Turk J Pharm Sci 2021;18(4):410-419
DOI: 10.4274/tjps.galenos.2020.70962

ORIGINAL ARTICLE

Development and Validation of a Stability-Indicating RP-HPLC Method for the Simultaneous Estimation of Bictegravir, Emtricitabine, and Tenofovir Alafenamide Fumarate

Biktegravir, Emtrisitabin ve Tenofovir Alafenamid Fumaratın Eşzamanlı Kestirimi için Stabilite Göstergeli RP-HPLC Yönteminin Geliştirilmesi ve Validasyonu

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ABSTRACT

Objectives: The focal intent of the current research work is to develop and validate a novel and reliable stability-indicating reverse-phase high performance liquid chromatographic method for the simultaneous estimation of a few anti-retrovirals, i.e., bictegravir, emtricitabine, and tenofovir alafenamide fumarate (AF).

Materials and Methods: The novel method employs inertsil octyldecylsilyl C_{18} (4.6×250 mm, 5 mm) using 0.2% triethylamine buffer and methanol in a ratio of 40:60% (v/v) as the mobile phase to attain optimal elution. The detection wavelength was 260 nm with a 1.2 mL/min flow rate and a 20 µL injection volume.

Results: The linearity ranges for bictegravir, emtricitabine and tenofovir AF were 25-125 µg/mL, 100-500 µg/mL, and 12.5-62.5 µg/mL, respectively. The retention times for bictegravir, emtricitabine, and tenofovir AF were found to be 5.998 min, 2.805 min, and 4.537, min respectively. The percent recoveries of bictegravir, emtricitabine, and tenofovir AF were within the range of 98-102% w/w.

Conclusion: The novel method was successfully validated as per International Conference on Harmonization guidelines. In forced degradation studies, emtricitabine was found to be sensitive to thermal conditions; bictegravir and tenofovir AF, to oxidative conditions. The developed method is economical and reliable for routine analysis concerning all validated parameters.

Key words: Bictegravir, emtricitabine, tenofovir AF, RP-HPLC, validation, forced degradation studies

ÖZ

Amaç: Mevcut araştırma çalışmasının odak amacı, birkaç anti-retroviralin [biktegravir, emtrisitabin ve tenofovir alafenamid fumarat (AF)] eş zamanlı tahmini için yeni ve güvenilir bir stabilite gösteren ters fazlı yüksek performanslı sıvı kromatografik yöntemi geliştirmek ve doğrulamaktır.

Gereç ve Yöntemler: Yeni yöntem, optimal elüsyona ulaşmak için mobil faz olarak %0,2 trietilamin tamponu ve %40:60 (h/h) oranında metanol kullanan inertsil octyldesilsilil C_{18}'i (4,6×250 mm, 5 mm) kullanmaktadır. Deteksiyon dalga boyu 260 nm, akış hızı 1,2 mL/dk ve enjeksiyon hacmi 20 µL idi.

Bulgular: Biktegravir, emtrisitabin ve tenofovir AF'nin doğrusallık ranjı sırasıyla 25-125 µg/mL, 100-500 µg/mL ve 12.5-62.5 µg/mL idi. Biktegravir, emtrisitabin ve tenofovir AF'nin retansiyon zamanları sırasıyla 5,998 dk, 2,805 dk ve 4,537 idi. Biktegravir, emtrisitabin ve tenofovir AF'nin yüzde gerikazanımları %98-102 a/a aralığında idi.

Sonuç: Yeni yöntem, Uluslararası Uyumlaştırma Konferansı yönergelerine göre başarıyla doğrulanmıştır. Zorla bozunma çalışmalarında, emtrisitabin termal koşullara biktegravir ve tenofovir AF ise, oksidatif koşullara duyarlı olduğu bulunmuştur. Geliştirilen yöntem, tüm valide edilmiş parametrelerle ilgili rutin analizler için ekonomik ve güvenilirdir.

Anahtar kelimeler: Biktegravir, emtrisitabin, tenofovir AF, RP-HPLC, validasyon, zorla bozunma çalışmaları

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Received: 26.04.2020, **Accepted:** 08.09.2020

INTRODUCTION

Human immunodeficiency virus (HIV) is a fatal viral infection that targets and alters the immune system, increasing the risk and impact of other infections and diseases. If left untreated, the infection might progress to an advanced disease stage called acquired immunodeficiency syndrome (AIDS). With the use of multiple specialized anti-retroviral medications that are commercially available, the state of HIV/AIDS infection can be controlled, de-escalated, and treated. Among numerous anti-retroviral formulations and combinations available, bictegravir is an oral tablet that contains three anti-retroviral drugs [bictegravir + emtricitabine + tenofovir alafenamide fumarate (AF)] under the brand name "BIKTARVY". Bictegravir belongs to the class of HIV-1 integrase strand transfer inhibitors (INSTIs); emtricitabine and tenofovir alafenamide, to the class of HIV-1 nucleoside analog reverse transcriptase inhibitors. Hence "BIKTARVY" can be considered as the sole regimen for HIV-1 (type 1) infected patients.[1-3] The INSTIs comprise two nucleoside reverse transcriptase inhibitors and recommended components during the initial stages of anti-retroviral therapy. Bictegravir is an effective INSTI with a high *in vitro* barrier that shows strong resistance toward the clinically relevant drug-drug interactions and possesses specific activity against HIV-1 and HIV-2. Bictegravir is metabolized by cytochrome P450 3A4 and a uridine diphosphate glucuronosyl transferase 1A1. It binds to the active site of HIV integrase and prevents HIV replication. Compared with other INSTIs, bictegravir possesses a high barrier to in vitro resistance and a lower potential to drug interactions among other readily available anti-retrovirals.[4,5] Emtricitabine and tenofovir AF act on DNA synthesis via HIV reverse transcriptase, resulting in viral DNA chain termination and preventing the replication of HIV.[6,7] The US Food and Drug Administration has approved "bictegravir" as a fixed-dose regimen (once daily) to treat HIV-1 infection.[8,9] The chemical structures of the three active pharmaceutical ingredients are shown in Figure 1-3. The dosage regimen is as follows:

Bictegravir (50 mg) + emtricitabine (20 mg) + tenofovir AF (25 mg).

According to the "Department of Health and Human Services",[10,11] the current combination regimen is intended to treat HIV-1 patients. Biktarvy can be administered with or without food and is not recommended with other anti-retrovirals.[12] A literature survey was performed, and very few stability-indicating reverse-phase high performance liquid chromatographic (HPLC) isocratic elution methods to estimate the drugs of interest are reported.

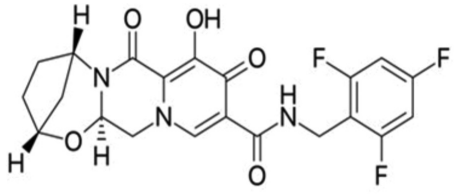

Figure 1. Bictegravir

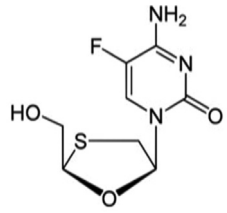

Figure 2. Emtricitabine

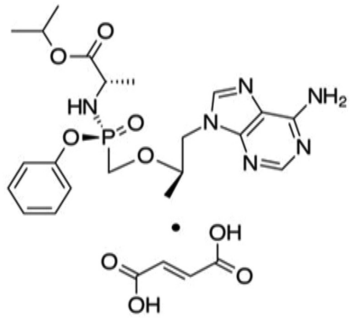

Figure 3. Tenofovir alafenamide fumarate

However, the available methods for determination of bictegravir, emtricitabine, and tenofovir AF in pharmaceutical formulations are scanty and vary in establishing multiple experimental variables during the validation of the method; the current method developed was found to be more sensitive and reliable. The details are outlined in Table 1.

Therefore, in the present work we developed a novel, reliable, and efficient method for the quantification of the drugs of interest. The stability of the drug indicates its shelf-life and bio-availability, which affect the chemical, pharmacological, and toxicological characteristics of the drug moieties; hence, stability studies were performed according to the International Conference on Harmonization (ICH) guidelines, and the results are reported.

MATERIALS AND METHODS

Experiment

Collection of drugs

Bictegravir of purity 99%w/w, emtricitabine of purity 99% w/w, and tenofovir AF of purity 99% w/w were procured from Hetero Labs, Hyderabad.

Chemicals and reagents

HPLC grade methanol (Rankem), Milli-Q grade water for HPLC (Merck), and HPLC grade triethylamine (Fine Chem Industries Research Laboratory) were used.

Apparatus

The HPLC WATERS system (2695 separation module 7 auto sampler) used in this method was equipped with a photodiode array (PDA) detector. Empower chromatography software (EMPOWER-2) was used for liquid chromatogram peak integration. Empower-2 software was used in data acquisition and processing.

Table 1. Comparison with similar existing methods

Kokkirala and Suryakala[13]	Sneha and Valli Kumari[14]	Sattar and Achanta[15]	Meenaksh and Shyam Sunder[16]	Current method	Remarks
Buffer and acetonitrile in a ratio of 50:50	Buffer and acetonitrile in a ratio of 55:45 v/v	Mobile phase ratio was (30:70 v/v) ortho-phosphoric acid buffer (adjust the pH 2.5 with NaOH solution): methanol	Buffer phosphoric dihydrogen phosphate as mobile phase A and methanol and water (70:30) as mobile phase B of the gradient program	0.2% triethylamine buffer and methanol in the ratio of 40:60 %v/v	Less expensive solvents were employed to obtain substantial results
C_{18} column (150 mm×4.6 mm, 5 µm)	Zodiac C_{18} 150×4.6 mm, 5 µm	Inspire C_{18} column (150×4.6 mm) 5.0 µm	Inertsil 30V C_{18} Column (250×4.6 mm, 5 µm)	Octyldecylsilyl C_{18} (4.6×250 mm, 5 µm)	Good peak shape and resolution acquired
272 nm	272 nm	272 nm	265 nm	260 nm	Detection of eluted peaks acquired at a shorter wavelength

a- The inertsil octadecylsilica (ODS) C_{18} (4.6×250 mm, 5 µm) column was found to be ideal for analyzing the selected drugs.

b- A rheodyne injector (20 µL loop) was used to inject the samples.

c- A ultraviolet (UV)-visible spectrophotometer (LABINDIA UV 300^{0+}) with UV Win software was used to establish the analytical wavelength.

d- Other instruments included an afcoset ER-200A electronic weighing balance, micropipettes, pipettes, burettes, micro-pore filtration assembly, ultra-sonic water bath for sonication of the mobile phase, and pH meter (Adwa-AD 1020).

Optimized chromatographic conditions

Once several trials had been conducted for optimization, the appropriate conditions were selected for the study, the details of which are as follows:

Instrument: HPLC (waters) with auto sampler

Detector: PDA detector

Temperature: Ambient

Column: ODS C_{18} (4.6×250 mm, 5 µm)

Mobile phase: 0.2% triethyl amine (TEA), buffer: Methanol (40:60 v/v)

Flow rate: 1.2 mL/min

Run time: 15 min

Wavelength: 260 nanometers (nm)

Preparation of 0.2% TEA buffer solution

TEA (2 mL) was measured accurately by pipetting into 1000 mL HPLC grade water and dissolved. The pH was adjusted to 3.5 with dilute formic acid.

Preparation of the mobile phase

Four hundred milliliters (40%) of the above-prepared buffer and 600 mL (60%) of methanol were measured accurately and mixed well.

Standard and sample preparation (emtricitabine, tenofovir AF, and bictegravir)

Standard preparation

Emtricitabine (100 mg), tenofovir AF (12.5 mg), and bictegravir (25 mg) working standards were weighed into a volumetric flask and added to 100 mL of diluent to makeup the volume. From the prepared stock solution, 3 mL was diluted to 10 mL. The resulting solution contained each of 300 ppm of emtricitabine, 37.5 ppm, of tenofovir AF, and 75 ppm of bictegravir.

Sample preparation

Ten tablets [prepared in-house by weighing the quantities as stated in the marketed formulation of emtricitabine (200 mg), tenofovir AF (25 mg), and bictegravir (50 mg)] were weighed accurately, and quantities equal to emtricitabine (100 mg), tenofovir AF (12.5 mg), and bictegravir (25 mg) samples were diluted to 100 mL. Three milliliters of each stock solution was diluted to 10 mL containing 300 ppm of emtricitabine, 37.5 ppm, of tenofovir AF, and 75 ppm of bictegravir.

Procedure

The % assay was estimated from the obtained peak areas of standard and sample using the formula:

$$\%\,Assay = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{Average\ weight}{Label\ Claim} \times \frac{P}{100} \times 100$$

Where;

AT: Average area counts of test (sample) preparation.

AS: Average area counts of standard preparation.

WS: Weight of working standard taken in mg.

DS: Dilution of working standard in mL.

DT: Dilution of test (sample) in mL.

WT: Weight of test (sample) taken in mg.

P: Percentage purity of working standard.

Method validation

The analytical method validation for the developed method was implemented to ensure that the method meet the intended requirements as stated in the respective guidelines.[17] The results obtained for the method validation can be considered to determine the reliability and consistency of the developed method. The proposed method was validated according to the ICH guidelines with respect to the following parameters.[18-21]

Calibration curves were obtained at concentrations of 25-125 µg/mL for bictegravir, 100-500 µg/mL for Emtricitabine, and 12.5-62.5 µg/mL for tenofovir AF.

Linearity

Linearity can be illustrated by examining different concentrations of active pharmaceutical ingredients. The linearity of a method can be evaluated from the calibration plots of bictegravir, emtricitabine, and tenofovir AF constructed from peak response vs. concentration, which approaches a straight line.

Emtricitabine (100 mg), tenofovir AF (12.5 mg), and bictegravir (25 mg) were diluted to 100 mL. From this stock solution, 1-5 mL was pipetted into five different 10 mL volumetric flasks, and a series of aliquots was prepared and analyzed.

Accuracy

Accuracy was illustrated from the % recovery of standard containing known concentrations of active pharmaceutical ingredients.

Emtricitabine (100 mg), tenofovir AF (12.5 mg), and bictegravir (25 mg) working standards were diluted to 100 mL.

Three milliliters of the resulting stock solution was diluted to 10 mL. This solution thus contained emtricitabine (300 ppm), tenofovir AF (37.5 ppm), and bictegravir (75 ppm). The standard solutions for accuracy determination, 50%, 100%, and 150%, were prepared and injected, and the recovery values for emtricitabine, tenofovir AF, and bictegravir were calculated.

Precision

Precision was evaluated on the basis of the closeness between the obtained results.

Emtricitabine (100 mg), tenofovir AF (12.5 mg), and bictegravir (25 mg) working standards were diluted to 100 mL. Three milliliters of this stock solution was diluted to 10 mL.

Specificity

Specificity can be illustrated by ensuring that the peaks are free from interference.

It is determined by injecting a blank and a standard into the chromatographic system and corroborating that no interference exists.

Detection limit (DL) and quantification limit (QL)

DL and QL values deal with the method's sensitivity. DL is the analyte's lowest detectable concentration, while QL is the lowest quantifiable concentration.

DL

Emtricitabine (100 mg), tenofovir AF (12.5 mg), and bictegravir (25 mg) working standards were weighed and diluted separately to 100 mL. From this stock solution, 3 mL was diluted to 10 mL. From the above, 1 mL of each of the above stock solutions (emtricitabine, tenofovir AF, and bictegravir) was dispensed into different 10 mL volumetric flasks and diluted with diluent. Emtricitabine stock solution (0.35 mL), tenofovir AF (1 mL), and bictegravir stock (1 mL) solutions were further diluted to 10 mL.

QL

Emtricitabine (100 mg), tenofovir AF (12.5 mg), and bictegravir (25 mg) working standards were diluted to 100 mL. From the stock solution, 3 mL was diluted to 10 mL. Further, emtricitabine (1 mL), tenofovir AF stock (1 mL) solutions, and bictegravir stock solution (3 mL) were diluted to 10 mL. Further pipette emtricitabine stock (1.1 mL) solution, tenofovir AF (4.1 mL) and bictegravir stock solution (3.9 mL) were diluted to 10 mL.

Robustness

Robustness can be illustrated by evaluating the impact of deliberate changes on the proposed method.

Degradation studies

The guideline of the "ICH" entitled "Stability testing of new drug substances and products" states that stress testing is performed to evaluate the instinct stability attributes of the active pharmaceutical substance. Stress degradation studies on emtricitabine, tenofovir AF, and Bbictegravir[22-27] were performed in the current work.

Preparation of stock

Emtricitabine (100 mg), tenofovir AF (12.5 mg), and bictegravir (25 mg) working standards were weighed and diluted to 100 mL. The resulting stock solution was used for stability testing. All the stress conditions were applied, and the percent degradation was studied for the selected drugs bictegravir, emtricitabine, and tenofovir AF. The stress conditions include acidic, alkali, thermal, oxidative, and photolytic conditions to study the nature of drugs and their stability against the above-mentioned conditions.

Statistical analysis

The data were processed through the "EMPOWER-2". The results were calculated as mean ± standard deviation (SD) for accuracy, and the relative SD (RSD) was calculated for precision. The coefficient of regression was also calculated for the linearity parameter.

RESULTS AND DISCUSSION

Optimization of the method

For the selection of a suitable mobile phase for simultaneous estimation of the selected drugs, various solvents such as water, ACN, TEA, and methanol varying in polarity were used in different combinations of concentrations to obtain high peak resolutions within a shorter runtime. Among all the different

mobile phase combinations employed, the mobile phase comprising 0.2% TEA buffer and methanol in the ratio of 40:60 v/v exhibited well-defined peaks.

Different flow rates from 0.5 to 1.2 mL/min have been studied to achieve a good peak resolution. Among all the flow rates employed, 1.2 mL/min was selected as optimal for the study.

The column temperature was set at 25, 30, and 35°C for optimization, according to its effect on peak resolutions and RT of the drug samples.

During the method optimization, the selected combinations of three drugs were analyzed using different columns, the column [ODS C_{18} (4.6×250 mm, 5 μm)] that exhibited good peak shape and resolution was selected for current study. The details are specified in Table 2.

Also, based on the UV-absorption spectra of the three drugs scanned over the range of 200-400 nm, the wavelength of 260 nm was selected as the ideal wavelength for the study.

System suitability

According to the optimized experimental conditions, the retention times obtained for bictegravir, emtricitabine, and tenofovir AF are 5.998 min, 2.805 min, and 4.537 min. The optimized chromatogram with tailing factor (<2), theoretical plates (>2000), resolution (>2), capacitance factor (>1) is shown in (Figure 4). Hence, the proposed method proved "selective" to determine the drugs (bictegravir, emtricitabine, and tenofovir AF). The system suitability results of the standard injections are tabulated in Table 3.

Assay of marketed formulation

The assay results obtained for the three drugs (bictegravir, emtricitabine, and tenofovir AF) are detailed in Table 4. No interference of the excipients was noticed in the current method; hence, the method is "specific". The typical chromatogram for assay of the commercial formulation (in-house preparation) is shown in Figure 5.

Linearity

To construct the calibration curve, different concentration ranges of bictegravir (25-125 μg/mL), emtricitabine (100-500 μg/mL), and tenofovir AF (12.5-62.5 μg/mL) were considered. The correlation coefficient (r^2) values obtained were found satisfactory. The results obtained are summarized in Table 5. The calibration plots of three drugs are as shown in (Figure 6-8).

Table 2. Comparison of optimum conditions

S. no.	Column used	Specification	Remarks
1	Hypersil	5.0×250 mm, 10 μm	Deformed peak shape was observed
2	Lichrosorb	4.6×250 mm, 5 μm	Low resolution observed
3	Inertsil ODS C_{18}	4.6×250 mm, 5 μm	Peak shape is sharp and free from Tailing with high resolution

ODS: Octyldecylsilyl

Accuracy

Accuracy was determined at 50%, 100%, and 150% of the test concentrations by calculating the individual recovery and mean recovery values of emtricitabine, tenofovir AF, and bictegravir. The recoveries ranged from 99.26% to 100.30% for bictegravir, 99.06% to 100.79% for emtricitabine, and 99.66% to 100.06% for tenofovir AF. The recovery values obtained were found to meet the acceptance criteria (not less than 98.0% and not more than 102.0%). The RSD values obtained were <2 with respect to three drugs. The accuracy results are outlined in Table 6.

Precision

The precision for the developed method is estimated as follows:

- System precision
- Intermediate precision
- Method precision

System precision

The % RSD of six standard injection areas were found to be less than 2% (acceptance criteria: Not more than 2%), hence the method is "precise". The results for emtricitabine, tenofovir AF, and bictegravir are summarized in Table 7.

Intermediate precision/ruggedness

No significant effect was observed in the recoveries, the peak area responses of all the three drugs, thus indicating that the proposed and developed method is rugged.

The results are summarized for emtricitabine, tenofovir AF, and bictegravir in Table 8.

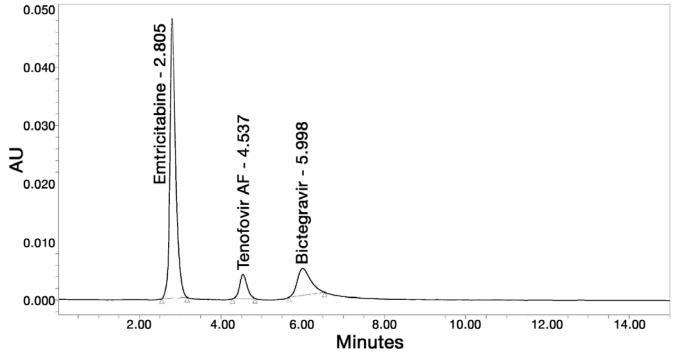

Figure 4. Optimized chromatogram showing the simultaneous elution of bictegravir, emtricitabine and tenofovir alafenamide fumarate

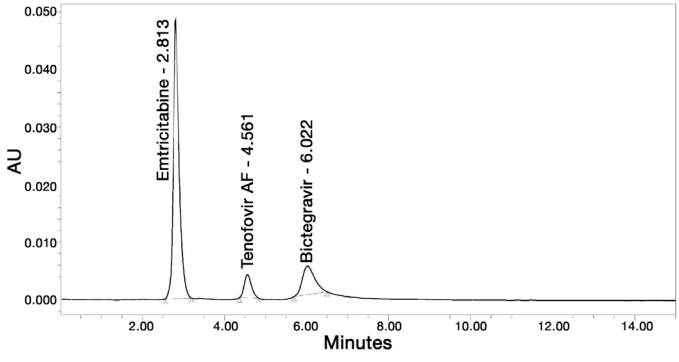

Figure 5. Assay chromatogram for marketed formulation of bictegravir, emtricitabine and tenofovir alafenamide fumarate

Table 3. System suitability results

S. no.	Parameter	Acceptance criteria	Bictegravir	Emtricitabine	Tenofovir alafenamide fumarate
1	Tailing factor	Not more than 2.0	1.33	1.30	1.13
2	Theoretical plates	Not less than 2000	2214.41	2185.90	2973.76
3	Resolution for the Tenofovir AF and bictegravir	Not less than 2	3.14	-	6.05
4	Capacitance factor	Not less than 1.0	4.36	1.54	3.09
5	Selectivity	Not more than 2.0	0.5	-	0.7

AF: Alafenamide

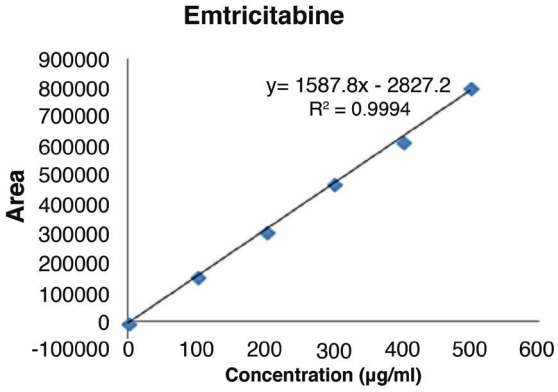

Figure 6. Linearity graph of emtricitabine

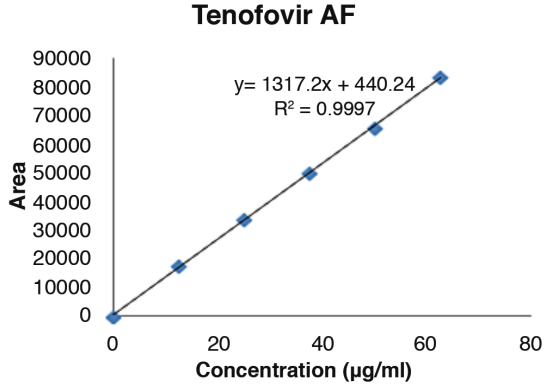

Figure 7. Linearity graph of tenofovir AF
AF: Alafenamide

Table 4. Assay results for % recoveries of marketed formulation

S. no.	Parameter	% Recovery of bictegravir	% Recovery of emtricitabine	% Recovery of tenofovir AF
1	Assay (specification: NLT 98.0 and NMT 102.0% w/w) (n=3)	99.97%	100.48%	99.82%

n: Number of determinations, NLT: Not less than, NMT: Not more than, AF: Alafenamide

Table 5. Results of linearity

S. no.	Parameters	Bictegravir	Emtricitabine	Tenofovir AF
1	Linearity range (µg/mL)	25-125	100-500	12.5-62.5
2	Correlation coefficient (r^2)	0.999	0.999	0.999
3	Slope	1287.3	1587.8	1317.2
4	Intercept	1224.6	2827.2	440.24

AF: Alafenamide

Table 6. Results of accuracy

S. no.	% Concentration (at specification level) (n=3)	% Recovery of bictegravir	% Recovery of emtricitabine	% Recovery of tenofovir AF
1	50%	99.26	100.44	100.06
2	100%	100.30	100.79	99.66
3	150%	100.01	99.06	99.75

n: Number of determinations, AF: Alafenamide

Method precision

To evaluate the method precision, the % assay was calculated from six individual samples solutions analyzed on same day. The % RSD obtained with respect to the results of the method precision met the acceptance criteria (not more than 2%), and the details of peak areas and % RSD values are summarized in Table 9.

Robustness

Robustness is defined as how the method can resist (less impact) small and deliberate changes in analytical procedure parameters such as the flow rate (±10%) and the organic phase composition (±10%). Minor changes did not affect the peak area responses of the method significantly; hence, the proposed method is robust.

The flow rate (1.08 mL/min and 1.32 mL/min) and organic phase composition (lesser to more organic) were altered, and there was no significant variation in the results obtained when deliberate changes were made to the developed method. The results obtained for the parameter robustness are summarized in Table 10-15.

DL and QL

DL and QL values were estimated using the formulas:

DL=3.3×(σ/S)

QL=10×(σ/S)

where;

σ=standard deviation;

S=slope.

The DL values for bictegravir, emtricitabine, and tenofovir AF obtained were 2.7, 1.05, and 1.35 µg/mL, with signal to noise ratio of 3:1, and the QL values for bictegravir, emtricitabine, and tenofovir AF obtained were 8.78, 3.30, and 4.61 µg/mL, with a signal to noise ratio of 10:1, which indicates that the "sensitivity" of the method is adequate. The results are summarized in Table 16.

Hydrolytic degradation under acidic conditions

To 3.0 mL of the stock solution, 3 mL of 1N HCl was added, diluted to 10 mL, and incubated at 60°C for 6 hours. The resulting solution was neutralized with 1N NaOH and adjusted to the mark with the diluent. There was no remarkable acid degradation with respect to the subject drugs, and the chromatogram is shown in Figure 9.

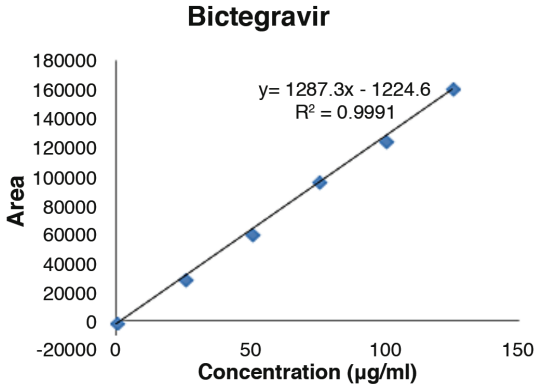

Figure 8. Linearity graph of bictegravir

Table 7. Results of system precision

Injection	Peak areas		
	Emtricitabine	Tenofovir AF	Bictegravir
Injection-1	4,74.652	50.304	97.274
Injection-2	4,70.806	50.532	96.658
Injection-3	4,79.900	50.680	97.574
Injection-4	4,73.621	50.727	97.021
Injection-5	4,75.167	50.255	98.232
Injection-6	4,76,538	50.235	97,987
Average	4,75,114.0	50,455.5	97,457.7
Standard deviation	3,031.1	219.9	592.8
% RSD (n=6)	0.6	0.4	0.6

n: Number of determinations, RSD: Relative standard deviation, AF: Alafenamide

Hydrolytic degradation under alkaline conditions

To 3.0 mL of the stock solution, 1N NaOH (3 mL) was added, diluted to 10 mL, and incubated at 60°C for 6 hours. Later, the solution was neutralized with 1N HCl. There was no significant degradation with respect to the three drugs, and the chromatogram obtained for alkali degradation is shown in Figure 10.

Thermal-induced degradation

The subject samples were placed separately in Petri dishes and remained in an oven at 110°C for a period of 24 hours. There was a minimal effect of thermal degradation

Table 8. Results of intermediate precision/ruggedness

Injection	Peak areas		
	Emtricitabine	Tenofovir AF	Bictegravir
Injection-1	4,77.752	49.821	97.234
Injection-2	4,74.159	50.388	96.991
Injection-3	4,69.272	50.289	95.433
Injection-4	4,69,317	50.176	96.414
Injection-5	4,77.171	50.337	97.491
Injection-6	4,73.102	50.073	97.166
Average	4,73,462.2	50.180.7	96,788.2
Standard deviation	3,674.6	209.8	755.4
% RSD (n=6)	0.8	0.4	0.8

n: Number of determinations, RSD: Relative standard deviation, AF: Alafenamide

Table 9. Results for method precision

Parameter	Sample weight (mg)	Peak areas		
		Emtricitabine	Tenofovir AF	Bictegravir
Method precision-1	174.2	4,75.652	50.166	97.455
Method precision-2	174.5	4,76.888	50.425	97.563
Method precision-3	174.1	4,75.988	50.253	97.234
Method precision-4	174.3	4,75.377	50.497	97.331
Method precision-5	174.2	4,76.765	50.556	97.548
Method precision-6	174.3	4,76.653	50.335	97.397
Average	-	4,76,220.5	50,372.0	97,421.3
Standard deviation	-	635.3	148.5	127.4
% RSD (n=6)	-	0.1	0.3	0.1

n: Number of determinations, RSD: Relative standard deviation, AF: Alafenamide

with respect to the drug emtricitabine and no significant effect with respect to bictegravir and tenofovir AF. The chromatogram obtained for thermal degradation is shown in (Figure 11).

Oxidative degradation

To the above stock solution, 3 mL of 3% (w/v) hydrogen peroxide (1 mL) was added in a 10 mL, and the flask was retained at ambient temperature for 12 minutes. There was a minimal effect of thermal degradation on bictegravir and tenofovir AF and no significant effect noticed with respect to emtricitabine. The chromatogram obtained for the oxidative degradation is shown in (Figure 12).

Photo degradation

The sample solution was exposed to external sunlight. No significant degradation was noticed with respect to the subject drugs, and the chromatogram obtained for the photolytic degradation is shown in (Figure 13).

A stability study was conducted for the drugs emtricitabine, tenofovir AF, and bictegravir under the respective stress conditions. The peak areas obtained, the % assay calculated,

Table 10. System suitability results for emtricitabine at a flow rate variation of ±10%

S. no.	Flow rate (mL/min)	System suitability results	
		USP tailing (T_f)	USP plate count (N)
1	1.08	1.37	2371.09
2	1.2	1.30	2185.90
3	1.32	1.31	2231.87

T_f: Tailing factor

Table 11. System suitability results for tenofovir AF at a flow rate variation of ±10%

S. no.	Flow rate (mL/min)	System suitability results		
		USP resolution (R)	USP tailing (T_f)	USP plate count (N)
1	1.08	6.32	1.25	3223.82
2	1.2	6.05	1.13	2973.76
3	1.32	6.07	1.06	2863.39

AF: Alafenamide, T_f: Tailing factor

Table 12. System suitability results for bictegravir at a flow rate variation of ±10%

S. no.	Flow rate (mL/min)	System suitability results		
		USP resolution (R)	USP tailing (T_f)	USP plate count (N)
1	1.08	3.28	1.31	2143.54
2	1.2	3.14	1.33	2214.41
3	1.32	3.20	1.40	2183.37

T_f: Tailing factor

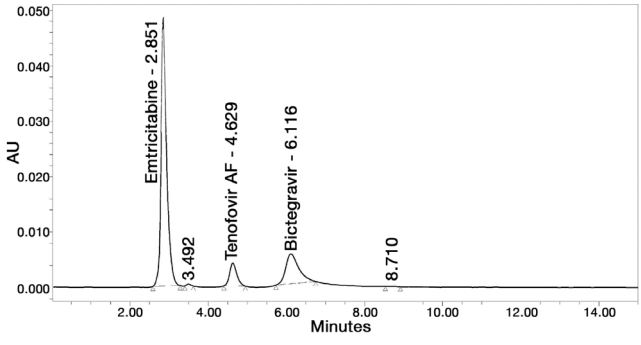

Figure 9. Acidic degradation chromatogram of bictegravir, emtricitabine and tenofovir alafenamide fumarate

AF: Alafenamide

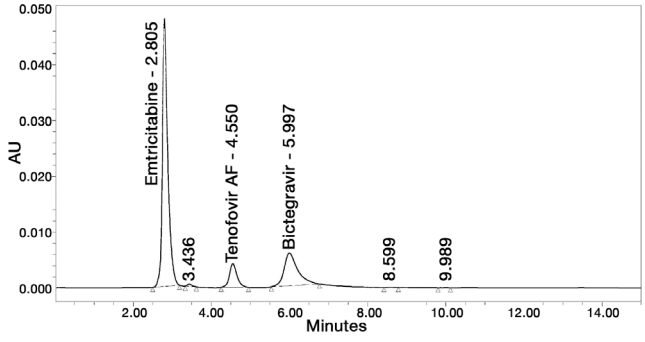

Figure 10. Alkali degradation chromatogram of bictegravir, emtricitabine and tenofovir alafenamide fumarate

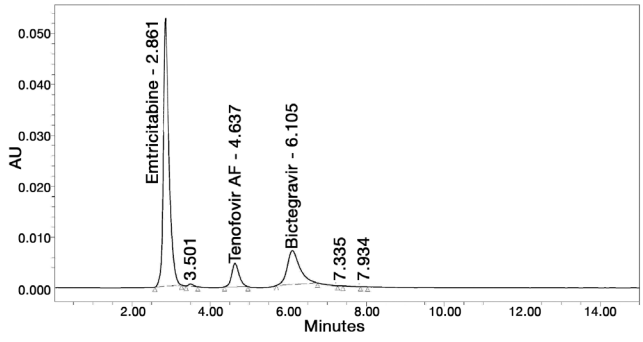

Figure 11. Thermal degradation chromatogram of bictegravir, emtricitabine and tenofovir alafenamide fumarate

AF: Alafenamide

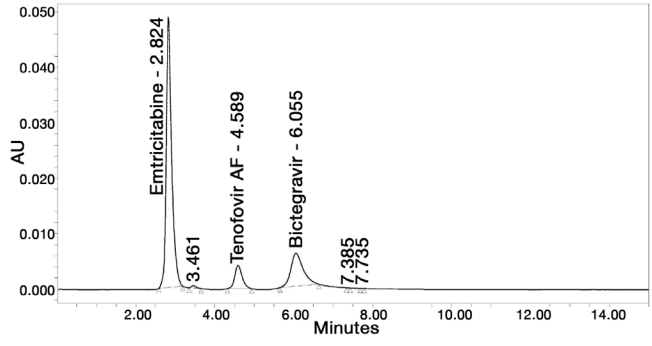

Figure 12. Oxidative degradation chromatogram of bictegravir, emtricitabine and tenofovir alafenamide fumarate

AF: Alafenamide

Table 13. System suitability results for emtricitabine at variation of the organic phase ±10%

S. no.	Organic phase ratio	System suitability results	
		USP tailing (T_f)	USP plate count (N)
1	Less organic	1.38	2254.66
2	Actual	1.30	2185.90
3	More organic	1.32	2263.23

T_f: Tailing factor

Table 14. System suitability results for tenofovir AF at variation of the organic phase ±10%

S. no.	Organic phase ratio	System suitability results		
		USP resolution (R)	USP tailing (T_f)	USP plate count (N)
1	Less organic	9.22	1.05	3228.79
2	Actual	6.05	1.13	2973.76
3	More organic	4.09	1.26	2672.79

T_f: Tailing factor, AF: Alafenamide

Table 15. System suitability results for bictegravir at variation of the organic phase ±10%

S. no.	Organic phase ratio	System suitability results		
		USP resolution	USP tailing	USP plate count
1	Less organic	4.65	1.10	2113.59
2	Actual	3.14	1.33	2214.41
3	More organic	2.16	1.49	2195.87

Table 16. Results of detection limit and quantification limit

S. no.	Sample	DL (µg/mL)	QL (µg/mL)	DL S/N ratio	QL S/N ratio
1	Bictegravir	2.7	8.78	3.02	10
2	Emtricitabine	1.05	3.30	3	9.98
3	Tenofovir AF	1.35	4.61	2.96	10.02

DL: Detection limit, QL: Quantification limit, S: Selectivity

Table 17. Results of % degradation by stability testing

	Emtricitabine			Tenofovir AF			Bictegravir		
	Area	% assay	% degradation	Area	% assay	% degradation	Area	% assay	% degradation
Standard	4,71,374.3	100	-	50,381.7	100	-	97,131.3	100	-
Acid	4,62.673	98.15	1.85	49.565	98.38	1.62	95.766	98.59	1.41
Base	4,59.782	97.54	2.46	48.566	96.40	3.60	94.866	97.67	2.33
Peroxide	4,52.736	96.05	3.95	47.687	94.65	5.35	93.145	95.90	4.10
Thermal	4,47.733	94.98	5.02	48.446	96.16	3.84	94.577	97.37	2.63
Photo	4,53.888	96.29	3.71	48.675	96.61	3.39	93.766	96.54	3.46

AF: Alafenamide

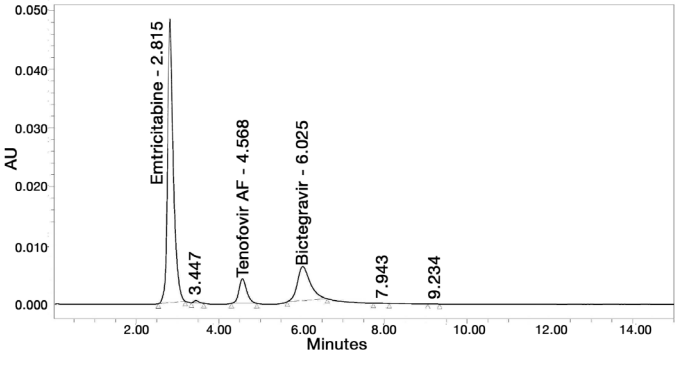

Figure 13. Photolytic degradation chromatogram of bictegravir, emtricitabine & tenofovir alafenamide fumarate

AF: Alafenamide

and the % degradation observed are summarized and detailed in Table 17. From the above data, it is evident that the drug "emtricitabine" is sensitive to thermal conditions, "tenofovir AF" and "bictegravir" are sensitive to oxidative conditions.

CONCLUSION

The newly developed method affirms good resolution between the three drugs bictegravir, emtricitabine, and tenofovir AF. The current method, method validation, and stability studies were found to be in line with the ICH guidelines and with official methods. The method requires no core extraction techniques; moreover, economical solvents are employed for the analysis, and good resolution is attained. No interference from any pharmaceutical dosage form or any remarkable impurities of degraded substance(s) was observed. Since the subject drugs of interest were analyzed by employing less expensive solvents and obtaining high resolution and shorter retention times with respect to the current method, the new proposed method is recommended for routine quality control analysis to provide simple, reliable, economical, and reproducible quantitative analysis for simultaneous estimation of the selected anti-retroviral fixed-dose regimen (bictegravir, emtricitabine, and tenofovir AF).

ACKNOWLEDGMENTS

Authors extend intense gratitude to M/S Pharma Train Labs, Hyderabad for providing the required research facilities and to Hetero Labs, Hyderabad for providing gift samples of bictegravir, emtricitabine and tenofovir AF.

Conflict of interest: No conflict of interest was declared by the authors. The authors are solely responsible for the content and writing of this paper.

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