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RP-HPLC Method For The Simultaneous Estimation Of Tenofovir Disoproxil Fumerate And Emtricitabine In Combined Tablet Dosage Form

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Abstract: A new, accurate and reliable RP-HPLC method has been developed and validated for simultaneous estimation of Tenofovir disoproxil fumerate (TDF) & Emtricitabine (EMT) in a combined tablet dosage form. First of all, maximum absorbance was found to be at 270nm and the peak purity was excellent. Injection volume was selected to be 20µl which gave a good peak area. The column used for study was PHENOMENEX LUNA C18 chosen. Ambient temperature was found to be suitable for the nature of drug solution. The flow rate was fixed at 1.0ml/min because of good peak area and satisfactory retention time. Mobile phase of Acetonitrile : Phosphate pH 3.5 buffers in the ratio of 60:40 was fixed due to good symmetrical peak. The retention times for TDF & EMT were found to be 2.84 min and 3.55 min respectively. The precision of the System and method were checked and found to be within limits. This indicates that the method is precise. Linearity study, correlation coefficient and curve fitting was found to be 0.999. The recovery value of pure drug was found between 99.4 % to 101.7 %. This indicates that the developed method is accurate, precise and economical and can be used for the routine analysis of tablets in quality control.
Keywords : RP-HPLC method, Tenofovir disoproxil fumerate, Emtricitabine.

INTRODUCTION AND EXPERIMENTAL

Tenofovir disoproxil fumerate is a white to off white crystalline powder. It is a salt of bis (isopropyloxy carbonyloxymethyl ester of (*R*)-9-(2 phosphonomethoxypropyl)adenine with fumaric acid. It is soluble in water : methanol (1:1) with empirical formula $C_{19}H_{30}N_5O_{10}P,C_4H_4O_4$ having molecular weight of 635.5. currently it is used as an anti-HIV agent.it comes under the category of Nucleoside and Nucleotide Reverse Transcriptase Inhibitors. The structure of TDF was shown in the figure-1[1-4].

Emtricitabine is a white to off-white crystalline powder. Chemically it is 4-amino-5-fluoro-1-[(2*R*,5*S*)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-2(1*H*)-pyrimidone with empirical formula $C_8H_{10}FN_3O_3S$ having molecular weight of 247.3 it is Soluble in water and sparingly soluble in methanol. It comes under the category of Anti-HIV Agent. The structure of emt was shown in the figure-2[1-4].

Literature survey reveals that few methods like HPLC, UV-Spectrophotometric, HPTLC were available for the simultaneous estimation of Tenofovir disoproxil fumerate and Emtricitabine in combined tablet dosage form[5-8]. Though few RP-HPLC methods are available there is some lacunae either in the form of retention times or mobile phases.

So, in the current study it is designed to develop a new, simple, accurate, less time consuming method of analysis for the simultaneous estimation of TDF and EMT in combined tablet dosage form by RP- HPLC to overcome the earlier lacunae.

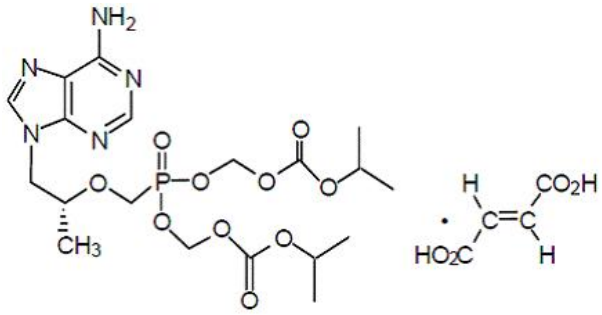


Figure-1 (Structure of tenofovir disoproxil fumerate)

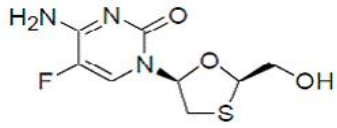


Figure-2 (Structure of emtricitabine)

MATERIALS & METHODS [8-12]

Associated Instruments and Chemicals

S. No.	Instruments
1.	SHIMADZU HPLC with UV detector LC 10 AT *VP*
2.	PHENOMENEX Luna (C18) A 100 RP Column, 250 mm x 4.6
3.	ELICO LI 120 P^H meter
4.	SHIMADZU Electronic Balance AUX-220
5.	HAMILTON HPLC syringe 20 µl
6.	PCi Ultra Sonicator

S. No	Chemicals and Solvents
1	Tenofovir disoproxil fumerate working standard
2	Emtricitabine working standard
3	0.03M potassium dihydrogen phosphate pH-3.5
4	Acetonitrile(HPLC Grade)
5	Methanol (HPLC grade)
6	Water (HPLC Grade)
7	Tenvir-EM tablets (sample)

Selection of Mobile Phase:

i) Separation using Acetonitrile: methanol: phosphate buffer (pH-6.5) (30:50:20) Inertsil ODS 3V,250*4.6,5µ 1ml/min

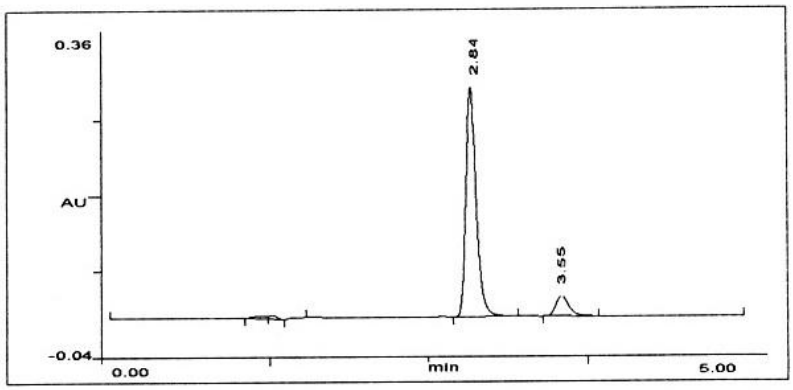
This trial was done by using mobile phase consisting of Acetonitrile: methanol: phosphate buffer (pH-6.5) (30:50:20) Phenomenex luna C18 A,250*4.6,5µ 1ml/min, resolution was not observed.

ii) Separation using Acetonitrile: methanol: phosphate buffer (pH-5) (30:50:20) Phenomenex luna C18 A, 250*4.6,5µ 1ml/min

Next trial was done by using mobile phase consisting of Acetonitrile: methanol: phosphate buffer (pH-5) (30:50:20) Phenomenex luna C18 A,150*4.6,5µ 1ml/min but the resolution between tenofovir disoproxil fumerate and Emtricitabine is too less.

iii) Separation using ACN : 0.03M potassium dihydrogen phosphate, Buffer Ph 3.5 (60:40) phenomenex luna C18 A, 250*4.6, 5µ 1 ml/min.

This trial was done by using mobile phase consisting of ACN : 0.03M potassium dihydrogen phosphate, Buffer3.5 (60:40) phenomenex luna C18 A, 250*4.6,5µ 1ml/min In this system good symmetrical peaks were obtained. So, this mobile phase was selected for further study. The chromatogram was shown in the figure-3.



No.	R.T.	Ht.	Area	Ht. %	Area %	Pk Ty	Area/ Ht
1	2.84	66797	9060931	91.8867	89.8669	BB	0.093
2	3.55	5898	1021681	8.1133	10.1331	BB	0.118
		7e+04	10082612				

Figure-3 (Optimized Chromatogram Of TDF & EMT Using Acetonitrile : Phosphate Buffer (pH-3.5) (60:40))

Selection of Detection Wavelength

The sensitivity of method that uses UV detector depends upon the proper selection of wavelength. An ideal wavelength is that gives maximum absorbance and good response for both drugs to be detected.

A UV spectrum of Tenofovir disoproxil fumerate and Emtricitabine was recorded by scanning between 190-400nm. From this spectrum λ_{max} at 270 nm was selected for the proper study.

Selection of Chromatographic method for separation

Proper selection of chromatographic method depends upon the nature of the drugs, molecular weight and solubility. Polar compounds can be separated by using Reverse Phase Chromatography on non-polar stationary phase and Non-polar compounds can be separated by using Normal Phase Chromatography on polar stationary phase . Since Tenofovir disoproxil fumerate and Emtricitabine are polar in nature, Reversed Phase Chromatography has been used.

Analysis of Formulation :

Preparation of Mobile Phase:

Preparation of Buffer solution: 4.08g of potassium dihydrogen phosphate is dissolved in 1000 ml of volumetric flask and make up with water and adjust the pH to 3.5. Filtered through a finer porosity membrane filter and degassed.

Mobile Phase: Mixed ACN and Buffer in the ratio of 600:400v/v respectively and degassed by sonication.

Preparation of diluent: Taking into consideration the solubility of the drugs in different solvents, the common diluent was selected for all the two drugs which is nothing but the Water.

Preparation of Standard: (TDF & EMT standards)

Standard stock solution of TDF and EMT were prepared separately in mobile phase with suitable

dilution to get the concentration of 100 µg / ml. From the standard stock solution of drugs, different dilutions were prepared, injected and their peak area was measured. Calibration curves were drawn between concentration against their respective peak area for TNF (3- 15µg / ml) and EMT (2 - 10µg / ml) respectively. Unknown samples were determined by using these regression equations of (Y= mx + c) calibration curves.

Sample preparation: (TENVIR-EM tablets)

Twenty tablets were weighed and their average weight was determined. The tablets were then crushed to a fine powder and the tablet powder equivalent to 30 mg of TNF and 20 mg of EMT was transferred into a volumetric flask and extracted with HPLC grade methanol. The solution was shaken for 5 min and sonicated for 15-20 min. The solution was filtered through Whatman filter paper 41. This filtrate was further diluted with mobile phase to get the final concentration of 15 µg / ml for TNF and 10 µg / ml for EMT theoretically. 20 µL of the sample solution was injected for quantitative analysis. Identification is done by comparing retention times of the sample solution with those of standard solution. The amount of TNF and EMT per tablet was calculated from Regression Plot.

Optimized Chromatographic Conditions

The following parameters were used for RP-HPLC analysis of assay of pharmaceutical dosage form.

Mode of operation	: Isocratic
Stationary phase	: PHENOMENEX Luna C18 A,250*4.6,5µ
Mobile phase	: Acetonitrile : 0.03M Potassium dihydrogen phosphate pH(3.5)
Ratio	: 60:40
Diluent	: Water
Detection wavelength	: 270nm
Flow rate	: 1 ml/min
Temperature	: 25°C
Sample volume	20 µl

Procedure: The chromatographic conditions were set as per the established parameter and mobile phase allowed to equilibrate with the stationary phase. Working standard solution was injected separately and chromatograms have been reproduced.

Study of system suitability parameters:

Procedure: The chromatographic conditions were set as per the optimized parameters and mobile phase was allowed to equilibrate with stationary phase as was indicated by the steady baseline. Five replicate injections of working mixed standard solution were injected and the chromatograms were recorded for the drugs and the results were shown in **the Table-9.**

Table-1 (Optical Charecterstics Of TDF & EMT BY RP-HPLC Method)

PARAMETERS	TDF	EMT
λ max	270 nm	270 nm
Beer's law limit(µg/ml)	3-15	2-10
Correlation Co- efficient (r^2)	0.9998	0.9999
Slope (m)	272159.04	187484.83
Intercept (c)	30617.3	-5990.75
Standard Error	329160.45	209542.88
Regression equation (Y= mx + c)	Y= 272159.04x + 30617.3	Y=187484.83x -5990.75

Table-2 (Assay of commercial formulation (tenvir-em™) by RP-HPLC method)

DRUG	SAMPLE NO.	LABELED AMOUNT (mg/tab)	Amount found (mg/tab)	PERCENTAGE OBTAINED	Average %	S.D (+/-)	% R.S.D
TDF	1 2 3 4 5 6	300 300 300 300 300 300	300.44 300.26 300.48 299.93 299.44 299.72	100.146 100.086 100.160 99.977 99.814 99.906	99.899	0.13	0.14
EMT	1 2 3 4 5 6	200 200 200 200 200 200	200.32 199.50 200.56 197.86 195.84 198.30	100.16 99.75 100.28 98.93 97.92 99.15	98.666	0.88	0.89

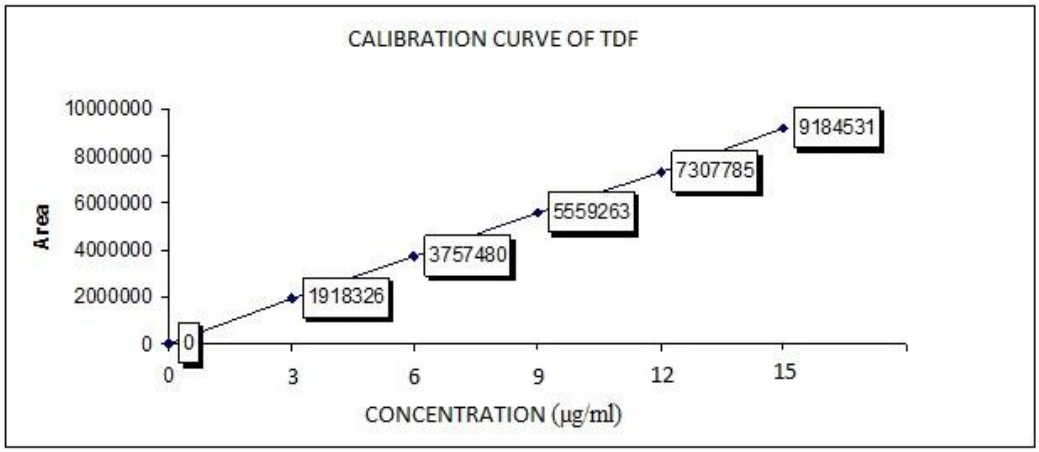


Figure -4 (Calibration Curve For TDF BY RP-HPLC)

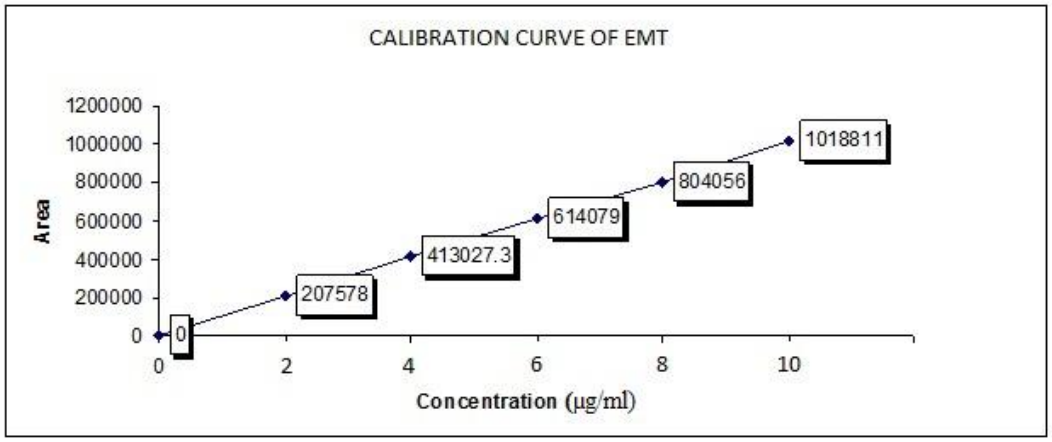


Figure-5 (Calibration Curve For EMT RP-HPLC)

VALIDATION OF PROPOSED METHOD[13]

1. Linearity: Accurately measured volume of mixed standard stock solution was diluted with diluent to get the final concentration of standard as 3-15 µg/ml. Five point linearity was determined.

Procedure: The chromatographic conditions were set as per the optimized parameters and mobile phase was allowed to equilibrate with stationary phase as was indicated by the steady baseline. Standard solutions of different concentration were injected separately and the chromatograms were recorded.

Peak areas were recorded for each injected concentration of drugs and the calibration curves, concentration vs. peak area were constructed for the drugs. Linearity performance parameters are depicted below. Peak areas were recorded and the graphs, concentration vs. peak area were constructed for the drugs. The calibration graphs were shown in figure 4 & 5. The statistical data's for Tenofovir disoproxil fumerate and Emtricitabine are in the table 3 & 4.

Table-3 (Linearity Range And Area (TDF))

Concentration (µg/ml)	Area
3	1918326
6	3757480
9	5559263
12	7307785
15	9184531
Slope	272159.04
Correlation coefficient (r^2)	0.9998

Table-4 (Linearity Range And Area (EMT))

concentration (µg/ml)	Area
2	207578
4	413027
6	614079
8	804056
10	101881
Slope	187484.83
Correlation coefficient (r^2)	0.9999

2.Accuracy:

Accuracy of proposed method was ascertained on the basis of recovery studies performed by standard addition at different level of labeled claim (i.e., 80 to 120 % of labeled claim).

Standard solution: Weighed accurately and transferred about 100mg of Tenofovir disoproxil fumerate working standard, 100mg of Emtricitabine working standard was taken in to 100ml volumetric flask separately. Added about 80ml of water, sonicated to dissolve and diluted to volume with the same. Filtered the solution.

Sample Solution: Accurately weighted quantity of placebo was taken in five different 100 mL volumetric flasks. To these flasks accurately weighed quantities of API equivalent to percent of 100 mg of Tenofovir disoproxil fumerate, 100mg of Emtricitabine are added to their respective flasks. About 80mL of diluent was added to all the flasks and then sonicated for 30min with intermediate shaking. The volume was made up to mark with diluent. Filtered the solutions

Procedure: The chromatographic conditions were set as per the optimized parameters and the mobile phase was allowed to equilibrate with stationary phase. Five replicate injections of the standard solution and three replicate injections of each sample solution were injected separately and chromatograms were recorded. The concentration of each drug was estimated by comparing sample peak area with standard. The results of accuracy are **shown in table-5.**

Table-5 (Recovery studies of TDF & EMT by RP-HPLC method)

Analyte	Amount Taken (µg/ml)	Amount Added (µg/ml)	Amount found (µg/ml)	Amount recovered (µg/ml)	% Recovery*	% RSD
EMT	20	16 20 24	36.011 39.899 44.411	16.011 19.899 24.411	100.0±1.1 99.4 ±0.8 101.7±0.3	1.20 0.85 0.56
TNF	30	24 30 36	53.911 60.029 66.177	23.911 30.029 36.177	99.6±0.6 100.0±1.1 100.4±0.5	0.63 1.10 0.49

The percent recovery was calculated by using following formula:

$$\%\text{Recovery} = \frac{\text{Amount Recovered}}{\text{Amount Added}} \times 100$$

$$\text{Amount recovered} = \frac{As \times D \times P}{AT \times 100}$$

Where, A_S = area of unknown
A_T = area of standard
D = dilution factor
P = Potency of Tenofovir disoproxil fumerate or Emtricitabine

$$\text{Amount added} = \frac{\text{Potency}}{100} \times \text{Weight of API Spiked}$$

Table-6 (LOD & LOQ)

	TDF	EMT
LOD (µg/ml)	0.1919119	0.6294184
LOQ (µg/ml)	0.5815496	1.9073285

3. Precision:

Standard Solution: Tenofovir disoproxil fumerate and Emtricitabine Standard Solution was used.

Sample Solution: Twenty tablets were accurately weighted and average was calculated. The tablets were then crushed to obtain a fine powder. An accurately weighted quantity of tablet powder equivalent to about 20 mg of Emtricitabine and 30mg of Tenofovir disoproxil fumerate was transferred to 100 mL volumetric flask, sonicated with 80 mL of diluent with intermediate shaking for 30 min. The volume was made up to the mark with diluent and the resulting solution was filtered.

Procedure: The chromatographic condition were set as per the optimized parameters and mobile phase was allowed to equilibrate with stationary phase, five replicate injection of standard solution and each of sample solutions were made separately and the chromatograms were recorded chromatograms of one of the sample solution is shown below and the results were shown in **the table 7 & 8.**

Amount of drug was calculated using formula

$$\textbf{Mg/unit of drug} = \frac{As \times D \times P \times AverageWeight}{AT \times 100}$$

Where,

A_S = area of unknown
A_T = area of standard
D = dilution factor
P = Potency of Emtricitabine or tenofovir disoproxil fumerate

The percentage label claim of was calculated using following formula:

$$\textbf{\% of Label Claim} = \frac{amountofdrug}{labelclaim} \times 100$$

4. Selectivity & Specificity:

The selectivity of the method was performed by injecting drugs solutions into the optimized system and it was observed that two sharp peaks of TNF and EMT were obtained at retention time of 2.84 and 3.55 min, respectively with reference to placebo solution. Comparing the chromatograms obtained from standard drugs, with the chromatogram obtained from tablet solutions, the specificity of the method was assessed. As the retention time of standard drugs and the retention time of the drugs in sample solution was same, so the method was specific. The developed method was found specific and selective, as there was no interference of excipients found.

Table-7 (Precision studies (TDF) interday & intraday)

Description	Mean area of TDF	% Assay	%RSD
Set 1	9184531	100.1	
Set 2	9189514	100.0	0.59
Set 3	9164099	99.7	
Set 4	9171008	100.0	
Set 5	9180098	100.9	
Set 6	9181987	101.2	

Table-8 (Precision studies (EMT) interday & intraday)

Description	Mean area of TDF	% Assay	%RSD
Set 1	1018811	100.0	
Set 2	1049110	101.974	0.96
Set 3	1010220	99.96198	
Set 4	1010334	99.96534	
Set 5	1028145	100.9833	
Set 6	1058123	101.9157	

Table-9 (System suitability parameters)

System suitability parameters			
	TDF	EMT	LIMITS
Retention time	2.85	3.55	-
Tailing factor	1.30	1.22	<2
Asymmetrical factor	1.44	1.33	<2
Capacity factor	1.82	4.37	>1
Theoretical plates	3626	5354	>2000

SUMMARY & CONCLUSION

RP-HPLC method for the simultaneous estimation of TDF & EMT was developed by studying different parameters. First of all, maximum absorbance was found to be at 270nm and the peak purity was excellent. Injection volume was selected to be 20µl which gave a good peak area. The column used for study was PHENOMENEX LUNA C18 chosen. Ambient temperature was found to be suitable for the nature of drug solution. The flow rate was fixed at 1.0ml/min because of good peak area and satisfactory retention time. Mobile phase of Acetonitrile : Phosphate pH 3.5 buffers in the ratio of 60:40 was fixed due to good symmetrical peak. So this mobile phase was used for the proposed study. Acetonitrile was selected because of maximum extraction and all the drug particles were completely soluble and showed good recovery. Run time was selected to be 5min because analyze gave peaks for TDF & EMT around 2.84 min and 3.55. After the development of the method, it was validated for accuracy, linearity, precision, robustness, LOD and LOQ studies. The precision of the System and method were checked and found to be within limits. This indicates that the method is precise. Linearity study, correlation coefficient and curve fitting was found to be 0.999. From the results shown in the accuracy table, it was found that recovery value of pure drug was between 99.4 % to 101.7 %.

This indicates that the developed method is accurate, precise and economical and also reveals that commonly used excipients and additives present in the pharmaceutical formulation were not interfering in the proposed methods.

It could be concluded from the results obtained in the present investigation that the method for the simultaneous estimation of TDF & EMT in tablet dosage form are simple, rapid, accurate, precise and economical and can be used, successfully in the quality control of pharmaceutical formulations and other routine laboratory analysis.

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