An Elsevier Indexed Journal ISSN-2230-7346

Journal of Global Trends in Pharmaceutical Sciences

SIMULTANEOUS HPLC METHOD DEVELOPMENT AND VALIDATION FOR QUANTIFICATION EMTRICITABINE, BICTEGRAVIR AND TENOFOVIR ALAFENAMIDE IN COMBINED DOSAGE FORM WITH STABILITY STUDIES

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ARTICLE INFO

Key Words

Emtricitabine, Bictegravir, Tenofovir alafenamide, Mobile phase, Validation

Access this article online Website: https://www.jgtps.com/ Quick Response Code:

ABSTRACT

A simple and rapid high performance liquid chromatographic method was developed and validated for simultaneous estimation of Emtricitabine, Bictegravir and Tenofovir alafenamide in their pharmaceutical dosage form. The chromatographic separation was achieved on Agilent C18 150 x 4.6 mm, 5µ.column, mobile phase as Water: Acetonitrile (60:40 v/v) at a flow rate of 0.6 ml/min and injecting 10µl sample into the chromatographic system. The eluted compounds were detected by using PDA detector at detection wavelength of 300 nm and temperature was maintained at 30°C. Retention times of the three compounds were found to be 2.285 min, 2.840min and 3.276 min for Emtricitabine, Bictegravir and Tenofovir alafenamide respectively. The linearity range was 50-300 ppm, 12.5-75 ppm and 6.25-37.5 ppm with values of LOD found to be 0.22 µg/ml, 0.57 µg/ml, 0.00 µg/ml and LOQ were found to be 0.67 µg/ml, 1.72 µg/ml and 0.01µg/ml for Emtricitabine, Bictegravir and Tenofovir alafenamide respectively which were linear enough showing correlation coefficient 0.999 in all the cases. The present method was specific, precise, accurate and robust.

INTRODUCTION

Emtricitabine 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 [1]. 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 antiretrovirals in a single tablet [2]. Tenofovir alafenamide fumarate (TAF) is a nucleotide reverse transcriptase inhibitor (NRTI) and a novel ester prodrug of the antiretroviral tenofovir. Following oral administration, TAF is converted in vivo to tenofovir, an acyclic nucleoside phosphonate (nucleotide) analog of adenosine 5′-monophosphate. Although tenofovir (available as tenofovir disoproxil fumarate) has a good safety profile and efficacy, and is currently a cornerstone of HIV antiviral treatment, its use has been associated with nephrotoxicity and reduced bone mineral density [3]. Emtricitabine, Bictegravir and Tenofovir alafenamide chemical structures were shown in fig 1.

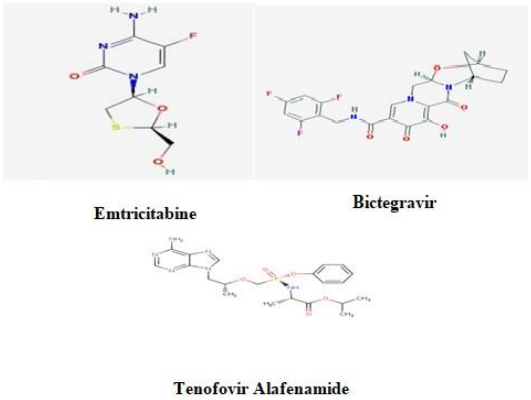

Figure No 1: Chemical structures of Emtricitabine, Bictegravir and Tenofovir alafenamide

Literature survey reveals that few HPLC and UV methods have been reported for the estimation of Emtricitabine, Bictegravir and Tenofovir alafenamide in individual and combination with other drugs [6,7,8]. The present attempt was made to develop an accurate, precise, sensitive, selective, reproducible and rapid analytical technique for cost effective estimation of Emtricitabine, Bictegravir and Tenofovir Alafenamidein combination by changing of few chromatographic conditions.

MATERIALS AND METHODS

Materials used:

The following materials used were either AR/LR grade or the best possible Pharma grade available as supplied by the manufacturer or supplier without further purification or investigation. Drug samples were obtained from Spectrum pharma research solutions pvt. Ltd. HPLC grade water, acetonitrile and AR grade orthophosphoric acid, tri ethyl amine and potassium hydrogen phosphate were purchased from Ranchem labs, Mumbai.

Instruments used:

Waters HPLC2695 series with quaternary pumps, Photo diode array detector and auto sampler integrated with empower software. UV double beam spectrophotometer with Uwin 5 manufactured by Lab india and BVK enterprises made pH meter and ultra sonicator were used.

Preparation of Standard stock solutions: Accurately weighed 50mg of Emtricitabine, 12.5mg of Bictegravir and 6.25mg of Tenofovir Alafenamide and transferred to three 25ml volumetric flasks separately. 10ml of Diluent was added to flasks and sonicated for 20mins. Flasks were made up with water and methanol (50:50) and labeled as Standard stock solution 1, 2 and 3.

Preparation of Standard working solutions (100% solution): 1ml from each stock solution was pipette out and taken into a 10ml volumetric flask and made up with Water methanol.

Preparation of Sample stock solutions: 5 tablets were weighed and calculate the average weight of each tablet then the weight equivalent to 1 tabletwas 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.

Preparation of Sample working solutions (100% solution): From the filtered solution 1ml was pipette out into a 10 ml volumetric flask and made upto 10ml with diluents. (200ppm, 50ppm & 25 ppm).

Preparation of Mobile phase:

The mixture of mobile phase consisting of 600 ml of water and 400 ml of acetonitrile degassed and sonicate with 30 min.

Optimized Chromatographic conditions:

Column Used	: Agilent C18 150 x 4.6 mm, 5µ.
Mobile phase	: Water : Acetonitrile (60:40 v/v)
Flow rate	: 0.6 ml/min
Diluent	: First dissolved in Acetonitrile and made up with water (50:50 v/v).
Wavelength	: 300.0 nm
Temperature	: 30°C
Injection Volume	: 10µl
Run time	: 6.0 min

Observation: Emtricitabine, Bictegravir and Tenofovir Alafenamide were eluted at 2.285 min, 2.840min and 3.276 min respectively with good resolution. Plate count and tailing factor was very satisfactory, so this method was optimized and to be validated.

Degradation studies:

To 1 ml of stock solutions of Emtricitabine, Bictegravir and Tenofovir Alafenamide. 1 ml of 20% hydrogen peroxide (H_2O_2), 1ml of 2N Hydrochloric acid, 1 ml of 2N sodium hydroxide and also exposed to the light. All the

solutions were added and prepared separately. The solutions were kept for 30 min at 60^0C. For HPLC study, the resultant solution was diluted to obtain 200µg/ml, 50µg/ml and 25µg/ml of all components and 10µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Method validation

Method validation was carried on according to ICH guidelines Q_2R_1. The validation parameters include System suitability, specificity, linearity, accuracy, precision, LOD& LOQ and robustness [17, 18].

RESULTS AND DISCUSSION

System suitability:

The system suitability parameters were determined by preparing standard solutions of Emtricitabine, Bictegravir and Tenofovir Alafenamide and the solutions were injected six times and the parameters like peak tailing, resolution and USP plate count were determined. Plate count of the Emtricitabine Was 5108, Bictegravir was 7472, and of Tenofovir Alafenamide was 9039, tailing factor of Emtricitabine was 1.18, Bictegravir was 1.22, and of Tenofovir Alafenamide was 1.15, resolution between Emtricitabine and Bictegravir was 4.0 and resolution between Bictegravir and Tenofovir Alafenamide was 3.0. According to ICH guidelines plate count should be more than 2000, tailing factor should be less than 2 and resolution must be more than 2. All the system suitable parameters were passed and were within the limits. The results were shown in table 1 and fig 3.

Specificity:

Retention times of Emtricitabine, Bictegravir and Tenofovir Alafenamide were 2.288 min, 2.828 min and 3.258min respectively. We did not found and interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific.

Linearity:

Six linear concentrations of Emtricitabine.(50-300µg/ml), Bictegravir(12.5-75µg/ml) and Tenofovir Alafenamide(6.25-37.5µg/ml) were injected in a triplicate maner. Average areas were mentioned above and linearity equations obtained for Emtricitabine was y = 16188x + 8406, Bictegravire was y = 26254x + 6541. and of Tenofovir Alafenamide was y = 17798x + 159.1. Correlation coefficient obtained was 0.999 for all the three drugs. The results were given in table 2 and calibration curves were shown in fig 4, 5 & 6.

Precision:

Repeatability: Multiple sampling from a sample stock solution was done and six working sample solutions of same concentrations were prepared, each injection from each working sample solution was given and obtained areas were mentioned in the above table. Average area, standard deviation and % RSD were calculated for three drugs and obtained as 0.4%, 0.5% and 0.5% respectively for Emtricitabine, Bictegravir and Tenofovir Alafenamide. As the limit of Precision was less than "2" the system precision was passed in this method. The data was shown in table 3.

Intermediate precision (Day_ Day Precision): Multiple sampling from a sample stock solution was done and six working sample solutions of same concentrations were prepared, each injection from each working sample solution was given on the next day of the sample preparation and obtained areas were mentioned in the above table. Average area, standard deviation and % RSD were calculated for three drugs and obtained as 0.7%, 0.7% and 0.7% respectively for Emtricitabine, Bictegravir and Tenofovir Alafenamide. As the limit of Precision was less than "2" the system precision was passed in this method.

Accuracy:

Three levels of Accuracy sample were prepared by standard addition method. Triplicate injections were given for each level of accuracy and mean %Recovery was obtained as 100.09%, 99.83% and 99.39% for Emtricitabine, Bictegravir and Tenofovir Alafenamide respectively. The results were tabulated in table 5,6 & 7.

LOD & LOQ:

0.25ml each from three standard stock solutions was pipette out and transferred to 3 separate 10ml volumetric flask and made up with diluents from the above solutions 0.1ml, 0.1ml and 0.1ml of Emtricitabine, Bictegravir and Tenofovir Alafenamide solutions respectively were transferred to 10ml volumetric flasks and made up with the same diluents. The results were depicted in table 8.

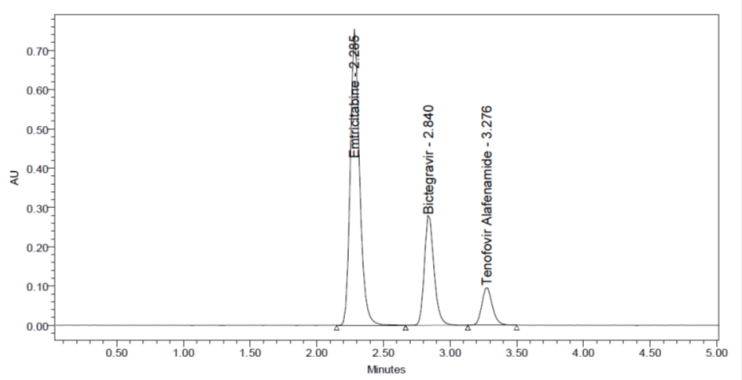

Figure No 2: Optimized chromatogram of Emtricitabine, Bictegravir and Tenofovir alafenamide

Table No 1: Results for system suitability

S no	Emtricitabine.			Bictegravir			Tenofovir Alafenamide		
Inj	RT(min)	TP	Tailing	RT(min)	TP	Tailing	RT(min)	TP	Tailing
1	2.288	5108	1.18	2.828	7472	1.22	3.257	9039	1.15
2	2.306	4177	1.16	2.837	7603	1.23	3.258	8426	1.17
3	2.310	4465	1.17	2.846	8185	1.18	3.262	8656	1.16
4	2.310	4580	1.17	2.849	7982	1.19	3.265	8065	1.17
5	2.311	4915	1.17	2.849	7706	1.20	3.265	8337	1.15
6	2.312	4813	1.18	2.854	7878	1.22	3.298	8560	1.15

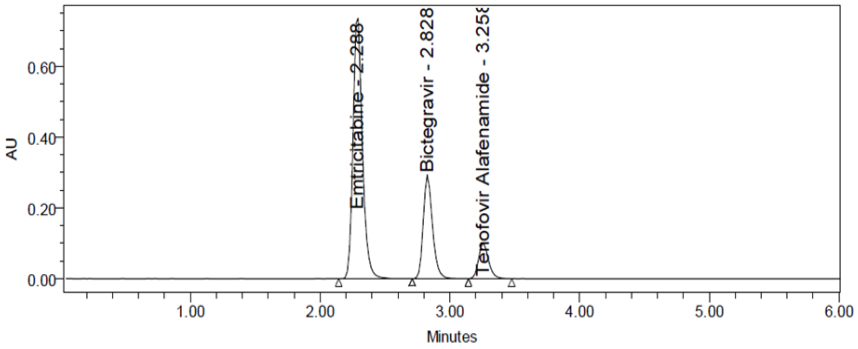

Figure No 3: System suitability chromatogram

Table No 2: Linearity Results

Emtricitabine.		Bictegravir		Tenofovir Alafenamide	
Conc (µg/mL)	Peak area	Conc (µg/mL)	Peak area	Conc (µg/mL)	Peak area
50	805543	12.5	333204	6.25	108327
100	1644434	25	662110	12.5	226190
150	2422404	37.5	997175	18.75	335288
200	3283121	50	1335900	25	443932
250	4066325	62.5	1645900	31.25	556604
300	4834234	75	1963289	37.5	666803

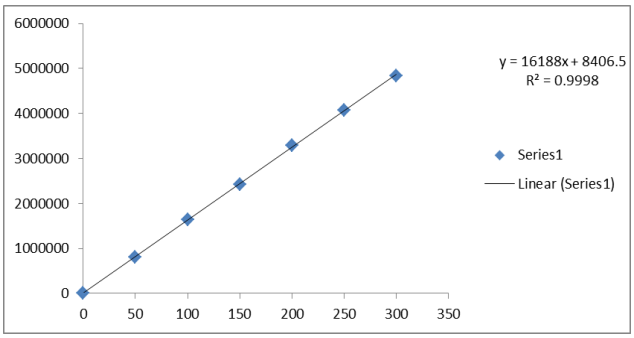

Figure No 4: Calibration curve of Emtricitabine

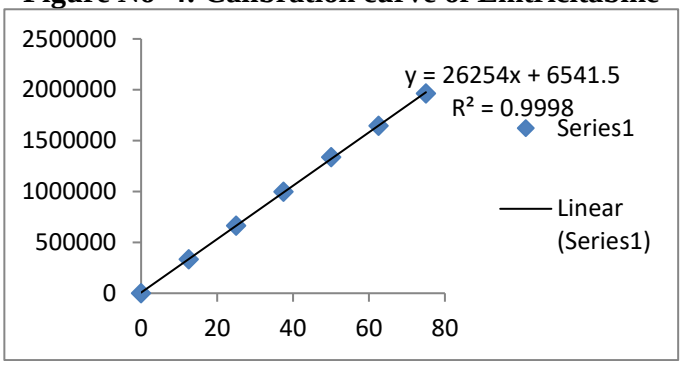

Figure No 5: Calibration curve of Bictegravir

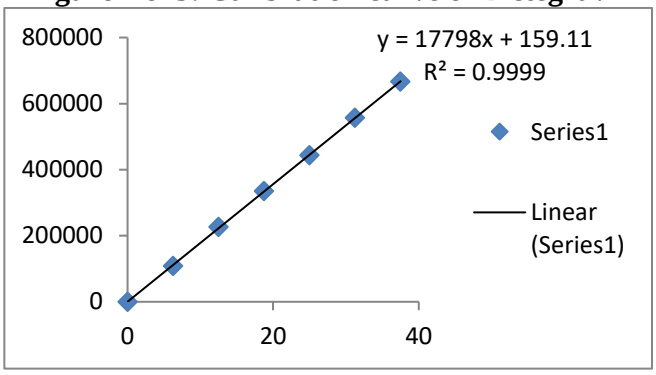

Figure No 6: Calibration curve of Tenofovir Alafenamide

Table No 3: Repeatability table of Emtricitabine, Bictegravir and Tenofovir Alafenamide.

S. No	Area of Emtricitabine.	Area of Bictegravir	Area of Tenofovir Alafenamide
1.	3232100	1329523	437902
2.	3236665	1345357	435509
3.	3213226	1326352	434953
4.	3211320	1328271	432306
5.	3229827	1331771	436432
6.	3203217	1328815	432706
Mean	3221059	1331682	434968
S.D	13541.6	6927.3	2156.3
%RSD	0.4	0.5	0.5

Table No 4: Intermediate precision results

S. No	Area of Emtricitabine.	Area of Bictegravir	Area of Tenofovir Alafenamide
1.	3204228	1304965	434529
2.	3198594	1327544	435263
3.	3204137	1303508	433630
4.	3168688	1318269	432381
5.	3194537	1323123	430861
6.	3242554	1314515	439470
Mean	3202123	1315321	434356
S.D	23795.2	9658.2	2954.6
%RSD	0.7	0.7	0.7

Table No 5: Accuracy table of Emtricitabine

% Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean %Recovery
50%	100	99.57	99.57	100.09%
	100	100.98	100.98	
	100	100.71	100.71	
100%	200	198.04	99.02	
	200	199.82	99.91	
	200	200.54	100.27	
150%	300	300.07	100.02	
	300	300.72	100.24	
	300	300.34	100.11	

Table No 6: Accuracy table of Bictegravir

% Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean %Recovery
50%	25	24.90	99.59	**99.83%**
	25	24.80	99.18	
	25	24.81	99.25	
100%	50	49.81	99.61	
	50	49.64	99.27	
	50	49.59	99.19	
150%	75	75.71	100.95	
	75	75.45	100.60	
	75	75.60	100.80	

Table No 7: Accuracy table of Tenofovir Alafenamide

% Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean %Recovery
50%	12.5	12.43	99.45	99.39%
	12.5	12.40	99.17	
	12.5	12.49	99.95	
100%	25	24.76	99.05	
	25	24.75	99.00	
	25	24.78	99.14	
150%	37.5	37.70	100.53	
	37.5	37.17	99.12	
	37.5	37.18	99.14	

Table No 8: Sensitivity table of Emtricitabine, Bictegravir and Tenofovir Alafenamide.

Molecule	LOD(µg/ml)	LOQ(µg/ml)
Emtricitabine.	0.22 µg/ml	0.67 µg/ml
Bictegravir	0.57 µg/ml	1.72 µg/ml
Tenofovir Alafenamide	0.00 µg/ml	0.01 µg/ml

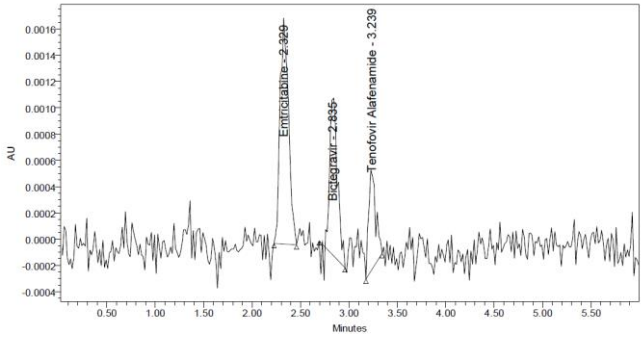

Figure No 7: LOD Chromatogram of standard

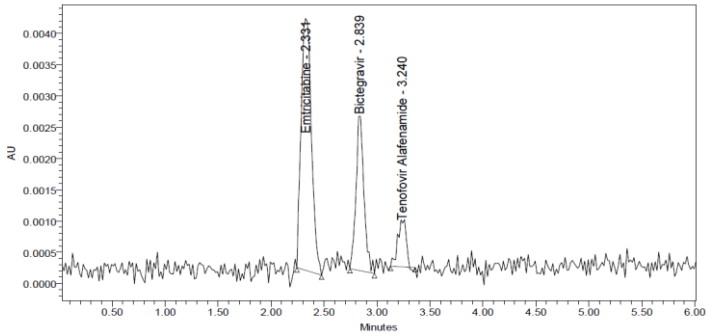

Figure No 8: LOQ chromatogram of standard

Table No 9: Robustness Results

S.no	Condition	%RSD of Emtricitabine.	%RSD of Bictegravir	%RSD of Tenofovir Alafenamide
1	Flow rate (-) 0.5ml/min	0.4	0.1	0.2
2	Flow rate (+) 0.7ml/min	0.2	0.2	0.2
3	Mobile phase (-) 35A:65W	0.8	0.7	0.6
4	Mobile phase (+) 45A:55W	0.5	0.5	0.8
5	Temperature (-) 25°C	0.5	0.4	0.6
6	Temperature (+) 35°C	0.2	0.3	0.3

Table No 10: Degradation Data of Emtricitabine.

S.No	Degradation Condition	% Drug Degraded	Purity Angle	Purity Threshold
1	Acid	6.45	0.269	0.328
2	Alkali	4.37	0.289	0.310
3	Oxidation	4.23	0.279	0.313
4	Thermal	1.23	0.207	0.305
5	UV	1.40	0.216	0.312
6	Water	0.48	0.207	0.295

Table No 11: Degradation Data of Bictegravir

S.No	Degradation Condition	% Drug Degraded	Purity Angle	Purity Threshold
1	Acid	6.56	0.296	0.372
2	Alkali	4.08	0.316	0.368
3	Oxidation	4.68	0.297	0.352
4	Thermal	1.46	0.294	0.356
5	UV	1.16	0.309	0.373
6	Water	0.57	0.319	0.345

Table No 12: Degradation Data of Tenofovir Alafenamide

S. No	Degradation Condition	% Drug Degraded	Purity Angle	Purity Threshold
1	Acid	6.60	0.444	0.474
2	Alkali	4.97	0.461	0.476
3	Oxidation	4.21	0.421	0.430
4	Thermal	1.54	0.437	0.446
5	UV	1.73	0.457	0.471
6	Water	0.76	0.440	0.442

Robustness:

Robustness conditions like Flow minus (0.5ml/min), Flow plus (0.7ml/min), mobile phase minus (35A:65W), mobile phase plus (45A:55W), 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. %RSD was within the limit. The results were shown in table 9.

Assay:

Biktarvy (200+50+25) the label claim Bictegravir 200mg, Emtricitabine 50mg, Tenofovir Alafenamide 25mg per unit formulation assay was performed with the above formulation. Average % Assay for Emtricitabine, Bictegravir and Tenofovir Alafenamide obtained was 99.78%, 99.81% and 100.07% respectively.

Degradation Studies:

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. The results were given in table 10, 11 & 12.

CONCLUSION

A simple, accurate, precise and robust method has been developed for the simultaneous estimation and stability studies of of the Emtricitabine, Bictegravir and Tenofovir Alafenamide in tablet dosage form. The validation results were found to be well with in the limit. Hence it can be concluded that the new developed method can be used for the

analysis of above mentioned formulation in the laboratory on regular basis.

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