

STABILITY INDICATING METHOD FOR THE SIMULTANEOUS ESTIMATION OF TENOFOVIR, EMTRICITABINE AND EFAVIRENZ IN PURE AND PHARMACEUTICAL DOSAGE FORM BY RP-HPLC

**Dr.Srinivasa Rao A¹, Naveen Kumar G²,
Srilekha K³, Dr. Aruna Kumari N⁴**

^{1,2,3} *Department of Pharmaceutical Analysis and Quality Assurance,*

Shri Vishnu College of Pharmacy, Vishnupur, Bhimavaram, West Godavari-D.T.,

Andhra Pradesh, (India)

⁴ *GIET(A), Rajamundry, Andhra Pradesh, (India)*

ABSTRACT

*A new simple, sensitive and stability indicating-HPLC method for the determination of Emtricitabine, Tenofovir and Efavirenz in pure and pharmaceutical dosage form was developed. Chromatographic separation was carried on Thermosil C18 (100*4.6 mm, 5 μ), with a mobile phase composed of Methanol and pH 7 Triethylamine (70:30 V/V) at an absorption maxima 260 nm. Linearity for detector response was observed in the concentration range of 10-50 μ g/ml for Emtricitabine, 15-75 μ g/ml for Tenofovir and 10-50 μ g/ml for Efavirenz of test concentration. Correlation coefficient found to be 0.999. Retention time was found to be 3.706 min-Emtricitabine, 4.632 min-Tenofovir and 8.121 min-Efavirenz. Percent recovery studies were found in the range 99.0 – 101.0 % of test concentration. Drug product was exposed to acid, base, heat, oxidation and photolytic stress conditions and the samples were analysed by the proposed validated method. Results of the analysis were validated statistically and by recovery studies. The developed method was found to be precise for the determination of Emtricitabine, Tenofovir and Efavirenz pure and its pharmaceutical dosage form.*

Keywords: *Emtricitabine, Tenofovir, Efavirenz, RP-HPLC, Stress degradation.*

I. INTRODUCTION

Antiviral drugs are a class of medication used specifically for treating viral infections. Like antibiotics and broad-spectrum antibiotics for bacteria, most antivirals are used for specific viral infections, while a broad-spectrum antiviral is effective against a wide range of viruses. Unlike most antibiotics, antiviral drugs do not destroy their target pathogen; instead they inhibit their development. Anti-retroviral therapies are medications that treat HIV (Human Immunodeficiency Virus). The drugs do not kill or cure the virus. However, when taken in combination

they can prevent the growth of the virus. Antiretroviral drugs are referred to as ARV. Combination ARV therapy (cART) is referred to as highly active ART (HAART).

Tenofovir (TDF), is $\{[(2R)-1-(6\text{-amino-9}H\text{-purin-9-yl})\text{propan-2-yl}]\text{oxy}\}\text{methyl}\}$ phosphonic acid. It is a drug under Anti-HIV agents, mainly used with other HIV medications to help control HIV infection; it mainly acts on reverse transcription enzyme and prevents formation of 5'- to 3'-phosphodiester linkage essential for DNA chain elongation. It causes premature termination of DNA transcription. TDF typical dose is 150-300 mg in the tablet and oral powder. Chemically emtricitabine (ETC) is, 4-amino-5-fluoro-1-[(2R,5S)-2-(hydroxyl methyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one, acts by inhibiting reverse transcriptase enzyme. It helps in lowering the amount of viral load and can indirectly increase the number of immune system cells (called T cells or CD4^+ T-cells) in patient's body. ETC typical dose is 200 mg (capsules), 10 mg/ml (oral solutions). Efavirenz (EFV), is (4S)-6-chloro-4-(2-cyclopropylethynyl)-4-(trifluoromethyl)-2,4-dihydro-1H-3,1-benzoxazin-2-one (fig. 1). It is a non-nucleoside reverse transcriptase inhibitor (NNRTI) of HIV virus type-I. Main action of Efavirenz is through non-competitive HIV-I reverse transcriptase. Efv typical dose is 200 mg, 600 mg, 50 mg in the dosage forms of tablets and capsules.

Literature review reveals that there are few analytical method reported for the analysis of Tenofovir, Emtricitabine and Efavirenz by simultaneous estimation using RP-HPLC. Spectrophotometer, HPLC and HPTLC ^[1-4] are the reported analytical methods for compounds either individually or in combination with other dosage form ^[5-10]. Present work is aimed to develop a new, simple, fast, rapid, accurate, efficient stability indicating and reproducible RP-HPLC method for the simultaneous analysis of tenofovir, emtricitabine and efavirenz.

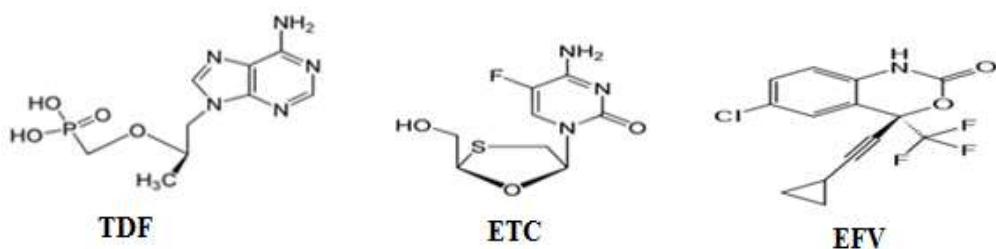


Fig. 1: Structures of Tenofovir (TDF), Emtricitabine (ETC), Efavirenz (EFV)

II. MATERIALS and METHODS

Chemicals and reagents

TDF, ETC, Efv gift samples were obtained from M/s Pharmadeep Remedies, Hyderabad, India. HPLC grade methanol and other analytical grade reagents were purchased from Merck, India. Water-HPLC grade was prepared using Milli-Q water purification system. ATRIPLA® tablets were purchased from local market of Hyderabad. Class A glassware is used throughout the experiment.

Equipment and chromatographic conditions

WATERS HPLC ALLIANCE-2695 with UV-Visible detector was used for method development, forced degradation studies and method validation. Chromatographic separation was carried out using Thermosil C18 (100*4.6 mm, 5 μ) in this method, 0.8 ml/min flow rate was used with detection wavelength at 260 nm, the injection volume was 10 μ l and runtime was set to 20 min and temperature was maintained at 30 °C.

Preparation of Solutions

Stock solution

Accurately weigh and transfer 200 mg of Emtricitabine and 300 mg of Tenofovir and 600 mg of Efavirenz working standard into a 100 ml clean dry volumetric flask add about 70 ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. Further pipette 1.5 ml of the above stock solution into a 100 ml volumetric flask and dilute up to the mark with diluent. The concentrations of ETC and EFV varied in the range of 10-50 μ g/ml each and of TDF is 15-75 μ g/ml. The calibration curves for LC analysis were constructed by plotting the peak area of the drug to that corresponding drug concentration.

Mobile Phase

The HPLC grade solvents were used for the preparation of mobile phase. pH 7 triethylamine (1 ml in 250 ml water, adjust to pH 7 with ortho-phosphoric acid). Mobile phase was prepared by mixing 70 ml of methanol and 30 ml of pH 7 triethylamine. The mobile phase was filtered through 0.45 μ membrane filter and then sonicated for 30 min.

Calibration standards and Quality controls

Calibration standards of ETC, TDF and EFV were prepared at concentrations of 10-50 μ g/ml, 15-75 μ g/ml and 10-50 μ g/ml respectively from a standard solution by appropriate dilution with mobile phase.

Generation of stressed samples for establishment of the stability-indicating assay:

Degradation studies were performed with stress conditions of acid and base hydrolysis, oxidation, photolysis and thermal degradation to evaluate the ability of the proposed method to separate ETC, TDF and EFV from their degradation products ^[11]. The reaction was carried out at concentrations of 30 μ g/ml of Emtricitabine, Tenofovir and Efavirenz. The stressed conditions were as followed acidic hydrolysis-drug solution in 1N HCl and kept for 10 h at 60 °C; alkaline hydrolysis-drug solution in 1N NaOH and kept for 10 h at 60 °C; Oxidative conditions-drug solution in 3% v/v hydrogen peroxide solution and kept for 24 h; Photolytic degradation-drug solution is exposed to UV light for 6 h; Thermal degradation-solid drug exposed at 80 °C for 60 min and at 220 °C for 5 min.

III. VALIDATION OF THE DEVELOPED METHOD

The method validation was done by evaluating system suitability, linearity & range, specificity, selectivity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), ruggedness and robustness as indicated in the ICH guidelines ^[12-17]. A similar method validation protocol was followed for all drugs.

System suitability

The system suitability was assessed by six replicates analyses of both drugs at concentrations 30 $\mu\text{g}/\text{ml}$ each of ETC, TDF and EFV. The % CV of peak area and retention time for all the drugs are within 2% indicating that the suitability of the system (fig. 3 and table 1) ^[12].

Specificity

Specificity is the ability of the method to measure the analyte response in the presence of other drugs, excipients and their potential impurities (fig.4) ^[13-17].

Linearity

Linearity of the method was established by triplicate injections of the solutions containing the drugs in the range of 10-50 $\mu\text{g}/\text{ml}$ for ETC and EFV; 15-75 $\mu\text{g}/\text{ml}$ for TDF. The calibration curves were constructed and the acceptable fit to the linear regression was demonstrated and reported by the necessary parameters (fig. 5, table 2) ^[13-17].

Accuracy and Precision

To determine the precision, triplicate injections of selected concentrations were analyzed, and the values of %RSD were calculated. In order to demonstrate applicability and accuracy of the proposed method, recovery tests are also carried out by analyzing the synthetic mixtures of the ETC, TDF and EFV. After five repeated experiments, the recoveries from these synthetic mixtures were calculated for each compound (table 3).

Assay of ATRIPLA® tablets

Twenty ATRIPLA® tablets (200 mg of ETC, 300 mg of TDF and 600 mg of EFV) made into fine powder, an equivalent weight of powder containing 200 mg of ETC, 300 mg of TDF and 600 mg of EFV was accurately weighed (1462 mg) and transfer into a 100 ml clean dry volumetric flask and add about 70 ml of diluent and sonicate to dissolve it completely. The clear supernatant liquid was sonicated in ultrasonic bath for 5 minutes and filtered through 0.45 μm membrane filter; final volume was made up with diluent. Further pipette 1.5 ml of the above stock solution into a 100 ml volumetric flask and dilute up to the mark with diluent. The sample solutions were prepared within the linearity range of both the drugs using same mobile phase.

IV. RESULTS

Method Development and Optimization

The standard solutions of ETC, TDF and EFV were scanned in UV-Visible Spectrometer and the λ_{max} of ETC, TDF and EFV were found to be 241, 247 and 260 nm respectively. The iso-absorptive point of the combined spectrum of all the drugs at 260 nm was chosen for the detection of the drugs. Different permutations and combinations at different pH (3 to 11), using various columns [Hypersil-BDS-C18, Thermosil C18, Symmetry C18, Sperisorb C18, Phenomenex C18], different buffers using ammonium acetate, ortho phosphoric acid, acetic acid and potassium dihydrogen phosphate along with acetonitrile and methanol were used as mobile phase for optimizing the method along with some peak modifiers. Efficient separation with good resolution factors obtained with Thermosil C18 column, methanol:pH 7 triethylamine (70:30 v/v) as mobile phase, at a

flow rate of 0.8 ml/min. Under these conditions ETC, TDF and EFV were eluted at 3.706, 4.632 and 8.121 min respectively with a run time of 5 min. A chromatogram of ETC, TDF and EFV was shown in fig. 2.

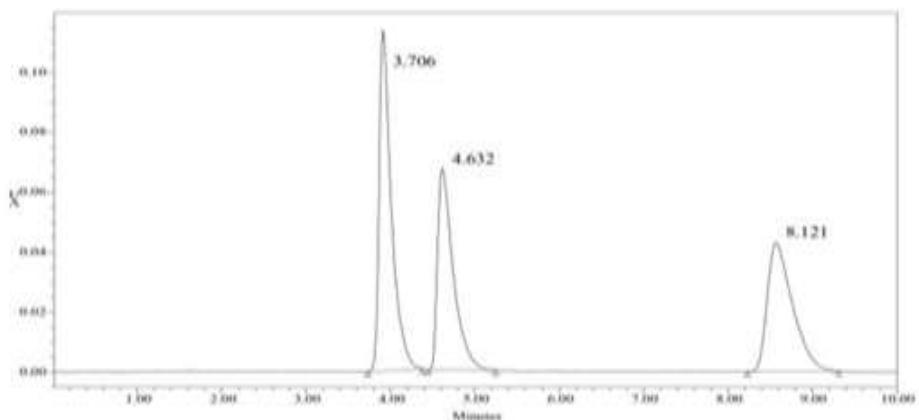


Fig. 2: Chromatogram of optimised method

System suitability

The U.S. Pharmacopeia (USP) suggests that system suitability tests be performed prior to analysis. System suitability for the proposed method was evaluated. A system suitability test can be defined as a test to ensure that the method can generate results of acceptable accuracy and precision. The requirements for system suitability are usually designed after method development. And also the method developed has been validated as per ICH guidelines. The parameters include tailing factor, retention factor, theoretical plate number, retention time, asymmetry factor, selectivity factor and RSD of peak height or area for repeated injections. Typically, at least two of these criteria are required to demonstrate system suitability for the proposed method. Some of the tests were carried out on the freshly prepared standard solutions including three drugs. System suitability test results are reported in fig. 3 and table 1 and found to satisfy the USP requirements.

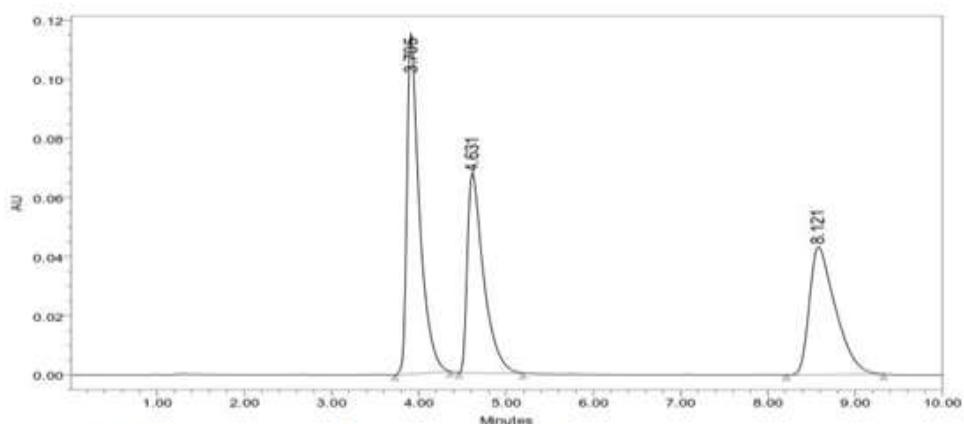


Fig. 3: Chromatogram of system suitability

Table 1: System suitability studies of the proposed RP-LC method

System Suitability Parameters	Compounds			Recommended value
	Emtricitabine	Tenofovir	Efavirenz	
Tailing factor (Tf)	1.8	1.7	1.6	<2
Resolution (Rs)	2.2	8.4	-	> 2
Retention time (Rt), (min)	3.706	4.632	8.121	-
Theoretical plates (N)	2903	2013	2788	>2000
%RSD (for retention time)	1.89	1.75	1.81	<2

*Average of six injections each.

Specificity

The specificity of the method is performed by separate injections of the blank, ETC, TDF and EFV and combined ETC, TDF and EFV samples. The specificity chromatogram was shown in fig. 4, where there is no interference in their retention times was found in between these three drugs.

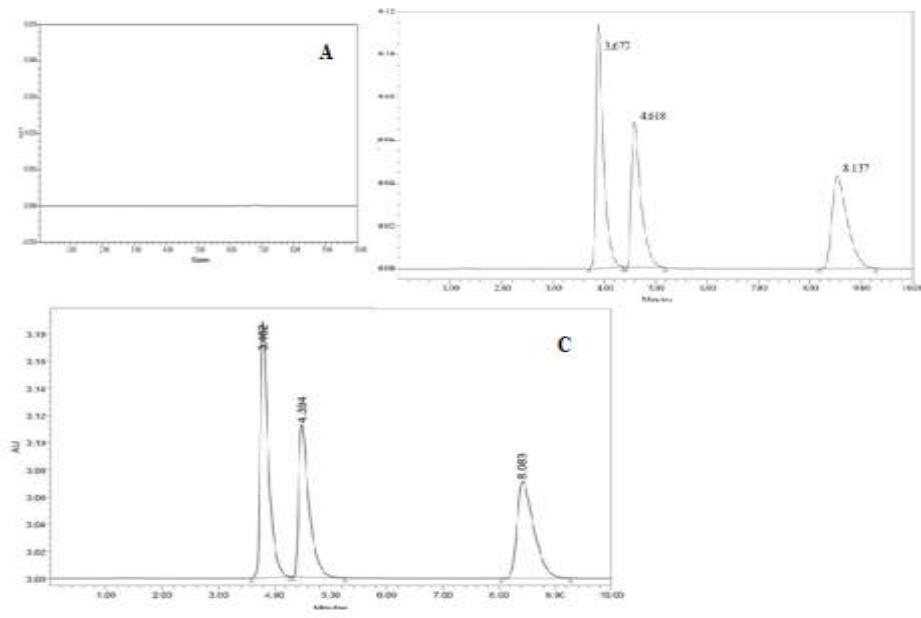


Fig. 4: Typical chromatograms: A) Blank B) Standards C) Samples

Linearity

Linearity was established least squares linear regression analysis of the calibration curve. The constructed calibration curves were linear over the concentration range of 10-50 $\mu\text{g/ml}$ for Emtricitabine, 15-75 $\mu\text{g/ml}$ for Tenofovir and 10-50 $\mu\text{g/ml}$ for Efavirenz. Peak areas of ETC, TDF and EFV were plotted versus their respective concentrations in the mobile phase, and linear regression analysis performed on the resultant curves and was confirmed by the high value of the correlation coefficient 0.999 for the three drugs (table 2). Calibration curves of ETC, TDF and EFV were shown in fig. 5.

Table 2: Statistical evaluation of the calibration data of ETC, TDF and EFV by RP-LC

Parameters	Compounds		
	ETC	TDF	EFV
Linearity range, $\mu\text{g/ml}$	10-50	15-75	10-50
Slope	12066	13566	12570
Intercept	7854	6754	8564
Correlation coefficient	0.999	0.999	0.999
LOD, $\mu\text{g/ml}$	2.96	3.0	3.09
LOQ, $\mu\text{g/ml}$	9.96	10.04	10.09
Precision (RSD %)	0.49	0.53	0.15

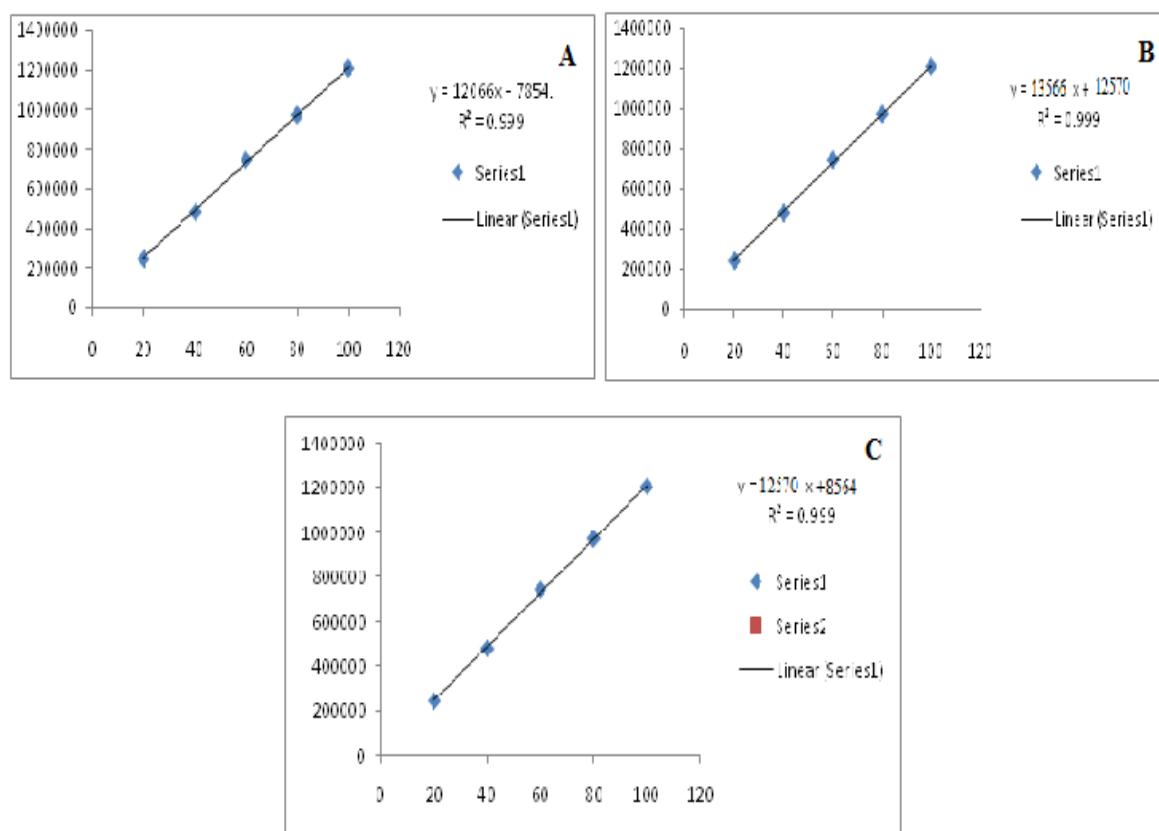


Fig. 5: Linearity graph: A) ETC B) TDG C) EFV

Limit of Detection and Limit of Quantification (LOD & LOQ)

The parameters LOD & LOQ were calculated from signal-to-noise ratio. The limit of detection (LOD) value was found to be 2.96, 3.0 and 3.09 $\mu\text{g/ml}$, and limit of quantification (LOQ) was 9.96, 10.04 and 10.09 $\mu\text{g/ml}$ for ETC, TDF and EFV respectively. The results were shown in table 2.

Accuracy

Accuracy study was performed by making three different standard concentrations at 50%, 100% and 150% levels of known amounts of studied drugs and their percentage recovery and % RSD was calculated. All the data

were within the acceptance criteria of 5% and the % recovery was found to be 100.5%, 99.8% and 100.1% for ETC, TDF and EFV respectively (table 3).

Precision

Precision data is summarized in table 3 and was assessed by using quality control samples prepared at three different concentrations (LQC, MQC and HQC) in the mobile phase. Precision was investigated by injecting triplicate aliquots of the samples. All the data were within the acceptance criteria of 5%.

Table 3: Accuracy and precision data

	LQC			MQC			HQC		
	ETC	TDF	EFV	ETC	TDF	EFV	ETC	TDF	EFV
Mean	465505	358821	363176	948732	725760	738275	138724	106045	109893
SD	5029	7065	3107	2028	59594	3078	17525	15130	4596
% RSD	1.11	0.19	0.85	0.21	0.82	0.41	1.26	1.42	0.41
% Recovery	98.6	102.0	99.4	98.9	99.5	98.5	100.0	100.0	101.3

*Average of three injections each

Recovery

The recovery analysis results were shown in table 3. The results of the recovery assay indicated that the method was selective, accurate and precise for ETC, TDF and EFV, without interference of excipients, used to formulate and produce the tablet dosage form studied.

Ruggedness

Ruggedness was determined by using the data obtained by the analysis performed by two different analysts. Each analyst prepared three samples of the same batch and the results obtained are shown in table 4.

Table 4: Ruggedness studies of ETC, TDF and EFV

	Drug	Retention time (min)	Tailing Factor	Theoretical plates
Analysis 1	ETC	3.636	0.49	2903
	TDF	4.472	1.15	2013
	EFV	8.112	1.6	2788
Analyst 2	ETC	3.656	1.2	2829
	TDF	4.595	1.7	3615
	EFV	8.096	1.6	2675

*Average of three injections each

Robustness

Robustness is the measure of method capacity to retain unaffected by deliberate small changes in the chromatographic conditions. The impact of flow rate ($\pm 0.1\text{mL}$) and effect of mobile phase composition ($\pm 5\%$) was evaluated on the important system suitability factors such as retention time; theoretical plates and tailing factor were studied. Results were shown in table 5.

Table 5: Robustness studies of ETC, TDF and EFV

Parameters	Variation	Retention time (min)	Tailing factor	Theoretical plates
ETC	Flow rate	0.7 ml/min	4.4112	3354.35
		0.9 ml/min	3.125	2974
	Mobile phase	75% organic phase	2.909	1851
		65% organic phase	2.345	578
TDF	Flow rate	0.7 ml/min	4.956	5567
		0.9 ml/min	4.175	2132
	Mobile phase	75% organic phase	4.881	1228
		65% organic phase	4.644	1668
EFV	Flow rate	0.7 ml/min	9.214	6587
		0.9 ml/min	7.254	4120
	Mobile phase	75% organic phase	10.541	2021
		65% organic phase	21.400	5643

*Average of three injections

Degradation Studies:

Degradation studies were performed by exposing the drug solution to different stress conditions (acidic, alkaline, H_2O_2 , U.V light and heat). Possible degradation products were observed in emtricitabine, tenofovir and efavirenz and peak purity test results were derived which were given in fig. 6 and table 6.

Table 6: Results for Stability studies

Stress Condition	Purity Angle			Purity Threshold		
	ETC	TDF	EFV	ETC	TDF	EFV
Acid Degradation	0.441	0.276	0.167	1.073	1.245	1.083

Alkali Degradation	0.531	1.495	0.213	1.096	2.662	1.179
Oxidative Degradation	0.202	0.417	0.136	0.286	0.659	0.276
Photolytic Degradation	0.192	0.153	0.096	0.274	0.310	0.265
Thermal Degradation	0.202	0.417	0.136	0.286	0.659	0.276

*Each value is the mean of three experiments

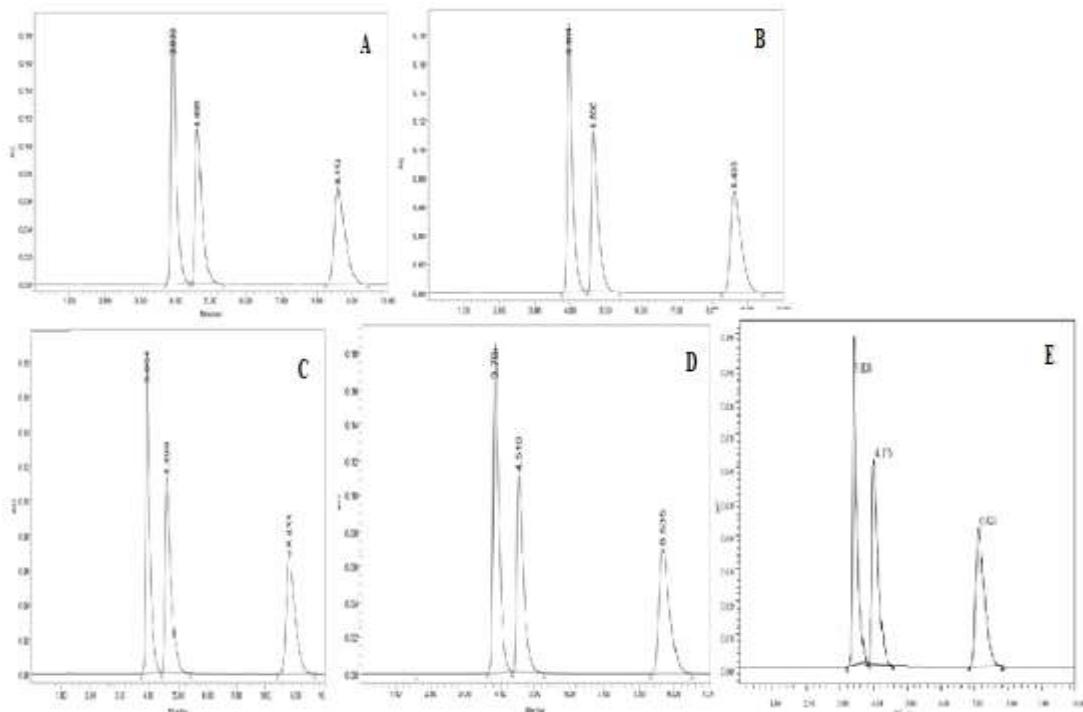


Fig.6: Typical degradation chromatograms of ETC, TDF and EFV: A) Acid hydrolysis B) Base hydrolysis C) Oxidation D) UV light E) Thermal

Assay of Atripla® tablets:

After working on synthetic mixtures, results encouraged that the use of the proposed method for the simultaneous assay of ETC, TDF and EFV in a commercial tablet dosage form. The results corresponding to the tablet dosage form of ETC, TDF and EFV are shown in Table 7. The proposed RP-LC method could be used for the simultaneous determination of ETC, TDF and EFV in the presence of each other and without prior separation of the excipients. Each tablet contained 200 mg of ETC, 300 mg of TDF equivalent to tenofovir disoproxil fumarate and 600 mg of EFV and the inactive ingredients. Removal of the excipients before analysis was found to be unnecessary. Fig. 7 shows a typical chromatogram obtained for the analysis of ETC, TDF and EFV in the tablets with well-shaped, symmetrical single peaks, well-separated from the solvent front. No interfering peaks were obtained in the chromatogram due to tablet excipients. The determined amount of drugs indicates that the active ingredients in samples were present at level included within the requirements with respect to the label claimed by the manufacturer. The utility of the proposed method was verified by means of triplicate analyses of the pharmaceutical preparation. Results obtained from the proposed method of the analysis of these three drugs in the tablet formulation indicated that the proposed technique can be used for simultaneous quantification and routine QC analysis of this ternary mixture in the pharmaceutical product.

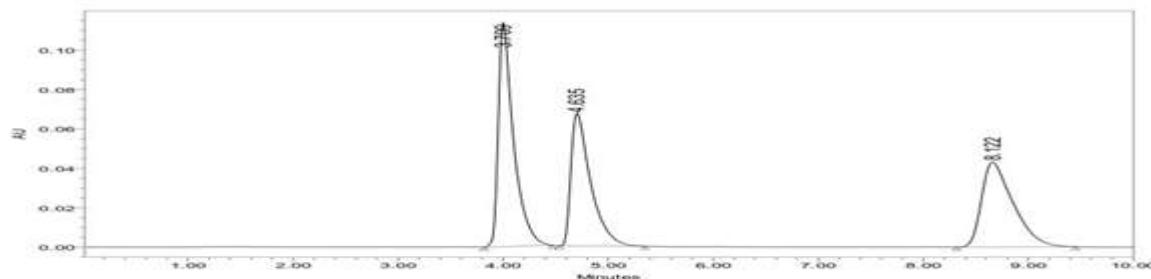


Fig. 7: Assay of Atripla® tablets

Table 7: Peak results of Standard & Test Chromatograms for Assay

	Emtricitabine		Tenofovir		Efavirenz	
	Rt	Peak Area	Rt	Peak Area	Rt	Peak Area
Standard	3.706	1146449	4.632	873159	8.121	893419
Sample	3.709	1147453	4.635	874114	8.122	894891
% Purity		99.9%		99.9%		99.6%

*Average for three injections

V DISCUSSION

The composition, pH and flow rate of the mobile phase were changed to optimize the separation conditions using standard substances of the three drug analytes. In order to effect the simultaneous separation of the working compounds, mixtures of methanol and pH 7 triethylamine and acetonitrile in different combinations with different ratios and at various flow rates were evaluated. Finally, an Thermosil 18 (150 x 4.6 mm, 5 μ m) column and the mobile phase methanol:pH 7 triethylamine (70:30 V/V) was found to be most suitable for LC analysis. A flow rate of 0.8 ml/min was selected for further studies after several preliminary investigatory chromatographic runs. The selected operating conditions were found to be optimal for sharp and symmetric peak shapes as well as to achieve minimal background noise. Under the described experimental conditions, all the peaks were well-defined and free from tailing. The proposed method was successfully used for the simultaneous determination of ETC, TDF and EFV in a tablet dosage form ^[2,3].

With the optimized operating conditions, the retention times corresponding to ETC, TDF and EFV were 3.706 min, 4.632 min and 8.121 min respectively and were stable among injections. However, the analysis time was set to 10 min, allowing elution of possible excipients and degradation products that could be retained, without the need of a further stabilization time between injections. The proposed RP-LC method provided simple, simultaneous determination of the ETC, TDF and EFV in the drug by UV detection at 260 nm. After determining the optimum conditions, a satisfactory resolution was obtained in a short analysis time (about 8 min). For three compounds sharp, symmetrical and well-resolved peaks were obtained (fig. 2).

Having optimized the efficiency of a chromatographic separation the quality of the chromatography was monitored by applying the system suitability tests. The acceptance criterion was $\pm 2\%$ for the percent coefficient

of variation for the peak area and retention times for ETC, TDF and EFV. The number of theoretical plates should not be less than 2000 and the tailing factor should not be more than 2.0. The peak purity of ETC, TDF and EFV were assessed by comparing the retention time (R_t) of standard and sample. A chromatogram obtained from reference substance solution is presented. The linearity range for ETC and EFV was found to be 10-50 µg/ml and for TDF was found to be 15-75 µg/ml (table 2). Both precision and accuracy were determined with standard quality control samples prepared in triplicates at different concentration levels covering the linearity range. The repeatability and intermediate precision are reported as % RSD in table 3 and the minimum variation in the % RSD indicates that the present method is precise. The accuracy of the proposed method was assessed by adding known amount of the drug to a drug solution of known concentration and subjecting the samples to the proposed HPLC method. All solutions were prepared and analysed in triplicate. The above procedure is adopted for ETC, TDF and EFV with high recovery values obtained (table 3) indicate that the proposed method is highly accurate.

To evaluate the robustness of the developed RP-HPLC method, small deliberate variations in the optimized method parameters were done. The effect of change in flow rate and mobile phase ratio on the retention time and tailing factor were studied. The values for proposed method are well within acceptance limits with a %RSD of less than 2.0. Above experiments indicated that the method is rugged and provides consistent and reliable results. The method specificity was assessed by studying the chromatograms obtained for a mixture of the drugs and the common excipients. As none of the excipients interfered with the analytes of interest, the method was found to be suitable for analysing the commercial formulation of these drugs.

VI CONCULSION

In this work, a simple, efficient, LC-MS compatible and stability-indicating RP-HPLC method has been developed for the simultaneous determination of ETC, TDF and EFV from bulk and tablet dosage form. The method was validated fully as per ICH guidelines, and validation acceptance criteria were met in all cases. Application of this method for simultaneous determination of ETC, TDF and EFV from tablet dosage form showed that neither the degradation products nor the excipients interfered in the estimation of all three drugs; therefore this method was specific and stability-indicating, and can be employed successfully for the simultaneous estimation of TDF, ETC and EFZ in commercial tablet dosage form.

REFERENCES

- [1] International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human use. Validation of Analytical Procedures: Text and Methodology ICH Q2 (R1), 2005.
- [2] Arun Ramaswamy, Smith AGD. Development and validation of analytical method for quantitation of emtricitabine, tenofovir, efavirenz based on HPLC. Arabian J of Chem. 2014.
doi:10.1016/j.arabjc.2014.08.007
- [3] Prashant SD, Roshan Borkar, NaliniShastri, Surendranath KV. A validated stability-indicating RP-HPLC method for the simultaneous determination of tenofovir, emtricitabine, and efavirenz and statistical approach to determine the effect of variables. ISRN Chromatography.

[4] Sudha T, Manjeera KK. Stability indicating rp-hplc method for the simultaneous estimation of the anti-retroviral drugs and in tablet dosage forms. International Journal of Biology, Pharmacy and Allied Sciences 2012;1(9):1322-1335.

[5] Prabhakar Reddy A, Chandra Teja U, sk. Ashraf Sultana Sk, Vijayalakshmi M, Buchi N. Nalluri. Development and validation of RP-HPLC-PDA method for the simultaneous estimation of emtricitabine, tenofovir disoproxil fumarate and rilpivirine hydrochloride in bulk, pharmaceutical dosage forms and in dissolution samples. Indo American journal of pharmaceutical research 2014; 4(11):5226-5234.

[6] Akula Srinath A, Sneha B, Akhila A, Rayees A, Kulkarni RG. Method development and validation for simultaneous estimation of lamivudine, tenofovir and efavirenz in combined tablet dosage form by RP-HPLC and UVspectroscopic method. International Journal of Pharmaceutical Sciences and Research 2014;5(12):5491-5497.

[7] Kavitha KY, Geetha G, Hariprasad R, Venkatnarayana R, Subramanian G. Development and validation of RP-HPLC analytical method for simultaneous estimation of emtricitabine, rilpivirine, tenofovir disoproxil fumarate and its pharmaceutical dosage forms. Pharmacie Globale 2013; 4(1):1-6.

[8] Deepthi K, Nagarjuna Reddy G, Dhanalakshmi K. Method development and validation for simultaneous estimation of emtricitabine and tenofovir disoproxil fumarate in pure and tablet dosage form by using RP-HPLC. International Journal of Pharma Research & Review 2013;2(10):1-11.

[9] Appala Raju N, Venkateswara Rao J, Vanitha Prakash K, Mukkanti K, Srinivasu K. Simultaneous estimation of tenofovir disoproxil, emtricitabine and efavirenz in tablet dosage form by RP-HPLC. Oriental Journal of Chemistry. 2008; 24(2): 645-650.

[10] Varma DP, Lakshmana Rao A. Stability-indicating RP-HPLC method for the simultaneous estimation of efavirenz, tenofovir and emtricitabine in pharmaceutical formulations. Research Communication. ISSN 2393 9079(Print) e-ISSN 2393 9087(Online).

[11] ICH Q1AR, Stability Testing of New Drug Substances and Products, International Conference on Harmonization IFPMA, Geneva, Switzerland;2000.

[12] U.S.Pharmacopeia, 24th ed. U.S. Pharmacopeial Convention, Inc., Rockville, MD;2000.

[13] Ermer J, Miller JH. Method Validation in Pharmaceutical Analysis, 1st Ed., Wiley-VCH Publishers, Weinheim, Germany, 2005. <http://dx.doi.org/10.1002/3527604685>.

[14] Swartz ME, Krull IS. Analytical Method Development and Validation, Marcel Dekker, New York, NY; 1997.

[15] ICH Q2A, Validation of Analytical Procedures, Methodology. International Conference on Harmonization, Brussels, Belgium, 1995.

[16] Riley CM, Rosanske TW. Development and Validation of Analytical Methods, Elsevier, Amsterdam, The Netherlands; 1996.

[17] Raul SK, Aravelli AB, Jhansi D. RP-HPLC method development and validation for the simultaneous estimation of atorvastatin and ezetimibe in pharmaceutical dosage form. Asian J Pharm Clin Res 2015;8:178-81.