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Determination of Lamivudine and Tenofovir in Combined Pharmaceutical Dosage Formusing High-Performance Liquid Chromatography

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ABSTRACT

A new,simple and accurate high performance liquid chromatography method is developed and validated for the estimation of two anti-viral drugs, Lamivudin and Tenofovir in tablet dosage form. Separation and quantification was performed on Thermohypersil BDS (150 x 4.6 mm; 5 µm particle size) analytical column using 0.02M KH_2PO_4 and acetonitrile (60:40 v/v) as the mobile phase. A UV detector set at 260 nm was used for evaluation. The developed method is validated according to ICH guidelines. The correlation coefficients of calibration curves were found to be 0.9999 in the concentration range of 37.5-225 for Lamivudine and tenofovirr. The method had an acceptable precision and accuracy. The method had the potential to quantify the selected drugs simultaneously from tablet dosage forms.

Keywords: *High-performance liquid chromatography; Lamivudine; Tenofovir.*

INTRODUCTION

Lamivudine [1, 2] is a Human Immunodeficiency Virus Type 1 (HIV-1) hepatitis B(HBV) Reverse Transcriptase Inhibitors. Chemically, lamivudine is described as 4-amino-1-[(2 R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one. Lamivudine is a synthetic nucleoside analogue and is phosphorylated intracellularly to its active 5'-triphosphate metabolite, lamivudine triphosphate (L-TP). This nucleoside analogue is incorporated into viral DNA by HIV reverse transcriptase and HBV polymerase, resulting in DNA chain termination.

Tenofovir [3, 4] belongs to a class of antiretroviral drugs known as nucleotide analogue reverse transcriptase inhibitors (NtRTIs) Chemically,tenofovir is known as ({[(2R)-1-(6-amino-9H-purin-9-yl)propan-2-yl]oxy}methyl) phosphonic acid. Tenofovir exerts its activity by inhibiting the HIV viral protease enzyme. Tenofovir inhibits the activity of HIV reverse transcriptase by competing with the natural substrate deoxyadenosine 5'-triphosphate and, after incorporation into DNA, by DNA chain termination. Specifically, the drugs are analogues of the naturally occurring deoxynucleotides needed to synthesize the viral DNA and they compete with the natural deoxynucleotides for incorporation into the growing viral DNA chain. Fig. 1 & 2 shows the chemical structure of lamivudine and tenofovir, respectively.

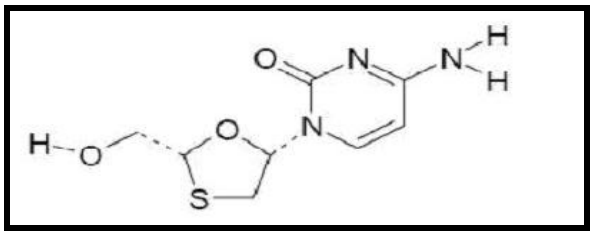

Fig. 1: Chemical structure of lamivudine

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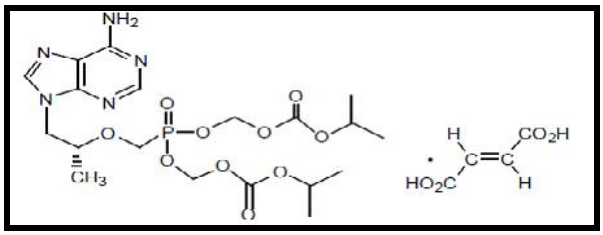

Fig. 2: Chemical structure of Tenofovir

The main goal of the treatment of hepatitis B virus (HBV) infection is the sustained suppression of HBV replication, because suppression is associated with a normalization of transaminase levels and improvement in histologic findings [5]. Lamivudine inhibits HBV replication in >80% of HIV/HBV—coinfected patients. However, HBV resistance to lamivudine is seen in 50% and 90% of patients after 2 and 4 years of continuous therapy, respectively [6]. Therefore, the use of combination therapy should be more effective than the use of monotherapy in reducing the viral load and in decreasing the emergence of resistance. Tenofovir is a nucleotide reverse-transcriptase inhibitor with excellent activity in vitro and in vivo against wild-type HBV and lamivudine-resistant HBV [7-8]. In the present pilot study, we prospectively evaluated the long-term anti-HBV activity of the combination of lamivudine and tenofovir as part of HAART for all HIV-1-infected patients who were chronically coinfected with wild-type HBV and who were followed during treatment at our institution from May 2001 through January 2002.

Literature survey reveals different methods for their analysis in formulations [2-10]. Our present plan is to develop a new, simple, precise RP-HPLC method and validated the same as per ICH norms [9-11].

The literature reports, many methods for simultaneous quantitative determination of tenofovir and lamivudine in bulk, tablet dosage form, capsule dosage form and human plasma. These methods include simultaneous estimation of tenofovir and lamivudine by UV spectrophotometry [12], HPTLC [13], HPLC [14-17] and LC-MS [16]. The summary of reported methods is shown in Table 1.

MATERIALS AND METHODS

Apparatus:

A Waters 2695 alliance with binary HPLC pump equipped with Waters 2998 PDA detector and Waters Empower2 software was used in the present investigation.

Mobile phase:

The solvents and chemicals used in the preparation of mobile phase were of HPLC grade and analytical grade, respectively. The mobile phase used was 0.02M KH_2PO_4 and acetonitrile in the ratio of 60:40 *v/v*. Before use, the mobile phase was filtered through millipore membrane filter and degassed for 15 minutes by sonication.

Chromatographic conditions:

Thermo Hypersil BDS (150 x 4.6 mm; 5 µm particle size) analytical column was used for separation and simultaneous analysis of lamivudine and tenofovir. The column temperature was maintained at 30±1^{0}C. The separation was carried out under isocratic elution. The flow rate was maintained as 1.0 ml/min. The injection volume was 10 µl. The eluents were detected at 260 nm.

Standard solutions:

The standard stock solution was prepared by dissolving 300 mg of lamivudine and 300 mg of tenofovir in 100 ml mobile phase. Working standard solutions equivalent to 37.5-225 µg/ml both lamivudine and tenofovir were prepared from stock solution by appropriately diluting the stock standard solution with the mobile phase.

Sample Solution:

Ten tablets were weighed and crushed to a fine powder. The powder equivalent of 300 mg of lamivudine and 300 mg was taken in a 100 ml volumetric flask containing 20 ml of mobile phase, sonicated for 20 minute and made up to mark with the same solvent. The resultant mixture was filtered through 0.45 µm filter paper. The filtrate was diluted appropriately with the mobile phase to get a final concentration of 1500 µg/ml lamivudine and1500 µg/ml tenofovir.

RESULTS AND DISCUSSION

HPLC parameters optimization:

So as to study the simultaneous elution of more than one drug under isocratic conditions, different chromatographic conditions (type of the column, mobile phase composition, flow rate and pH) have been investigated. The objective of the simultaneous HPLC method development was to achieve a peak tailing factor <2, USP plate count ≥ 2000, retention time in between 3 and 5 minutes, along with good resolution. This objective was obtained using mobile phase consisting of 0.02M potassium dihydrogen phosphate-acetonitrile in the proportion of (60/40, *v/v*). The pH of the mobile phase was adjusted to 4.5 with orthophosphoric acid. Under the above described conditions, the analyte peaks were well defined, resolved and free from tailing. The tailing factors were <2 for both the peaks. The elution orders were lamivudine (retention time- 2.915 min) and tenofovir (retention time- 4.613 min) at a flow rate of 1.0 ml/min (Fig. 3). The optimum wavelength for detection was 260 nm at which much better detector responses for the selected drugs were obtained.

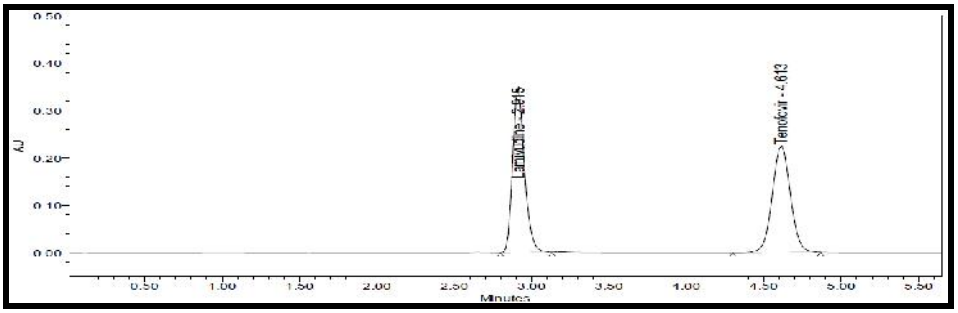

Fig. 3: Typical chromatogram of lamivudine and tenofovir

Method validation:

The optimized RP-HPLC method for simultaneous assay of Lamivudine and tenofovirr was validated according to ICH guidelines [19] with respect to system suitability, linearity, sensitivity, accuracy, precision and robustness.

System suitability:

In relation to U.S. Pharmacopeia, system suitability tests are an integral part of a liquid chromatographic method. System suitability tests are used to confirm that the column efficiency, resolution and reproducibility of the chromatographic system are sufficient for the analysis. System suitability test was assessed from five replicate injections of the standard solution containing 1500µg/ml lamivudine and 1500 µg/ml tenofovir. The results of system suitability in comparison with the required limits are shown in Table 2. The results are found to be suitable and are within the accepted limits.

Table No. 2: System suitability test of the HPLC method

Parameters	Results		Recommended limits
	Lamivudine	Tenofovir	
Retention time	2.915	4.613	-
Peak area	1832992 (%RSD – 0.6)	1815334 (%RSD–0.2)	RSD ≤1
USP resolution	-	6.543	> 1.5
USP plate count	6990	7513	> 2000
USP tailing factor	1.21	1.03	≤ 2

Linearity and range:

The linearity of the method was determined by analyzing five different concentrations of each drug. The calibration curve was plotted by area under the peak responses of the drugs against their corresponding concentrations. Calibration curves were linear over the concentration range of 37.5-225 µg/ml for both lamivudine and tenofovir. The parameters such as a regression equation and regression coefficient are given in Fig. 4 & 5. The results show a good correlation between the peak areas of the drugs and their corresponding concentrations.

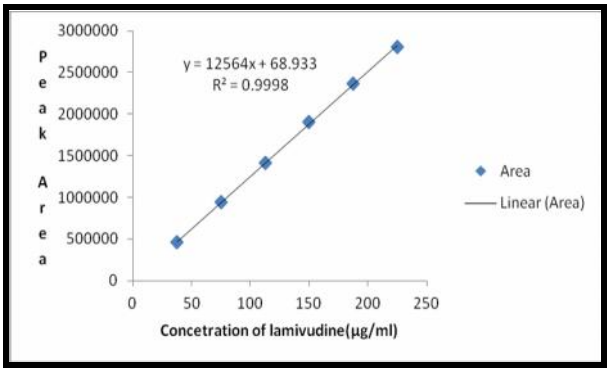

Fig. 4: Linearity curve of lamivudine

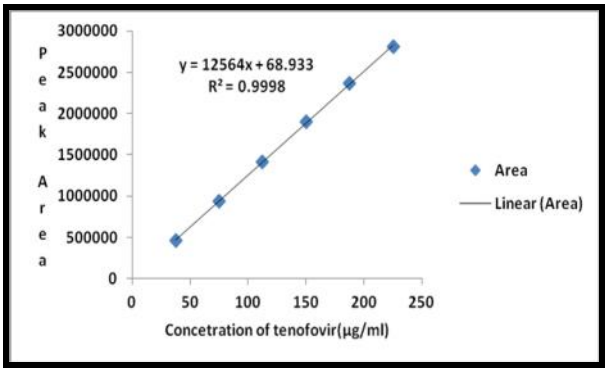

Fig. 5: Linearity curve of tenofovir

Sensitivity:

The sensitivity of the method was assessed by calculating limit of detection (LOD) and limit of quantification (LOQ) according to ICH guidelines. The results are summarized in Table 3. The low values of LOD and LOQ demonstrate the sufficient sensitivity of the method. The chromatograms of lamivudine and tenofovir at LOD and LOQ levels are presented in Fig. 6 & 7.

Table No. 3: Sensitivity of the HPLC method

Parameters	Results	
	Lamivudine	Tenofovirr
LOD	0.25	**0.01**
LOQ	**0.76**	**0.03**

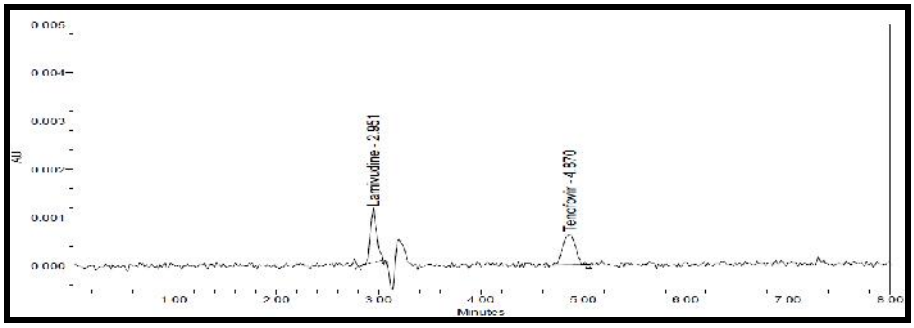

Fig. 6: Chromatogram of lamivudine and tenofovir at LOD level

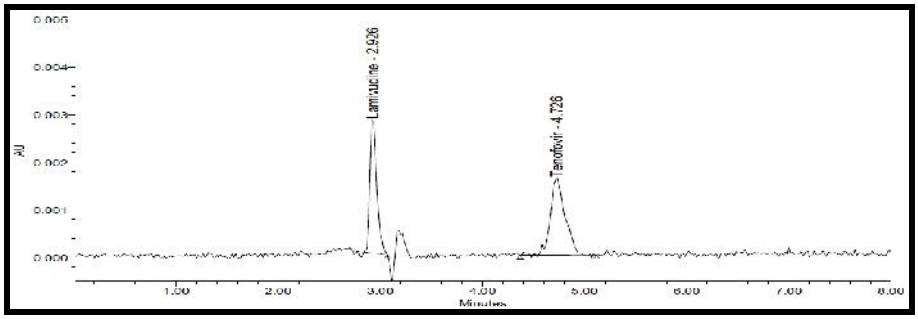

Fig. 7: Chromatogram of lamivudine and tenofovir at LOQ level

Precision:

Precision was determined by injecting six standard solutions of lamivudine(1500 µg/ml) and tenofovir (1500 µg/ml). The peak areas were determined. Relative standard deviation of peak areas of the two drugs was then calculated to represent precision. The results are summarized in Table 4. The low % RSD values indicated that the method was precise.

Table No. 4: Precision of the HPLC method

Lamivudine		Tenofovirr	
Peak area	%RSD	Peak area	%RSD
1824123	0.68	1807050	**0.28**
1827839		1813907	
1850618		1821372	
1845864		1819534	
1816215		1813854	
1833295		**1816286**	

Accuracy:

Accuracy of the method was evaluated by recovery studies at three concentration (75%, 150%, and 225%) levels by standard addition method. The mean percentage recoveries obtained were shown in Table 5. The good % recovery values showed that the method was highly accurate.

Table No. 5: Accuracy of the HPLC method

Drug	Amount added (µg/ml)	Amount Recovered (µg/ml)	Recovery (%)	% RSD
Lamivudine	75	75.15	100.2	0.71
	150	149.94	99.96	0.63
	225	225.16	100.07	0.68
Tenofovir	75	75.08	100.11	0.23
	150	149.52	99.68	0.52
	225	224.93	99.97	0.38

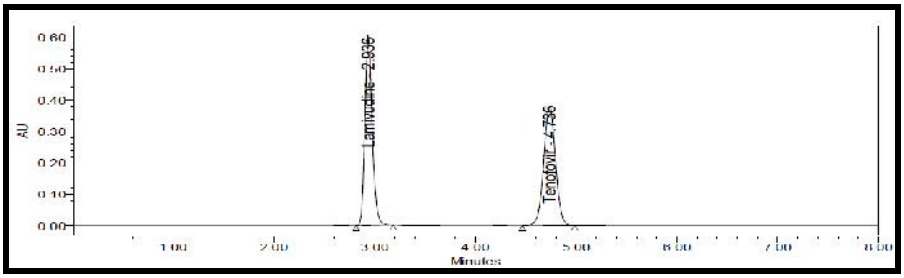

Fig. 8: Chromatogram of lamivudine and tenofovir at 75% level

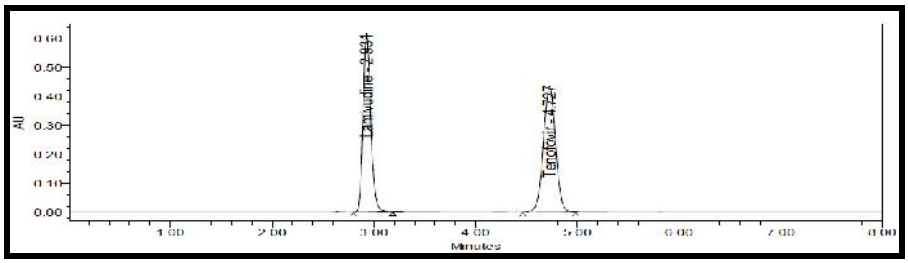

Fig. 9: Chromatogram of lamivudine and tenofovir at 150% level

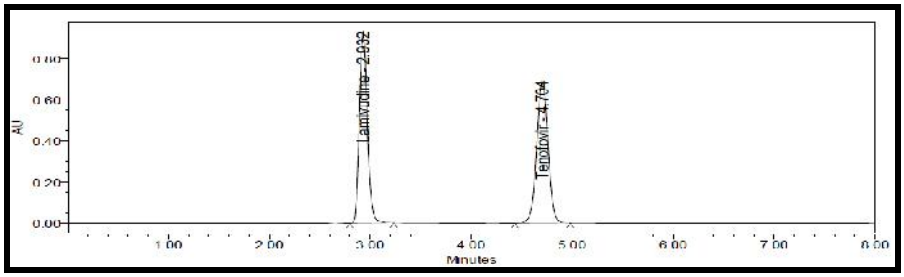

Fig. 10: Chromatogram of lamivudine and tenofovir at 225% level

Robustness:

In order to show the robustness of the method, system suitability parameters were evaluated at different flow rate and different column temperature. The parameters used to define robustness are retention time, USP tailing factor and USP plate count. The results showed (Table 7) that slight variations in method parameters had a negligible effect on the analysis.

Table No. 7: Robustness of the method

Drug	Parameter	Retention time	USP Plate Count	USP Tailing
Lamivudine	**Flow 1**	3.266	7985	**1.21**
	Flow 2	2.683	5932	**1.20**
	Temperature 1	2.192	7835	**1.23**
	Temperature 2	2.965	5742	**1.24**
Tenofovir	**Flow 1**	5.326	8545	**1.03**
	Flow 2	4.419	6234	**1.10**
	Temperature 1	4.706	8526	**1.15**
	Temperature 2	**4.685**	**6278**	**1.25**

CONCLUSION

A RP-HPLC method has been reported for simultaneous lamivudine and tenofovir. The proposed method gives good resolution of the two drugs. The validation of developed method was done as per ICH guidelines and proved that method to be simple, sensitive, precise, accurate and robust. The validated method was successfully applied to the determination of commercially available tablet dosage. The method can be used for the routine quality control analysis of tablet dosage forms containing lamivudine and tenofovir.

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