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Research Article

HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF EFAVIRENZ, EMTRICITABINE AND TENOFOVIR IN COMBINED DOSAGE FORM

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ABSTRACT

This paper describes the development and validation of a HPLC method for the quantization of Emtricitabine, Tenofovir, and Efavirenz in combined pharmaceutical formulations. The thermo C_{18} (250 × 4.6 mm, 5 µm) column was used. UV detection was performed at 254 nm. The mobile phase consisted of 10mM KH_2PO_4: Methanol (pH 3.0 with OPA) in the ratio of 20:80v/v, at the flow rate was 1.0 ml/min in ambient temperature. The injection volume of sample was 20 µl. The validity and reliability of proposed methods were assessed by recovery studies. The recovery of added standards (80 %, 100 % and 120 %) was found at three replicate and three concentrations level. The value of % means just close to 100, SD and % RSD are less than two, indicate the accuracy of method. The Simplicity, Rapidly and Reproducibility of the proposed method completely fulfill the objective of this research work.

Keywords: HPLC, Method development, Simultaneous, Validation, Emtricitabine, Tenofovir, Efavirenz.

1. INTRODUCTION

Around 33.4 million people were living with HIV in year 2008 and around 2 million people have died in the same year [1]. Atripla, a combination of a fixed dose of tenofovir, emtricitabine, and efavirenz was approved for the treatment of this disease by the Food and Drug Administration (FDA) on July 12, 2006. In the United States, Atripla was the first fixed dose formulation available to combine two distinct groups of antiviral drugs into a single tablet. Also available are several generic Atripla drugs, such as Viraday from Cipla Ltd. and Vonavir from Emcure Ltd. Efavirenz (EFV, brand names Sustiva and Stocrin) is a non-nucleoside reverse transcriptase inhibitor (NNRTI) and is used as part of highly active anti retroviral therapy (HAART) for the treatment of a human immune deficiency virus (HIV) type 1. Efavirenz is chemically described as (S)-6-chloro-(cyclopropylethynyl)-1,4-dihydro-4-(trifluoromethyl)-2H-3, 1-benzoxazin-2-one. Its empirical formula is C14H9ClF3NO2. 1. Efavirenz is a white to slightly pink crystalline powder with a molecular mass of 315.68 g/mol. It is practically insoluble in water (<10 µg/ mL) [2]. Emtricitabine (ETB) is a nucleoside reverse transcriptase inhibitor (NRTIs). Chemically it is 5-fluoro-1-(2R, 5S)-[2- (hydroxymethyl)-1,3-oxathiolan-5-yl] cytosine (fig. 1). FTC is the (-) enantiomer of thio analog of cytidine which differs from other cytidine analogs, in that it has fluorine in 5th position. FTC is an antiviral agent used for the prevention of perinatal HIV-1 reverse transcriptase [3]. It is also active against Hepatitis B virus [4, 5]. Tenofovir disoproxil fumarate (a prodrug of tenofovir), marketed by Gilead Sciences under the trade name Viread, belongs to a class of antiretroviral drugs known as nucleotide analogue reverse transcriptase inhibitors [6] (nRTIs), which block reverse transcriptase, an enzyme crucial to viral production in HIV infected people. *In vivo* tenofovir disoproxil fumarate is converted to tenofovir, an acyclic nucleoside phosphonate (nucleotide) analog of adenosine 5'-monophosphate. IUPAC is ({[(2R)-1-(6-amino-9H-purin-9-yl)propan-yl] oxy}methyl) phosphonic acid. Tenofovir belongs to a class of antiretroviral drugs known as nucleotide analogue reverse transcriptase inhibitors (NtRTIs) [7], which block reverse transcriptase; an enzyme crucial to viral production in HIV-infected people. Tenofovir is currently in late stage clinical trials for the treatment of hepatitis B. Tenofovir disoproxil fumarate is an acyclic nucleoside phosphonate diester analog of adenosine monophosphate. Tenofovir inhibits the activity of HIV reverse transcriptase by competing with the natural substrate deoxyadenosine 5'-triphosphate and, after incorporation into DNA, by DNA chain termination.

Specifically, the drugs are analogues of the naturally occurring deoxynucleotides needed to synthesize the viral DNA and they compete with the natural deoxynucleotides [8] for incorporation into the growing viral DNA chain. Various spectrophotometric [9-12], HPLC [13-22], HPTLC [23] methods are reported in the literature for the estimation of EFV individually and in combination with other drugs. However, no spectrophotometric method has yet been reported for simultaneous estimation of EFV, EMT, and TDF in pharmaceutical dosage forms. The methods mentioned in the literature, especially the chromatographic techniques, are time-consuming, costly, and require expertise. A simple, accurate, cost effective high performance liquid chromatography method development can be highly useful for the routine analysis of synthetic mixture formulations. Hence, an attempt has been made to develop and validate in accordance with ICH guidelines [24].

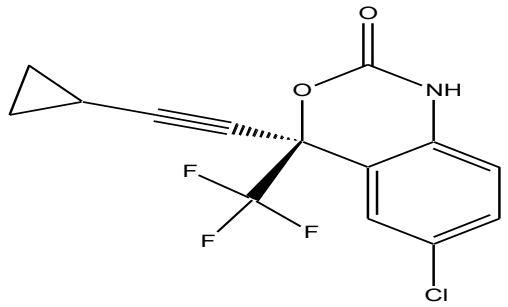

Fig. 1: Structure of Efavirenz

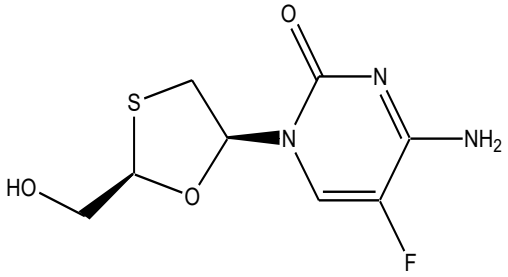

Fig. 2: Structure of Emtricitabine

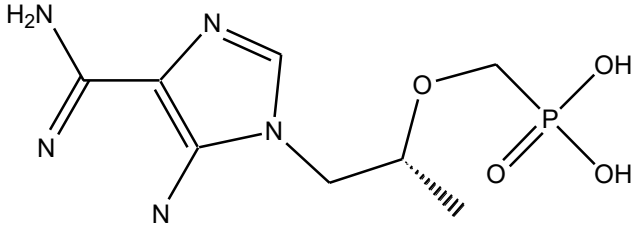

Fig. 3: Structure of Tenofovir

2. MATERIAL AND MATHODS

2.1. Reagents and chemicals

Reference standard of EFV, EMT and TDF was obtained from Scan Research Laboratories, Bhopal. Methanol, HCl was procured from Rankem, RFCL Limited, New Delhi, India. NaOH was procured from Himedia laboratory Pvt. Ltd. All solvents and reagents were of analytical grade. All the solutions were protected for light and were analyzed on the day of preparations. Triple distilled water was generated in house. Distilled water was obtained by Mili Q apparatus by Millipore (Milliford, USA) for whole experimental work.

2.2. Instrument

Liquid chromatographic system from Waters model no 784 comprising of manual injector, water 515 binary pump for constant flow and constant pressure delivery and UV-Visible detector connected to software Data Ace for controlling the instrumentation as well as processing the generated data.

2.3. Method development

2.3.1. Selection of Mobile Phase

Initially to estimate EFV, EMT and TDF in fix dosage form number of mobile phase in different ratio were tried. Taking into consideration the system suitability parameter like RT, Tailing factor, No. of theoretical plates and HETP, the mobile phase found to be most suitable for analysis was 10mM KH_2PO_4: Methanol (pH 3.0 with OPA) in the ratio of 20:80v/v. The mobile phase was filtered through 0.45µ filter paper to remove particulate matter and then degassed by sonication. Flow rate employed for analysis was 1.0 ml/min.

2.3.2. Linearity and Calibration Graph

To establish the linearity of analytical method, a series of dilution ranging from 5-25 µg/ml for EFV, 1-5µg/ml for EMT and 1-5µg/ml for TDF were prepared. All the solution were filtered through 0.2µm membrane filter and injected, chromatograms were recorded at 254 nm and it was repeat for three times. A calibration graph was plotted between the mean peak area and respective concentration and regression equation was derived.

2.3.3. Specificity

Specificity of the method was carried out to assess unequivocally the analyte presence of the components that might be expected to be present, such as impurities, degradation products and matrix components fig.4 and 5.

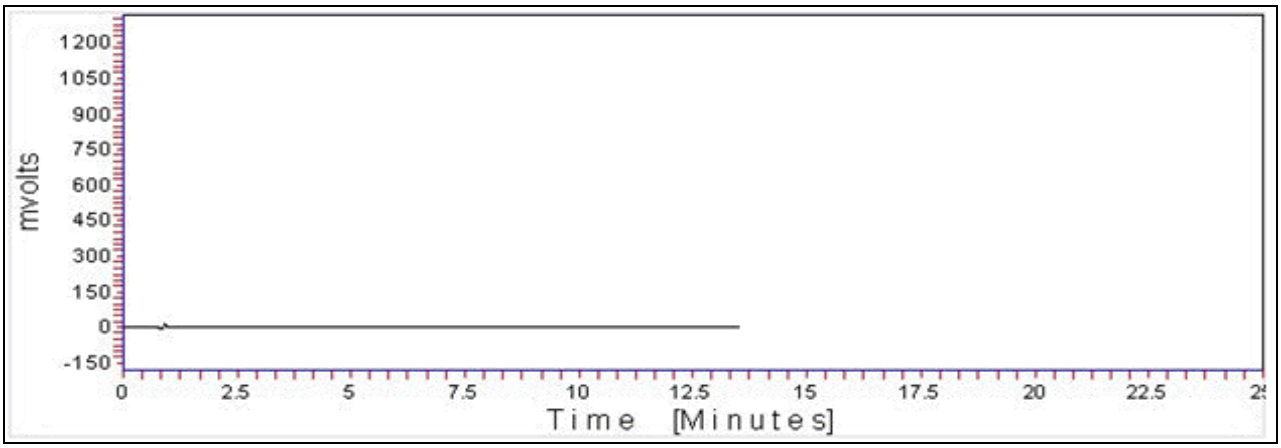

Fig. 4: Chromatogram of Blank

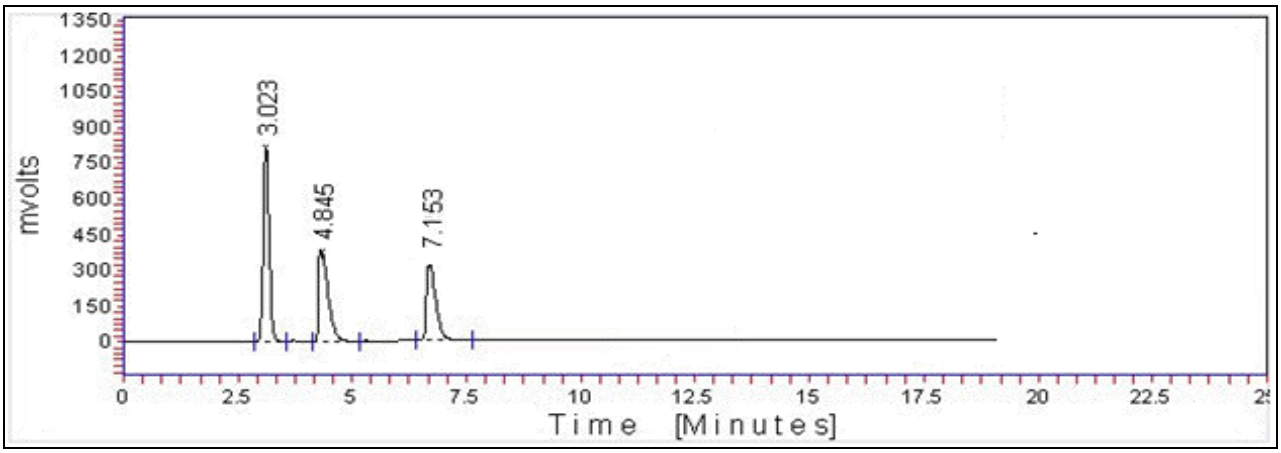

Fig. 5: Chromatogram of EFV, EMT, TDF

2.3.4. Accuracy

The accuracy of the proposed methods was assessed by recovery studies at three different levels i.e. 80%, 100%, 120%. The recovery studies were carried out by adding known amount of standard solution of EFV, EMT and TDF to preanalysed tablets powder. The resulting solutions were then re-analysed by proposed methods. Whole analysis procedure was repeated to find out the recovery of the added drug sample. This recovery analysis was repeated at 3 replicate of 5 concentrations levels.

2.3.5. Precision

Precision of the methods was studied at three level as at repeatability, intermediate precision (Day to day and analyst to analyst) and reproducibility. Repeatability was performed by analyzing same concentration of drugs for five times. Day to day was performed by analyzing 5 different concentration of the drug for three days in a week.

2.3.6. Detection Limit and Quantitation Limit

The LOD and LOQ of developed method were calculated based on the standard deviation of response and slope of the linearity curve.

2.4. Analysis of tablets formulation

Tablet powders were weighed and ground to a fine powder; amount equal to 30mg of EFV (10mg EMT and 12.2 mg TDF) was taken in 10 ml volumetric flask. Five ml of methanol was then added and the flask was sonicated for about 10 min to solubilize the drug present in tablet powder and the volume was made up to the mark with methanol. After sonication, filtration was done through 0.45µ membrane filter. Filtrate was collected and further diluted with methanol to get the final concentrations of both drugs in the working range. The mean area of final dilutions was observed the concentrations were obtained from calibration curve method. The procedure was repeated for five times.

3. RESULTS AND DISCUSSION

The linearity of analytical method was carried out to check its ability to elicit test results that are proportional to the concentration of analyte in sample within a given range. Different levels of standard solutions were prepared and estimated by HPLC and the results were recorded. The results of linearity are reported in table 1. The validity and reliability of proposed methods were assessed by recovery studies. The recovery of added standards (80%, 100% and 120%) was found at three replicate and three concentrations level. The value of % means just close to 100, SD and % RSD are less than 2 indicate the accuracy of method. Result of recovery study is shown in table 2. Precision was determined by repeatability and intermediate precision of drug. Repeatability result indicates the precision under the same operating condition over short interval of time. The intermediate precision study is expressed within laboratory variation on different days and analyst to analyst variation by different analyst. The value of SD and %RSD less than 2 indicate the precision of method. Result of precision is shown in table 3. The results of the analysis of tablets formulation were reported. The assay value of drugs was close to 100, SD and % RSD less than 2 indicate the no interference of excipient in the estimation of drugs (table 5). The LOD and LOQ of developed method were calculated based on the standard deviation of response and slope of the linearity curve table 4.

Table 1: Results of linearity of Efavirenz (EFV), Emtricitabine (EMT) and Tenofovir disoproxil fumarate (TDF)

Parameter	EFV	EMT	TDF
Concentration (µg/ml)	5-25	1-5	1-5
Correlation Coefficient (r^2)*	0.999	0.999	0.999
Slope (m)*	44.22	28.58	21.24
Intercept (c)*	5.075	2.312	-2.120

**Value of three replicates*

Table 2: Results of recovery study

% Level	% MEAN±SD*		
	EFV	EMT	TDF
80%	98.75±0.507	99.15±0.223	98.56±1.498
100%	99.59±0.415	98.26±0.791	98.09±1.192
120%	99.21±0.404	99.48±1.197	99.91±0.956

***** *Value of three replicate and five concentrations.*

Table 3: Results of precision

Parameter	% MEAN±SD*		
	EFV	EMT	TDF
Repeatability	98.737±0.116	97.043±0.081	96.563±0.093
Intermediate precision			
Day to day precision	99.371±0.059	97.716±0.039	96.889±0.069
Analyst-to-Analyst	99.365±0.055	96.835±0.082	97.250±0.057
Reproducibility	98.736± 0.135	96.535±0.066	97.012±0.050

** Value of five replicate and five concentrations*

Table 4: LOD and LOQ of EFV, EMT and TDF

Name	LOD (µg/ml)	LOQ (µg/ml)
EFV	0.95	2.75
EMT	0.015	0.045
TDF	0.020	0.060

Table 5: Assay of tablets formulation

	% Concentration Found		
	EFV	**EMT**	**TDF**
Replicate 1	99.28	98.34	97.38
Replicate 2	99.47	97.2	98.37
Average	99.38	97.77	97.88
S. D.	0.134	0.806	0.700
% RSD	0.135	0.824	0.715

4. CONCLUSION

Liquid chromatographic system from waters comprising of manual injector, waters 515 pump for constant flow and constant pressure delivery and U.V. Vis. detector connected to data ace software for controlling the instrumentation as well as processing the data generated were used. Drug sample was extracted by precipitating method using 5ml of methanol for each ml of plasma sample. The proposed methods were found to be linear with correlation coefficient close to one. Precision was determined by repeatability, Intermediate precision and reproducibility of the drugs. The robustness of developed method was checked by changing in the deliberate variation in solvent. The result obtained shows the developed methods to be Cost effective, Rapid (Short retention time), Simple, Accurate (the value of SD and %RSD less than 2), Precise and can be successfully employed in the routine analysis of these drugs in bulk drug as well as in tablet dosage form. The Simplicity, Rapidly and Reproducibility of the proposed method completely fulfill the objective of this research work.

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