RESEARCH ARTICLE

Development and Validation of RP-HPLC Method for the Simultaneous Estimation of Lamivudine and Tenofovir Disproxil Fumerate in Combined Dosage Form

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ABSTRACT

Objective: A highly sensitive isocratic reverse phase high performance liquid chromatographic method was developed and validated for the estimation of Lamivudine and Tenofovir disproxil fumerate in Bulk drug and Pharmaceutical dosage forms.

Method: Separation of Lamivudine and Tenofovir successfully achieved on symmetry COLUMN-ENABLE C_{18} G 5 µM, 250×4.6mm or equivalent utilizing HPLC Methanol and Water ph adjusted with 3.2 in the ratio of 70:30 v/v as mobile phase at a flow rate of 1mL/min and the eluates was monitored at260 nm.

Result: Chromatogram showed peak at a retention time of 3.048min and 5.354min. The method was validated for system suitability, linearity, precision, accuracy, specificity, ruggedness, robustness, LOD and LOQ. Recovery of Tenofovir disproxil fumerate and Lamivudine were found to be in the range of 100.46% and 100.66% and showing linearity in the range of 10-50 µg / ml. The S/N for LOD and LOQ for estimation of Lamivudine and Tenofovir disproxil fumerate were found to be 1.488µg / ml and 4.510 µg /ml and 3.552µg/ml and 9.763µg/ml respectively.

Conclusion: Proposed method can be successfully applied for the quantitative determination of Tenofovir disproxil fumerate and Lamivudine in Bulk drug and Pharmaceutical dosage form.

Keywords: Lamivudine, Tenofovir disoproxil fumerate, RP-HPLC.

1. INTRODUCTION:

Lamivudine is a nucleoside reverse transcriptase inhibitor (NRTIs). Chemically it is 4 – amino – 1 – [(2R, 5S) – 2 – (hydroxyl methyl) – 1, 3 – oxathiolan – 5 – yl] – 1, 2 – dihydro pyrimidin – 2– one. It can inhibit both types (I and II) of HIV reverse transcriptase and also the reverse transcriptase of Hepatitis B.Tenofovir disproxil Fumarate is fumaric acid salt of the bis isopropoxy carbonyl oxy methyl ester derivative of tenofovir. Chemically it is 9-[(R)-2-[[(isopropoxcarbonyl)-oxy] methoxy] phosphinyl] methoxy] propyl] adenine fumarate[1-4]. Lamivudine is official in IP[5], BP[6] and USP[7]. Tenofovir disoproxil fumerate is official in IP[8]. Literature survey reveals that Tenofovir disoproxil fumerate is estimated sensitive determination in plasma by-HPLC[9], Plasma LC/MS/MS[10]and in human peripheral blood mononuclear cells methods[11], Similarly for Lamivudine estimation in

human serum by HPLC[12-13-14], the simultaneous estimation of Lamivudine and Tenofovir disoproxil fumerate in RP- HPLC[15-16], HPTLC[17-18] and LC-MS/MS[19] were reported.

To the best of our knowledge, there is no reported RP - HPLC method for simultaneous estimation of Lamivudine and Tenofovir disoproxil fumerate in pharmaceutical formulations, previous to our work. Thus, efforts were made to develop fast, selective and sensitive analytical method for the estimation of Lamivudine and Tenofovir disoproxil fumerate in their combined dosage form using reverse phase high performance liquid chromatographic method. Now the authors report a simple, reliable and reproducible RP-HPLC method which was duly validated by statistical parameters precision, accuracy and recovery. The method has been satisfactorily applied to the

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simultaneous estimation of Lamivudine and Tenofovir disproxil fumerate in bulk and pharmaceutical dosage forms.
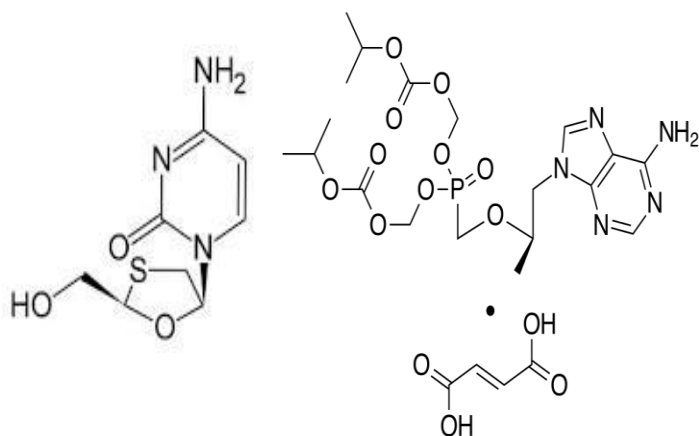

Fig1: Chemical structure of Lamuvidine and Tenofovir disoproxil fumerate

2. MATERIALS AND METHODS
EXPERIMENTAL
Apparatus
Rp-hplc was performed with a shimadzu LC-20AD Prominence Liquid Chromatography solvent –delivery system, a shimadzu SPD-20A Prominence UV-visible detector and a rheodyne 7725i universal loop injector of injection capacity 20µl. the monitor software was LC solution version 1.25. Compound was separated on an ENABLE C_{18} G column (250mm×4.6mm i.d, 5- µm particle) under reversed-phase chromatographic condition .ultrsonicator model Aeroflex-2200MH was used .this work was carried out in an air conditioned room maintained at temperature 25±2°c. the flow rate was 1ml/min and the Analytes were monitored at 260 nm.

Chemical and Reagents
Working standards of pharmaceutical grade Tenofovir disproxil fumerate and Lamivudine was obtained as a gift sample from Hetero drugs Hyderabad. Tablet dosage forms, TENOVIR-L manufactured by Cipla limited, Mumbai, India (L.C: LAM 300mg and TDF 300mg) was procured from Hetero pharmacy. HPLC grade Methanol (Merck), Orthophospharic Acid (Merck) and Milli-Q water. Waters High Performance Liquid Chromatography with auto sampler and UV-visible detector and manual injector mode was used with lc-solution version1.25, Electronic balance shimadzu (type- bl-22OH), Sonicator (Lab India), and Vaccum pump.

Preparation of phosphate buffer (3.2):
Adjust a 35.8 g/l solution of disodium hydrogen phosphate to ph 3.2 with dilute phosphoric acid. Dilute 100.0 ml of the solution to 2000.0 ml with water.

Preparation of mobile phase:
Mix a mixture of Methanol (HPLC grade) 700 mL (70%) and 300 mL of buffer HPLCgrade (30%) and degas in ultrasonic water bath for 10 minutes. Filter through 0.45 µ filter under vacuum filtration. Mobile phase used as Diluent.

Preparation of Standard Stock solution and construction of calibration curve:
Accurately 100mg of Tenofovir disoproxil fumerate and Lamivudine standards were weighed and taken in100 ml volumetric flask. Stock was dissolved by sonication in 100 ml of Diluent. (in a ratio of 70:30 Methanol and HPLC water adjusted with PH 3.2) from the standard stock solution of drugs, different dilution were prepared, to get the final concentration 10mg/ml and different dilution are prepared, injected and their peak area was measured. A calibration curve was constructed by plotting concentration in x axis and peak area in y axis. The amount of Lamuvidine and Tenofovir were calculated by using their respective calibration curves.

Preparation of working Standard solution:
0.1 ml of the above standard stock solution was taken in 10 ml volumetric flask and made up to 10 ml with diluents to get a concentration of10µg/ml for Tenofovir disproxil fumerate and Lamivudine.

Preparation of sample solution:
20 Tablets of TENVIR-L commercially available tablets (Tenofovir and Lamivudine formulation) were weighed and powdered in glass mortar. The powder equivalent to the 10mg of active ingredient was transferred into a 10 ml 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 and allowed to stand until the residue settles before taking an aliquot for further dilution. 0.1 ml of upper clear solution was transferred to a 10 ml volumetric flask and diluted with diluent up to the mark and the solution was filtered through 0.45 µm filter before injecting into HPLC system.

Chromatographic conditions:
Chromatographic separations were achieved by symmetry C_{18} G 5 µm, 250×4.6mm , (Make: Enable) or equivalent utilizing HPLC Methanol and buffer(PH:3.2)in the ratio of 70:30 v/v as mobile phase at a flow rate of 1mL/min and the eluates was monitored at 260 nm.

Method Validation:
The validation of the method has been performed in as per USP requirements for assay determination, which include system suitability, accuracy, precision selectivity, linearity, range, robustness and ruggedness.

System Sutability:
The standard stock solution was injected six times into rp-Hplc as per test procedure. System suitability parameters were estimated from standard chromatograms, by calculating the %RSD of retention times, tailing factor,

theoretical plates, and peak areas from six replicate injections.

Acuuracy:

The accuracy was determined by use of slandered addition at different levels, sample stock solution of tablet formulation at a concentration of 10 µg/ml and 50µg/ml of Lamuvidine and tenofovir disproxil Fumarate was prepared. A 10 and 50µg/ml of standards solution were added to the sample solution and% recovery was calculated values were found to be as per ich guidelines**.** (Table: 1**)**

Drug	%concentration(at specification level)	area	Amount added	Amount found (µg/ml)	%recovery	Mean recovery
LAM	10		10	10.123	101.23	100.469
	50		50	49.854	99.708	
TDF	10		10	10.111	101.11	100.668
	50		50	50.113	100.22	

Recovery studies of TENOVIR- L (tablet) n=2

Table 1: Accuracy

Precision:

Repeatability (intra-day precision):

The formulation was analysed in the same day for repeatability and the results were subjected to statistical analysis. The %rsd for Lamuvidine and tenofovir was 1.1735, 0.3855.according to ich norms. The results of analysis are shown in. (Table: 2)

Concentration (ug/ml)	Injection	Peak area	
		LAM	TEN
Lamivudine	1	2957671	1495787
	2	2932123	1475323
(20 ug/ml)	3	2912978	1463245
	4	2994568	1472145
Tenofovir Disoproxil Fumarate	5	2958789	1453243
	6	2914573	1493245
(50 ug/ml)			
MEAN		2863683	1475509
S.D		164304.516	1528.233
% R.S.D		0.0266	0.1035
MEAN ASSAY		100.5	102.23

Table 2: Repeatability of Injection

Intermediate precision:

Intermediate precision was calculated from six replicate injections of freshly prepared Lamuvidine and tenofovir combined test solution at concentration of 10 µg/ml and 30 µg/ml on same day. Peak areas of drugs were determined and %RSD value calculated. (Table: 3)

Parameter	Lamivudine	Tenofovir
AREA	2544977	1475799
	2946795	1477723
	2946987	1476721
	2946589	1474831
	2946872	1473711
	2947882	1474272
MEAN	2863683	1475509
S.D	164304.516	1528.233
% R.S.D	0.0266	0.1035
MEAN ASSAY	100.5	102.23

Table 3: Intermediate Precision

Reproducibility:

Reproducibility was calculated from six replicates injections of Lamuvidine and tenofovir combined sample solution. Peak areas were determined and %RSD value calculated. (Table: 4)

Parameter	Lamivudine	Tenofovir
AREA	2546877	1475399
	2946755	1477823
	2746677	1476321
	2546549	1474231
	2545712	1473211
	2947812	1474232
MEAN	2713487	1475202.83
S.D	197064.045	1673.8236
% R.S.D	0.0266	0.1134
MEAN ASSAY	102.45	105.23

Table 4: Reproducibility

Linearity:

Adequate dilutions were made from stock solution to get concentration ranging from 10-50 µg/ml for Lamivudine and Tenofovir disoproxil fumerate respectively. Evaluation was performed with UV-visible detector at 260 nm and Peak area was recorded for all the peaks and a Calibration graph was obtained by plotting peak area versus concentration of Lamivudine (Fig: 2) and Tenofovir disoproxil fumerate (Fig: 3).The plot of peak area of each sample against respective concentration was found to be linear in the range of 10-50 µg/ml with correlation coefficient of 0.999 and 0.999.
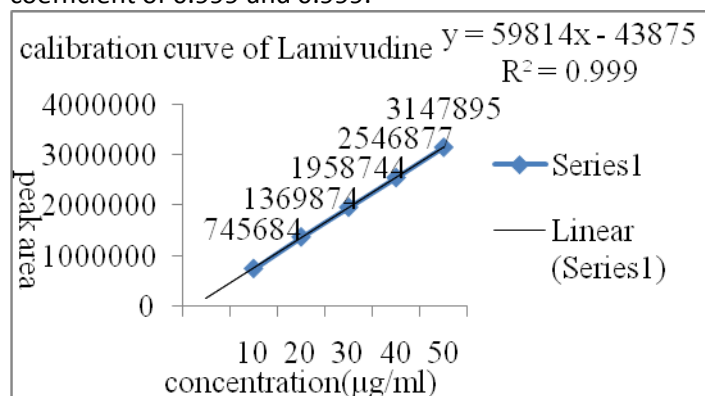

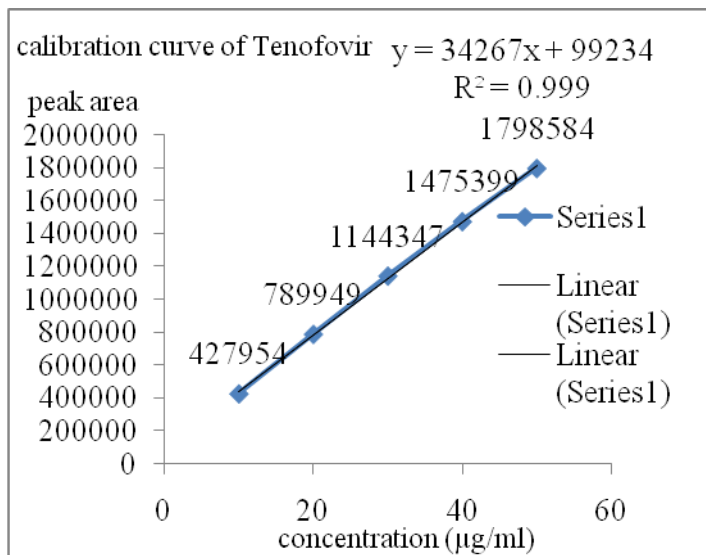**Fig: 2 linearity of Lamuvidine**

Fig: 3 linearity of Tenofovir

SPECIFICITY AND SELECTIVITY:

Specificity was performed to exclude the possibility of interference with excipients in the region of elution on lamivudine. 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 Lamuvidine and tenofovir disoproxil fumerate

3. RESULTS And DISCUSSION

As per the USP-XXVI system suitability tests were carried out on freshly prepared standard stock solution of Lamivudine and Tenofovir disoproxil fumerate. Parameters that were studied to evaluate the suitability of the system are given in Table 5. These parameters indicate good sensitivity, more ruggedness and robustness of the method.

Parameters	Lamuvidine	Tenofovir
Linearity range	10-50µg/ml	10-50µg/ml
Slope	59814	34267
Intercept	-43875	99234
Rention time	3.048	5.354
Area	2546877	1475399
Tailing factor	1.2	1.4
Theoretical plates	26478.23	5687.56
L.O.D	1.488341	3.552008
L.O.Q	4.510123	9.76366
Standard Deviation	0.681687	0.426779
R.S.D	0.678299	0.424776
Correlation coefficient (r^2)	0.9999	0.9999
Accuracy (mean±s.d)	100.499±0.681687	100.4715±0.426779

Table 5: Validation Parameters

From the typical chromatogram of Lamivudine and Tenofovir disoproxil fumerate as shown in Fig: 4, 5 & 6 it

was found that the retention times 3.048 min For Lamivudine and 5.354 min for Tenofovir disoproxil fumerate. Methanol and water in a ratio 70:30 v/v as mobile phase was found to be most suitable mobile phase combination to obtain well defined peaks with sharp peak shapes, high theoretical plates and less tailing.
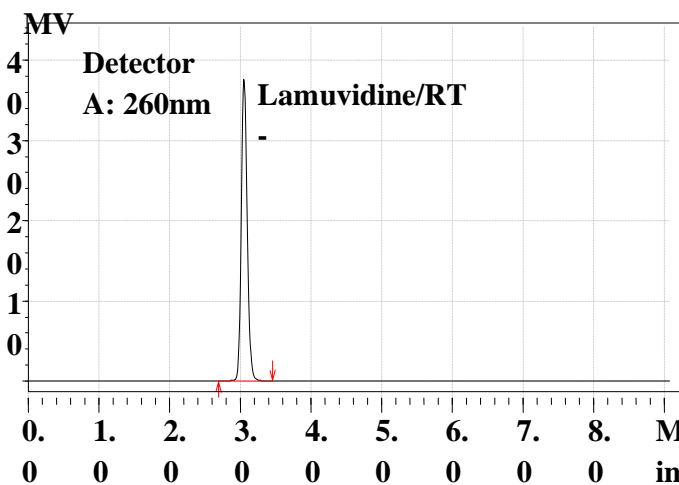

Fig 4: Standard chromatogram of Lamuvidine Standard chromatogram of Lamuvidine
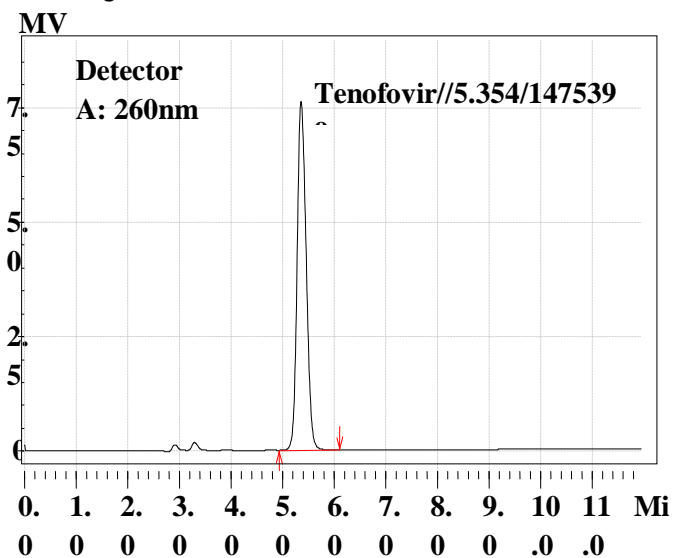

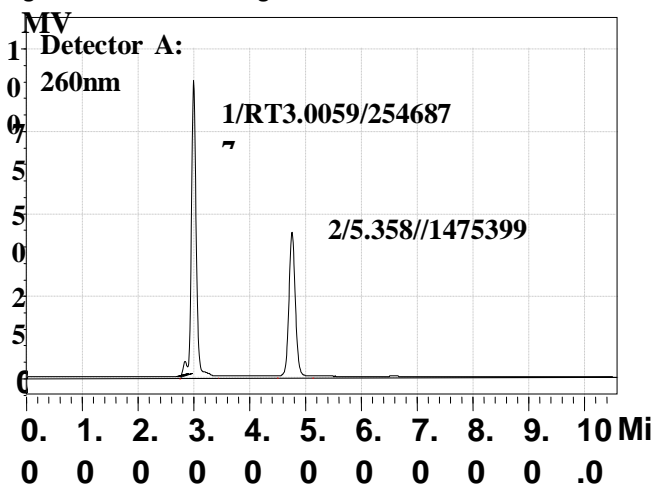
Fig 5: Standard chromatogram of Tenofovir

Fig 6: Standard chromatogram of Lamuvidine & Tenofovir (TENVIR-L)

In the present developed HPLC method, the standard and sample preparation involve very simple extraction procedure and required very less time. A good linear relationship (r=0.999) was observed for Lamivudine and Tenofovir Disoproxil fumerate in the concentration range of 10-50 μg/ml.

The percentage assay was found to be 99.8% for Lamivudine and 99.9 % for Tenofovir disoproxil fumerate in tablets. Recovery studies shows good extraction and recovery from 10% to 50% of test concentration. It was found percentage recovery was about 100.469% for Lamivudine and 100.668 % for Tenofovir disoproxil fumerate indicates good extraction and good recovery and accuracy of the method.

There is no additional peaks in the chromatogram at the main peak Retention times indicates non-interference of the common excipients used in the tablets. This demonstrates that the developed HPLC method is simple, linear, accurate, sensitive, rugged and reproducible.

4. CONCLUSION

A method was developed for the simultaneous estimation of Lamivudine and Tenofovir disproxil fumerate in bulk and pharmaceutical dosage forms which is simple, quick, reliable, inexpensive and simple. The results indicate that the described method can be used for quantitative analysis of the compound.

5. ACKNOWLEDGEMENTS

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