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Simultaneous Estimation of Lamivudine and Tenofovir Disoproxil Fumarate by RP-HPLC in the Pharmaceutical Dosage Form

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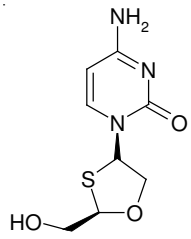
A high performance liquid chromatographic method was developed and validated for the simultaneous quantitative estimation of two antiretroviral drugs *viz.* lamivudine and tenofovir disoproxil fumarate in tablet dosage form. Chromatography was carried on Agilent eclipse XDB-C18 (5 µm, 4.6 mm × 150 mm) column with mobile phase based comprising of methanol and potassium di-hydrogen orthophosphate (0.02 M) in the ratio 60:40 v/v adjusted to pH 3.0 with formic acid. The flow rate was adjusted to 0.9 mL /min with UV detection at 260 nm. The retention times of lamivudine and tenofovir were found to be 1.9 and 3.4 min, respectively. The different analytical parameters such as linearity, precision, accuracy, ruggedness and robustness, limit of detection and limit of quantification were determined according to the International Conference on Harmonization (ICH) Q2B guidelines. The detector response is linear from 5-50 µg/mL for both lamivudine and tenofovir. The proposed method is highly sensitive, precise and accurate and hence was successfully applied for the reliable quantification of active pharmaceutical present in the commercial formulations.

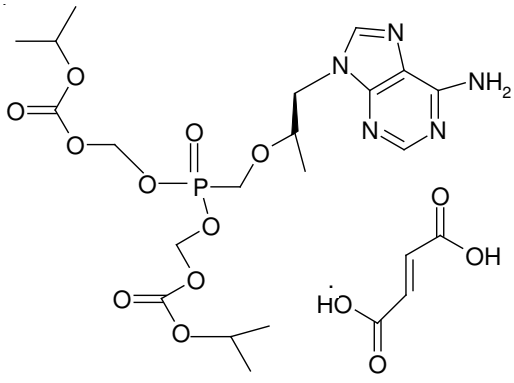
Key Words: Lamivudine, Tenofovir, RP-HPLC, Simultaneous estimation.

INTRODUCTION

Human immunodeficiency virus (HIV)[1,2] is a harmful virus which causes acquired immune deficiency syndrome or acquired immunodeficiency syndrome (AIDS), a condition in which human being immune system begins to slow down, leads to life dangerous and time serving infections. This condition progressively reduces the effectiveness of the immune system and leaves individuals susceptible to opportunistic infections[3]. For these conditions drugs are developed to disrupt the action of HIV known as antiretroviral[4]. These drugs or formulations made according to the different stages of the HIV life-cycle. Lamivudine (**I**) (4-amino-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one), commonly called 3TC) is a potent nucleoside analog reverse transcriptase inhibitor[5] (nRTI). It is the first class of drug introduced as antiretroviral agent for treatment of HIV. It is active against HIV-1, HIV-2 and HBV[6]. It has the molecular weight of 229.30; off-white crystalline solid with solubility in methanol and water at higher temperature. Lamivudine is administered orally, and it is rapidly absorbed with a bio-availability of over 80 %. Tenofovir (**II**) ({[(2R)-1-(6-amino-9*H*-purin-9-yl) propan-2-yl]oxy}methyl)phosphonic acid, belongs to the nucleotide



Lamivudine (**I**)



Tenofovir (**II**)

analogue reverse transcriptase inhibitors (nRTI) class of antiretroviral drugs, tenofovir was approved by the U.S. Food and Drug Administration for the treatment of HIV in the year 2001[7]. Tenofovir is the best combination with other anti-retroviral agents for the treatment of HIV-1 infection in adults. The pharmacokinetics of tenofovir is similar in healthy and HIV infected people. Lamivudine[8-15] in combination with other compounds has been determined in different pharmaceutical preparations by, HPLC and LC-MS. Tenofovir[16,17] was also determined by the HPLC and LC-MS. Combination of these two drugs into fixed dose combination (FDCs) has been an essential constituent of highly active anti-retroviral (HAART)[12] therapy. The present work involves simultaneous estimation of lamivudine and tenofovir by HPLC. Validation of the current method will be performed according to the ICH guidelines which include accuracy, precision, selectivity, linearity, range and robustness[18].

EXPERIMENTAL

Chromatography was performed using a JASCO HPLC instrument (Japan) equipped with a PU-2080 pump and detection was achieved by UV-2075 detector (JASCO) using a column Agilent Eclipse XDB-C18 (5 µm, 4.6 × 150 mm). Data acquisition and processing was performed using JASCO BORWIN software (Japan). Sample injection was performed with a Rheodyne 7725 injection valve *via* a 20 µL loop. Dissolution of the compound was enhanced by sonication on Bandelin sonerex sonicator. Degassing of the mobile phase and other solvents was achieved through by helium purging before the use. The pH of the solution was adjusted by using a pH meter (Cyber scan ph 2100) made by EUTECH and analytical balance (Model DI 707 of Digisum Electronic).

Pure samples of lamivudine and tenofovir disoproxil fumarate were obtained from Aurobindo Pharma limited. The commercial samples were obtained from Tenolam (Hetero Drugs Pvt. Ltd.) in the form of tablets containing 300 mg each respectively were purchased from local pharmacy. Methanol (HPLC grade), potassium dihydrogen ortho phosphate (G.R) and formic acid (G.R) were a product of a Merck limited. Purified water was prepared using a Millipore Milli-Q water purification system.

Chromatographic conditions were achieved by using column Agilent eclipse XDB-C18 (5 µm, 4.6 mm × 150 mm) analytical column. The mobile phase used in this study was a mixture of potassium dihydrogen orthophosphate (0.02 M) and methanol in the ratio (40:60 v/v); pH was adjusted to 3.0 with formic acid. The mobile phase was filtered through a 0.45 µ membrane filter and degassed using helium before use. The mobile phase was pumped from the solvent reservoir to column at a flow rate of 0.9 mL/min with injection volume of 20 µL and the retention times obtained for lamivudine and tenofovir were 1.9 and 3.4 min, respectively at UV detection point 260 nm. The identification of the separated lamivudine and tenofovir was confirmed by running the chromatograms of the individual compounds under identical conditions.

Recommended procedure for the standard graph: Primary stock solutions of lamivudine and tenofovir were weighed 10 mg each, respectively and dissolved in 10 mL methanol and sonicated for 10 min, from the above 1 mg/mL solution make the concentrations in range 5-50 µg/mL and the fixed concentration (50 µg/mL) were made with the mobile phase so as to obtain the linearity. Before injecting drug solution, the column was equilibrated for at least 30-45 min with the mobile phase flowing through the system. Each of the samples (20 µL) prepared were injected three times into the column. The amount of drug is calculated by the peak area ratio, standard graph was plotted by taking concentration of drug on X-axis and peak area ratio of drug to Y-axis.

Assay determination of lamivudine and tenofovir in dosage form: Twenty tablets of tenolam (each containing 300 mg of lamivudine and tenofovir) were made into fine powder, an amount equivalent to 25 mg of tablet powder accurately weighed and then extracted with methanol in a 25 mL volumetric flask, filtered through 0.45 µ filter and sonicated for 0.5 h. the solution was centrifuged and the supernatant was taken into a thoroughly cleaned and dried volumetric flask.

Simultaneous quantification of lamivudine and tenofovir in dosage form: From the above 1 mg/mL solution suitable dilutions (5-50 µg/mL) were made with the mobile phase so as to obtain the linearity of the two drugs previously determined, the sample volume of 20 µL was injected into the column. All the samples were made in triplicate according to the ICH guidelines.

Method validation: Validation of the current method will be according to the International Conference on Harmonization ICH Q2B guidelines[19,20]. Values are shown in Table-1.

TABLE-1
HPLC PARAMETERS

Parameters	Lamivudine	Tenofovir
Retention time (min)	1.9	3.4
Resolution	0	9.4
Theoretical plates	4250	6280
HETP	27.471	34.455
Tailing factor	1.2	1.5
LOD (ng/mL)	17.89	42.98
LOQ (ng/mL)	54.23	130.26

Specificity: The method specificity was validated by comparing the chromatogram obtained from the drug and the most commonly used excipient mixtures with those obtained from the blank. The excipients selected based on the commonly in the tablet formulation, includes lactose, starch, cellulose and stearate. The drug to excipients ratio used was similar to that in the commercial formulations.

Linearity: Method validation is done according to the International Conference on Harmonization Q2B guidelines for validation of analytical procedures, calibration curves were generated with appropriate volumes of working standard solutions for HPLC method. Linearity was determined by plotting the standard curve in the concentration range (5-50 µg/mL). The linearity of these methods was evaluated by linear regression analysis, using least square method.

Precision: The assay of the precision was determined by repeatability (intra-day) and intermediate precision (inter-day) and reported as % RSD. The intra and inter-day variation in the peak area of drug solution containing (5, 30 and 50 µg/mL)

of lamivudine and tenofovir, respectively were calculated in terms of coefficient of variation (CV), standard deviation and % RSD (Table-2).

TABLE-2
PRECISION OF PROPOSED HPLC METHOD

Drug name	Conc. (µg/mL)	Measured concentration (µg/mL) ± SD (n = 5)		% CV	
		Intra-day	Inter-day	Intra-day	Inter-day
Lamivudine	5	4.94±0.76	4.91±1.21	0.81	1.25
	30	30.02±0.62	29.95±0.68	0.68	0.71
	50	49.92±0.86	49.95±0.94	0.85	0.99
Tenofovir	5	4.92±0.78	4.92±1.28	0.87	1.27
	30	30.04±0.66	29.84±0.16	0.71	0.71
	50	49.91±0.89	49.99±0.91	0.78	0.77

Accuracy: Accuracy[21] is the presence of analyte recovered by assay from a known added amount. Accuracy of the HPLC method was done by adding known amount of 5 µg/mL of the drug (lamivudine and tenofovir) to drug solution of known concentration 80, 100 and 120 %, respectively. All the solutions were prepared and analyzed in triplicate (Table-3).

Robustness and ruggedness: Robustness was done by small changes in the chromatographic conditions such as percentage change in methanol in the mobile phase, flow rate, buffer strength, pH, filtration and sonication time was varied and the effects of parameters were observed and calculated for mean standard deviation and relative standard deviation. Results remained unaffected by small variations in these parameters. Ruggedness is being determined by the varying the analyst, instrument and different column of different grades. The relative standard deviation of the results obtained from different analysts and instruments was < 1.0 %.

Limit of detection (LOD) and limit of quantification (LOQ): Limit of detection and limit of quantification are calculated for the sensitivity of the method. They are quantified based on the signal to noise ratio. LOD is the lowest detectable concentration of the analyte by the method while LOQ is the minimum quantifiable concentration. LOD and LOQ are calculated according to the ICH guidelines.

$$LOD = 3.3 \times s/S$$

$$LOQ = 10 \times s/S$$

RESULTS AND DISCUSSION

A RP-HPLC method was developed for the antiretroviral drugs (lamivudine and tenofovir). This method can be used for routine quality control in pharmaceutical dosage forms. A typical chromatogram of lamivudine and tenofovir is shown in Fig. 1, which shows good base line separation and resolution. Individual chromatograms are also recorded. The order of elution was lamivudine followed by the tenofovir at 1.9 and 3.4 min, respectively. Calibration curves for lamivudine and tenofovir was later used to determine the concentrations of the drug in the tablets. The calibration curves were plotted by using 5-50 µg/mL concentrations of the drug sample. Linear regressions were obtained within the limits for lamivudine and tenofovir were y = 0.0036x + 0.0027 (r = 0.9994) and y = 0.003x + 0.002 (r = 0.9997).

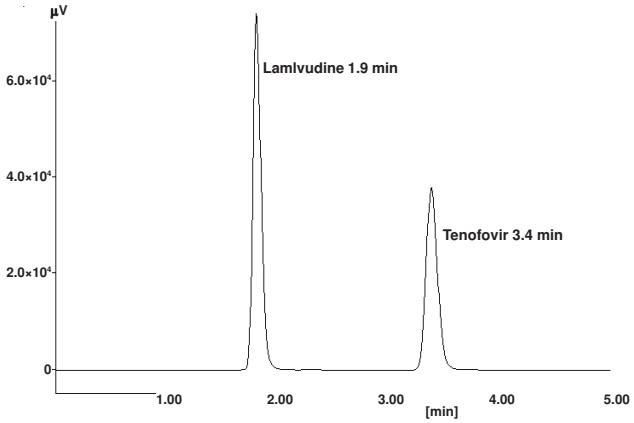


Fig. 1. Chromatogram of lamivudine and tenofovir

The chromatographic conditions were optimized in order to provide good performance in assay, various ratios and combinations of methanol with phosphate buffer were tried for lamivudine and tenofovir. Buffers of different concentrations (0.01-0.04 M) and pH (2.8-3.8) are trailed. Finally mobile phase was use of 60:40 v/v mixture methanol and potassium dihydrogen orthophosphate (0.02 M) at pH 3.0 at flow rate 0.9 mL/min and UV detection at 260 nm, at these conditions peaks obtained are with good shape and resolution.

The HPLC method development is accurate, precise, reproducible and sensitive. All the validation parameters of the two drugs were in the specific limits. Chromatographic parameters are similar for the pure samples and tablet extract, which indicated the robustness of the HPLC method. The validation of the method developed should be carried out for 3 replicates of chromatographic runs under identical conditions.

Conclusion

The proposed RP-HPLC method is simple, reliable and selective providing satisfactory accuracy and precision with lower limit of detection and quantification. Moreover the shorter duration of analysis for lamivudine and tenofovir make these reported method suitable for routine quantitative analysis in pharmaceutical dosage forms. Hence it can be easily and conveniently adopted for routine quality control analysis.

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TABLE-3
ACCURACY RESULTS

Drug	Label claim (mg)	Mean ± SD (amount recovered) (n = 5)	Estimated % of label claim ± SD (n = 5)	% Recovery ± SD (n = 5)		
				80	100	120
Lamivudine	300	294 ± 0.01	98.4 ± 0.2	99.06 ± 0.34	99.8 ± 0.33	101.09 ± 0.30
Tenofovir	300	291 ± 0.01	97.5 ± 0.6	99.89 ± 0.19	101.8 ± 0.58	102.15 ± 0.45

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