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ORIGINAL ARTICLE

Development of validated stability- indicating RP-HPLC method for simultaneous estimation oflamivudine and tenofovir disoproxil fumarate in tablet dosage form

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ABSTRACT
A simple, specific, accurate, and rugged reverse phase high performance liquid chromatographic method was developed for simultaneous estimation of lamivudine and tenofovir disoproxil fumarate in tablet dosage form. A reverse phase gradient program has been developed to separate the all active ingredients (lamivudine and tenofovir disoproxil fumarate). A gradient programming has been done, on a reverse phase Agilent C18 column (4.6 x 100 mm and 2.5 µm) with with mobile phase containing acetonitrile + 0.1% ortho phosphoric acid (80:20), adjusted to pH 6.2 using tetraethyline amine with a flow rate 1 mL/min, monitored at 260 nm.The retention time of lamivudine and tenofovir disoproxil fumarate was 2.9 min and 7.3 min respectively. The linearity for of lamivudine and tenofovir disoproxil fumarate was found to be in the range of 5-25 µg/ml. The recovery of lamivudine and tenofovir disoproxil fumarate was found in the range of 98.05-105.06%and 98.14-105.41 % respectively. The proposed method was validated in terms of linearity, range, accuracy, precision, specificity, robustness and stability studies and the method is successfully applies to the estimation oflamivudine and tenofovir disoproxil fumarate in tablet dosage form.
Keywords:*Lamivudine, Tenofovir Disoproxil Fumarate, RP-HPLC, Simultaneous estimation, Validation.*

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INTRODUCTION

Human immunodeficiency virus is a harmful virus which causes acquired immunodeficiency syndrome (AIDS), due to AIDS human immune system begins to disturb, and leads to number of infections. This condition reduces the capability of the immune system and leaves individuals susceptible to various infections. For treatment of these conditions drugs are developed to disrupt the action of HIV on body known as antiretroviral drugs. These drugs or prepared according to the different stages of the HIV life-cycle [1, 2].

Lamivudine is reverse transcriptase. According to study it was reported to be active against HIV-1, HIV- 2 and hepatitis B virus. It is chemically 4 - amino - 1 - [(2R, 5S) – 2 - (hydroxyl methyl) - 1, 3 – oxathiolan – 5 - yl] - 1, 2-dihydropyrimidin-2-one. Figure.1.Lamivudine is a synthetic nucleoside analogue incorporated into viral DNA by HIV reverse transcriptase an HBV polymerase, resulting in DNA chain termination[3].Tenofovir disoproxil fumarate (TDF) belongs to the class of antiretroviral drugs known as nucleotide analogue reverse transcriptase inhibitors (nRTIs), which blocks reverse transcriptase, an enzyme crucial to viral production in HIV-infected people. Chemically TDF is 9[(R)-2-[[bis [[(isopropoxycarbonyl) oxy] methoxy] phosphinyl] methoxy] propyl] adenine fumarate. Figure.2 TDF is the first nucleotide analog approved for HIV-1 treatment [4,5].

Literature survey reveals lamivudinein combination with other compounds has been determined various analytical methods by, UV, HPLC and LC-MS. Tenofovir was also determined by the HPLC, HPTLC and LC-

MS[6-14].To the best of our knowledge, there is some reported RP –HPLC method for simultaneous estimation of lamivudine and tenofovir disoproxil fumarate in pharmaceutical formulations, previous to our work. Thus, efforts were made to develop fast, selective and sensitive stability indicating analytical method for the estimation of lamivudine and tenofovir disoproxil fumarate in their combined dosage form using reverse phase high performance liquid chromatographic method. In the current work author developed a simple, reliable and reproducible stability –indicating RP-HPLC method which was duly validated by statistical parameters precision, accuracy and recovery. The method has been satisfactorily applied to the simultaneous estimation of lamivudine and tenofovir disoproxil fumarate in bulk and pharmaceutical dosage forms.

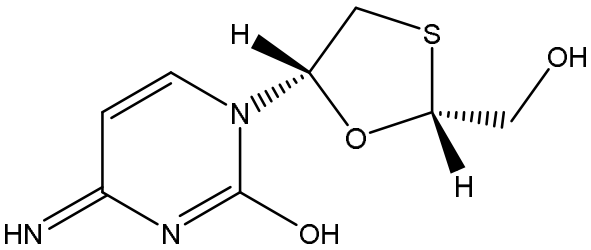

Figure 1: Chemical structure of Lamivudine

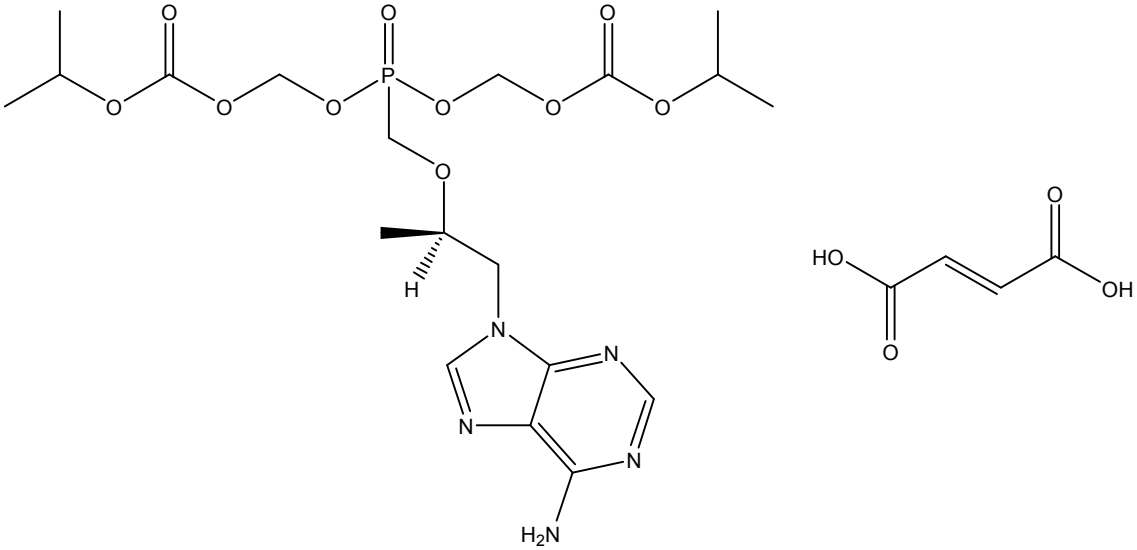

Figure2: Chemical structure of Tenofovir disoproxil fumarate

MATERIALS AND METHODS

Materials

Lamivudine and Tenofovir disoproxil fumarate were obtained as gift sample from Cipla Ltd. Kurkumbh, Pune.All chemicals and reagents used were analytical-grade and purchased from Qualigen's fine chemicals (Mumbai, India). The marketed preparation was purchased from the local market having brand name Tenvir-L which containing 300 mg of lamivudine and 300 mg tenofovir disoproxil fumarate.

Instrumentation and Chromatographic condition

A HPLC system of Younglin gradient system pump SP930Dwith UV 730Ddetectorwas used working via Chemstation10.1software. The separation was carried on Agilent column with C18 packaging and 4.6 x100 mm dimensions,2.5µm particle size. The mobile phase consist acetonitrile: 0.1 % OPApH6.2with 0.1% TEA in the ratio of 80:20with flow rate of 1ml/min. wavelength selected for the determination oflamivudine and tenofovir disoproxil fumaratewas 260.0nm according to observation.

Preparation of standard solution

The standard stock solution of 1000 µg/ml each oflamivudine and tenofovir disoproxil fumarate was prepared by accurately weighing 10 mg of lamivudine and tenofovir disoproxil fumarate transfer in 10 ml methanol.

Validation of lamivudine and tenofovir disoproxilfumarate HPLC assay

The RP-HPLC method for lamivudine and tenofovir disoproxilfumarate assay was validated in term of accuracy, linearity, specificity, LOD, LOQ, and robustness according to ICH Harmonized Guidelines.

System specificity

The system suitability was assessed by six replicate analyses of lamivudine and tenofovir disoproxil fumarate at a concentration of 5 µg/ml. The acceptance criterion was ±2% for the percent relative standard deviation (% RSD) for the peak area and retention times for lamivudine and tenofovir disoproxil fumarate

Linearity and range

Linearity was determined by three injections of differentlamivudine and tenofovir disoproxil fumarate (5-25 µg/ml). Linearity is the ability to obtain test results that are directly proportional to the concentration of the analyte. The average peak areas were plotted against concentrations. Then linearity was evaluated using the calibration curve to calculate coefficient of correlation, slope and intercept. In general, a value of correlation coefficient r2) > 0.998 is considered as the evidence of an acceptable fit for the data to the regression line.

Accuracy

The accuracy of an analytical method expresses the nearness between the expected value and the value found. In this case, to evaluate the accuracy of the developed method, successive analysis (n = 3) for three different concentrations (80%, 100 % and 120 %) of standard lamivudine and tenofovir disoproxil fumarate solution were performed using 10µg/ml concentration in the developed method. The data of the experiment were statistically analyzed using the formula [% Recovery = (Recovered conc. /Injected conc.) -100] to study the recovery and validity of the developed method. The mean recovery should be within 90–110% to be accepted.

Precision

The precision of the method was assessed by studying intra-day and inter-day variation. The intraday precision was assessed by analyzing the calibration curves of replicates of different concentrations (10, 15, 20µg/ml) of both drugs within the same day. The inter-day precision was determined by analyzing of six replicates of different concentrations (10, 15, 20µg/ml of both drugs on three different days. The total precision of the method was expressed as the relative standard deviation (%RSD).

Limit of detection and limit of quantification

Limit of detection (LOD) and limit of quantification (LOQ) for related substances are determined by injecting a series of solutions of known concentration till the signal-to-noise ratio became as 3:1 and10:1, respectively,

Robustness

The robustness of an analytical procedure is the measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. The robustness was studied by evaluating the effect of small but deliberate variations in the chromatographic conditions.

Analysis of a marketed formulation

To determine the content oflamivudine and tenofovir disoproxil fumarate in conventional tablet (Brand name:Tenvir-L), twenty tablets were weighed, 36.23 mgpowder and transferred into a 100ml volumetric flask, about 10 ml of methanol was added, the solution was sonicated for 15 min with intermittent shaking and diluted it up to the mark with remaining quantity of methanol and mixed well (1000 µg/ml lamivudine & tenofovir disoproxil fumarate1000 µg/ml). It was filtered through 0.45µ nylon filter.Peak areas were measured at 260 nm and concentrations in the samples were determined using multilevel calibration developed on the same HPLC system under the same conditions using linear regression equation.

Forced degradation study:

All stress decomposition studies were performed at a drug concentration 20µg/ml of lamivudine and tenofovirdisoproxil fumarate under conditions hydrolysis (acid, base), oxidation condition as mentioned in ICH guidelines Q1A (R2).Acid, base and oxidation degradation were performed by adding 1 ml of 0.1 N HCl, 1 ml of 0.1 N NaOH, and 1 ml of 3 % peroxide solution, respectively, to the sample solution, and these samples were kept on a bench for 60min and 120 min.

RESULTS AND DISCUSSION

Method Development

For the RP-HPLC method for estimation of lamivudine and tenofovir disoproxil fumarate, chromatographic conditions were optimized to obtain good resolution and proper peak shape. In chromatographic conditions the selection of mobile phase was totally depend upon peak parameters like symmetry, theoretical plates and capacity factor. Symmetrical peaks with good separation, retention time for lamivudine is 2.8 and tenofovir disoproxil fumarate is 7.38 were obtained with reverse phase Agilent

column with C18 packaging and 4.6 x100 mm dimensions,2.5µm particle size**.** The mobile phase was used containing acetonitrile + 0.1 % Ortho-phosphoric acid with pH-6.2 with TEA in the ration of 80:20 at a flow rate of 1.0ml/min. The optimum wavelength for detection and quantification was at 260nm, at which good response was obtained for both drugs. The results were summarized in (Table 1).

Table1: Details of optimized RP**-**HPLC method for estimation of LAMI and TDF

Parameters	Chromatographic conditions
HPLC System	Younglin (S.K)
Pump	SP930 D
Detector	UV 730 D
Column	4.6 x 100 mm 2.5µm
Column temperature	Ambient
Mobile phase	Acetonitrile (80) : (0.1 % Ortho-phosphoric acid with pH-6.2 with TEA)(20)
Concentration of standard stock Solution	1000 µg/ml of Lamivudine and 1000µg/ml of Tenofovir Disoproxil Fumarate
Detection of Wavelength	260nm
Flow rate	1ml/min
Sample volume	20µl
Run time	10 min
Retention time	Lamivudine: 2.8 min ,Tenofovir Disoproxil Fumarate: 7.3min

System Suitability

Before performing the completed analysis, the system suitability parameter was evaluated. For this purpose, various parameters were calculated as per their standard procedure e.g. retention time for drugs lamivudine and tenofovir disoproxil fumarate, theoretical plate's number of the column (for column efficiency), tailing factor, relative standard deviation of peak area and retention time. The column efficiency was more for analysis i.e. ≥2000. The tailing factor was also found within range. Table2 shows the details of System Suitability parameters of standard mixture of lamivudine and tenofovir disoproxil fumarate and chromatogram obtained from the analysis of pure drugs (lamivudine and tenofovir disoproxil fumarate) using the developed RP-HPLC method shown in (**Figure 3**).

Table2: Details of System Suitability parameters of standard mixture of LAMI and TDF

S.N	Name	RT (min)	Area (µV.sec)	TP	Resolution
1	LAMI	2.8	747.6716	2776.2	0000
2	TDF	7.38	282.5911	6439.3	12.5714

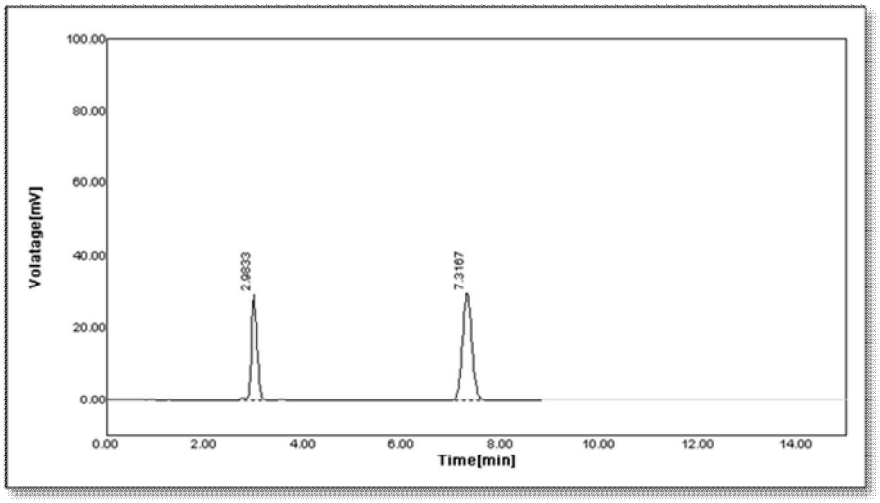

Figure3: Chromatogram of standard mixture of LAMI and TDF

Linearity and range

Linearity was established by method of least squares linear regression analysis of the calibration curve. The calibration curves were linear over the concentration range of 5-25 µg/ml for both lamivudine and

tenofovir disoproxil fumarate respectively. Calibration curve were plotted peak areas versus respective concentrations and linear regression analysis was performed on the resultant curves. Correlation coefficients were found to be 0.9981 for lamivudine which is given in Table3 and Figure4.And Correlation coefficients for tenofovir disoproxil fumarate found to be 0.9987, result given in Table4 and Figure 5.

Table 3: Linearity **of** Lamivudine

Standard conc.→	5µg/ml	10 µg/ml	15 µg/ml	20 µg/ml	25µg/ml
Replicates	**Peak area**				
1	141.59	285.61	415.71	555.91	658.02
2	144.83	279.66	422.16	547.05	665.1
Mean	143.21	282.64	418.94	551.48	661.56
±SD	2.29	4.21	4.56	6.26	5.01
%RSD	1.60	1.49	1.09	1.14	0.76

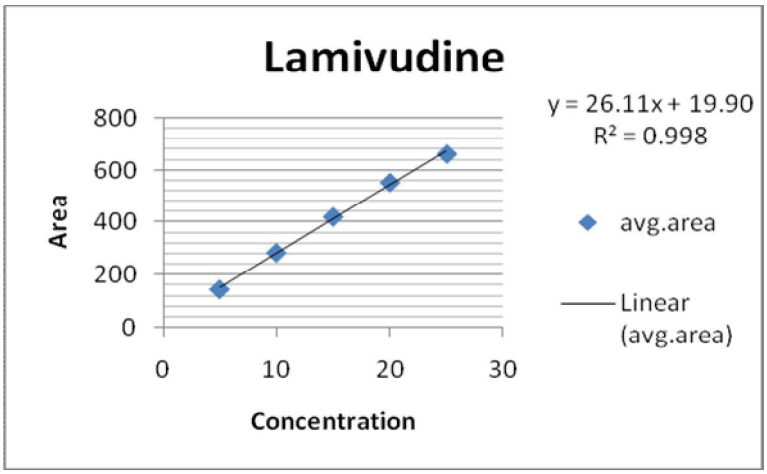

Figure 4: Calibration plot of Lamivudine

Table 4: Linearity of Tenofovir disoproxil fumarate

Standard conc.→	5 µg/ml	10 µg/ml	15 µg/ml	20 µg/ml	25 µg/ml
Replicates	**Peak area**				
1	140.25	309.47	478.61	625.34	772.04
2	146.26	303.03	475.63	616.33	761.28
Mean	145.26	306.25	477.12	620.84	766.66
±SD	1.42	4.55	2.11	6.37	7.61
%RSD	0.98	1.49	0.44	1.03	0.99

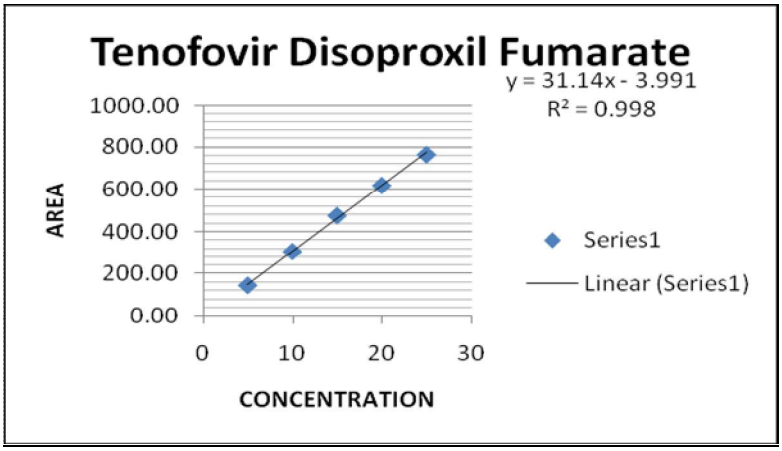

Figure 5: Calibration plot of Tenofovir Disoproxil Fumarate

Precision

Precision is the analytical method was studied by multiple sampling of the homogenous sample. The precision was done at two levels (intraday and inter day). Intraday precision was done by analyzing the intermediate concentration of both drug LAMI and TDF for three times. The results of intraday precision given Table.5and Table.6 for both drug LAMI and TDF respectively. Interday precision was measured over three consecutive days for the same drug concentrations for three times. The % RSD was calculated and was found to be within the limits (<2).RSD values indicate that the method is precise. The results of Interday precision given in Table7 and Table8 for LAMI and TDF respectively.

Table5: Intra-day variability of lamivudine

Conc. (µg/ml)	Peak area (µV. sec)		Mean area (µV.sec)	± SD	% RSD
	Area I	Area II			
10	280.51	283.72	282.12	2.27	0.80
15	410.45	413.46	411.96	2.13	0.52
20	550.89	548.69	549.79	1.56	0.28

Table6: Intra-day variability of Tenofovir Disoproxil Fumarate

Conc. (µg/ml)	Peak area (µV. sec)		Mean area (µV.sec)	± SD	% RSD
	Area I	Area II			
10	310.84	304.78	306.31	4.99	1.30
15	473.83	470.86	480.35	0.73	0.15
20	621.05	625.39	623.22	3.07	0.49

Table7: Inter-day variability of lamivudine

Conc. (µg/ml)	Peak area (µV. sec)		Mean area (µV.sec)	± SD	% RSD
	Area I	Area II			
10	284.51	288.72	286.62	2.98	1.04
15	412.45	417.46	414.96	3.54	0.85
20	558.89	551.69	555.29	5.09	0.92

Table 8: Inter-day variability of Tenofovir Disoproxil Fumarate

Conc. (µg/ml)	Peak area (µV. sec)		Mean area (µV.sec)	± SD	% RSD
	Area I	Area II			
10	309.84	302.78	306.31	4.99	1.31
15	479.83	480.86	480.35	0.73	0.15
20	621.05	625.39	623.22	3.07	0.49

Accuracy

Accuracy studies were carried out by applying the method to drug sampleto which known amount of standard Lamivudine and Tenofovir disoproxil fumarate corresponding level of recovery carried out at 80, 100 and 120% of the concentration as per standard addition method. The recoveries of lamivudine and tenofovir Disoproxil Fumarate were found in the range of 98.05-105.06 %and 98.14-105.41 %. The results are given in Table 9.

Table 9: Recovery Studies of Tablet Sample

Tablet sample	Level of recovery %	Amount of sample drug taken (µg/ml)		Amount of standard drug added (µg/ml)		Total amount recovered (µg/ml)		% Recovery	
	Drug	LAMI	TDF	LAMI	TDF	LAMI	TDF	LAMI	TDF
Tenvir-L	80	10	10	8	8	8.40	8.02	105.06	100.29
	80	10	10	8	8	8.22	8.15	102.84	101.90
	100	10	10	10	10	9.94	10.10	99.48	101.00
	100	10	10	10	10	10.6	10.54	100.60	105.41
	120	10	10	12	12	11.76	11.77	98.05	98.14
	120	10	10	12	12	12.01	12.07	101.11	100.58

Sensitivity

The sensitivity study oflamivudine and tenofovir disoproxil fumarate by use of the proposed method was estimated in the form of the Limit of quantification (LOQ) and Limit of Detection (LOD). Limit of detection

(LOD) and quantification (LOQ) were estimated from both linearity calibration curve method and signal to noise ratio method. The results are shown in Table.10 LOD values for LAMI and TDF were found to be 0.00106912µg/mL and 0.00071267 µg/mL respectively. Similarly, LOQ values for LAMI and TDF were calculated and were observed to be 0.00317007 µg/mL and 0.002152µg/mL respectively. This shows that the sensitivity of the method is adequate.

Table 10: Results of Sensitivity (LOD and LOQ)

Drug	LOD	LOQ
Lamivudine	0.00106912µg/mL	0.00317007 µg/mL
Tenofovir disoproxil fumarate	0.00071267 µg/mL	0.002152µg/mL

Robustness:

Robustness of the method was determined by carrying out the analysis under conditions during which mobile phase ratio and pH was altered. Variation of mobile phase pH and ratio were seemed to have greater impact on resolution and hence it should be meticulously controlled.

3.8Analysis of a marketed formulation

Experimental results of the amount oflamivudine and tenofovir disoproxil fumaratein the selected commercial tablets, expressed as a percentage of label claims were in good agreement with the label claims thereby suggesting that there is no interference from any of the excipients which are normally present. The drug content was found to be 103.65% and 99.40%forlamivudine and tenofovir disoproxil fumarate, different lots of lamivudine and tenofovir disoproxil fumarate tablets were analyzed using the proposed procedures as shown in Figure 6 and Table.11.

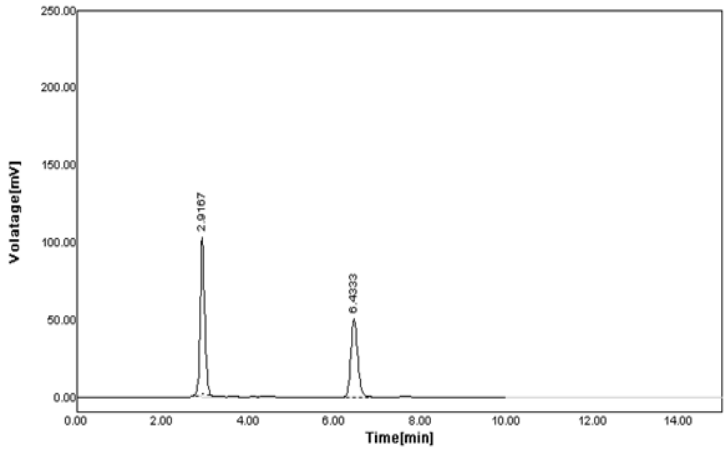

Figure6: Chromatogram of marketed formulation (TENVIR-L Tablet)

Table11: Analysis of a marketed formulation (TENVIR-L Tablet)

Sr. N	Amount present in(mg)		Amount found in(mg)		% Label claim	
	LAMI	TDF	LAMI	TDF	LAMI	TDF
1	20	20	20.64	19.81	103.20	99.05
2	20	20	20.82	19.95	104.10	99.75
Mean			20.73	24.85	103.65	99.40
SD			0.13	0.10	0.64	0.02
%RSD			0.61	0.40	0.61	0.02

Force degradation of lamivudine and tenofovir disoproxilfumarate

The stability of the sample was checked by forced degradation in different conditions and % of lamivudine and tenofovir disoproxil fumarate degradation was calculated. The values are given in Table12and Table 13 indicates that any other impurity is not merging with the main peak. The analyte solution was found to be stable. A method was developed for the determination of lamivudine and tenofovir disoproxil fumarate in tablets which is rapid, stable & specific. The results indicate that the described method can be used for quantitative analysis of the compounds.

Table 12: Force degradation data for Lamivudine

Tests	**Lamivudine**	
Conditions	**% Assay**	**% difference w.r. t reference**
Untreated sample	103.65	NA
Acid treated sample		
(0.1 N HCL After 1 Hrs)	102.59	1.06
(0.1 N HCL After 2 Hrs)	102.56	1.09
Base treated sample		
(0.1 N NaOH After 1 Hrs)	102.42	1.23
(0.1 N NaOH After 2 Hrs)	101.23	1.65
Peroxide-treated sample		
(3% H2O2 After 1 Hrs)	102.58	1.07
(3% H2O2 After 2 Hrs)	102.56	1.09

Table 13: Force degradation data for Tenofovir disoproxil fumarate

Tests	**Tenofovir disoproxil fumarate**	
Conditions	**% Assay**	**% difference w.r. t reference**
Untreated sample	99.75	NA
Acid treated sample		
(0.1 N HCL After 1 Hrs)	97.94	1.81
(0.1 N HCL After 2 Hrs)	97.90	1.84
Base treated sample		
(0.1 N NaOH After 1 Hrs)	98.56	1.19
(0.1 N NaOH After 2 Hrs)	98.89	0.86
Peroxide-treated sample		
(3% H2O2 After 1 Hrs)	98.59	1.16
(3% H2O2 After 2 Hrs)	99.23	0.52

CONCLUSION

A simple, precise, reliable, sensitive, and accurate stability -indicating RP-HPLC method has been developed for the estimation of lamivudine and tenofovir disoproxil fumarate in table dosage form. The developed method was good accuracy and precision. The method has a relatively short run time that allows quantifying a large number of samples in routine and quality control analysis of tablet in order to reduce cost of analysis reported method suitable for routine analysis.

CONFLICT OF INTEREST

The author declares no conflict of interest.

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