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A NOVEL STABILITY INDICATING RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE DETERMINATION OF TENOFOVIR DISOPROXIL FUMARATE AND EMTRICITABINE IN BULK AND PHARMACEUTICAL FORMULATIONS

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Keywords:

Tenofovir disoproxil fumarate, Emtricitabine, RP-HPLC, Method validation and Stability

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ABSTRACT: The prime aim and object of present investigation is to develop and validate a novel, precise, accurate, specific, rapid and economic stability- indicating isocratic reverse phase liquid chromatography method for the quantitative simultaneous estimation of Tenofovir disoproxil fumarate and Emtricitabine in bulk and marketed formulations. Estimation of drugs in this combination was achieved with a C18 column [Agilent TC-C18 (2) column. 5µm, 4.6×250 mm]kept at ambient temperature, using mobile phase of composition Methanol and phosphate buffer (30:70 v/v, pH 4).The flow rate was 1.0 ml/min and the effluents were monitored at 261 nm, using variable wavelength UV detector. The retention time of Tenofovir disoproxil fumarate and Emtricitabine were 2.81min and 4.72min respectively. Validation of the method was done according to the ICH guidelines for different analytical parameters. The method was found to be linear over a range of 40-80µg/ml for Tenofovir disoproxil fumarate and Emtricitabine. The established method proved as reproducible one with a %RSD value of less than 2 and having the robustness and accuracy within the specified limits. Assay of marketed formulation was determined and find with 97.58% and 97.88% for Tenofovir disoproxil fumarate and Emtricitabine respectively. The stressed samples were analyzed and this proposed method was found to be specific and stability indicating as no interfering peaks of degradation compounds and excipients were noticed. Successfully employed in the estimation of commercial formulations. This liquid chromatographic method can be applied for the qualitative and quantitative determination of selected drugs by the modern chemist.

INTRODUCTION: Tenofovir disoproxil fumarate is chemically designated as (9-[(R)-2-[[bis[[(iso propoxy carbonyl)oxy] methoxy] phosphinyl] methoxy] propyl] adenine fumarate 1:1 Tenofovir disoproxil fumarate) is an orally bioavailable ester prodrug of Tenofovir (also known as PMPA), an acyclic nucleotide analog with activity *in vitro* against retroviruses, including HIV-1, HIV-2, and hepatitis B virus (HBV).

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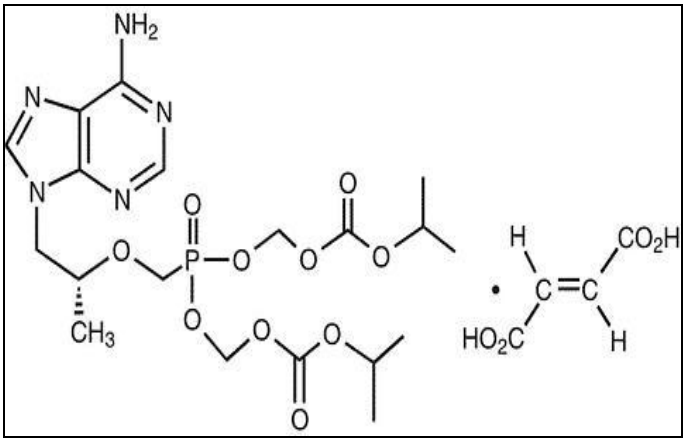
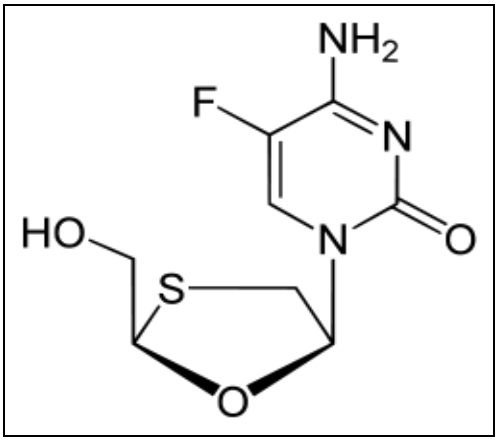
Due to the presence of a phosphonate group, tenofovir is negatively charged at neutral pH, which limits its oral bioavailability.

It is official drug in Indian Pharmacopoeia 2007 and 2014. Emtricitabine is chemically designated as 5-fluoro – 1 - [(2R,5S) - 2 - (hydroxymethyl) - 1,3-oxathiolan-5-yl] cytosine.

Emtricitabine is a nucleoside analogue and reverse transcriptase inhibitor used in combination with other agents for treatment and prevention of human immunodeficiency virus (HIV) infection and the acquired immunodeficiency syndrome (AIDS). It is official drug in Indian Pharmacopoeia 2007 and 2014 [1-3].



a. TENOFOVIR DISOPROXIL FUMARATE



b. EMTRICITABINE

FIG. 1: CHEMICAL STRUCTURES OF a) TENOFOVIR DISOPROXIL FUMARATE b) EMTRICITABINE

Extreme literature survey proved that very few methods were reported for the determination of Tenofovir disoproxil fumarate and Emtricitabine by RP-HPLC [4-24]. So in the present research we aimed to develop a novel, precise, accurate, sensitive, stable and economically viable liquid chromatographic method for the simultaneous determination of selected drugs.

MATERIALS AND METHODS:

Equipment used: The chromatographic separation was performed on Agilent 1120 compact isocratic liquid chromatographic system integrated with a variable wavelength programmable UV detector and a Rheodyne injector equipped with 20 µl fixed loop. A reverse phase C18[Agilent ODS UG 5 column, 250 mm × 4.5 mm]was used. Elico SL-218 double beam UV visible spectrophotometer was used for wavelength detection Axis AGN204-PO electronic balances were used for weighing purpose.

Reagents and chemicals: HPLC grade Methanol and Water were procured from Merck specialties private limited, Mumbai. Pharmaceutical grade pure Tenofovir disoproxil fumarate and Emtricitabine gift samples were procured from Mylan Laboratories, Hyderabad. Marketed formulation Tablets with dose of 300 mg of Tenofovir disoproxil fumarate and 200 mg of Emtricitabine were procured from local market. (Mfd. by Emcure Pharmaceuticals Ltd).

Chromatographic conditions: Agilent ODS-C_{18} column 5µm [250 mm x 4.6 mm] was used for the chromatographic separation at a detection wave length of 261 nm, under ambient temperature. Mobile phase of composition Methanol and Phosphate buffer pH-4 in a ratio of 30:70 v/v which was degassed under ultra-sonication was selected for elution and same mixture was used in the preparation of standard and sample solutions. Flow rate was optimized to 1.0 ml/min and the injection volume was 20 µl.

Preparation of Mobile phase: Phosphate buffer pH 4 was prepared by dissolve 0.504 gm of disodium hydrogen phosphate and 0.301 gm of Potassium dihydrogen phosphate of HPLC grade water and adjusts the pH to 4.0 with glacial acetic acid and sufficient water was added to produce100 ml filtered through 0.45 µ membrane filter and sonicated for 10 minutes.

Preparation of Standard solutions: 25 mg each of Tenofovir disoproxil fumarate and Emtricitabine were accurately weighed and transferred into two 25 ml volumetric flasks, dissolved using mobile phase and the volume was made up with the same solvent to obtain primary stock solutions A (Tenofovir disoproxil fumarate) and B (Emtricitabine) of concentration 1000µg/ml of each drug. From the primary stock solutions,0.8 ml of each was pipette out from A and B respectively, transferred to a 10 ml volumetric flask and the volume was made up with the mobile phase to obtain final concentrations of 80 µg/ml of each was Tenofovir disoproxil fumarate and Emtricitabine respectively and this solution is (working stock solution A).

Preparation of Sample Solution: Twenty tablets of Tenofovir disoproxil fumarate and Emtricitabine

were weighed and crushed. Tablet powder equivalent to 300 mg of Tenofovir disoproxil fumarate and 200 mg of Emtricitabine was weighed accurately and transferred to a 25 ml volumetric flask. The content was dissolved with 10 ml of mobile phase and then sonicated for 15 min. The volume was made up with the mobile phase and filtered with 0.45µ membrane filter and sonicated for 20 min. 0.1 ml of this solution was pipette out and transferred to a 10mlvolumetric flask and the volume was made up with the mobile phase to obtain a concentration of 150 µg/ml of Tenofovir disoproxil fumarate and 100 µg/ml of Emtricitabine (working stock solution B).

Optimization of RP-HPLC method: The HPLC method was optimized with an aim to develop a simultaneous estimation procedure for the assay of Tenofovir disoproxil fumarate and Emtricitabine. For the method optimization, different mobile phases were tried, but acceptable retention times, theoretical plates and good resolution were observed with Methanol, Phosphate buffer pH 4 (30:70 v/v) using Agilent TC-C_{18} (2) column 5µm [250mm x 4.6mm].

Validation of the RP-HPLC method: Validation of the optimized method was performed according to the ICH Q2 (B) guidelines.

System suitability: System suitability was carried out with five injections of solution of 100% concentration having 80 µg/ml of Tenofovir disoproxil fumarate and 80 µg/ml of Emtricitabine in to the chromatographic system. Number of theoretical plates (N) obtained and calculated tailing factors (T) were reported in **Table 1**.

Linearity: For the determination of linearity, appropriate aliquots were pipette out from working stock solution A to a series of 10 ml volumetric flasks and volume was made up with the solvent to obtain concentration ranging from 40-80 µg/ml of Tenofovir disoproxil fumarate and 40-80 µg/ml of Emtricitabine. Each solution was injected in triplicate. Calibration curves were plotted with observed peak areas against concentration followed by the determination of regression equations and calculation of the correlation coefficients. The calibration curves for Tenofovir disoproxil fumarate and Emtricitabine were shown in **Fig. 3** and **Fig. 4** their corresponding linearity parameters were given in **Table 2.**

Limit of Detection (LOD) and Limit of Quantitation (LOQ): The LOD and LOQ were calculated from the slope(s) of the calibration plot and the standard deviation (SD) of the peak areas using the formulae LOD = 3.3 σ/s and LOQ = 10 σ/s. The results were given in **Table 2**.

Precision: The repeatability of the method was verified by calculating the %RSD of six replicate injections of 100% concentration (80 µg/ml of Tenofovir disoproxil fumarate and 80 µg/ml of Emtricitabine) on the same day and for intermediate precision % RSD was calculated from repeated studies on different days. The results were given in **Table 3**.

Accuracy: To ensure the reliability and accuracy of the method recovery studies were carried out by standard addition method. A known quantity of pure drug was added to pre-analyzed sample and contents were reanalyzed by the proposed method and the percent recovery was reported. The results were given in **Table 4**.

Specificity: Specificity of a method was determined by testing standard substances against potential interferences. The method was found to be specific when the test solution was injected and no interferences were found because of the presence of excipients. The optimized chromatogram of Tenofovir disoproxil fumarate and Emtricitabine without any interference was shown in **Fig. 2.**

Robustness: Robustness of the method was verified by altering the chromatographic conditions like mobile phase composition, wavelength detection, flow rate, etc. and the % RSD should be reported. Small changes in the operational conditions were allowed and the extent to which the method was robust was determined. A deviation of ±2 nm in the detection wave length and ±0.2ml/min in the flow rate, were tried individually. A solution of 100 % test concentration with the specified changes in the operational conditions was injected to the instrument in triplicate. % RSD was reported in the **Table 5**.

Assay of Marketed Formulations: 20 µl of sample solution of concentration 150 µg/ml of Tenofovir disoproxil fumarate and 100 µg/ml of Emtricitabine was injected into chromatographic system and the peak responses were measured. The solution was injected three times in to the column.

The amount of drug present and percentage purity was calculated by comparing the peak areas of the standards with that of test samples. A typical chromatogram for assay of marketed formulation was shown in **Fig. 5** and the obtained values were reported in the **Table 6**.

Stability Studies:

Acid degradation studies: Prepared each 1mg/ml stock solution of Tenofovir disoproxil fumarate and Emtricitabine by using mobile phase as solvent, and then filtered through 0.45 µm membrane filter paper. Stock solutions of 0.8 ml and 0.8 ml of Tenofovir disoproxil fumarate and Emtricitabine were transferred into 10 ml volumetric flask and added 1 ml of 0.1N HCL and diluted to volume with mobile phase. The resultant solution was injected into the system; there was no acid degradation products were found and the obtained chromatogram was shown in **Fig. 6**.

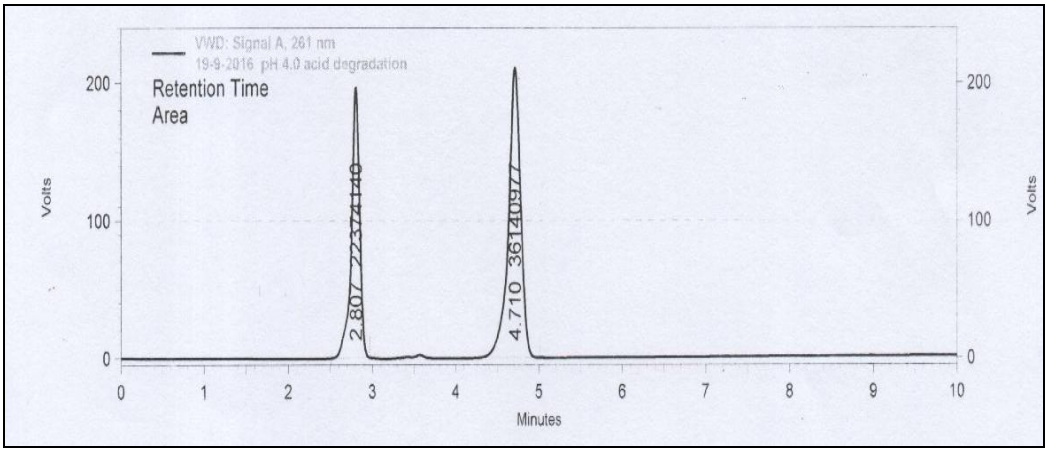


FIG. 6: CHROMATOGRAM OF ACID DEGRADATION

Alkaline degradation studies: 20 µl of Tenofovir disoproxil fumarate and Emtricitabine was injected having concentrations of 80 µg/ml Premixed with 1ml of 0.1N NaOH solution then filtered through 0.45 mm membrane filter paper. The obtained non interfered chromatogram was represented in **Fig. 7.**

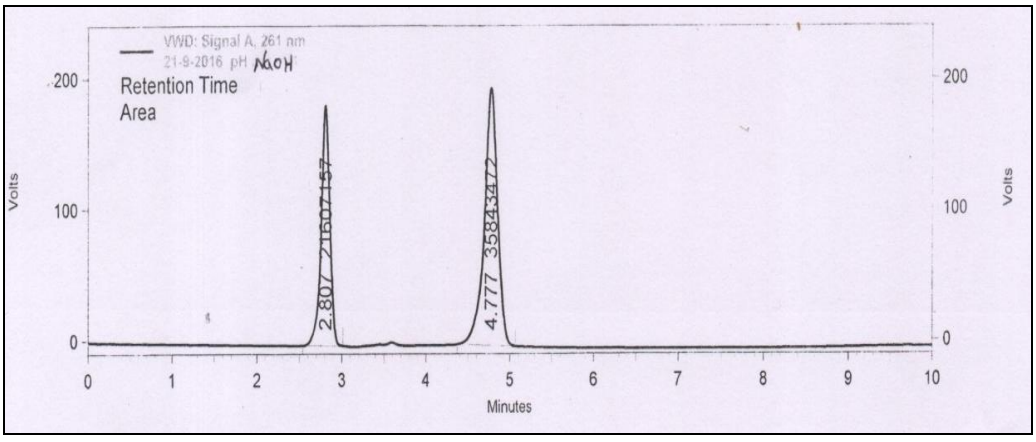


FIG. 7: CHROMATOGRAM OF ALKALINE DEGRADATION

Oxide degradation studies: Tenofovir disoproxil fumarate and Emtricitabine were prepared by dissolving 25 mg/25 ml of mobile phase in two different 25 ml volumetric flask then filtered through 0.45 µm membrane filter paper. Stock solutions of 0.8 ml and 0.8 ml of Tenofovir disoproxil fumarate and Emtricitabine was transferred into 10 ml volumetric flask and added 1 ml of H_2O_2 and diluted to volume with mobile phase. In this investigation no identifiable oxidative degradants were found and the chromatogram was shown in **Fig. 8**.

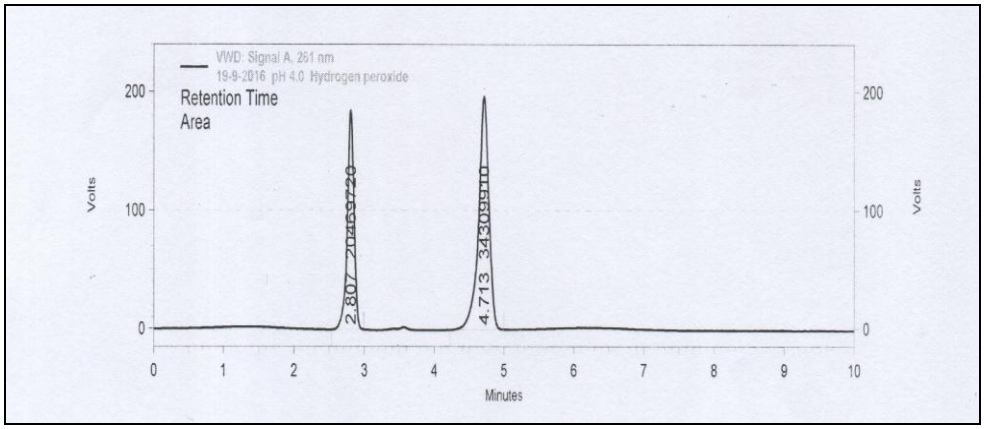


FIG. 8: CHROMATOGRAM OF HYDROGEN PEROXIDE DEGRADATION

Thermal degradation studies: From the primary stock solutions of 1mg/ml 80 µg/ml Tenofovir disoproxil fumarate and Emtricitabine was prepared in 10ml volumetric flask and diluted to volume with mobile phase and kept for 60 min at 60 ^{0}C in hot air oven. 20 µl of above solution was injected in to system and from the obtained chromatogram it was proved that the selected samples were stable against thermal degradation. The obtained chromatogram was shown in **Fig. 9**.

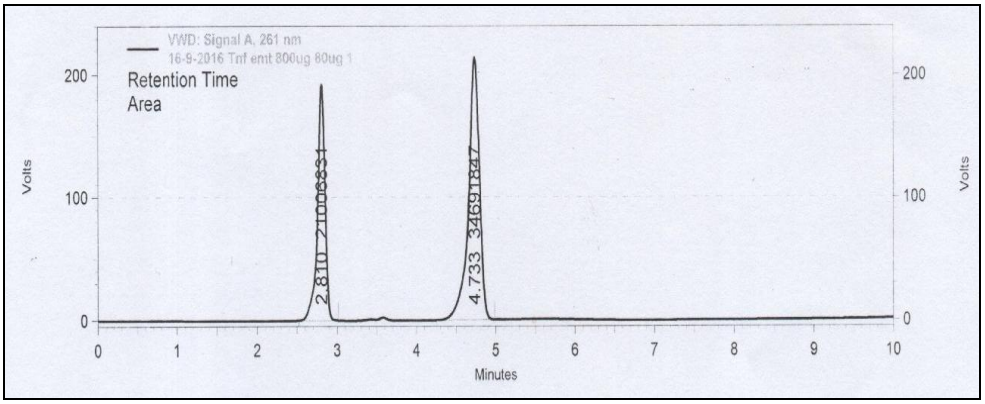


FIG. 9: CHROMATOGRAM OF THERMAL DEGRADATION

RESULTS AND DISCUSSION: After a number of trials with mobile phases of different composition, Methanol, Phosphate buffer pH 4.0 in the ratio 30:70 v/v was selected as mobile phase because of better resolution more no. of Theoretical plates and symmetric peaks. Tenofovir disoproxil fumarate and Emtricitabine were found to show appreciable absorbance at 261 nm when determined spectrophotometrically and hence it was selected as the detection wavelength. An optimized chromatogram showing the separation of Tenofovir disoproxil fumarate and Emtricitabine at different R_Ts was shown in **Fig. 2**.

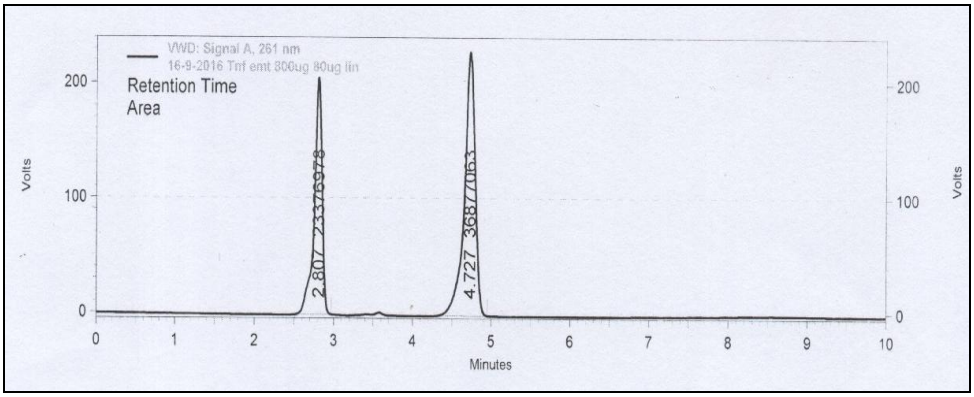


FIG. 2: OPTIMIZED CHROMATOGRAM OF TENOFOVIR DISOPROXIL FUMARATE AND EMTRICITABINE

System suitability was carried out by injecting 5 replicate injections of 100 % test concentration, number of theoretical plates, HETP and resolution were satisfactory. The chromatograms confirm the presence of Tenofovir disoproxil fumarate and Emtricitabine at 2.8 min and 4.7 min respectively without any interference. The parameters were given in **Table 1**.

TABLE 1: SYSTEM SUITABILITY PARAMETERS (n=5)

Parameters	Tenofovir disoproxil fumarate	Emtricitabine
Retention time (min)	2.8	4.7
Theoretical plates (N)	8012	7854
Tailing factor (T)	1.71	1.63
Resolution (R_s)	2.1	

Concentration range of 40-80 µg/ml for Tenofovir disoproxil fumarate and 40-80 µg/ml of Emtricitabine were found to be linear with correlation coefficients 0.999 and 0.999 for Tenofovir disoproxil fumarate and Emtricitabine respectively. The results were given in **Table 2**.

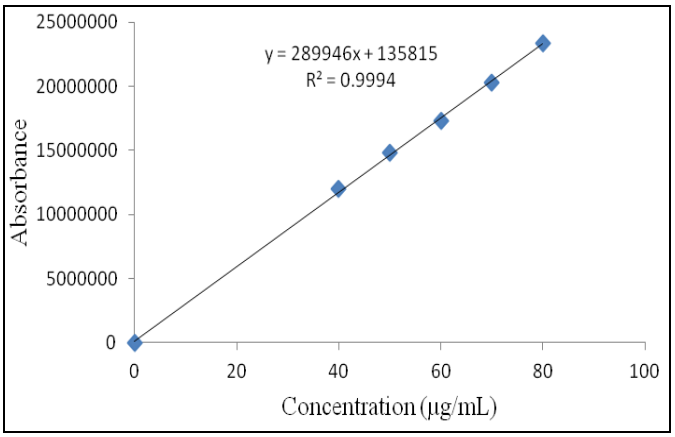


FIG. 3: CALIBRATION PLOT OF TENOFOVIR DISOPROXIL FUMARATE

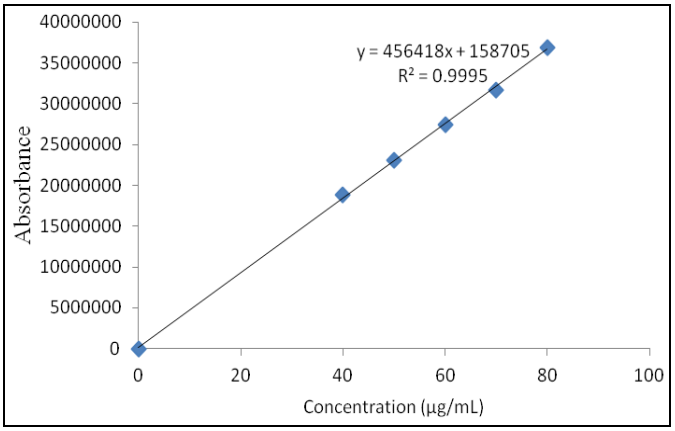


FIG. 4: CALIBRATION PLOT OF EMTRICITABINE

The limits of detection for Tenofovir disoproxil fumarate and Emtricitabine were found to be 1.9µg/ml and 3.5 µg/ml respectively and the limit of Quantitation were 6.2 µg/ml and 11.5 µg/ml respectively. Values were represented in **Table 2**.

TABLE 2: RESULTS FOR LINEARITY (n=3)

Parameters	Tenofovir disoproxil fumarate	Emtricitabine
Slope	28995	45641
y intercept	13581	15870
Correlation coefficient r^2	0.999	0.999
Regression Equation	y = 28995x + 13581	y = 45641x + 15870
Linearity range	40-80µg/ml	40-80µg/ml
LOD	1.9µg/ml	3.5µg/ml
LOQ	6.2µg/ml	11.5µg/ml

*n= No. of determinants

The proposed method was found to be precise and reproducible with % RSD of 0.7 and 0.5 for Tenofovir disoproxil fumarate and Emtricitabine respectively. % RSD was reported in **Table 3**.

TABLE 3: RESULTS OF PRECISION (n=6)

Drug	Intraday Precision (%RSD)	Interday Precision (%RSD)
Tenofovir disoproxil fumarate	0.7	0.8
Emtricitabine	0.5	0.7

*n= No. of determinants

Accuracy of the method was verified by performing recovery studies by standard addition method. The percent recovery of the standard added to the pre-analysed sample was calculated and it was found to be 97.05 % to 98.5 % for Tenofovir disoproxil fumarate and 96.9 to 98.4 % for Emtricitabine. This indicates that the method was accurate. Values obtained were given in **Table 4**.

The method was found to be robust after changing the conditions like detection wavelength (± 2nm) and flow rate (± 0.2 ml). % RSD was calculated for each variation and reported. Values obtained were given in **Table 5**.

TABLE 4: RESULTS FOR ACCURACY (n=3)

Recovery level	Tenofovir disoproxil fumarate				Emtricitabine			
	Amount Added (µg/ml)		Amount Found (µg/ml)	% Recovery	Amount Added (µg/ml)		Amount Found (µg/ml)	% Recovery
	std	test			Std	Test		
50%	10	20	29.31	97.7	10	20	29.52	98.4
100%	40	20	58.23	97.05	40	20	58.15	96.9
150%	70	20	88.69	98.5	70	20	88.15	97.9
Mean recovery			97.75%				97.7%	

∗n= No. of determinant

TABLE 5: RESULTS FOR ROBUSTNESS (n=3)

Parameters (n=3)	%RSD	
	Tenofovir disoproxil fumarate	Emtricitabine
Detection wavelength at 263nm	0.29	0.53
Detection wavelength at 259nm	0.69	0.59
Flow rate 0.8ml/min	0.52	0.93
Flow rate 1.2ml/min	0.82	0.72

∗n= No. of determinants

The method was found to be specific for the combination of interest after verifying the chromatograms showing no interference of the excipients present. Hence, the method was well suitable for the estimation of the commercial formulations of the selected combination with a percentage purity of 97.58 % for Tenofovir disoproxil fumarate and 97.88 % for Emtricitabine. The typical chromatogram for assay of marketed formulations was shown in **Fig. 5** and Values obtained were given in **Table 6**.

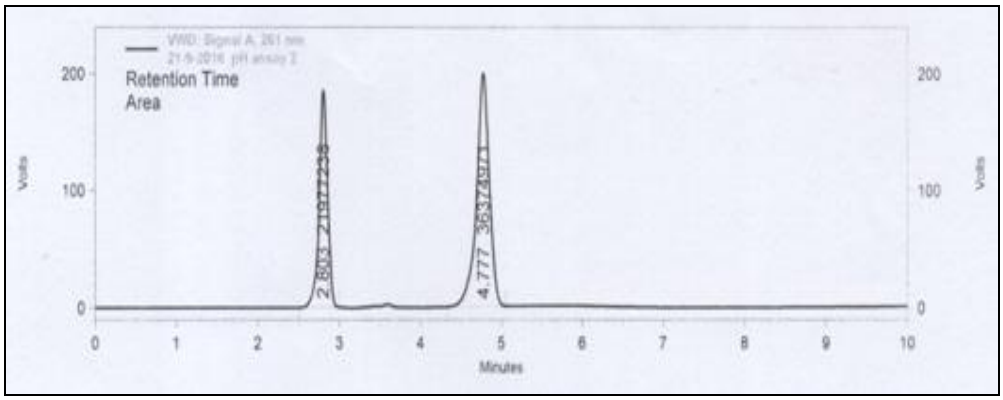


FIG. 5: A TYPICAL CHROMATOGRAM FOR ASSAY OF MARKETED FORMULATION CONTAINING 150 µg/ml OF TENOFOVIR DISOPROXIL FUMARATE AND 100 µg/ml OF EMTRICITABINE

TABLE 6: RESULTS FOR ASSAY (n=3) OF MARKETED FORMULATION

Drug	Label claim (mg/tab)	Amount recovered	% Amount found in drug
Tenofovir disoproxil fumarate	300	292.76	97.58%
Emtricitabine	200	195.77	97.88%

∗n= No. of determinants

Forced Degradation Study: Degradation studies indicated the specificity of developed method in presence of degradation products. Degradation was carried out in combination of two drugs and purity of drug peaks was confirmed by purity angles. Their combination drug products were exposed to acid, alkali, oxidative and thermal stress conditions. Then found to be no degradable substances presence and proved that the proposed method was stable towards acid, alkali, peroxide and thermal conditions. The obtained values were reported in **Table 7**.

TABLE 7: RESULTS FOR STABILITY STUDIES OF TENOFOVIR DISOPROXIL FUMARATE AND EMTRICITABINE COMBINED FORM (n=3)

Parameters	Peak area		% of degradation	
	Tenofovir disoproxil fumarate	Emtricitabine	Tenofovir disoproxil fumarate	Emtricitabine
Acid degradation	22374140	36140977	0.125	0.196
Alkali degradation	21607157	35843472	0.112	0.156
Peroxide degradation	20469720	34309910	0.268	0.341
Thermal degradation	21006831	34691847	0.262	0.192

*n= No. of determinants

CONCLUSION: The RP-HPLC method developed and validated allows a simple and rapid quantitative determination of Tenofovir disoproxil fumarate and Emtricitabine from their formulations. All the validation parameters were found to be within the limits according to ICH guidelines. The proposed method was found to be specific for the drugs of interest irrespective of the excipients present and the short retention times allows the analyst to analyze large no. of samples in short period and method was found to be simple, accurate, precise, rugged, robust and stable under forced degradation stress conditions. So the established method can be successfully applied for the routine analysis of the marketed formulations. Hence the proposed method is a productive one.

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