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A Validated Stability Indicating RP-HPLC Method for the Determination of Emtricitabine, Tenofovir Disoproxil Fumarate, Elvitegravir and Cobicistat in Pharmaceutical Dosage Form

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Abstract

A new simple, rapid stability indicating assay method has been developed and validated for the determination of emtricitabine, tenofovir disoproxil fumarate, elvitegravir and cobicistat using reverse-phase high-performance liquid chromatography in their pharmaceutical dosage form. The chromatographic separation was performed on an ODS column (250 × 4.6 mm, 5 µm) using mobile phase A (potassium dihydrogen orthophosphate, pH adjusted to 2.5) and mobile phase B (acetonitrile) in the ratio of 55:45% v/v at a flow rate of 1 mL/min. The analytes were detected at 250 nm. The method was found to be linear in the concentration range of 2–12 µg/mL for EMT, 3–18 µg/mL for TNDF, 1.5–9 µg/mL for ELV and COB, with the coefficient value (R^2) of >0.9990. The accuracy was measured via recovery studies and found to be acceptable, and the percentage recoveries were found in the range of 99.93–100.08 ± 0.5%. Forced degradation studies were also conducted, and the drugs were subjected to various stress conditions such as acid hydrolysis, base hydrolysis, oxidative, photolytic and thermal degradation. The proposed method was successfully validated and applied for the quantitative estimation of these drugs in both bulk and tablet dosage forms.

Introduction

Emtricitabine (EMT) is synthetic deoxycitidine analogue and has been approved as an anti-retroviral agent in the treatment of HIV-type 1 infections (1, 2). It is a nucleoside reverse transcriptase inhibitor that competitively inhibits the HIV reverse transcriptase enzyme and blocks the HIV replication. The chemical name of the EMT (Figure 1A) is 5-fluoro-1-[(2*R*,5*S*)-2 (hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine (3). Tenofovir disoproxil fumarate (TNDF) is an adenine derivative and is a pro-drug of tenofovir, chemically known as (Figure 1B) 9-[(*R*)[(bis)[(isopropoxy carbonyl)oxy]-phosphinyl]methoxy]propyl] adenine fumarate (4). It blocks the HIV reverse transcriptase enzyme and incorporated into viral DNA (5, 6). Elvitegravir (ELV) is a novel class of anti-retroviral agents, belongs to integrase strand transferase inhibitor, inhibits the integrase enzyme and prevents the virus replication (7). The chemical name of ELV (Figure 1C) is 6(3-chloro-2-fluorobenzyl)-1-[(2*S*)-1-hydroxy-3-methylbutane-2-yl]-7-methoxy-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (4). Cobicistat (COB) was used in patients with HIV infection. Co-administration of COB (Figure 1D) inhibits the cytochrome p450 3A4 enzyme and helps in improving bioavailability and half-life of ELV in HIV-infected patients (8–12). A literature survey revealed that there are various analytical methods such as UV (13), HPLC (14–16), HPTLC (17), UPLC (18) and LC–MS (19), which were reported for the estimation of individual drugs and in combination with other drugs. However, to the best of our knowledge, a simple, rapid method has not been published for the above-mentioned components. The reason for selecting this combination is to provide a validated HPLC method which can separate four different drugs in a single formulation that may be useful in the pharmaceutical industry. This study also deals with stability studies for developing a

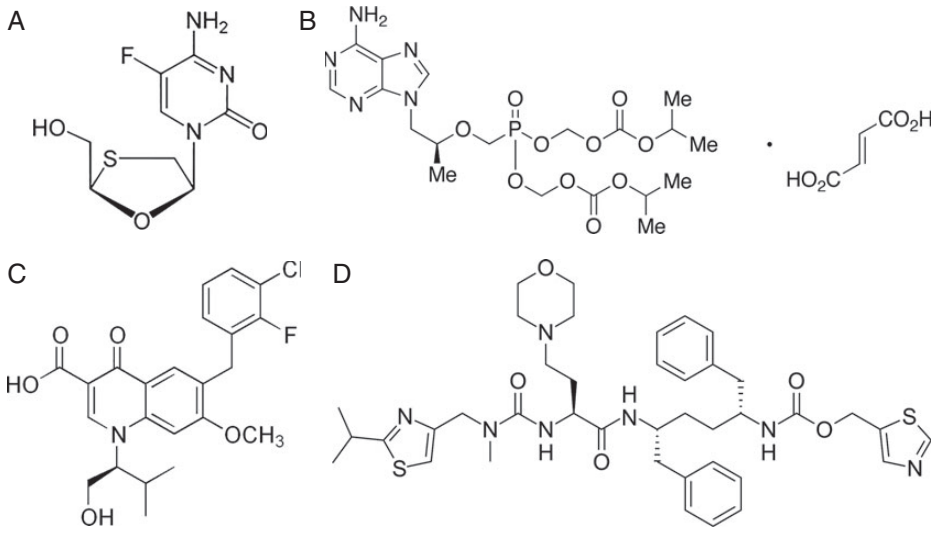

Figure 1. Chemical structures of (A) emtricitabine, (B) tenofovir disoproxil fumarate, (C) elvitegravir and (D) cobicistat.

degradation pathway and to isolate the impurities which are likely to be present.

Experimental section

Chemicals and reagents

A reference standard sample of ELV, COB, EMT and tenofovir was obtained as a gift sample from Spectrum Pharma Research Solution (Hyderabad, India), and the fixed dose combination was purchased from a local market. Acetonitrile (ACN) and water of HPLC grade were supplied by E. Merck (Mumbai, India). Potassium dihydrogen orthophosphate (PDOP), hydrochloric acid, sodium hydroxide and hydrogen peroxide (HP) of analytical grade were obtained from RANKEM Chemical (Mumbai, India). All other reagents of analytical grade were purchased from RANKEM (Mumbai, India).

Instrument and chromatographic separation

Chromatography was performed on a WATERS 2695 HPLC column (Waters Corporation, Milford, USA) with an autosampler and equipped with a waters 2996 series of PDA detector with a spectral bandpass of 1.2 nm. Components were detected using 250 nm, and data processing was achieved by Empower 2 software. A hot air oven was used for thermal degradation of the samples, and a UV cross-inker, with series of 234100 model UV chamber, equipped with a UV fluorescence lamp with the wavelength range between 200 and 300 nm was selected for photolytic degradation. The chromatographic separation was performed on an Intertsil ODS C18 (250 × 4.6 mm, 5 µm) column at an ambient column temperature. The samples were eluted using phosphate buffer (pH 2.5):ACN (55:45% v/v) as the mobile phase at a flow rate of 1 mL/min. The mobile phase was filtered through a 0.45-µm nylon filter, and it was degassed before use. Then, 10 µL of sample solutions were injected into the HPLC system.

Standard and working solutions

EMT (8 mg), TNDF (12 mg), ELV (6 mg) and COB (6 mg) were accurately weighed and transferred into a 10-mL clean and dry volumetric flask. Then, 7 mL of the diluent (water–ACN, 50:50) was added. It was sonicated for ~30 min and diluted to the final volume with the diluent. From the above stock solution, 1 mL of the solution was transferred into another 10 mL volumetric flask and then diluted to the final volume with the diluent.

Table I. Results of Percentage Recovery Data

Compound	Quantity (mg/mL)		Mean percentage recovery	% RSD
	Amount added	Amount found		
EMT	4	4.03	100.08	1.11
	8	7.95	99.43	
	12	11.98	99.86	
TNDF	6	5.99	99.93	0.99
	12	11.98	99.84	
	18	18.10	100.61	
ELV	3	2.96	98.68	1.28
	6	6.04	100.69	
	9	9.01	100.16	
COB	3	2.98	99.34	1.18
	6	5.94	99.61	
	9	9.00	100.06	

EMT, emtricitabine; TNDF, tenofovir disoproxil fumarate; ELV, elvitegravir; COB, cobicistat; RSD, relative standard deviation.

Sample solution preparation

Twenty tablets were weighed and powdered finely. The average weight of each tablet was calculated, and the weight of the powder equivalent to one tablet was transferred into a 100-mL volumetric flask. Then, 70 mL of the diluents were added, the flask was sonicated for 25 min and the volume was made up with the diluent and filtered. Finally, 0.4 mL of the filtered solution was pipetted out, transferred into a 10-mL volumetric flask and made up the final volume with diluents.

Results

Analytical method validation

The validation of an analytical method confirms the accuracy and characteristics of the method to satisfy requirements of the application. The proposed method was validated according to the ICH

Table II. Statistical Data of Calibration Curve

Parameter	EMT	TNDF	ELV	COB
Linearity range	2–12 µg/mL	3–18 µg/mL	1.5–9 µg/mL	1.5–9 µg/mL
Regression equation	$y = 12{,}681x + 4{,}187$	$y = 8{,}574x + 4{,}187$	$y = 17{,}059x + 2{,}953$	$y = 1{,}792x + 227.7$
Limit of detection	0.25 µg/mL	0.13 µg/mL	0.02 µg/mL	0.17 µg/mL
Limit of quantification	0.77 µg/mL	0.40 µg/mL	0.07 µg/mL	0.15 µg/mL

EMT, emtricitabine; TNDF, tenofovir disoproxil fumarate; ELV, elvitegravir; COB, cobicistat; RSD, relative standard deviation.

Table III. Results of Intra-Day and Inter-Day Precision

Sample	Concentration (µg/mL)	Mean area		% RSD	
		Intra-day	Inter-day	Intra-day	Inter-day
EMT	80	1,133,022	1,131,519	0.914	1.101
TNDF	120	1,014,157	1,018,501	0.664	1.113
ELV	60	1,010,134	1,011,482	0.821	1.336
COB	60	102,633	104,258	1.327	1.766

EMT, emtricitabine; TNDF, tenofovir disoproxil fumarate; ELV, elvitegravir; COB, cobicistat; RSD, relative standard deviation.

guidelines (20, 21) for specificity, recovery, precision, linearity, system suitability, robustness, limit of detection (LOD) and limit of quantification (LOQ). Under the validation study, the following parameters were studied.

Recovery

The percentage recovery was calculated by preparing standard drug concentrations of EMT, tenofovir, ELV and COB with concentration levels of 50%, 100% and 150%. A known amount of the standard drug was added to the blank sample at each level. Good recovery of the spiked drugs was obtained at each added concentration, and the mean percentage recovery of EMT, TNDF, ELV and COB was achieved between 99.93–100.08 ± 0.5%. The results are given in Table I.

Linearity, LOD and LOQ

Linearity of an analytical method was evaluated over the concentration range of standard solutions. A minimum of six standard drug concentrations ranging between 2 and 18 µg/mL were prepared, and their peak areas were recorded. The linearity of the calibration curve was checked by constructing a plot of area versus concentration. The LOD and LOQ were measured from the calibration curve method. The LOD value of EMT, TNDF, ELV and COB was found to be 0.25, 0.13, 0.02 and 0.17 µg/mL, respectively. The LOQ values were found to be 0.77 µg/mL of EMT, 0.40 µg/mL of TNDF, 0.07 µg/mL of ELV and 0.51 µg/mL of COB. The statistical linearity data are presented in Table II.

Precision

Precision is expressed as the closeness of agreement between a series of measurements obtaining from multiple sampling of the same homogeneous sample. Six replicate injections of a known concentration of EMT (80 µg/mL), TNDF (120 µg/mL), ELV (60 µg/mL) and COB (60 µg/mL) have been analyzed by injecting them into a HPLC column on the same day and on consecutive days. From the results obtained, % RSD was calculated and was found to be within the limits (<2). The results of precision are given in Table III.

Table IV. Optimized Chromatographic Conditions

Instrumentation	Optimized chromatographic conditions
Equipment	RP-HPLC Waters 2695 equipped with auto PDA detector
Column	Symmetry ODS 18 (250×4.6 mm, 5 µm)
Mobile phase	Phosphate buffer (pH 2.5) and ACN in the ratio of 55:45% v/v
Wavelength	250 nm
Flow rate	1 mL/min
Injection volume	10 µL
Runtime	10 min

Table V. Results of Stress-Induced Degradation Studies

Stress condition	% Degradation			
	EMT	TNDF	ELV	COB
Acid degradation	6.04	6.25	6.70	6.51
Base degradation	5.19	5.08	5.76	5.57
Oxidative degradation	6.99	7.05	7.55	7.48
Thermal degradation	3.59	2.54	3.04	3.42
Photolytic degradation	1.16	1.85	2.32	1.77

EMT, emtricitabine; TNDF, tenofovir disoproxil fumarate; ELV, elvitegravir; COB, cobicistat; RSD, relative standard deviation.

Table VI. Percentage Assay Results of Stribild Tablets

Tablet	Drug	Dosage (mg)	Amount found (mg)	% Assay
STRIBILD	Emtricitabine	200	198.42 mg	99.21
	Tenofovir	300	299.16 mg	99.72
	Elvitegravir	150	149.46 mg	99.64
	Cobicistat	150	147.54 mg	98.36

EMT, emtricitabine; TNDF, tenofovir disoproxil fumarate; ELV, elvitegravir; COB, cobicistat.

Robustness

Robustness of the proposed analytical method is a measure of its capacity to remain unaffected, and it reflects the reliability of the analysis with respect to deliberate changes in the parameters such as flow rate (0.8 ± 0.2 mL), column temperature (30 ± 5°C), mobile phase ratio and pH of the mobile phase. The parameters chosen for the study of robustness is the flow rate and column temperature. From the results obtained, there were no significant changes observed at the end of the study.

Forced degradation studies

The assay method was used to test the drug stability by conducting forced degradation studies for the drug substances under various stress

conditions. Stress degradation studies were carried out for acid hydrolysis (1 mL HCl heated for 30 min at 60°C), alkali hydrolysis (2 N NaOH heated for 30 min at 60°C), oxidative degradation (20% H_2O_2 heated at 60°C for 30 min) and thermal degradation (samples placed in an oven at 105°C for 6 h). For photolytic stress studies, samples were exposed to UV light by keeping them in a UV chamber for 7 days.

Discussion

Method development and optimization of chromatographic conditions

The main objective of this study is to develop and validate a stability indicating assay method for simultaneous estimation of EMT, TNDF,

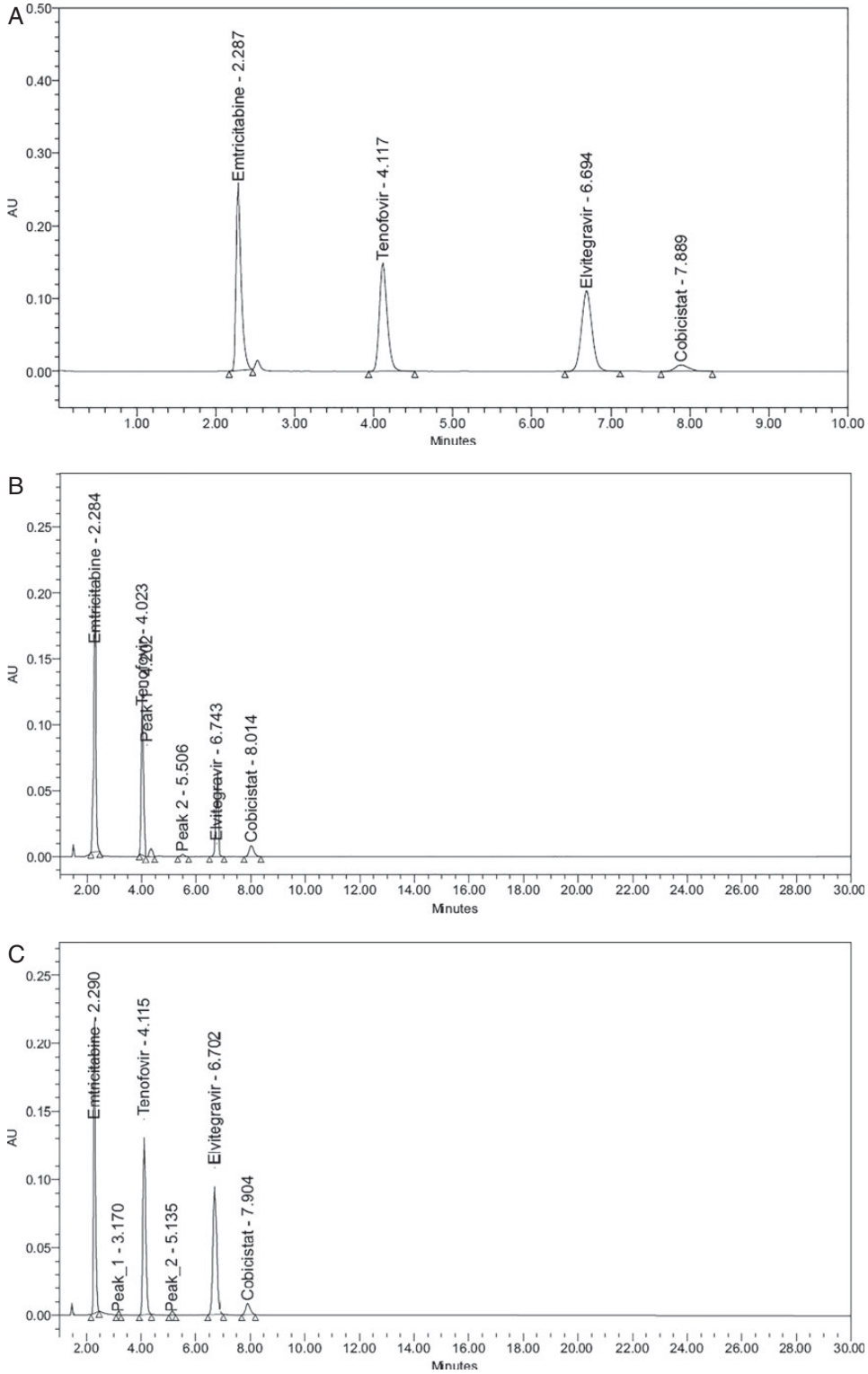

Figure 2. Chromatograms of forced degradation studies. (A) Undegraded standard chromatogram of EMT, TNDF, ELV and COB. (B) Acid degradation. (C) Alkali degradation. (D) Peroxide degradation. (E) Thermal degradation. (F) Photolytic degradation.

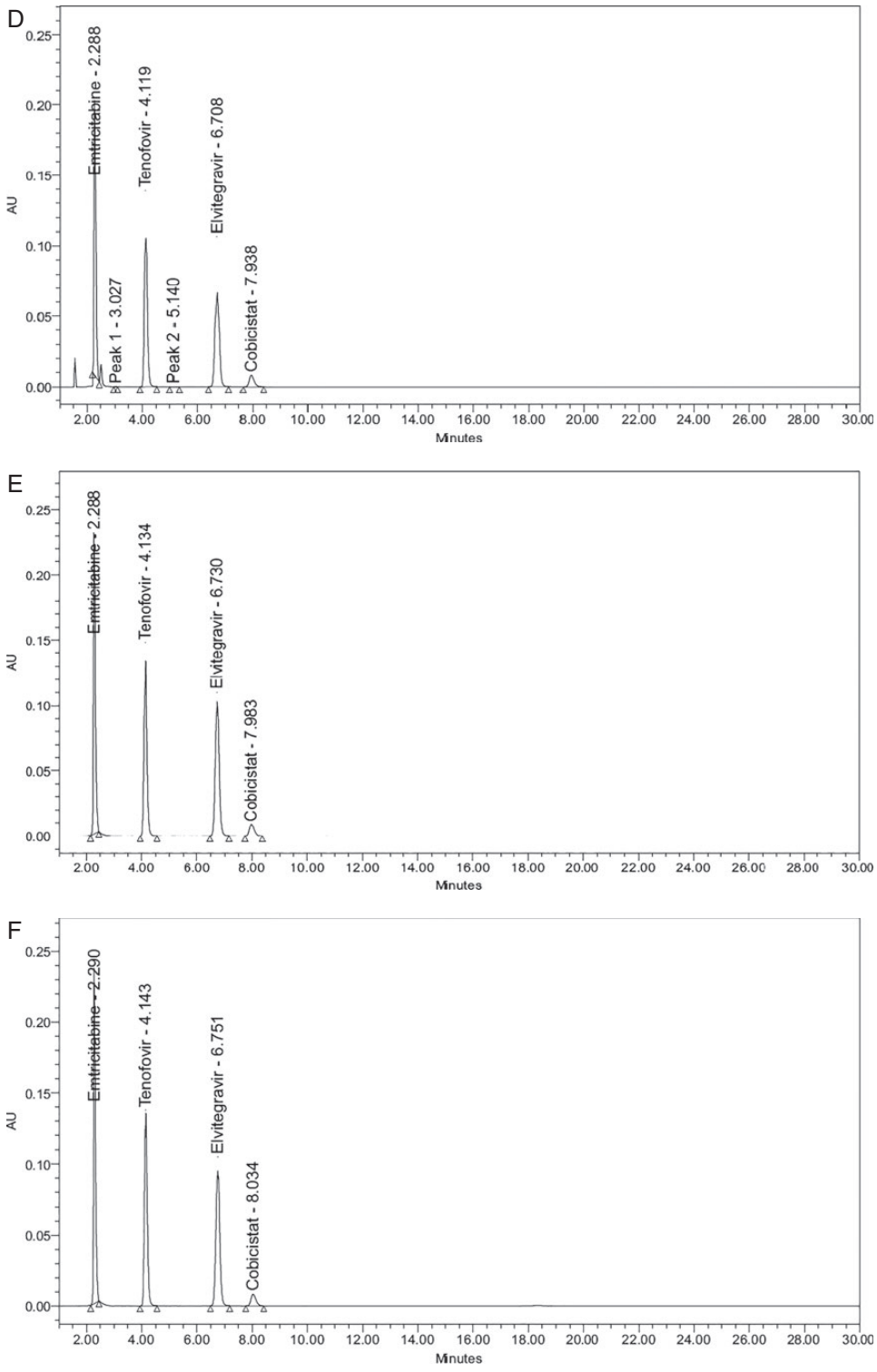

Figure 2. Continued

ELV and COB by reverse-phase high-performance liquid chromatography. Several chromatographic trials were conducted using various solvents such as methanol, ACN, water at different ratios, but the peak purity and resolution were not achieved properly. During the mobile phase selection, it was found that buffer could help in separating four drugs with good resolution. Then, the trials were conducted at different buffer pH levels 2.5, 3.5, 4.5 and 6. The best results were achieved by using the Inertsil ODS C18 asymmetry column with mobile phase A consisting of phosphate buffer (pH 2.5) and mobile phase B consisting of ACN in the ratio of 55:45% v/v at 250 nm using a PDA detector. The retention time of EMT, TNGF, ELV and COB was found to be 2.28, 4.11, 6.69 and 7.88 min,

respectively. The optimized chromatographic conditions are given in Table IV.

Forced degradation studies

Degradation studies were performed and compared with undegraded samples (Figure 2A) to demonstrate the stability of the drug substances. Forced degradation studies of EMT, TNDF, ELV and COB were conducted under various stress conditions. In all the stress conditions, EMT was found to be stable. However, tenofovir and ELV were undergone degradation only under acid, base and peroxide conditions. Under acid and alkali conditions (Figure 2B and C), both tenofovir and ELV have undergone slightly higher degradation with degradation products, retention time (RT) value of 4.202 min of tenofovir and 5.506 min of ELV. The peroxide degradation was carried out at different concentrations (3,10 and 20%) of hydrogen peroxide, and finally at 20% concentration (Figure 2D), it was observed that tenofovir and ELV were comparatively more susceptible to undergo degradation with degradation products with RT values of tenofovir and 3.027 min of ELV and whereas the ELV and COB were less susceptible. Under photolysis and dry heat conditions (Figure 2E and F), all the four drugs showed very less degradation. In all the degradation studies, the purity of angle was found to be less than the purity of threshold. The degradation results are given in Table V.

Analysis of Stribild tablet dosage form

The developed method was applied for the estimation of drugs in the commercial tablet dosage form (STRIBLID tablets: EMT 200 mg/TNDF 300 mg/ELV 150 mg/COB 150 mg). The chromatograms show good separation of the samples with acceptable limits such as tailing factor and USP plate counts. The % assay was calculated and found to be satisfactory, and the results are given in Table VI.

Conclusion

In this study, a simple, specific, accurate and robust stability indicating assay method was developed by using RP-HPLC. The developed stability indicating method was validated successfully for simultaneous estimation of EMT, TNDF, ELV and COB. The proposed method was tested for its accuracy, linearity, precision, robustness, LOD and LOQ. The forced degradation studies were carried out, and the reported method effectively separates the drug substances without interference of excipients and degradation products. Hence, it can be concluded that the developed method can be used for the routine analysis of EMT, TNDF, ELV and COB in the pharmaceutical dosage form.

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