Research Article

# Method Development and Validation for Simultaneous Estimation of Emtricitabine, Bictegravir and Tenofovir Alafenamide by RP-HPLC

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**ABSTRACT**

A simple, accurate, precise method was developed for the simultaneous estimation of the Emtricitabine (ECB), Bictegravir (BTG) and Tenofovir Alafenamide (TAF) in solid dosage form. Chromatogram was run through BDS (C8 150x4.6mm, 5m) Mobile phase containing Buffer and Acetonitrile in the ratio of 58:42 v/v was pumped through column at a flow rate of 1ml/min. Buffer used in this method was 0.01N KH2PO4 buffer at pH adjusted to 3.47 with dil. Ortho-phosphoric acid solution. Temperature was maintained at 30°C. Optimized wavelength for Emtricitabine, Bictegravir and Tenofovir Alafenamide was 272.0nm. Retention time of Emtricitabine, Bictegravir and Tenofovir Alafenamide were found to be 2.229min, 2.958min and 3.568min %RSD of system precision for Emtricitabine, Bictegravir and Tenofovir Alafenamide were and found to be 0.6, 0.9and 0.7 respectively. %RSD of method precision for Emtricitabine, Bictegravir and Tenofovir Alafenamide were and found to be 0.7, 0.8 and 0.5 respectively. % Recovery was Obtained as 100.17%, 100.14% and 100.17% for Emtricitabine, Bictegravir and Tenofovir Alafenamide respectively. LOD, LOQ values are obtained from regression equations of Emtricitabine, Bictegravir and Tenofovir Alafenamide were 1.40µg/ml, 4.24µg/ml, 0.12µg/ml and 0.36µg/ml, 0.15µg/ml, 0.47µg/ml respectively. Regression equation of Emtricitabine was y = 15921x + 10733, Bictegravir was y = 16753x + 2572 and of Tenofovir Alafenamide was y = 12838x + 3003.

**Keywords:** Emtricitabine, Bictegravir, Tenofovir Alafenamide, RP-HPLC

## INTRODUCTION

Chemically Emtricitabine (ECB) was 4-amino-5-fluoro-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one. Molecular weight and molecular formula of ECB were 247.247 g/mol and $C_8H_{10}FN_3O_3S$ respectively. Emtricitabine is a nucleoside reverse transcriptase inhibitor (NRTI) for the treatment of HIV infection in adults. Emtricitabine is a cytidine analogue. The drug works by inhibiting HIV reverse transcriptase, preventing transcription of HIV RNA to DNA. Structure of the ECB was shown in figure 1(A) [1].

Chemically Bictegravir (BTG) was (1S,11R,13R)-5-hydroxy-3,6-dioxo-N-[(2,4,6-trifluorophenyl)methyl]-12-oxa-2,9-diazatetracyclo[11.2.1.0^{2,11}.0^{4,9}]hexadeca-4,7-diene-7-carboxamide. Molecular weight and molecular formula of BTG were 449.386 g/mol and $C_{21}H_{18}F_3N_3O_5$ respectively. Bictegravir is a recently approved investigational drug that has been used in trials studying the treatment of HIV-1 and HIV-2 infection. It has been approved for HIV-1 monotherapy combined with 2 other anti-retrovirals in a single tablet. Structure of the BTG was shown in figure 1 (B) [2].

Chemically Tenofovir alafenamide (TAF) was propan-2-yl (2S)-2-{[(S)-({[(2R)-1-(6-amino-9H-purin-9-yl)propan-2-yl]oxy}methyl)(phenoxy)phosphoryl]amino}propanoate. Molecular weight and molecular formula of TAF were 476.474 g/mol and $C_{21}H_{29}N_6O_5P$ respectively. Tenofovir alafenamide is an alanine ester form characterized for presenting low systemic levels but high intracellular concentration. It has been reported to produce a large antiviral efficacy. Structure of the TAF was shown in figure1(C) [3].

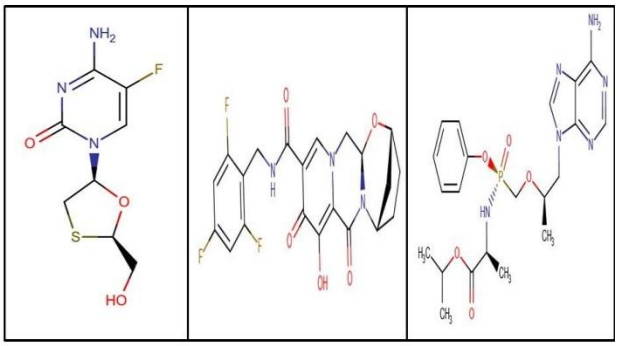


**Figure 1:** Structure of (A) Emtricitabine (B) Bictegravir(C) Tenofovir alafenamide

Literature survey reveals there are several methods to estimated these drugs in single or in combination of two drugs[4-7]but there is only very few HPLC methods[8] are available for simultaneous estimation of ECB, BTG and TAF, so the scope of developing and validating an analytical method is to ensure a suitable method for a particular analyte to be more specific, accurate and precise. The main objective for that is to improve the conditions and parameters, which should be followed in the development and validation processes.

## MATERIALS AND METHODS

### Reagents and Chemicals

The active pharmaceutical ingredient samples of Emtricitabine, Bictegravir and Tenofovir Alafenamide were obtained from Spectrum Pharma Pvt. Ltd., Hyderabad. All the chemicals and solvents used were HPLC grade. The tablet pharmaceutical dosage of combination of these drugs was purchased from local pharmacy.

### Instrumentation

Waters HPLC (2695 series) with quaternary pumps, Photo Diode array detector and auto sampler integrated with empower software-2 was used for separation of these drugs.

### Chromatographic conditions

Reverse phase HPLC was used for the method development, equipped with UV-Visible detector with Empower software by using BDS $C_8$ (150 x 4.6 mm, 5µ.) and 0.01N $KH_2PO_4$ (pH-3.47): Acetonitrile (50:50 v/v) used as stationary and mobile phases, respectively. Flow rate was optimized to 1.0ml/min and injection volume of 10µl. The results were monitored at 272.0 nm and at ambient column temperature.

### Preparation of potassium dihydrogenortho phosphate buffer (pH: 3.47)

Accurately weighed 1.36gm of Potassiumz dihyrogen Ortho phosphate in a 1000ml of Volumetric flask add about 900ml of milli-Q water added and degas to sonicate and finally make up the volume with water then pH adjusted to 3.47 with dil. Orthophosphoric acid solution.

### Preparation of mobile phase

Mixture of 500 ml of 0.01N $KH_2PO_4$ buffer (pH-3.47) and 500 ml of Acetonitrile in the ratio of 50:50 v/v were mixed and degassed in ultrasonic water bath for 15 minutes and filtered through 0.45 µ filter paper. Mobile phase was used as a diluent.

### Preparation of mixture Standard stock solution(ECB 2000 µg/ml, BTG 500 µg/ml, TAF 250 µg/ml)

Accurately weighed 50 mg of Emtricitabine, 12.5 mg of Bictegravir and 6.25 mg of Tenofovir Alafenamide and transferred to three 25ml volumetric flasks separately. 10ml of Diluent was added to flasks and sonicated for 20mins. Each flask was made up with diluent up to the mark.

### Preparation of Sample (Tablet) stock solutions

20 tablets were weighed and calculate the average weight of each tablet then the weight equivalent to 1 tablet (ECB 200mg, BTG 50 mg & TAF 25mg) was transferred into a 100 mL volumetric flask, 25mL of diluent added and sonicated for 50 min, further the volume made up with diluent and filtered. From the filtered solution 1ml was pipette out into a 10 ml volumetric flask and made up to 10ml with diluent. (ECB 2000µg/ml, BTG 500µg/ml, TAF 250µg/ml).

### Optimized chromatographic conditions:

**Column Used** : BDS $C_8$ (150 x 4.6 mm, 5µ.)

**Mobile phase** : 0.01N $KH_2PO_4$ (pH-3.47): Acetonitrile (50:50 v/v)

**Flow rate** : 1.0ml/min

**Wavelength** : 272.0 nm

**Temperature** : 30° C

**Injection Volume**: 10µl

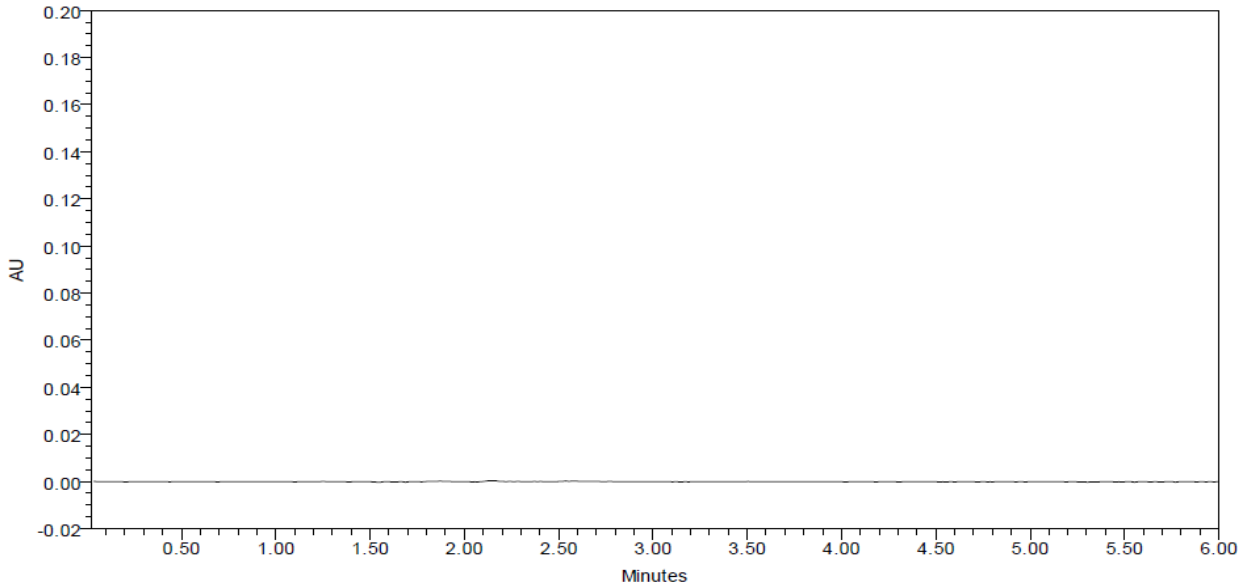


**Figure:** Blank chromatogram

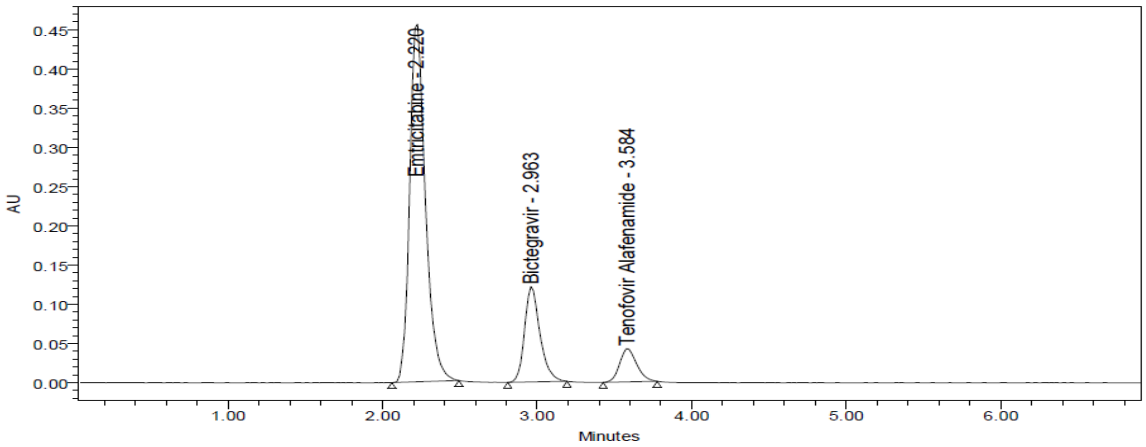


**Figure 3:** Chromatogram of standard mixture of ECB, BTG & TAF

| | Peak Name | RT | Area | USP Plate Count | USP Resolution | USP Tailing |
|---|---|---|---|---|---|---|
| 1 | Emtricitabine | 2.220 | 3216805 | 2415.3 | | 1.2 |
| 2 | Bictegravir | 2.963 | 842138 | 4508.4 | 4.0 | 1.2 |
| 3 | Tenofovir Alafenamide | 3.584 | 323859 | 4939.9 | 3.2 | 1.2 |

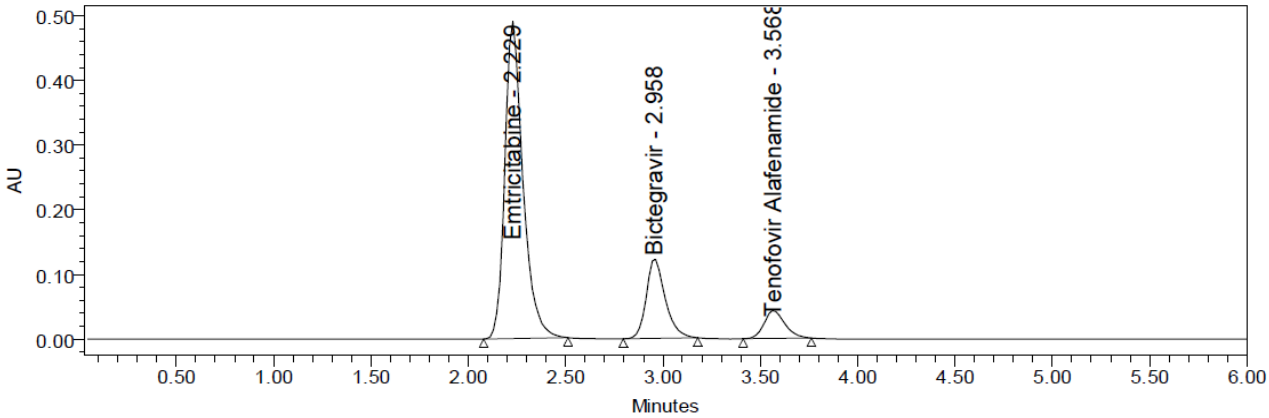


**Figure 4:** Chromatogram of sample mixture of ECB, BTG & TAF

**Validation**

The above optimized chromatographic method has been validated for the assay of ECB, BTG & TAF using the following parameters [International Conference on Harmonization (ICH) 1995]. Linearity was studied to find out the relationship of concentration with Peak area. Six different concentrations of ECB, BTG & TAF drug mixtures (50-300 µg/ml of ECB, 12.5-75 µg/ml of BTG and 6.25-37.5 µg/ml respectively). Each concentration of solution was injected into the HPLC and chromatogram was recorded. The calibration graph was constructed by plotting the peak area versus concentration of the each drug (µg/ml) and the corresponding regression equation derived. Precision was studied to find out variations in the test methods of mixtures of ECB, BTG and TAF150 µg/ml, 37.5µg/ml, 18.75µg/ml respectively. The precision of each method was ascertained separately from the peak area by actual determination of five replicates of a fixed amount of drug (of ECB, BTG and TAF 150 µg/ml, 37.5 µg/ml, and 18.75µg/ml respectively. The %RSD (percentage relative standard deviation) was calculated for precision and ruggedness. The accuracy of the method was shown by analyzing the model mixtures containing 50,100 and 150% of ECB, BTG & TAF. After the measurement, the Amount found and individual recoveries were calculated. Limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated based on the linearity data using the formulae LOD = 3.3×standard deviation /slope; LOQ = 10×standard deviation /slope. Robustness was performed by following the same method with different flow rate.

## RESULTS AND DISCUSSION

The regression equation for ECB was found to be y = 15921x + 10733 (slope, intercept and correlation coefficient were found to be 15921, 10733 and 0.999 respectively) and linear over beer's range of 50-300 µg/ml. The regression equation for BTG was found to be y = 16753x + 2572 (slope, intercept and correlation coefficient were found to be 16753, 2572 and 0.999 respectively) and linear over beer's range of 12.5-75

µg/ml. The regression equation for TAF was found to be y = 12838x + 3003 (slope, intercept and correlation coefficient were found to be 12838, 3003 and 0.999 respectively) and linear over beer's range of 6.25 – 37.5 µg/ml. Linearity graph of ECB, BTG & TAF were shown in Figure 5, 6 & 7 respectively. Linearity data was shown in table 1. The percentages of content of ECB, BTG and TAF in tablet dosage form was100.06 ±0.7%, 99.79 ±0.8% and 100.06 ±0.5% respectively.

**Table 1:** Linearity table for Emtricitabine, Bictegravir and Tenofovir Alafenamide.

| S. NO | Emtricitabine | | Bictegravir | | Tenofovir Alafenamide | |
|---|---|---|---|---|---|---|
| | Conc (µg/mL) | Peak area | Conc (µg/mL) | Peak area | Conc (µg/mL) | Peak area |
| 1 | 50 | 794232 | 12.5 | 217486 | 6.25 | 89862 |
| 2 | 100 | 1594364 | 25 | 413327 | 12.5 | 155999 |
| 3 | 150 | 2431201 | 37.5 | 635572 | 18.75 | 241995 |
| 4 | 200 | 3218742 | 50 | 841431 | 25 | 324871 |
| 5 | 250 | 3942187 | 62.5 | 1037649 | 31.25 | 404393 |
| 6 | 300 | 4800209 | 75 | 1267596 | 37.5 | 485926 |
| Slope | | 15920 | | 16753 | | 12838 |
| Intercept | | 10733 | | 2572.67 | | 3003.2 |
| Correlation coefficient | | 0.999 | | 0.999 | | 0.999 |

**Table 2:** System precision table of Emtricitabine, Bictegravir and Tenofovir Alafenamide.

| S. No | Area of Emtricitabine | Area of Bictegravir | Area of Tenofovir Alafenamide |
|---|---|---|---|
| 1. | 3169647 | 831751 | 317184 |
| 2. | 3199652 | 835849 | 312602 |
| 3. | 3213791 | 848249 | 317712 |
| 4. | 3187746 | 836760 | 315730 |
| 5. | 3169337 | 829457 | 315816 |
| 6. | 3174921 | 829308 | 318509 |
| **Mean** | 3189089 | 835229 | 316259 |
| **S.D** | 18052.4 | 7109.4 | 2091.8 |
| **%RSD** | 0.6 | 0.9 | 0.7 |

**Table 3:** summary of validation data of ECB, BTG and TAF

| Validation | Parameters | Emtricitabine | Bictegravir | Tenofovir Alafenamide |
|---|---|---|---|---|
| Linearity | Range (µg/ml) | 50-300 µg/ml | 12.5-75 µg/ml | 6.25-37.5 µg/ml |
| | Regression coefficient | 0.999 | 0.999 | 0.999 |
| | Slope(m) | 15920 | 16753 | 12838 |
| | Intercept(c) | 10733 | 2572 | 3003 |
| Assay | Mean % content | 100.06 % | 99.79 % | 100.06 % |
| Specificity | | Specific | Specific | Specific |
| System precision | %RSD | 0.6 | 0.9 | 0.7 |
| Method precision | %RSD | 0.6 | 1.1 | 0.4 |
| Accuracy % recovery | % recovery | 100.17 % | 100.14 % | 100.17 % |
| LOD | | 1.40 µg | 0.12µg | 0.15µg |
| LOQ | | 4.24µg | 0.36µg | 0.47µg |

**Table 4:** Degradation data of ECB, BTG and TAF

| S.NO | Degradation Condition | Emtricitabine | | | Bictegravir | | | Tenofovir Alafenamide | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | % Drug Degraded | Purity Angle | Purity Threshold | % Drug Degraded | Purity Angle | Purity Threshold | % Drug Degraded | Purity Angle | Purity Threshold |
| 1 | Acid | 4.45 | 0.223 | 0.276 | 4.29 | 0.392 | 0.432 | 4041 | 0.259 | 0.401 |
| 2 | Alkali | 3.21 | 0.211 | 0.380 | 4.12 | 0.236 | 0.402 | 3.14 | 0.307 | 0.393 |
| 3 | Oxidation | 5.34 | 0.140 | 0.267 | 3.74 | 0.136 | 0.310 | 2.99 | 0.249 | 0.403 |
| 4 | Thermal | 2.25 | 0.106 | 0.275 | 2.89 | 0.136 | 0.307 | 2.62 | 0.247 | 0.398 |
| 5 | UV | 1.11 | 0.120 | 0.274 | 1.85 | 0.132 | 0.302 | 1.19 | 0.231 | 0.390 |
| 6 | Water | 0.89 | 0.155 | 0.276 | 0.69 | 0.134 | 0.301 | 0.31 | 0.238 | 0.386 |

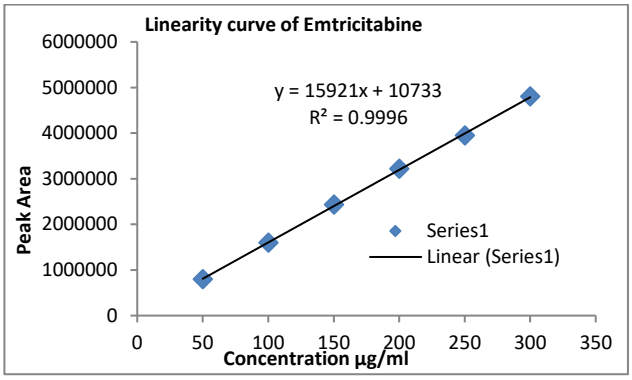


**Figure 5:** Linearity curve of Emtricitabine

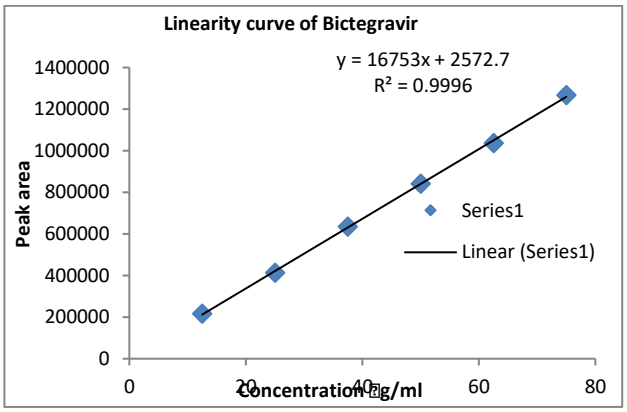


**Figure 6:** Linearity curve of Bictegravir

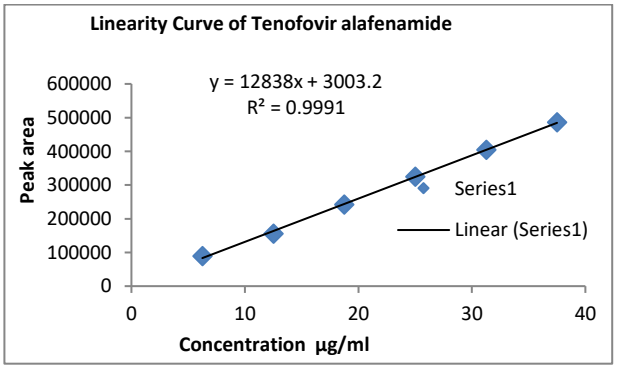


**Figure 7:** Linearity curve of Tenofovir Alafenamide

The precision and ruggedness were determined using the %RSD of the peak area for six replicate preparations of the drug. The %RSD of precision and ruggedness of ECB were found to be 0.6 and 0.6 respectively; for BTG were 0.9 and 1.1 and for TAF 0.7 & 0.4 respectively. The calculated RSD values were less than 2. Precision and ruggedness data are presented in Table 2. In order to verify the accuracy of the described method, recovery studies were carried out by analyzing model mixtures contained 50%, 100% and 150% of standard solution of drug ECB, BTG and TAF and along with 5 µg/mL of placebo solution with in the linearity ranges. The mean percentage recoveries were found to be 100.84±0.0435, 100.66±0.155 and 100.626±0.42 %w/w for 50%, 100% and 150% respectively for ECB. The mean percentage recoveries were found to be 99.91±0.44, 100.44±0.52 and 100.05 ±0.67 %w/w for 50%, 100% and 150% respectively for BTG the mean percentage recoveries were found to be 100.22±0.13, 100.39±0.92 %w/w and 99.9±0.67 for 50%, 100% and 150% respectively for TAF. The results of accuracy were shown that the developed method have a good percentage recovery at different concentrations of drugs. LOD for ECB, BTG and TAF was found to be 1.40 µg, 0.12 µg and 0.15µg respectively. LOQ for ECB, BTG and TAF was found to be 4.24µg, 0.36 µg and 0.47µg respectively. Summary of all the validation parameter shown in table 3.

### Degradation

Degradation studies were performed with the formulation and the degraded samples were injected. Assay of the injected samples was calculated and all the samples passed the limits of degradation. Degradation data shown in table 4.

## CONCLUSION

A simple, accurate, precise method was developed for the simultaneous estimation of the Emtricitabine, Bictegravir, and Tenofovir Alafenamide in Tablet dosage form was developed and the proposed method as suitable for routine analysis of ECB, BTG and TAF.

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