ijlpr 2020; doi 10.22376/ijpbs/lpr.2020.10.5.P90-101

International Journal of Life science and Pharma Research

Research Article

Pharmaceutical analysis for Novel drug formulation

Stability Indicating RP-UPLC Method for Simultaneous Estimation of Emtricitabine, Tenofovir and Bictegravir in Bulk and Pharmaceutical Dosage Form

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Abstract: The Proposed Sensitive, specific, Linear, accurate, precise and Robust method was developed for the simultaneous estimation of the Emtricitabine, Tenofovir Alafenamide and Bictegravir in bulk and Pharmaceutical tablet dosage form by Ultra performance liquid Chromatography. For the Optimised method, Chromatogram was run through AcquityBEH (Bridged Ethylene hybrid) C_{18} 130A° (100mm × 2.1 mm, 1.7µm) column at a flow rate of 0.5 mL/min and pH 3.0 buffer Triethylamine(TEA) was used in this method. The Column oven temperature was maintained at 40 °C and the working wavelength was selected at 280 nm. The retention time of Emtricitabine, Tenofovir Alafenamide and Bictegravir were found to be 2.6 min, 4.3 min and 5.2 min respectively. The percentage RSD (Relative standard deviation) of the method precision for Emtricitabine, Tenofovir Alafenamide and Bictegravir were found to be 0.31%, 0.02% and 0.17% respectively. Percentage Recovery was obtained as 99.8%, 100.2% and 100.7% for Emtricitabine, Tenofovir Alafenamide and Bictegravir respectively. Linearity was obtained as 0.999, 0.999 and 0.999 for Emtricitabine, Tenofovir, Alafenamide and Bictegravir respectively. Analytical Range was found from the linearity and accuracy for Emtricitabine was 100 µg/mL to 300µg/mL, Tenofovir Alafenamide was 12.5µg/mL to 37.5 µg/mL and Bictegravir was 25µg/mL to 75µg/mL. Method is stability indicating because in forced degradation studies all main analytes peak purities were passed and no blank and Placebo interferences at the retention time of the main analytes. Thus the present method was developed by using low-cost solvent in ratio of TEA buffer pH 3.0.: Methanol (45:55 v/v), detected by using photodiode array detector which was highly sensitive to detect at a lower concentration. The use of mobile phase as an extracting solvent makes it more compatible with the developed method. So the proposed research method was sensitive, specific, accurate, precise, linear, and robust and this method can be used for routine and stability analysis in quality control for reduction of time and cost. The existing methods were not cost effective due to the use of highly sophisticated detectors, Expensive (high cost) solvents such as Tetrahydrofuran and some methods were found to be less sensitive.

Keywords: simultaneous estimation**,** Emtricitabine, TenofovirAlafenamide, Bictegravir and Acquity BEH $C_{18;}$ PDA Detector; ICH Validation.

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Recieved On 15 June 2020
Revised On 21 September 2020
Accepted On 23 September 2020
Published On 02 December 2020

Funding This research did not receive any specific grant from any funding agencies in the public, commercial or not for profit sectors.

Citation Kishore Konam, Kanala Somasekhar Reddy , Stability Indicating RP-UPLC Method for Simultaneous Estimation of Emtricitabine, Tenofovir and Bictegravir in Bulk and Pharmaceutical Dosage Form.(2020).Int. J. Life Sci. Pharma Res.10(5), P90-101 http://dx.doi.org/10.22376/ijpbs/lpr.2020.10.5.P90-101

1. 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]-oxathiolane-5-yl)-1H-pyrimidin-2- one[1]. Bictegravir(Figure-2) 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-tetrafluorobenzyl)-12-oxa-2,9-diazabicyclo [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].Tenofoviralafenamidefumarate (TAF) (Figure-3) is a nucleotide reverse transcriptase inhibitor (NRTI) and a novel ester prodrug of the antiretroviral Tenofovir. Tenofovir mimics normal DNA building blocks, but lacks 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-purin9-yl) propan-2-yl]oxy}methyl) (phenoxy) phosphoryl] amino} propanoate[3]. Although Tenofovir (available as Tenofovir disoproxilfumarate) has a good safety profile and efficacy, and is currently a cornerstone of HIV antiviral treatment, but 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]. Thus , an attempt is made method for estimation and development of new chromatographic methods for the estimation of Emtricitabine, Bictegravir and Tenofoviralafenamide in pharmaceutical dosage forms by using RP-UPLC Technique. The objective of our research is to develop simple, precise, accurate, and economical and methods for the analysis of Emtricitabine, Bictegravir and Tenofoviralafenamide in pharmaceutical dosage forms. The literature survey reveals that few analytical methods were available for the estimation of Emtricitabine, Bictegravir and Tenofoviralafenamide in pharmaceutical dosage forms. The early reported methods available for the estimation of Emtricitabine and Tenofoviralafenamide are RP-HPLC[5-9] reverse phase high performance liquid chromatography method [TenofovirDisoproxilFumarate and Emtricitabine RPHPLC[9-11] reverse phase high performance liquidchromatography Method], Application of UV Spectrophotometric Methods for Simultaneous Estimation of Emtricitabine and TenofovirAlafenamideFumarate in Bulk[12], spectrofluorimetric analysis[13], reverse phase high performance liquid chromatography[14-17]. Since there are no official reported methods on Ultra-Performance Liquid Chromatographic (UPLC) methods for the simultaneous estimation of Emtricitabine, Bictegravir and Tenofoviralafenamide in the public domain and proposed method was cost effective and due to this,we have planned to develop a simple, precise, economic and accurate stability indicating Ultra-Performance Liquid Chromatographic (UPLC) method development and validation for the estimation of Emtricitabine, Bictegravir and Tenofoviralafenamide in pharmaceutical dosage form. The early reported methods were not cost effective due to the use of highly sophisticated detectors, costly solvents such as Tetrahydrofuran and some methods were found to be less sensitive. So in the present work a simple, precise, sensitive and stability-indicating method was developed by using low-cost solvent Methanol with buffer in ratio 50:50, detected by using photodiode array detector which was highly sensitive to detect at a lower concentration. The developed method was used for estimation of Emtricitabine, Bictegravir and Tenofoviralafenamide in pharmaceutical dosage form. The use of mobile phase as an extracting solvent makes it more compatible with the developed method.

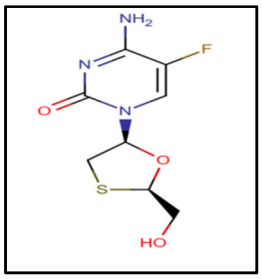

Fig1. Chemical Structure of Emtricitabine

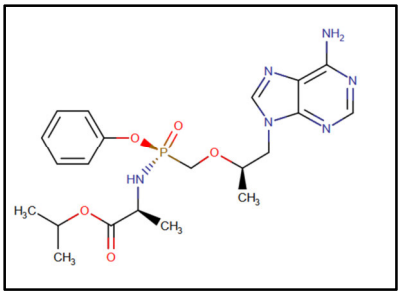

Fig2.Structure of TenofovirAlafenamide

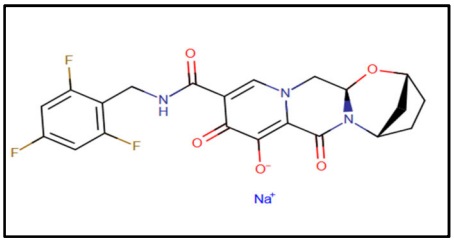

Fig 3.Chemical structure of Bictegravir

UPLC[12-14] is a substantial laboratory approach that reduces prices and improves the analytical overall performance to strengthen and validate the process. UPLC approach will increase the velocity of separation and improves efficiency, ensuring the fast improvement of approaches. UPLC approach reduces solvent consumption and improves pattern fine as properly as offering real-time trying out in line with manufacturing strategies.

2. MATERIALS AND METHODS

2.1 Instrumentation

The 1290 Series UPLC system (Agilent Technologies, Waldbronn, Germany) with PDA detection. Data processing was performed on EZCHROM Elite software package.

2.2 Chromatographic conditions

Acquity BEH C18 130A° (100 × 2.1 mm, 1.7µ) column, TEA buffer pH 3.0.: Methanol (45:55 v/v) mobile phase with a flow-rate of 0.5 mL/min. The column was placed at a temperature of 40^{O}C. 20 µL of sample was injected into the UPLC System. The retention time of Emtricitabine, TenofovirAlafenamide and Bictegravir were found to be 2.6 min, 4.3 min and 5.2 min

2.3 Drug Samples

The standard samples of Emtricitabine, TenofovirAlafenamide and Bictegravirwere procured from Dr.reddy's Laboratories, Hyderabad and Tablet dosage forms (Biktarvy 500 mg Emtricitabine, 200mg TenofovirAlafenamide and 25mg of Bictegravir were purchased from local Market.

2.4 Chemicals and Reagents

Triethylamine (Make :Merck and Grade: AR), Formic Acid (Make :Rankem and Grade: AR), Methanol (Make :Merck and Grade: HPLC) and Acetonitrile (Make :Merck and Grade: HPLC)

2.5 Analytical Methodology

As per ICH guidelines.[18]The method was validated and the parameters like Linearity, Specificity, Accuracy, Precision, Limit of Detection (LOD) and Limit of Quantitation (LOQ) were assessed.

2.6 Specificity

It is the ability of analytical method to measure the response of the analyte and have no interference from other extraneous components and well resolved peaks are obtained.

2.6.1 Linearity 25% Standard solution

0.25ml each from two standard stock solutions was pipetted out and made up to 10ml. (50µg/ml of Emtricitabine, 12.5µg/ml of Bictegravir and 6.25 µg/ml of TenofovirAlfanamide)

2.6.2 50% Standard solution

0.5ml each from two standard stock solutions was pipetted out and made up to 10ml. (100µg/ml of Emtricitabine, 25µg/ml of Bictegravirand 12.5 µg/ml of TenofovirAlfanamide).

2.6.3 75% Standard solution

0.75ml each from two standard stock solutions was pipetted out and made up to 10ml. (150µg/ml of Emtricitabine, 37.5µg/ml of Bictegravir and 18.75 µg/ml of TenofovirAlfanamide)

2.6.4 100% Standard solution

1.0ml each from two standard stock solutions was pipetted out and made up to 10ml. (200µg/ml of Emtricitabine, 50µg/ml of Bictegravir and 25 µg/ml of TenofovirAlfanamide)

2.6.5 125% Standard solution

1.25ml each from two standard stock solutions was pipetted out and made up to 10ml. (250µg/ml of Emtricitabine, 62.5µg/ml of Bictegravir and 31.25 µg/ml of TenofovirAlfanamide)

2.6.6 150% Standard solution

1.5ml each from two standard stock solutions was pipetted out and made up to 10ml (300µg/ml of Emtricitabine, 75µg/ml of Bictegravir and 37.5 µg/ml of TenofovirAlfanamide).

2.7 Accuracy

2.7.1 Preparation of Standard stock solutions

Accurately Weighed and transferred 50mgand12.5mgand6.25mg of Emtricitabine, Bectigravir and TenofovirAlfanamide working Standards into a 25 ml,25ml and25ml clean dry volumetric flask, add 10ml of diluent, sonicated for 15 minutes and make up to the final volume with diluents (2000µg/ml Emtricitabine and 500µg/ml Bictegravir and250µg/ml TenofovirAlfanamide. From the above stock solution.

2.7.2 Preparation of 50% Spiked Solution

0.5ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

2.7.3 Preparation of 100% Spiked Solution

1.0ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

2.7.4 Preparation of 150% Spiked Solution

1.5ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

2.8 Robustness

Small deliberatechanges in method like Flow rate, mobile phase ratio, and temperature are made but we found no recognized changes in the result and are within range as per ICH Guide lines. Robustness conditions like Flow minus (0.9ml/min), Flow plus (1.1ml/min), mobile phase minus (55:45) mobile phase plus (55:55) temperature minus (25°C) and temperature plus (35°C) was maintained and samples were injected in duplicate manner. System suitability parameters were not much affected and all the parameters were passed. Percentage RSD was within the limit.

2.8.1 LOD sample Preparation

0.25ml each from two standard stock solutions was pipetted out and transferred to two separate 10ml volumetric flasks and made up with diluents. From the above solutions 0.1ml each of Emtricitabine, Bictegravir and TenofovirAlfanamide solutions respectively were transferred to 10ml volumetric flasks and made up with the same diluents

2.8.2 LOQ sample Preparation

0.25ml each from two standard stock solutions was pipetted out and transferred to two separate 10ml volumetric flask and made up with diluent. From the above solutions 0.3ml each of Emtricitabin, Bectigravir and Tenofoviralfanamide solutions respectively were transferred to 10ml volumetric flasks and made up with same diluents.

2.9 Optimization of Chromatographic Conditions Working wavelength optimization

To develop and establish a suitable RP-UPLC method for simultaneous estimation of Emtricitabine, Bectegravir and Tenofoviralfanamide in bulk and Tablet dosage forms, different preliminary tests were performed and different chromatographic conditions were tested and optimized chromatographic conditions were developed which were given in Table-1.The final analysis was performed by using 45% : TEA Buffer :55% Methanol at a flow rate of 1.0 ml/min. samples were analyzed at 280nm detector wave length and at an injection volume of 10 µL using Acquity BEH C18 130A° (100 × 2.1 mm, 1.7µwith run time of 10 min. The proposed method was optimized to give sharp peak with good resolution and minimum tailing effect for Emtricitabine, Bictegravir and Tenofoviralfanamide, the optimized chromatogram was obtained as shown in (Figure- 3).

3. STATISTICAL ANALYSIS

We perfomed statistical analysis calibration curve, precision, Accuracy, LOD, LOQ, Robustness, Rugudnessand system suitability parameters. We used manually MS Excel for statistical analysis calculation.

4. RESULTS

4.1 Preparation of Standard solutions[12-14]

Weighed accurately 10mg of each Emtricitabine into 100mL volumetric flask and dissolved with 70mL of Methanol by sonication after cool to Room Temperature and Volume made up with Methanol and mixed well. Further diluted 1mL to 10mL this solution with methanol (10µg/mL of Emtricitabine) Prepared 10µg/mL of TenofovirAlafenamideand Bictegravir solutions in above preparation manner Scanned these solutions in UV Spectrophotometer at 200nm to 400nm using methanol as a Blank. Emtricitabine shown maximum absorbance at 258nm and Bictegravir shown maximum absorbance at 256nm, however Tenofovir has very less absorbance at the region of 256nm to 258nm hence 285nm selected as a Working wavelength. The UV graphs of the Emtricitabine, Bictegravir and Tenofovir were shown at 258 nm,256nm and 285 nm and graphs were shown in Fig 4, Fig 5 and Fig. 6.

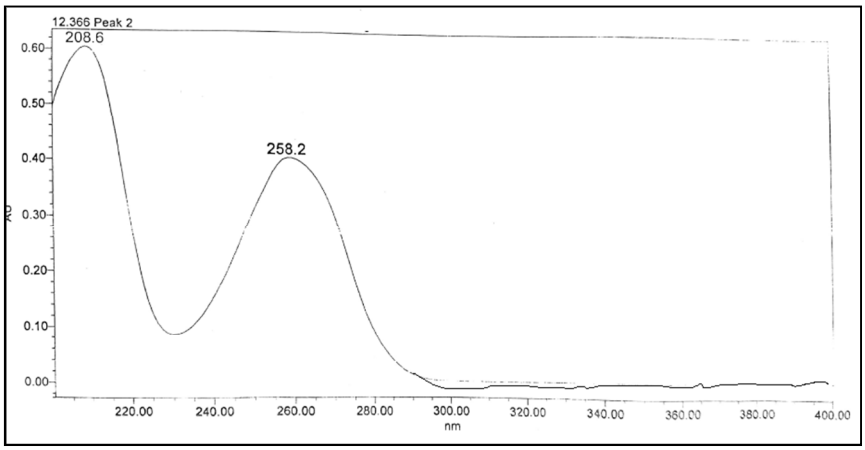

Fig 4: UV graph of Emtricitabine

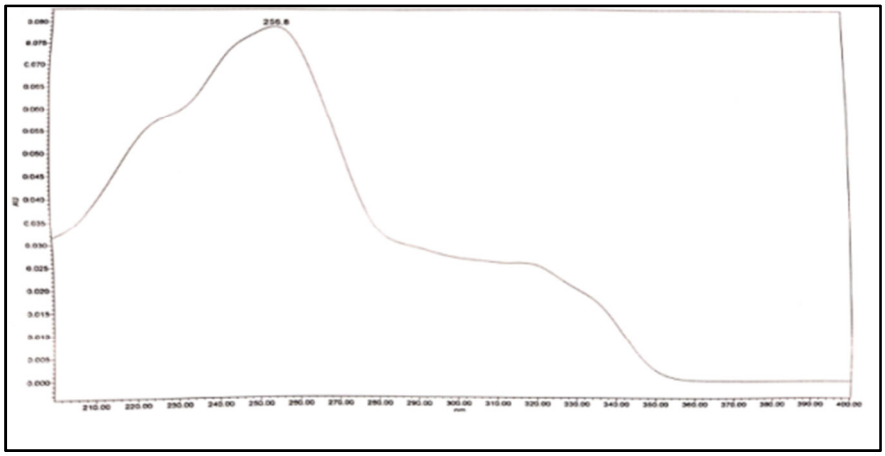

Fig 5: UV graph of Bictegravir

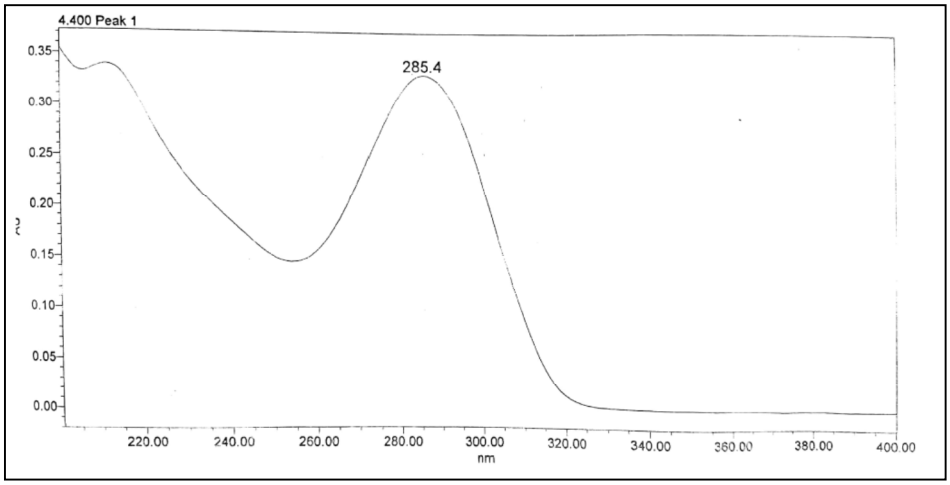

Fig 6: UV graph of TenofovirAlafenamide

4.2 Diluent

Mobile phase used as adiluent(pH 3.0 TEA Buffer: Methanol taken in the ratio of (45:55)

4.3 Preparation of Standard stock

Accurately weighed and transferred 200mg of Emtricitabine, 25mg of TenofovirAlafenamide and 50mg of Bictegravir in to 100mL Volumetric flask, added 70mL of Diluent then kept in sonicatortill the solute is dissolved. Final volume was made up to mark with diluents and mixed well. (Emtricitabine 2000µg/mL, TenofovirAlafenamide 250µg/mL and Bictegravir 500µg/mL)

4.4 Preparation of Standard working solution

Taken 5mL of above standard stock solution into 50mL volumetric flask and then diluted up to mark with diluents and mixed well. (Emtricitabine 200µg/mL, TenofovirAlafenamide 25µg/mL and Bictegravir 50µg/mL)

4.5 Preparation of Sample stock solution

20Tablets were taken and weighed. Average weight of each table calculated from the 20tablets weight and then and transferred into mortar and pestle, crushed into fine powder. Accurately weighed and transferred tablets fine powder equivalent to 200mg of Emtricitabine, 25mg of TenofovirAlafenamide and 50mg of Bictegravir in to 100mL Volumetric flask, added 70mL of Diluent then kept on sonicator up to 30min with intermediate shaking by maintain sonicator temperature 20°C to 25°C , Final volume made up to mark with diluents and mixed well. Centrifuged this sample solution at 5000RPM for 10min.

4.6 Preparation of Sample solution

Taken 5mL of supernatant of sample stock solution into 50mL volumetric flask then diluted up to mark with diluents and mixed well. Filtered through 0.45µ PVDF filter.

4.7 Preparation of pH 3.0Buffer

Accurately taken 2mL of Triethylamine and Transferred into 1000mL of water, mixed well and adjusted pH 3.00 with diluted formic acid. Filtered through 0.45µm Nylon membrane filter

4.8 Diluted formic acid Preparation

Dilute 5mL of Formic acid 20mL with water and mixed well.

4.9 Preparation of Mobile Phase

Weighed Accurately and Transferred in to 450mL of Buffer and 550mL of Methanol in a 1000mL of mobile phase bottle and mixed well. Degassed by sonication 5min.

4.10 Optimized Condition

The optimum chromatographic conditions were obtained with several trial and error methods and the optimum chromatographic conditions were shown in Table 1 and the optimum chromatogram was shown in Fig.7.

Table 1:*Optimised Chromatographic condition*

Column	Acquity BEH C18 130A° (100 × 2.1 mm, 1.7µ)
Mobile Phase and Composition	pH 3.0 TEA Buffer:Methanol(45:55)
Flowrate	0.5mL/min
Column ovenTemperature	40 °C
Injection volume	5µL
Detection wavelength	280nm
Autosampler Temperature	25°C
Retention Times	2.60min of Emtricitabine, 4.27min for Tenofovir 5.25min for Bictegravir (Total Runtime 8.0min)

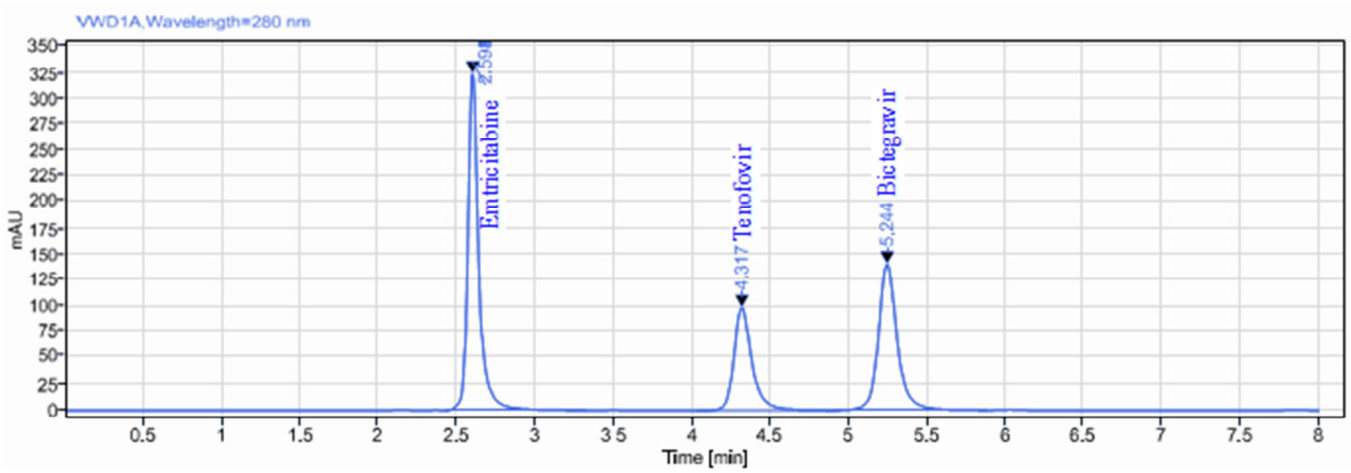

Fig7: Typical Optimized Chromatogram of EMT,TEN and BCT

4.11 Method Validation

Analytical method validation as per ICH: Method validation is a process of documenting/proving that an analytical method provides analytical data acceptable for the intended use

4.12 System Suitability and System Precision

By preparing standard solutions of Emtricitabine(200ppm), Bictegravir (50ppm)and Tenofoviralfanamide (25ppm) the system suitability parameters were determined and the solutions were injected six times and the parameters like peak tailing, resolution and the USP theoretical plate count were assessed to check whether the results complies with Recommended limits.

4.13 Specificity

So this method holds its specificity. Three levels of Accuracy samples 50%, 100%, 150% were prepared and triplicates of injections were given for each level of accuracy and mean percentage recovery was obtained as 100.02%, 99.99% and 99.99% for Emtricitabine, Bictegravir and Tenofoviralfanamide respectively. For System Precision, percentage for retention times and Area for the six replicate injections should not be more than 2.0 of each analyte and the results for the system suitability were summarized in Table 2 and the results for the system precision were summarized in Table 3.

Table 2: System Suitability Results

	EMT			TEN				BCT			
S.No	Rt (min)	USP Plate count	Tailing Factor	Rt(min)	Plate count	Tailing Factor	Resolution	Retention time (min)	Plate count	Tailing Factor	Resolution
1	2.601	3501	1.48	4.277	3105	1.40	5.7	5.248	4238	1.2	3.4
2	2.601	3503	1.48	4.281	3108	1.39	5.7	5.246	4239	1.2	3.4
3	2.600	3501	1.49	4.285	3106	1.40	5.7	5.246	4200	1.2	3.4
4	2.600	3548	1.47	4.289	3105	1.38	5.7	5.244	4239	1.2	3.4
5	2.600	3502	1.48	4.291	3108	1.40	5.7	5.243	4240	1.2	3.4
6	2.599	3508	1.48	4.294	3107	1.40	5.7	5.242	4240	1.2	3.4

Table 3: Precision results

	EMT		TEN		BCT	
S.No	Rt	Peak Area	Rt	Peak Area	Rt	Peak Area
1	2.601	1524.48	4.277	759.88	5.248	1157.69

2	2.601	1525.29	4.281	759.97	5.246	1157.47
3	2.600	1516.22	4.285	760.25	5.246	1156.93
4	2.600	1523.34	4.289	759.86	5.244	1156.77
5	2.600	1515.63	4.291	759.92	5.243	1161.60
6	2.599	1516.10	4.294	760.04	5.242	1156.09
AVG	2.600	1520.18	4.286	759.99	5.245	1157.75
%RSD	0.03	0.31	0.15	0.02	0.04	0.17

4.14 Observation

Percentage RSD for Retention times and Areas of six standard replicate injections was observed below 2.0%, hence the system is precise.

4.15 Specificity

Specificity is the ability to unequivocally assess the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrices, etc.The blank and Placebo solution was injected into the UPLC system and the Blank and placebo solution was not shown any interference at the retention time of the main peaks. The blank and placebo chromatograms were shown in fig.8 and Fig 9respectively.

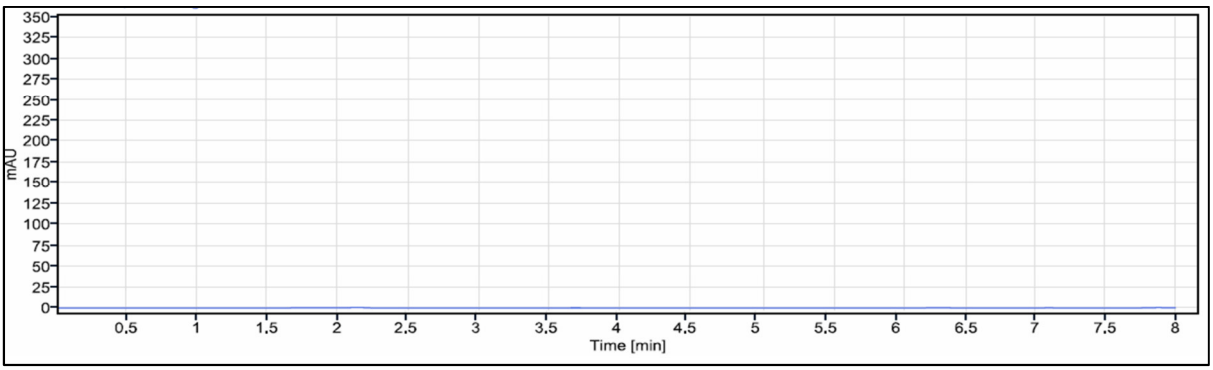

Fig 8: Blank Chromatogram

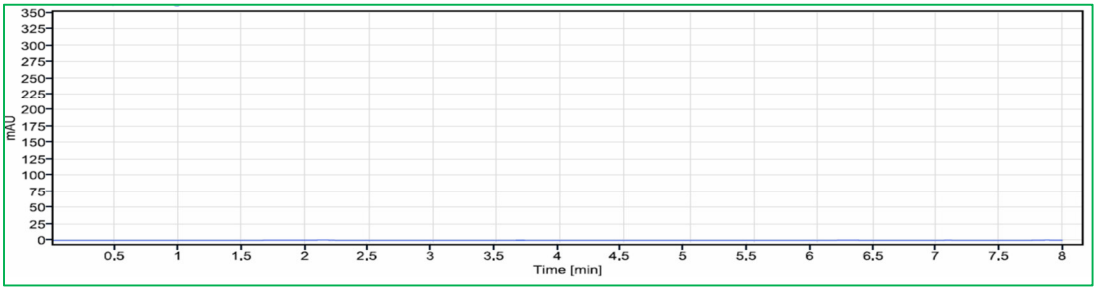

Fig 9:Placebo Chromatogram

4.16 Degradation study

Degradation studies also as a part of Specificity Parameter, Samples were treated with Acid (5N HCl/4Hrs/60°C), Base (5N NaOH/4Hrs/60°C), Peroxide (30% Hydrogen Peroxide for 4Hrs at Bench top), Thermal (80°C/24Hrs) and Photolytic condition. In base treatment, sample degradation occurred 28.6% for Emtricitabine, In remaining all conditions treated samples have shown less degradation (below 5%). Peak purity was also achieved for main analytes.(All Main Analytes showed a single point threshold value is less than peak purity index hence peaks were Pure) . The results for the degradation studies were conducted in different parameters and the results for the all samples were shown in Table-4 and the chromatographic graphs of the acid treated , base treated, Peroxide treated, Photolytic treated and thermal treated samples were shown in fig -10, fig-11,fig-12,fig-13 and Fig-14.

Table 4: *Degradation of Sample*

Degradation Condition	%Assay after Degradation		
	Emtricitabine	Tenofovir	Bictegravir
Control Sample	99.8	98.8	100.1
Acid	97.5	98.2	99.2
Base	72.4	97.6	97.8
Peroxide	100.2	97.2	98.4
Photolytic	99.4	100.1	99.1
Thermal	99.7	99.6	98.9

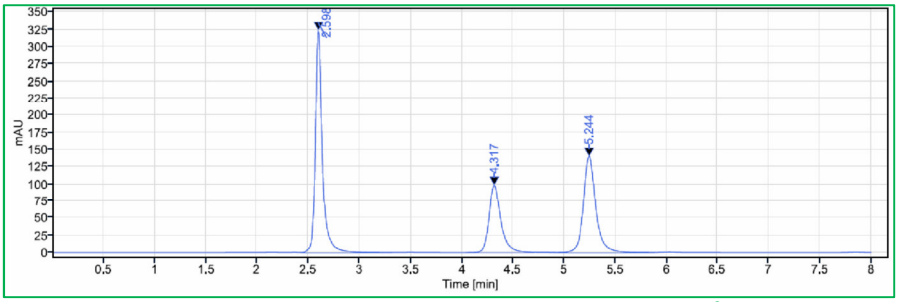

Fig 10: Acid Treated Sample (5N HCl/4Hrs/60°C)

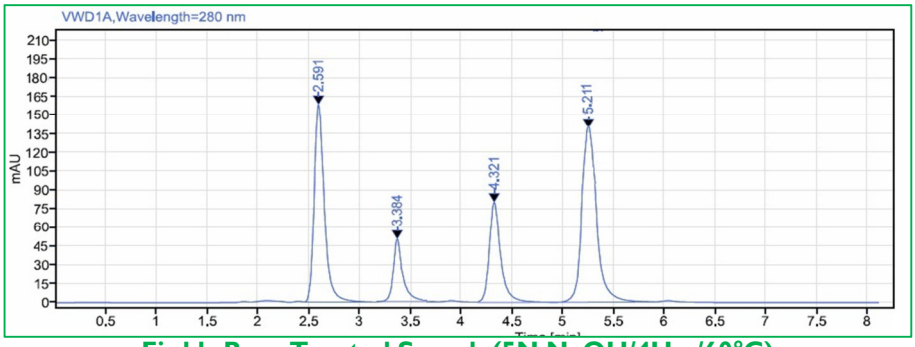

Fig11: Base Treated Sample(5N NaOH/4Hrs/60°C)

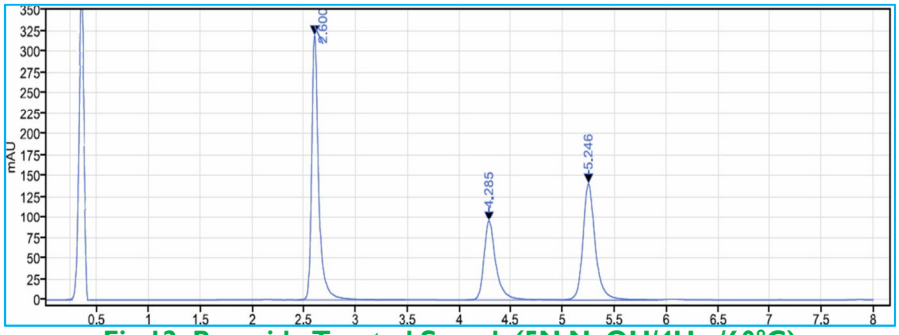

Fig 12: Peroxide Treated Sample(5N NaOH/4Hrs/60°C)

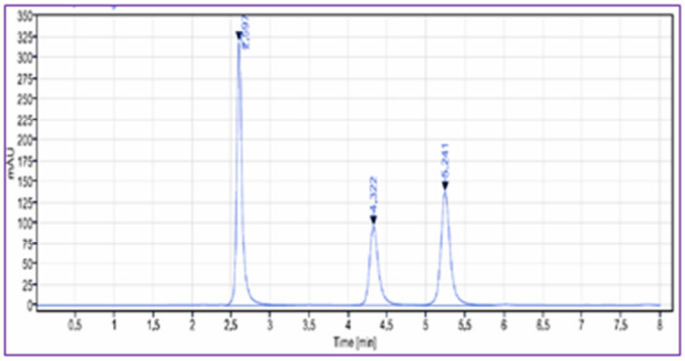

Fig13: Photolytic Treated Sample

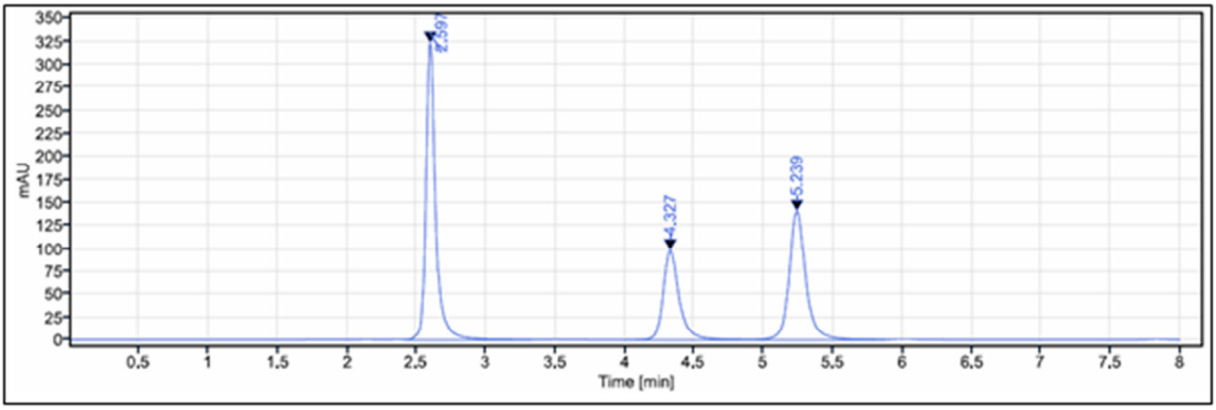

Fig.14: Thermal Treated Sample

4.17 Method Precision

Six sample solutions were prepared individually and injected into the UPLC System, Calculated percentageAssay against average area of Six Standards of Emtricitabine, Tenofovir and Bictegravir individually. Mean percentage Assay was obtained between 90 to 110% individual preparation and percentage RSD for percentageAssay of six replicate preparations was obtained below 2.0. The percentage RSD for percentage Assay of all peak area and retention time of Emtricitabine, Tenofovir and Bictegravir were calculated and summarized in the Table -5.

Table 5: Method Precision

Name of the Sample	%Assay of Emtricitabine	%Assay of Tenofovir	%Assay of Bictegravir
Method Precision-01	102.0	99.4	100.3
Method Precision-02	100.3	98.5	99.4
Method Precision-03	100.5	98.6	99.5
Method Precision-04	100.0	98.7	99.6
Method Precision-05	100.5	98.7	99.6
Method Precision-06	100.1	98.8	99.7
Standard Deviation	0.720	0.322	0.340
Average	100.545	98.787	99.673
%RSD	0.72	0.33	0.34

4.17.1 Accuracy

Three levels of accuracy samples were prepared in triplicate by standard addition method injected each level of Accuracy and mean percentage recovery was obtained 99.8% for Emitricitibine, 100.2% for Tenofovir and 100.7% for Bictegravir respectively. The Accuracy, Recovery and percentage RSD of Emtricitabine, Tenofovir and Bictegravir were calculated and summarized in the table -6.

Table 6: Details of Accuracy

Name of the Level	Emtricitabine	Tenofovir	Bictegravir
50% Accuracy	100.9	100.6	100.6
100% Accuracy	99.7	100.4	101.2
150% Accuracy	98.7	99.5	100.2
Mean	99.8	100.2	100.7
%RSD	0.94	0.57	0.42

4.17.2 Linearity

Five linearity solutions (50%, 80% 100%, 120% and 150%) were prepared from standard stock solution i.e., 100µg/mL to 300µg/mL for Emtricitabine, 12.5µg/mL to 37.5µg/mL for Tenofovir and 255µg/mL to 75µg/mL for Bictegravir. The Correlation coefficient was found 0.999 for Emtricitabine, Tenofovir, Alafenamide and Bictegravir. The linearity of different concentrations of the Emtricitabine, Tenofovir and Bictegravir were calculated and summarized in the Table -7 and graphs were shown in Fig.15-17.

Table 7: Calibration curve details of Emtricitabine,Tenofovir and Bictegravir

Emtricitabine		Tenofovir		Bictegravir	
Conc. in µg/mL	Peak Area	Conc. in µg/mL	Peak Area	Conc. in µg/mL	Peak Area
100	769.660	12.5	380.530	25	582.290
160	1225.960	20	611.190	40	934.520
200	1513.960	25	756.300	50	1155.200
240	1810.030	30	899.730	60	1380.190
300	2260.800	37.5	1129.990	75	1732.650
Correlation coefficient	0.999	Correlation coefficient	0.999	Correlation coefficient	0.999

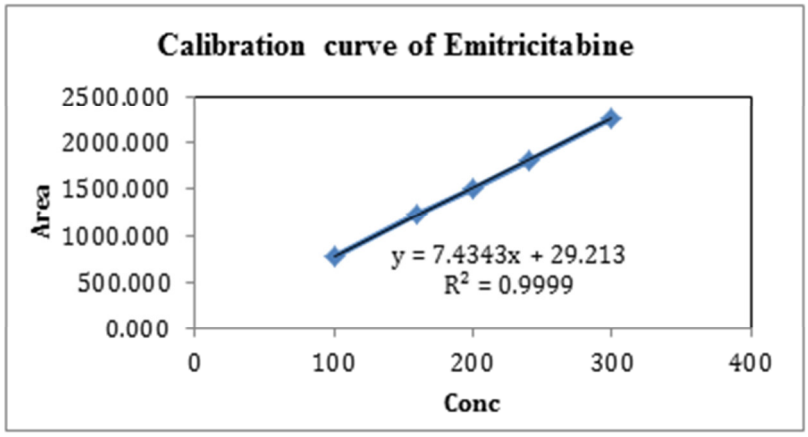

Fig 15: Calibration Curve of Emtricitabine

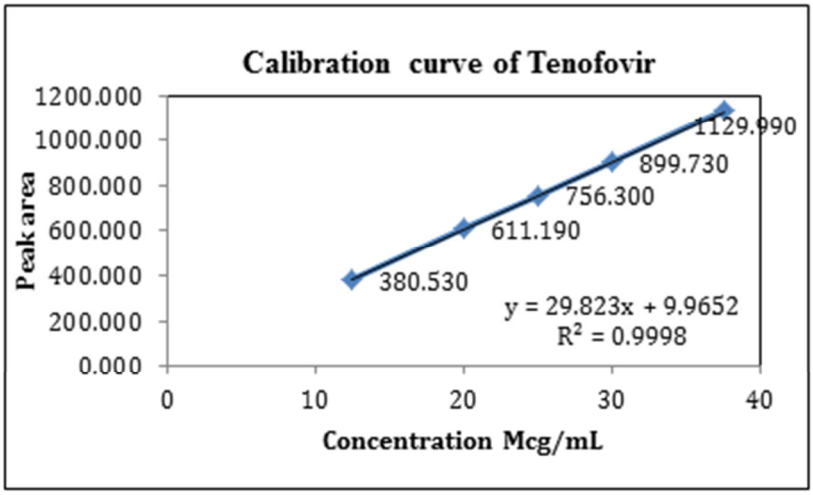

Fig16: Calibration Curve of Tenofovir

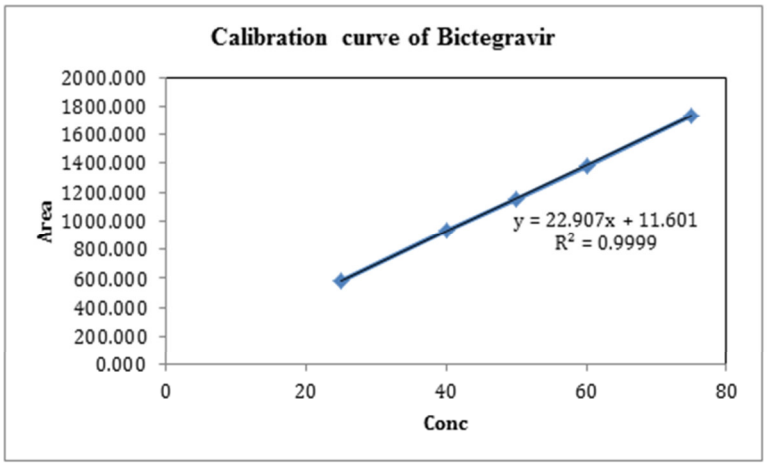

Fig 17: Calibration Curve ofBictegravir

4.17.3 LOD and LOQ

Name of Drugs	LOD (µg/mL)	LOQ(µg/mL)
Emtricitabine	3.1	9.1
Bectegravir	0.6	2.7
TenofovirAlfanmide	0.3	1.4

4.17.4 Robustness

Robustness conditions like flow (0.5mL/min±0.1mL) and Wavelength (280nm±5nm) were maintained in the UPLC System and injected Six standards replicate injections in each condition. System suitability parameters were within the acceptance criteria, percentageRSD for Area of six standard injections for within the limit. The Robustness of the different conditions (Flow rate and wavelength) were conducted, calculated and summarized in the table-8.

Table8: Robustness

S. No.	Condition	Percentage RSD for Emtricitabine	Percentage RSD for Tenofovir	Percentage RSD for Bictegravir
01	Flow Rate_0.4mL/min	0.3	0.2	0.2
02	Flow Rate_0.6mL/min	0.2	0.5	0.1
03	Wavelength (275nm)	0.7	0.4	0.6
04	Wavelength (285nm)	0.4	0.6	0.9

In order to develop and establish a suitable UPLC method for simultaneous estimation of Emtricitabine, Bictegravir and Tenofoviralafenamide in bulk and Tablet dosage forms, different preliminary tests were performed and different chromatographic conditions were tested and optimized chromatographic conditions were developed which were given in Table-1.The final analysis was performed by using 45% TEA Buffer:55% Methanol at a flow rate of 1.0 mL/min. samples were analyzed at 285 nm detector wavelength and at an injection volume ofwithm10 µL. using Acquity BEH(Bridged Ethylene hybrid) C_{18} 130A° (100mm × 2.1 mm, 1.7µm) column 5 run time of 10 min. The proposed method was optimized to give a sharp peak with good resolution and minimum tailing effect for Emtricitabine, Bictegravir and TenofovirAlafenamide, the optimized chromatogram was obtained as shown in (Figure-7).Mixtures of commonly used solvents like water, methanol and acetonitrile with or without buffers in different combinations were tested as mobile phases. The choice of the optimum composition is based on the chromatographic response factor, a good peak shape with minimum tailing. A mixture of Tetra Ethyl Amine buffer (pH 3.0 and methanol adjusted with OPA) in the ratio of 45:55 v/v was proved to be the most suitable of all the combinations since the chromatographic peak obtained was well defined, better resolved and almost free from tailing. The retention times of the Emtricitabine, TenofovirAlafenamide and Bictegravir was found to be 2.6 min, 4.3 min and 5.2min. The linearity was found satisfactory for the drugs in the range 50 to 250µg/ml for Emtricitabine, Tenofoviralafenamide 12.5µg/mL to 37.5µg/mL and Bictegravir was 25µg/mL to 75µg/mL. The regression equation of the linearity curve between concentrations of Emtricitabine over its peak areas were found to be y = 7.434x + 29.21 (where Y is the peak area and X is the concentration of TenofovirAlafenamidein µg/mL), y = 29.82x + 9.965 and y = 22.90x + 11.60 (where Y is the peak area and X is the concentration of Bictegravir in µg/mL) respectively. Precision of the method was studied by repeated injection of tablet solution and results showed lower percentage RSD values. This reveals that the method is quite precise. The percent recoveries of the drug solutions were studied at three different concentration levels. The percent individual recovery and the percentage RSD at each level were within the acceptable limits. This indicates that the method is accurate. The absence of additional peaks in the chromatogram indicates non-interference of the commonly used excipients in the tablets and hence the method is specific. The deliberate changes in the method have not much affected the peak tailing, theoretical plates and the percent assay. This indicates that the present method is robust. The system suitability studies were carried out to check various parameters such as theoretical plates and tailing factor. The lowest values of LOD and LOQ as obtained by the proposed method indicate that the method is sensitive. The solution stability studies indicates that both the drugs were stable up to 24 hours. The forced degradation studies indicate that both the drugs were stable in stability studies.

5. CONCLUSION

A simple, accurate, precise method was developed for the simultaneous estimation of the Emtricitabine, TenofovirAlafenamide and Bictegravir in Tablet dosage form. For the method development, percentage RSD of the Emtricitabine, TenofovirAlafenamide and Bictegravir were found to be 0.3, 0.1 and 0.2% respectively in system precision. In the method precision sample has shown precise percentage Assay results. The Assay also found between 90.01 to 110.02%. The percentage Recovery was obtained as 99.8%, 100.2% and 100.7% for Emtricitabine, TenofovirAlafenamide and Bictegravir respectively. Linearity was obtained as 0.9995, 0.9994 and 0.9997 for Emtricitabine, TenofovirAlafenamide and Bictegravir respectively. The developed stability indicating technique was once validated efficiently for simultaneous estimation of Emtricitabine, Tenofovir and Bictegravir. The proposed approach used to be examined for its accuracy, linearity, precision, robustness, and degradationstudies. The compelled degradation research had been carried out, and the mentioned technique efficiently separates the drug supplies besides interference of excipients and degradation products. Hence, it can be concluded that the developed technique can be used for the pursuit evaluation of Emtricitabine,Tenofovir and Bictegravir in the pharmaceutical dosage form.

6. ACKNOWLEDGEMENTS

Authors are very thankful to the Chandra Lab, my guide Dr.Somasekhar Reddy Konala and the management of Raghavendra Institute of Pharmaceutical Education and Research, Ananthapuramu, Andhra pradesh, for providing the necessary facilities to carry out the research work.

7. AUTHORS CONTRIBUTION STATEMENT

Concept and Supervision, Analysis and/or Interpretation and Critical Reviews were done by Dr. Y. Padmanabha Reddy and Dr.Kumaraswamy Gandla. Literature Search, Collection of Materials, datas, data processing and writing was done by Dr. Somasekhar Reddy Kanala.

8. CONFLICT OF INTEREST

Conflict of interest declared none.

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