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Analytical Method Validation Report for Assay of Tenofovir AF, Emtricitabine, and Bictegravir by RP-UPLC

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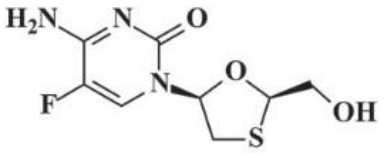
Abstract
A new simple, accurate, precise RP-UPLC [reverse phase ultra performance liquid chromatography] method was developed for the simultaneous estimation of the Emtricitabine, Bictegravir and Tenofovir alafenamide in pharmaceutical dosage form. Chromatogram was run through Denali C18 column (150 mm × 4.6 mm, 5 μm); mobile phase containing buffer and acetonitrile in the ratio of 50:50 was pumped through column at a flow rate of 1 ml/min [Buffer: 0.1% at pH 2.2, temperature 30°C]. Optimized wavelength was 272 nm. Retention times of Emtricitabine, Bictegravir and Tenofovir alafenamide were found to be 2.303, 3.219 and 3.754 min respectively. The %RSD of the Emtricitabine, Bictegravir and Tenofovir alafenamide were found to be 0.7, 0.8 and 0.4, respectively. The %recovery was obtained as 99.89%, 100.65% and 100.38% for Emtricitabine, Bictegravir and Tenofovir alafenamide, respectively. This method was accurate, precise and sensitive; hence, could be employed for routine quality control of Emtricitabine, Bictegravir and Tenofovir alafenamide in pharmaceutical industries and drug testing laboratories.
Key words: *Tenofovir, RP-UPLC, Emtricitabine and Bictegravir*

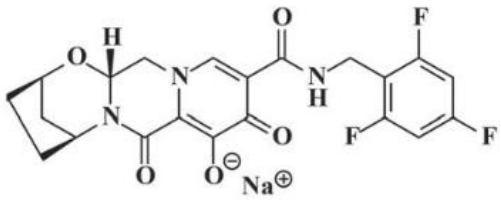
INTRODUCTION

Emtricitabine [Figure 1] is a nucleoside reverse transcriptase inhibitor (NRTI) for the treatment of HIV infection in adults. Emtricitabine is an analogue of cytidine. The drug works by inhibiting reverse transcriptase, the enzyme that copies HIV RNA into new viral DNA. Chemically it is known as 4-Amino-5-fluoro-1-((2R,5S)-2-hydroxymethyl-[1,3]-oxathiolan-5-yl)-1H-pyrimidin-2-one [1]. Bictegravir [Figure 1] is a recently approved investigational drug that has been used in trials studying the treatment of HIV-1 and HIV-2 infection. Chemically it is known as (1S,11R,13R)-5-Hydroxy-3,6-dioxo-N-(2,4,6-trifluorobenzyl)-12-oxa-2,9-diazatetracyclo [11.2.1.02,11.04,9] hexadeca-4,7-diene-7-carboxamide. It has been approved for HIV-1 monotherapy combined with 2 other antiretrovirals in a single tablet [2]. Tenofovir alafenamide fumarate (TAF) [Figure 1] is a nucleotide reverse transcriptase inhibitor (NRTI) and a novel ester prodrug of the antiretroviral Tenofovir. Tenofovir mimics normal DNA building blocks, but is lacking a 3'-OH molecule required for phosphodiester bond linkage. By competing with regular nucleotides for incorporation into proviral DNA and prevention of the formation of the 5' to 3' phosphodiester linkage required for DNA elongation, Tenofovir causes early chain termination and prevents proviral DNA transcription. Chemically it is known as propan-2-yl-(2S)-2-{[(S)({[(2R)-1-(6-amino-9H-purin-9-yl)propan-2-yl]oxy} methyl) (phenoxy)phosphoryl]amino} propanoate [3]. Although Tenofovir (available as Tenofovir disoproxil fumarate) has a good safety profile and efficacy, and is currently a cornerstone of HIV antiviral treatment, its use has been associated with nephrotoxicity and reduced bone mineral density. In comparison, TAF has been shown to have improved antiviral efficacy, enhanced delivery of TFV into peripheral blood mononuclear cells and lymphatic tissues, a higher barrier to resistance, and an improved safety profile. Improved renal safety is likely attributable to lower circulating plasma concentrations of tenofovir and therefore less exposure and damage to bone and the kidneys, where tenofovir is metabolized. Because HIV antiretroviral therapy is usually life-long, reduced toxicity and improved efficacy results in better patient outcomes and improved adherence in the long term [4]. The literature survey reveals that there is no analytical method available for the estimation of Emtricitabine, Bictegravir and Tenofovir alafenamide in pharmaceutical dosage forms. The reported methods available for the estimation of Emtricitabine and

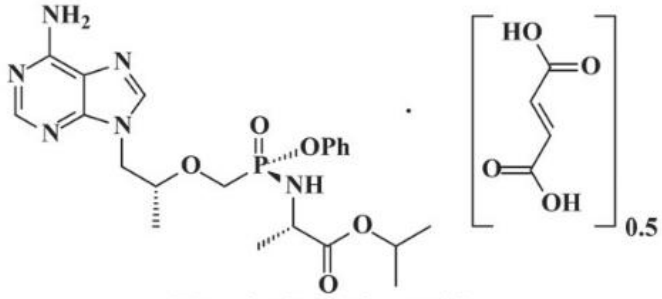
Tenofovir alafenamide are RP-HPLC [5–9 reverse phase high performance liquid chromatography method], Tenofovir Disoproxil Fumarate and Emtricitabine RP-HPLC [9–11 reverse phase high performance liquid chromatography Method], Application of UV Spectrophotometric Methods for Simultaneous Estimation of Emtricitabine and Tenofovir Alafenamide Fumarate in Bulk [12], spectrofluorimetric analysis [13], reverse phase high performance liquid chromatography [14,15].



Emtricitabine



Bictegravir



Tenofovir alafenamide

Since there are no official reported methods on high performance liquid chromatographic methods for the simultaneous estimation of Emtricitabine, Bictegravir and Tenofovir alafenamide in the public domain, we have planned to develop a simple, precise, economic and accurate stability indicating RP-HPLC reverse phase high performance liquid chromatography] method development and validation for the estimation of emtricitabine, bictegravir and tenofovir alafenamide in pharmaceutical dosage form. a gift sample from Spectrum Pharma research solutions, Hyderabad. The pharmaceutical dosage form (Biktarvy R© – Symphony Pharma Limited, Guwahati, Assam) was purchased from a local pharmacy. The solvents used in this work were of HPLC [reverse phase high performance liquid chromatography] grade and obtained from Merck Specialties Private Limited, Mumbai. Symphony Pharma Limited.

MATERIALS AND METHODS

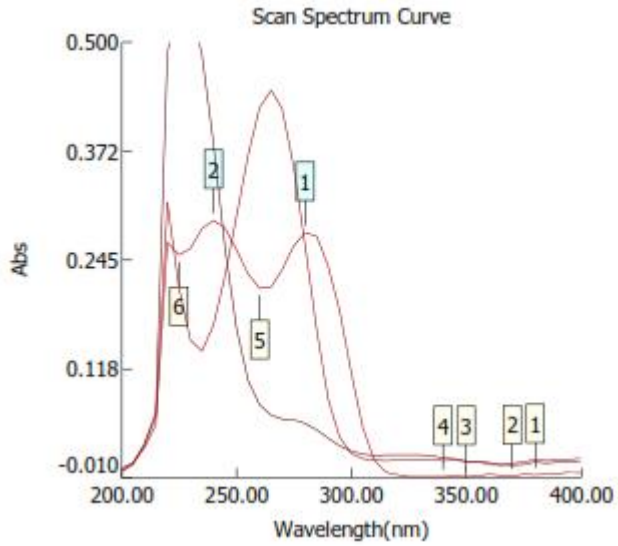
HPLC METHOD DEVELOPMENT:

Mobile Phase Optimization:

Initially, the mobile phase tried was methanol: OPA buffer and Methanol: phosphate buffer with various combinations of pH, as well as varying proportions. Finally, the mobile phase was optimized to Phosphate buffer with (pH 3.0) Acetonitrile in proportion 45:55 v/v respectively.

Wavelength selection:

UV spectrum of 10 µg/ml Tenofovir AF, Emtricitabine, and Bictegravir in diluents (mobile phase composition) was recorded by scanning in the range of 200nm to 400nm. From the UV spectrum wavelength selected as 260 nm. At this wavelength all these three drugs show good absorbance.



Optimization of Column:

The method was performed with various columns like C18 column, hypersil column, lichrosorb, and inertsil ODS column. BEH C_{18} (4.6 x 50mm, 1.7µm) was found to be ideal as it gave good peak shape and resolution at 0.3 ml/min flow.

OPTIMIZED CHROMATOGRAPHIC CONDITIONS:

Instrument used	:	Waters UPLC with auto sampler and PDA detector.
Temperature	:	Ambient
Column	:	Waters BEH C_{18} (4.6 x 50mm, 1.7 µm)
Buffer	:	3.4g potassium dihydrogen ortho phosphate was taken in a 1000ml volumetric flask and adjust the P^H with Diluted NaOH upto 3.
P^H	:	3.0
Mobile phase	:	45% buffer 55% Acetonitrile
Flow rate	:	0.3 ml per min
Wavelength	:	260 nm
Injection volume	:	2 µl
Run time	:	4 min.

PREPARATION OF BUFFER AND MOBILE PHASE:

Preparation of 0.025M Phosphate buffer:

3.4g of potassium dihydrogen ortho phosphate was weighed and taken in a 1000ml volumetric flask and adjust the PH with Diluted NaOH upto 3, finally the solution was filtered by using 0.45 Micron membrane filter, sonicate it for 10 mins.

Preparation of mobile phase:

Accurately measured 450 ml (45%) of above buffer and 550 ml of Acetonitrile UPLC (55%) were mixed and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation:

The Mobile phase was used as the diluent.

PREPARATION OF THE TENOFOVIR AF, EMTRICITABINE AND BICTEGRAVIR STANDARD & SAMPLE SOLUTION:

Standard Solution Preparation:

Accurately weigh and transfer 25 mg of Tenofovir AF, 200 mg of Emtricitabine and 50 mg of BICTEGRAVIR working standard into a 100 ml clean dry volumetric flask add about 70 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution) Further pipette 0.75 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Sample Solution Preparation:

Accurately weigh 10 tablets crush in mortor and pestle and transfer equivalent to 25 mg of Tenofovir AF, 200 mg of Emtricitabine and 50 mg of BICTEGRAVIR in (marketed formulation=467.8 mg of tablet Powder) sample into a 100 mL clean dry volumetric flask add about 70 mL of Diluent and sonicate it up to 30 mins to dissolve it completely and make volume up to the mark with the same solvent. Then it is Filtered through 0.45 micron Injection filter. (Stock solution).Further pipette 0.75 ml of Tenofovir AF, Emtricitabine and BICTEGRAVIR from the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure:

Inject 2 µL of the standard, sample into the chromatographic system and measure the areas for Tenofovir AF, Emtricitabine and BICTEGRAVIR peaks and calculate the % Assay by using the formulae.

SYSTEM SUITABILITY:

Tailing factor for the peaks due to Tenofovir AF, Emtricitabine and BICTEGRAVIR in Standard solution should not be more than 2.0.Theoretical plates for the Tenofovir AF, Emtricitabine and BICTEGRAVIR peaks in Standard solution should not be less than 2000. Resolution for the Emtricitabine and BICTEGRAVIR peaks in standard solution should not be less than 2.

METHOD VALIDATION SUMMARY:

PRECISION:

Preparation of stock solution:

Accurately weigh and transfer 25 mg of Tenofovir AF, 200 mg of Emtricitabine and 50 mg of BICTEGRAVIR working standard into a 100 ml clean dry volumetric flask add about 70 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).Further pipette 0.75 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure:

The standard solution was injected for six times and measured the area for all six. Injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.The results are summarized for Tenofovir AF, Emtricitabine and BICTEGRAVIR. Acceptance Criteria: The % RSD for the area of six standard injections results should not be more than 2%.

INTERMEDIATE PRECISION/RUGGEDNESS:

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different day.

Preparation of stock solution:

Accurately weigh and transfer 25 mg of Tenofovir AF, 200 mg of Emtricitabine and 50 mg of BICTEGRAVIR working standard into a 100 ml clean dry volumetric flask add about 70 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.75 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure:

The standard solutions prepared in the precision was injected on the other day, for six times and measured the area for all six injections in UPLC. The %RSD for the area of six replicate injections was found to be within

the specified limits. Acceptance Criteria: The % RSD for the area of six standard injections results should not be more than 2%.

SPECIFICITY:
For Specificity Blank and Standard are injected into system. There is no any inteferece of any peak in blank with the retiontime of the analytical peaks.

ACCURACY:
Preparation of Standard stock solution:

Accurately weigh and transfer 25 mg of Tenofovir AF, 200 mg of Emtricitabine and 50 mg of BICTEGRAVIR working standard into a 100 ml clean dry volumetric flask add about 70 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution) Further pipette 0.75 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Preparation Sample solutions: For preparation of 50% solution (With respect to target Assay concentration):
Accurately weigh and transfer 12.5 mg of Tenofovir AF, 100 mg of Emtricitabine and 25 mg of BICTEGRAVIR working standard into a 100 ml clean dry volumetric flask add about 70 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette 0.75 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.
For preparation of 100% solution (With respect to target Assay concentration): Accurately weigh and transfer 25 mg of Tenofovir AF, 200 mg of Emtricitabine and 50 mg of BICTEGRAVIR working standard into a 100 ml clean dry volumetric flask add about 70 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.75 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

For preparation of 150% solution (With respect to target Assay concentration): Accurately weigh and transfer mg of 37.5mg Tenofovir AF, 300 mg of Emtricitabine and 75 mg of BICTEGRAVIR working standard into a 100 ml clean dry volumetric flask add about 70 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.75 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure: Inject the standard solution, Accuracy -50%, Accuracy -100% and Accuracy -150% solutions. Calculate the Amount found and Amount added for Tenofovir AF, Emtricitabine and BICTEGRAVIR and calculate the individual recovery and mean recovery values.

LINEARITY:
Preparation of stock solution:
Accurately weigh and transfer 25 mg of Tenofovir AF, 200 mg of Emtricitabine and 50 mg of BICTEGRAVIR working standard into a 100 ml clean dry volumetric flask add about 70 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Preparation of Level – I
0.25 ml of above stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent.
Preparation of Level – II
0.5 ml of above stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level – III
0.75 ml of above stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent.
Preparation of Level – IV

1.0 ml of above stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent

Preparation of Level – V

1.25ml of above stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent

Procedure:

Inject each level into the chromatographic system and measure the peak area.

Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.

DETECTION LIMIT

LIMIT OF DETECTION: (for Emtricitabine)

Preparation of 150 µg/ml solution:

Accurately weigh and transfer 200 mg of Emtricitabine working standard into a 100 ml clean dry volumetric flask add about 70 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.75 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Preparation of 0.50 µg/ml solution:

Further pipette 0.5ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. Further pipette 0.7 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Calculation of S/N Ratio:

Average Baseline Noise obtained from Blank : 66 µV

Signal Obtained from LOD solution : 197 µV

S/N = 197/66 = 2.98

Acceptance Criteria:

S/N Ratio value shall be 3 for LOD solution.

LIMIT OF QUANTIFICATION:

Preparation of 150µg/ml solution:

Accurately weigh and transfer 200mg of Emtricitabine working standard into a 100 ml clean dry volumetric flask add about 70 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.75 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent. Preparation of 1.66 µg/ml solution: Further pipette 1.0ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Further pipette 1.2ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Calculation of S/N Ratio:

Average Baseline Noise obtained from Blank : 66 µV

Signal Obtained from LOQ solution : 659µV

S/N = 659/66 = 9.98

Acceptance Criteria:

S/N Ratio value shall be 10 for LOQ solution.

LIMIT OF DETECTION: (for BICTEGRAVIR)

Preparation of 37.5 µg/ml solution:

Accurately weigh and transfer 50 mg of BICTEGRAVIR working standard into a 100 ml clean dry volumetric flask add about 70 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.75 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Preparation of 1.02 µg/ml solution:

Further pipette 1.0ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents. Further pipette 2.7 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Calculation of S/N Ratio:

Average Baseline Noise obtained from Blank : 66 µV
Signal Obtained from LOD solution : 198 µV
S/N = 198/66 = 3.00

Acceptance Criteria:

S/N Ratio value shall be 3 for LOD solution.

LIMIT OF QUANTIFICATION:

Preparation of 37.5 µg/ml solution:

Accurately weigh and transfer 50 mg of BICTEGRAVIR working standard into a 100 ml clean dry volumetric flask add about 70 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.75 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Preparation of 3.4 µg/ml solution:

Further pipette 2 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents. Further pipette 4.5 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Calculation of S/N Ratio:

Average Baseline Noise obtained from Blank : 66 µV
Signal Obtained from LOQ solution : 660µV
S/N = 660/66 = 10.00

Acceptance Criteria:

S/N Ratio value shall be 10 for LOQ solution.

LIMIT OF DETECTION: (for Tenofovir AF)

Preparation of 18.75 µg/ml solution:

Accurately weigh and transfer 25 mg of Tenofovir AF working standard into a 100 ml clean dry volumetric flask add about 70 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.75 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Preparation of 0.22 µg/ml solution:

Further pipette 1.0ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents. Further pipette 1.1ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Calculation of S/N Ratio:

Average Baseline Noise obtained from Blank : 66 µV
Signal Obtained from LOD solution : 194 µV
S/N = 194/66 = 2.94

Acceptance Criteria:

S/N Ratio value shall be 3 for LOD solution.

LIMIT OF QUANTIFICATION:

Preparation of 18.75 µg/ml solution:

Accurately weigh and transfer 25 mg of Tenofovir AF working standard into a 100 ml clean dry volumetric flask add about 70 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).Further pipette 0.75 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Preparation of 0.74 µg/ml solution:

Further pipette 1.3ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents. Further pipette 3.0 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Procedure for LOD and LOQ:

The LOD and LOQ solutions was prepared injected, for three times and measured the area for all three injections in HPLC. The %RSD for the area of three replicate injections was found to be within the specified limits.

Calculation of S/N Ratio:

Average Baseline Noise obtained from Blank : 66 µV
Signal Obtained from LOQ solution : 658µV
S/N = 658/66 = 9.97

Acceptance Criteria:

S/N Ratio value shall be 10 for LOQ solution.

ROBUSTNESS:

As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method.

A. The flow rate was varied at 0.27 ml/min to 0.33 ml/min. Standard solution 150 ppm of Emtricitabine, 37.5 ppm of BICTEGRAVIR & 18.75 ppm of Tenofovir AF was prepared and analysed using the varied flow rates along with method flow rate.On evaluation of the above results, it can be concluded that the variation in flow rate affected the method significantly. Hence it indicates that the method is robust even by change in the flow rate ±10%.

B. The variation of ±10% mobile phase:

Standard solution of 18.75 ppm of Tenofovir AF, 150 ppm of Emtricitabine & 37.5 ppm of BICTEGRAVIR was prepared and analysed using the varied in ±10% mobile phase.

DEGRADATION STUDIES:

The International Conference on Harmonization (ICH) guideline entitled stability testing of new drug substances and products requires that stress testing be carried out to elucidate the inherent stability characteristics of the active substance. The aim of this work was to perform the stress degradation studies on the Tenofovir AFe, Emtricitabine and BICTEGRAVIR using the proposed method.

Preparation of stock:

Accurately weigh and transfer 25 mg of Tenofovir AF, 200 mg of Emtricitabine and 50 mg of BICTEGRAVIR working standard into a 100 ml clean dry volumetric flask add about 70 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Hydrolytic degradation under acidic condition

Pipette 0.75 ml of above solution into a 10ml volumetric flask and 3 ml of 0.1N HCl was added. Then, the volumetric flask was kept at 60°C for 6 hours and then neutralized with 0.1 N NaOH and make up to 10ml with diluent. Filter the solution with 0.22 microns syringe filters and place in vials.

Hydrolytic degradation under alkaline condition

Pipette 0.75 ml of above solution into a 10ml volumetric flask into a 10ml volumetric flask and add 3ml of 0.1N NaOH was added in 10ml of volumetric flask. Then, the volumetric flask was kept at 60°C for 6 hours and then neutralized with 0.1N HCl and make up to 10ml with diluent. Filter the solution with 0.22 microns syringe filters and place in vials.

Thermal induced degradation

Tenofovir AFe, Emtricitabine and BICTEGRAVIR sample was taken in petridish and kept in Hot air oven at 1100 C for 24 hours. Then the sample was taken and diluted with diluents and injected into HPLC and analysed.

Oxidative degradation

Pipette 0.75 ml above stock solution 2 into a 10ml volumetric flask solution into a 10ml volumetric flask 1 ml of 3% w/v of hydrogen peroxide added in 10 ml of volumetric flask and the volume was made up to the mark

with diluent. The volumetric flask was then kept at room temperature for 15 min. Filter the solution with 0.45 microns syringe filters and place in vials.

Results:

Emtricitabine	Area	% Degraded
Standard	956581	3.81
Acid	920116	3.02
Base	927681	3.61
Peroxide	922023	3.64
Thermal	921805	3.63
Photo	921845	3.81

BICTEGRAVIR	Area	% Degraded
Standard	154741	8.87
Acid	141008	7.88
Base	142549	6.58
Peroxide	144556	5.92
Thermal	145584	5.85
Photo	145686	8.87

Tenofovir AF	Area	% Degraded
Standard	89833	4.53
Acid	85764	3.92
Base	86313	3.03
Peroxide	87109	3.47
Thermal	86720	3.55
Photo	86646	4.53

RESULTS

System Suitability Results:

1. Tailing factor Obtained from the standard injection is 1.45
2. Theoretical Plates Obtained from the standard injection is 2112

The accuracy results for Emtricitabine

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	476290	100	9.38	99.38	99.98
100%	957024	200	199.69	99.85	
150%	1448027	300	302.15	100.72	

The accuracy results for BICTEGRAVIR

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	77719	25	25.06	100.25	99.78
100%	154381	50	49.78	99.57	
150%	231466	75	74.64	99.52	

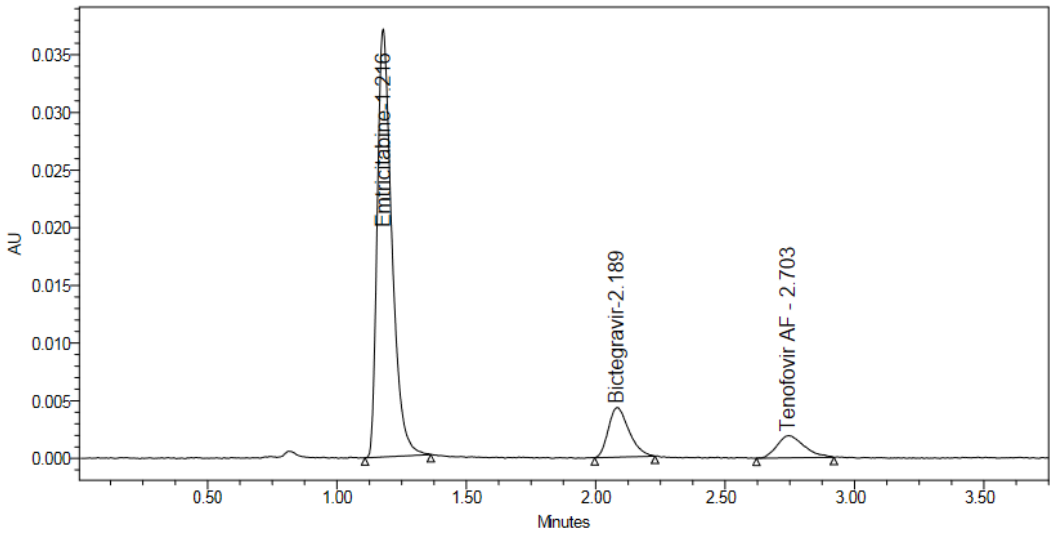
The accuracy results for Tenofovir AF

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	45290	12.5	12.58	100.63	99.99
100%	89720	25	24.92	99.67	
150%	134564	37.5	37.37	99.66	

Acceptance Criteria:
The % Recovery for each level should be between 98.0 to 102.0%

Linearity Results: (for Emtricitabine)

S. No	Linearity Level	Concentration	Area
1	I	50	522088
2	II	100	734633
3	III	150	950658
4	IV	200	1192066
5	V	250	1430452
Correlation Coefficient			0.999



Linearity Results: (for BICTEGRAVIR)

S. No	Linearity Level	Concentration	Area
1	I	12.5	65477
2	II	25	110790
3	III	37.5	153097
4	IV	50	193120
5	V	62.5	239955
Correlation Coefficient			0.999

Linearity Results: (for Tenofovir AF)

S. No	Linearity Level	Concentration	Area
1	I	6.25	47257
2	II	12.5	67723
3	III	18.75	89884
4	IV	25	109712
5	V	31.25	134068
Correlation Coefficient			0.999

Acceptance Criteria: Correlation coefficient should be not less than 0.99.

System suitability results for Emtricitabine:

S. No	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1	0.27	2263.65	1.40
2	0.3	2112	1.45
3	0.33	2151.29	1.44

System suitability results for BICTEGRAVIR:

S. No	Flow Rate (ml/min)	System Suitability Results		
		USP Plate Count	USP Tailing	USP Resolution
1	0.27	3331.30	1.29	7.38
2	0.3	3186.09	1.33	7.31
3	0.33	2971.64	1.41	7.11

System suitability results for Tenofovir AF:

S. No	Flow Rate (ml/min)	System Suitability Results		
		USP Plate Count	USP Tailing	USP Resolution
1	0.27	3035.38	1.40	3.87
2	0.3	3353.63	1.27	3.90
3	0.33	3465.98	1.41	3.89

* Results for actual flow (0.3 ml/min) have been considered from Assay standard.

System suitability results for Emtricitabine:

S. No	Change in Organic Composition in the Mobile Phase	System Suitability Results	
		USP Plate Count	USP Tailing
1	10% less	2445.83	1.40
2	*Actual	2112	1.45
3	10% more	2104.64	1.39

System suitability results for BICTEGRAVIR:

S. No	Change in Organic Composition in the Mobile Phase	System Suitability Results		
		USP Plate Count	USP Tailing	USP Resolution
1	10% less	3594.68	1.29	9.15
2	*Actual	3186.09	1.33	7.31
3	10% more	2935.13	1.48	6.02

System suitability results for Tenofovir AF:

S. No	Change in Organic Composition in the Mobile Phase	System Suitability Results		
		USP Plate Count	USP Tailing	USP Resolution
1	10% less	5094.60	1.32	4.68
2	*Actual	3353.63	1.27	3.90
3	10% more	3252.62	1.37	3.61

* Results for actual Mobile phase composition (45:55 Buffer : Acetonitrile has been considered from Accuracy standard.

CONCLUSION

The present developed RP-UPLC method was found to be simple, rapid, accurate and specific for the determination of Tenofovir AF, Emtricitabine and Bictegravir. The current method was validated according to ICH guidelines in terms of Linearity, Accuracy, Precision, Limit of detection, Limit of quantification, Robustness and Stability indicating capability. We have conducted the stability studies by exposing the drug product to different stress conditions like acid, alkali, peroxide, thermal, photolytic, hydrolytic conditions and observed the degradation of drug product in acidic medium, basic medium and in oxidation medium. Based on the chromatograms of the degradation studies, we have detected that the peak of the degradation products was not interfering with the peaks of drugs products and so we conclude that the current developed Reverse Phase Ultra Performance Liquid Chromatographic method is specific with respect to the pharmaceutical drug product containing Emtricitabine, Bictegravir and Tenofovir alafenamide. Hence the current developed method can be fruitfully applied for the estimation of Emtricitabine, Bictegravir and Tenofovir alafenamide in drug testing laboratories, pharmaceutical industries and also for quality control in any quality control and testing laboratory.

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