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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF TENOFOVIR DISOPROXIL FUMERATE AND EFAVIRENZ IN BULK AND FORMULATION

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ABSTRACT: The objective of the present work was to develop a simple, rapid, accurate RP-HPLC method for the simultaneous estimation of Tenofovir disoproxil fumerate (TDF), Efavirenz in bulk and formulation. The chromatographic separation was achieved by isocratic mode of elution by using Agilent Zorbax Eclipse XDB C_{18} (150 x 4.6mm, particle size 5µm) analytical column. The mobile phase consisting of Acetonitrile and phosphate buffer (0.03M KH_2PO_4, pH 2.5) the ratio of 70:30v/v with 0.6 ml/min flow rate and the eluents are monitored at 255nm.The retention times were 2.44min for TDF and 5.52 min for Efavirenz. The detector response was linear for TDF between 3-18µg/ml with R^2 =0.9987 and 6-36µg/ml for Efavirenz with R^2=0.9982 respectively. The % relative standard deviation (%R.S.D) values were found to be <2. The method was validated by determining accuracy, precision and linearity range. The results of the proposed RP-HPLC method is precise, rapid and accurate which is useful for quantitative determination and routine analysis of Tenofovir disoproxil fumerate and Efavirenz in bulk and in formulation.

INTRODUCTION: A novel antiretroviral formulation with combining doses of the non-nucleoside reverse transcriptase inhibitor Efavirenz (600mg) and nucleoside reverse transcriptase inhibitor Tenofovir disoproxil fumerate (300mg) are more effective. Tenofovir disoproxil fumerate [1, 2] is fumaric acid salt of bis isopropoxycarbonyloxy o-methyl ester derivative of Tenofovir is 9-[(R)-2-[[bis[[(isopropoxycarbonyl)oxy]methoxy]phosphinyl]methoxy]propyl]adeninefumerate.

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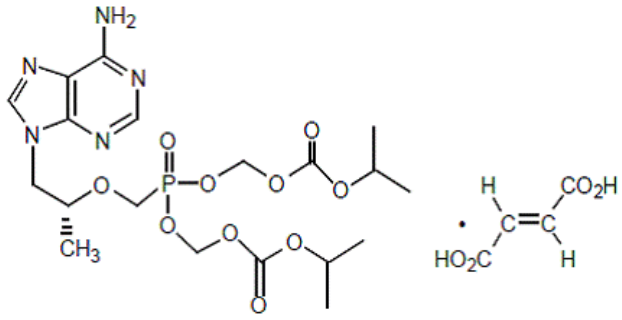
It is chemically the prodrug of Tenofovir. Efavirenz chemically (S)-6-chloro-4-(cyclopropylethynyl)-1, 4-dihydro4-(trifluoro methyl)-2H-3, 1-benzoxazin-2-one. Literature review [3-10] reveals a few chromatographic methods are developed for the determination of Tenofovir disoproxil fumerate Emtricitabine and Efavirenz, but there are no analytical methods for Tenofovir disoproxil fumerate and Efavirenz in biological fluids and pharmaceutical dosage forms along with other antiretroviral drugs.

The chemical structures of the drugs are furnished below. During formulation of a tablet dosage form, there is a possibility of formulation and process derived impurities generation and interference of such impurities in the analytical method.

Further there is a need to identify and control such impurities in the formulation as per the regulatory requirement. So studies were conducted to develop a validated analytical method for quantitative estimation of Tenofovir disoproxil fumerate and Efavirenz simultaneously from the pharmaceutically acceptable tablet dosage form and the results are reported here.



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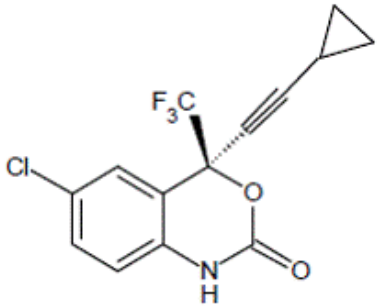

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FIGURE 1: CHEMICAL STRUCTURE OF TENOFOVIR DISOPROXIL FUMERATE AND EFAVIRENZ

MATERIALS AND METHOD: Tenofovir disoproxil fumerate, Efavirenz were obtained as a gift samples from NATCO Pharmaceuticals, Hyderabad.KH_2PO_4 was analytical grade supplied by S.D. Fine chemicals, Mumbai, Ortho phosphoric acid(OPA), Acetonitrile(Merck HPLC grade) and Water for HPLC method(in House).

Instrument: Quantitative HPLC was performed on Agilent technologies1200 series, PDA detector module equipped with auto injector with Ezchrome elite software. A reverse phase Agilent Zorbax Eclipse XDB C_{18} (150 x 4.6mm, particle size 5µm) analytical column was used.

HPLC Conditions: Preliminary studies were conducted and trails are made for the method development. The optimized mobile phase consists of Acetonitrile and phosphate buffer (0.03M KH_2PO_4, pH adjusted to 2.5 with orthophosphoric acid) in the ratio of 70:30 v/v. They were filtered through 0.45µm membrane filter using vaccum pump.

Flow rate was maintained at 0.6ml/min and run time for 10 min. prior to sample injection, column was saturated with mobile phase for 40 min and injection volume was 10µl injected by auto sampler. The detection response was measured at 255nm and maintained at ambient temperature.

Buffer preparation 0.03M: 10.08g of KH_2PO_4 was dissolved in 1000ml of double distilled water, pH adjusted to 2.5 with orthophosphoric acid.

Preparation of standard stock solution: Accurately 3mg of Tenofovir disoproxil fumerate and 6mg of Efavirenz standard drugs were weighed and transferred into 10ml volumetric flask and made up to the mark with Acetonitrile diluent-1(3000µg/ml and 6000 µg/ml).

Diluent1- Acetonitrile

Diluent-2 Mobile phase (Acetonitrile: Phosphate buffer, 70:30v/v)

Working standard solution: From above standard stock solution, working standard solution was prepared by pippeting 0.1ml into 10ml volumetric flask and made up to the mark with diluent-2. Final concentration of 3µg/ml and 6µg/ml of Tenofovir disoproxil fumerate and Efavirenz working standard solution were injected.

Preparation of in house tablet: Tenofovir disoproxil fumerate (300mg), Efavirenz (600mg), Starch paste(15mg), starch(60mg), Magnesium stearate (6mg), talc(6mg) were weighed.

Procedure: Tenofovir disoproxil fumerate (300mg), Efavirenz (600mg) were blended in mortar. The mixture was moistened with the binder starch paste. The damp mass was subjected to wet sieving (sieve no.12). The granules were dried at 60^0 for 4 hr and re-sieved.

The granules having a size of 44/60 were collected and compressed to form a tablet with 12mm compression tool by using Cad mach rotary tablet machine.

Preparation of sample solution for Assay: Ten tablets (in house) were accurately weighed and crushed into fine powder. The powder equivalent to Tenofovir disoproxil fumerate (300mg) and Efavirenz (600mg) was transferred to 10ml volumetric flask then sonicated for 10 min, filtered through whatmann filter paper no.1 and made up to the mark with diluent-1.From the above stock solution 0.1 ml solution was pippeted into 10 ml volumetric flask and makeup to mark with diluents-2. Final concentration of 3µg/ml and 6µg/ml of Tenofovir disoproxil fumerate and Efavirenz respectively was injected.

Validation of Analytical method: [11]

1. **Linearity and Range:** Appropriate aliquots of Tenofovir disoproxil fumerate and Efavirenz standard stock solution of 3000µg/ml and 6000µg/ml was transferred to series of 10 ml volumetric flasks (Each 0.1 ml contains 3µg/ml of Tenofovir disoproxil fumerate and 6µg/ml Efavirenz respectively.) 0.1-0.6ml of standard stock was pippeted and diluted to final volume with diluent-2.

2. **Accuracy studies:** Accuracy was determined by adding the known amount of standard drug to the pre fixed concentrations of assay samples by standard addition method. The percentage recovery studies were carried out in triplicate of three different levels 50%, 100%, 150% by spiking standard drug solution to the placebo.

3. **Precision Studies and System suitability:** The method precision of the proposed method is ascertained by injecting 6 replicates of test sample and % recovery, %RSD were calculated.

4. **LOD and LOQ:** The sensitivity of HPLC was determined from LOD and LOQ which were calculated from the calibration curve using the following equations

LOD = 3.3**σ**/S and

LOQ = 10 **σ**/S, where

σ= Standard deviation of y intercept of regression line

S = Slope of the calibration curve

5. **Robustness and Ruggedness:** Robustness is the measure of the ability of an analytical method to remain unaffected by small but deliberate variations in method parameters, pH change (±0.5), mobile phase composition (±0.2), flow rate (±1ml/min), wave length (2nm) were considered. The developed method is robust with deliberate changes with variation of analyst to analyst, column to column Intraday and Inter day precision and provides an indication of its reliability.

RESULTS AND DISCUSSION: The present study describes a new RP- HPLC method for the estimation of Tenofovir disoproxil fumerate and Efavirenz in bulk and formulation (in house). Preliminary studies were conducted, in first trail by using Acetonitrile: pH 4.0 buffer in 90:10 ratio split peak were observed. In the second trail using Acetonitrile: pH 6.8 buffer peak shape is not good. In the third trail by using

Acetonitrile: pH 2.5 buffer in 90:10 ratio peak shapes is good but poor resolution. In the fourth trail Acetonitrile: pH 2.5 buffer were used in 80:20 ratio good peaks were observed with low resolution. In the fourth trail by using Acetonitrile: pH 2.5 buffer in 70:30 ratio good peak shape with high resolution were observed and the corresponding chromatograms were shown in **figure 2** (working standard) and **figure 3** (sample extracted from tablet).

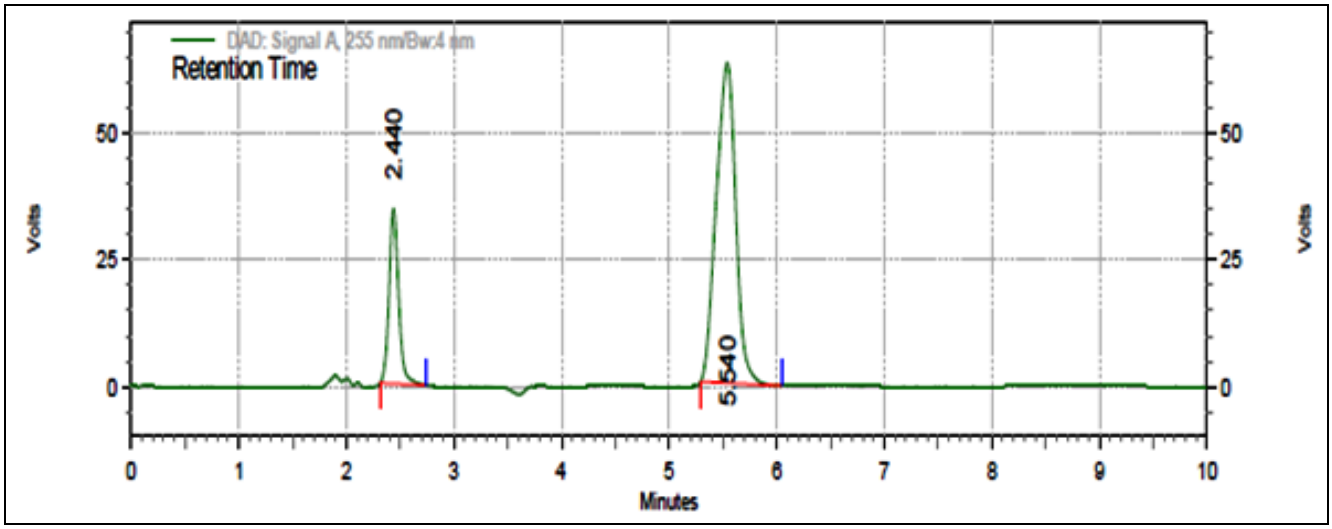

FIGURE 2: TYPICAL CHROMATROGRAM OF STANDARD CONTAINING TENOFOVIR DISOPROXIL FUMERATE AND EFAVIRENZ

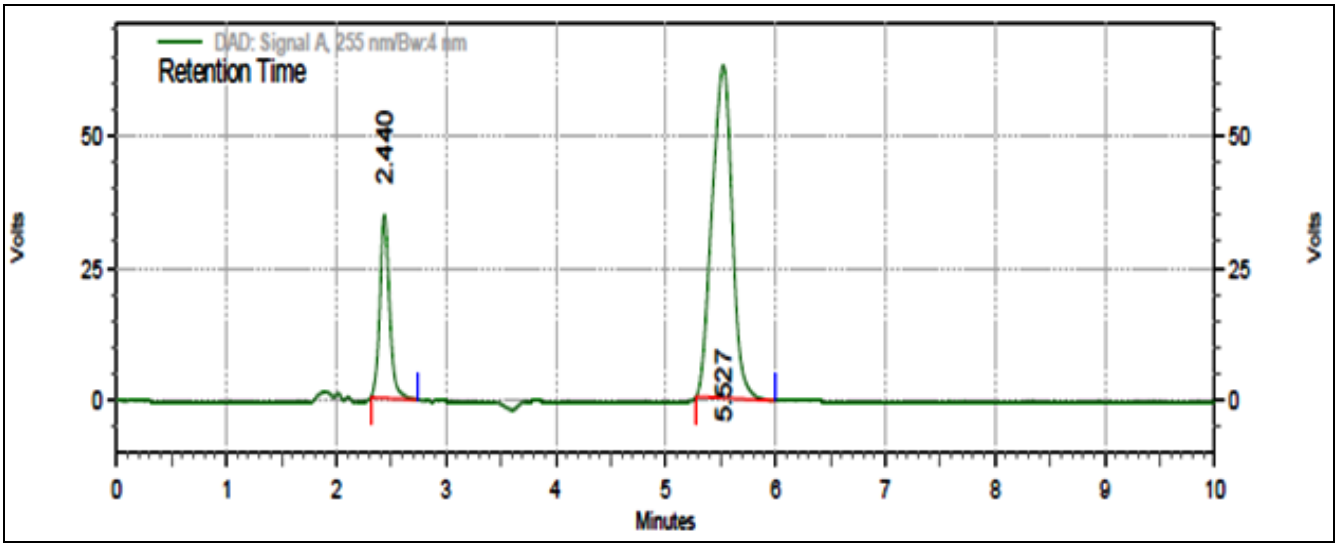

FIGURE 3: TYPICAL CHROMATROGRAM OF SAMPLE CONTAINING TENOFOVIR DISOPROXIL FUMERATE AND EFAVIRENZ

The developed method was validated in terms of Accuracy, Precision, Specificity, Detection limit Quantitation limit, Linearity Range Ruggedness, Robustness and System suitability parameters as per the recommendations of ICH guidelines.

From **Table 1 and 2,** the recovery studies demonstrated the better accuracy. The mean% recovery studies are within the assay limit100±1% with deliberate relative standard deviation (%RSD).

TABLE 1: ACCURACY STUDIES OF THE TENOFOVIR DISOPROXIL FUMERATE

%level	Amount of API Spiked (µg/ml)	Peak Area	Amount Found (µg/ml)	%Recovery	Mean %Recovery	Standard Deviation	%RSD
50%	9	442404	9.06	99.76	99.72	0.523	0.524
	9	445632	9.04	100.5			
	9	438821	9.07	98.92			
100%	12	590262	12.16	101.33	101.21	0.374	0.370
	12	591682	12.09	100.80			
	12	591024	12.18	101.5			
150%	18	868916	17.88	99.3	100.5	0.742	0.730
	18	889971	18.02	101.70			
	18	869971	19.09	99.43			

TABLE 2: ACCURACY STUDIES OF THE EFAVIRENZ

%level	Amount of API Spiked (µg/ml)	Peak Area	Amount Found (µg/ml)	%Recovery	Mean %Recovery	Standard Deviation	%RSD
50%	18	1621021	18.07	100.3	101.24	0.499	0.493
	18	1632910	18.20	101.15			
	18	1659184	18.05	100.27			
100%	24	2152642	24.19	100.81	100.93	0.28	0.277
	24	2150841	24.17	100.73			
	24	2161791	24.30	101.25			
150%	30	2623612	29.93	99.78	98.83	0.83	0.833
	30	2619139	29.37	98.50			
	30	2609932	29.46	98.22			

From **Table 3**, the %RSD of method and system precision parameter was found to be less than 2 for both repeatability and intermediate precision as per ICH guidelines. The mean recovery studies from the system and method precision were found to be 100.71 and 100.74 for Tenofovir disoproxil fumerate and Efavirenz respectively are within the assay limit100±1% with deliberate relative standard deviation (%RSD).

TABLE 3: PRECISION TABLE

S. No.	Sample area		%Assay on the label claim	
	Tenofovir	Efavirenz	Tenofovir	Efavirenz
Precision-1	448854	1639241	101.25	101.5
Precision -2	448929	1619817	101.27	100.31
Precision -3	449899	1629184	101.49	100.91
Precision-4	442404	1621021	99.76	100.3
Precision-5	445632	1632910	100.5	101.15
Precision-6	448821	1659184	100.01	100.27
			Mean = 100.71 **Standard deviation**=0.728 **%RSD** =0.722	**Mean** = 100.74 **Standard deviation**=0.524 **%RSD**=0.520

To determine specificity during the validation of blanks, sample matrix (placebo) and known related impurities are analyzed to determine whether interferences occur. It was observed that there were no analytical peaks observed in the blank indicating the method is specific. Moreover, the method is highly sensitive as evidenced by the LOD and LOQ values of Tenofovir disoproxil fumerate and Efavirenz are 0.0850 and 0.259, 0.199 and 0.605 respectively.

From **figure 4 and 5,** the standard calibration curve was linear over the concentration range of 3-18µg/ml and 6-36µg/ml for Tenofovir disoproxil fumerate and Efavirenz respectively. The linear regression equation was found to be Y=47873x+12581 for Tenofovir disoproxil fumerate with R^2 =0.9987 and Y=86782x+52813 for Efavirenz with R^2 =0.9982 and respectively.

Standard calibration curve was linear and best fit line with in the linear range.

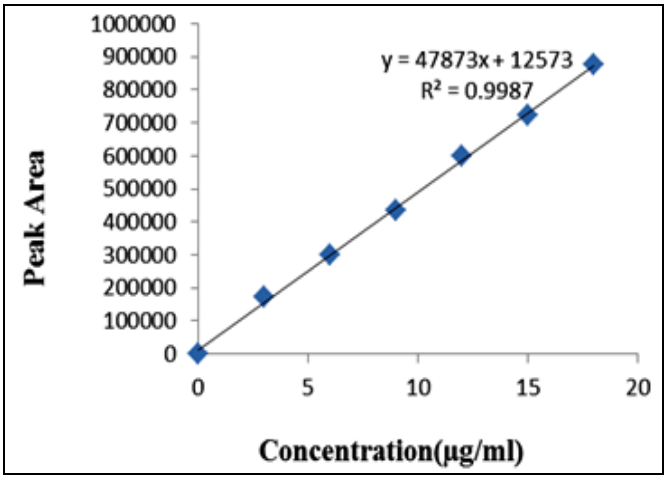

FIGURE 4: CALIBRATION CURVE OF TENOFOVIR DISOPROXIL FUMERATE

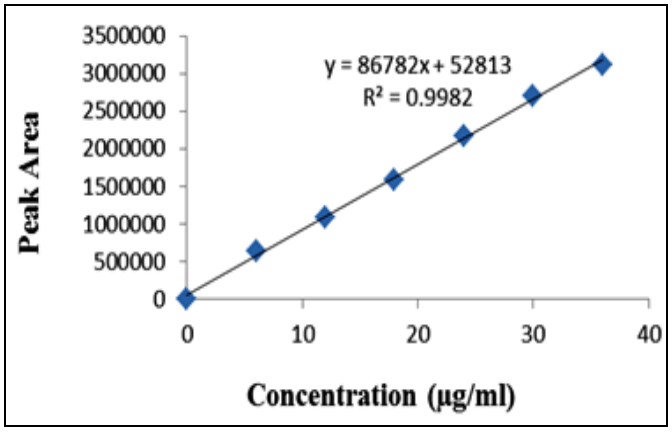

FIGURE 5: CALIBRATION CURVE OF EFAVIRENZ

As there are no significant change in the chromatographic parameters when variations in the optimized conditions indicates that the method is robust, less deviations were observed between analyst to analyst, column to column so the proposed method is rugged. The system suitability parameters shows that the method is highly selective and specific, as the retention time of Tenofovir disoproxil fumerate and Efavirenz were found to be 2.440 and 5.527min respectively so the method was rapid.

The theoretical plates were found to be >3000, thus demonstrating good column efficiency with significant resolution and better separation within accepted limits. As the asymmetry values are found to <1.5 it indicates the good peak shape. From the above results it was found that the optimized method is simple rapid, accurate, precise and within the limit of validation parameters.

CONCLUSION: The developed and validated HPLC method was found to be rapid, accurate, precise and reliable which is useful for routine quantification purpose in quality control. The system suitability parameters indicate good sensitivity, more ruggedness and robustness of the method. Therefore, the proposed method has proven simple, selective, specific which meets the ICH guidelines for analytical method validation.

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