RESEARCH ARTICLE

Advanced, Cutting-Edge RP-HPLC Methodology for the Comprehensive Quantitative Analysis of Assay of Bictegravir, Emtricitabine, and Tenofovir Alafenamide in Co-Formulated Pharmaceutical Products

Nitin Mehetre*, Inderbir Singh

Chitkara College of Pharmacy, Chitkara University, Rajpura, Punjab, India.

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ABSTRACT

In the current study, a simple and economic stability-indicating RP-HPLC method development and validation was carried out to estimate the bictegravir, emtricitabine and tenofovir from the pharmaceutical dosage form. 0.1M ammonium acetate in 0.5% v/v acetic acid solution and 1g of 1-octane sulfonic acid and adjustment of pH to 4.2 with dilute orthophosphoric acid done and methanol (40:60 v/v) was utilized as the mobile phase. The analysis was done using Inertsil ODS 3V (250 x 4.6 mm x 5 µm) column with column temperature kept at 30°C with an injection volume of 20 µL, flow rate of 1.0 ml/minute and detection was carried out at 260 nm. The method was developed and it was validated according to ICH Q2 (R1) guidelines. The RT of bictegravir, emtricitabine and tenofovir were determined to be 12.23, 3.0 and 8.5 minutes, providing a reliable marker for its identification. The method was found linear from about 50 To 150% Of the target concentration of bictegravir emtricitabine and tenofovir with of 0.999. Significant degradation was observed in acidic, basic and peroxide stress conditions. Hence, it can be concluded that tablets are sensitive to acidic, basic and oxidation stress conditions. RSD for the peak area responses of emtricitabine, 0.03 and 0.03, respectively, indicating that the system is precise. The testing procedure was accurate from about 50 to 150%. Of the target concentration of emtricitabine, tenofovir alafenamide and bictegravir. The method was robust In relation to flow variation, column oven temperature variation, mobile phase variation and pH variation. In conclusion, our proposed RP-HPLC method provides a sensitive, accurate, and precise means of analyzing emtricitabine, tenofovir alafenamide and bictegravir from pharmaceutical dosage forms.

Keywords: Emtricitabine, Tenofovir Alafenamide, Bictegravir, Assay, HPLC, Method development, Validation.

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INTRODUCTION

Antiviral drugs are most significantly used medical treatment for HIV-1 infection it reduce HIV-1 replication to extreme levels.[1,2] Bictegravir, emtricitabine and tenofovir are used as antiretroviral (HIV).[3] At the active site, integrase was bound and bictegravir inhibits HIV integrase and high potency and selectivity in antiviral tests are also demonstrated by inhibiting the strand transfer stage of retroviral deoxyribonucleic acid (DNA) integration, which is crucial for the HIV replication cycle.[4] Emtricitabine functions by preventing the enzyme reverse transcriptase from copying HIV RNA into viral DNA. The synthetic nucleoside analog of cytidine is emtricitabine. Emtricitabine 5'-triphosphate, which is in charge of inhibiting HIV-1 reverse transcriptase, is created when it is phosphorylated by biological enzymes.[5]

Bictegravir is an off–white or white to light yellow color powder, is chemical name is 4-amino-5 fluoro-1-(2R-hydroxymethyl-1,3 oxathiolan -5S-yl)-(1H) – pyrimidin -2- one, molecular formula $C_{20}H_{22}N_4O_{10}$ S molecular weight 471.40. Structure is given in Figure 1.[6,7] Emtricitabine is off white powder and the chemical name is (-) Cis-4 –amino-5-fluoro-1-(2-hydroxymethyl-1,3-oxathiolan-5-yl)-(1H) pyrimidin-2-one, molecular formula is C8H10FN3O3S, molecular weight is 247.25 and melting point range between 136–1400C. Its solubility in water and methanol is freely soluble and in methylene chloride, its insoluble. Its structure is given below.[8] Tenofovir alafenamide is whitish powder. Its chemical name is Isopropyl [N-{(S)-({{(2R)1-(amino-9H-purin-9-yl}-2-propanyl]oxy} methyl)(phenoxy) phosphoryl]-L-alafnate (2E) -2-butenedioate (2:1)and chemical formula is C21H29N6O5P, molecular weight is 1068.39. it

*Author for Correspondence: nitinmehetre03@gmail.com

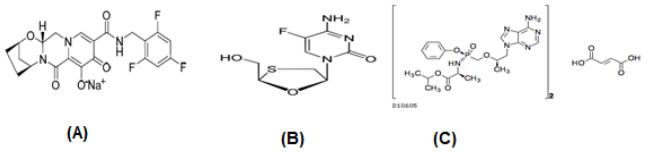


Figure 1: Structures of (A) Bictegravir sodium, (B) Emtricitabine and (C) Tenofovir alafenamide

is Soluble in dimethyl form amide and slightly soluble in methanol. The structure of it is given below in Figure 1.[9,10]

MATERIAL AND METHODS

Chemicals and Reagents

Working standards of emtricitabine, tenofovir alafenamide and bictegravir were received as gift samples from Hetero Lab. Ltd., Other reagents, including ammonium acetate, acetic acid solution, 1-Octane sulfonic acid, orthophosphoric acid and methanol (40:60 v/v) were Analytical grade.

Method Development for HPLC

Chromatographic conditions and instrument

In order to carry out chromatographic analysis, an RP-HPLC equipment with a UV detector was utilized. The Inert sil ODS 3V stationary phase is used (250 x 4.6 mm x 5 µm), (Part Number: 5020-01802), rate of flow was 1.0 ml/minute. A 30ºC temperature of the column and a 260 nm wavelength were chosen. The final concentration was selected as 60 ppm for bictegravir, 31 ppm for tenofovir alafenamide and 250 ppm for emtricitabine and an injection volume of 20 µL was selected. The pH meter, analytical balances, and ultra sonicator, among other instruments utilized in the validation, were calibrated.

Buffer solution preparation

Ammonium acetate (7.7 g) was taken and added into a beaker containing 1L of water, then about 5 mL of acetic acid was added and mixed to dissolve. Into this solution, 1 g of 1-octane sulfonic acid was added and pH to 4.2 ± 0.05 was adjusted by adding dilute orthophosphoric acid.

Mobile phase preparation

Established a 40:60% v/v degassed combination of the buffer and methanol.

Preparation of standard stock solution-1

About 66 mg of bictegravir sodium and 35 mg of tenofovir alafenamide hemifumarate working standards were transferred into flask of capacity 100 and 70 mL of diluent was added and sonication done for proper dissolving mixture. Use diluent to dilute to volume, then stir.

Standard stock solution-2 preparation

Emtricitabine working standard (50 mg) was added in a flask with 100 mL capacity and, solvent (70 mL) was added and sonication was done for proper dissolving of the mixture. With the help of a diluent, volume is made and mixed it properly.

Standard solution Preparation

Standard stock solution-1 and 2 (2 mL) were added into a flask of capacity 20 mL and dilution done with the help of diluent and mixed it properly.

Sample solution preparation

Tablet powder equivalent to about 50 mg of emtricitabine was taken into flask with a capacity 200 mL. After adding approximately 140 mL of diluent, sonication was done for at least 10 minutes, stirring it occasionally. Dilution was done and the desired volume was made and mixed properly. After passing the solution through a 0.45 µm membrane filter, the first few milliliters of the filtrate were discarded.

Procedure

Separately 20 µL of diluent as a blank, standard solution (5 injections), then the above-prepared solution was injected with chromatographic assembly. The graphs in chromatography were recorded and measured responses of a peak.

Method Validation[11,12]

This study's validation approach was verified for a number of criteria like specificity, force degradation study, precision, linearity, limit of detection LoD, LoQ, accuracy, robustness, stability in analytical solution (SIAS).

Forced Degradation

Bictegravir, emtricitabine and tenofovir alafenamide drug substances, placebo and bictegravir, emtricitabine and tenofovir alafenamide tablets 50/200/25 mg was stressed with a condition which includes acid, base, peroxide, thermal, photolytic and humidity degradation.

Linearity

Five linearity solutions were prepared by using bectegravir sodium,emtricitabine and tenofovir alafenamide hemifumarate working standard at levels of concentration ranging in between 50 to 150% of target standard concentration of bectegravir, emtricitabine and tenofovir alafenamide. Determined the area of peak response of solution at the lower level and higher level 6 times and another levels 2 times.

Specificity and system suitability

Standard solution was prepared, individual identification solution and sample solution according to the test method and in chromatographic assembly, samples were injected. Made blank and placebo solutions according to test method and in chromatographic assembly, samples were injected. The %RSD of retention time of bectegravir, emtricitabine and tenofovir alafenamide got from the ten replicate injections of the standard solution should not exceed 1.0 g.

Precision

Method precision

Prepared six sample solutions of bictegravir, emtricitabine and tenofovir alafenamide tablets 50/200/25 as per the test method and introduced into the system of chromatography.

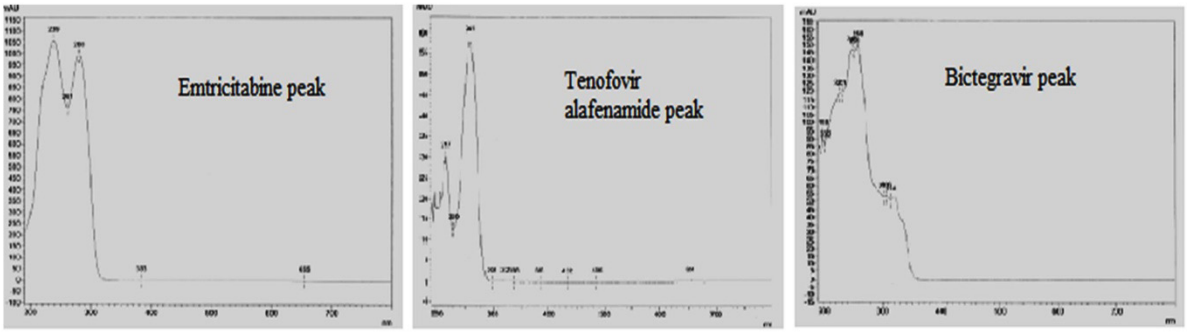


Figure 2: Chromatogram showing well-resolved peaks of Emtricitabine, Tenofovir and Bictegravir

System precision

Preparation of the standard solution was done according to test methods and in chromatographic assembly, injected samples 5 times into the system.

Intermediate precision

Intermediate precision was performed on bictegravir, emtricitabine and tenofovir alafenamide tablets 50/200/25 mg with another analyst on another day with use of various columns and various instruments. The percentage RSD for area of peak for response of bectagravir, emtricitabine and tenofovir alafenamide peaks from five replicate injections of standard solution should not exceed 2.0.

Accuracy

Accuracy was performed by spiking the bictegravir, emtricitabine and tenofovir alafenamide drug substance to the placebo at every step and analyzed according to test method. %Recovery should be not less than 98.0 and not more than 102.0.

Range

Data on linearity, precision, and accuracy can be used to generate a variety of analytical techniques. Describe the sample concentration range in percentage terms.

Stability of solution

In accordance with the test methods, the standard solution and sample solution were created and stored at Room temperature (25 + 2°C). Solution Stability was evaluated at initial, 12 hours, 24 hours and 48 hours. For the sample, the %Assay difference from primary and corresponding time intervals should not exceed 2.0.

Robustness

The standard solution was prepared in accordance with the test protocol and added it to the chromatographic apparatus under various varied circumstances.

RESULTS AND DISCUSSION

HPLC Method Development

Based on the method followed in the HPLC system by using PDA detector, maximum response was obtained at 260 nm for emtricitabine, tenofovir and bictegravir peaks. Hence, the wavelength selected at 260 nm (Figure 2). The desired peak shape and separation between emtricitabine, tenofovir and bictegravir peaks were obtained at 30ºC hence, column temperature was selected as at 30ºC and Flow rate was selected as 1.0 mL/minute.

Forced Degradation

Significant degradation was observed in acidic, basic and peroxide stress condition, hence it can be concluded that bictegravir, emtricitabine and tenofovir alafenamide tablets is sensitive to acidic, basic and oxidation stress condition, data represented in Table 1.

Linearity

The response of emtricitabine, tenofovir alafenamide and bictegravir was found linear from about 50 to 150% of the target concentration. From the results of Figure 3 and results shown in Table 2 it was concluded that residuals were within ± 2% of the 100% concentration response.

The observed theoretical plates for the emtricitabine, tenofovir and bictegravir peak from the reference solution exceed 2000. the tailing factor for the drugs from the standard solution is below 2.0. The response percentage for the peak areas of emtricitabine, tenofovir and bictegravir peak from 5 identical standard solution injections was found to be 0.25, 0.17 and 0.19. From the standard and sample chromatograms (Figure 4) it was determined that the retention times of bictegravir, emtricitabine and tenofovir alafenamide peak in standard and sample was comparable (Table 3). From the blank and placebo chromatograms, no peaks were observed at the time of retention of bictegravir, emtricitabine and tenofovir alafenamide. Therefore, it implies that the method is specific.

Method Precision

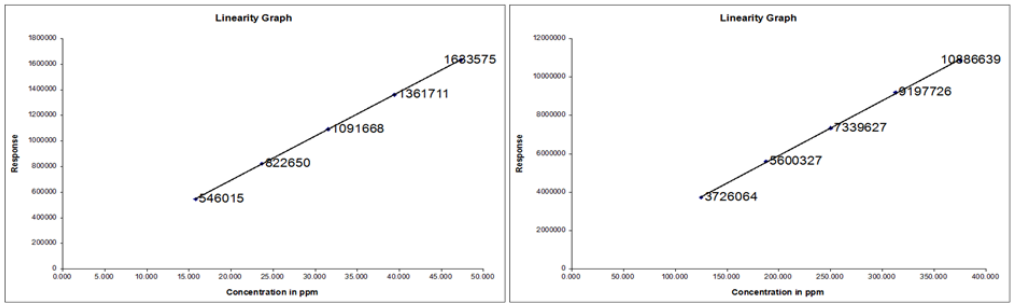
The theoretical plates for bictegravir, emtricitabine and tenofovir alafenamide peaks from a minimum of 2000 injections of the standard solution should be made in five replicates. The tailing factor for the five replicate injections of the standard solution of bictegravir, emtricitabine, and tenofovir alafenamide should not exceed 2.0. Outcomes are represented in Table 4. The test result demonstrates the precision of the system.

Table 1: Results for forced degradation for emtricitabine, tenofovir alafenamide and bictegravir

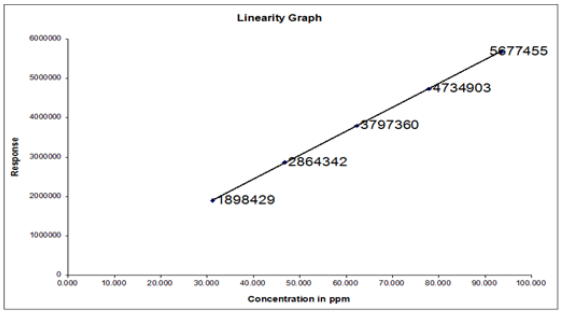
Degradation mechanism / condition	*%Assay*			*%Degradation*			*Single point threshold*		
Liquid State	*EMT*	*TAF*	*BEC*	*EMT*	*TAF*	*BEC*	*EMT*	*TAF*	*BEC*
Undegraded sample	98.7	99.6	99.5	---	---	---	0.999998	0.999998	0.999998
Acid/0.1N HCl, Bench Top For 5 minutes.	98.4	98.9	99.9	0.3	0.70	ND	0.999998	0.999998	0.999998
Base/0.01N NaOH, Bench Top For 5 minutes.	92.2	94.1	95.3	6.6	5.5	4.2	0.999998	0.999998	0.999998
Peroxide/1.0% H_2O_2, Bench top for 30 minutes.	95.8	98.0	99.0	3.0	1.60	0.5	0.999998	0.999998	0.999998
Solid state									
Thermal at 105ºC- 48 Hours	98.9	99.3	99.3	--	0.3	0.2	0.999998	0.999998	0.999998
Photolytic exposure	98.6	99.6	98.8	0.1	0.0	0.7	0.999995	0.999995	0.999995
Humidity at 90% RH-168 Hours	98.7	99.3	99.3	--	0.3	0.2	0.999998	0.999998	0.999998

Table 2: Results for linearity

Linearity level	*Concentration (ppm) as*			*Mean peak area*			*%RSD*		
Drug	*EMT*	*TAF*	*BEC*	*EMT*	*TAF*	*BEC*	*EMT*	*TEF*	*BEC*
Level-1	125.00	15.765	31.150	3726064	546015	1898429	0.02	0.05	0.03
Level-2	187.50	23.648	46.725	5600327	822650	2864342	---	---	---
Level-3	250.00	31.530	62.300	7339627	109166	3797360	---	---	---
Level-4	312.500	39.413	77.875	9197726	1361711	4734903	---	---	---
Level-5	375.000	47.295	93.450	10886639	1633575	5677455	0.01	0.03	0.01



Emtricitabine **Tenofovir**



Bictegravir

Figure 3: Linearity plots for emtricitabine, tenofovir and bictegravir specificity and system suitability

Table 3: Results for specificity and system suitability

S. No.	*Parameters*	*Emtricitabine*	*Tenofovir alafenamide*	*Bectegravir*
1	Retention time	3.051	8.545	12.251
2	Peak area	7304213	1028844	3598566
3	Theoretical plate	4151	7657	9361
4	Tailing factor	1.26	1.04	1.03

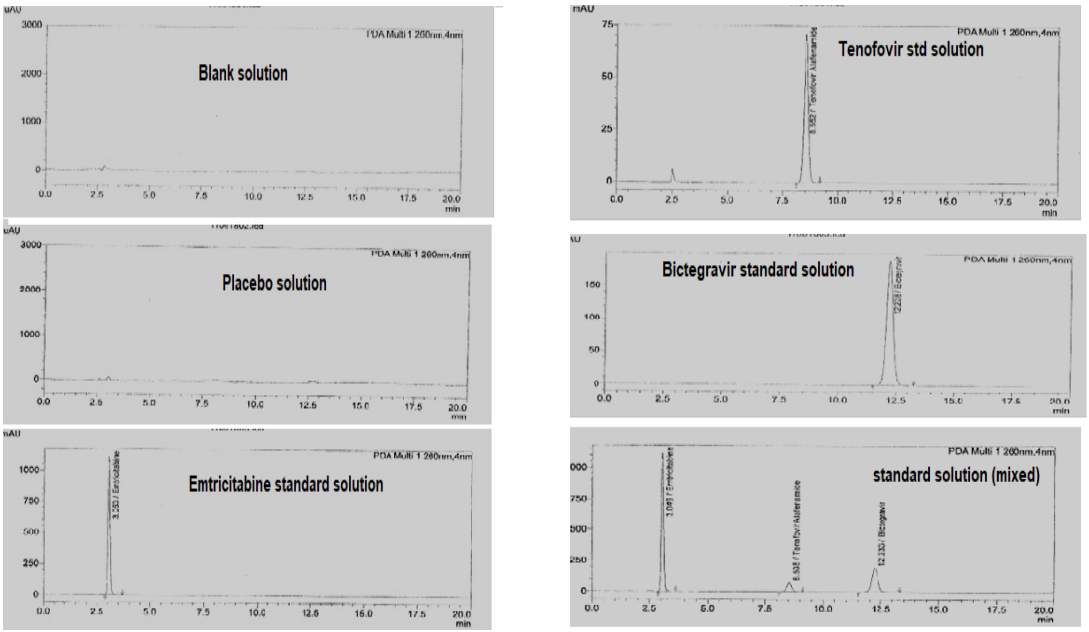


Figure 4: Chromatogram of blank, placebo solution, standard solution and emtricitabine, tenofovir alafenamide and bictegravir, precision

Intermediate Precision

The observed theoretical plates for the emtricitabine peak, tenofovir alafenamideand bectagravir from standard solution is above 2000. The observed tailing factor for emtricitabine peak, tenofovir alafenamide and bectagravir from standard solution is less than 2.0. Table 5 shows the percentage RSD for the peak area responses of the emtricitabine peak from five replicate injections of the reference solution. The test result demonstrates the precision of the system.

Accuracy

Results for accuracy at spike level 50, 100 and 100% are represented in Table 6, from the results. The test procedure was found to be accurate from about 50 to 150% of the intended target of emtricitabine, tenofovir alafenamide and bectagravir.

Range

It was concluded from the linearity, precision and accuracy data that this analytical method is linear, precise and accurate from about 50 to 150% of the target concentration of bictegravir, emtricitabine and tenofovir alafenamide.

Stability of Solution

For standard, the overall %RSD from the initial replicate standard peak area and the maximum bracketing standard peak area that is acceptable is 2.0. For the sample, the %Assay difference from initial and corresponding time intervals should be not more than 2.0. It was determined from the aforementioned data that the sample and reference solutions were stable for about 48 hours at 25 +2°C and It was determined from the aforementioned data that the mobile phase is stable for 48 hours. Table 7 represents result for the stability of the solution.

Table 4: Observations for method precision

Parameter	*Emtricitabine*	*Tenofovir alafenamide*	*Bectagravir*
Peak area	7296343	1072751	3760540
Theoretical plates	4061	6970	8182
Tailing factor	1.30	1.06	1.05
%RSD	0.05	0.03	0.03

Table 5: Results for intermediate precision

Parameter	*Emtricitabine*	*Tenofovir alafenamide*	*Bectagravir*
Peak area	7510185	1093941	3876756
Theoretical plates	4157	7175	8358
Tailing factor	1.32	1.10	1.10
%RSD	0.01	0.04	0.03

Table 6: Results for accuracy

Parameter	*Emtricitabine*			*Tenofovir alafenamide*			*Bectagravir*		
Spike level (%)	50	100	150	50	100	150	50	100	150
%Recovery	100.6	100.0	98.8	100.1	0.06	0.06	99.0	99.1	98.8
SD	0.29	0.06	0.06	100.2	0.15	0.15	0.25	0.12	0.15
%RSD	0.29	0.06	0.06	100.3	0.06	0.06	0.25	0.12	0.15

Table 7: Results for stability of solution

Standard solution						
Time (hours)	*%RSD*					
	Emtricitabine	*Tenofovir alafenamide*		*Bictegravir*		
Initial	0.07	0.06		0.08		
12	0.12	0.07		0.16		
24	0.17	0.12		0.29		
48	0.31	0.12		0.46		
Sample solution						
Time (hours)	*%Assay*			*Difference*		
	EMT	*TAF*	*BEC*	*EMT*	*TAF*	*BEC*
Initial	99.6	99.7	100.0	***	***	***
12	100.0	99.9	100.4	0.4	0.2	0.4
24	100.7	100.5	101.3	1.1	0.8	1.3
48	101.2	101.3	101.5	1.6	1.6	1.5

Table 8: Results for robustness

Parameter	*%RSD Emtricitabine*		*%RSD Tenofovir*		*%RSD Bictegravir*	
Flow variation 1.10 and 0.90 mL/min	0.04	0.02	0.10	0.10	0.06	0.03
Temperature 25 and 35°C	0.04	0.04	0.02	0.05	0.01	0.05
Mobile phase variation 380:620 and 420:580	0.02	0.02	0.03	0.04	0.03	0.07
pH variation 4.0 and 4.2	0.04	0.03	0.03	0.07	0.05	0.36

Robustness

Table 8 data led to the conclusion that the approach was robust to variations in flow, column oven temperature variation, mobile phase variation and pH variation.

CONCLUSION

This study underscores the rapidity, accuracy, and sensitivity of the developed analytical protocol. The method demonstrated a robust capability to effectively separate the drugs from their degradation products. Consequently, it is particularly well-suited for the analysis of stability samples and a diverse range of pharmaceutical formulations.

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