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Stability indicating UPLC method to quantify Emtricitabine, Tenofovir, and Efavirenz simultaneously in tablets: Method establishment

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ABSTRACT

An easy, precise, specific, and accurate UPLC method for the quantification of emtricitabine (ETC), tenofovir (TFR), and efavirenz (EVR) in their tablet dosage form was developed and validated. ETC, TFR, and EVR were separated and estimated using Waters UPLC with HSS C18 (100 × 3 mm, 1.7 μ) column. The mobile phase was 0.01 N potassium dihydrogen phosphate buffer (pH 4.5) and acetonitrile (40:60, vol/vol). The elution of ETC, TFR, and EVR was achieved using flow rate at 0.4 ml/min and detected at 265 nm using a photo-diode array detector. The detector response was linear from 75 to 450 μg/ml for TNF, 50 to 300 μg/ml for ETC, and 150 to 900 μg/ml for EVR. The limit of detection and limit of quantification were 0.601 μg/ml and 1.82 μg/ml, 0.330 μg/ml and 0.100 μg/ml, 0.911 μg/ml and 2.76 μg/ml for TNF, ETC and EVR respectively. Validation was carried out in compliance with ICH guidelines. It was noticed that all validation parameters were inside the permissible range.

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INTRODUCTION

Tenofovir (TNF), an antiretroviral drug, belongs to a drug class known as nucleoside blockers of reverse transcriptase (Chapman *et al.*, 2003), (Antoniou *et al.*, 2003) AntonioChemically, TNF is described as [(2*R*)-1-(6-aminopurin-9-yl)propan-2-yl]oxymethylphosphonic acid (Figure 1). TNF operates by blocking a vital enzyme, reverse transcriptase, which triggers viral replication in people infected with the human immunodeficiency virus (HIV). TNF is used in conjunction with certain other antiretroviral agents to treat infections caused by HIV and the hepatitis B virus (Ray *et al.*, 2016).

Emtricitabine (ETC) is a synthetic nucleoside analog of cytidine with activity against type 1 HIV reverse transcriptase Emtricitabine (ETC) is a cytidine-like synthetic nucleoside of type 1 HIV reverse transcriptase activity (Molina and Cox, 2005). ETC is defined in chemical terms as 4-amino-5-fluoro-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]pyrimidin-2-one(Figure 1). ETC has been authorized for usage in adult people with HIV infection in combo with certain antiretroviral agents. (Goicoechea and Best, 2007) ETC prevents replication of viruses through hindering type 1 HIV reverse transcriptase reverse behavior (Saravolatz and Saag, 2006), (Mandal *et al.*, 2017)

Efavirenz (EVR) is an antiretroviral non-analog nucleoside and non-competitive inhibitor of reverse transcriptase (Vrouenraets *et al.*, 2007). EVR is defined in chemical terms as (4S)-6-Chloro-4-

(2-cyclopropylethynyl)-4-(trifluoromethyl)-2,4-dihydro-1H-3,1-benzoxazine-2-one (Figure 1). EVR is used in the diagnosis of HIV infections as an antiretroviral agent (Homkham *et al.*, 2019), (Maggiolo, 2009). EVR is explicitly linked to the enzyme and restricts the operation of RNA and DNA-dependent DNA-polymerase triggering degradation of the catalytic enzyme site (Mcdonagh *et al.*, 2015).

ETC, EVR, and TNF combination are available as tablet formulation with the name Vonavir (Emcure Pharmaceuticals Ltd., India) (Vonavir tablets, 2019b) labeled claim of this (Vonavir tablets, 2019a) was 300 mg of TNF, 200 mg of ETC, and 600 mg EVR. The Vanover medication is meant for use in HIV-1 positive individuals who have been virologically repressed to their current antiretroviral treatment for more than 3 months (Deeks and Perry, 2010) , (Hodder *et al.*, 2010) medication doesn't cure HIV but only prevents viral multiplication in the body. The Vonavir is not advised for use in children under the age of 12.

Few methods based on HPLC technique were reported to quantify ETC, EVR and TNF combination, (Devrukhakar *et al.*, 2013; I H T Guideline, 2005) , (Ramaswamy and Dhas, 2018) , (Palavan *et al.*, 2013) , (Rezaei *et al.*, 2019) , (Raju *et al.*, 2008) , (Varma and Rao, 2014) , Atlas *et al.* (2016) . UPLC is a significant laboratory technique that reduces costs and improves the analytical performance needed to develop and validate the process. UPLC method increases the speed of separation and improves efficiency, resulting in the rapid development of approaches. UPLC method reduces solvent consumption and improves sample quality as well as providing real-time testing in line with production processes (Chawla and Ranjan, 2016), (Babu *et al.*, 2017) , (Tiwari *et al.*, 2010) , (Kiran *et al.*, 2017) , proposed methods based on UPLC technique. (Babu *et al.*, 2017) method was mainly concerned with quantification of related substances in ETC, EVR and TNF combination. (Tiwari *et al.*, 2010) method is not stability-indicating. (Kiran *et al.*, 2017) method is stability-indicating, but this method uses gradient elution, which increases solvent consumption and analytical expense. Therefore, the current investigation is aimed at developing and validating a cost-effective isocratic elution method using stability, indicating the UPLC technique to quantify ETC, EVR, and TNF combination in bulk and Vonavir tablets.

MATERIALS AND METHODS

Chemicals

The reference samples of ETC, EVR and TNF are obtained from M/s. Mylan labs Pvt. Ltd., India. Vonavir tablet (Emcure Pharmaceuticals Ltd., India) having 300 mg of TNF, 200 mg of ETC, and 600 mg EVR fixed dosage combination was purchased from the pharmacy market. The solvents used are UPLC grade, and the chemicals used are analytical grade. Acetonitrile, potassium dihydrogen phosphate, orthophosphoric acid, hydrochloric acid, hydrogen peroxide and sodium hydroxide were procured from M/s. Rankem Chemicals Ltd, India. All through the study, Milli-Q water was used, which is prepared with Milli-Q water system of purification (Millipore, Germany).

Instrumentation

The UPLC was carried out on Waters with empower 2695 separation module, autosampler, and PDA Detector. Labindia (Maharashtra, India) UV-Visible spectrophotometer was used for spectral measurements. The weighing was done on Afcoset (New Delhi, India) ER-200A, and pH meter Adwa (Szeged – Hungary) AD 1020 was used for adjustment of pH.

Chromatographic conditions

HSS C18 (100 $\times$ 3 mm, 1.7 μ) column was used for this study. The isocratic separation was achieved using 0.01 N potassium dihydrogen phosphate buffer (40% volume) with pH 4.5 and acetonitrile (60 % volume) combination as mobile phase. The mobile phase was degassed for 5 minutes in the ultrasonic water bath and filtered under vacuum filtering by 0.45 μ filter. The mobile phase stream rate was maintained at 0.4 ml/min. The temperature of the column was held at 25 °C, and the quantification and detection were performed at 265 nm. Water and acetonitrile in the ratio of 50:50 (volume by volume) was employed as a diluent for preparing standard solutions. The injection volume was 5 μl.

Stock and working solutions of ETC, EVR, and TNF

The stock solution of TNF (3000 μg/ml), ETC (2000 μg/ml) and EVR (6000 μg/ml) was prepared as follows: Accurately weighed 300 mg of TNF, 200 mg of ETC and 600 mg of EVR are transferred into a 100 ml clean dry volumetric flask, and then 40 ml of diluent was added, sonicated for 10 min and made the final volume to 100 ml with diluent. Calibration solutions of concentration range 75-450 μg/ml (TNF), 50-300 μg/ml (ETC) and 150-900 μg/ml (EVR) were prepared by proper dilution of stock solution (TNF - 3000 μg/ml, ETC - 2000 μg/ml and EVR - 6000 μg/ml) with diluent. Working solutions of concentration 300 μg/ml (TNF), 200 μg/ml (ETC) and 600 μg/ml were prepared for validation study by proper dilution of stock solution (TNF - 3000 μg/ml, ETC - 2000 μg/ml and EVR - 63000 μg/ml) with diluent.

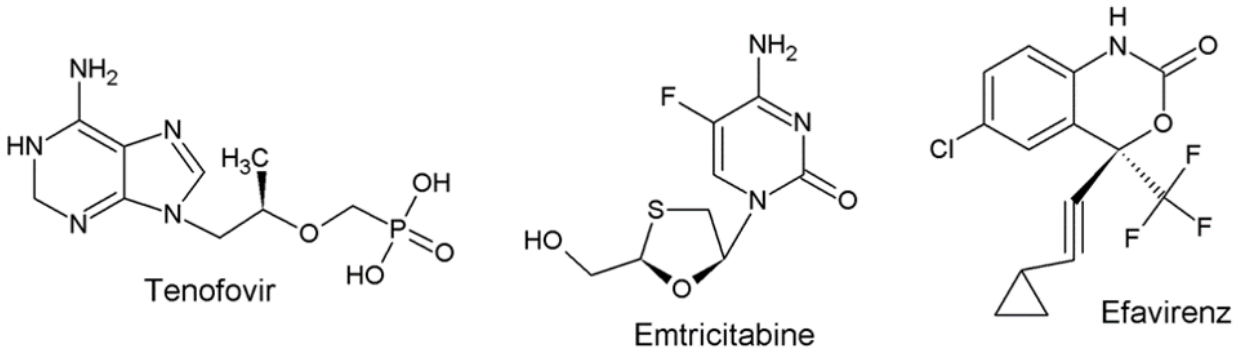

Figure 1: Structures of TNF, ETC, and EVR

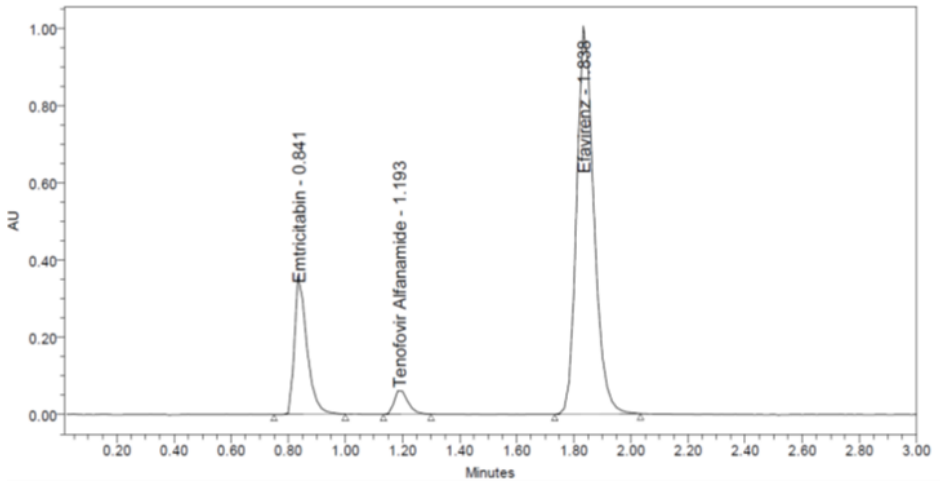

Figure 2: : Chromatogram with well separated ETC, EVR and TNF peaks

Table 1: ETC, EVR and TNF system suitability findings

S.No.	Emtricitabine		Tenofovir		Efavirenz	
	Plate Count	Tailing	Plate Count	Tailing		
1	5771	1.41	2810	1.23	4580	1.18
2	5727	1.28	2891	1.24	4417	1.19
3	5579	1.3	2816	1.25	4573	1.19
4	5414	1.4	2683	1.26	4554	1.17
5	5831	1.42	2829	1.25	4628	1.19
6	5824	1.42	2873	1.23	4472	1.19
Mean	5691	1.372	2817	1.243	4537	1.185
SD	168.101	0.066	75.827	0.011	79.400	0.009
RSD	2.954	4.847	2.692	0.917	1.750	0.755

Calibration curves of ETC, EVR, and TNF

Six calibration solutions of concentration range 75-450 μg/ml (TNF), 50-300 μg/ml (ETC), and 150-900 μg/ml (EVR) were injected into the system and evaluated under the conditions suggested. The ETC, EVR, and TNF peak area and concentration data were used in the development of the respective calibration curves.

Analysis of ETC, EVR, and TNF in Vonavir tablets

Twenty tablets are finely powdered and measured their weight. An accurately weighed portion of powdered sample equivalent to 200 mg of ETC, 300 mg of TNF and 600 mg of EVR are transferred into a 100 ml clean dry volumetric flask, and then 40 ml of diluent was added, sonicated for 10 min and made the final volume to 100 ml with diluent. Concentration of prepared tablet stock solution was TNF - 3000 μg/ml, ETC - 2000 μg/ml and EVR - 6000 μg/ml. This mixture has been filtered by a membrane filter of 0.45 μ. Working test solution of concentration

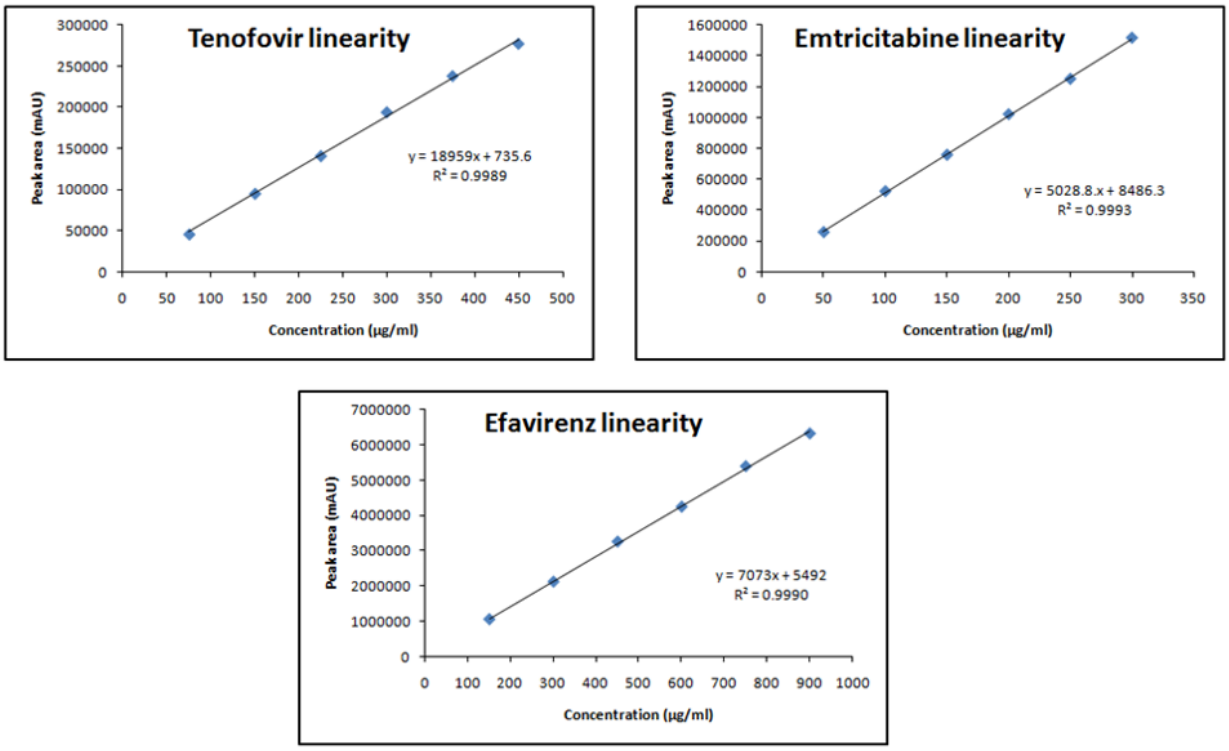

Figure 3: Linearity graphs of TNF, ETC, and EVR

Table 2: ETC, EVR and TNF intra-day findings

S.No.	Emtricitabine	Tenofovir	Efavirenz
		Peak area values	
1	1013584	198688	4213969
2	1018931	198947	4245455
3	1015804	199241	4208198
4	1014595	197814	4218495
5	1017275	197159	4211589
6	1017094	196381	4216703
Mean	1016214	198038	4219068
SD	2124.779	861.445	14963.077
RSD	0.209	0.435	0.355

SD – standard deviation; RSD – percent relative standard deviation

Table 3: ETC, EVR and TNF inter-day findings

S.No.	Emtricitabine	Tenofovir	Efavirenz
		Peak area values	
Day 1*	1008061	195169	4214229
Day 2*	1008059	195165	4214232
Mean	1008060	195167	4214231
SD	3424.8	775.3	11557.7
RSD	0.314	0.419	0.350

* - Average of six values; SD – standard deviation; RSD – percent relative standard deviation

Table 4: ETC, EVR, and TNF recovery findings

Spiking level (%)	Labeled claim (mg)	Spiked conc. (mg)	Total found (mg)	Recovery (%)	Average recovered (%)	SD	**RSD (%)**
			Tenofovir				
50	300	150	445.82	99.07	99.31	0.269	0.270
	300	150	446.67	99.26			
	300	150	448.20	99.60			
100	300	300	602.28	100.38	100.02	0.587	0.586
	300	300	601.98	100.33			
	300	300	596.04	99.34			
150	300	450	755.18	100.69	100.07	0.534	0.534
	300	450	748.35	99.78			
	300	450	748.13	99.75			
			Emtricitabine				
50	200	100	298.29	99.43	99.25	0.185	0.186
	200	100	297.75	99.25			
	200	100	297.18	99.06			
100	200	200	392.52	98.13	98.99	0.751	0.758
	200	200	398.12	99.53			
	200	200	397.20	99.30			
150	200	300	495.05	99.01	99.30	0.374	0.377
	200	300	495.80	99.16			
	200	300	498.60	99.72			
			Efavirenz				
50	600	300	892.44	99.16	99.24	0.898	0.905
	600	300	885.51	98.39			
	600	300	901.62	100.18			
100	600	600	1183.08	98.59	99.08	0.506	0.511
	600	600	1188.48	99.04			
	600	600	1195.20	99.6			
150	600	900	1476.60	98.44	98.98	0.479	0.484
	600	900	1490.40	99.36			
	600	900	1486.95	99.13			

Conc. – concentration; SD – standard deviation; RSD – percent relativestandard deviation

300 μg/ml (TNF), 200 μg/ml (ETC) and 600 μg/ml was prepared by proper dilution of stock tablet solution (TNF - 3000 μg/ml, ETC - 2000 μg/ml and EVR - 6000 μg/ml) with diluent for analysis by the method proposed. The peak areas of ETC, EVR, and TNF were measured. The labeled content of ETC, EVR, and TNF in Vonavir tablets was quantified employing respective calibration curves/regression equations.

ETC, EVR, and TNF degradation studies

ETC, EVR and TNF forced degradation was studied by degradation through exposure of tablet sample solution (TNF - 300 μg/ml, ETC - 200 μg/ml and EVR - 600 μg/ml) to acid hydrolysis, oxidation, base hydrolysis, photodegradation and heat as follows (Guideline, 2003) 10 ml of tablet sample solution was transferred to a 100 ml capacity volumetric flask. 10 ml of 0.1 N HCl was added, thoroughly mixed, and refluxed for 60 min at temperature 60°C. The volume was accomplished to mark (100 ml) using diluent. The solution was filtered, injected into the HPLC system, and analyzed employed the conditions suggested. The same procedure was repeated with the addition of 10 ml of 0.1 N NaOH (for base hydrolysis) and with 10 ml of 30% peroxide (for oxidation degradation).

An accurately weighed portion of powdered sample equivalent to 200 mg of ETC, 300 mg of TNF, and 600

Table 5: ETC, EVR, and TNF robustness findings

Parameter	Condition	Emtricitabine		Tenofovir		Efavirenz	
		Mean area*	% Assay	Mean area*	% Assay	Mean area*	% Assay
	Optimized	1078319	99.70	209914	99.10	4266264	98.90
Flow rate	0.3 ml/min	1075986	99.10	213148	100.90	4266014	98.60
	0.5 ml/min	1072518	98.60	212401	100.50	4249107	98.30
Buffer:	45:55	1082742	100.90	209789	98.60	4286926	100.20
acetonitrile	35:65	1081287	100.20	214442	101.30	4308627	100.90
Column	23°C	1094325	101.10	212144	100.10	4270193	99.30
Temperature	27°C	1103863	101.20	215357	101.70	4274522	99.70

* Average of six values

Table 6: ETC, EVR and TNF degradation outcomes

Sample	Emtricitabine		Tenofovir		Efavirenz	
	Peak area	% Degraded	Peak area	% Degraded	Peak area	% Degraded
Undegraded	1018525		198797		4222619	
Acid degraded	993684	2.73	191687	3.77	3984829	5.73
Base degraded	989884	3.10	189856	4.69	4030378	4.65
Peroxide oxidation	983443	3.73	190140	4.55	4074947	3.59
Thermal degraded	994225	2.68	192461	3.38	4126900	2.36
Photo degraded	1003483	1.77	195949	1.63	4175235	1.22

mg of EVR was exposed to 105 ^{o}C in the oven (to study thermal degradation) and direct sunlight for 6 hr (to study photodegradation). After the degradation period, the sample preparation and analysis were done as explained in section "Analysis of ETC, EVR, and TNF in Vonavir tablets."

In all conditions, the percent degradation and assay of ETC, EVR, and TNF were determined by comparison with the undegraded sample solution.

RESULTS AND DISCUSSION

Method development

Preliminary experiments included checking several mobile phase combinations for successful separation of ETC, EVR, and TNF on column HSS C18 (100 mm $\times$ 3 mm, 1.7 μ). Method development began with examining methanol combination with 0.01 N potassium dihydrogen phosphate buffer of pH 4.5 and acetonitrile combination with 0.01 N potassium dihydrogen phosphate buffer of pH 4.5 in isocratic elution. For the separation of ETC, EVR, and TNF, acetonitrile combination with potassium dihydrogen phosphate buffer has been effective.

Consequently, the same mixture was used in isocratic elution. Different ratios of acetonitrile and potassium dihydrogen phosphate buffer were examined. Good results were obtained with 0.01 N potassium dihydrogen phosphate buffer (40% volume) and acetonitrile (60% volume). Distinct pH values were examined. pH 4.5 was found as best because, at this pH, better separation of ETC, EVR, and TNF was achieved. Different flow rates (0.3, 0.4, and 0.5 ml/min) were examined and observed that 0.4 ml/min was the good one. Ambient temperature was perfect for this separation and was therefore used throughout the process. Ultraviolet detection at 265 nm was utilized because it was discovered to be the optimum wavelength for ETC, EVR, and TNF. At this wavelength, it provided the ETC, EVR,

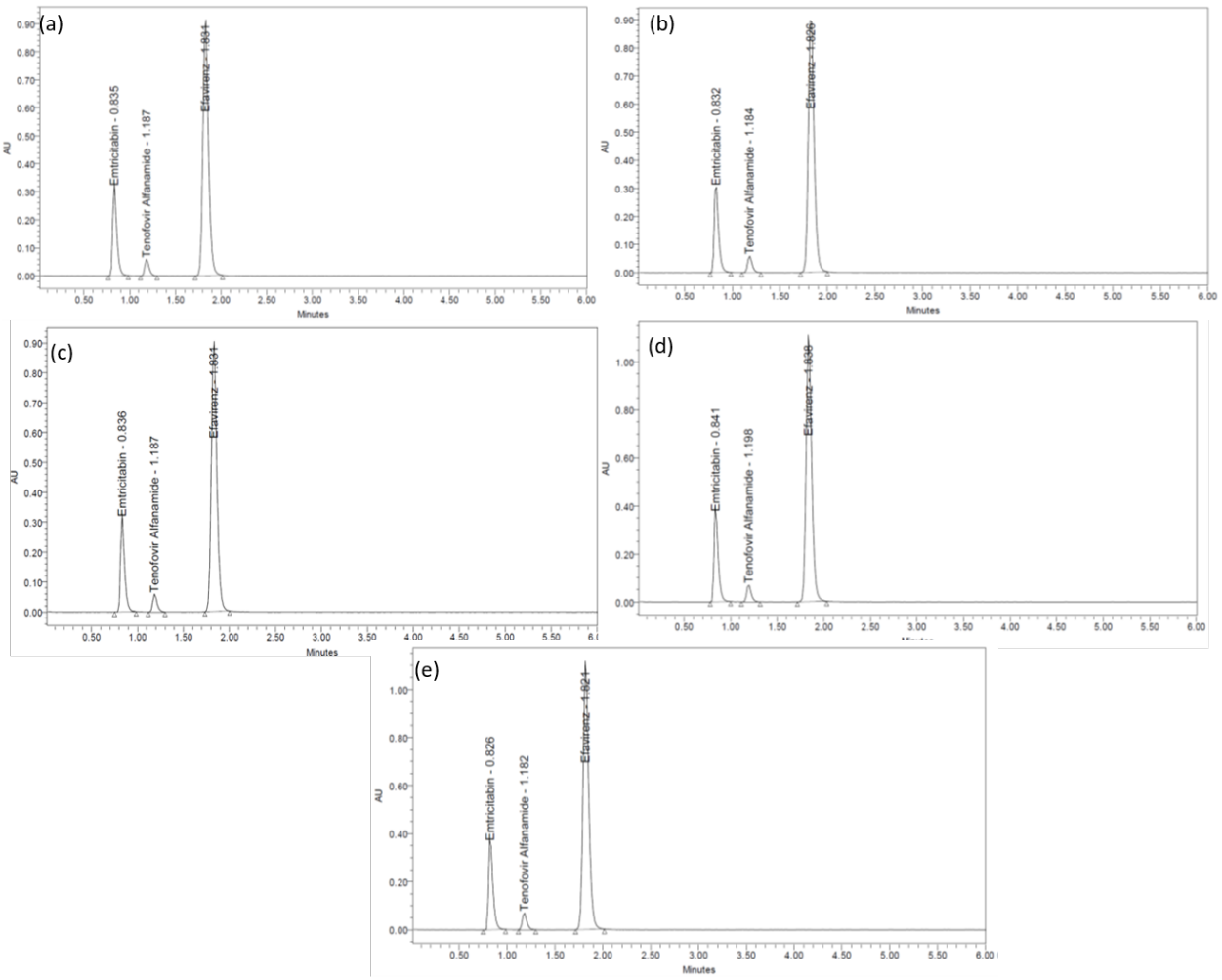

Figure 4: Photodegradation chromatogram

and TNF elevated peak area. Using these conditions, good separation of ETC, EVR, and TNF with acceptable peak shape, resolution, and sensitivity were obtained (Figure 2).

Method validation

The procedure developed to estimate ETC, EVR, and TNF simultaneously has been validated for different parameters (I H T Guideline, 2005; Atlas *et al.*, 2016) System suitability, linearity, specificity, accuracy, robustness, precision, detection limits and quantitation limit.

System suitability

The parameters for system suitability (Tailing factor and plate count) were checked through analysis of ETC, EVR, and TNF solution at a concentration of 200 μg/ml, 600 μg/ml, and 300 μg/ml, respectively. The findings are noticed in line with ICH guidelines in the acceptance criteria (Table 1).

Linearity

Linearity is found to be in the range of 75-450 μg/ml for TNF, 50-300 μg/ml for ETC, and 150-900 μg/ml for EVR. The correlation coefficient (R^2) values for ETC, EVR, and TNF are between 0.9989 and 0.9993. The linearity graphs and regression equations of ETC, EVR, and TNF obtained are shown in (Figure 3).

Detection limit

The detection limit was assessed using a standard deviation of intercept in the regression equation (a) and slope of a regression equation (b). The below equation was employed to compute the detection limit.

$$Detection\ limit\ =\ a/b \times 3.3$$

The level of detection was 0.601 μg/ml for TNF, 0.330 μg/ml for ETC and 0.911 μg/ml for EVR.

Quantification limit

The quantification limit was also assessed using a standard deviation of intercept in regression equation (a) and slope of a regression equation (b). Equation to compute quantification limit was:

$$Quantification\ limit\ =\ a/b \times 10$$

The level of quantification was 1.82 μg/ml for TNF, 1.00 μg/ml for ETC, and 2.76 μg/ml for EVR.

Precision

Six replicate injections of the same dilution (TNF - 300 μg/ml, ETC - 200 μg/ml, and EVR - 600 μg/ml) are analyzed on the same day for verifying inter-day precision and on two different days for verifying inter-day precision. The relative standard deviation for ETC, EVR, and TNF peak areas were determined (Table 2 and Table 3). Values less than 2.0% demonstrated excellent precision.

Accuracy

Recovery analysis using standard additional procedure was done for verifying method accuracy. The percentage of recovery investigation of ETC, EVR, and TNF has been achieved by spiking three varying quantities (50, 100, and 150%) of pure ETC, EVR, and TNF into the pre-analyzed tablet form. The recovery percentage of ETC, EVR, and TNF were calculated for each spiked level in three replicates. The percentage recovery outcomes (Table 4) demonstrated good accuracy and non-interference of excipients in the tablet.

Degradation study of ETC, EVR, and TNF

The study of degradation was performed by placing the tablet sample under stress conditions such as acid hydrolysis, base hydrolysis, oxidative degradation, thermal degradation, and photodegradation. This study was conducted to evaluate the stability-indicating efficiency and specificity of the proposed procedure, and also the stability of ETC, EVR, and TNF under the conditions applied. (Table 6) outlines the results. The chromatograms of degradation studies are depicted in (Figure 4 a –e). The order of stability of drugs in the applied conditions was:

1. ETC - sunlight > dry heat > HCl > NaOH > peroxide
2. TNF - sunlight > dry heat > HCl > peroxide > NaOH
3. EVR - sunlight > dry heat > peroxide > NaOH > acid

CONCLUSIONS

Easy and cost-effective stability-indicating isocratic UPLC method was established and validated for routine quantitative analysis of ETC, EVR, and TNF in the tablet dosage formulation. The method is stability-indicating and hence reliable and efficient during stability studies to show and identify any predicted change or deterioration in the drug product. The method is sufficiently accurate, precise, and robust to reproduce results under various conditions of method.

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