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Development and validation of a new stability indicating RP-UPLC method for the simultaneous estimation of Tenofovir Alafenamide, Emtricitabine and Bictegravir in presence of internal standard (Acyclovir)

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ABSTRACT

A new stability indicating RP-UPLC method has been proposed for the simultaneous estimation of Tenofovir Alafenamide, Emtricitabine and Bictegravir in presence of an internal standard, Acyclovir using Waters ACQUITY UPLC system with PDA detector and Hibar C18 (100 x 2.1 mm, 1.8µ) column. Mobile phase consisting of 0.01M Ammonium acetate and Acetonitrile (70:30, v/v) was used with flow rate 0.3 ml/min (Detection wavelength: 260 nm) (Injection volume: 1.0 µL) (Column temperature: 30°C) with run time 6 mins. The method was linear over the concentration range 2.5-15 µg/ml, 20-120 µg/ml and 5-30 µg/ml for Tenofovir Alafenamide, Emtricitabine and Bictegravir respectively. Forced degradation studies were performed and the method was validated as per ICH guidelines.

INTRODUCTION

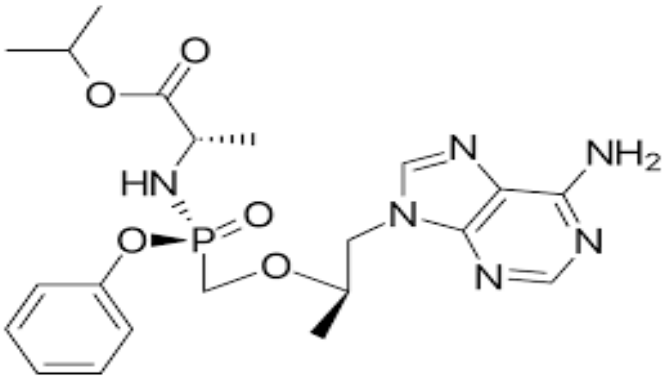
Tenofovir Alafenamide (CAS: 379270-37-8) is an antiviral prescription medication primarily used to treat chronic hepatitis B virus infection. It is chemically, propan-2-yl(2*S*)-2-[[[(2*R*)-1-(6-

aminopurin-9-yl) propan-2-yl] oxy methyl-phenoxy phosphoryl] amino] propanoate. The molecular weight of Tenofovir Alafenamide is 476.474 gm/mole with molecular formula $C_{21}H_{29}N_6O_5P$. It is a nucleotide reverse transcriptase inhibitor and is a prodrug of Tenofovir[1-2].

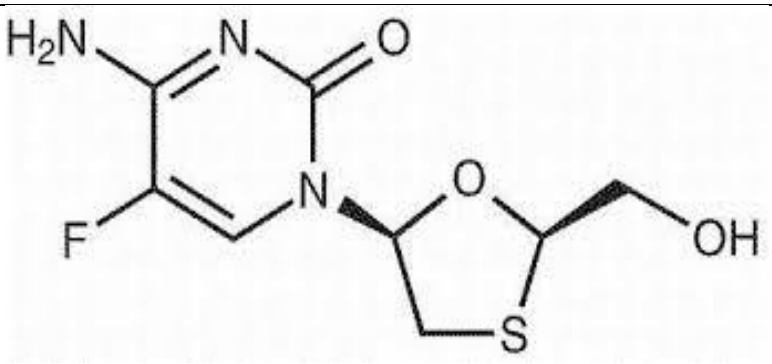
Emtricitabine (CAS: 143491-57-0) is a nucleoside reverse transcriptase inhibitor[3] indicated for the treatment of HIV infection in adults. It is chemically, 4-amino-5-fluoro-1-((2R, 5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl) pyrimidin-2(1H)-one. The molecular weight of Emtricitabine is 247.24 gm/mole with molecular formula $C_8H_{10}FN_3O_3S$.

Bictegravir (CAS: 1611493-60-7) is a human immunodeficiency virus integrase strand transfer inhibitor[4]. It is a second-generation integrase strand transfer inhibitor. It is chemically, (2R,5S,13aR)-2,3,4,5,7,9,13,13a-octahydro-8-hydroxy-7,9-dioxo-N-[(2,4,6-tri fluoro phenyl) methyl]-2,5-methano pyrido [1',2':4,5] pyrazino [2,1-b][1,3] oxazepine-10-carboxamide. The molecular weight of Bictegravir is 449.4 g/mol with molecular formula $C_{21}H_{18}F_3N_3O_5$.

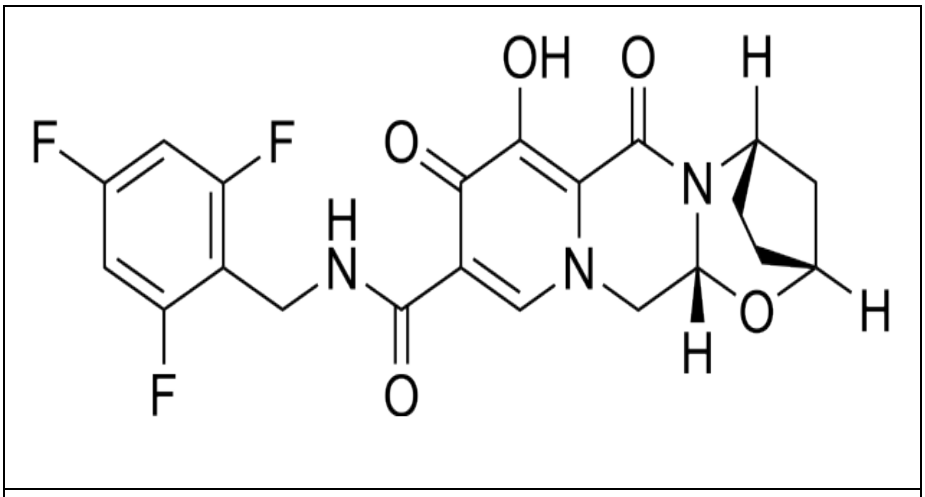
The combination[5] (Figure 1) of Tenofovir Alafenamide, Emtricitabine and Bictegravir is used to treat HIV.



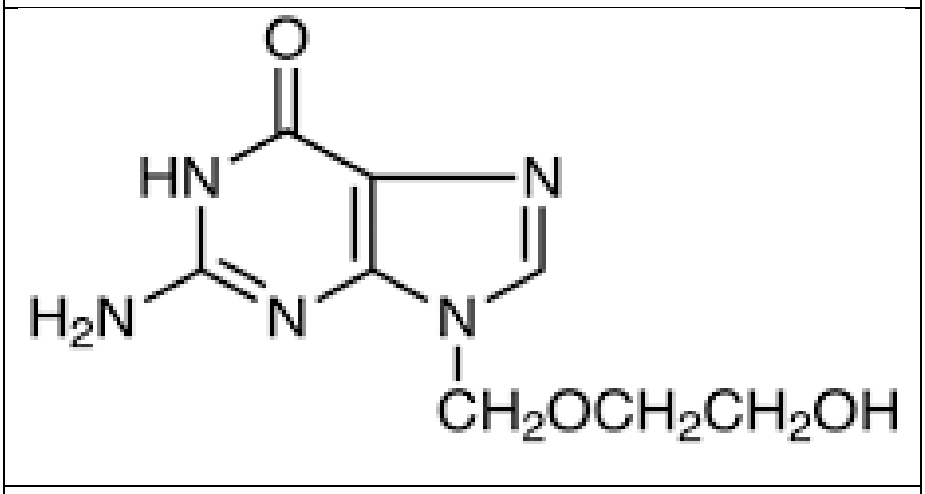
Tenofovir Alafenamide (TF)



Emtricitabine (EC)



Bictegravir (BG)



Acyclovir (Internal standard) (IS)

Figure 1: Chemical structures of Tenofovir Alafenamide, Emtricitabine, Bictegravir and Acyclovir (Internal standard)

Tanuja et al., have developed a stability-indicating RP-HPLC method[6] for the simultaneous estimation of Bictegravir, Emtricitabine and Tenofovir alafenamide fumarate using Inertsil octyl decylsilyl C18 column using a mixture of 0.2% Triethylamine buffer and Methanol (40: 60, v/v) as mobile phase with flow rate 1.2 mL/min (Detection wavelength: 260 nm). The linearity was found to be 25-125 µg/mL, 100-500 µg/mL and 12.5-62.5 µg/mL for Bictegravir, Emtricitabine and Tenofovir AF respectively and the retention times for Bictegravir, Emtricitabine and Tenofovir AF were found to be 5.998 min, 2.805 min and 4.537 min respectively.

Vamsi and Sowjanya have developed a RP-UPLC method[7] for the simultaneous determination of Emtricitabine, Bictegravir and Tenofovir alafenamide in pharmaceutical dosage form using HSS

C18 column with mobile phase consisting of 0.1% ortho phosphoric acid (pH 2.2) and Acetonitrile (70:30, v/v) with flow rate 0.3 ml/min (Detection wavelength 260 nm) (Column temp. 30°C) and the linearity was followed over the concentration range 50-300 µg/ml for Emtricitabine, 12.5-75 µg/ml for Bictegravir and 6.25-37.5 µg/ml for Tenofovir alafenamide respectively and the retention times for Bictegravir, Emtricitabine and Tenofovir AF were found to be 0.89 min, 0.62 min and 1.75 min respectively.

Nitin Mehetre and Inderbir Singh have developed a RP-HPLC method[8] for the simultaneous determination of Emtricitabine, Bictegravir and Tenofovir alafenamide in pharmaceutical dosage form using Inertsil ODS 3V column with mobile phase consisting of 0.1M ammonium acetate in 0.5% v/v acetic acid solution and 1g of 1-octane sulfonic acid (pH adjustment to 4.2 with dilute orthophosphoric acid) and methanol (40:60, v/v) with flow rate 1.0 ml/min (Detection wavelength 260 nm) (Column temp. 30°C) and the linearity was followed over the concentration range 125-375 µg/ml for Emtricitabine, 31.150-93.450 µg/ml for Bictegravir and 15.765-47.295 µg/ml for Tenofovir alafenamide and the retention times for Bictegravir, Emtricitabine and Tenofovir were found to be 12.23 min, 3.0 min and 8.5 min respectively.

Tej Kumar and Suryakala have developed a RP-HPLC method[9] for the simultaneous determination of Emtricitabine, Bictegravir and Tenofovir alafenamide in bulk and pharmaceutical dosage form using Denali C18 column with mobile phase consisting of 0.1% ortho phosphoric acid buffer (pH 2.2) and Acetonitrile (50:50, v/v) with flow rate 1.0 ml/min (Detection wavelength 272 nm) (Column temp. 30°C) and the linearity was followed over the concentration range 50-300 µg/ml for Emtricitabine, 12.5-75 µg/ml for Bictegravir and 6.25-37.5 µg/ml for Tenofovir alafenamide and the retention times for Emtricitabine, Bictegravir and Tenofovir alafenamide were found to be 2.303, 3.219 and 3.754 min respectively.

Raja Reddy have developed a RP-HPLC method[10] for the simultaneous determination of Emtricitabine, Bictegravir and Tenofovir alafenamide in pharmaceutical dosage form using Kromasil C18 column with mobile phase consisting of 0.1% OPA and acetonitrile (55:45, v/v) with flow rate 1.0 ml/min (Detection wavelength 272 nm) (Column temp. 30°C) and the linearity was followed over the concentration range 25-150 µg/ml for Emtricitabine, 6.25-37.5 µg/ml for Bictegravir and 3.125-18.75 µg/ml for Tenofovir and the retention times for Tenofovir, Bictegravir and Emtricitabine were 2.140 min, 2.432 min and 2.992 min respectively.

Gizem et al. have developed a novel HPLC and spectrophotometric methods[11] for the simultaneous estimation of Bictegravir, Emtricitabine and Tenofovir alafenamide fumarate. For liquid chromatographic method, XBridge C18 column and a mixture of Acetonitrile and Phosphate buffer at pH 6.8 (30:70, v/v) was used as mobile phase with flow rate 0.5 mL/min (Detection wavelength: 260 nm) and the linearity was followed over the concentration range 10-60 µg/ml for Emtricitabine, 2.5-15 µg/ml for Bictegravir and 1.24-7.4 µg/ml for Tenofovir and the retention

times for Tenofovir, Bictegravir and Emtricitabine were 1.804 min, 4.604 min and 0.581 min respectively.

In the present study the authors have proposed a new stability indicating RP-UPLC method for the simultaneous estimation of Tenofovir Alafenamide, Emtricitabine and Bictegravir in tablets in presence of an internal standard, Acyclovir, an antiviral drug and the method was validated as per ICH guidelines.

MATERIALS AND METHODS

Tenofovir Alafenamide, Emtricitabine and Bictegravir API were procured from Laurus Labs as gift samples. The combination of Tenofovir Alafenamide, Emtricitabine and Bictegravir is available as tablets with different brand names Taffic, Bictarvy, Lagmitaf (Laurus Labs) etc and MCSTAR, ALCIBICLA (Mylan Laboratories Ltd) with label claim of Tenofovir Alafenamide 25 mg, Bictegravir 50 mg and Emtricitabine 200 mg. HPLC grade Acetonitrile was procured from Merck (India) and all other chemicals Ammonium acetate, Sodium hydroxide, Hydrochloric acid and Hydrogen peroxide (30% w/v) were purchased from Merck (India) and Milli Q water was used from Millipore system.

Preparation of stock and standard solutions

5 mg of Tenofovir Alafenamide, 40 mg of Emtricitabine and 10 mg of Bictegravir were accurately weighed and transferred in to three different 50 ml volumetric flasks and diluted with the diluent (Water: Acetonitrile) (50:50, v/v), sonicated for 10 min and then make up to the final volume with the diluent (100 µg/ml of Tenofovir Alafenamide, 800 µg/ml of Emtricitabine, 200 µg/ml of Bictegravir).

1ml from each of the stock solutions was pipetted out and taken into a 10 ml volumetric flask and the volume was made up to volume with the diluent (10 µg/ml of Tenofovir, 80 µg/ml of Emtricitabine and 20 µg/ml of Bictegravir).

Preparation of 0.01M Ammonium acetate buffer solution

0.77 gm of Ammonium acetate was accurately weighed and transferred into a 1000 ml volumetric flask and about 900 ml of Milli-Q water was added and sonicated to degas and finally the volume was made up to volume with Milli-Q water by adjusting the pH to 3.0 with dilute acetic acid.

Instrumentation and Chromatographic conditions

Waters ACQUITY Ultra Performance Liquid Chromatography (UPLC) system with PDA detector and Hibar C18 (100 x 2.1 mm, 1.8µ) column were used for the chromatographic study. Mobile phase mixture consisting of 0.01M Ammonium acetate and Acetonitrile (70:30, v/v) was used with flow rate 0.3 ml/min (Detection wavelength: 260 nm) (Injection volume: 1.0 µL) (Column temperature: 30°C) with run time 3 mins for the chromatographic study. A mixture of water and Acetonitrile (50:50, v/v) was used as diluent.

Method validation[14]

Linearity study

A series of solutions containing a mixture of Tenofovir Alafenamide (2.5-15 µg/ml), Emtricitabine (20-120 µg/ml) and Bictegravir (5-30 µg/ml) were prepared from the stock and working standard solutions were prepared along with the internal standard, Acyclovir using the diluent (Water: Acetonitrile) (50:50, v/v) and each of these solutions were injected (n=3) into the UPLC system and the chromatograms were recorded. The peak area of each of the solutions injected was noted at its retention time and the mean peak area ratio (Analyte/IS) was calculated. A calibration curve was drawn by plotting the concentration of drug solution on the x-axis and the corresponding mean peak area ratio values on the y-axis. The LOD and LOQ were calculated from the signal to noise ratio (S/N). The LOD is 3.3 times the signal to noise ratio and that of LOQ is 10 times the signal to noise ratio.

Precision study

Precision of the method was evaluated intra-day and inter-day precision studies. A mixture of Tenofovir Alafenamide (10 µg/ml), Emtricitabine (80 µg/ml) and Bictegravir (20 µg/ml) solutions were prepared (n=6) along with the internal standard (Acyclovir) within the linearity range on the same day (intra-day precision) and on different consecutive days (inter-day precision) and the chromatographic study was performed. The mean peak area (n=3) and thereby the % RSD was calculated.

Accuracy study

Accuracy of the method was measured by spiking the formulation solution with a known concentration of standard drug (50, 100, 150%) containing Tenofovir Alafenamide, Emtricitabine and Bictegravir and were injected thrice into the UPLC system after the addition of internal standard and the chromatograms were recorded. The mean peak area ratio (Analyte/IS) was calculated from the chromatograms obtained and finally the % RSD was calculated.

Assay of tablets

20 tablets of two different brands were weighed, and the average weight of each tablet was calculated and then the weight equivalent to 1 tablet was transferred into a 100 mL volumetric flask, 50mL of diluent was added and then sonicated for 50 mins and further the volume made up with the diluent and filtered. The filtrate contains 250 µg/ml of Tenofovir Alafenamide, 2000 µg/ml of Emtricitabine and 500 µg/ml of Bictegravir respectively. 0.4 ml of the filtered solution was pipetted out into a 10 ml volumetric flask and the volume was made up to 10 ml with the diluent and the resultant solution contains 10 µg/ml of Tenofovir Alafenamide, 80 µg/ml of Emtricitabine and 20 µg/ml of Bictegravir respectively. 1µl of each of the marketed formulation solution along with the internal standard was injected into the UPLC system and the chromatogram was recorded and the amount of Tenofovir Alafenamide, Emtricitabine and Bictegravir was calculated from the respective calibration curves.

Forced degradation studies[15]

The specificity of the method can be known from the stability studies. Forced degradation studies were performed to determine the stability of Tenofovir Alafenamide, Emtricitabine and Bictegravir towards stress conditions such as acidic hydrolysis, alkaline hydrolysis, oxidation, neutral thermal and photolytic degradation.

Acid degradation

1 ml of stock solution of Tenofovir Alafenamide, Emtricitabine and Bictegravir was taken and 1 ml of 2N Hydrochloric acid was added and refluxed for 30mins at at 60^0C. The resultant solution was diluted to obtain 10 µg/ml Tenofovir Alafenamide, 80 µg/ml Emtricitabine and 20 µg/ml Bictegravir solution and 1µl of this solution was injected into the UPLC system and the chromatogram was recorded.

Alkaline degradation

1 ml of stock solution of Tenofovir Alafenamide, Emtricitabine and Bictegravir was taken and 1 ml of 2N sodium hydroxide was added and refluxed for 30mins at at 60^0C. The resultant solution was diluted to obtain 10 µg/ml Tenofovir Alafenamide, 80 µg/ml Emtricitabine and 20 µg/ml Bictegravir solution and 1µl of this solution was injected into the UPLC system and the chromatogram was recorded.

Thermal (Dry heat) degradation

The standard drug solution was placed in oven at 105^0C for 6 Hrs to study dry heat degradation. The resultant solution was diluted to obtain 10 µg/ml Tenofovir Alafenamide, 80 µg/ml Emtricitabine and 20 µg/ml Bictegravir solution and 1µl of this solution was injected into the UPLC system and the chromatogram was recorded.

Photolytic degradation

The photochemical stability was performed by exposing the solution containing 250 µg/ml Tenofovir Alafenamide, 2000 µg/ml Emtricitabine and 500 µg/ml Bictegravir solution kept in a beaker to UV light in in photo stability chamber (UV chamber) for 7 days or 200 Watt hours/m^2.

The resultant solution was diluted to obtain 10 µg/ml Tenofovir Alafenamide, 80 µg/ml Emtricitabine and 20 µg/ml Bictegravir solution and 1µl of this solution was injected into the UPLC system and the chromatogram was recorded.

Neutral degradation

Stress testing under neutral conditions was studied by refluxing the solution containing 250 µg/ml Tenofovir Alafenamide, 2000 µg/ml Emtricitabine and 500 µg/ml Bictegravir in water for 6 Hrs at a temperature of 60ºC and the resultant solution was diluted to obtain 10 µg/ml Tenofovir Alafenamide, 80 µg/ml Emtricitabine and 20 µg/ml Bictegravir solution and 1µl of this solution was injected into the UPLC system and the chromatogram was recorded.

Oxidative degradation

1 ml of stock solution of Tenofovir Alafenamide, Emtricitabine and Bictegravir was taken and 1 ml of 20% hydrogen peroxide (H_2O_2) was added separately and the solutions were kept for 30

min at 60^0C. The resultant solution was diluted to obtain 10 µg/ml Tenofovir Alafenamide, 80 µg/ml Emtricitabine and 20 µg/ml Bictegravir solution and 1µl of this solution was injected into the UPLC system and the chromatogram were recorded.

RESULTS AND DISCUSSION

The authors have proposed a new stability indicating RP-UPLC method for the simultaneous estimation of Tenofovir Alafenamide, Emtricitabine and Bictegravir in tablets and the method was validated as per ICH guidelines. Waters ACQUITY Ultra Performance Liquid Chromatography (UPLC) system with PDA detector and Hibar C18 (100 x 2.1 mm, 1.8µ) column were used for chromatographic study.

The present RP-UPLC method was compared with the previously published methods and some of the important observations were highlighted in Table 1.

Table 1: Comparison of previously published methods with the present methods

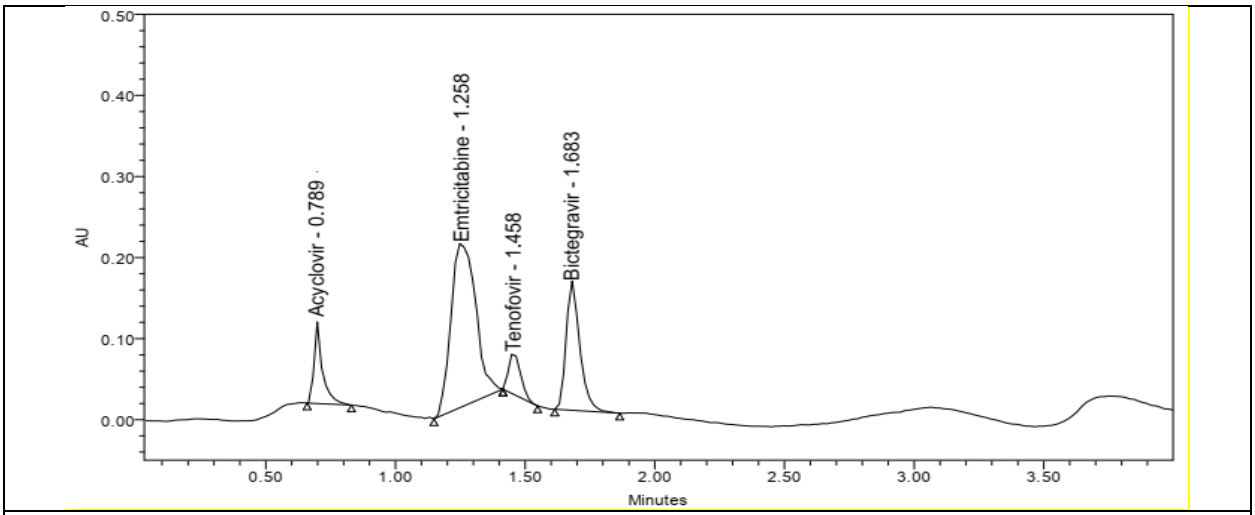
Mobile phase(v/v)	Column	Rt (min)	Linearity (µg/ml)	Ref
RP-HPLC 0.2% Triethylamine buffer: Methanol (40: 60)	Inertsil ODS C18	5.998 (BG) 2.805 (EC) 4.537 (TF)	25-125 (BG) 100-500 (EC) 12.5-62.5 (TF)	6
RP-UPLC 0.1% ortho phosphoric acid (pH 2.2): Acetonitrile (70:30)	HSS C18	0.89 (BG) 0.62 (EC) 1.75 (TF)	12.5-75 (BG) 500-300 (EC) 6.25-37.5 (TF)	7
RP-HPLC 0.1M Ammonium acetate in 0.5% v/v Acetic acid solution and 1g of 1- Octane sulfonic acid (pH adjustment	Inertsil ODS 3V	12.23 (BG) 3.0 (EC) 8.5 (TF)	31.15-93.45 (BG) 125-375 (EC) 15.765-47.295 (TF)	8

to 4.2 with dilute ortho phosphoric acid): Methanol (40:60)				
RP-HPLC 0.1% ortho phosphoric acid buffer (pH 2.2): Acetonitrile (50:50)	Denali C18	3.219 (BG) 2.303 (EC) 3.754 (TF)	12.5-75 (BG) 50-300 (EC) 6.25-37.5 (TF)	9
RP-HPLC 0.1% OPA and acetonitrile (55:45)	Kromasil C18	2.432 (BG) 2.992 (EC) 2.14 (TF)	6.25-37.5 (BG) 25-150 (EC) 3.125-18.75 (TF)	10
RP-HPLC Acetonitrile and Phosphate buffer at pH 6.8 (30:70)	XBridge C18	4.604 (BG) 0.581 (EC) 1.804 (TF)	2.5-15 (BG) 10-60 (EC) 1.24-7.4 (TF)	11
RP-HPLC 0.1 M Sodium perchlorate and methanol (65:35, v/v) (pH 4.8)	ProntoSIL Hypersorb ODS C18	4.6 (BG) 7.0 (EC) 10.1 (TF)	5-30 (BG) 20-120 (EC) 2.5-15 (TF)	12
RP-HPLC water, Acetonitrile and Methanol (15:35:50)	Symmetry C18	4.462 (BG) 1.590 (EC) 6.870 (TF)	50-150 (BG) 50-150 (EC) 50-150 (TF)	13
RP-UPLC 0.01M Ammonium acetate and Acetonitrile (70:30)	Hibar C18	1.512 (BG) 1.026 (EC) 1.322 (TF)	5-30 (BG) 20-120 (EC) 2.5-15 (TF)	Present

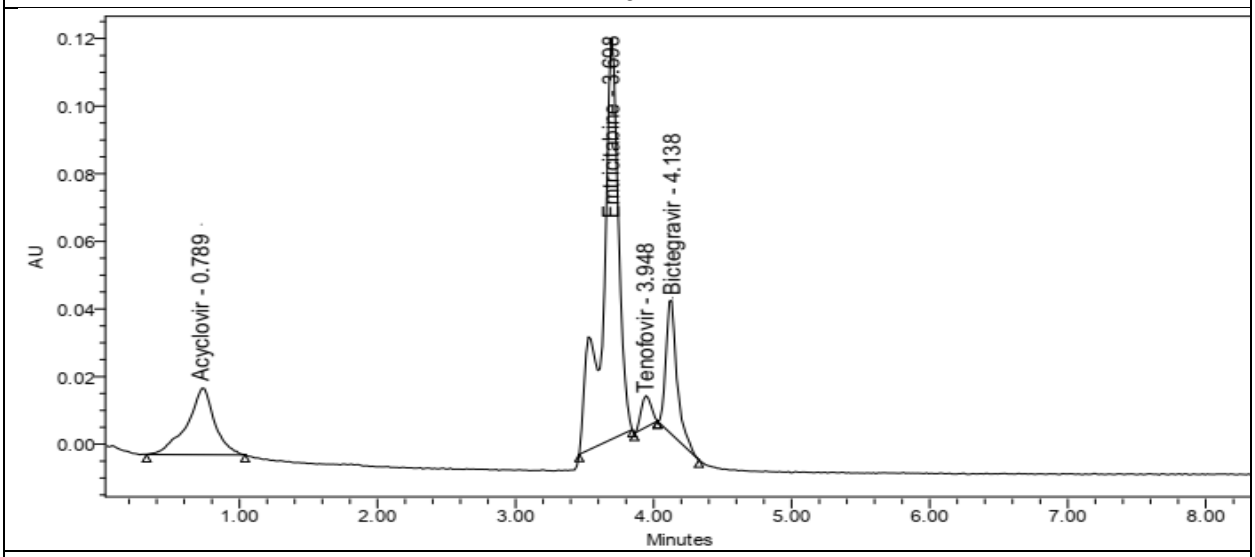
(Internal standard: Acyclovir)		0.784 (IS)		meth od

Method optimization

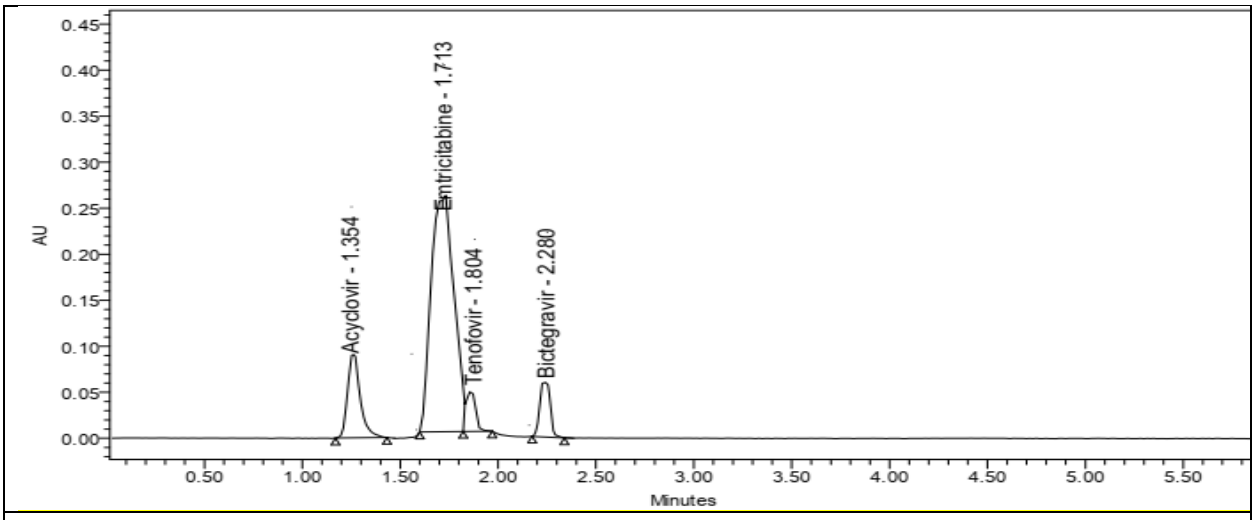
Initially, different columns and chromatographic conditions were applied to optimize the method and the trial runs (Figure 2) were shown in Table 2.



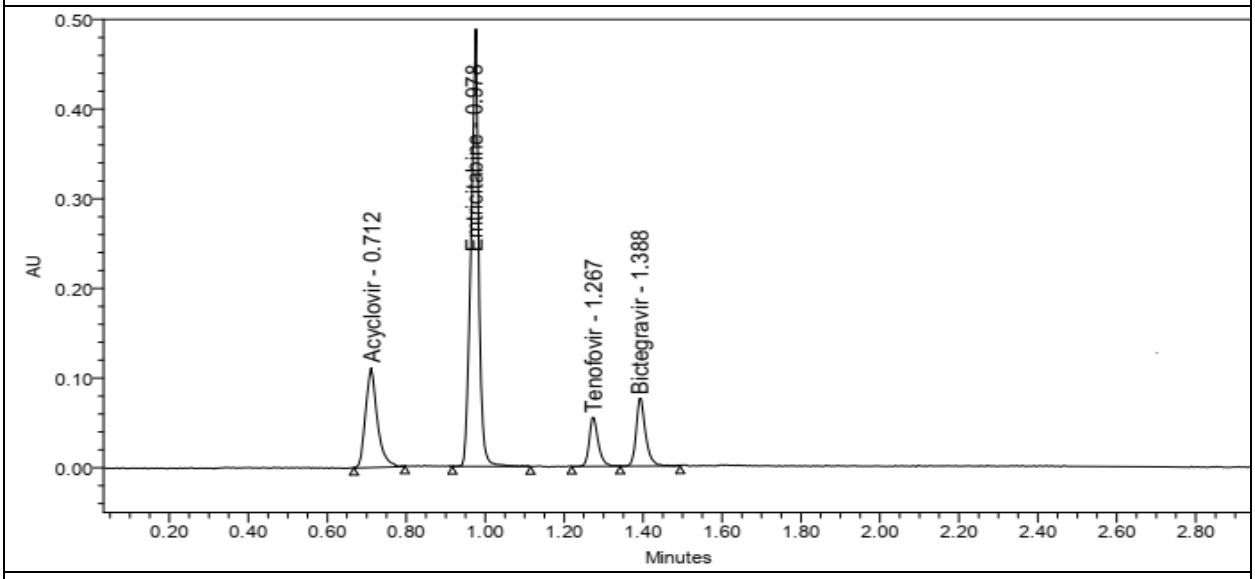
Trial 1



Trial 2



Trial 3



Trial 4 (Optimized)

Figure 2: Typical chromatograms obtained during method optimization (Trial runs)

Table 2: Method optimization

Trial	Mobile phase (v/v)	Column	Result

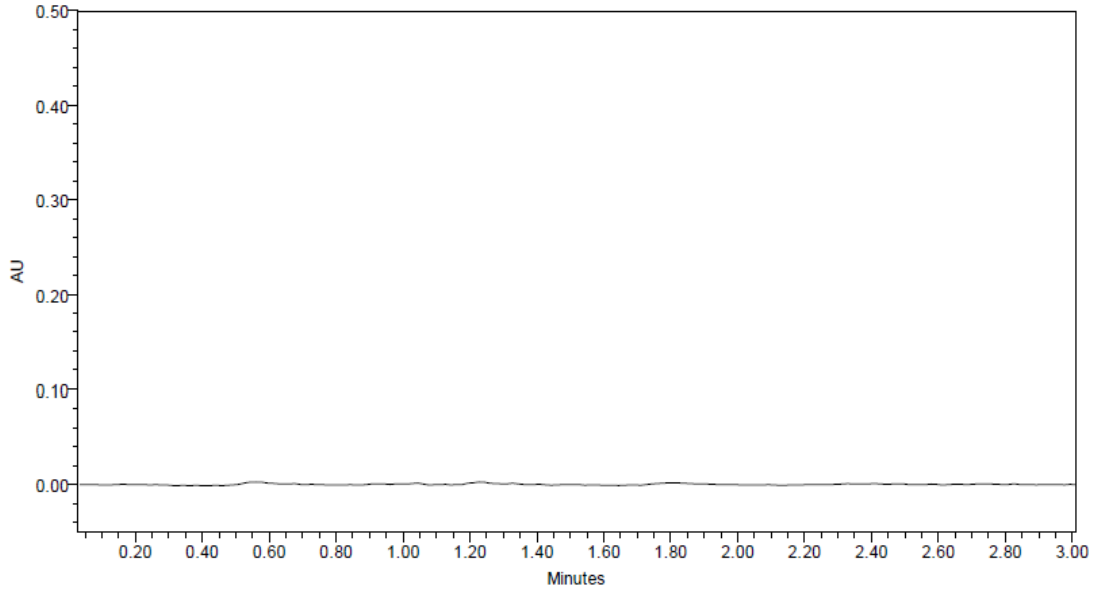
1	Acetonitrile: Water (50:50)	SB C8 80Å	Peaks eluted but baseline was improper and emtricitabine with less plate count and Tenofovir resolution was less.
2.	Acetonitrile: OPA (50:50)	Hibar	Column was changed and peak were eluted but with less resolution and peak merge was seen.
3	Acetonitrile: Water (50:50)	Hibar	Peak were eluted but peak merge was seen in emtricitabine and Tenofovir
4	Ammonium Acetate: Acetonitrile: (70:30)	Hibar	Peaks was eluted with good shape and all parameters were passed. (Method optimized)

Method validation

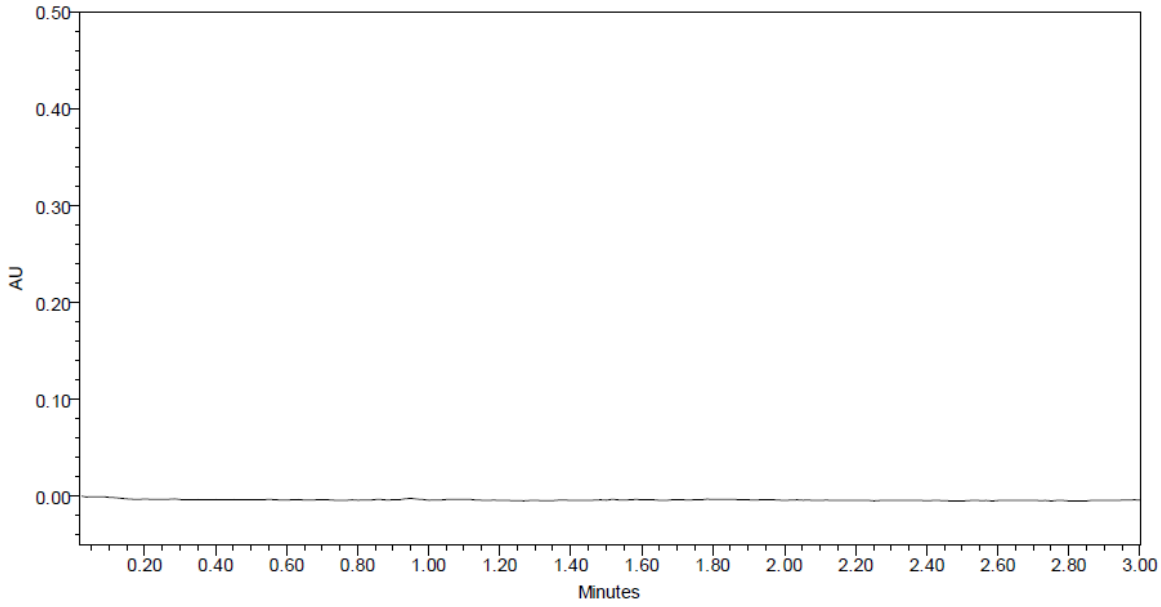
The representative chromatograms of blank, placebo and the combination of Tenofovir Alafenamide, Emtricitabine and Bictegravir in presence of an internal standard, Acyclovir were shown in Figure 3.

Tenofovir Alafenamide has shown linearity over the concentration range 2.5-15 µg/ml with linear regression equation, $y = 0.0179x + 0.0017$ ($R^2 = 0.9997$). Emtricitabine has shown linearity over the concentration range 20-120 µg/ml with linear regression equation, $y = 0.0151x + 0.0072$ ($R^2 = 0.9998$) and Bictegravir has shown linearity over the concentration range 5-30 µg/ml with linear regression equation, $y = 0.024x + 0.0044$ ($R^2 = 0.9998$) (Table 3). The LOD and LOQ were found to be 0.04 µg/ml and 0.11 µg/ml for Tenofovir Alafenamide; 0.25 µg/ml and 0.77 µg/ml for Emtricitabine and 0.48 µg/ml and 1.46 µg/ml for Bictegravir respectively. The representative calibration curves were shown in Figure 4.

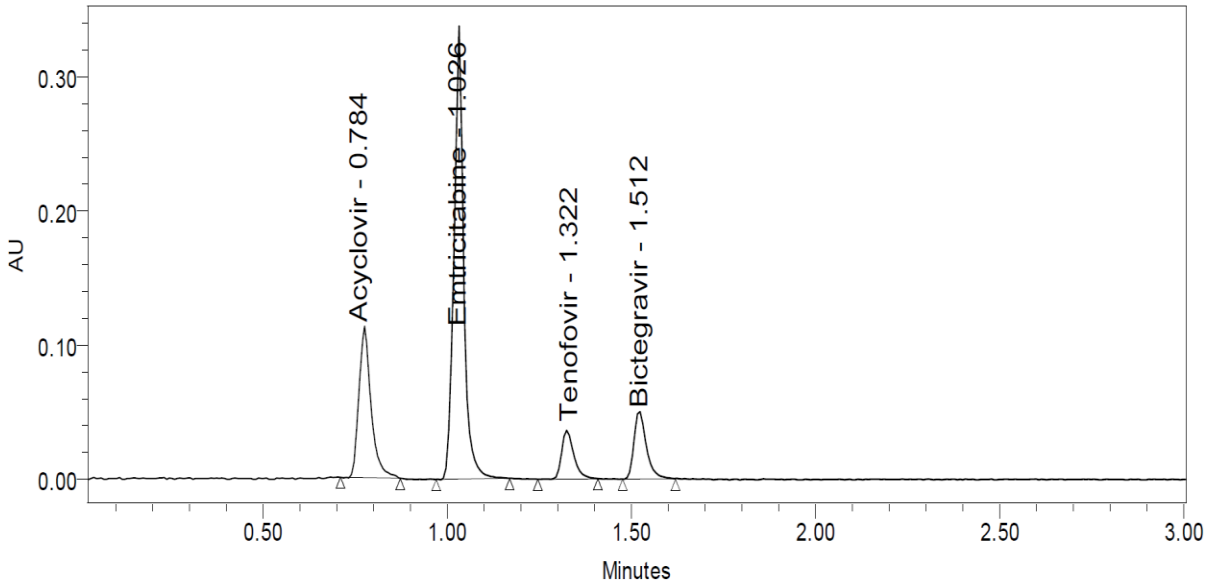
The % RSD in intraday and inter day precision studies was found to be less than 2.0 (Table 4) indicating that the method is precise. The % RSD in accuracy studies was found to be less than 2 (Table 5) indicating that the method is accurate.



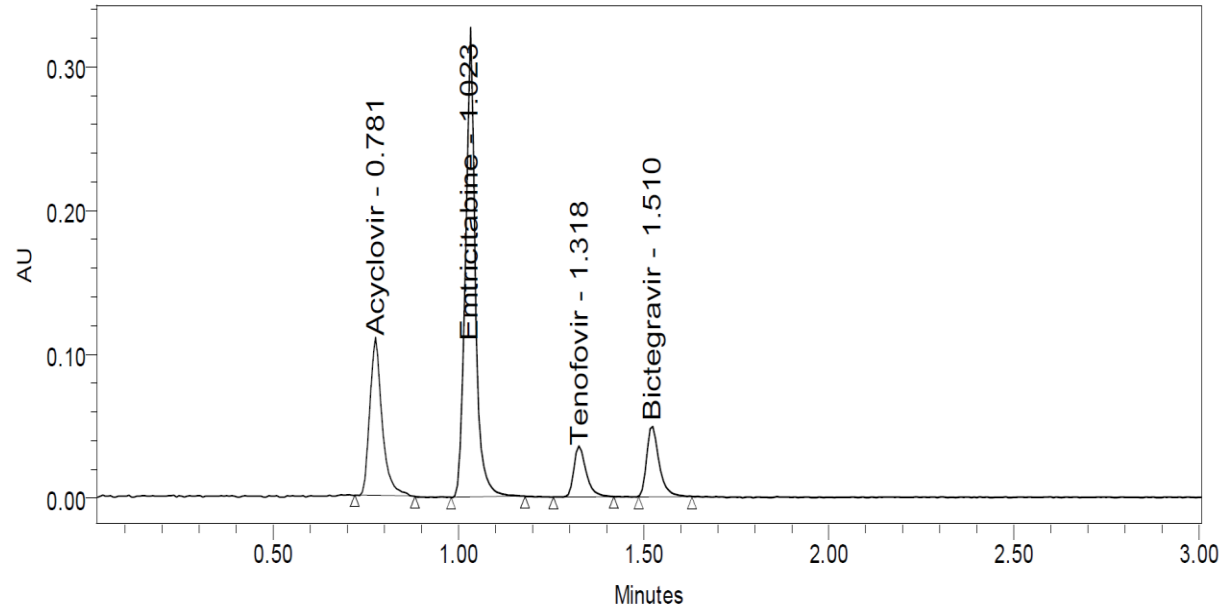
Blank



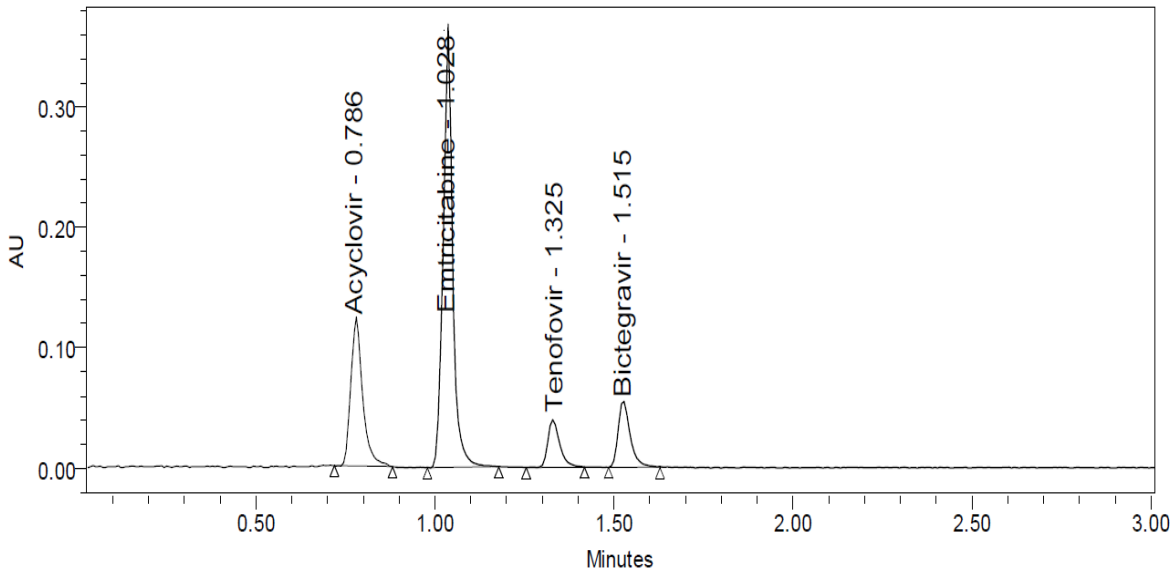
Placebo



Typical chromatogram of Tenofovir, Emtricitabine and Bictegravir (API) in presence of internal standard (Acyclovir)



Typical chromatogram of Tenofovir, Emtricitabine and Bictegravir tablet formulation in presence of internal standard (Acyclovir) (Brand I)



Typical chromatogram of Tenofovir, Emtricitabine and Bictegravir tablet formulation in presence of internal standard (Acyclovir) (Brand II)

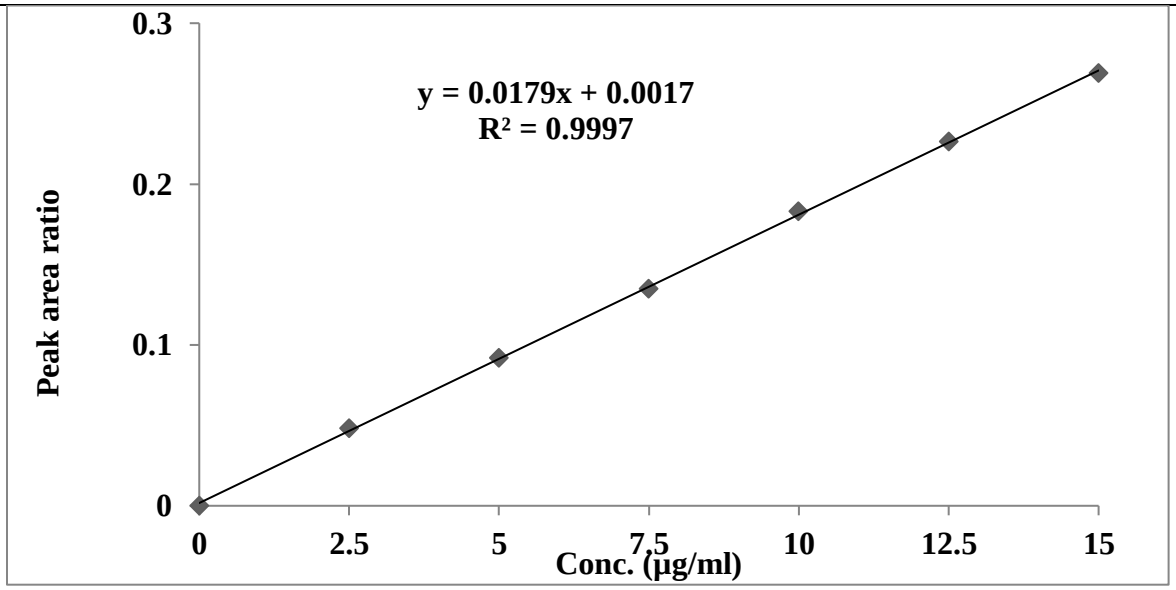
Figure 3: Typical chromatogram of Tenofovir, Emtricitabine and Bictegravir in presence of internal standard (Acyclovir)

Table 3: Linearity study

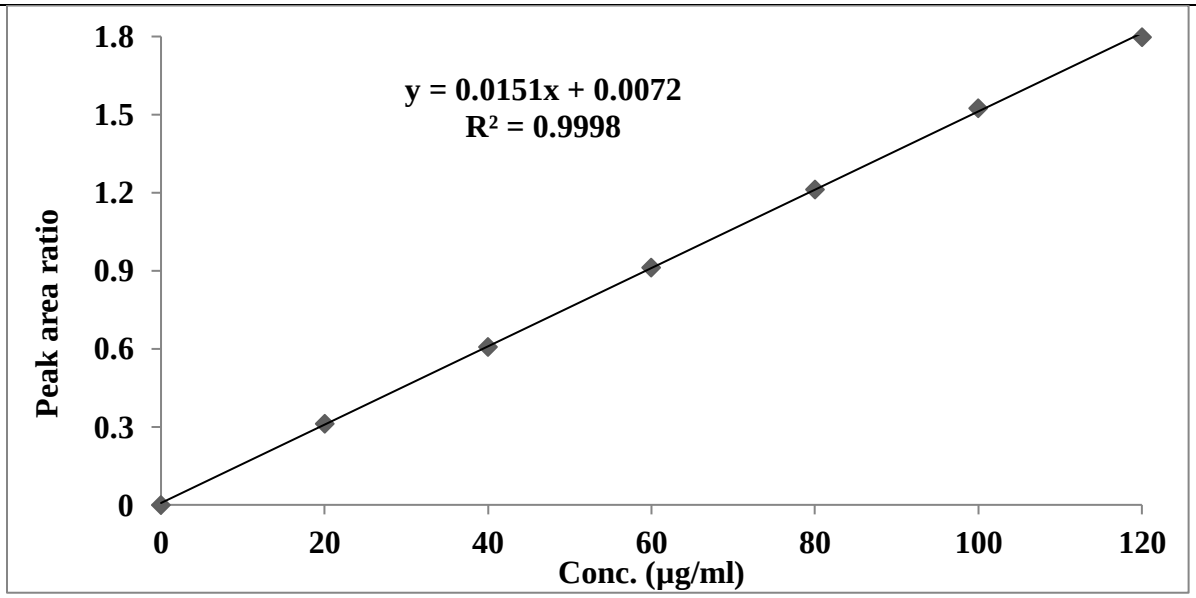
Conc. (µg/ml)			Peak area ratio (Analyte/IS)		
Tenofovir Alafenamide	**Emtricitabine**	**Bictegravir**	**Tenofovir Alafenamide / IS**	**Emtricitabine / IS**	**Bictegravir / IS**
2.5	20	5	0.048	0.312	0.1303
5	40	10	0.092	0.609	0.2507
7.5	60	15	0.135	0.912	0.3773
10	80	20	0.183	1.212	0.4980

12.5	100	25	0.226	1.526	0.6275
15	120	30	0.269	1.800	0.7376

*Mean of three replicates



Tenofovir Alafenamide (TA)



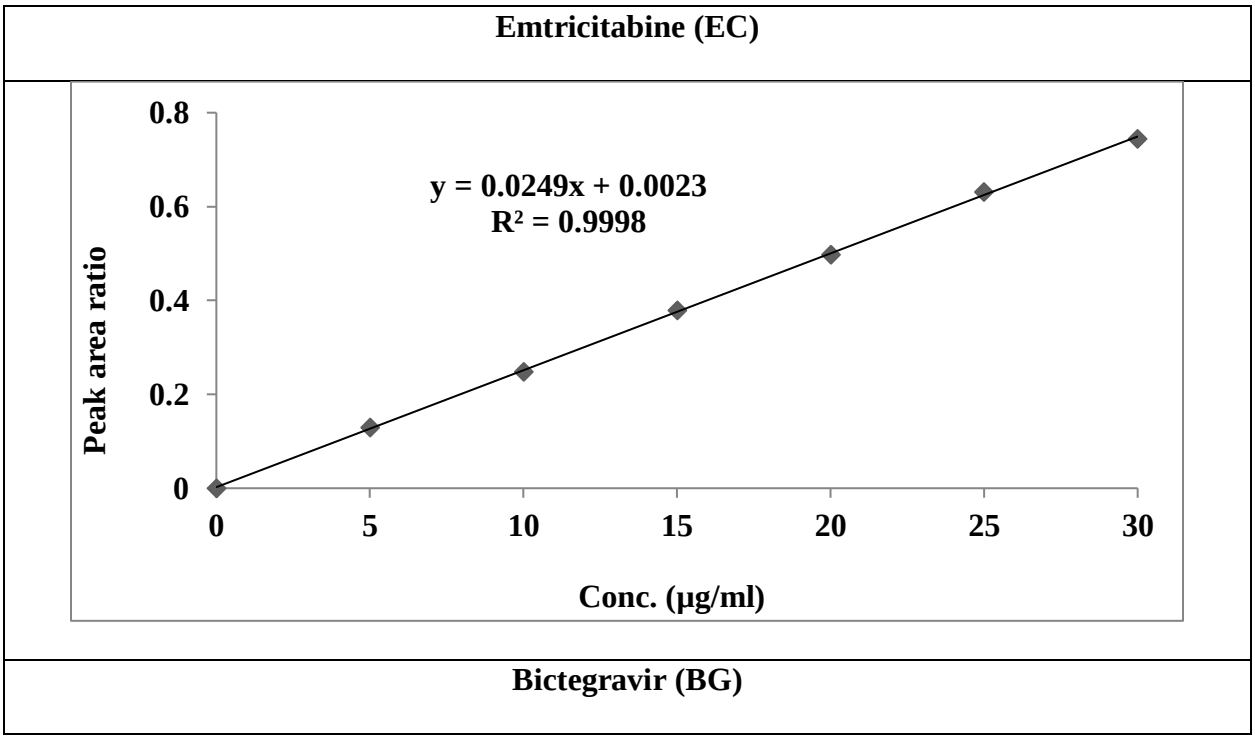


Figure 4: Calibration curves

Table 4: Precision study

Emtricitabine							
Intraday precision					Interday precision		
Conc. (µg/ml)		Peak area		Peak area ratio	Peak area		Peak area ratio
EC	IS	EC	IS	Analyte / IS	EC	IS	Analyte / IS
80	50	1431825	1153614	1.24116	1395448	1150488	1.21292
80	50	1425320	1154654	1.23441	1396858	1147608	1.21719
80	50	1406460	1153914	1.21886	1391827	1147388	1.21304
80	50	1414929	1157724	1.22216	1392105	1156818	1.20339

80	50	1423546	1157721	1.22961	1392742	1156809	1.20395
80	50	1421384	1153466	1.23227	1409420	1144044	1.23196
*Mean peak area ratio ± SD (% RSD) = 1.23 ± 0.01 (0.7)					*Mean peak area ratio ± SD (% RSD) 1.2137 ± 0.01 (0.9)		
Tenofovir Alafenamide							
Intraday precision					**Interday precision**		
Conc. (µg/ml)		**Peak area**		**Peak area ratio**	**Peak area**		**Peak area ratio**
TA	IS	TA	IS	Analyte / IS	TA	IS	Analyte / IS
20	50	205430	1153614	0.17808	199257	1150488	0.1731935
20	50	206082	1154654	0.17848	199220	1147608	0.1735959
20	50	205239	1153914	0.17786	198921	1147388	0.1733686
20	50	203425	1157724	0.17571	199865	1156818	0.1727713
20	50	206141	1157721	0.17806	199944	1156809	0.172841
20	50	205939	1153466	0.17854	200172	1144044	0.1749688
*Mean peak area ratio ± SD (% RSD) = 0.18 ± 0.0 (0.6)					*Mean peak area ratio ± SD (% RSD) 0.17 ± 0.0 (0.5)		
Bictegravir							
Intraday precision					**Interday precision**		
Conc. (µg/ml)		**Peak area**		**Peak area ratio**	**Peak area**		**Peak area ratio**

BG	IS	TA	IS	Analyte / IS	TA	IS	Analyte / IS
10	50	588578	1153614	0.51020	579761	1150488	0.50393
10	50	576535	1154654	0.49931	570818	1147608	0.49740
10	50	580504	1153914	0.50307	577029	1147388	0.50291
10	50	581830	1157724	0.50256	575231	1156818	0.49725
10	50	585229	1157721	0.50550	582287	1156809	0.50336
10	50	583354	1153466	0.50574	578873	1144044	0.50599
*Mean peak area ratio ± SD (% RSD) = 0.5 ± 0.0 (0.7)					*Mean peak area ratio ± SD (% RSD) = 0.5 ± 0.01 (0.7)		

*Mean of three replicates

Table 5: Accuracy study

Emtricitabine			
Spiked drug Conc. (µg/ml)	**Drug Formulation (µg/ml)**	***Drug recovered (µg/ml) ± SD (% RSD)**	**% Recovery**
40	80	39.21053836	98.03
	80	39.14091944	97.85
	80	39.99174018	99.98
80	80	79.14372591	98.93
	80	79.09157982	98.86
	80	79.96432903	99.96

120	80	118.2947132	98.58
	80	118.9213103	99.10
	80	119.1627533	99.30
*Mean % Recovery ± SD (% RSD) = 98.95 ± 0.74 (0.8)			
Tenofovir Alafenamide			
Spiked drug Conc. (µg/ml)	**Drug Formulation (µg/ml)**	***Drug recovered (µg/ml) ± SD (% RSD)**	**% Recovery**
5	10	4.944791033	98.90
5	10	4.934921559	98.70
5	10	4.985499247	99.71
10	10	9.883471574	98.83
10	10	9.875985177	98.76
10	10	10.00020124	100.00
15	10	14.82901883	98.86
15	10	14.75708009	98.38
15	10	14.86056763	99.07
*Mean % Recovery ± SD (% RSD) = 99.02 ± 0.51 (0.52)			
Bictegravir			

Spiked drug Conc. (µg/ml)	Drug Formulation (µg/ml)	*Drug recovered (µg/ml) ± SD (% RSD)	% Recovery
10	20	9.884566529	98.85
10	20	9.8669792	98.67
10	20	9.918595591	99.19
20	20	19.83244217	99.16
20	20	19.83771268	99.19
20	20	19.99062378	99.95
30	20	29.67768978	98.93
30	20	29.706	99.02
30	20	29.865	99.55
*Mean % Recovery ± SD (% RSD) = 99.72 ± 0.342 (0.3)			

*Mean of three replicates

Assay of tablets

The tablet formulations contain 25 mg of Tenofovir Alafenamide, 50 mg of Bictegravir and 200 mg of Emtricitabine. The assay for the estimation of Tenofovir Alafenamide, Emtricitabine and Bictegravir was performed for two of the marketed brands with optimized chromatographic conditions and the percentage of purity was found to be 99.55-99.87 for Tenofovir Alafenamide, 99.50-99.72, for Emtricitabine and 99.81-99.84 for Bictegravir (Table 6). The typical chromatograms obtained for the two tablet formulations were shown in Figure 2 in presence of the internal standard, Acyclovir.

Table 6: Assay of tablets

S. No.	Brand name	Label claim (mg)			*Observed amount (%w/w)			% Recovery*		
		TF	EC	BG	TF	EC	BG	TF	EC	BG
1	Brand I	25	200	50	24.97	199.44	49.92	99.87	99.72	99.84
2	Brand II	25	200	50	24.89	199.00	49.91	99.55	99.50	99.81

*Mean of three replicates

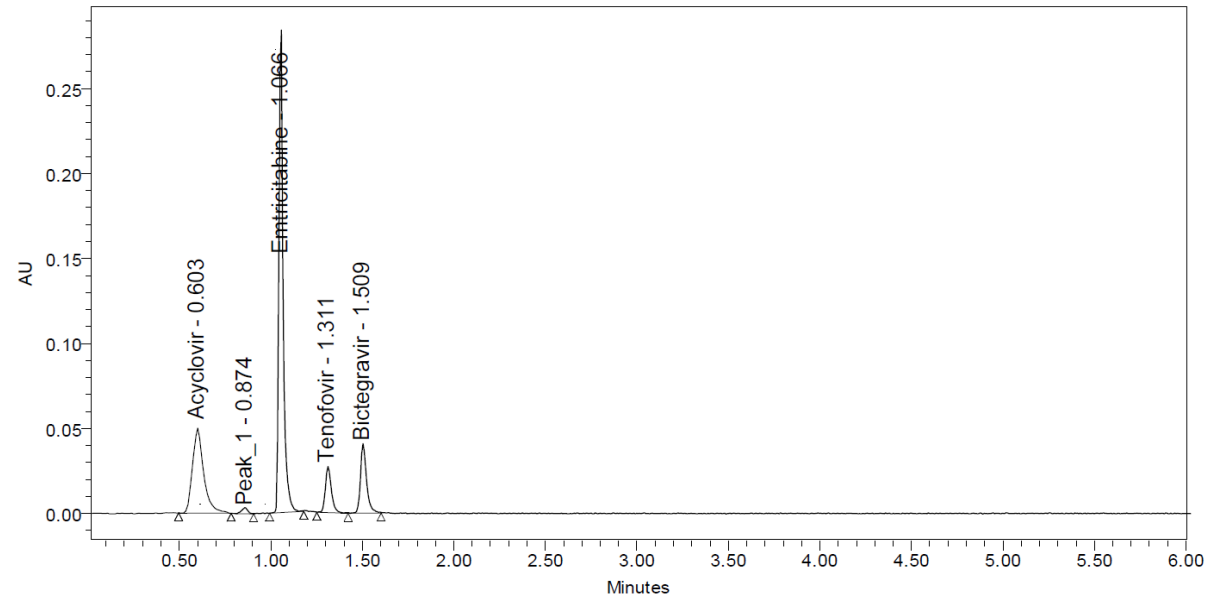
Forced degradation studies

The respective chromatograms obtained during the forced degradation studies were shown in Figure 5 and the other details were shown in Table 7.

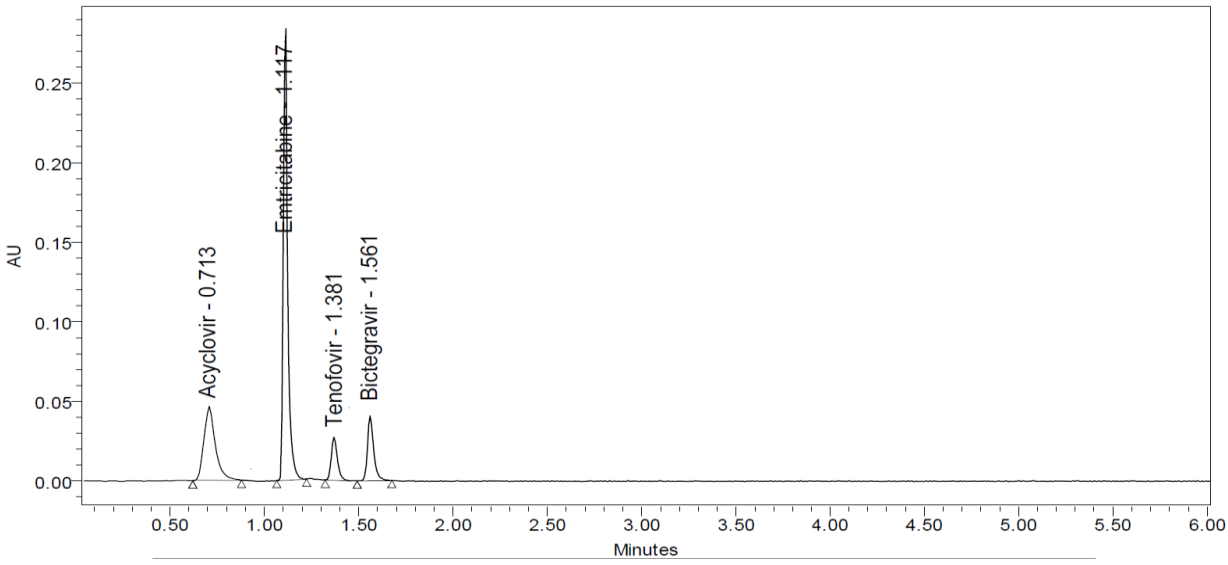
Table 7: Forced degradation studies

Stress Conditions	Rt (min)				Peak area ratio (Analyte/IS)			*Drug recovered (%)			*Drug decomposed (%)		
	IS	EC	TF	BG	TF	EC	BG	TF	EC	BG	TF	EC	BG
Standard	0.784	1.026	1.322	1.512	0.178	1.230	0.504	100	100	100	-	-	-
Acidic degradation	0.603	1.066	1.311	1.509	0.169	1.145	0.478	94.82	92.91	94.64	5.18	1.09	5.36
Alkaline degradation	0.790	1.056	1.301	1.598	0.145	0.978	0.416	81.55	79.34	82.23	18.45	20.66	17.77
Oxidative degradation	0.713	1.117	1.381	1.561	0.169	1.150	0.489	94.65	93.29	96.84	5.35	6.71	3.16
Thermal degradation	0.653	1.075	1.369	1.536	0.176	1.210	0.502	98.78	98.20	99.28	1.22	1.80	0.72
Photolytic degradation	0.783	1.125	1.444	1.513	0.177	1.211	0.502	99.11	98.28	99.40	0.89	1.72	0.60
Neutral degradation	0.776	1.153	1.427	1.519	0.177	1.213	0.503	99.38	98.44	99.47	0.62	1.56	0.53

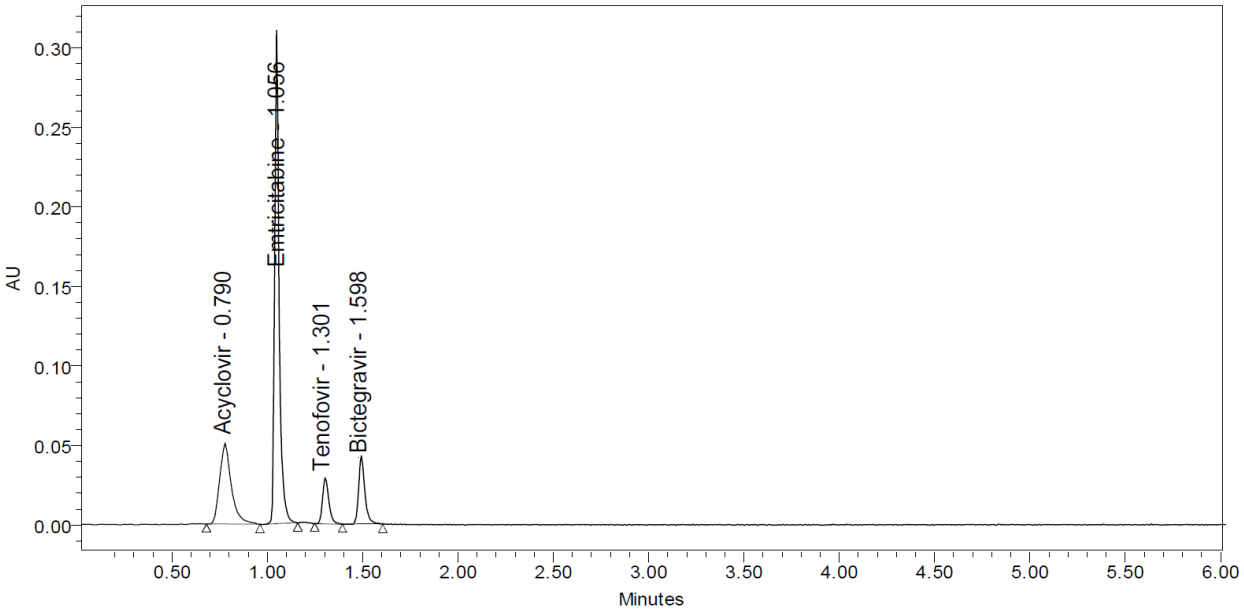
*Mean of three replicates



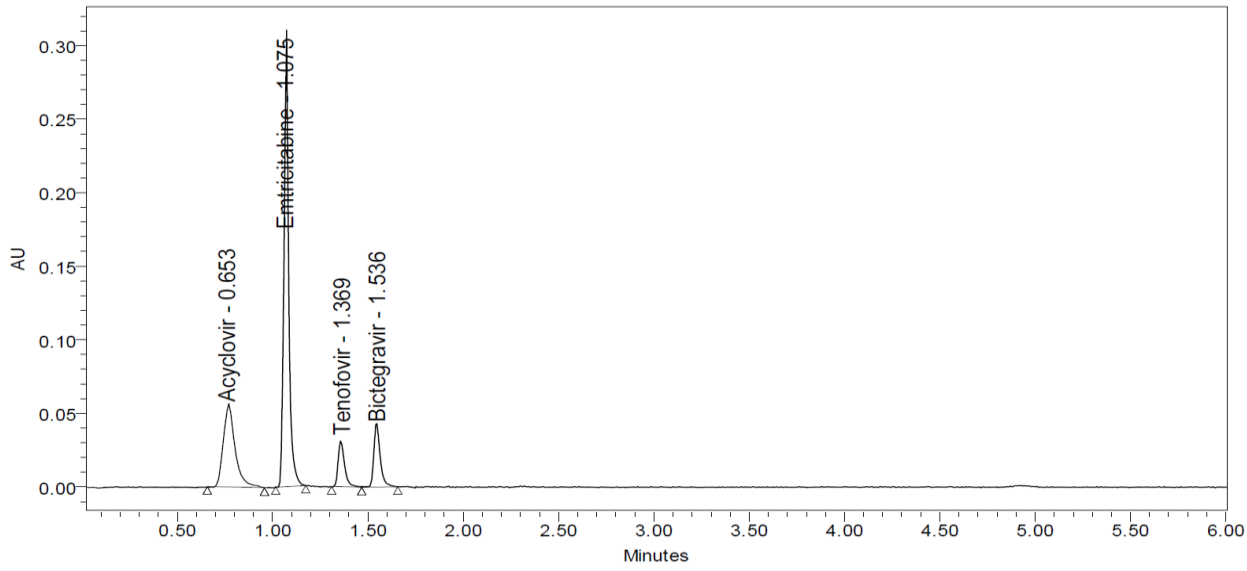
Acidic degradation



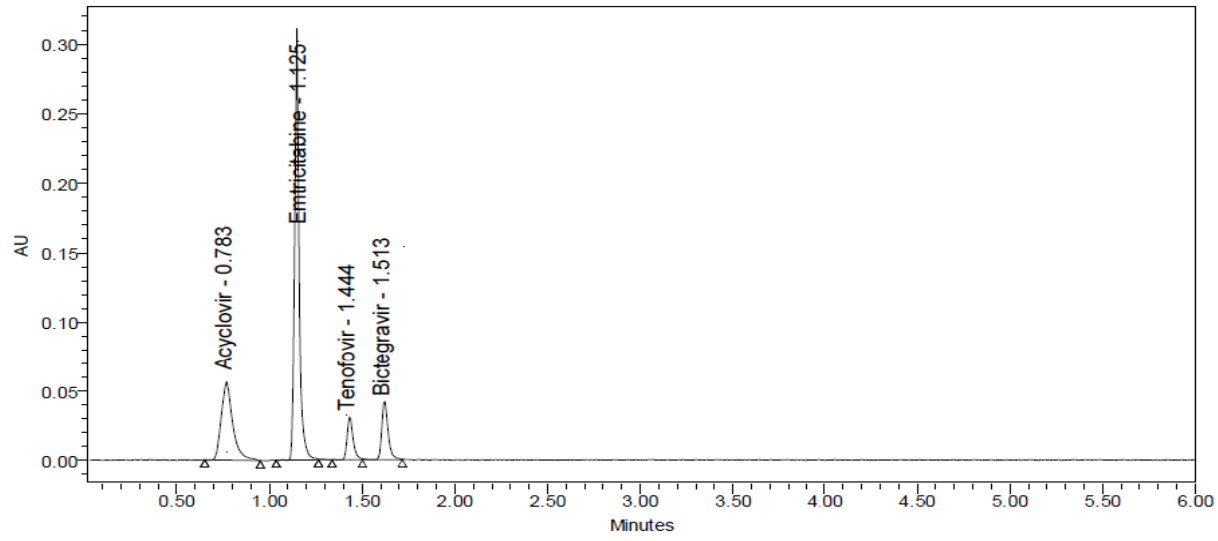
Oxidative degradation



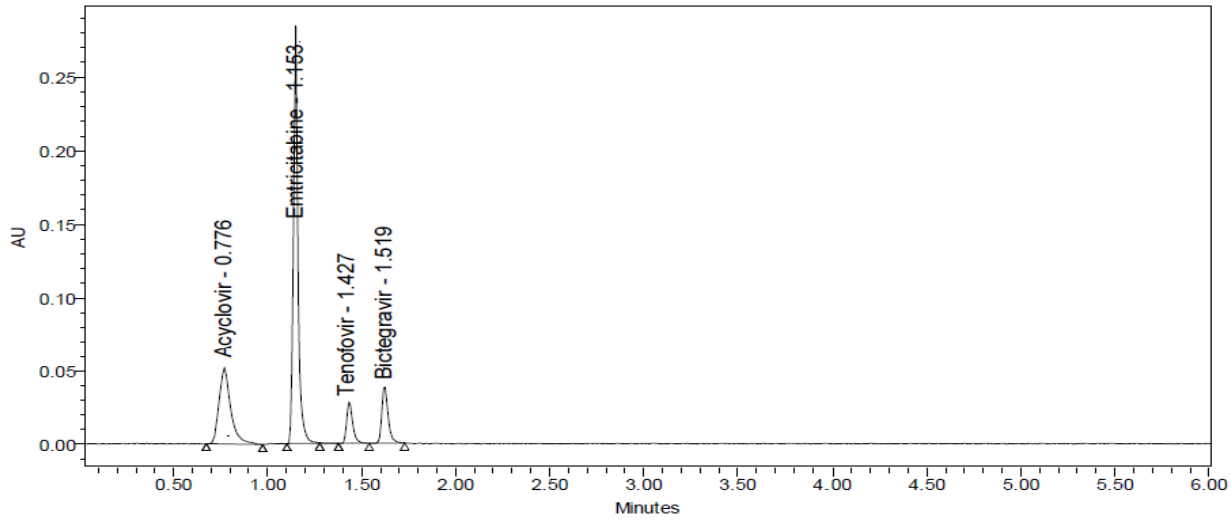
Alkaline degradation



Thermal degradation



Photolytic degradation



Neutral degradation

Figure 5: Chromatograms of Tenofovir Alafenamide, Emtricitabine and Bictegravir in presence of the internal standard (Acyclovir) during forced degradation studies

CONCLUSIONS

A new stability indicating RP-UPLC method has been developed for the estimation of the combination of Tenofovir Alafenamide, Emtricitabine and Bictegravir and validated as per ICH guidelines. The method is specific and there is no interference of excipients used in the tablet formulation. The proposed method is simple precise, accurate and robust and can be applied for the pharmaceutical formulations successfully.

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