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Development and Validation of Stability Indicating RP-HPLC Method for the Simultaneous Estimation of Emtricitabine Tenofovir Alafenamide Bulk and their Combined Dosage Form

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ABSTRACT

The present work describes development and validation of stability indicating RP-HPLC method for the simultaneous estimation of Emtricitabine tenofovir alafenamide bulk and their combined dosage form. The chromatographic separation was performed on Column: Inertsil ODS (4.6 × 250 mm, 5 µm) using phosphate buffer: Acetonitrile (80:20) as mobile phase at a flow rate of 1 mL/min and column oven temperature of 30ºC. The detection was carried out using a Diode array detector at 259 nm. The linearity of the method was determined in concentration range of 20-100 µg/ml for Emtricitabine, 0.25-12.5 µg/ml for Tenofovir alafenamide. The retention times of EMT and TAF were found to be 3.314 and 5.068 respectively. Average correlation coefficient R^2=0.999 for all the drugs with %RSD values ≤2.0 across the concentration ranges studied, was obtained from regression analysis. Recovery studies was found to be 98.86% for Emtricitabine and 99.96% Tenofovir alafenamide with the value of RSD less than 1% indicating that the proposed method is accurate for the simultaneous estimation of all drugs from their combination drug products in presence of their degradation products. The LOD that were found to be 0.1µg/ml for Emtricitabine and 0.0125 µg/ml for Tenofovir alafenamide drug. The LOQ for Emtricitabine and Tenofovir alafenamide were found to be 0.3 µg/ml and 0.0375µg/ml respectively. Total run time was 10 minutes within which main compounds and their degradation products were separated. The developed method was successfully applied to the simultaneous quantitative analysis of the title drugs in tablet dosage forms.

Keywords: Stability indicating assay; RP-HPLC; Emtricitabine; Tenofovir alafenamide; Forced degradation studies

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INTRODUCTION

Emtricitabine

Chemically 4-amino-5-fluoro-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one [1]. It has a molecular formula of $C_8H_{10}N_3O_3S$ and a molecular weight- 247.24 g/mol. Emtricitabine works by inhibiting reverse transcriptase the enzyme that copies HIV RNA into new viral DNA. Emtricitabine is a synthetic nucleotide analogue of cytidine. Freely soluble in methanol and in water, practically insoluble in methylene chloride. It has the following structural formula as shown in Figure 1.

Tenofovir Alafenamide

Chemically propan-2-yl (2S)-2-{[(S)-({[(2R)-1-(6-amino-9H-purin-9-yl)propan-2-yl]oxy}methyl)(phenoxy)phosphoryl]amino}propanoate [2]. It has a molecular formula of $C_{21}H_{29}N_6O_5P$ and a molecular weight- 476.47 g/mol. TAF is a nucleotide reverse transcriptase inhibitor (NRTII) and a novel ester prodrug of the antiretroviral Tenofovir. Soluble in water and methanol and also soluble in dimethyl sulfoxide. It has the following structural formula as shown in Figure 2**.**

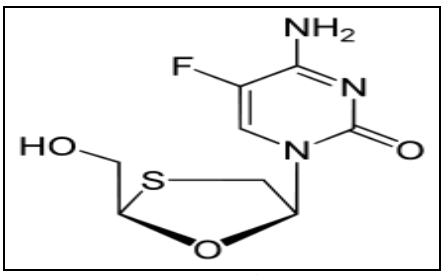

Figure 1: Structure of emtricitabine

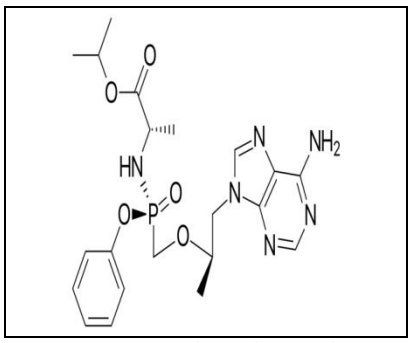

Figure 2: Structure of Tenofovir alafenamide

The literature survey reveals that there was only one HPLC [3] method was developed in combination of Emtricitabine and Tenofovir alafenamide in bulk and dosage forms. There were no reported analytical methods for simultaneous estimation Emtricitabine and Tenofovir alafenamide in bulk and their combined dosage forms in presence of their degradation products. Hence an author made an attempt to develop stability indicating specific, sensitive, accurate and precise RP-HPLC method for simultaneous estimation of these drugs. The developed method was validated as per ICH Q2 guidelines.

MATERIALS AND METHODS

Chemicals and Reagents

Reference standards of EMT and TAF were obtained as gift samples from Hetero pharma limited, Hyderabad, India. The formulation used was Descovy tablets containing (Label claim: 200 mg of EMT, 12.5 mg of TAF,) was procured from the local market. Potassium dihydrogen phosphate (AR Grade), Finer chemical limited, acetonitrile (HPLC grade) Ortho phosphoric acid (AR Grade), water and methanol used for of HPLC.

Instrumentation

The development and validation was carried out by using HPLC waters 2695 separation module model, variable wavelength UV detector module equipped with auto-sampler with injection volume 20 µl, column used was inertial ODS (4.6 × 250 mm, 5 µm) column and data recorded using Empower software.

Chromatographic Conditions

Various combinations of mobile phases were screened and finally, the mobile phase consisting of phosphate buffer (solvent A) and 0.1% Ortho acetonitrile (solvent B) was set with gradient programming for 12 min was optimized at a flow rate of 1 ml/min, 259 nm wavelength, injection volume of 20 µL and

ambient temperature was maintained during the entire process to obtain symmetric peaks of Emtricitabine and Tenofovir alafenamide.

Preparation of Solutions

Diluent:
Mobile phase was used as the diluent.

Mobile phase:
3.4 g of phosphate buffer (0.1 N) and acetonitrile is programmed as RP HPLC method.

Preparation of standard solution:
Standard stock solution was prepared accurately weigh and transfer 100 mg of Emtricitabine and 12.5 mg of Tenofovir alafenamide working standard into a 10 ml clean dry volumetric flask add about 7 mL of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution). Further pipette 0.6 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Method development:
The optimized chromatographic conditions (Figure 3).The best peak shape and maximum separation was achieved with mobile phase composition of phosphate buffer: Acetonitrile (80:20) using, peak symmetry and reproducibility were obtained on Inertial ODS (4.6 × 250 mm, 5 µm) column. The optimum wavelength for detecting the analytes was found to be 259 nm, a flow rate of 1ml/min yielded optimum separation and peak symmetry. The optimized chromatographic conditions were shown Table 1.

To saturate the column, the mobile phase was pumped for about 30 minutes thereby to get the base line corrected. The separate standard calibration lines were constructed for each drug. A series of aliquots were prepared from the above stock solutions using diluent to get the concentrations 20-100 µg/ml for Emtricitabine (EMT) and 2.5-12.5 µg/ml Tenofovir alafenamide (TAF). Each concentration 6 times was injected in to chromatographic system. Each time peak area and retention time were recorded separately for all the drugs. Calibration curves were constructed as by taking average peak area on Y-axis and concentration on X-axis separately for both drugs. From the calibration curves regression equations were calculated, these regression equations were used to calculate drug content in formulation. The obtained results were shown Table 2.

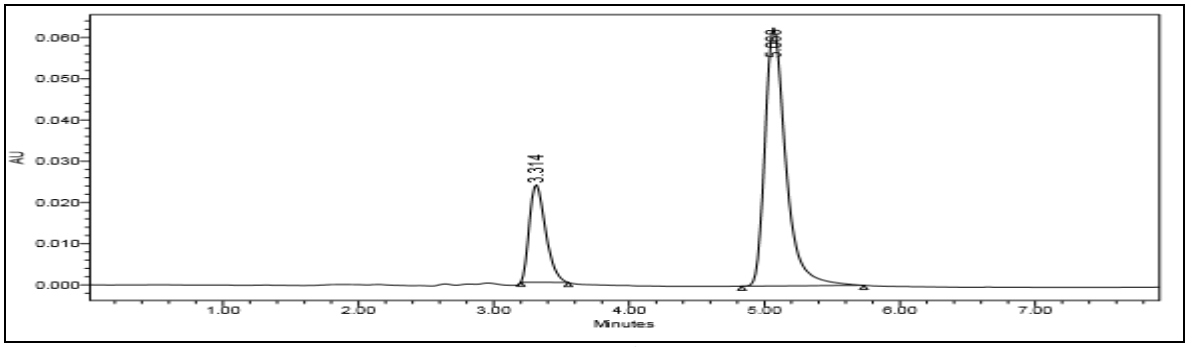

Figure 3: Chromatogram of EMT and TAF

Table 1: Optimized chromatographic conditions

Column	**ODS (4.6 × 250 mm, 5 mm)**
Mobile phase	Acetonitrile+phospate buffer 80+20
Flow rate	1 ml/min
Column temperature	Ambient
Injection volume	20 µl
Detection Wavelength	259 nm
Run time	10 mins
Retention time	3.314, 5.068.
Remarks	This method is suitable for validation

Preparation of sample solution:

Accurately weigh 10 tablets crush in mortor and pestle and transfer equivalent to 100 mg of emtricitabine and 12.5 mg tenofovir alafenamide sample into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate it up to 15 mins to dissolve it completely and make volume up to the mark with the same solvent. Then it is filtered through 0.45 micron injection filter (stock solution). Further pipette 0.6 ml of Emtricitabine and Tenofovir AF from the above stock solution into a 10 ml volumetric flask and dilute up to the mark with diluent to obtain concentration of 100 µg/ml, and 12.5 µg/ml for Emtricitabine and Tenofovir alafenamide respectively. The assay procedure was repeated 6 times (n=6) the drug content was estimated using above calculated regression equation; the results of tablet dosage form are shown in the Table 2.

Table 2: Results of tablet dosage form

Compound name	**Brand name**	**Label claim (mg)**	**Test concentration (µg/ml)**	**Mean amount estimated (µg/mL) (n=6)**	**%Assay**	**%RSD**
Emtricitabine	Discovery	200	600	600.1	100.35	0.362
Tenofovir alafenamide (TAF)		25	75	75.61	100.68	0.095

METHOD VALIDATION

The developed method for simultaneous estimation of Emtricitabine and Tenofovir alafenamide has been validated in accordance with the ICH guidelines.

Specificity and Selectivity

Selectivity test determines the effect of excipients on the assay result. To determine the selectivity of the method, standard solution of Emtricitabine and Tenofovir alafenamide, commercial product solution and blank solutions were run in the instrument one after another. The results of the tests proved that the components other than the drug did not produce any detectable signal at the retention time of EMT and TAF as shown in Figures 4-6. There were no interfering peaks at retention time of Emtricitabine and Tenofovir alafenamide.

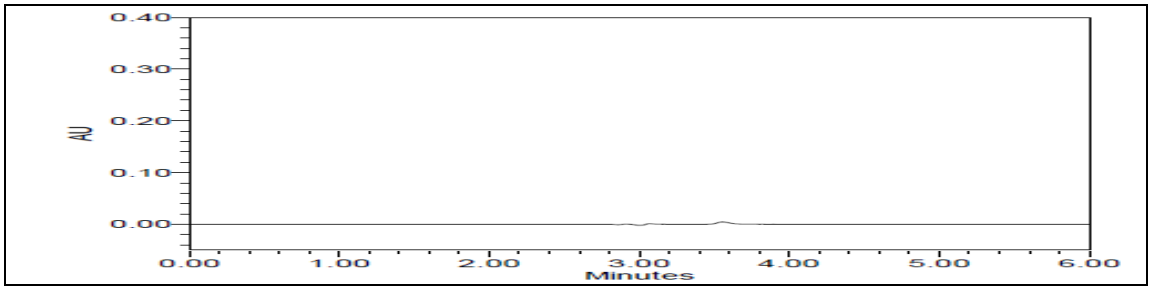

Figure 4: Specificity chromatogram of blank

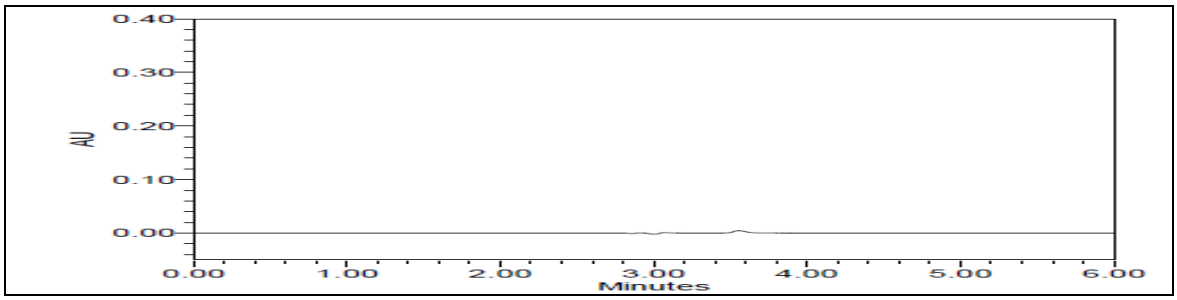

Figure 5: Specificity chromatograph of placebo

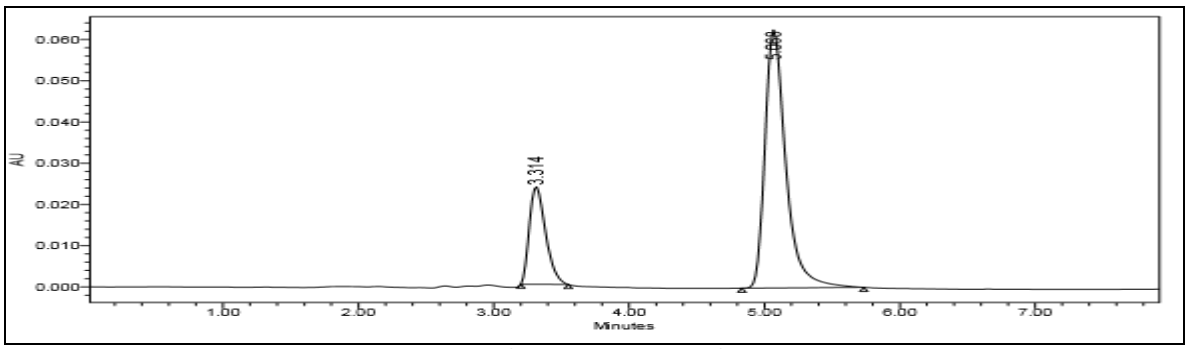

Figure 6: Specificity chromatograph of sample

Linearity

Several aliquots of standard stock solution of EMT and TAF were taken in different 10 ml volumetric flask and diluted up to the mark with mobile phase such that their final concentrations was 20-100 µg/ml for EMT, 2.5-12.5 µg/ml for TAF respectively. Peak areas were plotted against the corresponding concentrations to obtain the calibration graph for each compound. The linearity regression co-efficient (R2) values were found to be 0.999 for EMT and TAF, respectively. Linearity equation obtained for EMT and TAF were y = 1547.9x - 1967.5 and y = 1547.9x - 1967.5, respectively (Table 3). Figures 7 and 8 shows linearity graphs for EMT and TAF respectively.

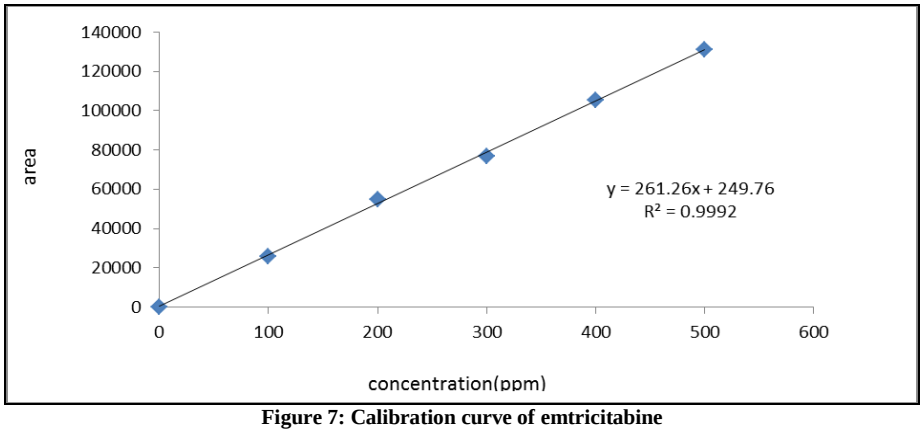

Figure 7: Calibration curve of emtricitabine

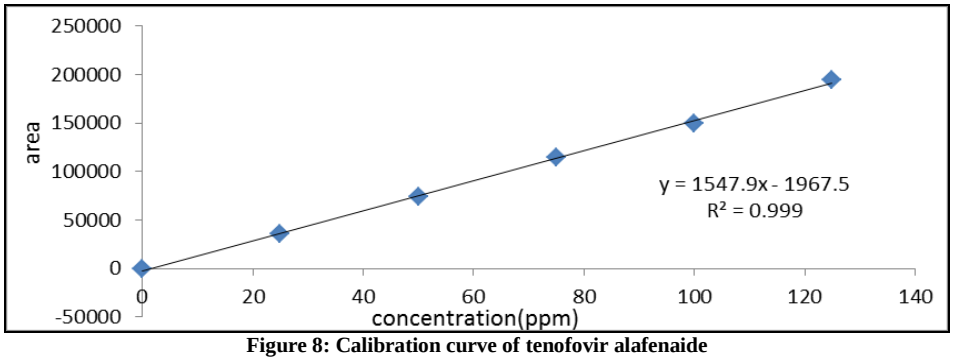

Figure 8: Calibration curve of tenofovir alafenaide

Accuracy:

The accuracy of the method for assay determination was achieved at three concentration levels of 50%, 100%, and 150% for EMT and TAF known amount of standard drug concentration was added to the sample and peak area was determined. The mean percentage recovery values are shown in Table 4.

Precision:

Precision is the degree of repeatability of an analytical method under normal operation condition.
Precision is of 3types.

1. System precision.
2. Method precision.
3. Intermediate precision.
   a. Intraday precision.
   b. Inter day precision.

Method precision was achieved by repeating the same procedure of preparation solution six times and injecting.

System precision is checked by injecting using standard chemical substance to ensure that the analytical system is working properly. In this peak area and percentage of drug of six determination is measured and percentage relative standard deviation should be calculated.

In method precision, a homogenous sample of single batch should be analyzed 6 times. The indicates weather a method is giving constant result for a single batch. In this analyze the sample six times and calculate the % RSD and the results are shown in the Table 5.

LOD and LOQ

LOD:

It is lowest amount of analyte in a sample that can be detected but not necessarily quantities ass an extract value under the stated, experimental conclusion. The detection limit is usually expressed as the concentrated analyte. The standard deviation and response of the slope.

LOD = 3.3*standard deviation (σ)/s

LOQ:

The quantitation limit of an analytical procedure is the lowest amount of an analyte of a sample which can be quantitatively determined with suitable precision and accuracy. The standard deviation and response of the slope and the results obtained.

LOQ= 10* standard deviation (σ)/s

The results of LOD and LOQ are shown in the Table 2.

System suitability:

Six replicate of sample containing EMT and TAF were given to evaluate equipment, electronics, and analytical operations and samples suitability. Parameters calculated for system suitability were percentage

of relative standard deviation of retention time and area, number of theoretical plates and resolution. Results found are given in Table 6 and within acceptable limits.

Robustness:
To evaluate the robustness of the method, the chromatographic conditions were deliberately altered and degree of reproducibility was evaluated. During robustness testing each condition was varied separately, all other conditions being held constant at the optimized values. Robustness of the proposed method was assessed with respect to small alterations in the flow rate (1.0 ± 0.2 ml/min), organic composition and wavelength (259 ± 2) and the results obtained from as shown the Table 7.

Forced Degradation Studies

Preparation of sample stock solution:
Accurately weigh 10 tablets crush in mortar and pestle and transfer equivalent to 100 mg of Emtricitabine and 12.5 mg Tenofovir AF in sample into a 10ml clean dry volumetric flask add about 7 ml of diluent and sonicate it up to 5 mins to dissolve it completely and make volume up to the mark with the same solvent. Then it is filtered through 0.44 micron injection filter (stock solution).

Hydrolytic degradation under acidic condition:
Pipette 0.6 ml of above solution into a 101 ml volumetric flask and 3 ml of 0.1 N HCl d. Then, the volumetric flask was kept at 60ºC for 24 hours and then neutralized with 0.1 N NaOH and make up to 10 ml with diluent. Filter the solution with 0.44 microns syringe filters and place in vials.

Hydrolytic degradation under alkaline condition:
Pipette 0.6 ml of above solution into a 10ml volumetric and add 3 ml of 0.1 N NaOH was added in 10 ml of volumetric flask. Then, the volumetric flask was kept at 60ºC for 24 hours and then neutralized with 0.1 N HCl and make up to 10ml with diluent. Filter the solution with 0.44 microns syringe filters and place in vials.

Thermal induced degradation:
Emtricitabine and Tenofovir alafenamide sample was taken in petridish and kept in Hot air oven at 110°C for 3 hours. Then the sample was taken and diluted with diluents and injected into HPLC and analyzed.

Oxidative degradation:
Pipette 0.6 ml above stock solution into a 10ml volumetric flask and 1 ml of 12.5% w/v of hydrogen peroxide added in 10 ml of volumetric flask and the volume was made up to the mark with diluent. The volumetric flask was then kept at room temperature for 15 min. Filter the solution with 0.45 microns syringe filters and place in vials.

Photo degradation:
Pipette 0.6 ml above stock solution into a 10ml volumetric flask and expose to sunlight for 24 hrs and the volume was made up to the mark with diluent. Filter the solution with 0.45 microns syringe filters and place in vial.

RESULTS AND DISCUSSION

Optimization of Chromatographic Conditions
A gradient, rapid and simple RP-HPLC method was developed and validated for the simultaneous estimation of EMT and TAF. Mobile phase consisting of acetonitrile (solvent A) and 0.1% phosphate buffer (solvent B) was set with gradient programming for 10 min. Chromatographic conditions were optimized for mobile phase using inertial ODS (250 × 4.6 mm, 5 µm) column at a flow rate of 1 ml/min. Effluents were detected at 259 nm by variable wavelength UV detector. Column compartment temperature was in ambient. Chromatogram of EMT and TAF at optimized chromatographic condition is shown in Figure 3.

Specificity and Selectivity

Figure 4-6 shows the chromatogram of blank, working placebo and sample solution. There were no interfering peaks at retention time of EMT and TAF.

Linearity, LOD and LOQ

The linearity regression co-efficient (R2) values were found to be 0.999 for EMT and 0.999 for TAF Linearity equation obtained for EMT and TAF were y =1547.9x - 1967.5, and y =1547.9x - 1967.5, respectively. Figures 7 and 8 show linearity graphs for EMT and TAF respectively. The LOD that were found to be 1.68 µg/ml for Emtricitabine and 2.03 µg/ml for Tenofovir alafenamide drug. The LOQ for Emtricitabine and Tenofovir alafenamide were found to be 5.58 µg/ml and 6.57 µg/ml respectively. The Regression results indicate that method was linear in the concentration range studied and can be used for detection and quantification of Emtricitabine and Tenofovir alafenamide in a very wide concentration range.

Table 3: Linearity studies of proposed method

Parameters	Emtricitabine	Tenofovir alafenamide
Linearityrange (ug/ml)	20-100	2.5-12.5
Regression equation	Y=261.26x+ 249.76	Y=1547.9x-1967.5
Slope	261.26	1547.9
Intercept	249.76	-1967.5
Correlation coefficient	0.999	0.999
LOD (µg/ml)	0.1	0.0125
LOQ (µg/ml)	0.3	0.0375

Accuracy and Precision

Accuracy as recovery was evaluated by spiking previously analyzed test solution with additional Placebo at three different concentration levels (Table 4). Recovery of previously analyzed test solution drug concentration added was found to be 98.86% for Emtriciabine and 99.96% for Tenofovir Alafenamide with the value of RSD less than 1% indicating that the proposed method is accurate for the simultaneous estimation of all drugs from their combination drug products in presence of their degradation products. The low RSD values indicate the repeatability and reproducibility of the Method (Tables 6 and 7).

Table 4: Recovery studies of emtriciabine and tenofovir alafenamide

Drugs	% of Recovery levels	Preanalyzed concentration (ug/ml)	Amount Added (ug/ml)	Amount Found (ug/ml)	%Recovery	% RSD
	50	20	10	29.98	99.96	
EMT	100	20	20	39.86	99.86	0.11
	150	20	30	49.66	99.77	
	50	5	2.5	7.67	100.28	
TAF	100	5	5	10.04	100.04	0.9
	150	5	7.5	12.17	99.56	

Table 5: Results for precision of the standard

	Emtricitabine				Tenofovir Alafenamide	
S.no	Concentration (ug/ml)	Retention Time	Area	concentration (ug/ml)	Retention Time	Area
Injection 1	60	3.602	111368	7.5	5.137	852828
Injection 2	60	3.603	112717	7.5	5.138	852337
Injection 3	60	3.604	112655	7.5	5.168	858355
Injection 4	60	3.606	113839	7.5	5.143	852839
Injection 5	60	3.609	1112.513	7.5	5.156	858513
Injection 6	60	3.609	112282	7.5	5.156	857582
Mean			112662.3			855404
SD			845.7			12.524
%RSD			0.8			0.4

Table 6: Results for precision of the Sample

S.no	Emtricitabine				Tenofovir Alafenamide	
	concentration (ug/ml)	Retention Time	Area	concentration (ug/ml)	Retention Time	Area
Injection 1	60	3.602	111368	7.5	5.137	852828
Injection 2	60	3.603	112717	7.5	5.138	852337
Injection 3	60	3.604	112655	7.5	5.168	858355
Injection 4	60	3.606	113839	7.5	5.143	852839
Injection 5	60	3.609	1112.513	7.5	5.156	858513
Injection 6	60	3.609	112282	7.5	5.156	857582
Mean			112662.3			855404
SD			845.7			12.524
%RSD			0.8			0.4

Robustness

Results of the robustness (Table 7). The elution order and resolution for all components were not significantly affected. RSD of peak areas were found to be well within the limit of 2.0%.

Table 7: Results of Robustness by variation in flow rate and organic rate

Parameters	Retention Time		Peak area		%Recovery	
	EMT	TAF	EMT	TAF	EMT	TAF
Flow Minus(0.8)	4.02	5.79	700908	88677	100	100
Flow Plus(1.2)	3.3	4.72	823857	119810	100	100
Organic Minus	3.91	6251	846897	113202	100	100
Organic Plus	3.37	4.5	815110	118785	100	100

System Suitability

Six replicates of sample containing EMT and TAF were given to evaluate equipment, electronics, and analytical operations and samples suitability. Parameters calculated for system suitability were a number of theoretical plates, tailing factor, resolution, retention time, and area (Table 8).

Table 8: System suitability results for emtriciabine and tenofovir alafenamide

S.no	Parameters	EMT	TAF
1	Theoretical plates	4361	5749
2	Tailing factors	1.3	1.25
3	Resolution	-	6.64
4	Relative retention time (minutes)	3.11	5.12

Degradation Studies

Results are tabulated in Table 9.

Acid hydrolysis:

Upon performance of acid degradation studies 7% of Emtricitabine and 6.6% of Tenofovir alafenamide were degraded (Figure 9a).

Base hydrolysis:

Upon performance of base degradation studies 4.4% of Emtricitabine and 4.7% of Tenofovir alafenamide were degraded (Figure 9b).

Peroxide hydrolysis:
Upon performance of peroxide degradation studies 4.7% of Emtricitabine and 5.7% of Tenofovir alafenamide were degraded (Figure 9d).

Thermal degradation:
Upon performance of Thermal degradation studies 4.6% of Emtricitabine and 4.8% of Tenofovir alafenamide were degraded (Figure 9c).

Photolytic degradation:
Upon performance of Thermal degradation studies 6.5% of Emtricitabine and 2.5% of Tenofovir alafenamide were degraded (Figure 9e).

Table 9: Stability studies for emtricitabine and tenofovir alafenamide

Conditions	Drugs	% Degradation	% of Assay After Degradation
Acid	Emtricitabine	7	93
	Tenofovir alafenamide	6.6	93.4
Alkali	Emtricitabine	4.4	95.6
	Tenofovir alafenamide	4.7	95.3
Peroxide	Emtricitabine	4.7	95.3
	Tenofovir alafenamide	5.7	94.3
Thermal	Emtricitabine	4.6	95.6
	Tenofovir alafenamide	4.8	95.2
photolytic	Emtricitabine	6.5	93.5
	Tenofovir alafenamide	2.5	97.5

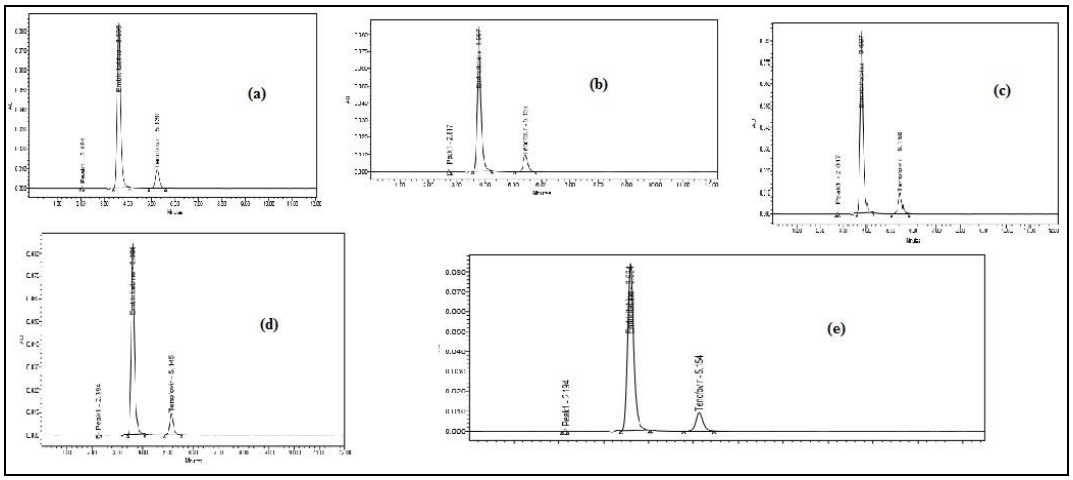

Figure 9: Chromatogram of (a) acid degradation (b) base degradation (c) thermal degradation (d) peroxide degradation (e) photolytic degradation

CONCLUSION

A simple, rapid, accurate and precise stability-indicating RP-HPLC analytical method has been developed and validated for the quantitative analysis of Emtricitabine and Tenofovir alafenamide in bulk drugs and combined dosage forms. The newly developed RP-HPLC method for separation of different degradation products along with the pure drugs were found to be capable of giving faster retention times while still maintaining good resolution than that achieved with conventional HPLC. This method exhibited an excellent performance in terms of sensitivity and speed. The results of stress testing undertaken according to the ICH guidelines reveal that the method is specific and stability-indicating. The proposed method has

the ability to separate these drugs from their degradation products in tablet dosage forms and hence can be applied to the analysis of routine quality control samples and samples obtained from stability studies.

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