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Development and Validation of Absorption Ratio Method for Simultaneous Estimation of Tenofovir Disoproxil Fumarate and Lamivudine in Bulk and Combine Dosage Form

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ABSTRACT

A simple, robust, precise, UV spectroscopic method has been developed for the simultaneous estimation of Tenofovir disoproxil fumarate and Lamivudine in bulk and tablet dosage forms. In this paper, the estimation of those drugs was carried out by the absorbance ratio method. This method was based on measurement of isosbestic point absorption at 264 nm and 280 nm i.e. λmax of Lamivudine respectively. The linearity observed for Tenofovir disoproxil fumarate at an isosbestic point was in the range of 10-50 µg/ml and at λmax of Lamivudine was in the range of 10-50 µg/ml. The accuracy of the method was found to be within the range of 99.69%-99.97% for both Lamivudine and Tenofovir disoproxil fumarate respectively. The developed methods were validated concerning linearity, accuracy (recovery), and precision. The method can be employed for the estimation of pharmaceutical formulations with no interference from any excipients and diluents. The results were validated as per ICH guidelines.

INTRODUCTION

Tenofovir disoproxil fumarate is chemically known as (2E)-but-2-enedioic acid; bis ({[(propan-2-yloxy) carbonyl]oxy}methyl){[(2R)-1-(6-amino-9H-purin-9-yl)propan-2yl]oxy}methane phosphonate. This is used to treat chronic hepatitis B and to prevent and treat HIV/AIDS. Tenofovir disoproxil is a nucleotide analog reverse-transcriptase inhibitor (NtRTI). It selectively inhibits viral reverse transcriptase, a crucial enzyme in retroviruses such as human immunodeficiency virus (HIV), while showing limited inhibition of human enzymes, such as DNA polymerases α, β, and mitochondrial DNA polymerase γ. In vivo, Tenofovir disoproxil fumarate is converted to Tenofovir, an acyclic analog of deoxyadenosine 5'-monophosphate (d-AMP). Tenofovir lacks a hydroxyl group in the position corresponding to the 3' carbon of the d-AMP, preventing the formation of the 5′ to 3′ phosphodiester linkage essential for DNA chain elongation. Once incorporated into a growing DNA strand, Tenofovir causes premature termination of DNA transcription, preventing viral replication.[1,2]

The IUPAC nomenclature for Lamivudine is 4-amino-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one as an antiretroviral medication used to prevent and treat HIV/AIDS. Lamivudine is an analog of cytidine. It can inhibit both types (1 and 2) of HIV reverse transcriptase and also the reverse transcriptase of hepatitis B virus. It is phosphorylated to active metabolites that compete for incorporation into viral DNA. They inhibit the HIV reverse transcriptase enzyme competitively and act as a chain terminator of DNA synthesis. The lack of a 3'-OH group in the incorporated nucleoside analogue prevents the formation of the 5' to 3' phosphodiester linkage essential for DNA chain elongation, and therefore, the viral DNA growth is terminated. It is also used to treat chronic hepatitis B when other options are not possible.[3,4]

From the extensive literature review, few analytical methods were reported for individual analysis such as UV[5--8] HPLC and by other methods but there are very few analytical methods were reported in combination UV. [9-13] HPLC.[14-17]

The absorption ratio method is a modification of the simultaneous equation procedure. It depends on the property that, for a substance which obeys Beer's Law at all wavelengths, the ratio of absorbance at any two wavelengths is a constant value independent of concentration or path length.[18]

There are several challenges for the quality control of TDF and LAM in several formulations produced in India inspired the authors to develop simple methods for quantification of TDF and LAM in bulk and formulation by absorption ratio method. The developed method was validated according to the International conference of harmonization (ICH) guidelines.[19-20]

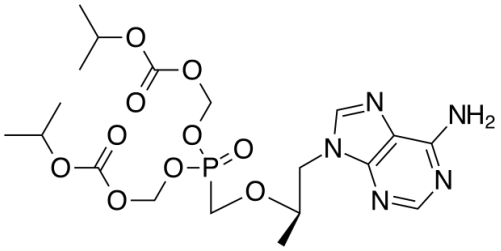

Figure No. 1. Structure of Tenofovir disoproxil fumarate

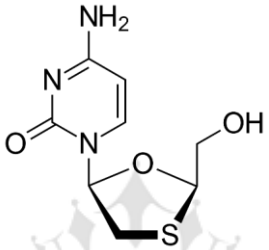

Figure No. 2. Structure of Lamivudine

MATERIALS AND METHODS

Instruments

Shimadzu UV-1800 double beam spectrophotometer was used to record the spectra of sample and reference solutions using a pair of quartz cells of 10mm path length. All weighing was carried out on Shimadzu AUX220 weighing balance. Sonicator of Ultrasonic is used for sonication, Filter papers of Sartorius Stedim Biotech of grade 292 are used for filtration purpose.

Chemicals

The bulk drug of tenofovir disoproxil fumarate was provided by Hetero Drugs Ltd as a gift sample and lamivudine was provided from Cipla India Pvt. Ltd. pharma as a gift sample. Fixed-dose combination tablets (Tenvir L) containing TDF 300 mg and LAM 300 mg were

procured from local Market All chemicals and reagents of analytical grade and HPLC grade were purchased from USV LTD, Mumbai, India.

Preparation of stock solution and selection of wavelength

A) Tenofovir disoproxil fumarate standard stock solution [T]:

An accurately weighed quantity of TDF (10 mg) was taken in a 10 mL volumetric flask and dissolved in water (9 mL) with the help of ultrasonication for about 10 min. Then the volume was made up to the mark using water to get a standard stock solution (1 mg / mL).

B) Tenofovir disoproxil fumarate working standard solution [T_1]:

TDF standard stock solution [T] (1 mL) was diluted to 10 mL using 40% aqueous methanol adjusted to pH 4 using a phosphoric acid buffer to get a working standard solution (100 µg / mL).

C) Lamivudine standard stock solution [L]:

An accurately weighed quantity of LAM (10 mg) was taken in a 10 mL volumetric flask and dissolved in water (9 mL) with the help of ultrasonication for about 10 min. Then the volume was made up to the mark using water to get a standard stock solution (1 mg / mL).

D) Lamivudine working standard solution [L_1]:

LAM standard stock solution L_1 (1 mL) was diluted to 10 mL using 40% aqueous methanol adjusted to pH 4 using a phosphoric acid buffer to get a working standard solution (100 µg / mL).

Procedure for Phosphoric Acid Buffer at pH 4.0:

Dissolve sodium dihydrogen phosphate dihydrate (M.W. =156.01) 6.24 gm and phosphoric acid (85%) was added to make up the volume of one-liter solution to attain pH 4.

Determination of λ Max of Individual Component

An appropriate aliquot portion working standard of TDF and LAM were transferred to two separate 10 mL volumetric flasks, the volume was made up to the mark using 40 % v/v aqueous methanol adjusted to pH 4 using phosphoric acid buffer to obtain TDF (30 µg/mL)

and LAM (30 µg/mL). Drug solutions were scanned separately between 200 nm to 400 nm. TDF shows the λ_{max} at 260 nm and LAM shows λ_{max} at 280nm while the isosbestic point was found at 264 nm as shown in fig No. 3.

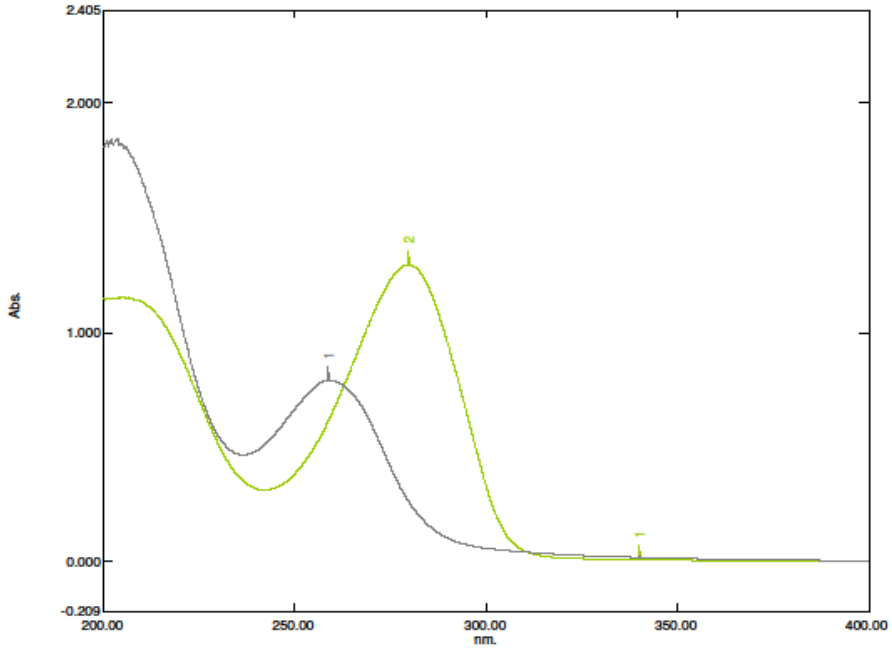

Figure no. 3 Overlay spectra of Tenofovir disoproxil fumarate and Lamivudine

Linearity study for Tenofovir disoproxil fumarate

An accurately measured aliquot portion of the working standard solution of TDF was transferred to five separate 10 mL volumetric flasks. The volume was made up to the mark using 40 % v/v aqueous methanol adjusted the pH 4 by using a phosphoric acid buffer to obtain concentrations (10µg/ml, 20µg/ml, 30µg/ml, 40µg/ml, 50µg/ml). The absorbance of these solutions was plotted as absorbance vs concentration, absorbance measured at 260 nm, the results are shown in Table No. 1.

Linearity study for Lamivudine

Accurately measured aliquot portions of the working standard solution of LAM were transferred to five separate 10 mL volumetric flasks. The volume was made up to the mark using 40 % v/v aqueous methanol solution adjusted the pH 4 by using a phosphoric acid buffer to obtain concentrations (10 µg/ml, 20µg/ml, 30µg/ml, 40µg/ml and 50µg/ml). The

calibration curve was plotted, absorbance vs concentration, absorbance measured at 280 nm, the results shown in Table No. 1.

Table No. 1 Regression and Optical characteristics of TDF and LAM

Parameters	Value for Tenofovir disoproxil fumarate	Value for Lamivudine
Beer's law limit (µg/ml)	10-50 µg/ml	10-50 µg/ml
Regression Coefficient(R^2)	0.9983	0.9981
Slope	0.0099	0.042
Intercept	0.0063	0.0194

The study of regression and optical characteristics of TDF and LAM are carried out in which Regression Coefficient (R^2) of TDF is 0.9983 and of LAM is 0.9981. Concentration vs Absorbance is fairly linear between both co-ordinates by the statistical manner and obey ICH guidelines.[19,20]

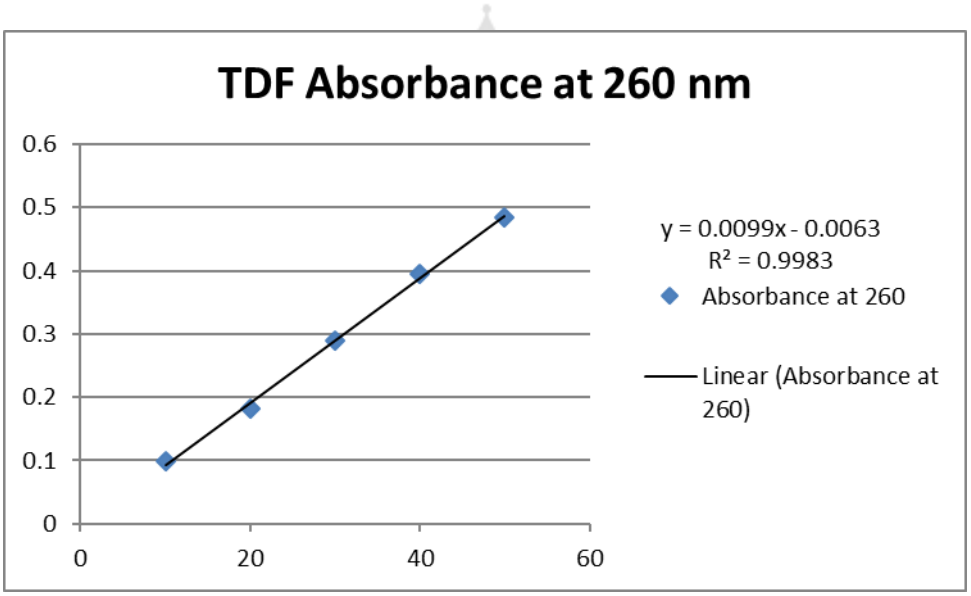

Figure no. 4 Calibration curve of Tenofovir disoproxil fumarate at 260nm

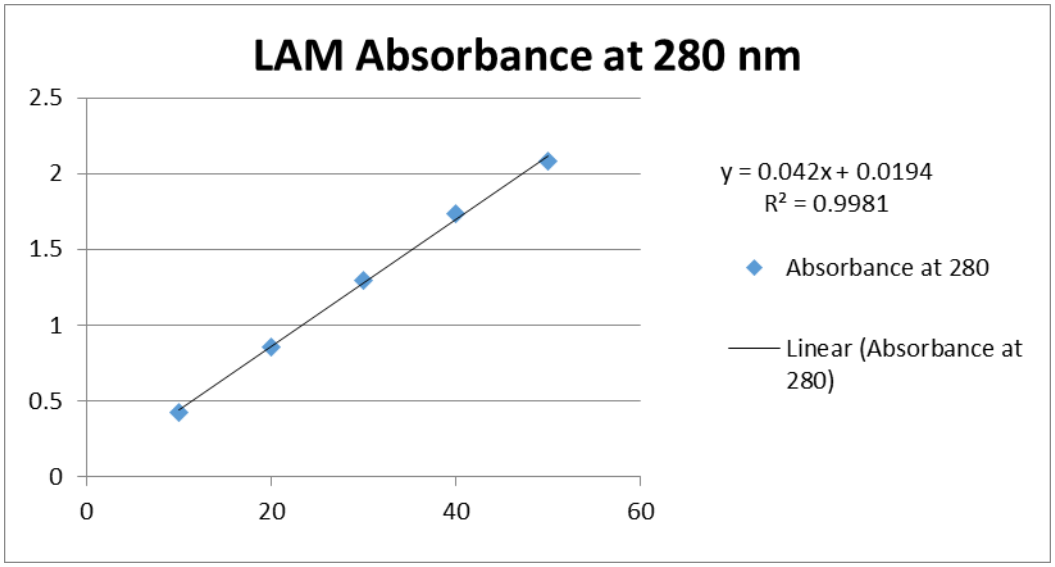

Figure no. 5 Calibration curve of Lamivudine at 280nm

Absorbance Ratio Method

The absorbance ratio method[19] is a modification of the simultaneous equations procedure. It depends on the property that, for a substance that obeys Beer's Law at all wavelengths, the ratio of absorbance at any two wavelengths is a constant value independent of concentration or path length.

Absorbance ratio method uses the ratio of absorbance at two selected wavelengths, one at is an absorptive point and other being the λmax of one of the two drug Tenofovir disoproxil fumarate and Lamivudine have λmax at 260 and 280 nm respectively and isosbestic point 264nm. The wavelengths selected for analysis were 260 and 280 nm, respectively. (1%,1cm) values of Tenofovir disoproxil fumarate and Lamivudine were determined at 260 and 280 nm.

The concentration of two drugs in the mixture was calculated by using the following equations,

$$C_x = \frac{Qm - Qy}{Qx - Qy} \times \frac{A}{ax1} \qquad \text{----------(1)}$$

$$C_y = \frac{Qm - Qx}{Qy - Qx} \times \frac{A}{ay1} \qquad \text{----------(2)}$$

Where,

$$Qm = \frac{\text{The absorbance of the sample at 280 nm}}{\text{The absorbance of the sample at 260nm}}$$

$$Qx = \frac{\text{(1 \% 1cm) of TDF at 260nm}}{\text{(1 \% 1cm) of TDF at 260nm}}$$

$$Qy = \frac{\text{(1 \% 1cm) of LAM at 280 nm}}{\text{(1 \% 1cm) of LAM at 280nm}}$$

Estimation of Laboratory Mixture

Exact quantity pure drug TDF (300 mg) and LAM (300 mg) was weighed and transferred into 100 mL volumetric flask containing water (100 mL), sonicated for 15 min and the volume was made up to the mark and filtered through Whatman filter paper (No. 45). This solution was (1 mL) transferred to 100 mL volumetric flask, dissolved and volume was adjusted to the mark with 40% aqueous methanol adjusted the pH 4 by using a phosphoric acid buffer. The absorbance of the solutions was measured at 260 nm and 280 nm against a blank. The concentrations of the two drugs in the sample were determined**.** The results are reported in Table No.2.

Table No.2 Results of estimation of TDF and LAM in standard laboratory mixture

Analyte	**% Concentration estimated (Mean ± S.D)**	**% R.S.D**
Tenofovir disoproxil fumarate	99.94±0.1277	0.1278
Lamivudine	99.92±0.1673	0.1674

The estimation of TDF and LAM in Standard Laboratory Mixture are carried out in which the percentage concentration of TDF and LAM were found to be 99.94% and 99.92% respectively. Those values are fairly accurate in a statistical manner and are as per ICH guidelines.

Application of the proposed method for estimation of drugs in tablet

Twenty (Tenvir L) tablets containing TDF (300 mg) and LAM (300 mg) weighed and ground to a fine powder. A quantity of powder equivalent to TDF (300 mg) and LAM (300 mg) was transferred into 100 mL volumetric flask containing water (100 mL), sonicated for 15 min and the volume was made up to the mark and filtered through Whatman filter paper (No. 45). This solution was (1 mL) transferred to 100 mL volumetric flask, dissolved and volume was adjusted to the mark with 40 % aqueous methanol adjusted the pH 4 by using a phosphoric acid buffer. The absorbance of the solution was measured at 260 nm and 280 nm against a blank. The concentrations of the two drugs in the sample were determined**.** The results are reported in Table No.3.

Table No. 3. Results of estimation of TDF and LAM in the tablet dosage form.

Analyte	**Label claim (mg/Tab)**	**% Label claim estimated (Mean ± S.D)**	**% R.S.D**
Tenofovir disoproxil fumarate	300	99.97 ± 0.1816	0.1820
Lamivudine	300	99.69 ± 0.1299	0.1303

n=5

The results of the Estimation of TDF and LAM in tablet dosage shows form the % purity 99.97 and 99.69 with SD and RSD bellow 2 which is fairly accurate in a statistical manner and are as per ICH guidelines.

Validation of the proposed method

The proposed method was validated as per ICH guidelines[19,20].

Accuracy (Recovery study)

Accuracy of the proposed method was ascertained based on a recovery study performed by the standard addition method. A known amount of standard drug solution was added to the tablet powder to make final concentrations in the range of 80%, 100%, and 120% and re-analyzed it by the proposed method. The absorbance recorded and the % recoveries were calculated using the following formula.

% Recovery = [A - B/ C] X 100

Where,

A = Total amount of drug estimated

B = Amount of drug found on the pre-analyzed basis

C = Amount of Pure drug added.

The results are reported in Table No. 4

Table No. 4. Recovery study

The drug in mixture solution (µg/ml)		% Recovery ± S.D.	
Tenofovir disoproxil fumarate	**Lamivudine**	**Tenofovir disoproxil fumarate**	**Lamivudine**
20	20	99.80 ± 0.1278	99.82±0.1277
30	30	99.63 ± 0.1673	99.77±0.1673
40	40	99.78 ± 0.1673	99.94±0.1677

The results of the recovery study of TDF and LAM are found to be fairly accurate between 99.63 to 99.80 % for TDF and 99.77 to 99.94 % for LAM between various concentrations under observation by the statistical way and obeys ICH guidelines.

Precision

Precision was determined as intra-day and inter-day variations. Intra-day precision was determined by analyzing Tenofovir disoproxil fumarate (20, 30 and 40 µg/mL) and Lamivudine (20, 30 and 40 µg/mL) for three times on the same day. Inter-day precision was determined by analyzing the same concentration of solutions for three different days over a week. The results are shown in Table No. 5.

Table No. 5. Precision study

Drug	Conc. [µg/mL]	Intra-day Amount Found		Inter-day Amount Found	
		Mean ±S.D [*n* = 5]	% R.S.D.	Mean ± S.D. [*n* =5]	% R.S.D.
TDF	20	19.83±0.0858	0.4327	19.81± 0. 0944	0.4768
	30	29.81±0.2062	0.6917	29.67± 0.3113	1.0494
	40	39.73±0.2141	0.5390	39.70± 0.1997	0.5029
LAM	20	19.85± 0.0630	0.3172	19.84± 0.1759	0.8867
	30	29.72± 0.3764	1.2665	29.57± 0.4131	1.3969
	40	39.76± 0.0535	0.1347	39.69± 0.3341	0.8417

The Precision Study of TDF and LAM were carried out and results are found to be fairly accurate in a statistical manner as per ICH guidelines.

Ruggedness

The ruggedness of the proposed method was determined by the analysis of aliquots from the homogenous slot by two different analysts using the same operational and environmental conditions. The results are reported in Table No. 6.

Table No. 6. Ruggedness study

	Tenofovir disoproxil fumarate 300 mg		Lamivudine 300 mg	
	Amount found in mg Mean ± S.D. (n=3)	% R.S.D	Amount found in mg Mean ± S.D. (n=3)	% R.S.D
Analyst I	299.95± 0.2059	0.0686	299.84± 0.0595	0.0198
Analyst II	299.73± 0.4543	0.1515	300.00± 0.8197	0.2732
Day I	299.95 ± 0.9089	0.3030	300.02± 0.2426	0.0808
Day II	299.81 ± 0.5412	0.1805	299.99± 0.1159	0.0386
Instrument I	299.86 ± 0.1216	0.0405	299.99± 0.2064	0.0688
Instrument II	299.77 ± 0.1357	0.0452	299.98± 0.1761	0.0587

n= 3

The Ruggedness study of TDF and LAM are carried out and results are found to be fairly accurate by the statistical manner and obey ICH guidelines.

The lower limit of detection and Lower limit of quantitation

The limit of detection for Tenofovir disoproxil fumarate and Lamivudine was found to be 0.1087 µg /ml and 0.0219 µg /ml respectively and the limit of quantitation of was found to be Tenofovir disoproxil fumarate and Lamivudine 0.3294 µg /ml and 0.0732 µg /ml respectively for a given method.

RESULTS AND DISCUSSION

An Absorbance Ratio Method in UV Spectroscopy was developed for Tenofovir disoproxil fumarate and Lamivudine, the method employs 260 nm as λ1 and 280 nm as λ2 for the formation of equations. Tenofovir disoproxil fumarate and Lamivudine obeys Beer's law in the concentration range 10-50 µg/ml (R^2=0.9983) and 10-50 µg/ml (R^2=0.9988) respectively at given wavelengths. The assay estimation in given formulation for Tenofovir disoproxil fumarate and Lamivudine was found to be 99.97 % and 99.69 % respectively. The developed method was validated according to ICH guidelines and values of accuracy, precision and other statistical analyses were found to be in good accordance with the prescribed values.

CONCLUSION

The proposed absorbance ratio method in UV Spectroscopy in this paper has advantages of simplicity, accuracy, precision, and convenience for quantitation of Tenofovir disoproxil fumarate and Lamivudine but authors suggest prior research before the implementation of the method.

ACKNOWLEDGMENTS

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Conflict of interest

Hereby authors declare that there is no conflict of interest for this publication.

ABBREVIATIONS

Selected drug

TDF	Tenofovir Disoproxil Fumarate
LAM	Lamivudine

Symbols

λ max	Wavelength of maximum absorbance
R^2	Correlation coefficient
mL	Milliliter
µg	Microgram
mg	Milligram
g	Gram
nm	Nanometer
%	percentage
>	Greater than
<	Less than
Sec	Second
Min	Minute
Fig	Figure
Temp	Temperature
pH	Concentration of hydrogen ion

Other

HPLC	High-Performance Liquid Chromatography
UV	Ultra Violet
IP	Indian Pharmacopoeia
ICH	International Conference on Harmonization
RSD	Relative Standard Deviation
SD	Standard Deviation
LOD	Limit of Detection
LOQ	Limit of Quantitation

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