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METHOD DEVELOPMENT AND VALIDATION BY RP-HPLC FOR SIMULTANEOUS ESTIMATION OF EMTRICITABINE, BICTEGRAVIR, TENOFOVIR ALAFENAMIDE IN FIXED DOSAGE FORM

R. Meenakshi[1] and Dr. R. Shyam Sunder[2]

[1]Department of Pharmaceutical Chemistry, Sarojini Naidu Vanitha Pharmacy Mahavidyalaya, Affliated to Osmania University,

[2]University College of Technology, Osmania University, Hyderabad.

Abstract:

*Reverse phase HPLC method which is simple, precise, reproducible, accurate was developed for the simultaneous estimation of ETC, BTG, TFRA in fixed combination of formulation drug All the three analytes were separated efficiently using Inertsil 30V C 18 Column (250*4.6mm, 5 micron) using buffer phosphoric dihydrogen phosphate as mobile phase A and methanol and water (70:30) as mobile phase B. Acetonitrile and buffer are used as diluents. The analysis was performed at a wavelength of 265 nm, flow rate of 1.5ml/min and injection volume of 10µl.Linearity was good within the designed concentration range. System suitability was achieved as RSD values were <2. LOD values for ETC, BTG, TFRA were 0.2µg/ml, 0.5µg/ml, 0.0025µg/ml and LOQ values were 0.6µg/ml, 1.5µg/ml, 0.0075µg/ml respectively. Precision and accuracy was achieved as RSD values and percentage recovery was within in the limits i.e., <2 and within 100%. Robustness for the method was achieved using three parameters including pH of the mobile phase, flow rate and different column. In these varied conditions, all three analytes were eluted, resolved and there was no change in their retention times. This proves that this reverse HPLC method developed was simple, accurate, precise and robust for the simultaneous estimation of ETC, BTG, TFRA.*

Key words: *Emtricitabine, bictegravir, tenofovir alafenamide, gradient -HPLC*

Corresponding author:

R. Meenakshi,

Deparment of Pharmaceutical chemistry
Sarojini Naidu Vanitha Pharmacy Mahavidyalaya
H.No. 12-5-31 & 32; Vijaypuri colony
Secunderabad (T.S)
Pin code: 500017
Mobile No. 9052314825
Email id: meenakshi.rajapaga@gmail.com

QR code

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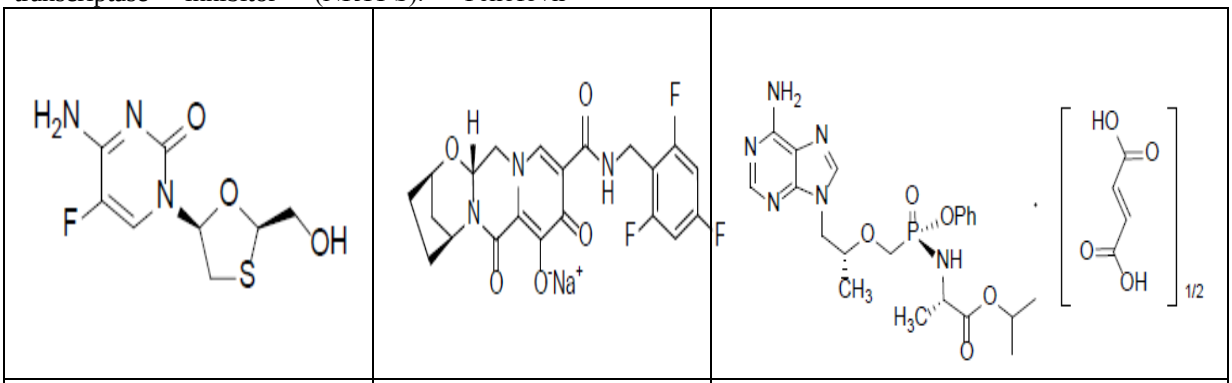
INTRODUCTION:

Biktarvy® is a trio fixed dose combination of a drug, one pill once a day prescription mainly used in the treatment of HIV-1 infection [1]. It is a combination of 50mg of bictegravir (equivalent to 52. mg of bictegravir sodium), 200mg of emtricitabine and 25mg of tenofovir alafenamide (equivalent to 28mg tenofovir alafenamide fumarate) [2]. Bictegravir (BTG) [3] i.e., bictegravir sodium chemically it is 2,5-Methanopyrido[1',2':4,5]pyrazino[2,1-b][1,3]oxazepine-10-carboxamide,2,3,4,5,7,9,13,13a-octahydro-8-hydroxy-7,9-dioxo-N-[(2,4,6-trifluorophenyl)methyl]-, sodium salt (1:1), (2R,5S,13aR) and it works as a integrase strand inhibitor. Emitricitabine (ETC) [4] is 4-amino-5-fluoro-1-(2R-hydroxymethyl-1,3 oxathiolan-5S-yl)-(1H)-pyrimidin-2-one. It is a levo isomer of a thio analog of cytidine and it is same as other cytidine analogs but only differs at position 5 having fluorine works as a nucleoside reverse transcriptase inhibitor (NRTI'S). Tenofovir alafenamide (TFRA) [5] is L-alanine,N-[(S)-[[(1R)-2-(6-amino-9H-purin-9-yl)-1methylethoxy]methyl]phenoxyphosphinyl]-,1-methylethyl ester, (2E)-2-butenedioate (2:1) and works as a nucleoside reverse transcriptase inhibitor(NRTI).

Study of literature has revealed many analytical techniques for method development and validation for simultaneous estimation of tenofovir and emtricitabine a combination drug [6-9]. On the contrary, to the best of our insight there is no method disclosing the simultaneous estimation of BTG, ETC, TFRA in pharmaceutical formulations. In this paper for the foretime we report reverse phase HPLC for simultaneous estimation of Emtricitabine, Bictegravir and Tenofovir alafenamide in fixed dosage form. The new method is effective in separating all three active ingredients. Validation of the method is performed according to ICH guidelines.



Emtricitabine	Bictegravir	Tenofovir alafenamide

Figure 1: Chemical structures of emtricitabine, bictegravir, tenofovir alafenamide.

EXPERIMENTAL:

Chemicals and reagents

ETC, BTG, TFRA are acquired as a munificent gift samples from Mylan Laboratories Limited, Hyderabad. Phosphoric dihydrogen phosphate and ortho phosphate are obtained from Merck, Mumbai, India. All the chemicals used were HPLC grade and prepared in Milli Q water (Millipore, USA). Test samples composed of Biktarvy® oral tablet contains 50mg of BTG (equivalent to 52.5mg of BTG sodium), 200mg of ETC and 25mg of TFRA (equivalent to 28mg of tenofovir alafenamide fumarate).

HPLC instrumentation and Optimized chromatographic conditions

Reverse phase HPLC was used for the method development, equipped with UV-Visible detector with Empower software by using Inertsil ODS 3V C 18 column (250*4.6mm, 5 micron). Gradient mixture of solvent A and solvent B were used as stationary and mobile phases, respectively. Buffer of 0.03M Potassium dihydrogen orthophosphate whose pH was adjusted to 3.2 with dilute ortho phosphoric acid was used as solvent A and methanol and water are used as solvent B. The gradient program (T/%B) was given in Table 1. Flow rate was optimized to 1.5ml/min and injection volume of 10µl. The results were monitored at 265nm and at ambient column temperature. Data acquiring, analysis and reporting was done using Empower 2 (Waters) chromatography software.

Table 1: Gradient Elution program for separation of emtricitabine, bictegravir, tenofovir alafenamide.

Time	Mobile phase-A	Mobile phase-B
0	90	10
3	90	10
4	30	70
6	10	90
11	10	90
13	90	10
15	90	10

Solutions for validation study:
Standard solutions**:** Standard solutions were prepared by taking 200mg of ETC, 50mg of BTG, 25mg of TFRA in a 50ml volumetric flask, dissolved and diluted with diluent (acetonitrile and buffer in the ratio of 70:30). From the above solution 5ml was taken and diluted with diluent to 50ml in the volumetric flask.

Sample solutions: Biktarvy® tablet powder equivalent to 485 mg containing 200mg of ETC, 50mg of BTG and 25mg of TFRA was weighed and transferred to 50ml volumetric flask and dissolved and diluted to volume with diluent. From the above solution 5ml was transferred to 50ml volumetric flask and diluted with diluent to obtain concentration of 400µg/ml ETC, 100µg/ml BTG, 50µg/ml TFRA.

Method validation
The developed chromatographic method was validated for selectivity, precision, linearity, accuracy, sensitivity, robustness and system suitability.

Specificity
Interference between drugs and tablet excipients were evaluated from the comparison of spectral purity obtained from the analysis for the standard solutions and sample solutions.

System suitability
The effectiveness of the system was analysed using drugs at concentrations of 400µg/ml ETC, 100µg/ml BTG, 50µg/ml TFRA. Reproducibility and repeatability for the method was acceptable for the parameters peak area, retention time and theoretical plates. % RSD was within the acceptance limits.

Linearity
Linearity was measured by taking five different standard concentrations in the range of 40% to 120% i.e., 160µg-480µg/ml of ETC, 40µg-120µg/ml of BTG, 20µg-60µg/ml of TFRA. Linear calibration graph was plotted using peak areas of drug to drug concentrations. It was evaluated by linear regression analysis and data is reported in Table 3.

Precision
Repeatability and reproducibility of the method was assessed for concentrations of drugs at 800µg/ml ETC, 100µg/ml BTG, 1200µg/ml TFRA and 4500µg/ml of formulation sample drug Biktarvy® in semestral injections. The means and % RSD was calculated from peak areas.

Accuracy
Actualness or credibility of the method was calculated using % recovery method by spiking known amount of standard concentrations at range of 80%, 100%, 120% to the formulation drug sample in triplicate injections.

LOD and LOQ
The lowest concentration of analyte that can be detected and lowest concentration of sample that can be quantified in the drug formulation is assessed using series of concentrations. LOD and LOQ were calculated as signal to noise ratio of 3:1 and 10:1 according to ICH guidelines.

Robustness
The efficiency of the method was studied by changing the HPLC parameters from normal procedural conditions including flow rate was 1.5ml/min which was changed by ±0.1 units to 1.4 to 1.6ml/min. Different LC columns from different batches were used to study the effect of stationary phase. The buffer effect was studied at pH 2.7 to 3.7 (±0.5).

These HPLC parameters were assessed for resolution between ETC, BTG, TFRA, in system suitability with respect to RT and % assay of drugs.

RESULTS AND DISCUSSION:
HPLC method development

All three drug solutions were prepared using diluent and scanned under UV –visible spectrophotometer which showed 265nm as the UV maxima for all the three drugs. Therefore detection at 265nm was selected for performing method development. For a good chromatographic separation, the parameters like pH of mobile phase, concentration of the acid or buffer solution, percentage and type of organic modifier were tested. Trials displayed that reverse phase HPLC column gives sharp and symmetric peaks with acidic mobile phase buffer. For this purpose, potassium dihydrogen orthophosphate buffer with pH adjustment with dilute orthophosphoric acid to 3.2 was preferred as acidic buffer solution. Methanol was used as organic modifier because of its good solubility parameters with drugs. Good resolution was observed for mobile phase composition in a gradient mode at flow rate of 1.5ml/min. Then to separate all three analytes the method was optimized by changing to gradient mode. Several gradient conditions were tried before optimizing the final gradient program as: time(min)/%solutionB:0/10,3/10,4/70,6/90,11/90,13/10,15/10. The chromatographic separation was satisfactory with good shape peaks achieved on Inertsil ODS 3V C18 column using 0.03M Potassium dihydrogen Orthophosphate buffer (adjustment of pH to 3.2 with dilute orthophosphoric acid) as solvent A and methanol and water as solvent B with flow rate of 1.5ml/min and injection volume of 10µl. Acetonitrile and buffer (70:30) was used as diluents. In this optimised gradient conditions ETC, BTG and TFRA were separated efficiently with resolution <2.

Method validation

The optimized method was validated for the following parameters selectivity, system suitability, linearity, accuracy, precision, robustness, LOD and LOQ.

Selectivity

Three analytes were separated efficiently depicting good selectivity of the method. The ideal chromatogram of fixed pharmaceutical dosage form containing excipients showed no peak interfering with analytes as they have no absorbance. Furthermore all the three analytes were separated with good resolution factor. This indicates the selectivity of the method for the analytes peaks as excipients did not interfered.

System suitability

RT and % RSD values of peak area for analytes were within <2% thus the system suitability was achieved.

Table 2: Results from regression analysis and System Suitability data

Parameter	Emitricitabine	Bictegravir	Tenofovir Alafenamide
Retention Time (min)	6.278	10.487	8.439
Tailing Factor	1.10	1.08	1.14
Theoretical Plates	52146.37	57811.40	32612.16
HETP	$4.79419*10^{-6}$	$4.3244*10^{-6}$	$7.66585*10^{-6}$
USP plates/meter	208585.48	231245.6	130448.64
Resolution	----	10.22	16.58
Linear range in (µg/mL)	160-480	40-120	20-60
Limit of Detection (LOD) (µg/mL)	0.2	0.5	0.0025
Limit of Quantification (LOQ) (µg/mL)	0.6	1.5	0.0075
Slope(m)	75492.65	51630.35	322519.46
Intercept©	387925	17571.2	349679
Correlation Coefficient (r)	0.997	0.999	0.995
Method Precision (RSD, %, n=6)	0.4	0.3	0.2
% of Assay	98.60	99.39	98.68

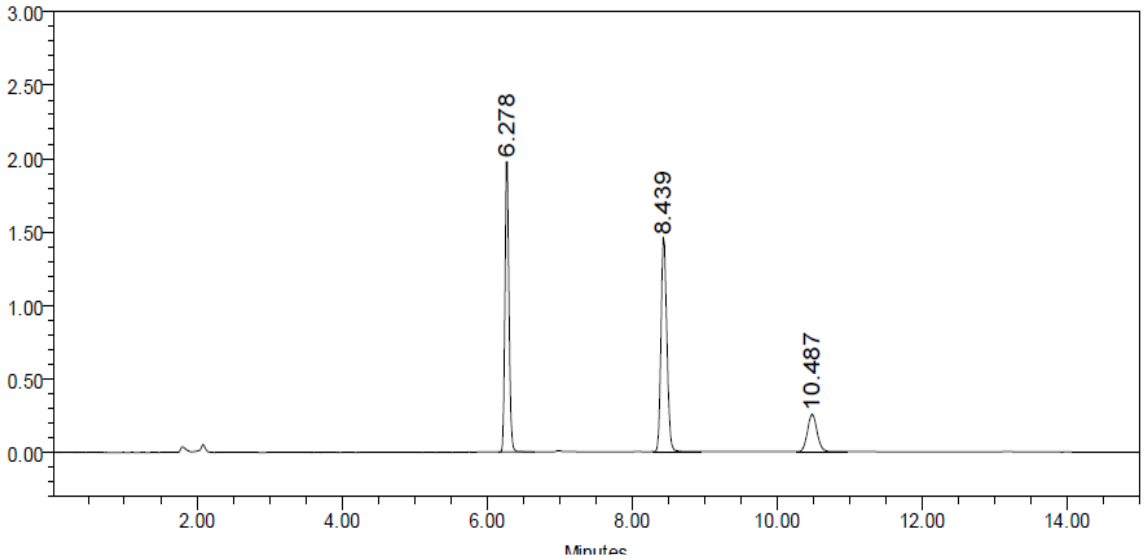


Figure 2: System suitability chromatogram of combined standard solution contains 200µg/ml of emtricitabine (RT: 6.278), 100µg/ml of bictegravir (RT:10.487), 50µg/ml of tenofovir alafenamide (RT:8.439) as standard solutions.

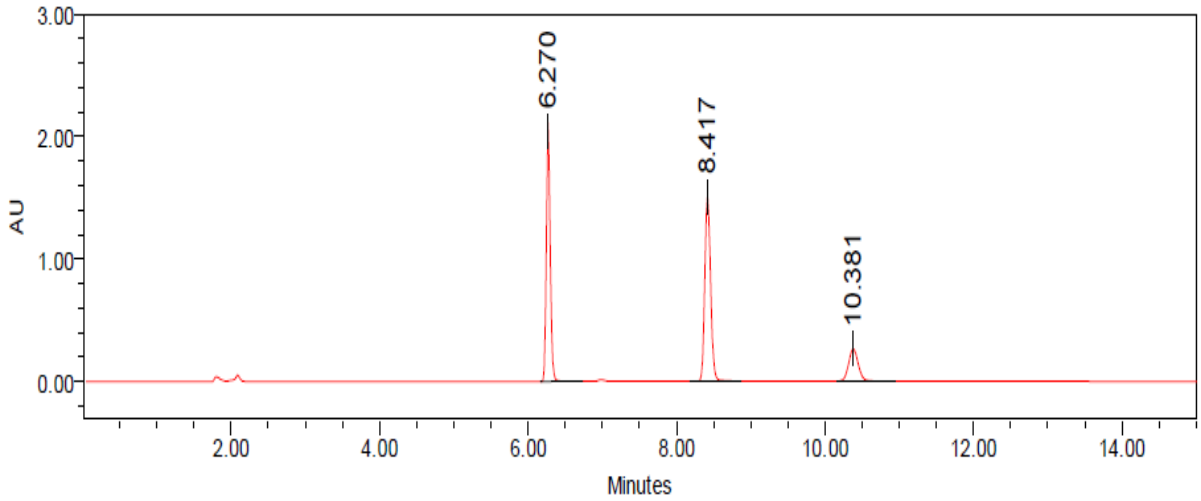


Figure 3: A typical Chromatogram Biktarvy® contains 200µg/ml of emtricitabine (RT:6.270), 100µg/ml of bictegravir (RT:10.381), 50µg/ml of tenofovir alafenamide (RT : 8.417) as standard solutions

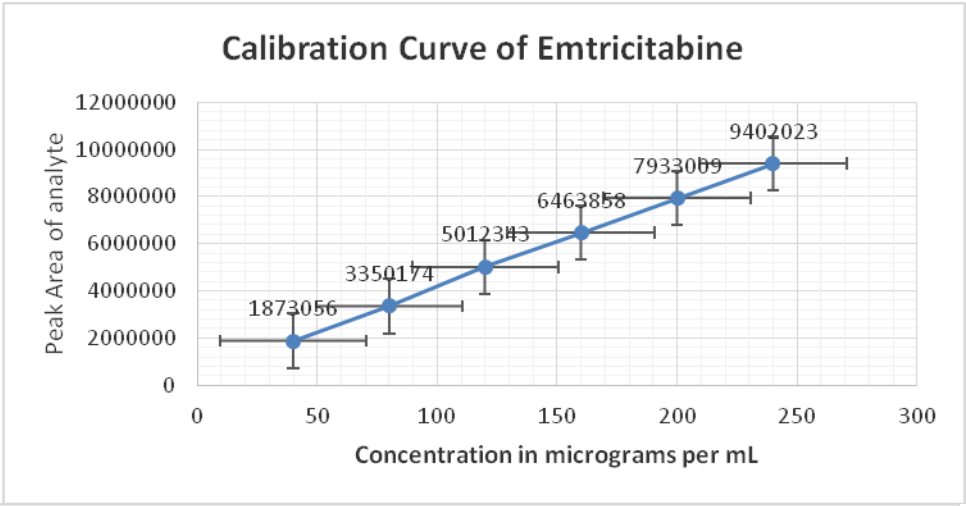
Linearity

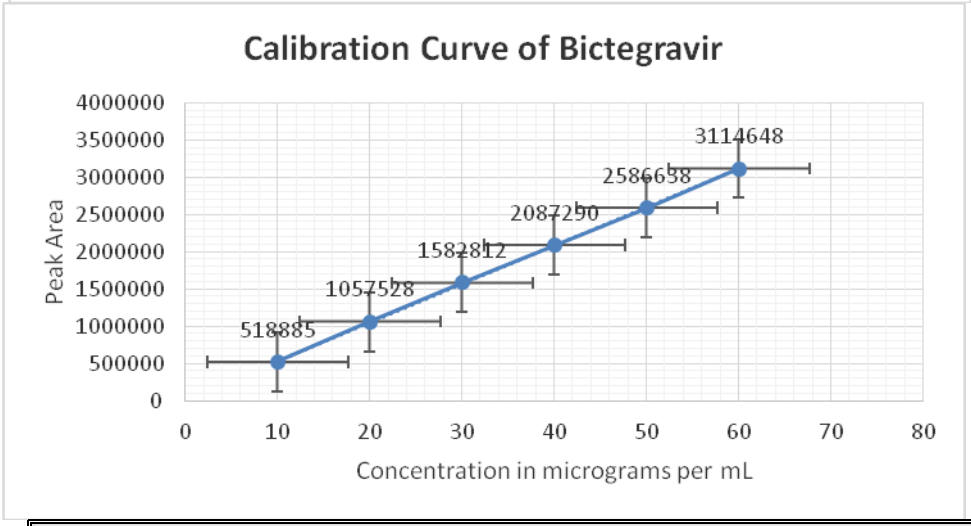
Linearity was studied by taking five variant concentration levels within 40%-120% of the target concentration range for analytes. Linear graph was obtained by plotting peak area of the drug to respective concentrations, 160µg-480µg/ml of ETC, 40µg-120µg/ml of BTG, 20µg-60µg/ml of TFRA. Calibration equations and correlation coefficients were calculated using peak areas of the active ingredients and concentrations were calculated using least square linear regression analysis .The square of the correlation coefficient (r^2 =0.999) established a significant correlation between concentration and detector response. The results (Table 3) depicted that there was an excellent correlation between peak ratios and concentrations of the drugs in the range tested (figure 4).

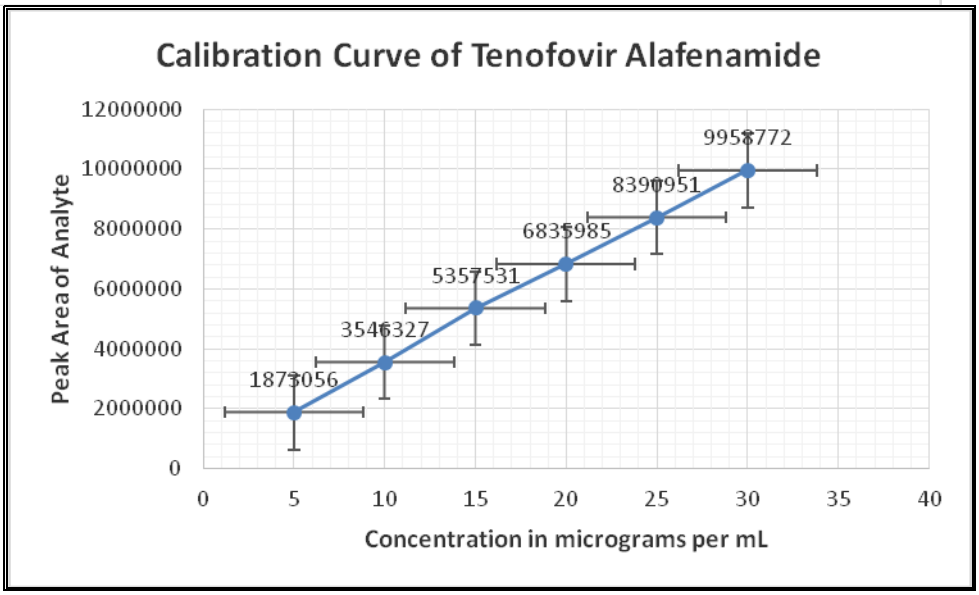
Table 3: Linearity data for Biktarvy® – fixed dosage form

Parameters	Emtricitabine	Bictegravir	Tenofovir alafenamide
Concentration range (µg/mL)	160-480	20-60	40-120
Regression equation	y=75492.65x+387925	Y=51630x+17571.2	Y=32219.46x+349679.73
Correlation coefficient	0.999	0.999	0.999
Limit of detection (LOD)	0.2 (µg/mL)	0.5 (µg/mL)	0.0025 (µg/mL)
Limit of Quantification (LOQ)	0.6 (µg/mL)	1.5 (µg/mL)	0.0175 (µg/mL)

Figure-4: Calibration Curves of (a) emtricitabine, (b) bictegravir, and (c) tenofovir alafenamide as standard dilutions







Precision

It was calculated using standard solutions of three analytes by injecting 6 times. The RSD of peak areas of six counterfiets was found to be <2. The results are depicted in Table 4 and 5. In all instances, the %RSD values were <2%.

Table 4: Results of Precision Studies (sample)

Injection	Emtricitabine		Tenofovir alafenamide		Bictegravir	
	RT	Area	RT	Area	RT	Area
1	6.270	7631294	8.417	8207710	10.381	2634114
2	6.268	7654884	8.402	8181076	10.362	2330163
3	6.261	7591030	8.394	8178072	10.320	2330239
4	6.254	7611878	8.386	8214311	10.302	2330659
5	6.255	7575501	8.397	8232932	10.296	2337993
6	6.250	7649795	8.413	8211530	10.338	2329106
Mean	6.260	7619063.7	8.402	8404271.8	10.333	2333212.3
SD	0.008	31975.9	0.012	21029.4	0.034	5036.1
%RSD	0.13	0.4	0.14	0.3	0.33	0.2

Table 5: Results for precision of the standard drug

Injection	Emtricitabine		Tenofovir alafenamide		Bictegravir	
	RT	Area	RT	Area	RT	Area
1	6.278	7696446	8.439	8209779	10.487	2341031
2	6.288	7683636	8.432	8277249	10.505	2339563
3	6.270	7810952	8.420	8233647	10.442	2357141
4	6.280	7771205	8.414	8262495	10.402	2340346
5	6.269	7692024	8.429	8330977	10.406	2358919
6	6.265	7697062	8.452	8202956	10.432	2371258
Mean	6.275	7725220.8	8.431	8252850.5	10.446	2351376.3
SD	0.009	52757.2	0.013	47969.7	0.042	13066.4
%RSD	0.14	0.7	0.16	0.6	0.40	0.6

Accuracy

Accuracy was determined using percentage recovery method for the fixed dose combination of tablet dosage form. The results for accuracy studies for standard solutions and sample matrix are shown in Table 6, the recovery values depict that the method was accurate within the desired range.

Table 6: Results of Recovery Accuracy Studies

Accuracy parameter	Spiked with 80% of the Working Std. Solution contains analytes in mcg/mL			Spiked with 100% of the Working Std. Solution contains analytes in mcg/mL			Spiked with 120% of the Working Std. Solution contains analytes in mcg/mL		
	Emitricitabine	Bictegravir	Tenofovir Alafenamide	Emitricitabine	Bictegravir	Tenofovir Alafenamide	Emitricitabine	Bictegravir	Tenofovir Alafenamide
Amount added	320	80	40	400	100	50	480	120	60
Amount Found	290.65	83.04	39.96	402	107	48.29	457.9	123.9	58.05
% Recovery	90.83	103.8	99.91	100.66	107.0	96.58	95.41	103.33	96.75
% RSD.	0.1	0.2	0.3	0.4	0.7	0.8	2.1	2.4	3.2

Senstivity

LOD and LOQ values for ETC, BTG, TFRA are listed in Table 3. Both LOD and LOQ values indicated high sensitivity of the method.

Robustness

Robustness of the method was studied by changing the flow rate between 1.4 -1.6ml/min (±0.1 units), effect of stationary phase is studied by changing LC Columns from different batches at ambient temperature. The effect of buffer pH was studied at 2.7-3.7(±0.5 units). All analytes were resolved successfully under these variations and there was no change in the elution order. All analytes maintained signal to noise ratio over 10 under all varied parameters. Results are given in Table 7.

Table 7: Results from testing of the Robustness of the method (n=3, 100% of the Sample Solution contains Emitricitabine 400 µg/mL, Bictegravir 100µg/mL and Tenofovir alafenamide 50µg/mL)

Condition Studied in Robustness	Modification in OFAT analysis	Mean Peak Areas ± S.D			% RSD			Mean Retention Time (in min) ± S.D		
		Emitricitabine	Bictegravir	Tenofovir Alafenamide	Emitricitabine	Bictegravir	Tenofovir Alafenamide	Emitricitabine	Bictegravir	Tenofovir Alafenamide
Different column	Phenomex C18	8762073±56212.5	2764749±9872.3	9175763±49267.6	0.6	0.4	0.5	6.248±0.03	9.956±0.004	8.437±0.003
Flow rate in ml/min	1.4	8368828.3±14031.9	2790187±1891.1	8877022.7±5152.4	0.2	0.1	0.2	6.418±0.002	10.265±0.013	8.666±0.001
	1.6	7919375±56417.9	2713178±25926.5	8435143.3±94213.4	0.7	1.0	1.1	6.071±0.003	9.615±0.004	8.201±0.003
Buffer pH	2.7	7905239.3±59423.8	2936545.6±17942.6	8323149±77212.9	0.7	0.6	0.9	6.223±0.009	8.913±0.007	8.282±0.008
	3.7	7850416.7±10154.6	2449562.7±8466.0	8320071±21303.8	0.1	0.3	0.3	6.240±0.008	10.078±0.009	8.354±0.008

The differences between the amount claimed and those assayed were very low and the RSD values were within acceptable range. The mean percentage recoveries obtained after six repeated experiments were found between 98.6 and 99.39(Table 8), indicating that the results are accurate and precise , and there is no interference from the excipients used in the pharmaceutical dosage form.

Table 8: Assay results of Emtricitabine, Bictegravir, Tenofovir alafenamide in Biktarvy® tablet.

Formulation	Label claim(mg/tablet)			Amount found in (mg)		
	ETC	BTG	TFRA	ETC	BTG	TFRA
Biktarvy® is a fixed dose combination tablet	200	50	25	197.2	49.9	24.67

Abbrevations used in the manuscript
ETC- Emtricitabine, BTG- Bictegravir, TFRA-Tenofovir alafenamide

CONCLUSION:

In the present study a method was developed, validated and procedure was described for the assay of a complex multi drug dosage regimen containing ETC, BTG, TFRA for oral administration, which is indicated as one pill, once a day prescription medicine for the treatment of HIV-1. All the three ingredients were successfully resolved and quantified. The developed method reported herein was validated by parameters according to ICH-Q2B guidelines. The proposed method provided good system suitability, resolution, precision, accuracy, LOD and LOQ, linearity and robustness and requires less expensive reagents.

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