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METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF LAMIVUDINE, TENOFOVIR AND EFAVIRENZ IN COMBINED TABLET DOSAGE FORM BY RP-HPLC AND UV-SPECTROSCOPIC METHOD

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Keywords:

RP-HPLC and UV spectroscopy, Lamivudine, Tenofovir, Efavirenz.

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ABSTRACT: A rapid and sensitive RP-HPLC method with UV detection and UV spectrophotometric method for the determination of Lamivudine, Tenofovir and Efavirenz simultaneously in combined tablet dosage form was developed. Chromatography was performed with mobile phase containing a mixture of methanol: Water (pH adjusted to 2.5) and 0.1 % TEA in the proportion of (68: 32 % v/v) the samples were injected onto Symmetry C18 Column (4.6 x 100mm, 5µm, Make: HYPERSIL ODS) column. The flow rate was 1.2ml.min–1. The samples were detected at 260nm. The UV spectrophotometric method was performed at 272nm for Lamivudine, 260 nm for Tenofovir and 247 nm for Efavirenz, and samples were prepared with a solution of Water methanol (30:70 % v/v). The assay was linear in range from 25% to 150% targeted concentration and regression coefficient for all three drugs was found to be 0.999 highly significant for the method. The proposed methods were simple, rapid, precise, accurate and sensitive, and can be used for the routine of the quality control in pharmaceuticals.

INTRODUCTION: Lamivudine is reverse transcriptase reported to be active against HIV-1, HIV- 2 and hepatitis B virus. Lamivudine, chemically 4 - amino - 1 - [(2R, 5S) – 2 - (hydroxyl methyl) - 1, 3 – oxathiolan – 5 - yl] - 1, 2-dihydropyrimidin-2-one. It 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 an HBV polymerase, resulting in DNA chain termination.

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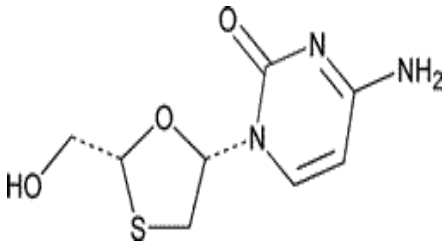

FIG 1: CHEMICAL STRUCTURE OF LAMIVUDINE

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. TDF is the first nucleotide analog approved for HIV-1 treatment [3, 4].

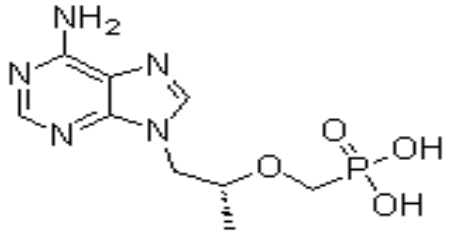

FIG 2: CHEMICAL STRUCTURE OF TENOFOVIR

Efavirenz is a human immunodeficiency virus type-I (HIV-I) specific non nucleoside reverse transcriptase inhibitor (NNRTI). Efavirenz is chemically described as (S) - 6 – chloro – 4 - (cyclopropylethynyl) - 1, 4 – dihydro – 4 - (trifluoromethyl) - 2H - 3, 1 – benzoxazin – 2 - one[5]

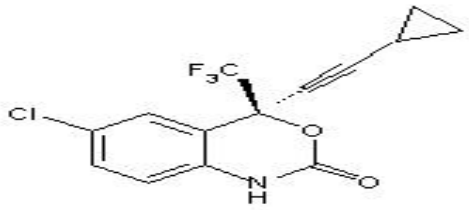

FIG 3: CHEMICAL STRUCTURE OF EFAVIRENZ

A literature survey reveals that analytical methods based on HPLC [18 -30], HPTLC [13-17], UV Spectrometry [7-12] are available for the determination of these drugs individually and in combination with other drugs in different dosage forms, there is one analytical method reported with Acetonitrile and phosphate buffer pH 4 for the simultaneous estimation of Lamivudine, Tenofovir and Efavirenz in a Combined Dosage Form. The aim of the present work is develop a simple, precise, accurate, and rapid method with less run time for the determination of Lamivudine, Tenofovir and Efavirenz in a Combined Dosage Form without lack of interference.

MATERIALS AND METHODS:

Instruments:

High Performance Liquid Chromatographic system (waters) equipped with auto sampler, UV detector. Glass Van Hypodermic injecting syringe and Symmetry C18 Column (4.6 x 100mm, 5µm, Make: HYPERSIL ODS) and UV- Visible Spectrophotometer (Lab India)

Reagents:

Working Standards of Lamivudine, Tenofovir and Efavirenz; Methanol of HPLC Grade (Merck); and Orthophosphoric acids are of reagent grade (Merck); Millipore water

Preparation of Mobile Phase:

Add 0.1 ml of Triethylamine to HPLC water in 1000 ml beaker, diluted to 1000 ml with HPLC water. pH of the solutions was adjusted to 2.5 with Orthophosphoric acid. 320 mL buffer (32%) and 680 mL of methanol HPLC (68%) were mixed, degassed in ultrasonic water bath for 5 minutes and filtered through 0.45 µ filter under vacuum filtrate ion.

Preparation of Standard solution:

For HPLC method: 10 mg of Lamivudine and 10 mg of Tenofovir and 20mg of Efavirenz working standards were accurately weighed and transferred into a 10mL clean dry volumetric flask about 7mL of diluents (mobile phase) was added and sonicated to dissolve it completely and volume was made up to the mark with the diluents, the solution was filtered through 0.45µ filter under vacuum filtration (Stock solution). Further 0.3ml of this solution was pipetted into a 10ml volumetric flask and diluted up to the mark with diluents (Standard Solution). The solution was filtered through 0.45µ filter under vacuum filtration.

For the UV Spectrophotometric method: 10mg of Lamivudine and 10mg of Tenofovir and 20mg of Efavirenz working standards were accurately weighted, transferred to a 100ml volumetric flask and dissolved in a methanol: water solution. 0.6 ml of this solution was diluted to 10.0 ml with a methanol: water. Concentrations of 6µg.ml Lamivudine, 6µg.ml Tenofovir and 12µg.ml Efavirenz were prepared.

Preparation of Sample Solution:

For HPLC Method: Accurately weighed 1754.5 mg of Lamivudine and Tenofovir and Efavirenz tablet powder transferred into a 100 mL clean dry volumetric flask, and sonicated to dissolve and volume made up to the mark with the diluents,. Pipette 0.1 ml of this solution was pipetted out into a 10 ml volumetric flask and diluted up to the mark with diluents (sample solution).

For UV-Spectrophotometric Method:

The powder equivalent to 10mg of Lamivudine, Tenofovir disoproxil fumarate and 20 mg of

Efavirenz was weighed accurately and transferred into a 100 ml standard volumetric flask. An aliquot of 0.6 ml of test solution was diluted to produce the concentration 6 mcg / ml of Lamivudine, Tenofovir disoproxil fumarate and 12 mcg/ml of Efavirenz.

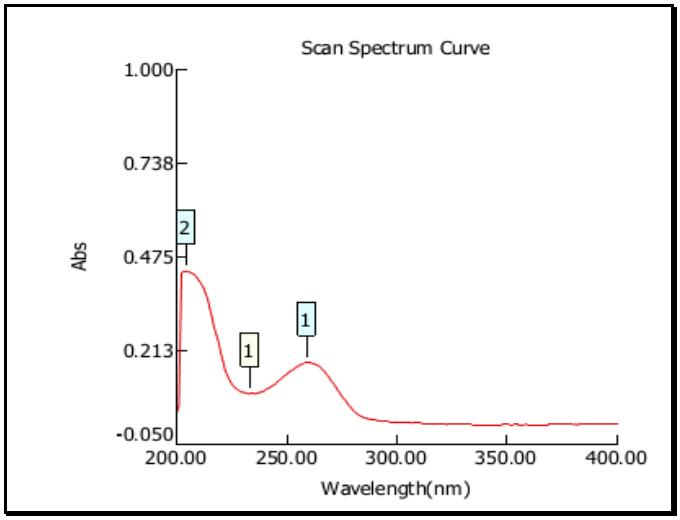

FIG 4: SPECTRUM OF LAMIVUDINE

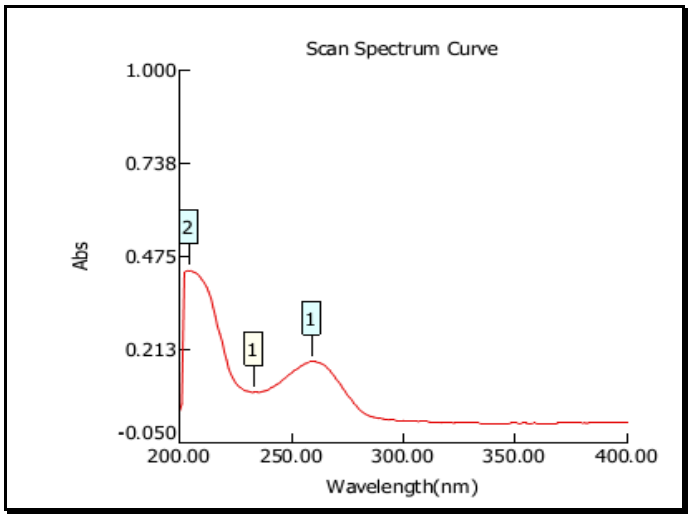

FIG 5: SPECTRUM OF TANOFOVIR

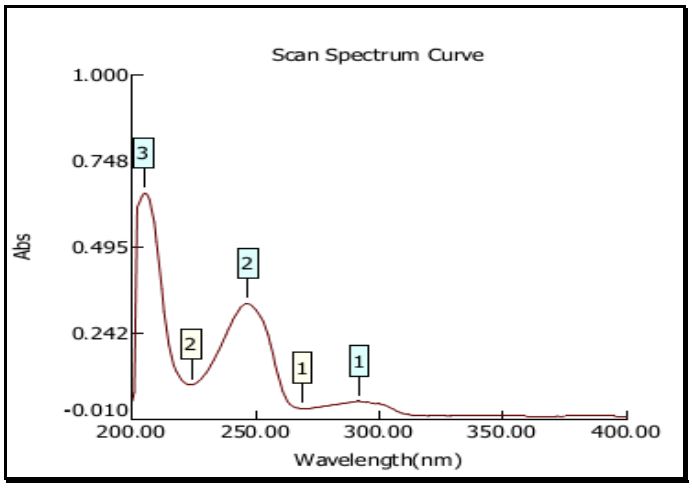

FIG 6: SPECTRUM OF EFFAVARENZ

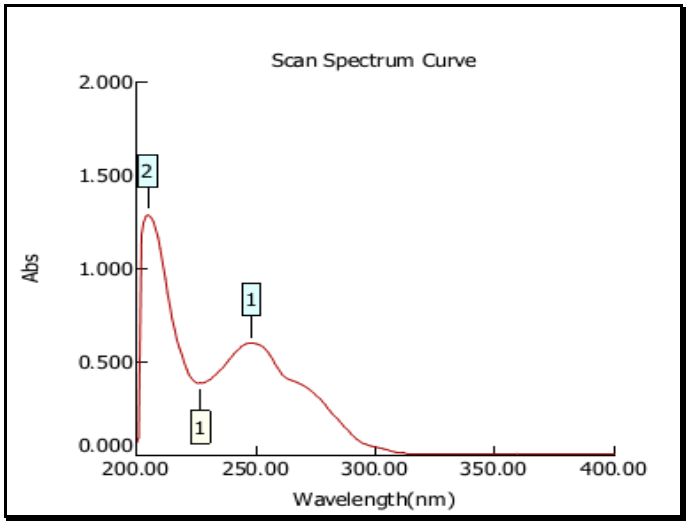

FIG 7: SPECTRUM OF ASSAY FORMULATION

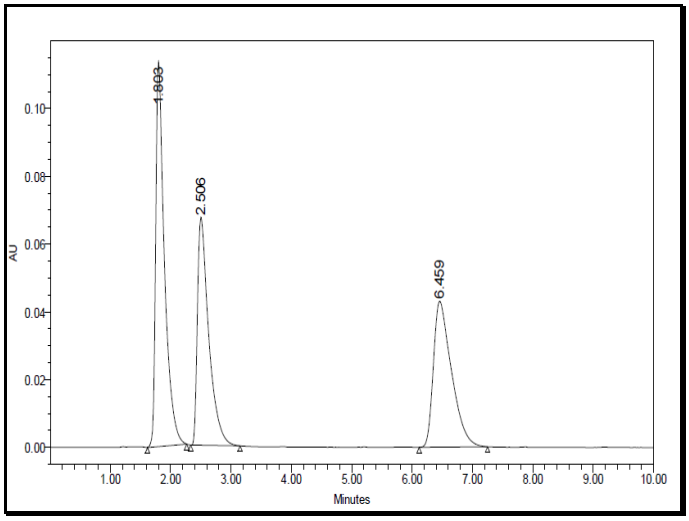

FIG 8: FORMULATION CHROMATOGRAM

RESULTS AND DISCUSSION:

Method Validation:

Specificity

To check the specificity placebo, standard and sample solutions were injected, verified that the there no interference of tablets excipients.

Linearity:

The calibration curve was obtained with five concentrations of the standard solution for 10-50 mcg / ml of Lamivudine, Tenofovir disoproxil fumarate and 20-100 mcg/ml of Efavirenz for HPLC method and 2-10 mcg / ml of Lamivudine, Tenofovir disoproxil fumarate and 4-20 mcg/ml of Efavirenz for UV spectrophotometric method. The linearity was evaluated by linear regression analysis, which was calculated by the least square regression method

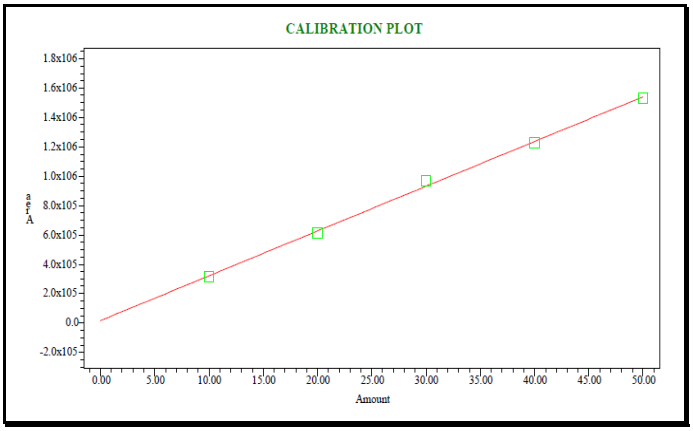

FIG 9: LAMIVUDINE CALIBRATION CURVE

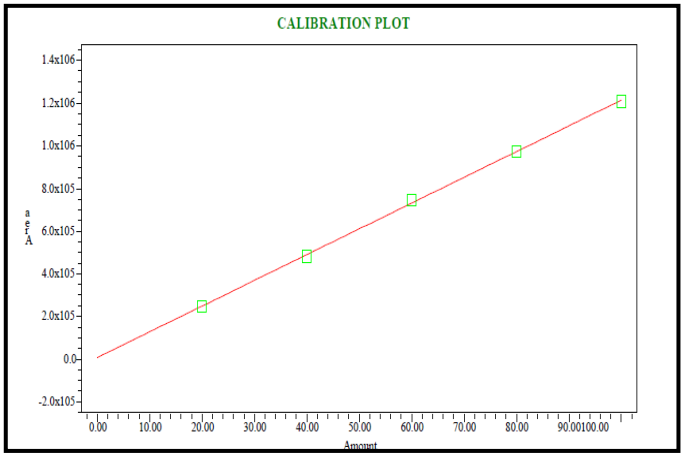

FIG 10: TENOFOVIR CALIBRATION CURVE

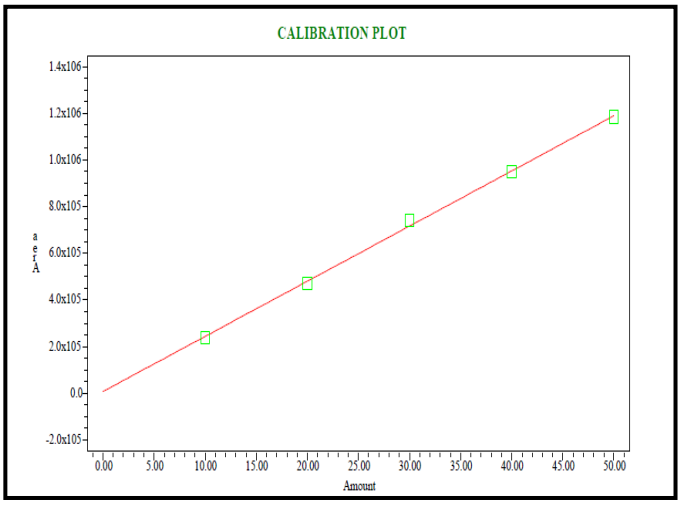

FIG 11: EFAVIRENZ CALIBRATION CURVE

Precision

The assay precision was carried out by repeatability (within-day) and intermediate precision (inter-day). five sample solutions (10 mcg / ml of Lamivudine, Tenofovir disoproxil fumarate and 20mcg/ml of Efavirenz for HPLC method and 6 mcg / ml of Lamivudine, Tenofovir disoproxil fumarate and 12mcg/ml of Efavirenz for UV-Spectrophotometric method. for UV spectrophotometric method) were prepared and assayed in triplicate.

TABLE I: PRECISION

Drug	Lamivudine		Tenofovir		Efavirenz	
	UV method	HPLC method	UV method	HPLC method	UV method	HPLC method
Precision Analyst – 1 % RSD	0.27	0.91	0.24	0.92	0.16	0.43
Intermediate precision Analyst – 2 % RSD	0.45	0.49	0.24	0.44	0.40	0.15

Accuracy: Sample solutions were prepared at three different concentrations 50%, 100% and 150% and known amount of sample was added to this solutions and recovery of added sample was studied.

TABLE 2: ACCURACY RECOVERY STUDIES

Concentration added	Lamivudine mean % recovery		Tenofovir mean % recovery		Efavirenz mean % recovery	
	UV method	HPLC method	UV method	HPLC method	UV method	HPLC method
50%	98.37	101.7%	100.38	99.8%	99.89	99.5%
100%	100.27	99.9%	99.09	99.7%	99.84	99.2%
150%	101.44	101.2%	101.03	100.6%	98.61	99.7%

Robustness: To evaluate the robustness of the developed method, small deliberate variations in optimized method parameters were done such as small changes in the percentage of methanol (10-15%) in the mobile phase, flow rate (1.08-1.32ml.min–1), effect of these changes on retention

time, peak symmetry, resolution and theoretical plates were evaluated.

Limit of Detection and Quantification

LOD was determined using the signal-to noise ratio, and then comparing the test results from the samples with known concentrations. The analyte concentration that produced a signal- to-noise ratio of 3:1 was accepted as LOD. Limit of quantification (LOQ) is defined as the lowest concentration of the analyte that can be determined with acceptable precision and accuracy under the stated experimental conditions.

To develop a simple, precise, accurate, and rapid Reverse Phase High Performance Liquid Chromatographic method and UV-Spectrophotometric for simultaneous estimation of Lamivudine, Tenofovir and Efavirenz, different chromatographic conditions were tried. The symmetry C18 column, mobile phases containing mixture of Water (pH adjusted to 2.5), methanol (32:68% v/v) and 0.1 % TEA and the flow rate of 1.2 mL/min found to resolve all three components with good peak symmetry and theoretical plates.

The retention times for Lamivudine, Tenofovir and Efavirenz were found to be 1.801 min, 2.506 min and 6.549 min respectively. The specificity of the method was assessed by comparing the retention time of standard Lamivudine, Tenofovir, Efavirenz and sample, good correlation was obtained between the retention time of standard and sample. Placebo and blank were injected and there were no peaks.

There are no interferences hence method is specific. The linearity range for Lamivudine, Tenofovir and Efavirenz were found to be as 10-50 ppm, 10-50 ppm and 20-100 ppm respectively. The regression equation for Lamivudine, Tenofovir and Efavirenz were found to be as y = 30493X+17236, y = 23639X+7866 and y = 24132X+7854 respectively and correlation coefficient (R2) for all three drugs found to be 0.999. Percentage relative standard deviation (%RSD) was found to be less than 2% for sample analysis that proves method is precise.

The recovery studies shown recovery of the sample is between 99-102% that proves methods accuracy. The analysis of sample by second analyst did not shown any effect on its performance. The small deliberate changes in mobile phase composition, pH of the buffer and flow rate did not show any impact on retention time, peak symmetry, resolution and theoretical plate count. The limit of detection for Lamivudine, Tenofovir and Efavirenz found to be 0.04µg/ml, 0.08µg/ml, and 0.25µg/ml. The limit of quantification for Lamivudine, Tenofovir and Efavirenz found to be 0.16µg/ml, 0.27µg/ml, and 0.84µg/ml.

The proposed spectrophotometric method allowed a rapid and accessible quantitation of Lamivudine, Tenofovir and Efavirenz in tablets without any time-consuming sample preparation. The absorption spectra was wavelength of 272 nm for Lamivudine, 260 nm for Tenofovir and 247 nm for Efavirenz used. The calibration curves were constructed in the range of and 2-10 mcg / ml of Lamivudine, Tenofovir disoproxil fumarate and 4-20mcg/ml of Efavirenz The representative equation analysis for Lamivudine, Tenofovir and Efavirenz were found to be as y = 0.054X+0.0086, y = 0.029X+0.0015 and y = 0.024X+0.0423 respectively and correlation coefficient (R2) for all three drugs found to be 0.999.

The limit of detection for Lamivudine, Tenofovir and Efavirenz found to be 0.97µg/ml, 0.1.65 µg/ml, and 2.5µg/ml. The limit of quantification for Lamivudine, Tenofovir and Efavirenz found to be 2.9µg/ml, 5.0µg/ml, and 7.6µg/ml. respectively, a good accuracy of the method was verified with a mean recovery of 99-101% within day and inter-day. Finally, the method showed to be specific for the determination of Lamivudine, Tenofovir and Efavirenz in tablets

CONCLUSIONS: The proposed method's are simple, specific, accurate and precise and hence can be used in routine for estimation of Lamivudine, Tenofovir and Efavirenz in tablet dosage. Statistical analysis of the results has been carried out revealing high accuracy and good precision. The percentage RSD for all parameters was found to be less than two, which indicates the validity of the method's and assay results obtained by this method are in fair agreement.

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