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A Validated RP - HPLC Method for Simulataneous Estimation of Emtricitabine and Tenofovir Disoproxil Fumarate in Pure and in Tablet Dosage Form

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ABSTRACT

A simple, rapid reverse - phase high performance liquid chromatographic method has been developed and validated for the simultaneous estimation of emtricitabine and tenofovir disoproxil fumarate in pure and in tablet dosage form. The estimation was carried out on a Phenomenax Luna C_{18} (150 mm x 4.6 mm i.d., particle size 5µm) column with a mixture of acetonitrile: methanol: water in the ratio of 30:50:20 (v/v) as mobile phase. UV detection was performed at 258 nm. The method was validated for linearity, accuracy, precision, specificity and sensitivity as per ICH norms. The developed and validated method was successfully used for the quantitative analysis of commercially available dosage form. The retention time was 2.77 and 3.49 min. for emtricitabine and tenofovir disoproxil fumarate, respectively. The flow rate was 0.6 mL/ min. The calibration curve was linear over the concentration range of 2 –12 µg mL^{-1} for emtricitabine and 1-6 µg/ mL for tenofovir disoproxil fumarate. The LOD and LOQ values were found to be 0.03003 and 0.091015 µg/ mL for emtricitabine and 1.4270 and 4.3243 µg/ mL for tenofovir disoproxil fumarate, respectively. The high percentage of recovery and low percentage coefficient of variance confirm the suitability of the method for the simultaneous estimation of emtricitabine and tenofovir disoproxil fumarate in pure and in tablet dosage form.

Keywords: Emtricitabine, Tenofovir disoproxil fumarate, RP-HPLC, Validation.

INTRODUCTION

Emtricitabine (EMT) is a nucleoside reverse transcriptase inhibitor (NRTIs). Chemically it is 5-fluoro-1- (2R,5S) - [2 - (hydroxymethyl) - 1,3 – oxathiolan – 5 -yl] cytosine. EMT is the (-)

enantiomer of thio analog of cytidine which differs from other cytidine analogs, in that it has a fluorine in 5 th position. EMT is an antiviral agent used for the prevention of perinatal HIV-1 reverse transcriptase [1]. It is also active against Hepatitis B virus [2, 3].Tenofovir disoproxil Fumarate (TDF) is fumaric acid salt of the bisisopropoxycarbonyl – oxymethyl ester derivative of tenofovir. Chemically it is 9 - [(R) - 2 - [[(isopropoxcarbonyl) - oxy] methoxy] phosphiny] ethoxy] propyl] adenine fumarate [1]. It is also the nucleotidereverse transcriptase inhibitor (NRTIs) used in combination with other antiretrovirals for the treatment of HIV infection [2]. Both the drugs are not official in any of the pharmacopoeias. These are listed in the Merck Index and Martindale: The complete drug reference. (Fig.1)

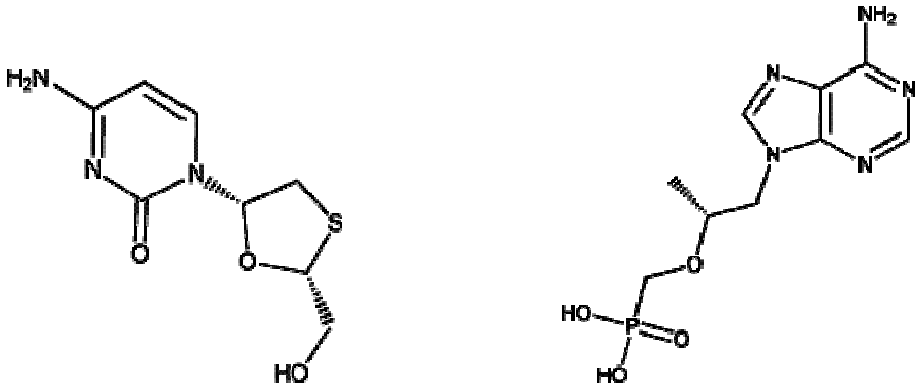

Fig 1.The chemical structures of EMT and TDF

Literature survey reveals that few RP-HPLC [4, 5, 6] methods are reported for estimation of EMT, TEN and efavirenz in pharmaceutical formulation. TEN is estimated individually by UV [7], derivative - HPLC [8], Plasma RP-HPLC [9,10] and Plasma LC/MS/MS [11,12,13] methods. Similarly for EMT, HPLC with Fluorometric detection [14] in human plasma and Stability indicating liquid chromatographic [15] methods were reported. HPLC [16] and LC-MS/MS [17] method is reported for simultaneous estimation of EMT and TEN in human plasma. HPTLC [18] method is also reported for simultaneous estimation of EMT and TEN in pharmaceutical formulation. But there is no method was reported for the simultaneous estimation of EMT and TEN in pure and in combined fixed dose combination RP – HPLC. Hence, the purpose of this study was to develop simple, rapid, precise and accurate RP – HPLC method for the simultaneous estimation of both the drugs in pure and in combined tablet dosage form.

MATERIALS AND METHODS

2.1 Apparatus

RP-HPLC was performed with a Shimadzu LC-10 AT $_{VP}$ solvent-delivery system, a Shimadzu SPD-10 A_{VP} UV–visible detector, and a Rheodyne 7725i universal loop injector of injection capacity 20 µL. The monitoring software was Winchrom. The equipment was controlled by a PC workstation. Compounds were separated on a Phenomenex Luna C_{18} column (150 mm × 4.6 mm i.d, 5-µm particle) under reversed-phase partition chromatographic conditions. Ultrasonicator Model Soltec – 2200 MH was used. The work was carried out in an air-conditioned room

maintained at temperature 25 ± 2°C. The flow rate was 0.6 mL/ min and the analytes were monitored at 258 nm.

2.2. Chemicals and Reagents

Working Standards of pharmaceutical grade EMT and TDF were obtained as gift samples from Strides Arcolabs Bangalore, India. The tablet dosage form, TENVIR, manufactured by Cipla Limited, Mumbai, India (Label claim: EMT 200 mg and TDF 300 mg), was procured from the local pharmacy. All the chemicals and reagents used were of HPLC grade and purchased from Qualigens Fine Chemicals, Mumbai, India.

2.3. Mobile phase

The mobile phase consisting of acetonitrile: methanol: water in the ratio of 30; 50; 20% v/v was prepared and degassed with ultrasonicator.

2.4. Standard stock solution and Construction of Calibration curve

Standard stock solution of EMT and TDF (25 mg of each) were prepared separately in 50 mL of mobile phase to get the concentration of 500 µg/ mL. From the standard stock solution of drugs, different dilutions were prepared, injected and their peak area was measured. After that, calibration curves were drawn between concentration against their respective peak area for EMT and TDF respectively. Unknown samples were determined by reference to these calibration curves.

2.5. Standard mixture solution

Mixed standard analysis was performed to validate the procedure. From the standard stock solutions of the drugs, different mixed standard solutions of 2:12, 4:10, 6:8, 8:6, 10:4, 12:2 of EMT and TDF respectively were prepared and analyzed, statistical results were within the range of acceptance i.e. %COV<2.0 and S.D.<1.0.

2.6. Sample preparation

For the analysis of tablet dosage form, twenty tablets (TENVIR) were weighed and their average weight was determined. The tablets were then crushed to a fine powder and the tablet powder equivalent to 25 mg of TDF was transferred to a 25 mL volumetric flask and dissolved in about 20 mL of methanol. The solution was shaken for 5 min. Sonicated for 15-20 min at 100 rpm and made up to the volume with methanol. The solution was filtered through Whatman filter paper # 41. This filtrate was further diluted with mobile phase to get the final concentration of 2 µg/ mL for EMT and 3 µg/ mL for TEN theoretically. 20 µL of the sample solution was injected for quantitative analysis. The identities of both the compounds were established by comparing retention times of the sample solution with those of standard mixed solution. The amount of EMT and TDF per tablet was calculated by extrapolating the peak area from the calibration curve. The results are reported in Table 1.

Table 1. Assay of Tablet Formulation

Drug	TENVIR - L Label Claim mg/ tab (n=6)	Amount Found		SD	%COV	SE
		mg	%			
EMT	200	199.56	99.78	0.7744	0.7761	0.0775
TDF	300	299.66	99.89	0.5979	0.5997	0.0598

S.D.: standard deviation, COV: coefficient of variance, S.E.: standard error.

RESULTS AND DISCUSSION

3.1. HPLC method development and optimization

Column chemistry, solvent type, solvent strength, detection wavelength and flow rate were varied to determine the chromatographic conditions giving the best separation. The mobile phase conditions were optimized so that the components were not interfered from the solvent and excipients. After trying column C_8 and C_{18}, the final choice of stationary phase giving satisfactory resolution and run time was the reversed phase column phenomenax Luna C_{18}. Mobile phase and flow rate selection was based on peak parameters (height, area, tailing, theoretical plates, capacity factor and resolution) and run time. The best result was obtained by use of 30: 50: 20 (v/v) ratio of acetonitrile,methanol and water with 0.6 mL/ min. From the overlain UV spectra (Shimadzu-1700), suitable wavelength considered for monitoring the drugs was 258 nm (Fig 2).

Solutions of EMT and TDF in diluents were also injected directly for HPLC analysis and the responses (peak area) were recorded. It was observed that there was no interference from the mobile phase or baseline disturbances and both the analytes absorbed well at 258 nm. The chromatogram of placebo and standard mixture is shown in Fig 3 and 4 respectively.

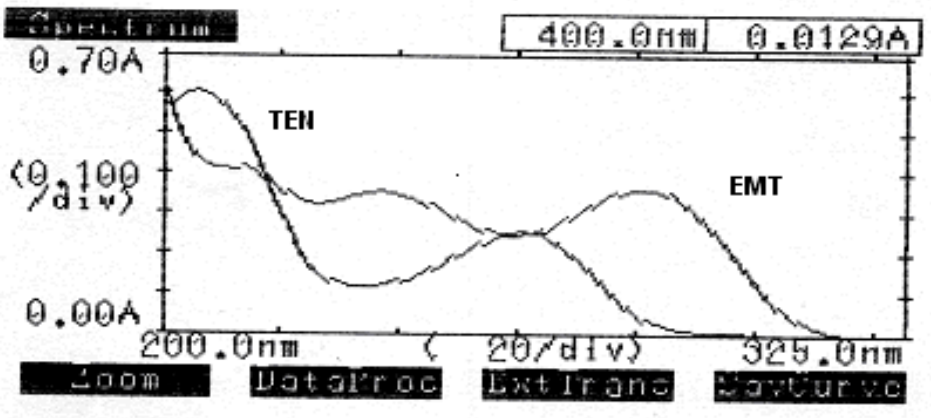

Fig 2. Overlain Spectra of EMT and TDF

Under the optimum chromatographic conditions, the retention time obtained for EMT and TDF were 2.77 and 3.48 min, respectively for sample preparation and is shown in Fig 5. The results of capacity factor, tailing factor, Number of theoretical plates and resolution are reported in Table 2. The values obtained for these properties ($1<k<10$, $Rs>2$) shows that, the chromatographic conditions are appropriate for separation and determination of compounds.

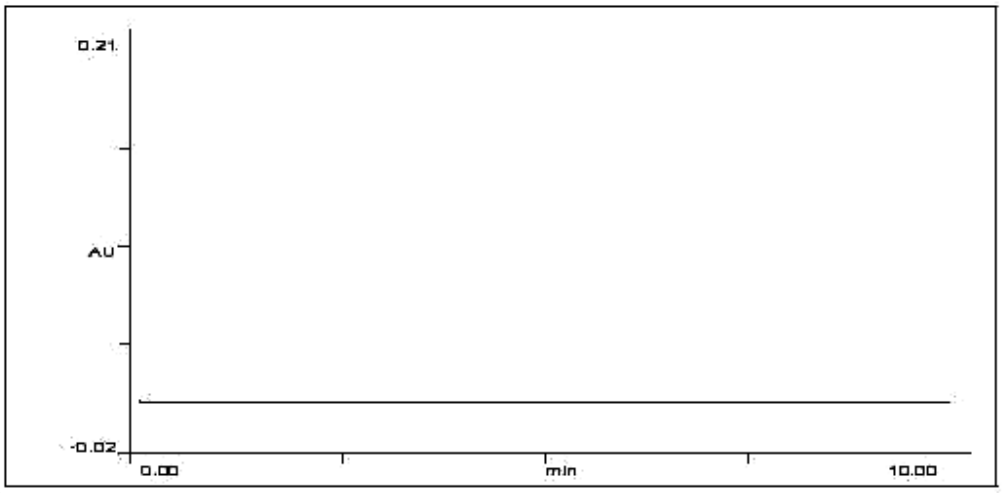

Fig 3. Chromatogram of Placebo

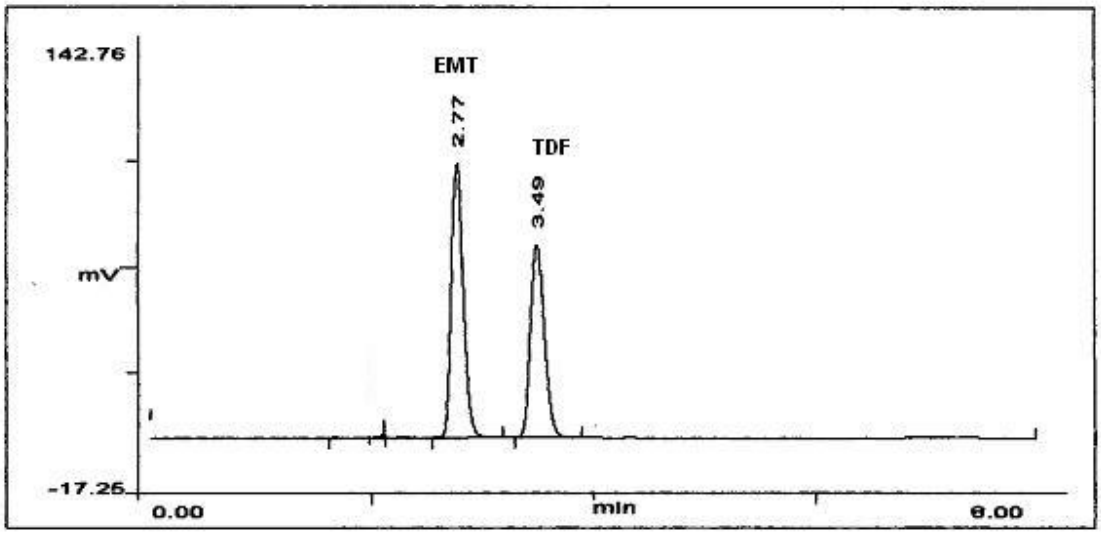

Fig 4. Chromatogram of mixed standard solutions

Table 2. System suitability parameters

Property	EMT	TDF
Rt	2.77	3.49
T f	1.24	1.21
As	1.41	1.37
K'	1.72	2.43
N	3092	3630
R s	2.05	

Rt-retention time; Tf - tailing factor; *k'*- capacity factor; N- number of theoretical plates; R_S- resolution

3.2. Validation of the developed method

The method was validated for linearity, accuracy, precision, repeatability, selectivity and specificity study as per ICH norms[19,20]. All the validation studies were carried out by replicate injection of the sample and standard solutions.

3.3. Linearity

Linearity was found to be 2 - 12 µg mL^{-1} for both EMT and TDF. The linear regression equations for EMT and TDF were

EMT y = 298567.59x + 3142.77 (n=6, r_2 = 0.9999)
TDF y = 936904.57x + (-6516.46) (n=6, r_2 = 0.9998)
Where *y* is response (peak area) and *x* is the concentration.

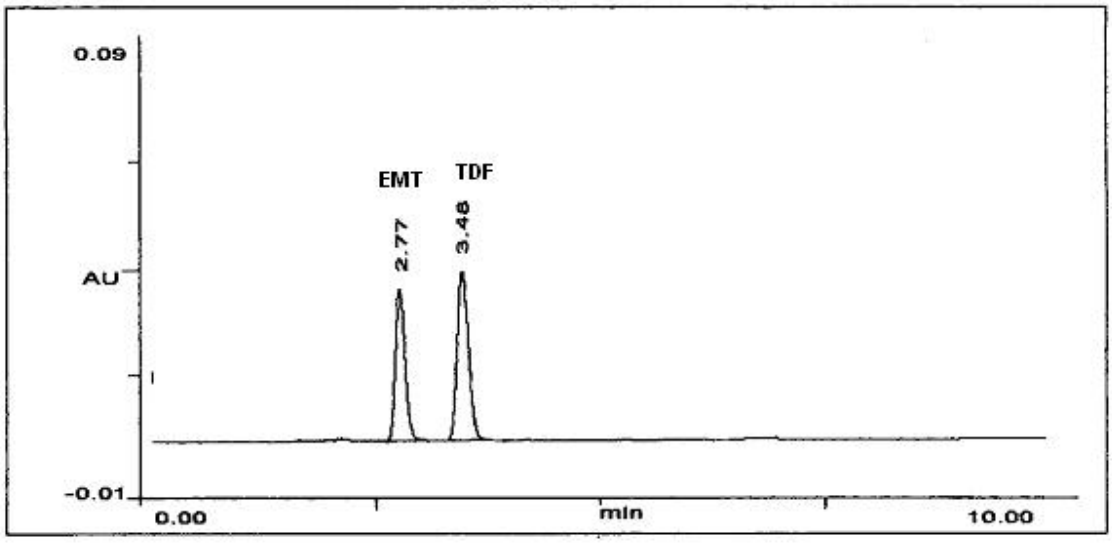

Fig 5. Chromatogram of EMT and TDF in sample solution with their retention time

3.4. Accuracy

Accuracy of developed method was confirmed by doing recovery study as per ICH norms at three different concentration levels by replicate analysis (n=3). The result of accuracy study was

reported in Table 3. From the recovery study it was clear that the method is very accurate for quantitative estimation of EMT and TDF in tablet dosage form as all the statistical results were within the range of acceptance i.e. %COV<2.0

3.5. Precision, Limit of Detection, and Limit of Quantitation

The concentrations of both the drugs were measured three times on the same day at intervals of 1 h and on three different days for intra and interday study respectively. LOD and LOQ were calculated by use of the equations LOD = 3.3σ/S and LOQ = 10σ/S, where σ is the standard deviation and S is the slope of the calibration curve. The results are reported in Table 4.

3.6. Selectivity and Specificity

The selectivity of the method was confirmed by injecting the solution of both the drugs into the system and it was observed that two sharp peaks of EMT and TDF having resolution of 2.05 were obtained at retention time of 2.77 and 3.49 min, respectively in reference to placebo solution.

Table 3. Recovery Studies

Drug	Amount Taken (µgmL-1)	Amount Added (µgmL-1)	% Recovery	% COV
EMT	1.9956	2 4 6	101.17 100.51 100.82	0.5704 0.4372 0.6101
TDF	2.9910	1 2 3	100.95 100.77 101.59	0.5595 0.2706 0.0715

COV: coefficient of variance

Table 4. Intra Day and Inter Day Precision, LOD and LOQ Studies

Drug	Intra day Precision % COV (n = 6)	Interday Precision % COV			LOD (µgmL-1)	LOQ (µgmL-1)
		Day 1	Day 2	Day 3		
EMT TDF	0.7738 0.6299	0.7561 0.2884	0.6753 0.5649	0.9773 0.5110	0.03003 1.4270	0.09105 4.3243

Mean of six determinations, COV: coefficient of variance, LOD: limit of detection, LOQ: limit of quantitation.

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.

CONCLUSION

A new, reversed-phase HPLC method has been developed for simultaneous analysis of EMT and TDF in a tablet formulation. It was shown that, the method was linear, accurate, reproducible, repeatable, precise, selective and specific proving the reliability of the method. The run time is relatively short, i.e. 5 min, which enable rapid determination of any samples in routine and quality control analysis of tablet formulations. The same solvent was used throughout the experimental work and no interference from any excipient was observed. Hence, the proposed method was successfully applied to analyze the tablet formulation containing EMT and TDF.

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