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Development, Optimisation, and Validation of a Stability-Indicating Reverse Phase HPLC Method for Simultaneous Estimation of Tenofovir and Emtricitabine in Drug Substance and Combination Formulations

Chetan Prakash Pulate*[1], Srujan Kumar M[2]

[1]Institute of Pharmaceutical Sciences & Research, Bhagwant University, Sikar Road, Ajmer-305023, Rajasthan, India

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KEYWORDS

Emitricitabine, Metformin HCL, RP-HPLC, ICH Q2R2, Method Validation, LOQ, and LOD establishment.

ABSTRACT:

The quantification of impurities and their degradants in the active pharmaceutical ingredients (APIs) of tenofovir and emtricitabine, as well as in their combinations, is essential for quality control analysis and for indicating product stability for product approval. The method was developed and validated in accordance with the International Conference on Harmonisation (ICH) Q2 R2 guidelines. This sensitive method was employed to detect and quantify impurities in both APIs and finished dosage forms for 50 mg/500 mg, 50 mg/850 mg, and 50 mg/1000 mg extended-releasetablet formulations. The same samples of the reference formulations were compared with the generic compositions. The analytical method developed for tenofovir and emtricitabine quantification utilised 50 mM ammonium acetate adjusted pH to 4.7 ± 0.05 with glacial acetic acid as mobile Phase A, consisting of as the mobile phase A, and Mobile Phase B, a mixture of methanol: buffer: acetonitrile in the ratio of 50:40:10%v/v/v/v. Mobile phase C is 100% acetonitrile in a gradient composition. The diluent in the mixing of 25mM ammonium acetate buffer was adjusted to 5.0 ± 0.05, and methanol in the ratio of 80:20 %v/v. This was conducted using a Ghost-Hunter column, 50*4.6mm YMC Pack ODS-AQ, 250*4.6mm, 5µm (Agela & GHC0505-0 A for Ghost Column) columns with a column oven temperature of 35°C, an injection volume of 10 µL, and a flow rate of 0.8 mL/min at 260 nm, employing The limit of detection was established at 0.05% relative to the test concentrations, ensuring the method's capability to detect all known and unknown impurities in both analytes within the APIs and the finished dosage forms.

1. Introduction

The fixed-dose combination of tenofovir and emtricitabine stands as one of the most significant advancements in modern antiretroviral therapy (ART) for managing and preventing human immunodeficiency virus (HIV) infection. [1] As nucleos(t)ide reverse transcriptase inhibitors (NRTIs), these agents work by inhibiting the HIV reverse transcriptase enzyme, thereby preventing viral replication and reducing viral load. When combined, they offer potent, synergistic antiviral activity with a favourable safety profile and the convenience of once-daily dosing. Marketed under brand names such as Truvada and Descovy, this combination has become a cornerstone in both HIV treatment and pre-exposure prophylaxis (PrEP). In therapeutic regimens, tenofovir/emtricitabine forms the backbone of many first-line combination therapies recommended by global guidelines, including those from the World Health Organisation and the Centres for Disease Control and Prevention. Beyond its role in treatment, the tenofovir/emtricitabine combination has revolutionised HIV prevention. [2,3] Clinical trials have shown that daily PrEP significantly reduces the risk of HIV acquisition in high-risk populations, marking a transformative step in public health strategies aimed at ending the HIV epidemic. Pharmacologically, the two agents differ slightly in their prodrug formulations—tenofovir disoproxil fumarate (TDF) and tenofovir alafenamide (TAF)—which affect renal and bone safety profiles. These distinctions have informed individualised patient selection and expanded therapeutic options across diverse populations. This

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article explores the pharmacology, clinical efficacy, safety considerations, approved indications, and evolving role of tenofovir/emtricitabine in contemporary HIV management. [4,5]

The Combination Pharmacological Mechanism:

Tenofovir and emtricitabine are antiretroviral agents that are classified as nucleos(t)ide reverse transcriptase inhibitors (NRTIs). They function by inhibiting the HIV reverse transcriptase enzyme, which is essential for viral replication. When used in combination, they offer potent synergistic suppression of HIV replication. Tenofovir is a nucleotide analogue of adenosine monophosphate and is administered as a prodrug, either as tenofovir disoproxil fumarate (TDF) or tenofovir alafenamide (TAF). Once absorbed, the prodrug is converted intracellularly to tenofovir diphosphate, an active metabolite. [6-9]

Tenofovir and emtricitabine are antiretroviral agents classified as nucleos(t)ide reverse transcriptase inhibitors (NRTIs). They work by inhibiting the HIV reverse transcriptase enzyme, which is crucial for viral replication. When combined, these compounds offer potent synergistic suppression of HIV replication. Tenofovir, a nucleotide analogue of adenosine monophosphate, is administered as a prodrug in the form of tenofovir disoproxil fumarate (TDF) or tenofovir alafenamide (TAF). Once absorbed, the prodrug is converted intracellularly to tenofovir diphosphate, an active metabolite. [10] This process involves competition with the natural substrate deoxyadenosine 5′-triphosphate (dATP), incorporation into viral DNA during reverse transcription, and the lack of a 3′-OH group, which causes premature DNA chain termination and prevents the conversion of viral RNA into the proviral DNA. After intracellular phosphorylation to emtricitabine triphosphate, the action involves competition with deoxycytidine 5′-triphosphate (dCTP), incorporation into viral DNA, and DNA chain termination due to the absence of a functional 3′-OH group, which inhibits HIV-1 and HIV-2 reverse transcriptase. When used together (e.g. in Truvada or Descovy), they target the same viral enzyme (reverse transcriptase) at different nucleotide sites, providing additive or synergistic antiviral activity. [11] This combination reduces the viral load to undetectable levels when used with other antiretroviral agents and decreases the risk of resistance as part of combination ART. The pharmacodynamic features include selective toxicity, with a greater affinity for viral reverse transcriptase than human DNA polymerases; a long intracellular half-life supporting once-daily dosing; a high barrier to resistance when used in combination regimens; and activity against HIV-1, HIV-2, and hepatitis B virus (HBV), particularly tenofovir and emtricitabine. Both tenofovir and emtricitabine act as competitive inhibitors and chain terminators of HIV reverse transcriptase. By blocking viral DNA synthesis, they prevent viral replication and form the backbone of many first-line antiretroviral regimens and pre-exposure prophylaxis (PrEP) strategies. [12-14]

Mechanism of action:

Emtricitabine is a synthetic cytidine nucleoside analogue that undergoes intracellular phosphorylation to form emtricitabine 5′-triphosphate, which inhibits HIV-1 reverse transcriptase (RT) by competing with deoxycytidine 5′-triphosphate and incorporating into viral DNA, resulting in chain termination; it exhibits minimal inhibition of mammalian DNA polymerases α, β, ε, and mitochondrial DNA polymerase γ. Tenofovir disoproxil fumarate (TDF), an acyclic nucleoside phosphonate analogue of adenosine monophosphate, is converted to tenofovir through hydrolysis and subsequently phosphorylated to tenofovir diphosphate, which inhibits HIV-1 RT by competing with deoxyadenosine 5′-triphosphate and causing DNA chain termination, with weak effects on host DNA polymerases. Both drugs demonstrate potent antiviral activity against HIV-1 across multiple clades (A–G and O) and exhibit no antagonism when used in combination or with other antiretroviral agents. Emtricitabine shows EC_{50} values ranging from 0.0013–0.64 μM, while tenofovir exhibits EC_{50} values between 0.04–8.5 μM. In nonhuman primate models, daily oral administration of emtricitabine and tenofovir significantly reduced infection rates and delayed viral replication. Resistance development is primarily associated with mutations in the HIV-1 RT gene, notably M184V/I for emtricitabine and K65R or K70E for tenofovir, leading to reduced drug susceptibility. [15-18]Clinical trials such as Study 934, iPrEx, and Partners PrEP demonstrated low resistance emergence during prophylactic use, although mutations were observed in individuals infected at baseline or during treatment. Cross-resistance among nucleoside reverse transcriptase inhibitors (NRTIs) has been documented, particularly involving M184V/I and K65R substitutions, which may reduce susceptibility to emtricitabine, lamivudine, and tenofovir. Despite this, viruses with resistance to zidovudine or stavudine often remain susceptible to

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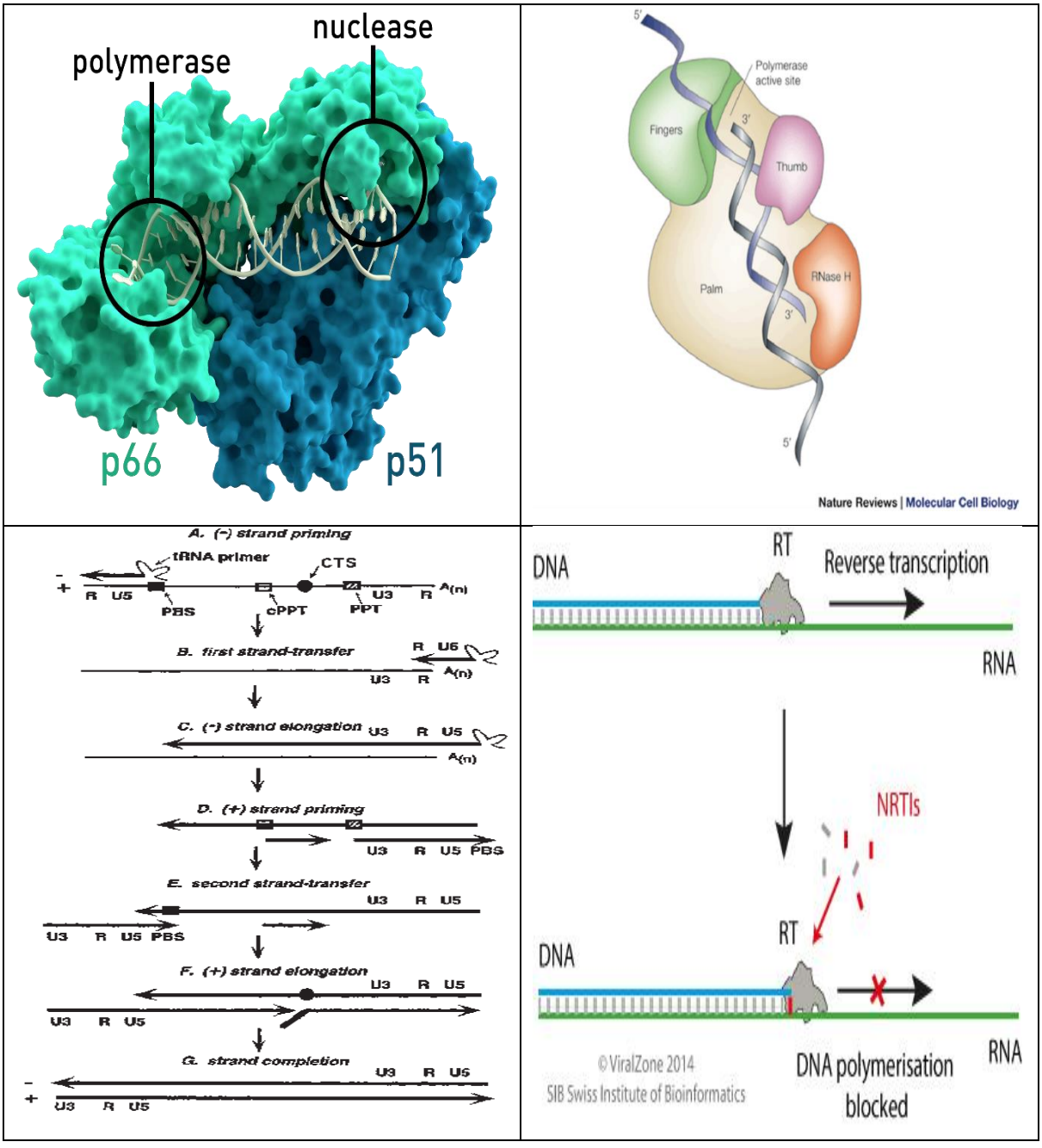
emtricitabine, highlighting its continued therapeutic relevance in combination antiretroviral therapy [19]



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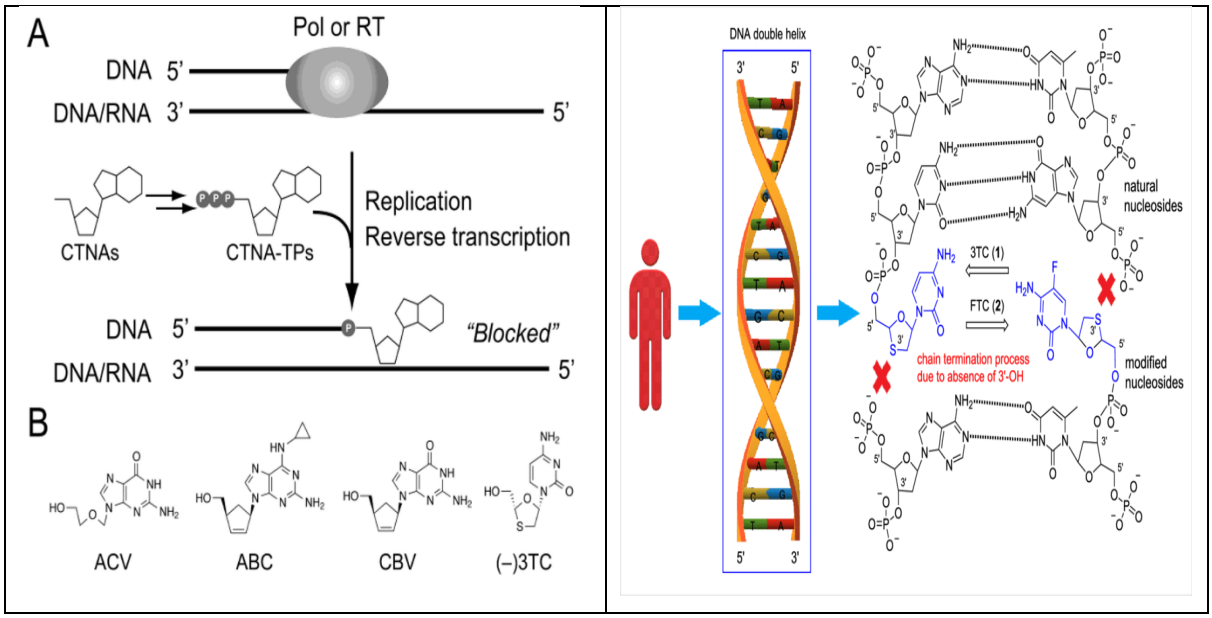

Figure 1: The physiological evaluation of drug actions

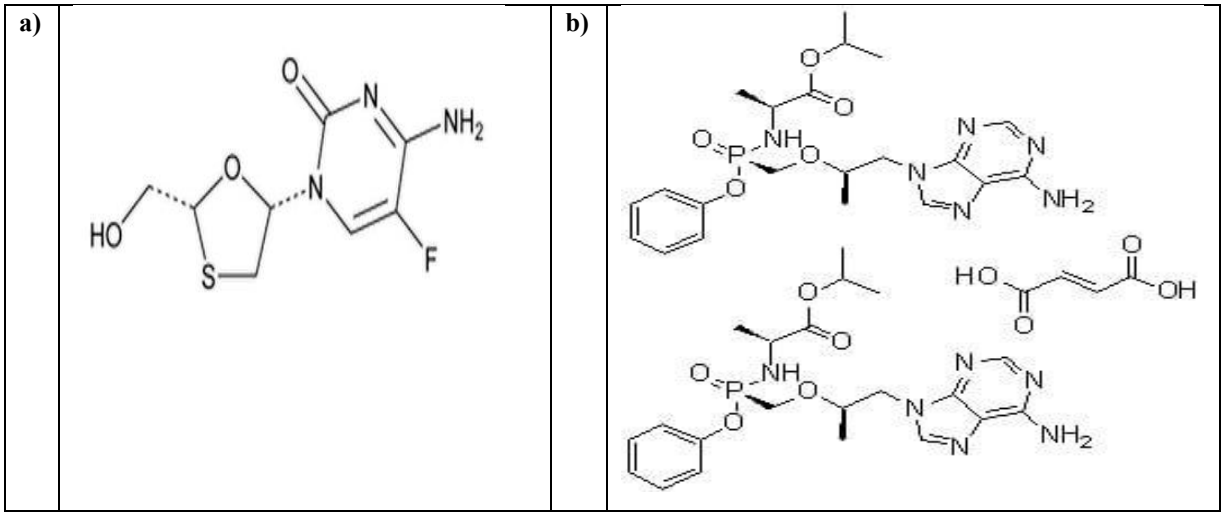

Figure 2: Chemical structures for a) Emtricitabine, b) Tenofovir Alafenamide Fumarate as Per USP

2. Materials and Methods

2.1 Chemicals and Reagents

The reference standards for Tenofovir alafenamide fumarate and Emtricitabine were obtained from the USP as reference material. Tenofovir and Emtricitabine tablets in strengths of 50 mg/500 mg, 50 mg/850 mg, and 50 mg/1000 mg extended-release dosage forms were utilised for validation studies. All reagents used were of high analytical purity to minimise background interference in mass spectrometric detection. Acetonitrile, Methanol, ammonium acetate (ACS grade), glacial acetic acid (Merck Life Sciences), and orthophosphoric acid (ACS grade) were procured from established commercial sources. High-purity water was generated using a Milli-Q water purification system. PVDF, Nylon membrane and Micro glass fibre filters with a pore size of 0.45 µm were employed for sample filtration, a crucial step often optimised to prevent analyte loss [20].

2.2 Instrumentation and Chromatographic Conditions

The analysis was conducted using a Waters HPLC with Empower-3 software, connected to UV detectors. The Chromatographic separation was achieved for Emtricitabine and Tenofovir on a Ghost Hunter column, 50*4.6mm, and YMC Pack ODS-AQ (5 µm particle

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size, 250 x 4.6 mm dimensions) maintained at a constant temperature of 35°C. 50 mM ammonium acetate adjusted pH to 4.7 ± 0.05 with glacial acetic acid as mobile Phase A, consisting of as the mobile phase A, and Mobile Phase B, a mixture of methanol: buffer: acetonitrile in the ratio of 50:40:10%v/v/v/v, Mobile phase C is 100% acetonitrile in a gradient composition. The diluent in the mixing of 25mM ammonium acetate buffer was adjusted to 5.0 ± 0.05, and methanol in the ratio of 80:20 %v/v. [21]

A gradient elution program was implemented to optimise the separation of the impurity from the main peak and matrix components. The flow rate was maintained at 0.8 mL/min with an injection volume of 10 µL. The autosampler temperature was controlled at 5°C to ensure the stability of the sample solutions during the analytical run. [22]

Table 1. Chromatographic Conditions

<table>
<tr><th>Parameter</th><th>Condition</th></tr>
<tr><td>Instrument</td><td>Waters Alliance e2695 equipped with UV Detectors</td></tr>
<tr><td>Column</td><td>Ghost-Hunter column, 50*4.6mm (Make & cat. No- Ageia & GHC0505-0) or Ghost-Buster column, 50*4.6mm; YMC Pack ODS-AQ, (250*4.6, 5µm) or equivalent</td></tr>
<tr><td>Column Temperature</td><td>35°C</td></tr>
<tr><td>Sample Temperature</td><td>5°C</td></tr>
<tr><td>Flow Rate</td><td>0.8 mL/min</td></tr>
<tr><td>Injection Volume</td><td>10 µL</td></tr>
<tr><td>Run Time</td><td>125 minutes</td></tr>
<tr><td>Mobile Phase A</td><td>Dissolved 5.8g ammonium acetate buffer into a beaker containing 1000 mL milli-Q water and adjusted the pH of the solution to 4.7±0.05 with dilute glacial acetic acid. Filtered the solution with 0.22µ membrane filter.</td></tr>
<tr><td>Mobile Phase B</td><td>Prepare a degassed mixture of methanol, buffer-1, and acetonitrile in the ratio of 50:40:10 %v/v/v.</td></tr>
<tr><td>Mobile Phase-C</td><td>Acetonitrile</td></tr>
<tr><td>Diluent</td><td>Dissolved 1.94 g ammonium acetate buffer into a beaker containing 1000 mL milli-Q water and adjusted the pH of the solution to 5.0±0.05 with dilute glacial acetic acid. Mix buffer and methanol in the ratio of 80:20% v/v.</td></tr>
<tr><td>Gradient Program</td><td>
<table>
<tr><th>Time (Minutes)</th><th>% of Mobile Phase -A</th><th>% of Mobile Phase -B</th><th>% of Mobile Phase -C</th></tr>
<tr><td>0</td><td>95</td><td>5</td><td>00</td></tr>
<tr><td>5</td><td>95</td><td>5</td><td>0</td></tr>
<tr><td>20</td><td>90</td><td>10</td><td>0</td></tr>
<tr><td>40</td><td>80</td><td>20</td><td>0</td></tr>
<tr><td>60</td><td>60</td><td>40</td><td>0</td></tr>
</table>
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	70	40	60	0
	90	10	70	20
	100	0	30	70
	110	0	30	70
	112	95	5	0
	125	95	5	0

2.4 Preparation of Standard and Sample Solutions

The emtricitabine stock solution was prepared by dissolving 40mg of standard into a 100 ml volumetric flask, adding 60 ml of diluent, and sonicating to dissolve (0.4mg/mL). For Tenofovir alafenamide fumarate, a standard stock was prepared as dissolved 32mg of the standard into 100ml volumetric flask by adding 60 mL of diluent and making up the volume with diluent, and mixing well(0.257mg/mL). Further transfer 10 mL of emtricitabine stock solution and 2 mL of Tenofovir stock solution into 100 mL volumetric flasks, add diluent, and mix well. [23-25] This solution was serially diluted to obtain a working standard solution with concentrations of 0.04mg/mL of emtricitabine (40ppm) and 0.005mg/mL of Tenofovir alafenamide (5ppm), corresponding to the specification level. Transfer the 2.0 mL of tenofovir alafenamide standard stock solution into a 100 mL volumetric flask and further diluted to 5.0 mL to 50 mL volumetric flasks and dilute to volume with diluent to achieve 0.0005mg/mL concentration as a sensitivity solution (0.5 PPM). For the placebo preparation, weigh the placebo powder equivalent to 100mg of tenofovir alafenamide into a 100mL volumetric flask, add 60 mL of diluent, and sonicate for 30 minutes (maintain the bath temperature between 20 and 25°C) with occasional shaking. [26,27] Make up the volume with diluent and mix. Centrifuge a portion of the solution at 5000 rpm for about 10 minutes. Filter a portion of the solution through a 0.45µm PVDF filter and discard the first 3mL of the filtrate. For the sample, weigh and powder not less than 10 tablets 10 tablets. Accurately weigh and transfer tablet powder equivalent to 100 mg of tenofovir alafenamide into a 100 mL volumetric flask, add 60mL of diluent, and sonicate for 30 minutes (maintain the sonicator bath temperature between 20 and 25°C) with occasional shaking. Make up the volume with diluent and mix well. Centrifuge a portion of the solution at 5000 rpm for about 10 minutes. Filter a portion of the solution through a 0.45µm filter and discard the first 3mL of the filtrate. [28]

2.5 Method Development Trials:

Initially, the USP methods were used to develop a method by using an ACE C18 150*4.6mm, 2.7µm column as a potassium phosphate monobasic buffer pH 3.0 with orthophosphoric acid. Mix the buffer and acetonitrile in the ratio of 75:25%v/v. Diluent is used as acetonitrile and water in the ratio of 40:60%v/v. Flow rate was 1.3mL and column oven temperature was 50°C injection volume as 10µL as auto sampler at 10°C at UV 260nm. The impurities were not well separated in this method, and developed by changing the buffer and get the separations for all impurities and with a better response. [29,30]

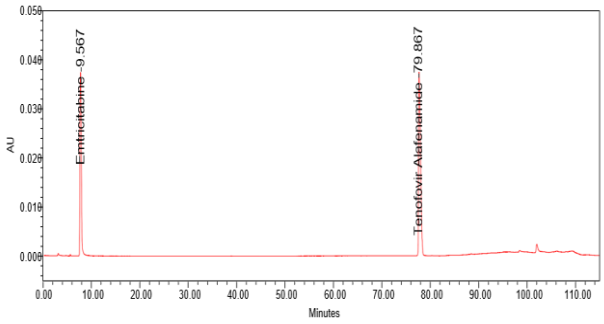

Figure:3 standard solution for Emtricitabine and Tenofovir Alafenamide as per the USP

3. Results and Discussion

3.1 System Suitability and Specificity

The system suitability of the method was assessed by analysing six replicate injections of the working standard solution. The relative standard deviation

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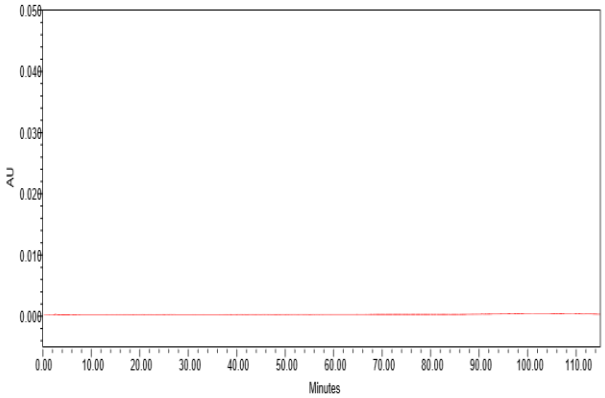
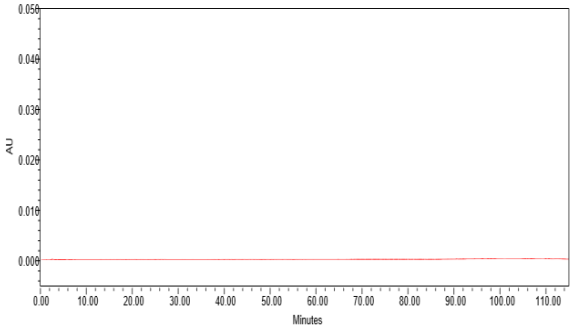
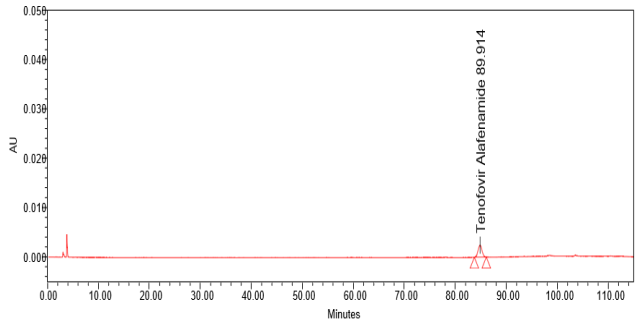
(RSD) for the peak area of Emtricitabine and Tenofovir alfenamide was calculated to be 0.2% and 0.3%, respectively, which is well within the acceptance limit of 10.0%. Moreover, the bracketing standards analysed throughout the sequence demonstrated recovery values between 100.0% and 100.1%, confirming the stability of the system during the analysis. The sensitivity solution of Tenofovir alafenamide signal-to-noise ratio is not less than 10.

Specificity was evaluated to verify that the method could unequivocally assess the analyte in the presence of components such as degradants, excipients, and the active pharmaceutical ingredient. Chromatograms of the blank diluent, placebo formulations for all strengths, and control samples were acquired. No interfering peaks were observed at the retention time of Emtricitabine and Tenofovir alafenamide (approximately Emtricitabine 34 and Tenofovir Alafenamide 89 minutes). The results confirmed that the placebo matrix and diluent contributed no interference, showing the high selectivity, which is essential for compliance with USP <621>.







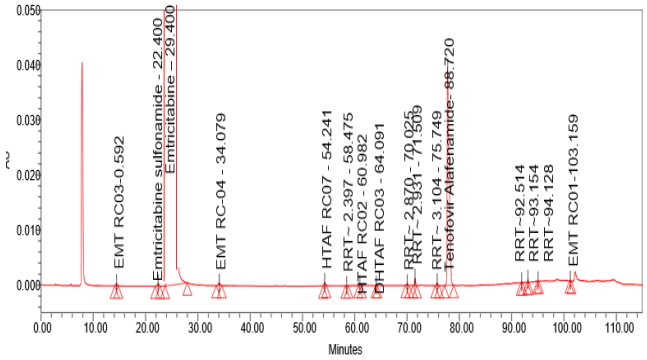


Figure 4. Typical Chromatograms for Specificity Analysis. Blank Diluent, Placebo, sensitivity and spiked sample.

3.2 Linearity and Range

The linearity of the analytical method was established by analysing a series of standard solutions ranging from the Limit of Quantification (LOQ) to 200% of the specification limit.

The concentrations range from 0.050920µg/ml to 0.708330µg/ml. Solutions were prepared and injected. Regression analysis yielded a correlation coefficient (r) of 1.00 and a coefficient of determination (r^2) of 1.00, indicating a perfect linear relationship between concentration and response within the studied range. The slope of the regression line was found to be with in limit and the y-intercept of is matched. The residual sum of squares was minimal, further validating the linear model. The method was deemed linear across the range of 20% to 200% of the target specification.

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Table 2: Linearity Results for Emtricitabine

Linearity Level	Concentration (mg/mL)	Area Response	Statistical Parameters
Linearity-1 (20%)	0.008	15974	Slope: 68303.7
Linearity-2 (40%)	0.016	100212	Intercept: 76.0079
Linearity-3 (70%)	0.028	158991	Residual Sum of Squares: 2362564
Linearity-4 (100%)	0.04	238886	Correlation Coefficient (r): 1.00
Linearity-5 (150%)	0.06	360078	Coefficient of Determination (r^2): 1.00
Linearity-6 (200%)	0.08	488879	

Table 3: Linearity Results for Tenofovir Alafenamide

S. No.	Linearity level (%)	Tenofovir Alafenamide-API	
		Concentration (µg/mL)	Area
1	Level-01	0.005358	12476
2	Level-02	0.0125	64059
3	Level-03	0.025	102987
4	Level-04	0.05	126950
5	Level-05	0.065	153031
6	Level-06	0.075	192555
7	Level-07	0.1004	231266
8	Level-08	0.2003	284372
Slope		23742.29523	
Intercept		508.98317	
Res sum of sq.		1341.44752	
CC (r)		0.99990	
RSQ(r^2)		0.9998	
RRF		1.0	
% y-Intercept		0.40	

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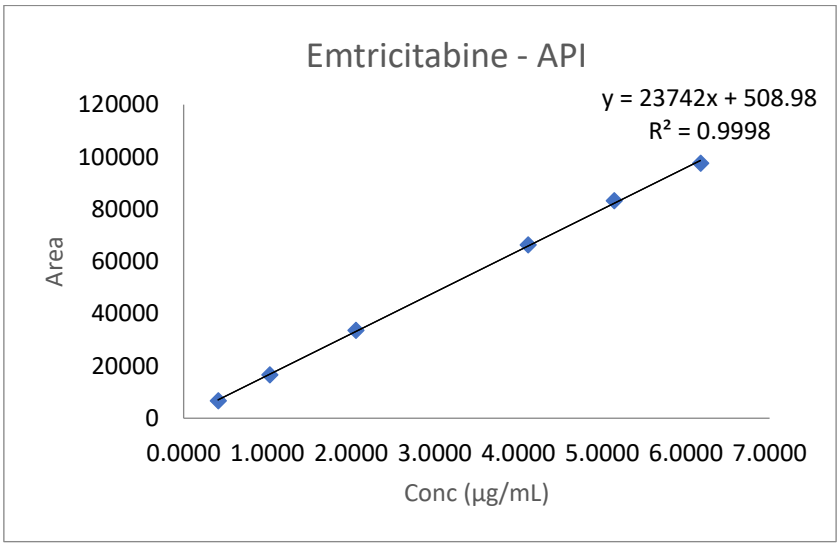

Figure 5: Linearity Graph for Tenofovir Alafenamide- API

Table 4: Linearity Results for Related Impurities

S.NO	**Name of the Impurity**	**Correlation coefficient**	**% Y intercept**	**RRF**
1	H_TAFRC01	0.9978	2.12	1.01
2	Emtricitabine 5-Fluorouracil analogue	0.9976	3.06	1.00
3	H-TAFRC07	0.9991	4.90	1.00
4	H-TAFRC02	0.9994	-3.59	1.00

3.3 Sensitivity (Limit of Detection and Quantification)

The sensitivity of the method was determined based on the Signal-to-Noise (S/N) ratio approach. The Limit of Quantification (LOQ) was established at 0.000509 µg/mL (0.5ppm), which corresponds to 10% of the specification limit. At this concentration, the S/N ratio was found to be greater than 10, and the precision (% RSD) of six replicate injections was 2.0%. The Limit of Detection (LOD) was determined to be 0.038 ng/mL, exhibiting an S/N ratio greater than 3. These values indicate that the method is sufficiently sensitive to quantify Emtricitabine and Tenofovir alafenamide at trace levels required by current regulatory standards.

3.4 Precision and Accuracy

Method precision was evaluated by preparing six independent samples of the drug substance and drug product (0.0005mg and 0.005mg/mL) spiked at the specification level. The % RSD for the drug substance and its impurities were given in the tables below. Intermediate precision was assessed by repeating the analysis on a different day, by a different analyst, and using a different column. The cumulative % RSD (n=12) for the drug product and its related impurities were performed, results met the acceptance criteria, showing the robust reproducibility of the method.

Accuracy was determined through recovery studies performed at three concentration levels: LOQ (20%), 100%, and 200% of the specification limit. The study was conducted in triplicate for both the API and the drug product. The mean recovery values for the drug

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product ranged from 95-105% while the drug substance recoveries ranged from 91.0 to 110.0% for both Emtricitabine and Tenofovir alafenamide. All individual and mean recovery values of all impurities were within the 85.0% to 115.0% acceptance range (70-130% for LOQ), confirming the method's accuracy and extraction efficiency.

Table 5: Precision results for impurities (100% Level)

S.No	Sample No	H_TAFRC01	Emtricitabine 5-Fluorouracil analogue	H_TAFRC07	H_TAFRC07
1	Spiked sample pre-01	0.265	0.223	0.201	0.211
2	Spiked sample pre-02	0.253	0.220	0.199	0.222
3	Spiked sample pre-03	0.253	0.201	0.205	0.203
4	Spiked sample pre-04	0.257	0.202	0.204	0.210
5	Spiked sample pre-05	0.255	0.205	0.211	0.225
6	Spiked sample pre-06	0.259	0.204	0.200	0.210
	Average	**0.257**	**0.209**	**0.203**	**0.214**
	%RSD	**1.77**	**4.64**	**2.17**	**3.89**

Table 6: % recovery results for impurities (LOQ Level)

S.No	Sample No	H_TAFRC01	Emtricitabine 5-Fluorouracil analogue	H_TAFRC07	H_TAFRC07
1	LOQ Precision-01	100.2	101.3	101.6	103.6
2	LOQ Precision-02	105.3	100.4	100.5	102.5
3	LOQ Precision-03	103.1	100.0	102.6	101.6
4	LOQ Precision-04	108.5	101.5	103.6	104.6
5	LOQ Precision-05	104.7	102.4	102.1	102.1
6	LOQ Precision-06	107.8	105.5	101.6	104.6
	Average	**104.9**	**101.9**	**102.0**	**103.2**
	%RSD	**2.92**	**1.94**	**1.03**	**1.25**

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3.5 Forced degradation study:

Acid sample preparation (1N HCl at Bechtop-60mins)

Weigh and crush not less than 10 tablets. Accurately weigh and transfer the tablet powder equivalent to 100mg of Tenofovir alafenamide into a 100 mL volumetric flask. Add about 60mL of diluent-1 and sonicate for 60 minutes with intermediate shaking (maintain the sonicator temperature between 20-25°C). added 10ml of 1N HCl and kept the flask on the bench top for 60 minutes, and cooled to room temperature and neutralised with 10 ml of 1N NaOH. Dilute to volume with diluent and mix. Centrifuge a portion of the solution at 4500 RPM for 10 minutes. Filter the solution through a 0.45µm PVDF syringe filter and discard the first 3mL of the filtrate.

Base sample preparation (5N Methanolic NAOH at Benchtop-3days):

Weigh and crush not less than 10 tablets. Accurately weigh and transfer the tablet powder equivalent to 100mg of Tenofovir alafenamide into a 100 mL volumetric flask. Add about 60 mL of diluent-1 and sonicate for 60 minutes with intermediate shaking (maintain the sonicator temperature between 20-25°C). added 10ml of 5N Methanolic NaOH and kept the flask on the bench top for 3days, and cooled to room temperature and neutralised with 10 ml of 5N Methanolic HCl. Dilute to volume with diluent-1 and mix. Centrifuge a portion of the solution at 4500 RPM for 10 minutes. Filter the solution through a 0.45µm PVDF syringe filter and discard the first 3mL of the filtrate.

Peroxide sample Preparation (1% H2O2 Bench top Intermediate):

Weight and crush not less than 10 tablets. Accurately weigh and transfer powder equivalent to 100mg of Tenofovir alafenamide into a 100mL volumetric flask, add about 60 mL of diluent-1 and sonicate for 60 minutes with intermediate shaking (maintain the sonicator bath temperature between 20-25ºC). Added 5 mL of 1% H202 and immediate preparation. Dilute to volume with diluent-1 and mix. Centrifuge a portion of the solution at 4500 RPM for 10 minutes. Filter the solution through a 0.45µm PVDF syringe filter and discard the first 3mL of the filtrate.

Table 7: Forced degradation results

S.NO	Condition	Total Impurities %w/w	%Assay	Mass balance
1	Control sample	0.025	99.9	-
2	1N HCl-60mins at Benchtop	0.255	98.2	100.21
3	5N Methane NAOH-3days at BT	3.864	97.2	101.18
4	1%H202-immediate	0.045	99.2	102.00

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5	Thermal-24hours at 60ºC	0.253	100.9	104.00

Robustness and Solution Stability

The robustness of the method was challenged by deliberately varying critical parameters, including flow rate (± 0.02 mL/min) and column temperature (± 5°C). Under all tested conditions, the system suitability criteria were met, with the % RSD of standard injections remaining below 5.0% (specifically between 1.0% and 4.7%), and the retention times varied predictably as shown in Table 5. This indicates that the method is reliable and unaffected by small, deliberate variations in operating parameters.

Table 8: Retention Time of Emtricitabine and Tenofovir Alafenamide

Variation Condition	% RSD (n=6)	Retention Time (min) Emtricitabine	Retention Time (min) Tenofovir Alafenamide
Control	1.0%	29.39	88.39
Low Flow (-0.02 mL/min)	2.0%	32.99	89.99
High Flow (+0.02 mL/min)	2.1%	28.21	87.21
Low Temperature (-5°C)	2.8%	31.35	89.35
High Temperature (+5°C)	1.0%	28.92	87.92

Note: Acceptance criterion for %RSD is NMT 15.0%.

Solution stability was assessed by storing standard and sample solutions under refrigerated conditions (2-8°C). Re-analysis at intervals up to 48 hours (Day 2) showed that the response did not deviate significantly from the initial analysis. As detailed in Tables 15 and 16, the difference in response remained within 10% for standards and within 10% for drug product samples, well within the 20% acceptance limit.

Table 9: Solution Stability Results for Emtricitabine (Refrigerated)

Solution Type	Time Point	% Initial / % Recovery	% Difference from Initial
Standard	Initial	100.0%	N/A
	Day-1	97.7%	2%
	Day-2	97.4%	3%
Drug Substance	Initial	97.6%	N/A
	Day-1	95.7%	2%
	Day-2	96.3%	1%
Drug Product	Initial	98.6%	N/A
	Day-1	97.9%	0.8%
	Day-2	98.9%	-0.3%

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Table 10: Solution Stability Results for Tenofovir Alafenamide (Refrigerated)

Solution Type	Time Point	% Initial / % Recovery	% Difference from Initial
Standard	Initial	100.0%	N/A
	Day-1	99.8%	0.2%
	Day-2	99.5%	0.5%
Drug Substance	Initial	100.5%	N/A
	Day-1	100.2%	0.1%
	Day-2	100.1%	0.1%
Drug Product	Initial	100.5%	N/A
	Day-1	100.9%	0.9%
	Day-2	100.5%	0.4%

4. Conclusion

A sensitive, specific, and robust RP-HPLC method was successfully developed and validated for the quantification of emtricitabine and tenofovir alafenamide-related impurities at the LOQ level to 200 % level for the targeted concentration and drug substance and tablet formulations. The method demonstrates excellent linearity, precision, and accuracy across the validation range of 0.0509 ng/mL to 0.7093 ng/mL. The LOQ of 0.0509 ng/mL allows for the quantification of these unknown impurities well below the quantification limits. The validation data confirms that the method complies with ICH Q2(R1) and ICH Q2(R2) guidelines and is suitable for routine quality control applications to ensure the safety and quality of Trimipramine Maleate pharmaceutical products.

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