine and Tenofovir Disoproxil Fumerate were 3.4min and 4.5min respectively. The method was validated for system suitability, accuracy, precision, linearity and ruggedness. The system suitability parameters were within limit, hence it was concluded that the method was suitable to perform the assay. It was also used for determining lower concentration of drug in its solid dosage forms. Therefore it was concluded that the proposed method can be used for analysis of Lamivudine and Tenofovir Disoproxil Fumerate in Pharmaceutical dosage forms.

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Table 1. Assay Data

S.no	Peak area of Lamivudine	Peak area of Tenofovir
1	1238232	4222121
2	1247565	4220120
3	1239665	4219232
Avg	**1241821**	**4220491**
Regression equation	y = 5633 x + 12910.	y = 5404.x + 1432.1
% Assay	98. 20%	98. 32%

Table 2: System suitability parameters for Lamivudine and Tenofovir

SAMPLE	R_t	Peak Area	USP plate count	USP Tailing
LAMIVUDINE	3.48	1112325	1161	1.0
TENOFOVIR	4.31	1193159	1302	1.0

Table 3: Linearity data of Lamivudine and Tenofovir.

Lamivudine		Tenofovir	
Conc (µg/mL)	**Peak area**	**Conc (µg/mL)**	**Peak area**
3.75	318579	50	1083182
7.50	632307	100	2140868
11.25	940714	150	3178742
15.00	1242080	200	4234699
18.75	1555290	250	5283960
22.50	1882846	300	6383864

Table 4: Accuracy data of Lamivudine and Tenofovir

	Accuracy-- 80%		**Accuracy-- 100%**		**Accuracy-- 120%**	
	Lam	Tenofovir	Lam	Tenofovir	Lam	Tenofovir
S No	**Area**	**Area**	**Area**	**Area**	**Area**	**Area**
Injection-1	989546	3393262	1238232	4222121	1495695	5072521
Injection-2	993025	3353232	1247565	4220120	1488513	5035654
Injection-3	994545	3366565	1239665	4219232	1477756	5091236
Avg	**992372**	**3371020**	**1241821**	**4220491**	**1487321**	**5066470**
amt Recovered	**79.41**	**79.35**	**99.20**	**99.32**	**119.02**	**119.27**
%Recovery	**99.26**	**99.19**	**99.20**	**99.32**	**99.18**	**99.39**

Table 5: Method Precision data of Lamivudine and Tenofovir

S No	Name	Lamivudine		Tenofovir	
		RT	Area	RT	Area
1	M-Precision-1	3.481	1220596	4.323	4201252
2	M-Precision-2	3.477	1226595	4.319	4199998
3	M-Precision-3	3.489	1230155	4.315	4222215
4	M-Precision-4	3.485	1229899	4.316	4201213
5	M-Precision-5	3.486	1221999	4.315	4215222
6	M-Precision-6	3.488	1245656	4.316	4212121
Average		3.484	1229150	4.317	4208670
Standard Deviation		0.0045	8998.4	0.003	9210.24
%RSD		0.1305	0.732	0.07	0.22

Table 6: System Precision data of Lamivudine and Tenofovir

S No	Name	Lamivudine		Tenofovir	
		RT	Area	RT	Area
1	S-Precision-1	3.486	1239517	4.313	4209541
2	S-Precision-2	3.486	1241754	4.312	4212874
3	S-Precision-3	3.486	1246030	4.312	4232293
4	S-Precision-4	3.486	1244401	4.312	4228294
5	S-Precision-5	3.485	1247129	4.311	4250605
6	S-Precision-6	3.486	1251757	4.311	4248839
Average		3.486	1245098	4.312	423408
Standard Deviation		0.0004	4293.1	0.001	17311.94
%RSD		0.0117	0.345	0.02	0.41

Table 7: Robustness data of Lamivudine and Tenofovir

S.No.	Lamivudine		Tenofovir	
	RT	Area	RT	Area
Standard				
1	3.487	1232691	4.314	4220012
Robust-1 Flow -1				
2	3.107	1122733	3.841	3791183
Robust-2 Flow-2				
3	3.971	1434649	4.915	4879613
Robust-3 Column Oven Temperature-1				
4	3.506	1253860	4.338	4252684
Robust-4 Column Oven Temperaure-2				
5	3.467	1250755	4.304	4273151

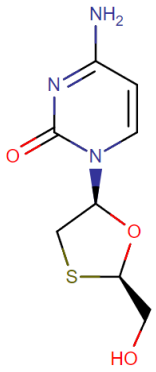

Fig 1: Structure of Lamivudine

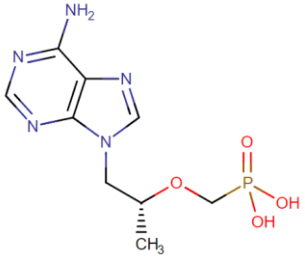

Fig.2: Structure of Tenofovir

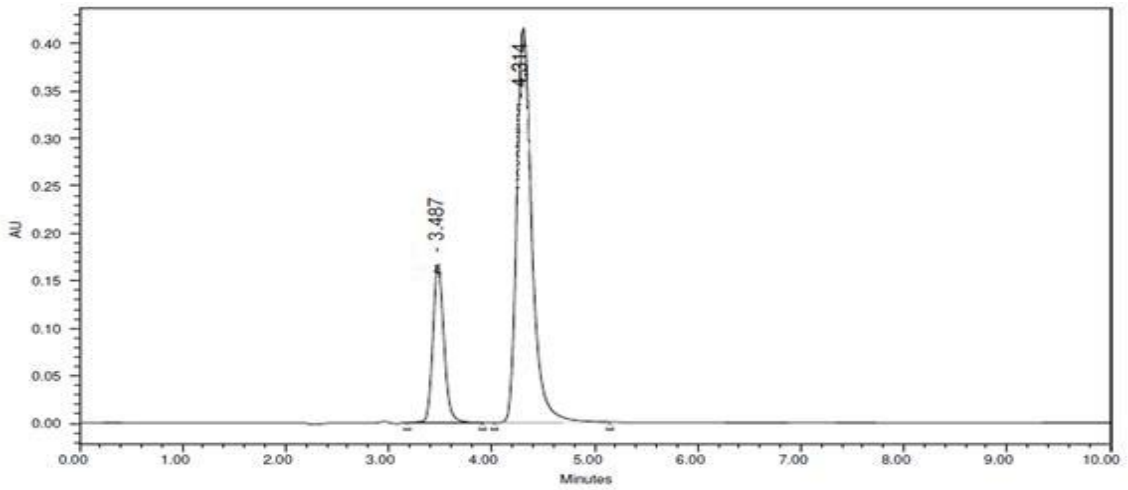

Fig 3: Representative Chromatogram of working standard solution

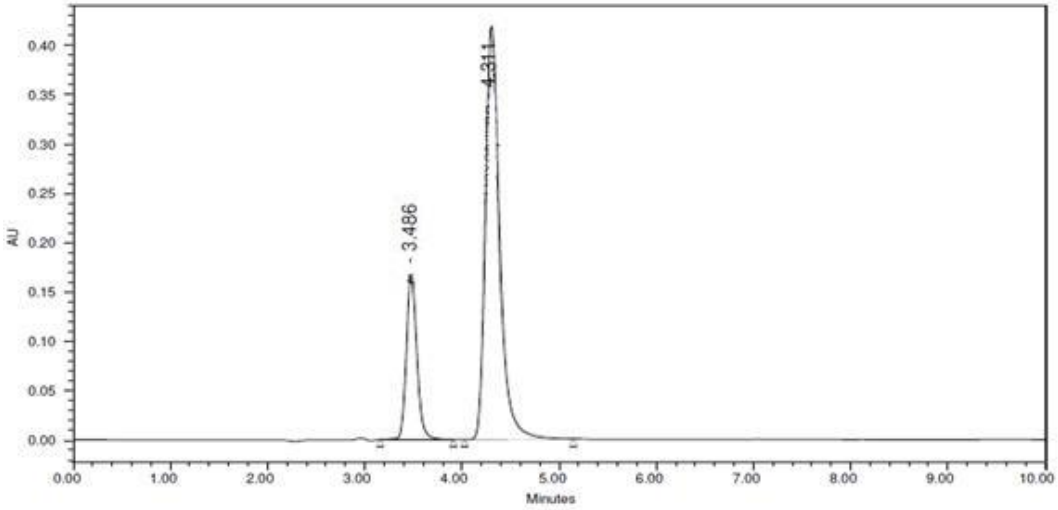

Fig 4: Representative Chromatogram of working sample solution

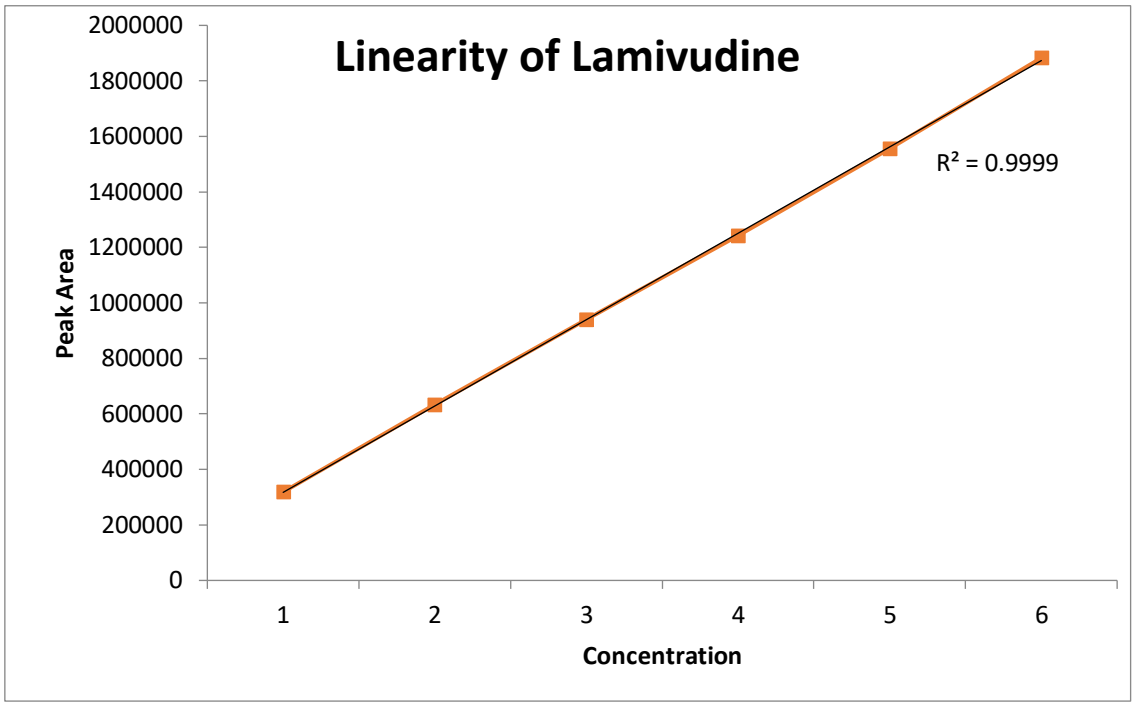

Fig 5: Standard graph of Lamivudine

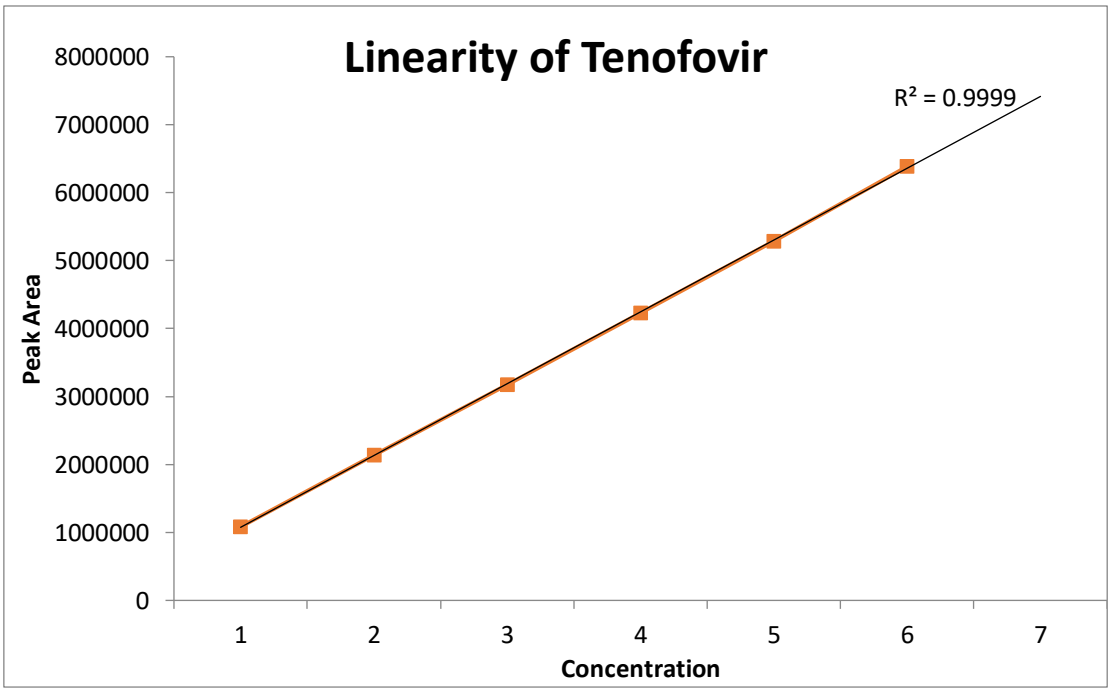

Fig 6: Standard graph of Tenofovir