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A validated RP - HPLC method for simulataneous estimation of Lamivudine and Tenofovir disoproxil fumarate in pure and in tablet dosage form

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Abstract

A simple, rapid reverse - phase high performance liquid chromatographic method has been developed and validated for the simultaneous estimation of lamivudine and tenofovir disoproxil fumarate in pure and in tablet dosage form. The estimation was carried out on a Phenomenax Luna C18 (150 mm x 4.6 mm i.d., particle size 5µm) column with a mixture of acetonitrile: methanol: water in the ratio of 30:50:20 (v/v) as mobile phase. UV detection was performed at 258 nm. The method was validated for linearity, accuracy, precision, specificity and sensitivity as per ICH norms. The developed and validated method was successfully used for the quantitative analysis of commercially available dosage form. The retention time was 3.27 and 4.15 min. for lamivudine and tenofovir disoproxil fumarate, respectively. The flow rate was 1.0 mL min^{-1}. The calibration curve was linear over the concentration range of 2 –12 µg mL^{-1} for both lamivudine and tenofovir disoproxil fumarate. The LOD and LOQ values were found to be 0.0099 and 0.0299 µg mL^{-1} for lamivudine and 0.0328 and 0.0994 µg mL^{-1} for tenofovir disoproxil fumarate, respectively. The high percentage of recovery and low percentage coefficient of variance confirm the suitability of the method for the simultaneous estimation of lamivudine and tenofovir disoproxil fumarate in pure and in tablet dosage form.

Keywords:

Lamivudine; Tenofovir disoproxil fumarate; RP-HPLC; Validation

1. Introduction

Lamivudine (LAM) 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 disoproxil Fumarate (TDF) 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] adeninefumarate [1-3]. Fig.1 show the nucleotide reverse transcriptase inhibitors (NtRTIs) used in combination for the treatment of HIV infection. Lamivudine is official in IP [4], BP [5] and USP [6]. TDF is official in IP [7]. Literature survey reveals that TDF is estimated individually by UV [8], derivative-HPLC [9], Plasma RP-HPLC [10-11] and Plasma LC/MS/MS [12-14] methods. Similarly for LAM, HPLC [15], Titrimetry [16-17] and HPLC in plasma [18-20] were reported. Few RP-HPLC [21-23] methods were reported

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for estimation of Emtricitabine, Tenofovir and efavirenz in pharmaceutical formulation. RP-HPLC [24] and LC-MS/MS [25] and HPTLC [26] methods were reported for the simultaneous estimation of Emtricitabine and TDF in human plasma and in formulations. Also UV [27-32], HPLC [33-39], LC – MS [40], HPTLC [41-42] and enzymatic assay [43] methods were reported for the simultaneous estimation of LAM with other antiretero viral drugs. The purpose of this study was to develop simple, rapid, precise and accurate RP-HPLC method for the simultaneous estimation of TDF and LAM in pure and in combined tablet dosage form.

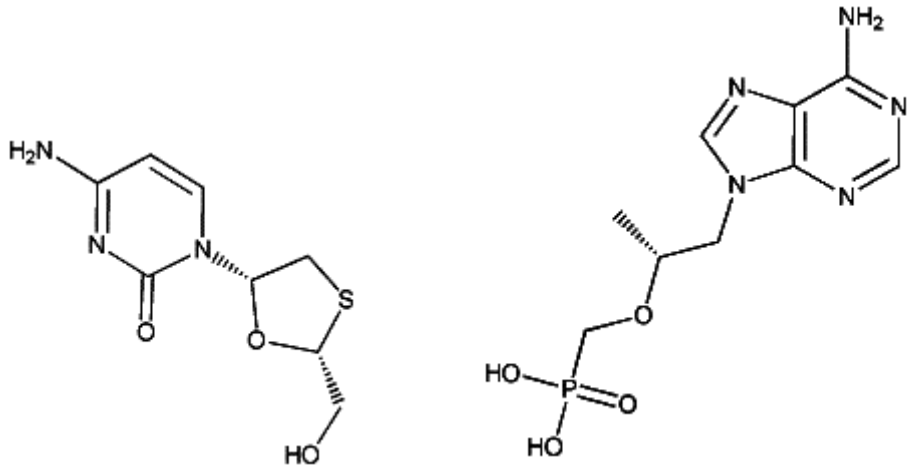

Fig 1. The chemical structures of LAM and TDF

2. Experimental

2.1 Apparatus

RP-HPLC was performed with a Shimadzu LC-10 AT $_{VP}$ solvent-delivery system, a Shimadzu SPD-10 A$_{VP}$ UV–visible detector, and a Rheodyne 7725i universal loop injector of injection capacity 20 µL. The monitoring software was Winchrom. The equipment was controlled by a PC workstation. Compounds were separated on a Phenomenex Luna C18 column (150 mm×4.6 mm i.d, 5-µm particle) under reversed-phase chromatographic conditions. Ultrasonicator Model Soltec – 2200 MH was used. The work was carried out in an air-conditioned room maintained at temperature 25 ± 2°C. The flow rate was 1mL min^{-1} and the analytes were monitored at 258 nm.

2.2. Chemicals and Reagents

Working standards of pharmaceutical grade LAM and TDF were obtained as gift samples from Strides Arcolabs Bangalore, India. The tablet dosage form, TENVIR - L, manufactured by Cipla Limited, Mumbai, India (Label Claim: LAM 300 mg and TDF 300 mg), was procured from the local pharmacy. All the chemicals and reagents used were of HPLC grade and purchased from Qualigens Fine Chemicals, Mumbai, India.

2.3. Mobile phase

The mobile phase consisting of acetonitrile: methanol: water in the ratio of 30: 50: 20% v/v was prepared and degassed with ultrasonicator.

2.4. Standard stock solution and Construction of Calibration curve

Standard stock solution of LAM and TDF (25 mg of each) were prepared separately in 25 mL of mobile phase to get the final concentration of 1 mg mL^{-1}. From the standard stock solution of drugs, different dilutions were 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 LAM and TDF were calculated by using their respective calibration curves.

2.5. Standard mixture solution

Mixed standard analysis was performed to validate the procedure. From the standard stock solutions of the drugs, different mixed standard solutions of 2:12, 4:10, 6:8, 8:6, 10:4, 12:2 of LAM and TDF respectively were prepared and analyzed, statistical results were within the range of acceptance i.e. %COV<2.0 and S.D.<1.0.

2.6. Sample preparation

For the analysis of tablet dosage form, twenty tablets (TENVIR - L) were weighed and their average weight was determined. The tablets were then crushed to a fine powder and the tablet powder equivalent to 25 mg of TDF was transferred to a 25 mL volumetric flask and dissolved in about 20 mL of methanol. The solution was shaken for 5 min. Sonicated for 15-20 min at 100 rpm and made up to the volume with methanol. The solution was filtered through Whatman filter paper # 41. This filtrate was further diluted with mobile phase to get the final concentration of 6 μg mL^{-1} for both the drugs theoretically. 20 μL of the sample solution was injected for quantitative analysis. The identities of both the compounds were established by comparing retention time of the sample solution with those of standard mixed solution. The amount of LAM and TDF per tablet was calculated by extrapolating the peak area from the calibration curve.

3. Results and Discussion

3.1. HPLC method development and optimization

Column chemistry, solvent type, solvent strength, detection wavelength and flow rate were varied to determine the chromatographic conditions giving the best separation. The mobile phase conditions were optimized so that the components were not interfered from the solvent and excipients. After trying column C8 and C18, the final choice of stationary phase giving satisfactory resolution and run time was the reversed phase column phenomenax Luna C18. Mobile phase and flow rate selection was based on peak parameters (height, area, tailing, theoretical plates, capacity factor and resolution) and run time. The best result was obtained by use of 30: 50: 20 (v/v) ratio of acetonitrile, methanol and water with 1.0 mL $min.^{-1}$ From the overlain UV spectra (Shimadzu-1700), suitable wavelength considered for monitoring the drugs was 258 nm (Fig 2). Solutions of LAM and TDF in diluents were also injected directly for HPLC analysis and the responses (peak area) were recorded. It was observed that there was no interference from the mobile phase or baseline disturbances and both the analytes absorbed well at 258 nm. The chromatogram of placebo and standard mixture is shown in Fig 3 and 4 respectively. Under the optimum chromatographic conditions, the retention time obtained for LAM and TDF were 3.27 and 4.15 min, respectively for sample preparation and is shown in Fig 5. The results of system suitability parameters [44, 45] of relative retention time, response factor, capacity factor, tailing factor, Number of theoretical plates and resolution are reported in Table 1. The values obtained for these properties ($1<k<10$, Rs>2) shows that, the chromatographic conditions are appropriate for separation and determination of compounds.

3.2. Validation of the developed method

The method was validated for linearity, accuracy, precision, repeatability, selectivity and specificity study as per ICH norms [46]. All the validation studies were carried out by replicate injection of the sample and standard solutions.

3.3. Linearity

Linearity was found to be 2 - 12 μg mL^{-1} for both LAM and TDF. The linear regression equations for LAM and TDF were

LAM Y = 594995.71x + 53624.08 (n=6, r^2 = 0.9993)

TDF Y = 908701x + 140693 (n=6, r^2 = 0.9991)

Where Y is response (peak area) and *x* is the concentration.

The standard deviation of the relative residuals [47] was calculated with n – 2 degrees of freedom. The calculated value was found to be 0.06 for LAM and 0.02 for TDF. These calculated values were in good agreement with standard limit, i.e., a good calibration curve has a standard deviation of relative residuals less than 0.1. The standard deviation of the relative residuals clearly better to interpret than r, because of their linear response to the random errors of the signals combined with possible systematic errors produced by the non linearity of the real calibration function. By using this concept, problems due to different numbers of degrees of freedom between calibration and analytical data could be avoided.

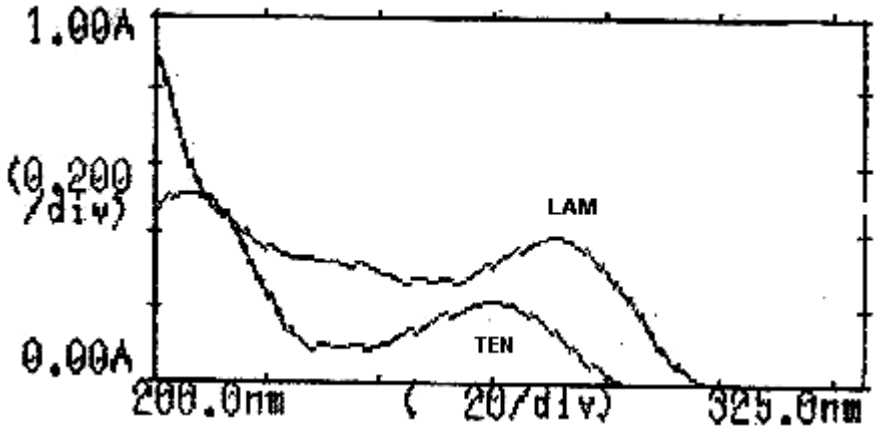

Fig 2. Overlain Spectra of LAM and TDF

Table 1. System suitability parameters

Property	LAM	TDF
Rt	3.27	4.15
RRT	1.009	0.998
RF	928024.7	595932.6
Tf	1.32	1.29
As	1.53	1.51
k'	1.11	1.68
N	4307	5562
R s	Between LAM and TDF 3.52	

Rt-Retention time; Tf - Tailing factor; *k'*- Capacity factor; N- Number of theoretical plates; R_S- Resolution

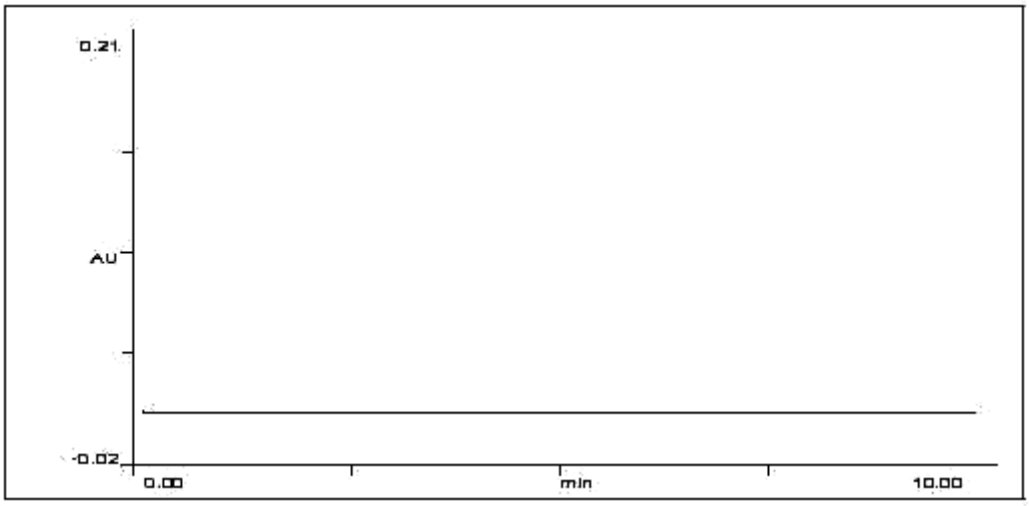

Fig 3. Chromatogram of Placebo

3.4. Precision

3.4.1. Repeatability

The formulation was analyzed in same day for repeatability and the results were subjected to statistical analysis. The %RSD for LAM was 1.1735 and for TEN it was 0.3855 which is according to ICH norms. The results of analysis are shown in table 2.

Table 2. Assay of Tablet Formulation

Drug	TENVIR - L Label Claim mg/ tab (n=6)	Amount Found		SD	RSD%	SE
		Mg	%			
LAM	300	301.97	100.66	1.1812	1.1735	0.1172
TDF	300	302.89	100.96	0.3892	0.3855	0.1589

S.D.: Standard deviation, COV: Coefficient of variance, S.E.: Standard error.

3.4.2. Intermediate Precision

This parameter was confirmed by intraday and inter day analysis of formulation. This was performed for three times in a same day and one time in three consecutive days. The % RSD values were found to be less than 2.

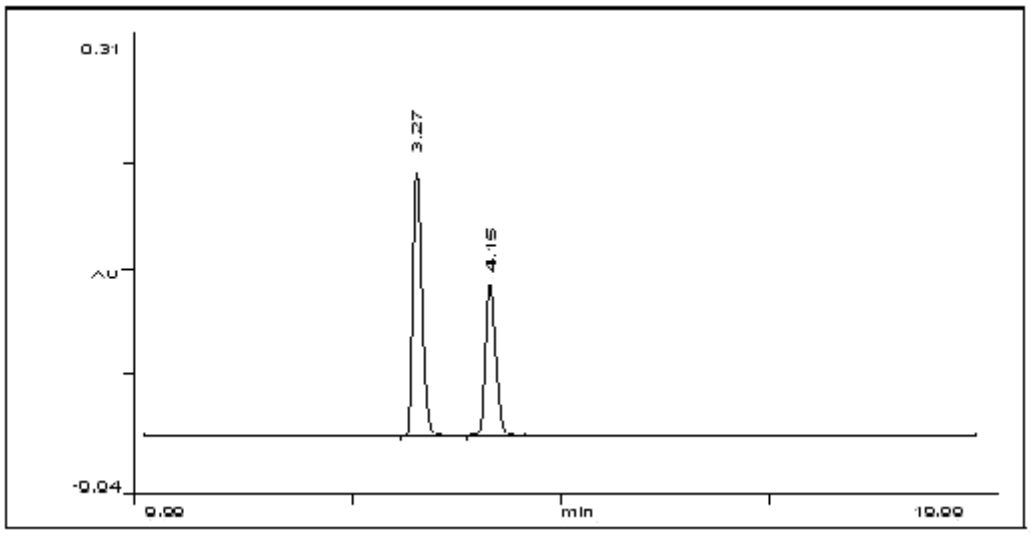

Fig 4. Chromatogram of mixed standard solutions

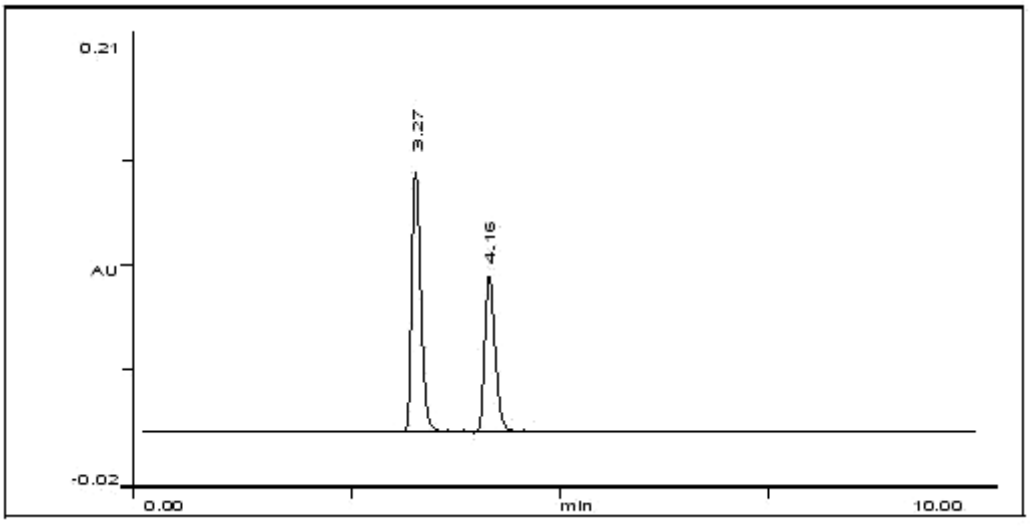

Fig 5. Chromatogram of LAM and TDF in sample solution with their retention time

3.5. Accuracy

Accuracy of developed method was confirmed by doing recovery study as per ICH norms at three different concentration levels by replicate analysis (n=3). The result of accuracy study was reported in Table 3. From the recovery study it was clear that the method is very accurate for quantitative estimation of LAM and TDF in tablet dosage form as all the statistical results were within the range of acceptance i.e. %COV< 2.0

3.6. Limit of Detection, and Limit of Quantitation

LOD is calculated by use of the equation LOD = 3.3σ/S and and LOQ were calculated by the use of the equation LOQ = 10σ/S, where σ is the standard deviation and S is the slope of the calibration curve. The results are reported in Table 4.

Table 3. Recovery Studies

Drug	Amount Present (μgmL^{-1})	Amount Added (μgmL^{-1})	Recovery%	RSD%
LAM	6.06	2	101.0241	1.732403
		4	101.1464	0.683219
		6	100.4808	0.517331
TDF	6.03	2	100.1127	1.354876
		4	100.8231	1.338464
		6	100.5526	0.92333

COV: Coefficient of variance

Table 4. Intra Day and Inter Day Precision, LOD and LOQ Studies

Drug	Intra day Precision RSD% (n = 6)	Interday Precision RSD%			LOD (μgmL^{-1})	LOQ (μgmL^{-1})
		Day 1	Day 2	Day 3		
LAM	0.416438	0.764084	0.772634	0.827447	0.0099	0.0299
TDF	0.651821	1.230288	1.147076	1.067622	0.0328	0.0994

Mean of six determinations, COV: Coefficient of variance, LOD: Limit of detection, LOQ: Limit of quantitation.

3.6.1. Instrument detection limit (IDL)

The IDL is treated as the minimum concentration of pure drug solution that can be reliably detected by the HPLC system used in this study under the stated conditions of analysis. For this blank solvent was used to compare the results from noise. The IDLs for LAM and TDF were estimated through 10 repetitive injections of a standard solution containing 10 μg mL^{-1} of each drug as follows:

$$IDL\ (\mu g\ mL^{-1}) = SD \times t_{95}$$

Where SD is the standard deviation of the peak areas for the replicate injections, and t_{95} is the Student's *t* at the 95% level of confidence. This theoretical IDL was finally injected in HPLC to confirm the detection limit. IDL was found to be 3.38 and 3.07 μg mL^{-1} for LAM and TDF, respectively.

3.6.2. Estimated method detection limit (EMDL)

The estimated method detection limit is defined as the approximate minimum concentration of drug that can be determined from a particular matrix by a particular method. It depends upon the recovery of drug by the given method and can be different for different matrices.

The EMDLs were estimated from the IDLs as follows:

$$EMD(\mu g\ g^{-1}) = \frac{IDLx100xV}{Mx\,\mathrm{Re}\,c\%}$$

With *M* being the mass of sample (g) and %Rec is the average percent recovery of the drug in the method. EMDL for LAM and TDF were found to be 7.95 and 7.5 μg g^{-1}, respectively.

3.7. Selectivity and Specificity

The selectivity of the method was confirmed by injecting the solution of both the drugs into the system and it was observed that two sharp peaks of LAM and TDF having

resolution of 3.52 were obtained at retention time of 3.27 and 4.15 min, respectively in reference to placebo solution.

Comparing the chromatograms obtained from standard drugs, with the chromatogram obtained from tablet solutions, the specificity of the method was assessed. As the retention time of standard drugs and the retention time of the drugs in sample solution was same, so the method was specific. The developed method was found specific and selective, as there was no interference of excipients found.

4. Conclusion

A new, reversed-phase HPLC method has been developed for simultaneous analysis of LAM and TDF in a tablet formulation. It was shown that, the method was linear, accurate, reproducible, repeatable, precise, selective and specific proving the reliability of the method. The run time is relatively short, i.e. 6 min, which enable rapid determination of any samples in routine and quality control analysis of tablet formulations. The same solvent was used throughout the experimental work and no interference from any excipient was observed. Hence, the proposed method was successfully applied to analyze the tablet formulation containing LAM and TDF.

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