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Simultaneous method development and validation for Tenofovir AF, Emtricitabine, and Bictegravir by RP-UPLC

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ABSTRACT
A new, accurate RP-UPLC method has been developed and validated for the simultaneous quantification of Bictegravir (BCG), Tenofovir Alafenamide (TAF), and Emtricitabine (ETB) in pharmaceutical formulations. Chromatographic separation was achieved on an Acquity UPLC BEH C18 column (2.1 × 50 mm, 1.7 μm) using an isocratic mobile phase of 10 mM phosphate buffer (pH 3.0): acetonitrile (60:40, v/v) at a flow rate of 0.3 mL/min and detection at 260 nm. Retention times for ETB, BCG, and TAF were 2.3, 3.2, and 3.7 minutes, respectively. The method was validated per ICH Q2 (R1) guidelines and demonstrated excellent linearity (r > 0.999), precision (%RSD < 1%), and high recovery (99.8–100.6%) for all analytes. Forced degradation studies indicated the method is stability-indicating. The proposed UPLC method is suitable for routine quality control analysis of these drugs in fixed-dose combination tablets.
Key words: *Tenofovir, RP-UPLC, Emtricitabine, and Bictegravir*

1. INTRODUCTION

Emtricitabine (ETB) is a nucleoside reverse transcriptase inhibitor and cytidine analog used to treat HIV infection. It exerts its antiviral effect by competitively inhibiting reverse transcriptase, thereby preventing the conversion of viral RNA into proviral DNA. Its chemical structure is designated as 4-amino-5-fluoro-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]pyrimidin-2(1H)-one, and the structure of ETB was depicted in Figure 1 [1]. Bictegravir (BCG) is a novel integrase strand transfer inhibitor (INSTI) permitted for the medication of HIV-1 and HIV-2 infections. Structurally, it is classified as (2R,5S,13aR)-8-hydroxy-7,9-dioxo-N-(2,4,6-trifluorobenzyl)-2,4,12-trioxa-10-azatetracyclo[11.2.1.0^{2},^{11}.0^{5},^{10}]hexadeca-1(15),6,8,13-tetraene-6-carboxamide and the structure of BCG was shown in Figure 1. It is commonly administered in combination with other antiretroviral agents as part of a single-tablet regimen for enhanced therapeutic efficacy [2]. Combination therapy with two or more drugs effectively suppresses the virus and regulates the immune system and prevents drug resistance. Tenofovir alafenamide (TAF) is a nucleotide reverse transcriptase inhibitor (NRTI) and a prodrug of tenofovir. Unlike conventional nucleotides, tenofovir lacks a 3'-hydroxyl group, leading to premature termination of viral DNA chain elongation during reverse transcription. Its chemical name is isopropyl (2S)-2-[[[(1R)-2-(6-amino-9H-purin-9-yl)-1-methylethoxy] methylphenoxy-phosphoryl]amino] propanoate, and the chemical structure is shown in Figure 1) [3]. Tenofovir disoproxil fumarate (TDF) has been extensively used as a key part of HIV antiretroviral therapy because of its proven effectiveness and safety. However, long-term use of TAF can cause adverse symptoms such as bone mineral depletion and nephrotoxicity. In comparison, tenofovir alafenamide fumarate (TAF) shows better antiviral activity, delivers tenofovir (TFV) more efficiently into peripheral blood mononuclear cells and lymphoid tissues, has a higher genetic barrier to resistance, and offers a better safety profile. The lower renal and bone toxicity linked to TAF is due to its lower systemic plasma tenofovir levels, which reduces exposure to sensitive tissues. Since antiretroviral therapy is usually lifelong, these benefits support better long-term patient outcomes and improved treatment adherence [4].

A comprehensive literature review indicates that no analytical method has been reported for the simultaneous quantification of ETB, BCG, and TAF in pharmaceutical formulations. Existing methods include reverse-phase high-performance liquid chromatography (RP-HPLC) for emtricitabine and TAF [5–9], RP-HPLC for TAF and ETB [9–11], UV spectrophotometric techniques for the simultaneous analysis of emtricitabine and TAF [12], spectroscopic and fluorimetric assays [13], and additional RP-HPLC methods [14,15]. Compared to HPLC, UPLC is a good choice because it has lower retention times, consumes less solvent, and can withstand higher column pressures.

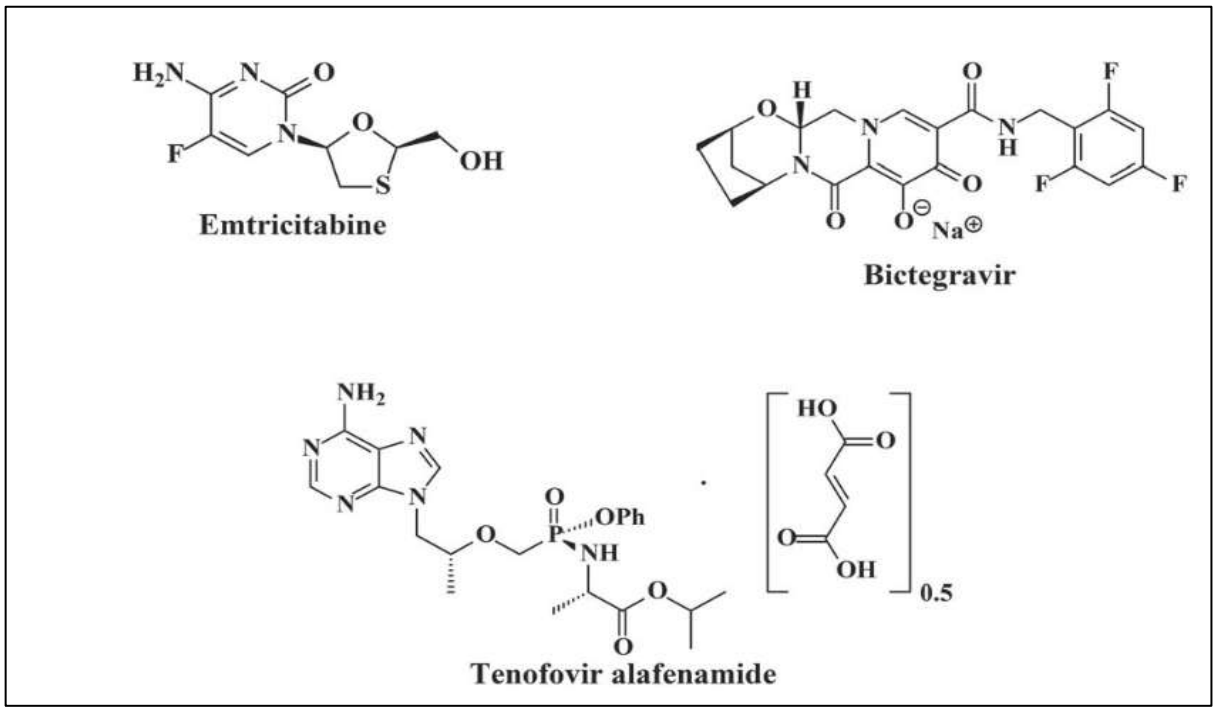

Figure 1. Chemical structures of ETB, BCG and, TAF

Currently, no official high-performance liquid chromatography (UPLC) methods for the simultaneous quantification of ETB, BCG, and TAF in pharmaceutical formulations have been reported in the literature. Therefore, this analysis aimed to develop and validate a novel, cost-effective, precise, and accurate stability indicating reverse-phase UPLC (RP-UPLC) method for the analysis of these antiretroviral drugs in combined dosage forms.

2. MATERIALS AND METHODS

Pure drug samples of ETB, BCG, and TAF were generously provided as gift samples by Spectrum Pharma Research Solutions, Hyderabad. The commercial pharmaceutical formulation (Biktarvy®, manufactured by Symphony Pharma Limited, Guwahati, Assam) was procured from a local pharmacy. All solvents, chemicals and reagents used in the study were of analytical grade and sourced from Merck Private Limited, Mumbai.

Method Development and Optimization

During method development, various mobile phase compositions were evaluated, including methanol mixed with orthophosphoric acid (OPA) aqueous buffer and methanol mixed with phosphate buffer at various pH levels and volumetric ratios. After systematic optimization, the final mobile phase was selected as a mixture of aqueous phase (phosphate buffer, pH 3.0) and organic phase (acetonitrile) in a 60:40 (v/v) ratio, which provided optimal chromatographic performance. The UV absorption spectra of TAF, ETB, and BCG (each at a concentration of 10 µg/mL in diluent matching the mobile phase composition) were recorded across a UV wavelength series of 200–400 nm. Based on spectral analysis, 260 nm was selected as the optimal detection wavelength, as all three compounds exhibited significant and consistent absorbance at this value. The chromatographic method was evaluated using multiple stationary phases, including C18, Hypersil, Lichrosorb, and Inertsil ODS columns. Among these, the BEH C18 column with dimensions 2.1 × 50 mm, 1.7 µm particle size was selected as optimal due to its superior peak symmetry and resolution at a flow rate of 0.3 mL/min.

This study was accomplished by using a Waters branded UPLC system equipped with autosampler technology and an inbuilt photodiode array detector. The chromatographic separation of the analytes was attained by using a UPLC BEH C18 column with a diameter of 50x4.6mm and a particle size of 5µm, and kept at 25 ±2 °C as column temperature. The mobile phase consisted of a phosphate buffer and acetonitrile in a ratio of 60:40 (v/v). The aqueous buffer was made by dissolving 3.4 g of potassium dihydrogen orthophosphate in 1.0 L of milli-Q-water, followed by pH adjustment to 3.0 using diluted orthophosphoric acid solution. The flow rate was set at 0.3 mL/min, and detection was carried out at a wavelength of 260 nm. The injection volume was 2 µL, and the total run time was 4 minutes. A homogeneous mobile phase was prepared by mixing 600 mL (60% v/v) of the previously made buffer solution with 400 mL (40% v/v) of UPLC-grade acetonitrile. The mixture was degassed for 10 minutes using an ultrasonic water bath to eliminate dissolved gases, followed by vacuum

filtration through a 0.45 µm membrane filter to ensure particulate-free solvent compatibility with the UPLC system. The mobile phase (buffer and ACN in the ratio of 65:35) v/v is directly used as a diluent for making the sample solutions.

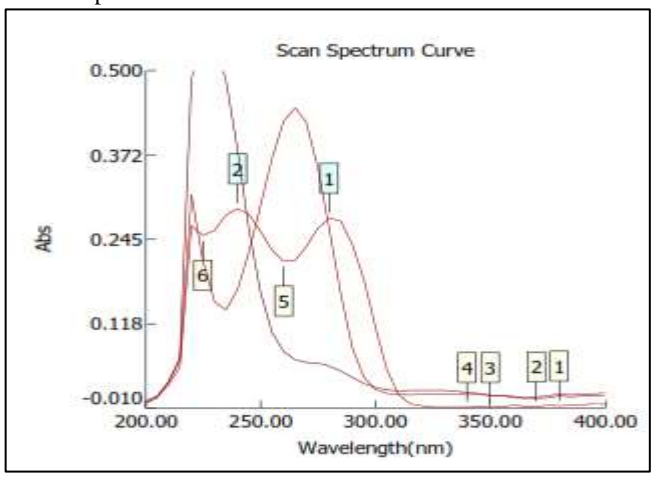

Figure 2. UV scan spectra for ETB, BCG and TAF

Preparation of standard solution

To prepare the mixed standard solution, TAF, ETB, and BCG were transferred into a 100 mL volumetric flask with TAF (25 mg), ETB (200 mg), and BCG (50 mg). Then, 50 mL of diluent was added, the mixture was sonicated to dissolve, and the volume was adjusted to the mark with diluent. Additionally, 750 µL of this stock solution was transferred into a 10 mL volumetric flask and made up to the volume with diluent. This solution was used as a working standard solution for further analysis.

Preparation of sample solution

To prepare the sample solution, ten tablets each of TAF 25 mg, ETB 200 mg, and BCG 50 mg were transferred into a mortar. These were crushed into a fine powder with a pestle. The powder was then transferred into a 100 mL volumetric flask, and some diluent was added. The mixture was shaken well and then sonicated for 30 minutes to dissolve. After sonication, the solution was diluted with diluent. The solution was filtered, and 750 µL of it was transferred to a 10 mL volumetric flask, which was then diluted to volume with diluent. This prepared solution was used as a working solution. Then, 2 µL of this solution was directly injected into the UPLC for analysis of the three drugs. The peak areas corresponding to TAF, ETB, and BCG were recorded. The assay percentages of the three drugs were calculated using the following formulas.

% Assay = (Sample Peak Area / Standard Peak Area) × (Standard Concentration / Sample Concentration) × 100

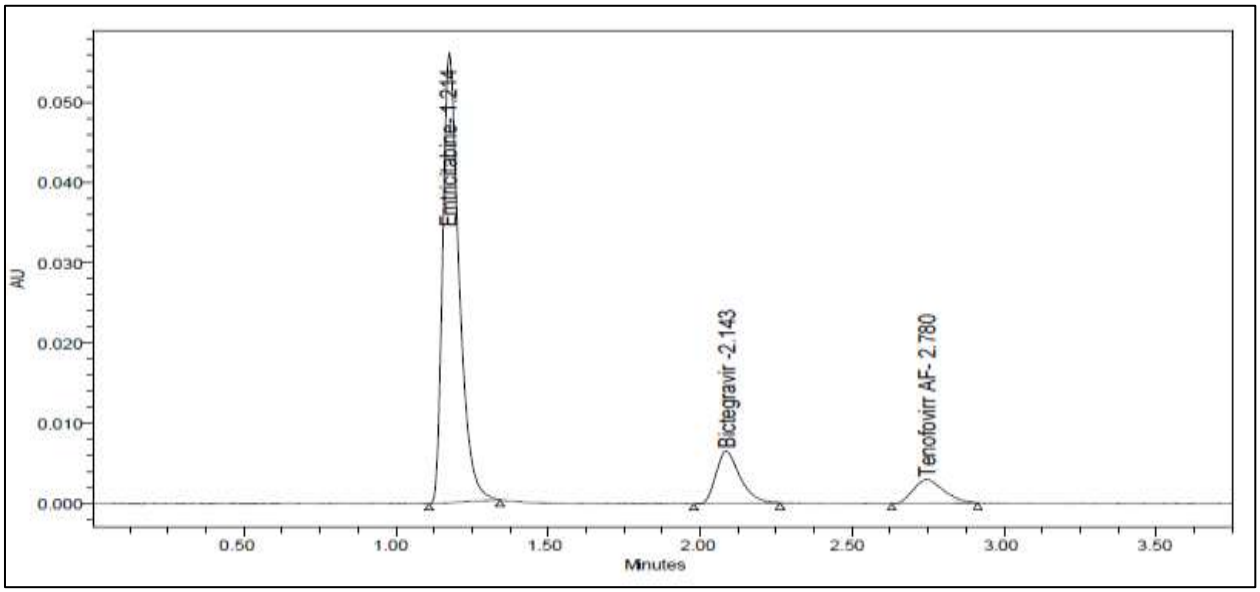

Figure 3. UPLC chromatogram of ETB, BCG and TAF

METHOD VALIDATION

System suitability

Prior to analyzing sample solutions, the system suitability test (SST) was conducted. During the SST, samples and standard solutions were injected into the UPLC. The results showed a tailing factor is 1.42, resolutions between the peaks greater than 2, and a theoretical plate count of 5575.

Specificity

To check the method specificity, blank, sample, and standard solutions were injected into the UPLC. No interference from blank peaks was observed with the RT's of analyte peaks in the sample and standard solutions.

Precision

To evaluate the method's precision, six injections of the standard solution at 100% concentration were performed using UPLC, and the % RSD values for TAF, ETB, and BCG were recorded. To assess intermediate precision, the samples were analyzed on different days by different personnel. The method demonstrated good precision with % RSD values below 0.4%.

Accuracy

To check the method's accuracy, three various concentrations (50%, 100%, and 150%) levels of the standard solution were injected in triplicate into the UPLC, and the resulting recovery values for TAF, ETB, and BCG were measured and depicted in Table 1.

Table 1. Results for accuracy

Drug	Concentration (%)	Area	Amount added	Amount found	% Recovery	Mean recovery
ETB	50%	476,290	100	99.38	99.38	**99.98**
	100%	154,381	50	49.78	99.57	
	150%	231,466	75	74.64	99.52	
TAF	50%	45,290	12.5	12.58	100.63	**99.99**
	100%	89,720	25	24.92	99.67	
	150%	134,564	37.5	37.37	99.66	
BCG	50%	77,719	25	25.06	100.25	**99.78**
	100%	154,381	50	49.78	99.57	
	150%	231,466	75	74.64	99.52	

Linearity

To assess the method's linearity, prepared five different concentrations of the sample solutions: 25, 50, 75, 100, and 125. Each concentration was injected in triplicate using UPLC. Then plotted a graph of the area versus concentration. The resulting linearity plot yielded a strong correlation coefficient value of greater than 0.999. The resulting linearity plots and their R^2 values for TAF, BCG and EMT depicted in Figure 4.

Table 2. Results for Linearity

Linearity Level	Concentration (µg/mL)	Area (EMT)	Area (BCG)	Area (TAF)
I	50 (ETB), 12.5 (BCG), 6.25 (TAF)	522088	65477	47257
II	100 (ETB), 25 (BCG), 12.5 (TAF)	734633	110790	67723
III	150 (ETB), 37.5 (BCG), 18.75 (TAF)	950658	153097	89884
IV	200 (ETB), 50 (BCG), 25 (TAF)	1192066	193120	109712
V	250 (ETB), 62.5 (BCG), 31.25 (TAF)	1430452	239955	134068
Correlation Coefficient (r^2)		**0.999 (All Analytes)**		

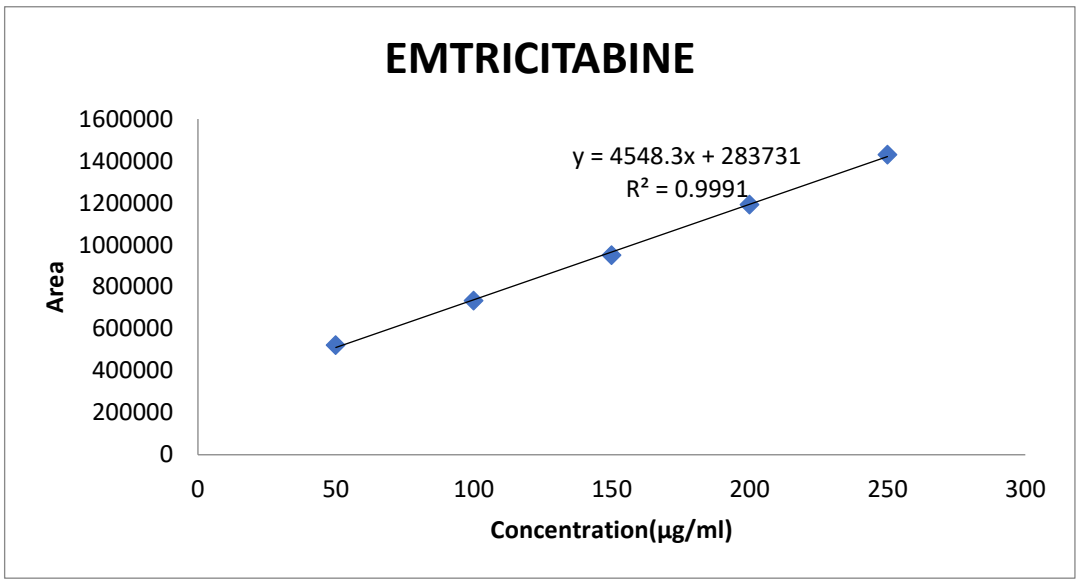

Figure 4. a) Linearity plot for ETB

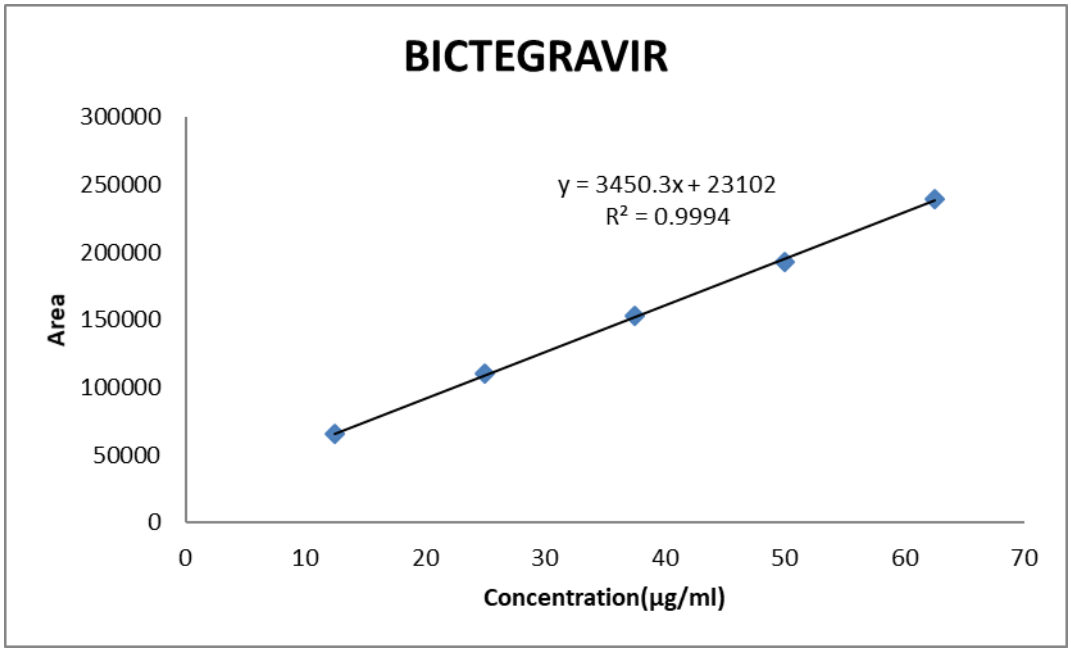

Figure 4. b) Linearity plot for BCG

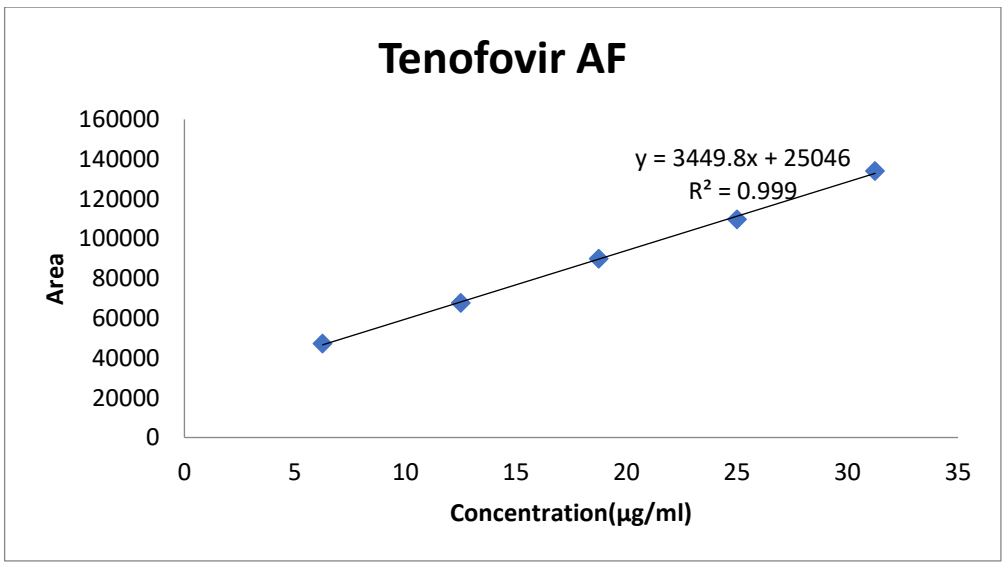

Figure 4. c) Linearity plot for TAF

LOD & LOQ

The method limit of detection (LOD) and limit of quantification were assessed by using the signal to noise (s/n) ratio. Standard stock solutions of the three drugs were serially diluted to prepare working solutions of known concentrations. The LOD and LOQ were known based on a signal-to-noise ratio (SNR) of 3:1 and 10:1, respectively. This method was established with required concentrations at the LOD and LOQ being 0.13 and 0.35 µg/mL.

Robustness

To assess the robustness of the method, deliberate variations in key chromatographic parameters were introduced, including flow rate, mobile phase composition, and column temperature. The flow rate was adjusted within a range of 0.27 mL/min to 0.33 mL/min, while the mobile phase composition was altered by ±10% of its original ratio. Additionally, the column temperature was systematically varied to evaluate its influence on method performance. These modifications were implemented to determine the analytical method's resilience to minor operational changes and its suitability for routine quality control applications. During the robustness, the resulting values for various parameters such as USP plate count, USP tailing, and USP resolutions are depicted in Table 3. Acceptance criteria for plate count is not ≤ 1500, tailing factor not ≥2, and resolution is not ≤ 2.

Table 3. Variation in flow rate

Analyte	Flow Rate (mL/min)	USP Plate Count	USP Tailing	USP Resolution
ETB	0.27	2263.65	1.40	-
	0.30*	2112.00	1.45	-
	0.33	2151.29	1.44	-
BCG	0.27	3331.30	1.29	7.38
	0.30*	3186.09	1.33	7.31
	0.33	2971.64	1.41	7.11
TAF	0.27	3035.38	1.40	3.87
	0.30*	3353.63	1.27	3.90
	0.33	3465.98	1.41	3.89

Table 4. Variation in Organic Mobile Phase Composition

Analyte	**Organic Phase Change**	**USP Plate Count**	**USP Tailing**	**USP Resolution**
ETB	10% less	2445.83	1.40	–
	Actual*	2112.00	1.45	–
	10% more	2104.64	1.39	–
BCG	10% less	3594.68	1.29	9.15
	Actual*	3186.09	1.33	7.31
	10% more	2935.13	1.48	6.02
TAF	10% less	5094.60	1.32	4.68
	Actual*	3353.63	1.27	3.90
	10% more	3252.62	1.37	3.61

Degradation Studies

According to the ICH were carried out the stress studies of TAF, ETB and BCG. In this study, stability educations were accompanied on TAF, ETB, and BCG using the developed analytical method to assess their degradation behavior under various stress conditions.

Acid hydrolysis

During alkaline hydrolysis, the sample solution (750 µL) was transferred into a clean volumetric flask (10 mL), to which 3.0mL of hydrochloric acid (HCl) was added. This solution was incubated at 60°C for 6 hours to induce acidic hydrolysis. After degradation, the solution was neutralized by adding 0.1 N sodium hydroxide (NaOH) to stop further degradation. The resulting solution was filtered and transferred into the UPLC for further analysis.

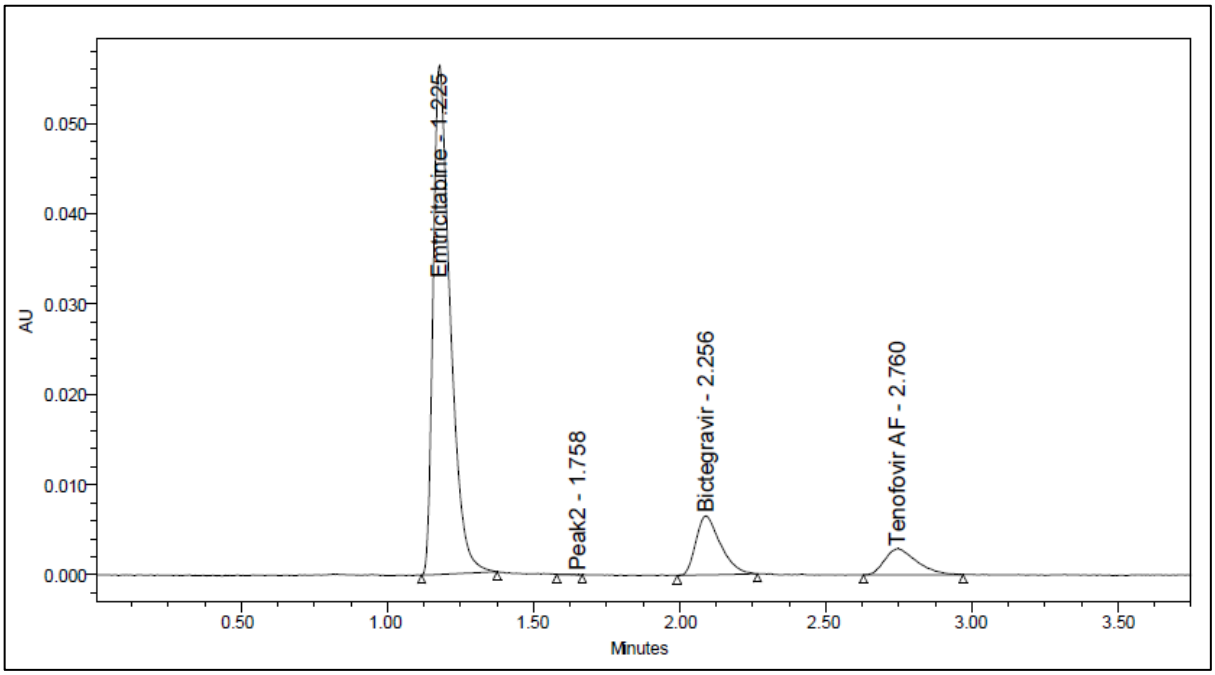

Figure 5. UPLC chromatogram for acidic degradation behavior of ETB, BCG, and TAF

Alkaline hydrolysis

During alkaline hydrolysis, the sample solution (750 µL) was transferred into a clean volumetric flask (10 mL), to which 3.0 mL of 0.1 N sodium hydroxide (NaOH) was added. This solution was incubated at 60°C for 6 hours to induce alkaline hydrolysis. After degradation, the solution was neutralized by adding 0.1N HCl to stop further degradation. The resulting solution was filtered and transferred into the UPLC for further analysis.

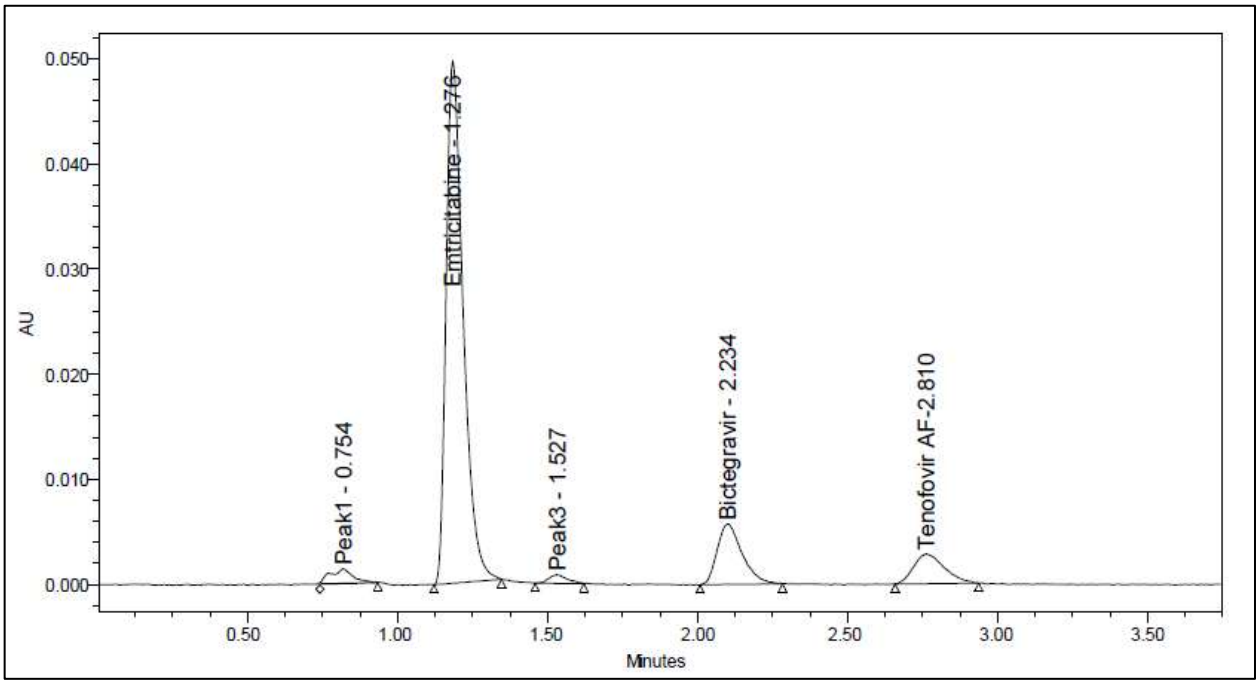

Figure 6. UPLC chromatogram for alkaline degradation behavior of ETB, BCG, and TAF

Thermal degradation

Forced degradation of TAF, ETB, and BCG was conducted under thermal stress conditions. The drug samples were placed in a petri dish and subjected to heating in a hot air oven at 110°C for 24 hours. Following thermal exposure, the degraded samples were allowed to cool, reconstituted with a suitable diluent, and subsequently analyzed using high-performance liquid chromatography (UPLC) to assess stability and degradation behavior.

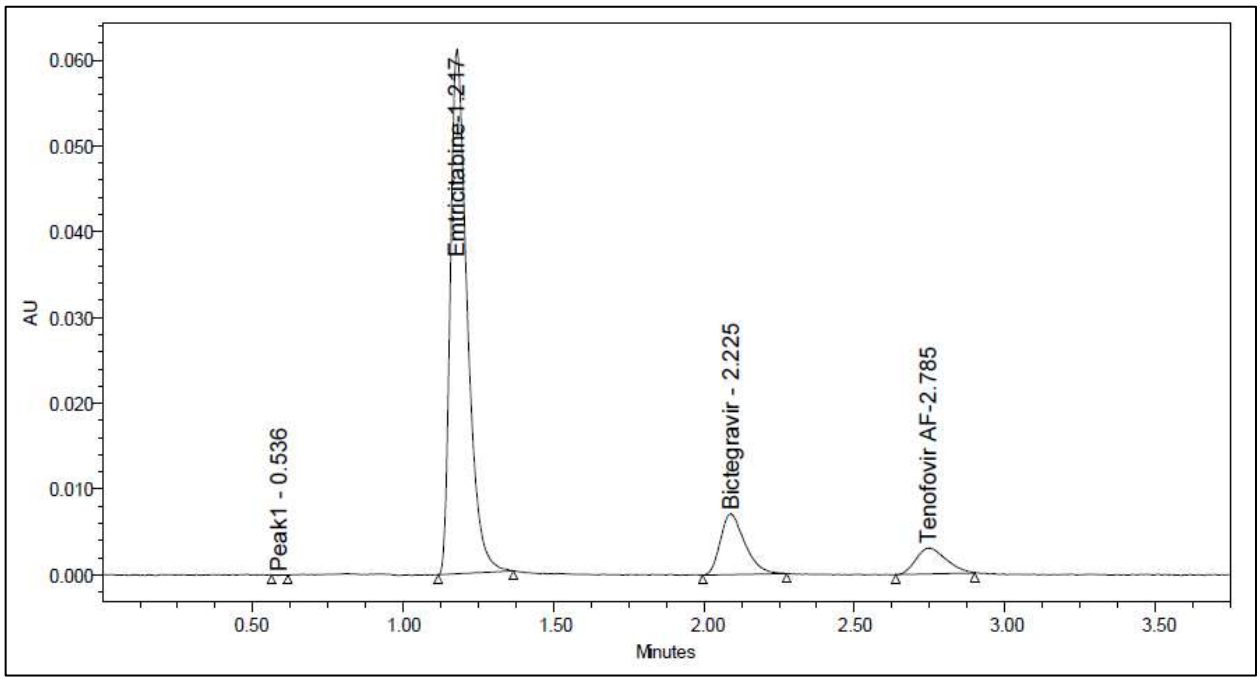

Figure 7. UPLC chromatogram for thermal degradation behavior of ETB, BCG, and TAF

Oxidative degradation

Accurately pipette 750 µL mL of the prepared stock solution into a 10 mL VF. Added 1000 µL of 3% (w/v) H_2O_2 (hydrogen peroxide solution) to the solution and diluted to the final volume (10 mL) with the appropriate diluent. To induce oxidative stress, the solution was left at room temperature for 24 h. The resulting solution was filtered and transferred into UPLC vials for further analysis.

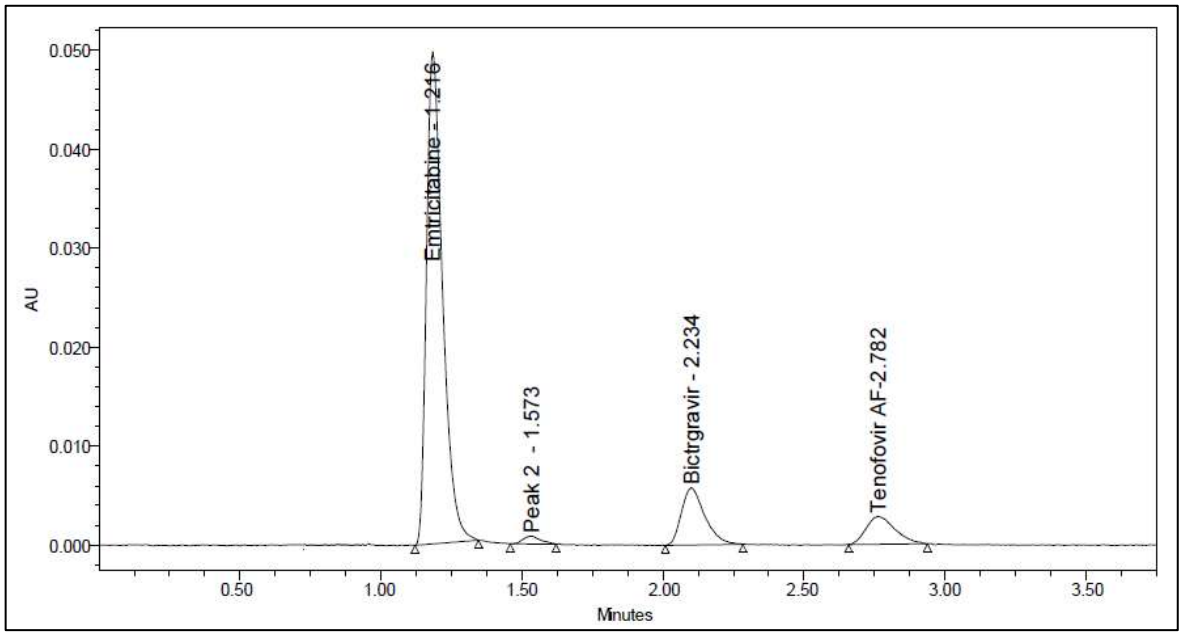

Figure 8. UPLC chromatogram for oxidative degradation behavior of ETB, BCG, and TAF

Photolytic degradation

Accurately pipette 750 µL mL of the prepared stock solution into a 10 mL and diluted to the final volume (10 mL) with the appropriate diluent. To induce photolytic stress, the solution was left at UV chamber for 24 h. The resulting solution was transferred into UPLC vials for further analysis.

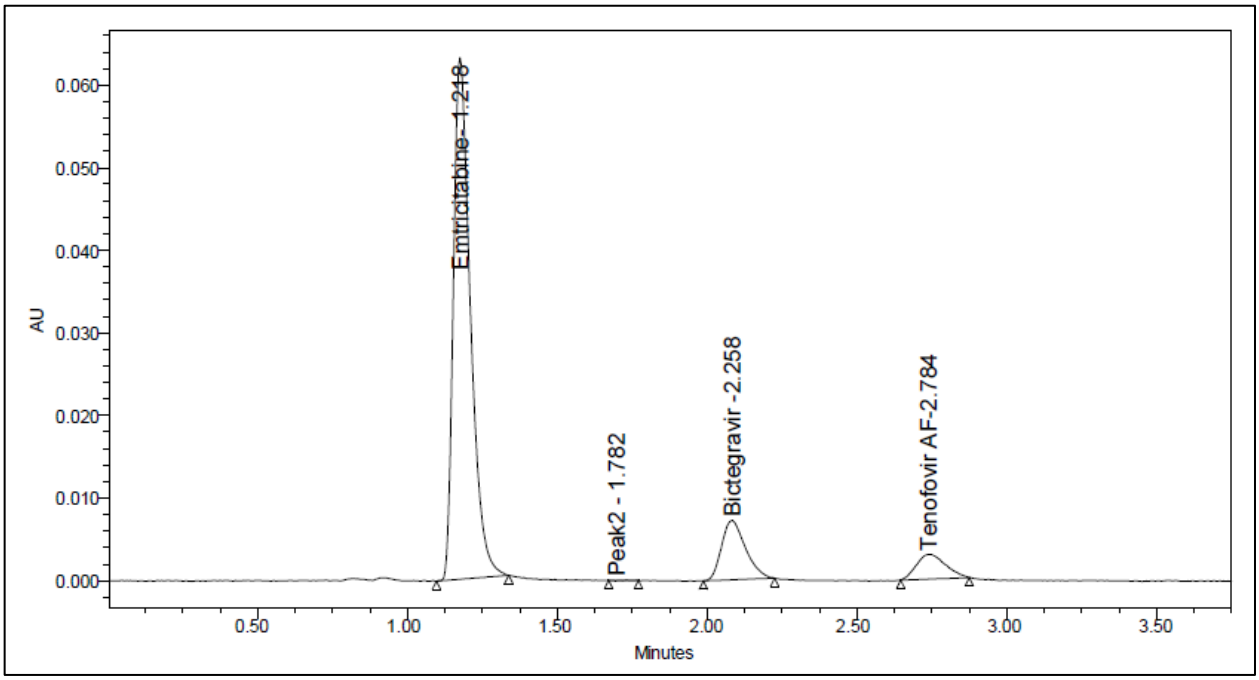

Figure 9. UPLC chromatogram for photolytic degradation behavior of ETB, BCG, and TAF

Table 5. Results for degradation studies

Drug	Condition	Area	% Degraded
ETB	Standard	956,581	-
	Acid	920,116	3.02
	Base	927,681	3.61
	Peroxide	955,023	-
	Thermal	951,805	-
	Photo	921,845	3.81
BCG	Standard	154,741	-
	Acid	141,008	7.88
	Base	142,549	6.58
	Peroxide	144,556	5.92
	Thermal	155,584	-
	Photo	145,686	8.87
TAF	Standard	89,833	-
	Acid	85,764	3.92
	Base	86,313	3.03
	Peroxide	87,109	3.47
	Thermal	89,720	-
	Photo	86,646	4.53

CONCLUSION

Successfully developed a simultaneous UPLC method for the identification of three antiviral drug molecules of Tenofovir alafenamide (TAF), Emtricitabine (ETB), and Bictegravir (BCG) in pharmaceutical formulations. This method is simple, accurate, and efficient for the determination of these three drugs, even it has been validated according to ICH guidelines. This method was shown to have good method accuracy, precision, linearity, LOD, LOQ, and robustness. These three drugs showed good degradation under stress conditions, including acidic, basic, oxidative, photolytic, and thermal stress conditions. And they formed new degradation impurities. Degradation studies also revealed that impurities formed in the stress studies did not interfere with the peaks of the three drug molecules. Importantly, chromatographic analysis confirmed that the degradation products did not interfere with the analyte peaks, confirming the specificity of the method for the target drugs. Given its reliability and reproducibility, the validated RP-UPLC method is robust, specific and suitable for simultaneous quantification of TAF, BCG, and ETB in bulk and tablet forms. Its successful application to

forced degradation conditions confirms its stability-indicating nature, making it ideal for routine QC (quality control) analysis in pharma manufacturing, drug testing laboratories, and regulatory compliance assessments.

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Authors Contribution
T. Raja Reddy was responsible for the literature review, a simultaneous UPLC method development, chromatographic analysis, drafting, revising, reviewing, and editing the manuscript.
Dr. Madhusudana Reddy M.B. provided overall guidance, conceptualization, and supervision of the project.

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