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

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ABSTRACT
Introduction: 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 (RP-HPLC) method for the simultaneous estimation of few Anti-Retrovirals i.e., Bictegravir, Emtricitabine and Tenofovir Alafenamide Fumarate.
Methods: The novel method employs Inertsil Octyldecylsilyl (ODS) C_{18} (4.6 x 250mm, 5 µm) using 0.2% Triethylamine (TEA) buffer and methanol in the ratio of 40:60 %v/v as mobile phase to attain the optimized elution. The Detection wavelength is 260 nm with 1.2 ml/min flow rate and 20 µl of injection volume.
Results: Linearity ranges for Bictegravir, Emtricitabine & Tenofovir Alafenamide Fumarate were 25 - 125 µg/ml, 100 - 500 µg/ml, 12.5 - 62.5 µg/ml respectively. The Retention Times for Bictegravir, Emtricitabine and Tenofovir Alafenamide Fumarate are found to be 5.998 min, 2.805 min & 4.537 min respectively. Percent Recovery results of Bictegravir, Emtricitabine & Tenofovir AF were found within the range of 98 - 102% w/w.
Discussion and Conclusion: The novel method was successfully validated as per ICH guidelines. Forced degradation studies are performed and based on the % degradation, Emtricitabine was found sensitive to Thermal conditions and the drugs Bictegravir & Tenofovir AF were found sensitive to Oxidative conditions. Based on the evaluation of obtained results, the

developed method is economical and reliable for regular analysis concerning all validated parameters.

Keywords: Bictegravir, Emtricitabine, Tenofovir Alafenamide Fumarate, RP-HPLC, Validation, Forced degradation studies.

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. The infection might progress to an advanced disease stage called Acquired-Immuno Deficiency Virus (AIDS), if left untreated.. With the use of multiple specialized anti-retroviral medications that are commercially available, the state of HIV/AIDS infection can be controlled, descended and treated. Among numerous anti-retroviral formulations and combinations available, Bictegravir, is an oral tablet that contains three antiretroviral medicines (Bictegravir+ Emtricitabine+ Tenofovir AF) with the brand name 'BIKTARVY'. Out of three drugs, a Bictegravir, is a class of HIV-1 integrase strand transfer inhibitor, the Emtricitabine and Tenofovir Alafenamide are class of HIV-1 nucleoside analog reverse transcriptase inhibitors. Hence 'BIKTARVY' can be considered as an absolute regimen for HIV-1 (Type-1) infected patients[1-3]. The Integrase strand transfer inhibitors (INSTIs) comprises of two nucleoside reverse transcriptase inhibitors and recommended components during the initial antiretroviral therapy. Bictegravir is an effective INSTI with a high in-vitro barrier that shows high resistance towards the clinically relevant drug-drug interactions and shows specific activity against HIV-1 and HIV-2. The Bictegravir is metabolized by cytochrome P450 3A4 and a uridine diphosphate glucuronosyl transferase1A1. It binds to the active site of HIV integrase, and prevents the HIV replication. Compared with other INSTIs, Bictegravir possesses an in-vitro resistance profile among the other markedly available anti-retrovirals[4-5]. Emtricitabine and Tenofovir Alafenamide Fumarate act on DNA synthesis by HIV reverse transcriptase, resulting in viral DNA chain-termination and preventing the replication of HIV[6-7]. The US Food and Drug Administration (FDA) approved the 'Bictegravir' as a fixed-dose regimen (daily-once) to treat HIV-1 infection[8-9]. The chemical structures of three active pharmaceutical ingredients are shown in Fig-1, Fig-2 & Fig-3. The dosage regimen is as follows;

Bictegravir (50mg) + Emtricitabine (20 mg) + Tenofovir Alafenamide Fumarate (25mg).

According to the 'Department of Health and Human Services',[10-12] the current combination of regimen is intended to treat HIV-1 patients. Biktarvy can be administered with/ without food and is not recommended with other anti-retrovirals[13]. The literature survey was done and very few stability-indicating RP-HPLC isocratic elution methods to estimate the drugs of interest are reported.

However, the methods obtainable for Bictegravir, Emtricitabine and Tenofovir AF from pharmaceutical formulation are scanty, varying in establishing multiple experimental variables, the developed method was found more sensitive and reliable. The details are as follows in Table-1;

Therefore, in the present work we developed a novel, reliable and efficient method for the quantification of the subject drugs. 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 are performed according to the ICH guidelines and the obtained results are reported.

EXPERIMENT

Materials and methods

Collection of drugs:
Bictegravir of purity 99%w/w, Emtricitabine of purity 99% w/w & Tenofovir Alafenamide Fumarate of purity 99% w/w, procured from Hetero Labs, Hyderabad.

Chemicals and Reagents:
Methanol - HPLC grade (Rankem), water for HPLC – Milli-Q grade (Merck), Triethylamine - HPLC grade (Research lab fine chem industries).

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

(a) The Inertsil Octadecyl-Silica (ODS) C_{18} (4.6 x 250mm, 5µm) was found ideal for the analyzing the selected drugs.

(b) The Rheodyne injector (20 µL loop) is used for injecting the samples.

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

(d) Other instruments included Afcoset ER-200A electronic weighing balance, micropipettes, pipettes, burettes, micro-pore filtration assembly, Ultra-sonic water bath for sonication of Mobile phase, pH -meter (Adwa – AD 1020) etc.

OPTIMIZED CHROMATOGRAPHIC CONDITIONS:

Upon several trials conducted for optimization, the appropriate conditions were selected for the study and the details are as follows;

Instrument : High Performance Liquid Chromatograph (Waters) with auto sampler.
Detector : PDA detector.
Temperature : Ambient
Column : Inertsil Octadecyl-silica (ODS) C_{18} (4.6 x 250mm, 5µm)
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% Triethyl amine (TEA) buffer solution:
Accurately pipette out 2ml of triethyl amine, into 1000 ml HPLC grade water and dissolve. The pH was adjusted to 3.5 with dilute formic acid.

Preparation of mobile phase:
Accurately measured the buffer (above prepared) of 400 ml (40%) and Methanol of 600 ml (60%) quantities 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 are taken in 100 ml of diluent. From the prepared Stock solution, 3 ml was diluted to 10 ml, the resulting solution contains 300 ppm of Emtricitabine, 37.5 ppm of Tenofovir AF and 75 ppm of Bictegravir.

Sample Preparation:
10 tablets (prepared in-house by weighing the quantities as stated in the marketed formulation of Emtricitabine (200 mg), Tenofovir AF (25 mg) & Bictegravir (50 mg)) were accurately weighed and the quantities that equals to Emtricitabine (100 mg), Tenofovir AF (12.5 mg) and Bictegravir (25 mg) samples were diluted to 100 ml. The 3 ml of Stock was made to 10 ml that contains 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;

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

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 developed method is implemented to ensure that the method meet the intended requirements as stated in the respective guidelines[23]. 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[14-17].
Calibration curves were obtained at 25-125 µg/ml for Bictegravir, 100-500 µg/ml for Emtricitabine & 12.5-62.5 µg/ml for Tenofovir Alafenamide Fumarate concentrations.

Linearity:
Linearity can be illustrated by examining different concentrations of active pharmaceutical ingredients. The linearity of a method can be evaluated by the calibration plots of Bictegravir, Emtricitabine, & Tenofovir Alafenamide Fumarate constructed from peak response vs. concentration that approaches a straight line.

Emtricitabine (100 mg), Tenofovir AF (12.5 mg), and Bictegravir (25 mg) taken and diluted to 100 ml. From this stock solution, pipette 1-5 ml into 5 different 10 ml volumetric flasks and the series of aliquots were 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 are diluted to 100 ml.

Pipette out 3 ml of the resulting Stock solution and dilute to 10ml, that contains Emtricitabine (300 ppm), Tenofovir AF (37.5 ppm) and Bictegravir (75 ppm). The standard solutions of Accuracy - 50%, 100%, and 150% were prepared and injected and the recovery values for Emtricitabine, Tenofovir AF & Bictegravir were calculated.

Precision:
It is 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 are made upto to 100 ml. The 3 ml of this Stock was diluted to 10 ml.

Specificity:
Specificity can be illustrated by ensuring that the peaks are free from interference.
It is determined by injecting blank and standard into the chromatographic system and corroborate that no interference exists to determine the developed method as Specific.

Detection Limit (DL) and Quantification Limit (QL):
DL and QL values deal with the method sensitivity. DL is the analyte's lowest detectable concentration, while QL is the lowest quantifiable concentration.

Detection Limit (DL):
Emtricitabine (100 mg), Tenofovir AF (12.5 mg) & Bictegravir (25 mg) working standards are weighed and diluted to 100 ml separately. From this stock solution, 3 ml was diluted to 10 ml. From the above, each 1 ml of the above stock solutions (Emtricitabine, Tenofovir AF & Bictegravir) into different 10 ml volumetric flasks and diluted with diluent. Further pipette out Emtricitabine stock solution (0.35 ml), Tenofovir AF (1 ml), and Bictegravir stock (1 ml) solutions are diluted to 10 ml.

Quantification Limit (QL):
Emtricitabine (100 mg), Tenofovir AF (12.5 mg) & 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) & Bictegravir stock solution (3.9 ml) were diluted to 10 ml.

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

DEGRADATION STUDIES
The guideline of 'International Conference on Harmonization (ICH)' entitled "Stability testing of new drug substances and products" states that the stress testing is performed to evaluate the instinct stability attributes of the active Pharmaceutical substance. The stress degradation studies of Emtricitabine, Tenofovir AF and Bictegravir[18-22,27] were determined in current work.

Preparation of stock:

Emtricitabine (100 mg), Tenofovir AF (12.5 mg), and Bictegravir (25 mg) working standards are weighed and diluted to 100 ml. This resulting Stock solution was used for stability testing. All the stress conditions were applied and the % degradation was studied for the selected drugs Bictegravir, Emtricitabine & Tenofovir AF. The stress conditions include Acidic, alkali, thermal, oxidative & Photolytic conditions to study the nature of drugs and their stability against the above-mentioned conditions.

RESULTS AND DISCUSSION:

Optimization of the Method:

For the selection of suitable Mobile phase for simultaneous estimation of the selected drugs, various solvents such as water, ACN, triethyl amine, and methanol varying in polarity were used in different combinations of concentrations to get high peaks resolutions within lesser runtime. Among all the different mobile-phase combinations employed, the mobile –phase comprising of 0.2% TEA Buffer and Methanol in the ratio of 40:60 v/v exhibited well-definitive 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 exercised, the flow rate of 1.2 mL/min was selected as optimized for the study.
The column temperature was set at 25°, 30°, and 35°C for optimizing according to its effect on peak resolutions and RT of the drug samples.
During the method optimization, the selected combination of three drugs were analyzed using different columns, the column (Inertsil Octadecyl-silica (ODS) C_{18} (4.6 x 250mm, 5µm) 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 ideal wavelength for the study.

System suitability:

According to the optimized experimental conditions, the retention times obtained for Bictegravir, Emtricitabine, and Tenofovir Alafenamide Fumarate are 5.998 min, 2.805 min, and 4.537 min. The optimized chromatogram with Taling factor (<2), theoretical plates (>2000), Resolution (>2), Capacitance Factor (>1) is shown in (Fig-4). Hence, the proposed method was proved as 'selective' to determine the drugs (Bictegravir, Emtricitabine & Tenofovir AF). The system suitability results of the standard injections are tabulated in Table-3.

Assay of marketed formulation:

The assay results obtained for three drugs (Bictegravir, Emtricitabine & Tenofovir Alafenamide Fumarate) are detailed in Table-4. No interference of the excipients was noticed in current method hence the method is 'specific'. The typical chromatogram for assay of commercial formulation (in-house preparation) is shown in (Fig-5).

Linearity:

To construct the calibration curve, different concentration ranges of Bictegravir (25-125 µg/ml), Emtricitabine (100-500 µg/ml) and Tenofovir Alafenamide Fumarate (12.5-62.5 µg/ml) are considered. The correlation coefficient (r^2) values obtained are found satisfactory. The results obtained are summarized in Table-5. The calibration plots of three drugs are as shown in (Fig-6, Fig-7 & Fig-8).

Accuracy:
Accuracy was determined at 50%, 100% and 150% of test concentrations by calculating the individual recovery and mean recovery values of Emtricitabine, Tenofovir AF, and Bictegravir. The recoveries ranged from 99.26 – 100.30% for Bictegravir, 99.06 - 100.79% for Emtricitabine, and 99.66 - 100.06% for Tenofovir Alafenamide Fumarate are determined. The recovery values obtained are found meeting the acceptance criteria (Not less than 98.0% & Not more than 102.0%). The RSD values obtained were < 2 with respect to three drugs. The obtained results were mentioned in the 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 injections area are found 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.

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 is meeting the acceptance criteria (Not more than 2%) and the details of peak areas and %RSD values are summarized in Table-9.

Robustness:
The robustness is illustrated as how the method can resist (less impact) to the small and deliberate changes in analytical procedure parameters like flow rate (±10%) and in 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 from the obtained results, it is evident that there is no significant variation in the results obtained for the deliberate changes made to the developed method. The results obtained for the parameter of Robustness are summarized in Table 10-15.

Detection Limit (DL) and Quantification Limit (QL):

DL and QL values were estimated using the formulae;
$DL = 3.3 \times (\sigma/S)$
$QL = 10 \times (\sigma/S)$

Where,
σ = Standard Deviation;
S = Slope.
The DL values for Bictegravir, Emtricitabine and Tenofovir Alafenamide Fumarate are obtained as 2.7, 1.05 & 1.35 µg/ml with signal to noise ratio 3:1 and QL values for Bictegravir, Emtricitabine and Tenofovir Alafenamide Fumarate are obtained as 8.78, 3.30 and 4.61 µg/ml with signal-to-Noise ratio 10:1, which indicates that the 'sensitivity' of the method is adequate. The results were summarized in Table-16.

Hydrolytic degradation under acidic condition:

To the 3.0 ml of Stock solution, add 3 ml of 1N HCl, diluted to 10 ml and kept at 60 ºC for 6 hours. The resulting solution was neutralized with 1N NaOH and made to the mark with the diluent. There is no remarkable acid degradation noticed with respect to the subject drugs and the chromatogram is as shown in (Fig-9).

Hydrolytic degradation under alkaline condition:

To the Stock solution (3.0 ml), add 1N NaOH (3 ml) and diluted to 10 ml, remained at 60 ºC for 6 hours. Later, neutralized with 1N HCl. There is no significant degradation noticed with respect to the three drugs and the chromatogram obtained for the alkali degradation is shown in (Fig-10).

Thermal induced degradation:

The subject samples were placed separately in Petri dish and remained in oven at 110° C for period of 24 hours. There is a minimal effect of thermal degradation with respect to the drug Emtricitabine and no significant effect with respect to Bictegravir and Tenofovir Alafenamide Fumarate. The chromatogram obtained for the thermal degradation is shown in (Fig-11).

Oxidative degradation:

To the above stock solution (3 ml), add 3% w/v of hydrogen peroxide (1 ml) in 10 ml and the flask was retained at ambient temperature for 12 minutes. There is minimal effect of thermal degradation over Bictegravir and Tenofovir Alafenamide Fumarate and no significant effect noticed with respect to Emtricitabine. The chromatogram obtained for the oxidative degradation is shown in (Fig-12).

Photo Degradation:

The sample solution was exposed to the external sunlight. No significant degradation was noticed with respect to the subject drugs and the chromatogram obtained for the photolytic degradation is shown in (Fig-13).
The stability study was conducted for the drugs Emtricitabine, Tenofovir AF and Bictegravir under respective stress conditions. The peak areas obtained, % Assay calculated and the %

degradation observed were 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 new developed method elucidates good resolution between the three drugs Bictegravir, Emtricitabine and Tenofovir Alafenamide Fumarate. The current method, method validation and stability studies were found in-line to the ICH guidelines and in-line to the official methods. The method requires no core extraction techniques, economical solvents are employed for analysis and the 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, high resolution with lesser retention times with respect to the current method, therefore, this proposed method is recommended for routine quality control analysis to provide simple, reliable, economical and reproducible quantitative analysis for simultaneous estimation of selected anti-retroviral fixed dose regimen (Bictegravir, Emtricitabine and Tenofovir Alafenamide Fumarate).

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Table 1: Comparison with similar existing methods

Kokkirala TK et.all [11]	Muthyala Sneha et.all [24]	Sattar MA et.all [25]	R Meenakshi et.all [26]	Current method	Remarks
Buffer and Acetonitrile in the ratio of 50:50	Buffer and Acetonitrile in the 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): Methano	Buffer phosphoric dihydrogen phosphate as mobile phase A and methanol and water (70:30) as mobile phase B of gradient program.	0.2% Triethylamine (TEA) buffer and methanol in the ratio of 40:60 %v/v	Less-Expensive solvents were employed to obtain substantial results.
C18 column (150 mm × 4.6 m	Zodiac C18 150x4.6mm, 5□	Inspire C18 column (150×4.6mm) 5.0µm	Inertsil 30V C 18 Column (250*4.6mm, 5	Octyldecylsilyl (ODS) C_{18} (4.6	Good peak shape and resolution

m, 5 µm)			micron)	x 250mm, 5µm)	acquired.
272 nm	272 nm	272 nm	265 nm	260 nm	Detection of eluted peaks acquired at lower wavelength.

Table 2: Comparison of Optimum conditions

S. No.	Column used	Specification	Remarks
1	Hypersil	5.0 x 250 mm, 10 µm	Deformed peak shape was observed
2	Lichrosorb	4.6 x 250 mm, 5 µm	Low Resolution observed
3	Inertsil ODS C18	4.6 x 250 mm, 5 µm	Peak Shape is sharp and free from tailing with high Resolution

Table 3: System suitability results.

S. No:	Parameter	Acceptance Criteria	Bictegravir	Emtricitabine	Tenofovir Alafenamide Fumarate
1	Tailing factor (T_f)	Not more than 2.0	1.33	1.30	1.13
2	Theoretical Plates (N)	Not less than 2000	2214.41	2185.90	2973.76
3	Resolution for the Tenofovir AF and Bictegravir (R)	Not less than 2	3.14	-	6.05

4	Capacitance Factor (k)	Not less than 1.0	4.36	1.54	3.09
5	Selectivity (S)	Not more than 2.0	0.5	-	0.7

Table 4: Assay results for %Recoveries of marketed formulation.

S. No	Parameter	%Recovery of Bictegravir	%Recovery of Emtricitabine	%Recovery of Tenofovir Alafenamide Fumarate
1	Assay (Specification NLT 98.0 and NMT 102.0 % w/w) *(n=3)*	99.97%	100.48%	99.82%

n= number of determinations

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

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

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

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

Table 9: Results of 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

Parameter	Sample Weight (mg)	Peak Areas		
		Emtricitabine	Tenofovir AF	Bictegravir
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

Table 10: System suitability results for Emtricitabine at 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

Table 11: System suitability results for Tenofovir AF at Flow Rate variation of ±10%.

S. No	Flow Rate	System Suitability Results

	(ml/min)	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

Table 12: System suitability results for Bictegravir at 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

Table 13: System suitability results for Emtricitabine at variation of Organic Phase ±10%.

S. N	Organic Phas 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

Table 14: System suitability results for Tenofovir AF at variation of 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

Table 15: System suitability results for Bictegravir at variation of 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 (DL) and Quantification Limit (QL).

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

Table 17: Results of % Degradation by Stability Testing.

	Emtricitabine			Tenofovir AF			Bictegravir		
	Area	% Assay	% Degradation	Area	% Assay	% Degradation	Area	% Assay	% Degradati
Standard	4,71,374	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

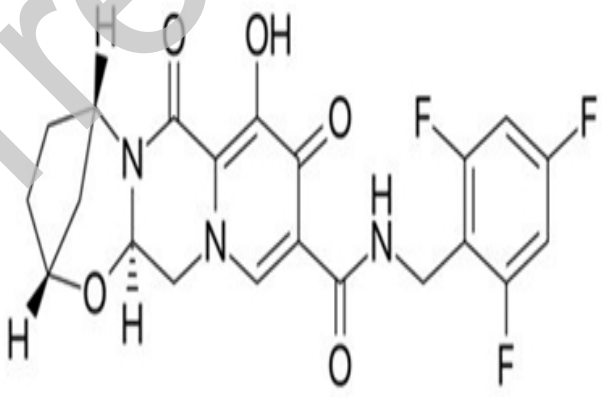

Figure 1: Bictegravir

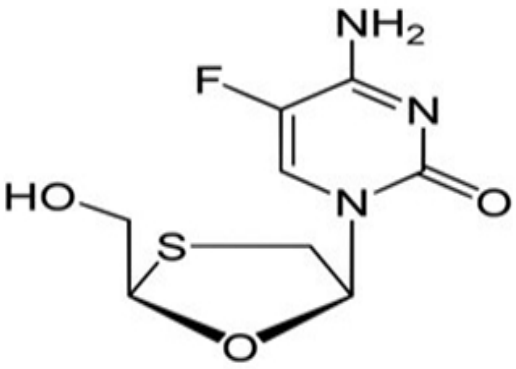

Figure 2: Emtricitabine

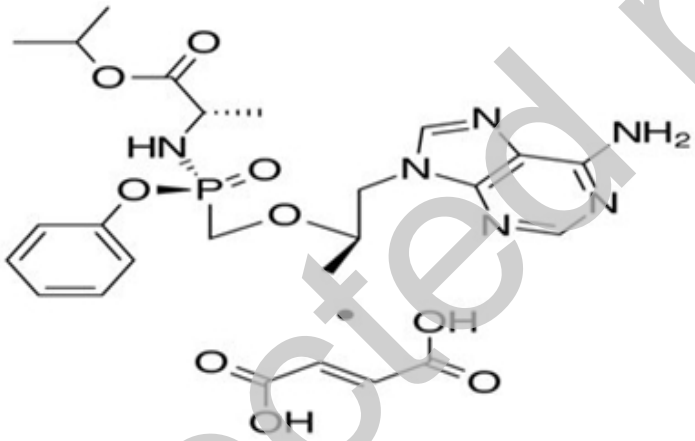

Figure 3: Tenofovir Alafenamide fumarate

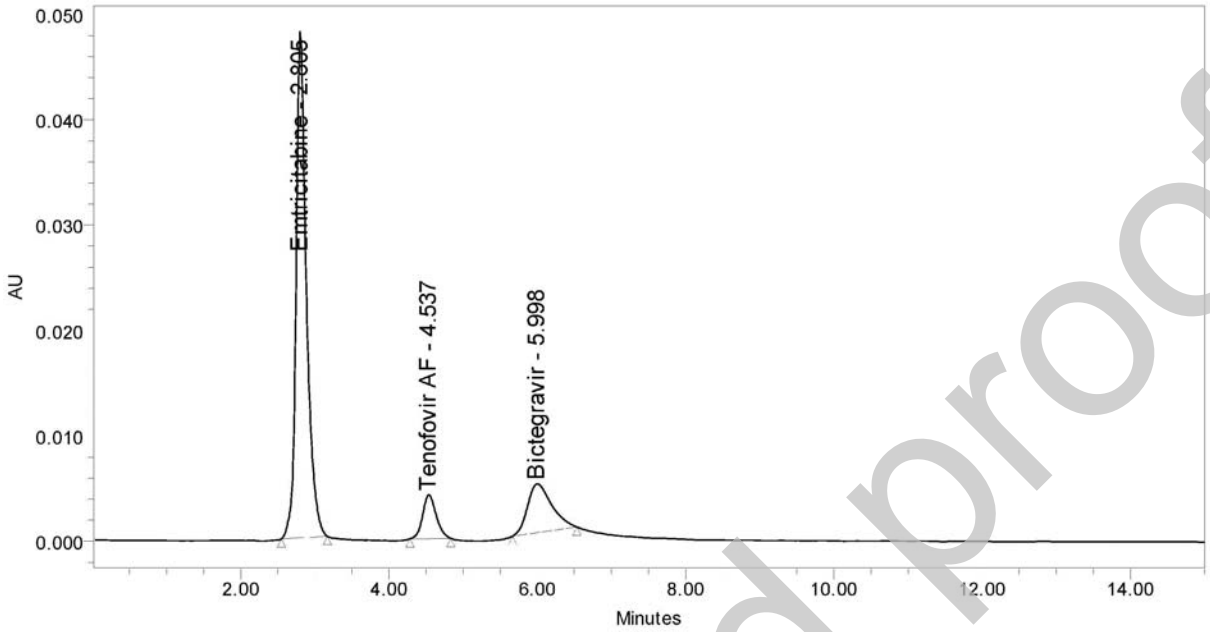

Figure 4: Optimized chromatogram showing the simultaneous elution of Bictegravir, Emtricitabine & Tenofovir Alafenamide Fumarate

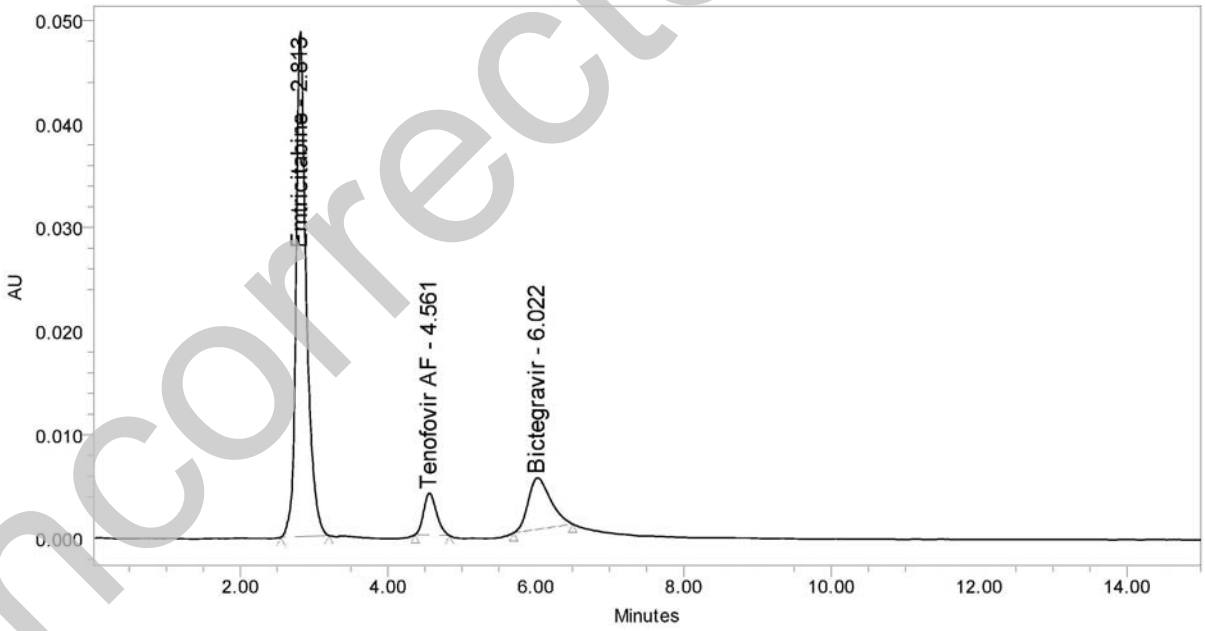

Figure 5: Assay chromatogram for marketed formulation of Bictegravir, Emtricitabine & Tenofovir Alafenamide Fumarate

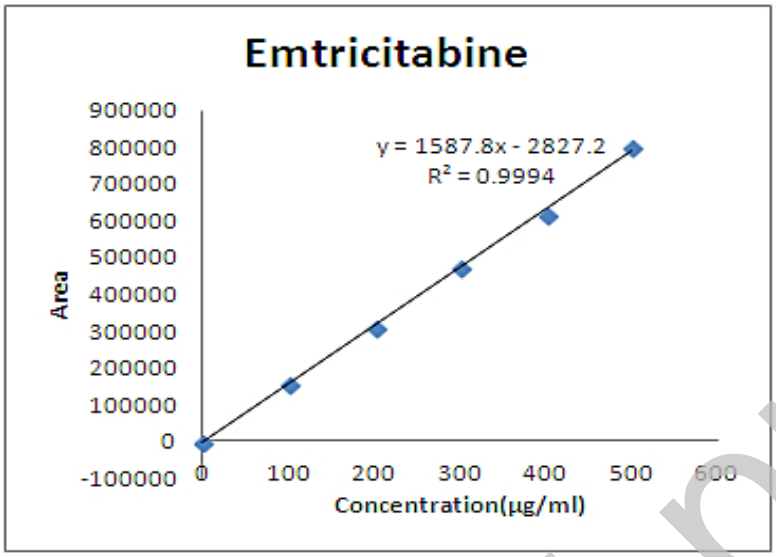

Figure 6: Linearity graph of Emtricitabine

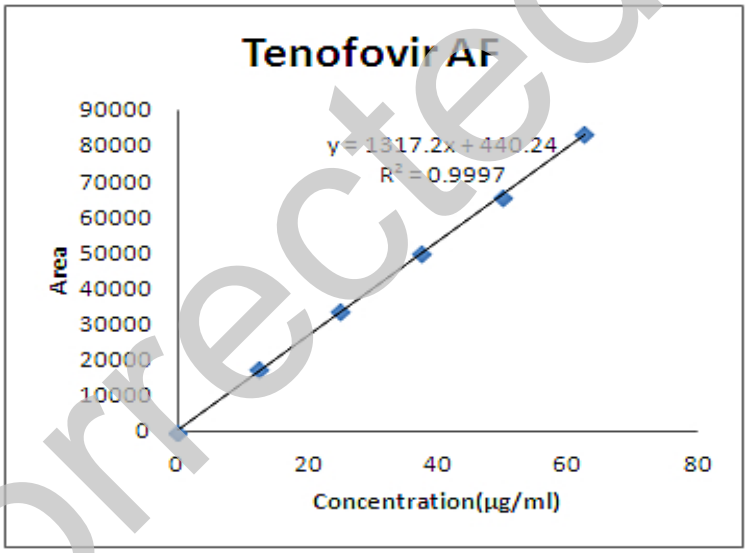

Figure 7: Linearity graph of Tenofovir AF

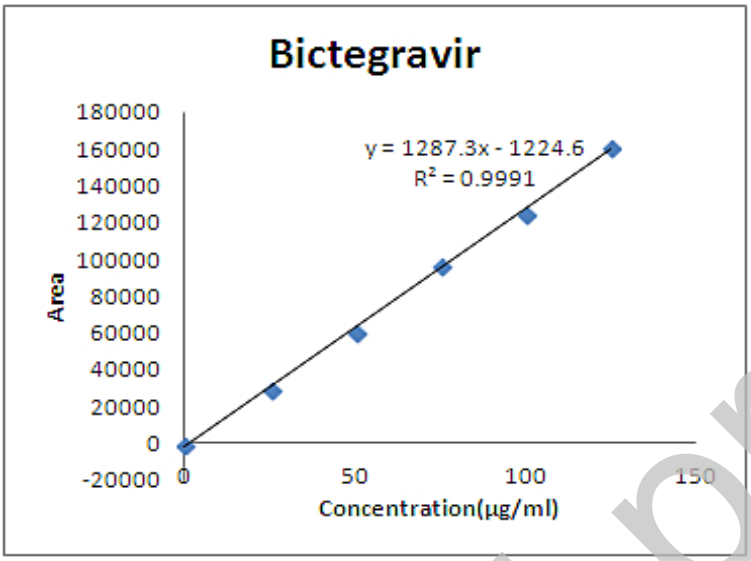

Figure 8: Linearity graph of Bictegravir

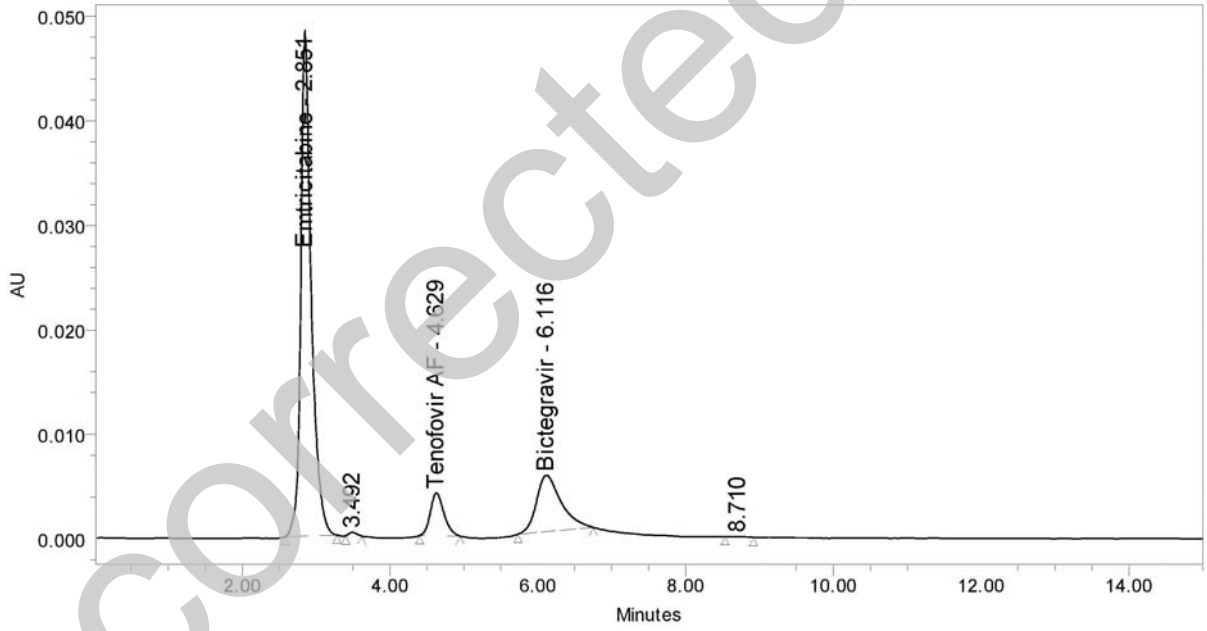

Figure 9: Acidic degradation chromatogram of Bictegravir, Emtricitabine & Tenofovir Alafenamide Fumarate

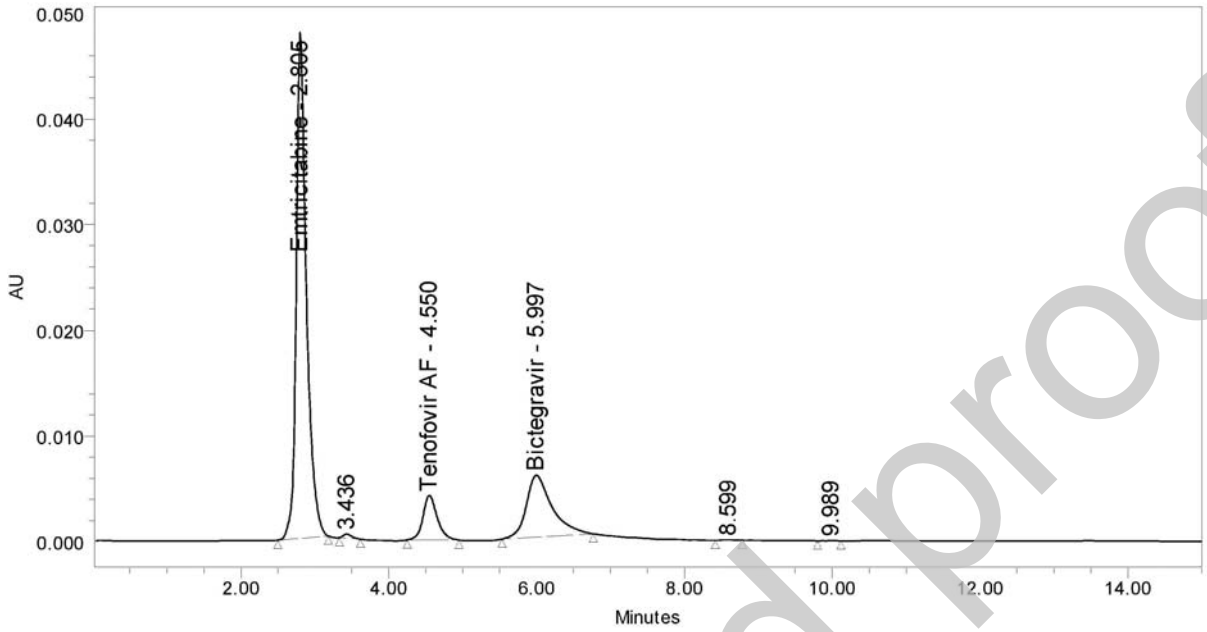

Figure 10: Alkali degradation chromatogram of Bictegravir, Emtricitabine & Tenofovir Alafenamide Fumarate

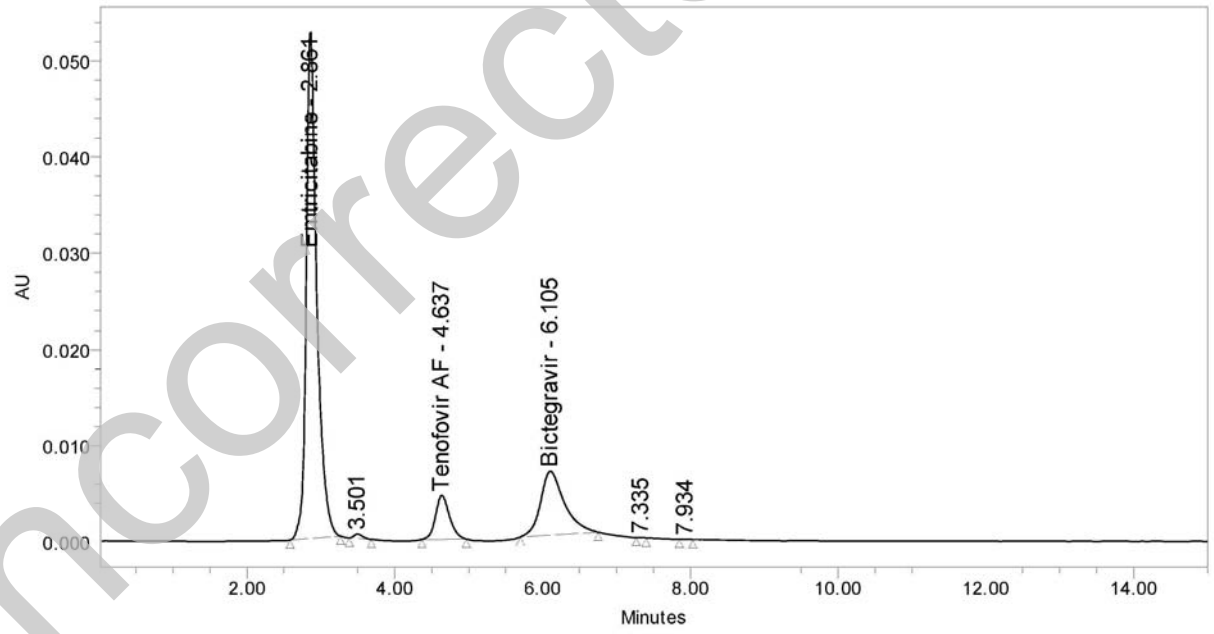

Figure 11: Thermal degradation chromatogram of Bictegravir, Emtricitabine & Tenofovir Alafenamide Fumarate

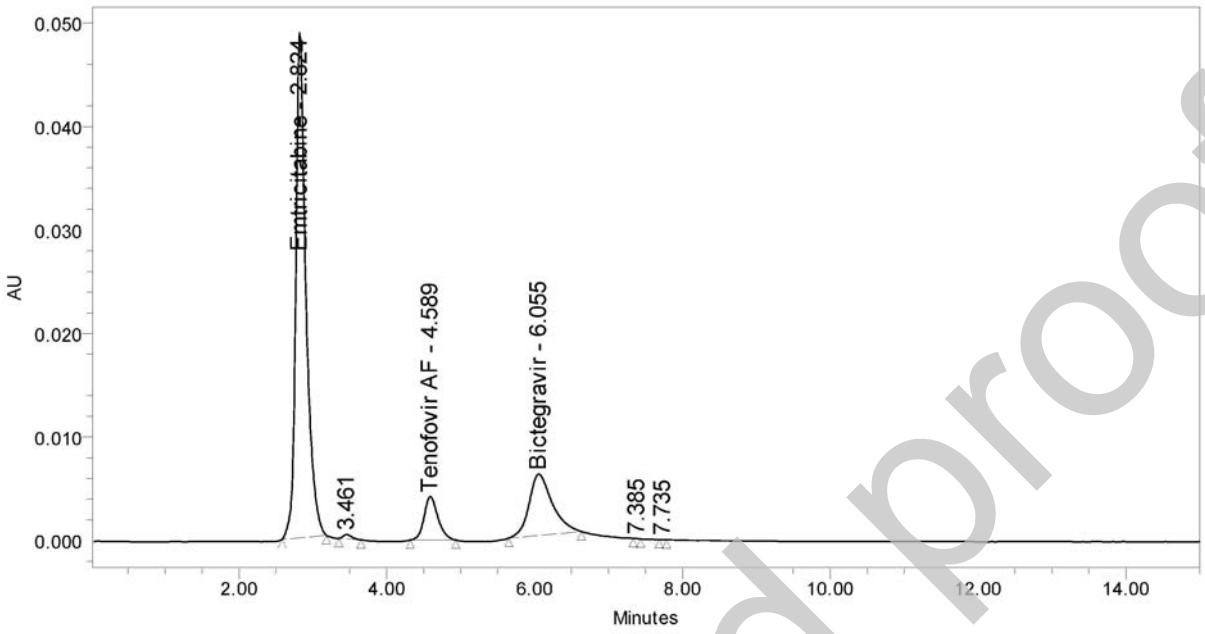

Figure 12: Oxidative degradation chromatogram of Bictegravir, Emtricitabine & Tenofovir Alafenamide Fumarate

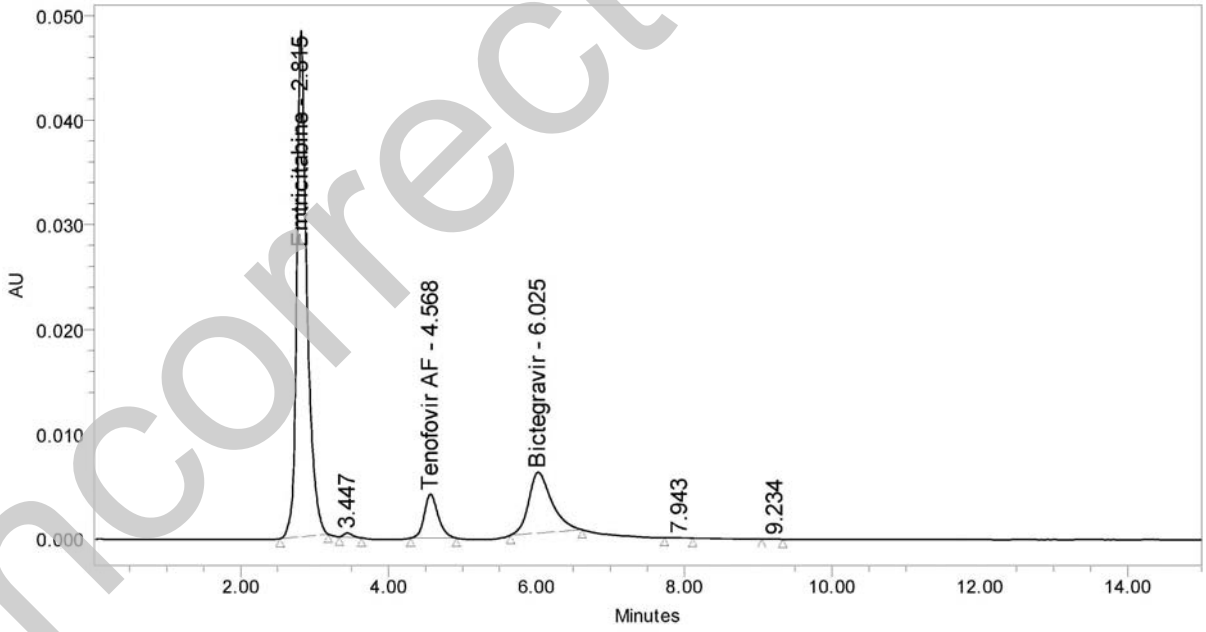

Figure 13: Photolytic degradation chromatogram of Bictegravir, Emtricitabine & Tenofovir Alafenamide Fumarate