Acta Pharm. Sci. Vol 63:(3), 2025
Original article
DOI: 10.23893/1307-2080.APS6339

Development and validation of a versatile ultra performance liquid chromatography method for simultaneous estimation of selected antiviral drugs in bulk and dosage form

Divya NARLA[1*], Nagaraju PAPPULA[2], Prakash Nathaniel Kumar SARELLA[1]

1 Department of Pharmaceutical Analysis, Aditya College of Pharmacy, ADB road, Surampalem-533437, India
2 Department of Pharmaceutical Analysis, Hindu College of Pharmacy, Amaravathi Road, Guntur-522002, India

ABSTRACT

This research aimed to develop and validate an accurate Ultra performance Liquid Chromatography (UPLC) method with Photo Diode Array detection to simultaneously estimate Bictegravir, Emtricitabine and Tenofovir Alafenamide Fumarate in their fixed dose combination. The developed method used Acetonitrile and pH 2.5 triethanolamine buffer in a 30:70 v/v ratio as the mobile phase at 1.0 mL/min flow rate and 0.50 µL injection volume. The analytes were separated on a BEH C18 column (1.8µ, 100×2.1mm) and detected at 265nm. Bictegravir, Emtricitabine and Tenofovir Alafenamide Fumarate obeyed Beer's law in the ranges of 5–75 µg/mL, 20-300 µg/mL and 2.50–37.50 µg/mL respectively. The recovery for accuracy was 99-101%. Precision and robustness met acceptable limits. This stability indicating method could distinguish and quantify the compounds even with degradants. Thus, a specific, accurate and robust stability indicating method was developed to simultaneously quantify Bictegravir, Emtricitabine and Tenofovir Alafenamide fumarate in their combined dosage form.

Keywords: bictegravir, emtricitabine, tenofovir alafenamide fumarate, UPLC

*Corresponding author: Divya NARLA
E-mail: divyanarla@gmail.com
ORCIDs:
Divya NARLA: 0000-0002-3255-7108
Nagaraju PAPPULA: 0000-0001-8315-2859
Prakash Nathaniel Kumar SARELLA: 0000-0002-7386-6222
(Received 3 May 2023, Accepted 5 Aug 2024)

INTRODUCTION

Bictegravir (BIC) is an antiretroviral agent used to treat HIV infection in combination with other drugs[1]. It is an integrase strand transfer inhibitor (INSTI). The chemical name of Bictegravir sodium 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). Bictegravir sodium has a molecular formula of $C_{21}H_{17}F_3N_3NaO_5$ and molecular weight 471.4. It appears as an off-white to yellow solid with 0.1 mg solubility in 1mL of water at 20°C. Bictegravir is an INSTI used only in combination with other antiretroviral drugs to treat HIV infection[2].

Emtricitabine (FTC) belongs to the nucleoside reverse transcriptase inhibitor (NRTI) class of antiretroviral drugs. It can be used with other antiretroviral agents to treat HIV infection and AIDS[3]. The chemical name of FTC is 4-amino-5-fluoro-1-(2R-hydroxymethyl-1,3-oxathiolan-5S-yl)-(1H)-pyrimidin-2-one. FTC is the (-) enantiomer of a thio analog of cytidine which differs from other cytidine analogs with a fluorine in the 5 positions. FTC has a molecular formula of $C_8H_{10}FN_3O_3S$ and molecular weight 247.2. It appears as a white to off-white powder with 112 mg solubility in 1mL of water at 25°C.

Tenofovir Alafenamide Fumarate (TAF) is a prodrug and HIV-1 reverse transcriptase inhibitor (NtRTI)[4].

The chemical name of Tenofovir alafenamide fumarate drug substance is L-alanine, N-[(S)-[[(1R)-2-(6-amino-9H-purin-9-yl)-1¬methylethoxy]methyl] phenoxyphosphinyl]-, 1-methylethyl ester, (2E)-2-butenedioate (2:1). It has an empirical formula of $C_{21}H_{29}O_5N_6P.½(C_4H_4O_4)$ and formula weight 534.5 g/mol. TAF appears as a white to off-white or tan powder with 4.7 mg solubility in 1 mL of water at 20°C. It is an NtRTI antiretroviral drug used with other drugs to treat HIV. The chemical structures of Bictegravir, Emtricitabine and Tenofovir Alafenamide Fumarate are shown in Figure 1.

The fixed dose combination of Bictegravir, Emtricitabine and Tenofovir alafenamide fumarate was approved by USFDA and is recommended for the treatment of patients suffering from chronic HIV infection with or without indication of compensated cirrhosis[5]. The objective of the present study is to develop and validate a simple, accurate and precise stability indicating UPLC method for the simultaneous estimation of BIC, FTC and TAF in pharmaceutical dosage forms, which would be applied for routine quality control of dosage form in the presence of degradants. UPLC was chosen over HPLC as it offers advantages of fast analysis, less solvent consumption, small sample size and increased sensitivity.

Various analytical methods have been reported for simultaneously estimating Bictegravir (BIC), Emtricitabine (FTC) and Tenofovir Alafenamide Fumarate (TAF) in bulk and pharmaceutical formulations using LC-MS/MS[6-8] and high-performance liquid chromatography (HPLC)[9-16]. Ultra Performance Liquid Chromatography (UPLC) methods are scarcely available in literature[17]. An attempt was made to develop a stability indicating UPLC method to quantify BIC, FTC and TAF in pharmaceutical formulations. Though HPLC methods exist for simultaneously estimating BIC, FTC and TAF, no UPLC method has been reported. The current work aims to develop a stability indicating UPLC method to quantify these drugs in pharmaceutical formulations.

The developed UPLC method would provide improved sensitivity, speed and resolution over the existing HPLC techniques for analysis of BIC, FTC and TAF. The stability indicating nature of the method also allows determining the drugs in the presence of degradation products ensuring the quality and stability of pharmaceutical formulations.

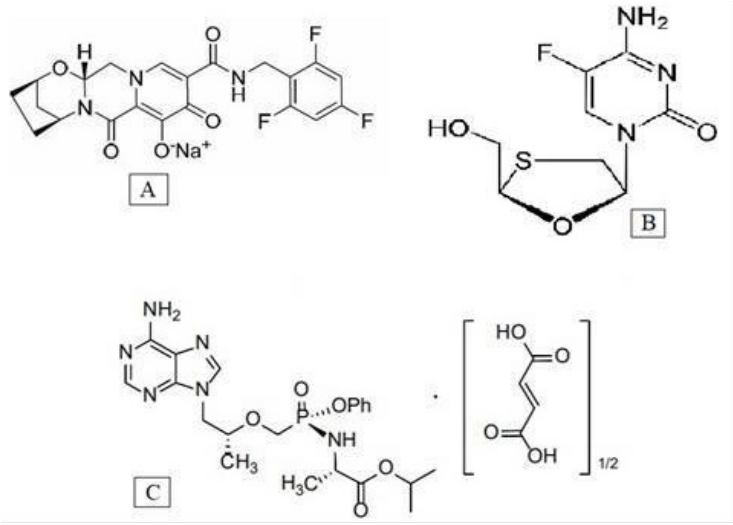

Figure 1. Chemical structures of Bictegravir (A), Emtricitabine (B), Tenofovir Alafenamide Fumarate (C)

METHODOLOGY

Reagents and chemicals

Reference standards of Bictegravir, Emtricitabine and Tenofovir Alafenamide fumarate were obtained as gift samples from Mylan, Hyderabad and Lupin Pharmaceuticals, Visakhapatnam. The fixed dose generic combination of Bictegravir/Emtricitabine/Tenofovir Alafenamide (50mg/200mg/25mg) was procured from commercial sources under the brand name Biktarvy®.

The following chemicals of chromatographic grade were used in the current study: Acetonitrile (UPLC Lichrosolv,Merck), Triethanolamine (Qualigens, India), Orthophosphoric acid (Merck, India) Hydrochloric acid (Finar, India), Sulphuric acid (Finar, India), Sodium Hydroxide (Finar, India), Hydrogen Peroxide (Finar, India).

The obtained reference standards and procured pharmaceutical products along with the chemicals were used in the present work to develop and validate the proposed UPLC method for simultaneous analysis of BIC, FTC and TAF.

Instrumentation and chromatographic conditions

Liquid chromatographic analysis to simultaneously estimate Bictegravir (BIC), Emtricitabine (FTC) and Tenofovir Alafenamide fumarate (TAF) was performed using a Waters Acquity UPLC system equipped with quaternary pump, an inbuilt auto injector, a PDA 2996 detector and controlled by Empower 2 software.

Other equipments used included: An electronic balance (Shimadzu), pH meter (Adwa AD1020), Ultra sonicator (Labsoul, India), hot air oven (BiTechno, India), UV chamber (cole parmer, US).

Chromatographic separation of BIC, FTC and TAF was carried out on a BEH C18 column (100×2.1 mm,1.8 µm) at ambient temperature. A mobile phase containing Acetonitrile and TEA buffer (pH 2.5) in 30:70 v/v ratio was used at 1.0 mL/min flow rate with 0.5 µL injection volume. Detection of separate analytes was performed at 265 nm with 7 min runtime.

Preparation of standard solutions

Accurately weigh and transfer standardized amounts of Bictegravir (50 mg), Emtricitabine (200 mg) and Tenofovir alafenamide fumarate (25 mg) into 100 mL clean, dry volumetric flasks. Add a diluent solution of acetonitrile and buffer in 30:70 ratio and sonicate for 10 minutes. Bring the final volume to the appropriate level with the diluent solution to achieve concentrations of

500 micrograms per milliliter of Bictegravir, 2000 micrograms per milliliter of Emtricitabine and 250 micrograms per milliliter of Tenofovir alafenamide. Extract an additional 5 milliliters of this solution and dilute it further with the diluent to a final volume of 50 milliliters. This will produce a standard solution containing 50 micrograms per milliliter of Bictegravir, 200 micrograms per milliliter of Emtricitabine and 25 micrograms per milliliter of Tenofovir alafenamide.

Preparation of sample solutions

Five tablets of Biktarvy were weighed and finely ground into a powder. 350 milligrams of the powdered tablets were transferred into a 100-milliliter volumetric flask. 70 milliliters of diluent were added, and the solution was sonicated for approximately 30 minutes to dissolve the contents. The volume was then adjusted to the proper level with the diluent and filtered through a 0.45-micron filter. Five milliliters of the filtered sample stock solution were transferred to a 50-milliliter volumetric flask. The volume was adjusted to 50 milliliters with diluent to achieve concentrations of 50 micrograms per milliliter of Bictegravir, 200 micrograms per milliliter of Emtricitabine, and 25 micrograms per milliliter of Tenofovir alafenamide.

Method validation

The developed analytical method was validated in accordance with ICH guidelines for the following parameters[13]:

System suitability

The standard solutions were injected into the UPLC system, and the system suitability parameters were evaluated, including:

Theoretical plates: A measure of the effectiveness of the separation process. The number of theoretical plates reflected column efficiency.

Tailing factor: The asymmetry of the chromatographic peaks is indicated by tailing factor. Values close to 1 indicated symmetric peaks.

Resolution: The ability of the system to separate the compounds of interest into discrete peaks. Resolutions greater than 2 were considered acceptable.

These system suitability parameters were assessed to ensure the UPLC system and analytical column were performing adequately and suitable for the sample analysis. Acceptable values indicate the system could produce reproducible and precise results for the analysis.

Specificity

Method specificity is unequivocally assessed for the presence of interfering peaks by injecting blank (diluent) and placebo solutions. Absence of additional peaks at the same retention times of analytes indicates absence of interference and specificity of the method.

Linearity

Series of six standard solutions of known concentrations in triplicate were used to assess linearity range from peak area versus concentration. Calibration curves are plotted, and correlation coefficient, slope and intercept are calculated by using straight line equation.

Precision

The system precision or Intraday precision (Repeatability) was studied by repeated injection of replicates of standard solution containing 50 μg/mL of BIC, 25 μg/mL of TAF and 200 μg/mL of FTC for six times. The method precision was determined by injecting six solutions of sample into the UPLC system and calculating the percent relative standard deviation (%RSD) values. Six replicate injections of the standard solutions were run on the second day to assess how precisely the method could measure the concentrations of the compounds. The %RSD was calculated as the standard deviation of the six measurements divided by the mean, expressed as a percentage. Lower %RSD values indicated higher precision. Acceptable %RSD limits were established to ensure the method precision was sufficiently accurate and consistent. %RSD values within the acceptable range showed the method could produce reliable and reproducible results.

Accuracy

The accuracy of the UPLC method was assessed through recovery studies. The standard solution containing 500 μg/mL of BIC, 2000 μg/mL of FTC and 250 μg/mL of TAF was used to prepare solutions of 3 concentrations (n=3) at 50%, 100% and 150% levels of target assay. The accuracy was analyzed by the standard addition method. The % recovery and RSD were evaluated.

LOD and LOQ

The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the calibration data using the following equations:

$$LOD = \frac{3{\cdot}3\,\sigma}{s}$$

$$LOQ = \frac{10\,\sigma}{s}$$

Where:

Standard deviation of intercept = Variability in the intercept value from the calibration equation.

Slope = The slope of the calibration curve, which represents sensitivity.

LOD indicates the lowest concentration at which the presence of an analyte could be detected with reasonable certainty. LOQ refers to the lowest concentration that could be measured with acceptable accuracy, precision, and variability. Both LOD and LOQ provide measures of sensitivity and quantitative ability for the method.

These parameters were determined to establish the minimum amounts of the compounds that could be reliably detected (LOD) and precisely quantified (LOQ) using the analytical method. LOD and LOQ values had to meet method validation acceptance criteria.

Robustness

The robustness of the method was determined by deliberately changing the optimized analytical conditions in a controlled manner, including:

Mobile phase composition: The composition of acetonitrile and buffer in the mobile phase was varied by ± 5% to assess the method's tolerance.

Flow rate: The flow rate of the mobile phase was increased and decreased by ± 0.1 milliliters per minute to evaluate the robustness.

Column temperature: The temperature of the analytical column was raised and lowered by ± 5 degrees Celsius to determine if the method could withstand minor changes.

These variables were intentionally perturbed from the optimized conditions to evaluate the impact on method performance parameters like peak shape, resolution, theoretical plates, accuracy and precision. Limited deviations from the optimal values that still yielded acceptable results indicated a robust method.

A robust analytical method is less prone to variations from operational and environmental factors that could compromise the results. By subjecting the key method parameters to small, controlled changes, the robustness assessment evaluated the overall ruggedness and reliability of the procedure.

Solution stability

The stability of prepared standard solution was estimated by analyzing the solutions after 24hrs of storage at room temperature.

Forced degradation studies

The stability indicating nature of the method and identification of possible degradants were achieved by performing degradation studies under stressful conditions such as:

Acid hydrolysis: The standard solution was mixed with 0.1N hydrochloric acid and refluxed at 60°C for 30 minutes. The solution was then neutralized and diluted to concentrations of 50 micrograms per milliliter of Bictegravir, 200 micrograms per milliliter of Emtricitabine and 25 micrograms per milliliter of Tenofovir alafenamide. The solution was injected into the UPLC system and chromatograms were assessed for sample stability.

Alkali degradation: 0.1N sodium hydroxide was added to the standard solution and refluxed at 60°C for 30 minutes. The solution was neutralized and diluted to concentrations of 50 micrograms per milliliter of Bictegravir, 200 micrograms per milliliter of Emtricitabine and 25 micrograms per milliliter of Tenofovir alafenamide. The solution was injected into the UPLC system and chromatograms were assessed for sample stability.

Dry heat degradation: The standard solution was placed in an oven at 105°C for 6 hours. For UPLC analysis, the solution was diluted to concentrations of 50 micrograms per milliliter of Bictegravir, 200 micrograms per milliliter of Emtricitabine and 25 micrograms per milliliter of Tenofovir alafenamide. The solution was injected into the UPLC system to obtain chromatograms which were then assessed to indicate sample stability.

Oxidative degradation was studied by reflexing 1mL of standard solution and 1mL of 10% H_2O_2 at 60°C for 30mins. The resultant solution was neutralized and diluted to obtain concentrations of 50 µg/mL of Bictegravir, 200 µg/mL of Emtricitabine & 25 µg/mL of Tenofovir alafenamide and injected into UPLC system. The sample stability was assessed from the chromatograms obtained.

Photochemical stability of analyte was assessed by exposing the solution containing 500 µg/mL of Bictegravir, 2000 µg/mL of Emtricitabine & 250 µg/mL to UV light in UV chamber for 7days. The resultant solution was diluted and injected into UPLC system to record and assess the chromatograms.

Neutral Hydrolysis was performed by refluxing the drug in water for 6hrs at 60°C and diluted solution is injected into UPLC system to record chromatograms for stability assessment.

The degradation studies subjected the compounds to acid, alkali, heat and oxidation to evaluate the ability of the method to separate degradants from the analytes of interest. The method could be considered stability indicating if it could detect the formation of degradants under stressful conditions. Analysis of chromatograms allowed for the identification of possible degradation products.

Stability indicating methods are more robust and suitable for long term stability testing and estimation of shelf life. Degradation studies provide evidence of method specificity for the desired compounds in the presence of potential impurities or break down products.

RESULTS and DISCUSSION

Optimization of method was achieved by considering mobile phases with various solvents at different ratios with changing flow rates over columns with suitable stationary phase. The developed UPLC-PDA method was validated according to ICH guidelines for various chromatographic parameters to ensure suitability for the intended purpose.

Specificity

The specificity of the developed method was assessed by determining the ability to measure the analytes in the presence of likely components such as excipients, impurities, matrix, degradants, etc. Chromatograms of the following were evaluated for peaks that could indicate a lack of specificity:

Mobile phase alone: The chromatogram of just the mobile phase solvents was checked for any peaks that could interfere with analyte peaks. No peaks demonstrated the mobile phase would not compromise specifically.

Placebo solution: The chromatogram of a placebo solution containing excipients but not the active ingredients was analyzed for peaks at the retention times of the analytes. No peaks at the analyte retention times indicated no interference from excipients or matrix.

Blank: A blank sample with no active ingredients or excipients was injected to detect any impurities or system peaks at the analyte retention times. No peaks showed the blank would not impact specificity.

The absence of peaks in the chromatograms of the mobile phase alone, placebo solution, and blank demonstrated the specificity of the method. The method could accurately measure the analytes without interference from other components likely to be present.

Method specificity is the ability to assess unequivocally the analyte of interest in the presence of potential interferences. By evaluating chromatograms for interference at the retention times of interest, the specificity of the developed UPLC-PDA method was validated.

Figures 2, mentioned in the text, likely showed the chromatograms from the mobile phase alone, placebo solution, and blank that exhibited no peaks at the retention times of the analytes, thereby proving method specificity.

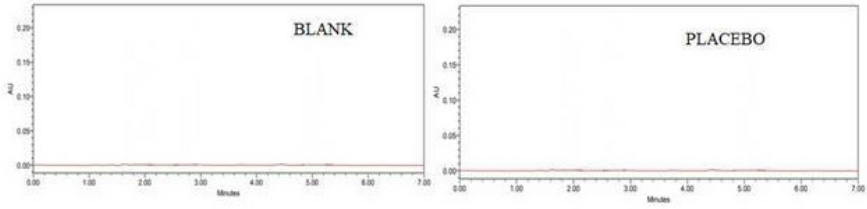

Figure 2. UPLC chromatogram of Blank and Placebo

System suitability

The performance of the UPLC system and suitability of the developed method for the intended purpose were verified by evaluating the system suitability parameters as mentioned in Table 1.

Acceptable limits for these parameters were established to ensure adequate system performance before proceeding to sample analysis. Results that fell within the acceptable range demonstrated the system could produce precise and accurate results. The results of system suitability and other validation parameters were reported in Table 1. As indicated, the results for resolution, plate count and tailing factor were found to lie within the acceptable limits. System suitability testing provides evidence that the system is capable of producing complete, separate, symmetrical and accurate measurements that meet specified requirements. By evaluating key performance indicators, system suitability verification confirms the system's quality, consistency and reliability for the intended analytical purpose. Only when system suitability was proven could the developed method be considered suitable and fit for the intended use of analyzing samples for the active ingredients Bictegravir, Emtricitabine and Tenofovir alafenamide.

Table 1. Results of system suitability and validation

Parameter	Bictegravir	Emtricitabine	Tenofovir alafenamide
USP Plate count	2874	8646	3383
USP tailing	1.11	1.04	1.06
Resolution	---	11.18	4.47
Retention time (Min)	1.910	5.020	2.739
Linearity range (µg/mL)	5-75	20-300	2.5-37.50
Correlation coefficient	0.9993	0.99906	0.99958
Slope	7303.16	8626.72	7639.59
Intercept	2892.52	14214.94	2997.09
LOD (µg/mL)	0.05	0.20	0.025
LOQ (µg/mL)	0.5	2.00	0.25
Flow rate Minus(%RSD)	0.15	0.53	0.66
Flow rate plus (%RSD)	0.06	0.35	0.15
Mobile Phase Minus (%RSD)	0.12	0.35	0.21
Mobile phase Plus (%RSD)	0.15	0.7	0.25
Assay	99.71%	99.04%	99.26%
Stability at room temperature (0-24 Hrs)	Stable	Stable	Stable
Stability at 2-8°C (0-24 Hrs)	Stable	Stable	Stable

%RSD-Percentage Relative Standard Deviation, LOD-Limit of Detection, LOQ-Limit of Quantification

Linearity

The linear relationship between analyte concentration and analytical response (peak areas) was determined for the method. Linearity was evaluated by obtaining the peak areas of replicate injections at different concentrations of the compounds within a specified range. The results were reported in Table 2 and calibration curves were shown in Figure 3. The correlation coefficients between concentration and response for the three analytes were above 0.999, indicating a high degree of linearity within the tested range. Linearity demonstrates the proportional and consistent response to increasing analyte amount across a defined concentration interval. It shows the method can accurately quantify the compounds over the expected or specified concentration range. Established linearity acceptance criteria, like minimum correlation coefficients, ensure the method has an adequate linear dynamic range for the intended application.

Results that meet and exceed the criteria substantiate the method's ability to quantify the compounds with acceptable accuracy at different concentrations.

Table 2. Linearity data

Bictegravir		Emtricitabine		Tenofovir alafenamide	
Concentration (µg/mL)	Peak area	Concentration (µg/mL)	Peak area	Concentration (µg/mL)	Peak area
0	0	0	0	0	0
5	46922	2.5	25879	20	233039
12.5	95366	6.25	50605	50	455421
25	179919	12.5	96490	100	867950
37.5	265022	18.75	150164	150	1218901
50	380055	25	192749	200	1771775
62.5	462116	31.25	237940	250	2186899
75	547335	37.5	291945	300	2610328

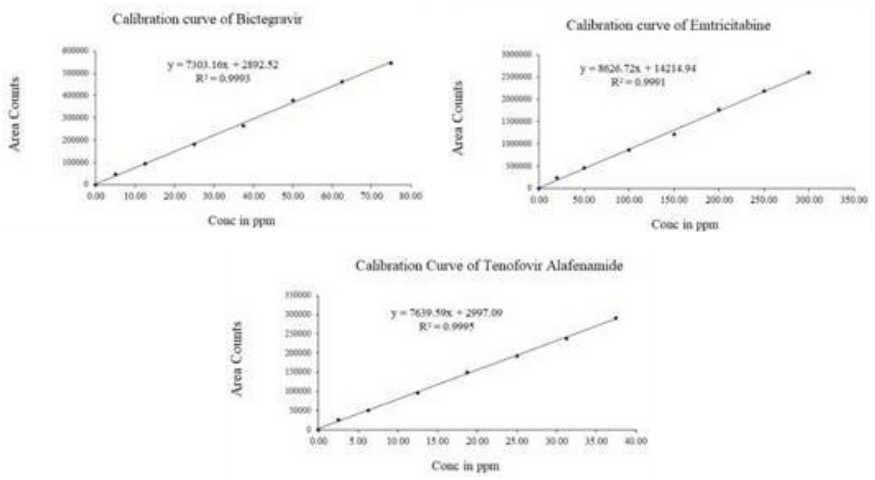

Figure 3. Calibration curves of BIC, FTC and TAF

Precision

The precision of an analytical method is typically expressed as either the standard deviation or percent relative standard deviation (%RSD). For this method, an acceptable %RSD value was established as not greater than 2.0. Repeatability (Intraday Precision) indicates the closeness of agreement between a series of measurements obtained under the same conditions. It

was determined by replicate injections of the same sample under a single set of conditions. The results of system precision and method precision were reported in Table 3.

Intermediate precision refers to the precision between laboratories or on different days. It was assessed by injecting the sample multiple times on different days. The results were provided in Table 4.

The percentage RSD values of 2% or less for precision and intermediate precision demonstrated good methods of precision and reproducibility. Higher %RSD values indicate greater variability and less reliable measurements. Methods with %RSD beyond the acceptable limit may not produce consistent or dependable results, especially for quantitative testing.

Table 3. Results of system and method precision

Sample No	Peak area response of drugs					
	Bictegravir		Tenofovir alafenamide		Emtricitabine	
	SP	MP	SP	MP	SP	MP
1.	384598	383598	192749	195483	1771809	1773276
2.	384223	381696	191930	194218	1773145	1761536
3.	377760	382323	193502	195368	1759786	1751822
4.	384743	382357	196369	194366	1780502	1744264
5.	381599	381758	194518	193369	1771283	1766622
6.	382243	382665	195558	193641	1764221	1783451
Average	382528	382399.5	194104	194407.5	1770124	1763495
STDEV	2673.66	695.84	1693.54	869.8158	7248.88	14238.09
%RSD	0.69	0.18	0.87	0.45	0.41	0.81

SP-System Precision, MP- Method Precision, STDEV – Standard deviation, %RSD – Percentage Relative Standard Deviation

Table 4. Results of intermediate precision

Inj No	Bictegravir				
	Day-1	Day-2	Average	STDEV	%RSD
1	384598	387759	386178.5	2235.16	0.58
2	384223	381695	382959	1787.57	0.47
3	377760	380724	379242	2095.86	0.55
4	384743	381596	383169.5	2225.27	0.58
5	381599	381691	381645	65.05	0.02
6	382243	383213	382728	685.89	0.18
	Emtricitabine				
1	192749	193985	193367	873.98	0.45
2	191930	192813	192371.5	624.38	0.32
3	193502	191740	192621	1245.92	0.65
4	196369	194196	195282.5	1536.54	0.79
5	194518	193532	194025	697.21	0.36
6	195558	194559	195058.5	706.40	0.36
	Tenofovir alafenamide fumarate				
1	1771809	1759787	1177199	8500.84	0.72
2	1773145	1763386	1178844	6900.66	0.59
3	1759786	1755593	1171794	2964.90	0.25
4	1780502	1768348	1182951	8594.18	0.73
5	1771283	1775920	1182403	3278.85	0.28
6	1764221	1756647	1173625	5355.63	0.46

Inj – Injection, STDEV – Standard Deviation, %RSD – Percentage Relative Standard Deviation

Accuracy

Accuracy of any developed analytical method indicates the degree of closeness between the measured value and the true value or reference value. Method accuracy was determined by performing recovery studies. The results of recovery studies in Table 5 confirm the adequate accuracy of developed method.

Table 5. Results of % recovery studies

% level	Bictegravir				Emtricitabine				Tenofovir alafenamide			
	A.A	A.R	%R	Mean %R ± RSD	A.A	A.R	%R	Mean %R ± RSD	A.A	A.R	%R	Mean %R ± RSD
50	25.1	24.75	98.6	98.43 ± 0.21	12.50	12.49	99.9	99.17 ± 0.6	100	98.94	98.9	98.81 ± 0.1
	25.2	24.83	98.5		12.50	12.35	98.9		100	98.7	98.7	
	25.1	24.64	98.2		12.50	12.34	98.7		100	98.8	98.8	
100	50.10	50.09	100	99.53 ± 0.43	25.0	24.89	99.6	98.77 ± 0.8	200	198.34	99.2	99.30 ± 0.3
	50.20	49.8	99.2		25.0	24.68	98.7		200	198.21	99.1	
	50.10	49.81	99.4		25.0	24.49	98.0		200	199.1	99.6	
150	75.20	73.78	98.1	98.16 ± 0.05	37.50	37.67	100.5	100.3 ± 0.3	300	295.14	98.4	99.30 ± 0.9
	75.40	74.02	98.2		37.50	37.48	99.9		300	197.77	99.3	
	75.30	73.97	98.2		37.50	37.67	100.5		300	300.53	100.2	

A.A – Amount Added, A.R – Amount Recovered, %R – Percentage Recovery, STDEV – Standard Deviation, %RSD – Percentage Relative Standard Deviation

Forced degradation studies

Degradation of analytes was induced by exposing the sample to various stress conditions and the chromatograms were studied for the presence of any degradant peaks without interfering with the analyte peaks. The degradation chromatograms were shown in Figure 4, and the results were given in Table 6.

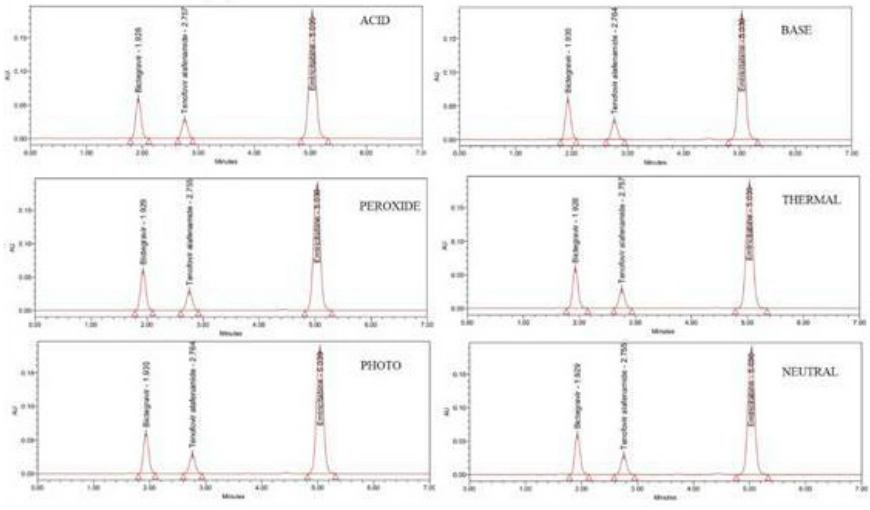

Figure 4. UPLC chromatograms of degradation studies

Table 6. Results of forced degradation studies

Stress Condition	Bictegravir			Emtricitabine			Tenofovir alafenamide		
	% D	P.A	P.T	% D	P.A	P.T	% D	P.A	P.T
Control	0.2	0.07	2.076	-0.7	0.07	2.022	0	0.483	2.199
Acid	1.8	0.071	2.107	2.7	0.087	2.033	3.2	0.233	2.209
Alkali	1.9	0.071	2.119	2.6	0.088	2.033	3.5	0.371	2.218
Oxidation	1.2	0.067	2.095	3.4	0.065	2.024	4.1	0.38	2.199
Thermal	0.8	0.083	2.115	3.4	0.088	2.032	4	0.325	2.226
Photo	1.6	0.076	2.122	3.4	0.088	2.035	3.4	0.454	2.219
Neutral	0.9	0.071	2.1	2.1	0.067	2.027	-3	0.438	2.213

%D – Percentage Degradation, P.A – Purity Angle, P.T – Purity Threshold

Assay

The developed method was applied for the assay of Biktarvy tablets. The assay values and the standard and sample chromatograms were shown in Table 7 & Figure 5.

Table 7. Assay results of marketed tablets

S.No	Parameter	Assay of Bictegravir %	Assay of Emtricitabine %	% Assay of TAF
1	Assay (Specification: NLT 98.00 % and NMT 102.00% w/w) (n=3)	99.82	99.63	99.57

NLT: Not less than, NMT: not more than, n=number of determination

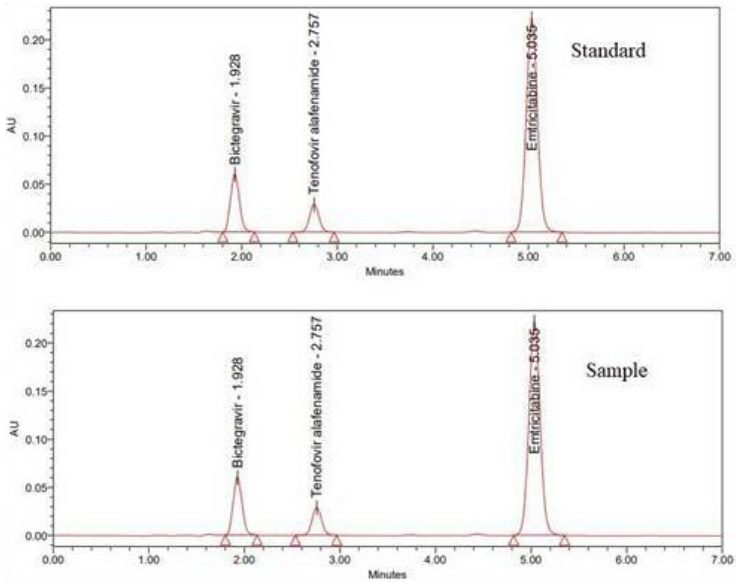

Figure 5. UPLC chromatograms of standard and sample

For the simultaneous estimation of BIC, FTC and TAF, a specific, accurate and suitable UPLC method was developed by applying different sets of conditions to achieve system suitability parameters within acceptable limits. A variety of mobile phase combinations in different proportions at different flow rates over different stationary phases were used to optimize the method.

The mobile phase consisting of acetonitrile and 0.1% TEA buffer pH 2.5 in a 30:70 volume ratio with a flow rate of 1.0 milliliters per minute was selected over a BEH C18 column as it provided better resolution and separation with an elution time of 3 minutes. Detection of the analytes was achieved at 265 nanometers using a PDA detector. The retention times were found to be 1.910, 5.020 and 2.739 minutes for Bictegravir (BIC), Emtricitabine (FTC) and Tenofovir alafenamide (TAF), respectively. The optimized chromatographic conditions were validated according to ICH guidelines to verify suitability for the intended use of the proposed method. Specificity of the developed method was indicated by the absence of interfering peaks. Key chromatographic parameters like plate count, tailing factor, resolution, peak area and retention times were evaluated for system suitability and found compliant with acceptable limits. Standard solutions in the concentration range of 18.75 to 112.5 µg/mL for BIC, 3.125 to 18.75 µg/mL for FTC, and 12.5 to 75 µg/mL for TAF showed a linear relationship with correlation coefficients above 0.999, demonstrating linearity. The proposed method was precise, accurate and robust for estimat-

ing BIC, FTC and TAF as %RSD values were less than 2.0%. When subjected to various stress conditions, the percentage degradation of BIC, FTC and TAF remained within limits. Degradation products were resolved from the analytes, indicating method specificity.

Table 8. Comparison between proposed method and reported method

Parameters	Proposed method			Reported method[17]		
	BIC	TAF	EMT	BIC	TA	EMT
Linearity (µg/mL)	5-75	2.5-37.5	20-300	12.5-75	6.25-37.5	50-300
LOD (µg/mL)	0.05	0.025	0.20	0.54	0.16	3.66
LOQ (µg/mL)	0.5	0.25	2.00	1.63	0.49	3.66

Upon comparison of the developed method with the reported method indicated that the developed method is more sensitive due to low values of LOD and LOQ, which can quantify the analytes in low concentrations (Table 8).

The proposed stability indicating UPLC method allows precise, linear, rapid and stable estimation of Bictegravir, Emtricitabine and Tenofovir Alafenamide fumarate in bulk and tablet dosage forms. The results of parameters validated were complying with the acceptance criteria given in ICH guidelines. Since the analytes were quantified with high sensitivity, better resolution and short retention times, the newly developed method can be choice for rapid determination of samples in routine quality control analysis of marketed formulation.

STATEMENT OF ETHICS

Not applicable.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

The authors significantly contributed to the present research work. The design, acquisition, analysis, drafting, critical revision, statistical analysis, and technical support of this work was contributed by Dr. Divya Narla. Supervision and critical revision of the manuscript was carried out by Dr. P. Nagaraju. Analysis and drafting of the manuscript were contributed by SPN Kumar.

FUNDING SOURCES

Not applicable.

ACKNOWLEDGMENTS

The authors take the privilege to express their gratitude to the Management, Principal of Aditya College of Pharmacy, for their continuous support.

REFERENCES

1. National Center for Biotechnology Information. PubChem compound summary for CID 90311989, Bictegravir [Internet]. 2023 [Mar 1, 2023]. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Bictegravir

2. Gilead Sciences, Inc. Biktarvy prescribing information. Foster City (CA): Gilead Sciences, Inc.; 2018 Feb.

3. National Center for Biotechnology Information. PubChem compound summary for CID 60877, Emtricitabine [Internet]. 2021 [Mar 2, 2021]. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Emtricitabine

4. National Center for Biotechnology Information. PubChem compound summary for CID 71492247, Tenofovir Alafenamide fumarate [Internet]. 2021 [Mar 2, 2021]. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Tenofovir-Alafenamide-fumarate

5. U.S. Food and Drug Administration. Biktarvy (bictegravir, emtricitabine, and tenofovir alafenamide) tablets prescribing information [Internet]. Silver Spring (MD): FDA; 2019 [Mar 2, 2021]. Available from: https://www.accessdata.fda.gov/drugsatfda_docs/label/2019/210251s006lbl.pdf

6. Raju VR, Ramana MV, Shyam T, Rajinikanth KN, Kumar VK, Raju NA. Bioanalytical method development and validation for simultaneous determination of bictegravir tenofovir and emtricitabine in human plasma by LC-MS/MS. World J Pharm Sci, 2018;7(12):441-463.

7. Prathipati PK, Mandal S, Destache CJ. Simultaneous quantification of tenofovir, emtricitabine, rilpivirine, elvitegravir and dolutegravir in mouse biological matrices by LC–MS/MS and its application to a pharmacokinetic study. J Pharm Biomed Anal, 2016;129:473-481. Doi: 10.1016/j.jpba.2016.07.040

8. Tanuja A, Ganapaty S. Bio-analytical method development and validation for simultaneous determination of Bictegravir, Emtricitabine, and Tenofovir Alafenamide Fumarate in human plasma by LC-MS/MS, Indian J Pharm Educ, 2022;56(4):1190-1205. Doi: 10.5530/ijper.56.4.201

9. Kokkirala TK, Suryakala D. RP-HPLC method development and validation for the estimation of Emtricitabine, Bictegravir and Tenofovir alafenamide in bulk and pharmaceutical dosage form. J Taibah Univ Sci, 2019;13(1):1137-1146. Doi: 10.1080/16583655.2019.1689601

10. Attaluri T, Ganapaty SE, Varanasi SNM. Development and validation of a stability-indicating RP-HPLC method for the simultaneous estimation of bictegravir, emtricitabine, and tenofovir alafenamide fumarate. Turk J Pharm Sci, 2021;18(4):410. Doi: 10.4274/tjps.galenos.2020.70962

11. Deepthi VSS, Narendra D. Method development and validation for simultaneous estimation of Emtricitabine, Bictegravir and Tenofovir alafenamide by RP-HPLC. Int J Pharm Sci Rev Res, 2019;58(2):54-59.

12. Sudha PDC, Sohail P, Avulapati U. Stability indicating RP-HPLC method for the simultaneous determination of emtricitabine, tenofovir, cobicistat and elvitegravir. Int J Pharm Sci & Res, 2020;11(3):1452-1466. Doi: 10.13040/IJPSR.0975-8232.11(3).1452-66

13. Yamana AV, Chandra Sekhar KB. Simultaneous HPLC method development and validation for quantification emtricitabine, bictegravir and tenofovir alafenamide in combined dosage form with stability studies. J Global Trends in Pharm Sci, 2020;11(3):8226-8234.

14. Meenakshi R, Shyam Sunder R. Method development and validation by RP-HPLC for simultaneous estimation of emtricitabine, bictegravir, tenofovir alafenamide in fixed dosage form. IAJPS, 2018;5(7):7123-7131.

15. Deepthi R, Gowri Sankar D. Novel stress indicating RP-HPLC method development and validation for the simultaneous estimation of Bictegravir, Emtricitabine and Tenofovir alafenamide. Indo Am J P Sci, 2019;6(6):13282-13291.

16. Abdul Sattar MD, Achanta S. Analytical method development and validation for the determination of Emtricitabine and Tenofovir Disoproxil Fumarate using reverse phase HPLC method in bulk and tablet dosage form. J Pharm Sci & Res, 2018;10(5):1207-1212.

17. Dadi V, Sowjanya G. Validated method for the simultaneous determination of emtricitabine, bictegravir and tenofovir alafenamide in pharmaceutical dosage form using UPLC. J Pharm Sci & Res, 2022;13(11):4536-4543. Doi: 10.13040/IJPSR.0975-8232.13(11).4536-43

18. International Conference on Harmonisation (ICH). ICH harmonised tripartite guideline Q2(R1): validation of analytical procedures – text and methodology. Geneva: ICH; 2005.