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BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS DETERMINATION OF BICTEGRAVIR, TENOFOVIR AND EMTRICITABINE IN HUMAN PLASMA BY LC-MS/MS

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ABSTRACT

A simple, sensitive and fast throughput liquid chromatography tandem mass spectrometry (LC-MS/MS) method has been developed for the simultaneous estimation of bictegravir, tenofovir and emtricitabine in human plasma, using respective didanosine, stavudine and abacavir as internal standards respectively. The method involved Liquid-Liquid Extraction of the analytes and internal standards from human plasma. The chromatographic separation was achieved on a Zorbax C18 column (150×4.6mm and 5µm particle size) analytical column using isocratic mobile phase, consisting of Methanol : 0.1% formic acid in water (85:15, v/v), at a flow-rate of 1.0 mL/min with 90% flow splitting. The parent→product ion transitions were monitored at m/z 268.2 → 127.1 (BTGR), m/z 237.1→137.1 (DDI), m/z 230.2→112.1 (TNFR), m/z 248.1→130.0 (D4T), m/z 267.2→226.1 (EMTB) and m/z

287.2→191.2 (ABC) on a triple quadrupole mass spectrometer, operating in the multiple reaction monitoring (MRM) positive ion mode. The method was validated over the concentration range of 5-1500 ng for Bictegravir and tenofovir and 10-3000 ng/mL for Emtricitabine. The mean recovery values for both the drugs from spiked plasma samples were reproducible. The method was rugged and rapid with a total run time of 4.0 minutes.

KEYWORDS: Bictegravir, Tenofovir and Emtricitabine, LC-MS, Extraction.

INTRODUCTION

Biktarvy® (bictegravir, emtricitabine, and tenofovir alafenamide) is a fixed dose combination tablet containing bictegravir (BIC), emtricitabine (FTC), and tenofovir alafenamide (TAF) for oral administration.[1-4] 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 a molecular weight of 471.4. Bictegravir sodium is an off-white to yellow solid with a solubility of 0.1 mg per mL in water at 20 °C.: 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 in that it has a fluorine in the 5 position. FTC has a molecular formula of $C_8H_{10}FN_3O_3S$ and a molecular weight of 247.2. Emtricitabine is a white to off-white powder with a solubility of approximately 112 mg per mL in water at 25°C. : 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] phenoxy phosphinyl]-, 1-methylethyl ester, (2E)-2-butenedioate (2:1). Tenofovir alafenamide fumarate is a white to off-white or tan powder with a solubility of 4.7 mg per mL in water at 20°C. Each tablet contains 50 mg of BIC (equivalent to 52.5 mg of bictegravir sodium), 200 mg of FTC, and 25 mg of TAF (equivalent to 28 mg of tenofovir alafenamide fumarate) and the following inactive ingredients: croscarmellose sodium, magnesium stearate, and microcrystalline cellulose. The tablets are film-coated with a coating material containing iron oxide black, iron oxide red, polyethylene glycol, polyvinyl alcohol, talc, and titanium dioxide.[5-8] Biktarvy is indicated as a complete regimen for the treatment of human immunodeficiency virus type 1 (HIV-1) infection in adults who have no antiretroviral treatment history or to replace the current antiretroviral regimen in those who are virologically-suppressed (HIV-1 RNA less than 50 copies per mL) on a stable

antiretroviral regimen for at least 3 months with no history of treatment failure and no known substitutions associated with resistance to the individual components of Biktarvy®. The tablets are purplish brown, capsule-shaped, film-coated, and debossed with "GSI" on one side and "9883" on the other side. There are some analytical methods that have been reported for the determination of Bictegravir, tenofovir and emtrcitabine in pharmaceutical formulations at the time of commencement of research work.[9-10] The objective of this study was to develop and validate a more sensitive and selective high throughput LC-MS/MS method that can be efficiently used in pharmacokinetic studies, to evaluate bioavailability and bioequivalence for this potent combination of Bictegravir, Tenofovir and Emtricitabine [11]. This method has been developed exclusively to study the pharmacokinetic parameters by non-compartmental design, which offers the good selectivity and specificity than the methods reported earlier. Also, the present method is sensitive in terms of LOD and LOQ by LC method with MS-detection.

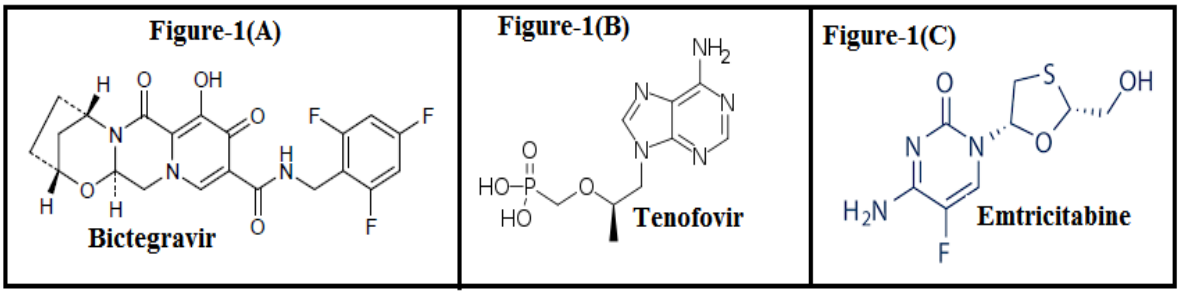


Figure- 1(a): Chemical Structure of Bictegravir

Figure- 1(b): Chemical Structure of Tenofovir

Figure-1(c): Chemical Structure of Emtricitabine

Reference and working standards: The reference standards of Bictegravir (BTGR), Tenofovir (TNFR) and Emtricitabine (EMTB) are procured from Mylan Laboratories, Hyderabad as gift samples. Didanosine (DDI), Stavudine (D4T) and Abacavir (ABC) are acquired from Hetero Drugs, Hyderabad and used as internal standards.

Reagents and Chemicals: All the chemicals and reagents used were of standard grade. Methanol and acetontirle (HPLC Grade) is obtained from Thermo Fisher Scientific India Private Limited (Sion East, Mumbai, India). Formic acid (GR Grade) is purchased from Merck (Worli, Mumbai, India). Blank plasma is Harvested K_2 EDTA blank plasma for method development and validation was obtained Sri Laxmi Sai Diagnostics (Hyderabad, India).

Table 1: Instruments used in LC-MS method development.

Name of the instrument	Model / Features	Manufacturers
Analytical balance	CP225D	Sartorius
Analytical Columns	C18, 150 x 4.6 mm i.d., 5 µm	Zorbax
Centrifuge	SW12R	Firlabo
Deep Freezer	-86°C & -20°C, VIP Series	Sanyo
HLB cartridges	30mg / 1CC	Waters
HPLC System	Prominence series with SIL HTc Autosampler	Shimadzu
Micro balance	CP2P	Sartorius
Micropipette	5-50µL, 100-1000µL	Brand
MS Spectrometer	API-4000	MDS, Sciex
Nitrogen Evaporator	TurboVap LV	Caliper Life sciences
pH Meter	Orion Star	Thermo Electronic Corporation
Solid phase extraction unit	SpeeDisk48	Orochem Technologies
Ultrasonic bath	Powersonic 510	Hwashin Technologies
Vortexer	Spinix	Spinix
Water purification system	Elix 10 & Milli-Q Gradient A10	Millipore

Equipment and LC-MS/MS Assay Conditions: HPLC system (Shimadzu, Kyoto, Japan), equipped with LC-20AD pumps for solvent delivery, DGU-20 A3 degasser, CTO-AS vp Column oven and a high throughput a SIL HTc autosampler was used for the analysis. Mass spectrometric detection was performed on an API-4000 triple quadrupole instrument (MDS-SCIEX, Toronto, Canada) using multiple reaction monitoring (MRM) mode. An optional low pressure gradient valve (LC-20AD/20AT), CBM-20Alite system controller, and a seal wash pump (LC-20AD) can now be installed inside the solvent delivery unit, enabling 4-solvent gradient elution in a compact space. The LC-20AB high-pressure binary gradient pump delivers the performance of two LC-20AD pumps in one compact unitA turbo ion spray interface in positive ionization mode was used and the data processing was performed on Analyst software, version 1.4.1 (SCIEX).

Table 2: The optimized chromatographic conditions.

Parameter	Condition
Mobile Phase	Methanol : 0.1% formic acid in water (85:15, v/v)
Cloumn	Zorbax C18 column (150 mm x 4.6 mm i.d., 5 µm, Make: Chromatopak analytical instrumentation, India)
Flow Rate	0.8 mL / minute
Injection Volume	3 µL
Column Oven Temperature	35 ± 1°C
Auto-sampler Temperature	10 ± 1°C
Retention Time(s)	Bictegravir: 1.5 minutes
	Didanosine: 1.4 minutes (ISTD for Bictegravir)

	Tenofovir: 1.2 minutes
	Stavudine: 1.4 minutes (ISTD for Tenofovir)
	Emtricitabine: 1.7 minutes
	Abacavir: 1.1minutes (ISTD for Emtricitabine)
Run Time	3 minutes
Splitness	25 : 75

Mass Spectrometry Conditions

The basic principle of MS is the production of ions which are subsequently separated according to their mass-to-charge ratio (m/z) and detected. Method development involves scanning of the analytes to find the parent and its respective fragment ions in mass spectrometer. For this purpose 200ng/mL solution of Analytes and Internal standards were prepared in acetonitrile: water mixture 90:10%, v/v. Solution of each analyte / ISTD was infused separately at a flow rate of 10µL using the Hamilton syringe pump and scanned parent and product masses in full scan mode. Mass spectra of each analyte and internal standard were recorded in the range of 100 to 600 amu. Once the parent ion was obtained it was further scanned for product ions using MS/MS mode. Nitrogen gas was used as collision gas, zero air as sheath gas and the resolution was set to unit mass. The fragment ion having higher intensity was selected for multiple reactions monitoring (MRM). After selecting the parent and product ion, compound parameters were optimized in infusion mode and the gas parameters were optimized in flow injection analysis with mobile phase. A "T" connector was used to connect the LC pump and syringe pump to the detector and optimized the gas parameters at a flow rate of 0.3ml/min. to get appropriate gas parameter values. A Turbo ion spray interface (TIS) operated in positive ionization mode was used for the detection. The MRM transitions monitored were m/z 268.2 → 127.1 (BTGR), m/z 237.1→137.1 (DDI), m/z 230.2→112.1 (TNFR), m/z 248.1→130.0 (D4T), m/z 267.2→226.1 (EMTB) and m/z 287.2→191.2 (ABC) with a dwell time of 200 ms per transition and the quadruples 1 and 3 were set at unit resolution.

Table 3: Mass Spectral Parameters used in Tandem Analysis of BTGR, TNFR, EMTB.

Compound Parameters						
Parameter	**Value**					
	BTGR	**DID**	**TNFR**	**D4T**	**EMTB**	**ABC**
Declustering potential	40	40	40	40	20	40
Entrance Potential	10	10	10	10	10	10
Collision Energy	15	12	10	15	50	30
Collision Cell Exit Potential	10	10	10	10	10	10

Gas / Turbo Ion Spray Source Parameters	
Parameter	**Value**
Ion source gas 1 (psi)	35
Ion source gas 2 (psi)	35
Curtain gas (psi)	30
Collision gas (psi)	4
Ion spray Voltage (v)	5500
Source temperature (°C)	450
Interface Heater (ihe)	ON

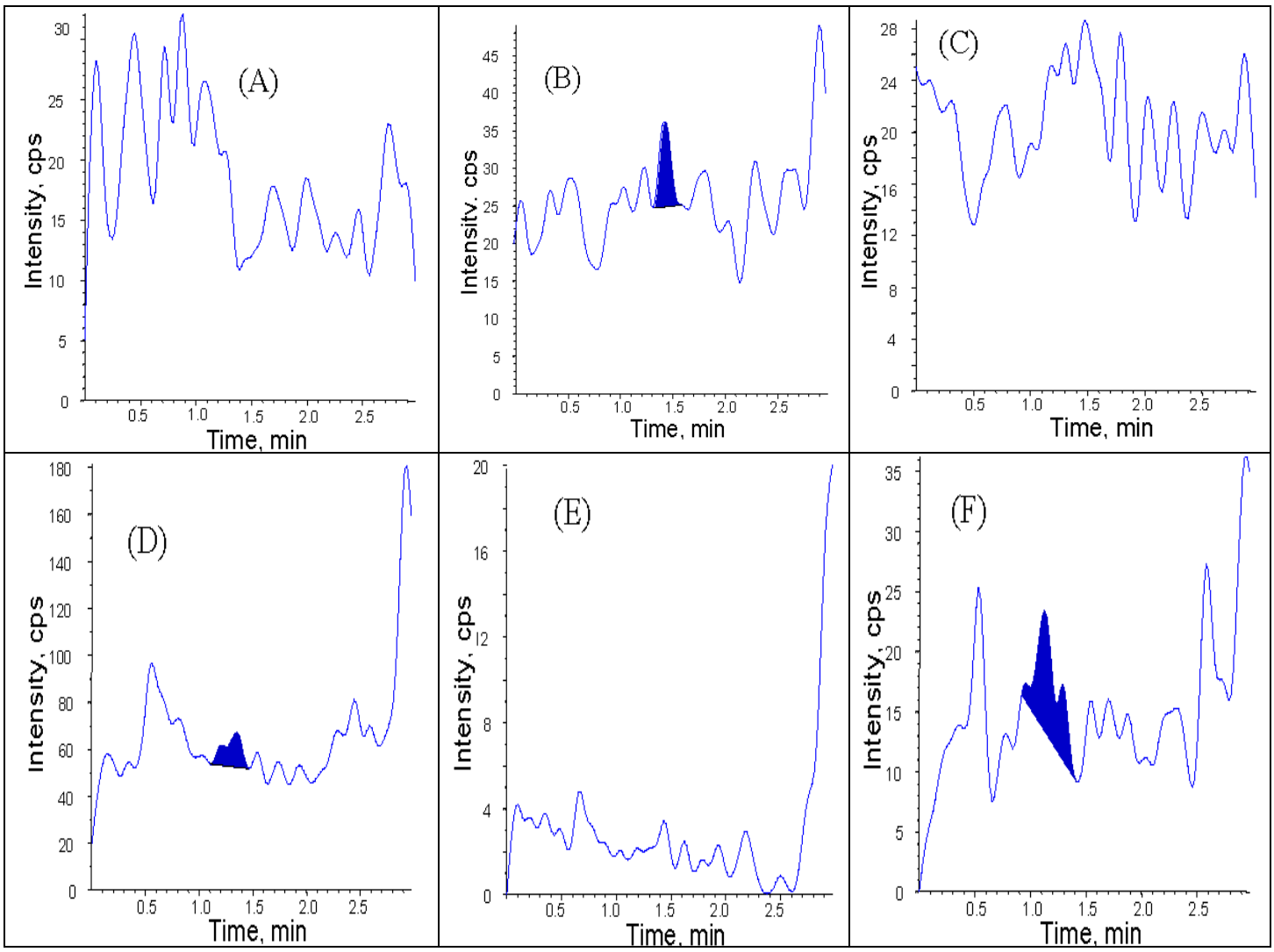


Figure 2: Representative chromatograms of (A) BTGR, (B) DDI (C) TNFR (D) D4T (E) EMTB and ABC (F) in Blank Plasma.

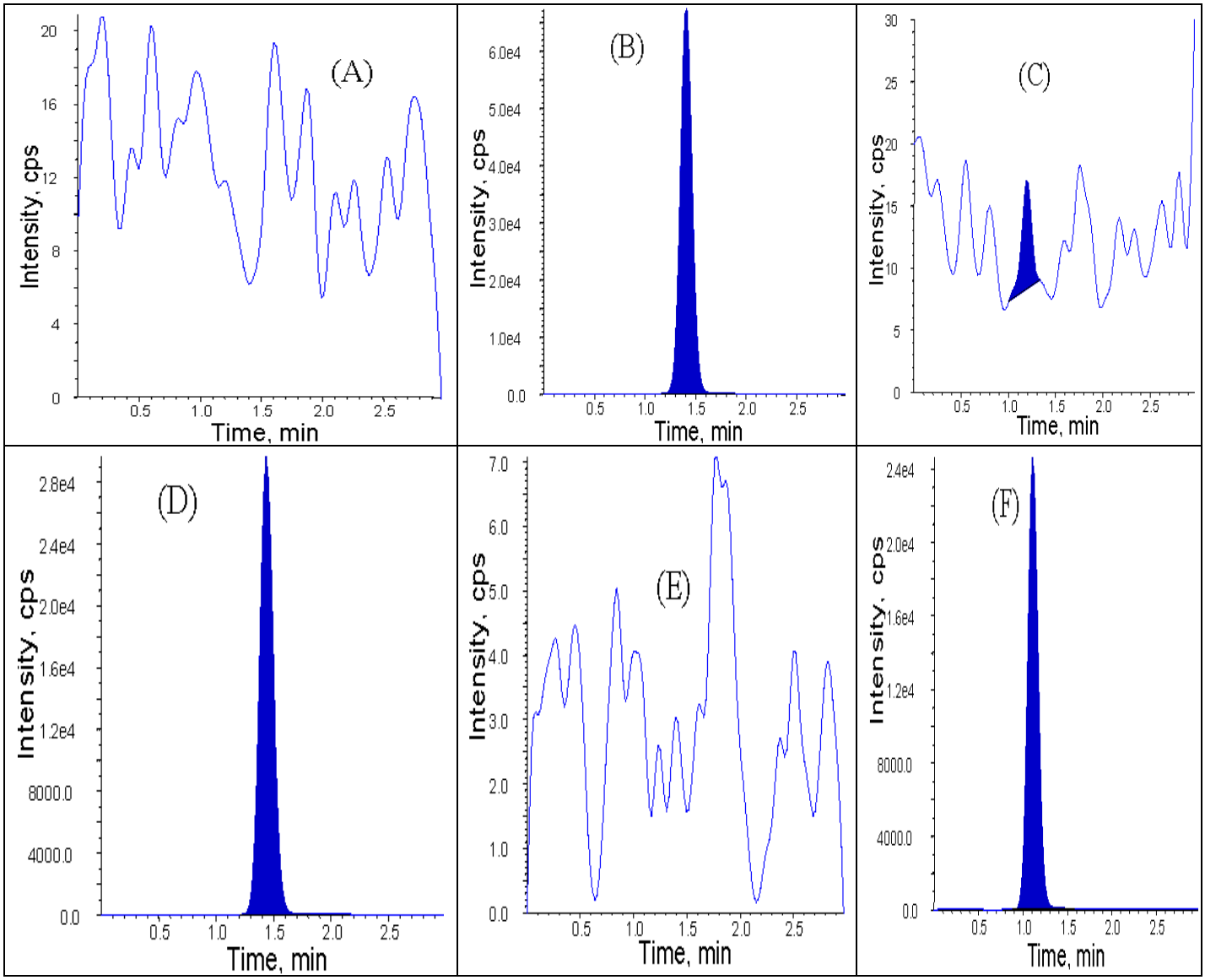
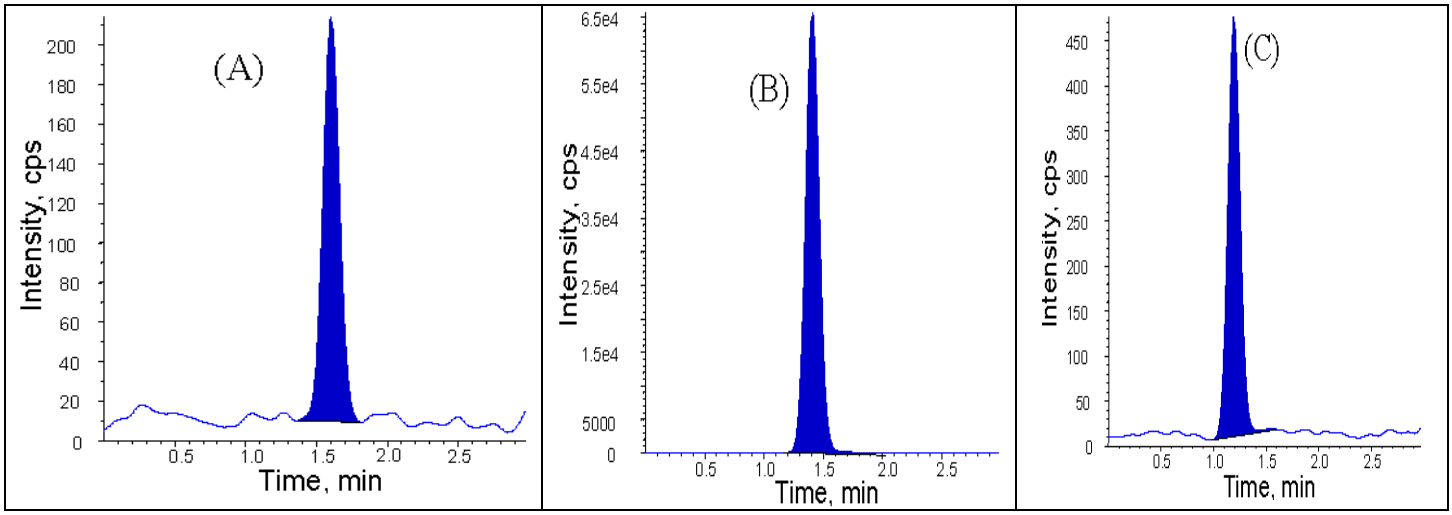


Figure-3: Representative chromatograms of (A) BTGR, (B) DDI (C) TNFR, (D) D4T (E) EMTB and ABC (F) in Blank Plasma with internal standards.



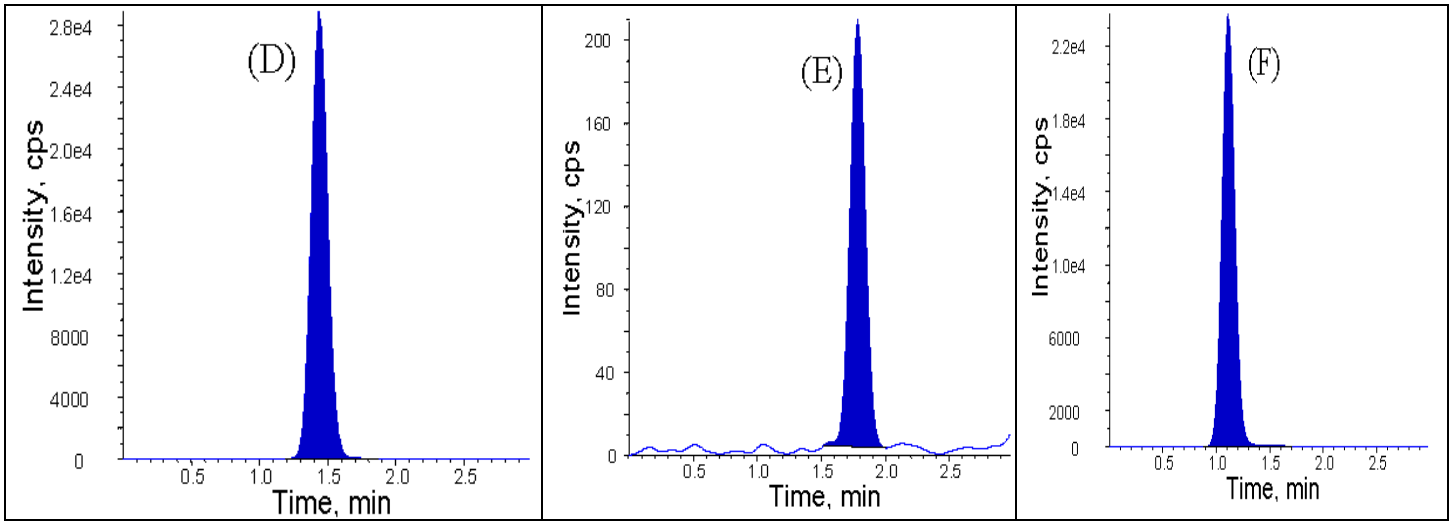


Figure-4: **Representative chromatograms of (A) BTGR, (B) DDI (C) TNFR, (D) D4T (E) EMTB and ABC (F) in LLOQ Samples**.

Preparation of Standard Stock Solutions: A 99.9% pure Tenofovir, Bictegravir and Emtricitabine obtained from M/S. Hetero research center were used as standard reference materials to prepare the stock solutions for both calibration and quality control standards. Stock solutions of BTGR, TNFR, EMTB and ISTDs were prepared by weighing each of the working standard equivalents to 10.0 mg into separate 10.0mL volumetric flasks, dissolved with 5.0 mL of methanol and diluted up to the mark with methanol. The stock solutions were stored in refrigerator at 1-10°C.

Preparation of Working Solutions: The combined working solutions of BTGR, TNFR and EMTB were prepared by diluting the stock solutions in methanol: water mixture (50:50, v/v). Internal standard working solution (containing 2000 ng/mL DDI, 500 ng/mL D4T and 250 ng/mL of ABC) was also prepared in methanol: water mixture (50:50, v/v) and is used in the assay. The prepared working solutions were stored at room temperature and daily fresh dilutions were made during the time of analysis. All the volumetric measurements were made using calibrated micropipettes.

Preparation of Plasma Spiked Calibration Standards and Quality Control Samples: Calibration standards and quality control (QC) samples were prepared by spiking blank plasma with freshly prepared working solutions. Blank plasma lots obtained from healthy, non-smoking volunteers were individually screened and pooled before use. Calibration standards were made at concentrations of 5, 10, 25, 100, 400, 800, 1200, 1500 ng/mL for Bictegravir and Tenofovir; and 10, 20, 50, 200, 800, 1600, 2400, 3000 ng/mL for

Emtricitabine. Quality control samples were prepared at 5 ng/mL (LLOQ QC), 15 ng/mL (LQC), 800 ng/mL (MQC) and 1200 ng/mL (HQC) for BTGR and TNFR; and 10 ng/mL (LLOQ QC), 30 ng/mL (LQC), 1600 ng/mL (MQC) and 2400 ng/mL (HQC) for EMTB. LLOQ QC samples were prepared only during validation batch runs). 0.5mL each of the plasma spiked calibration standards and Quality control samlpes were distributed in to single use polypropylene tubes and are stored at -70 ± 15°C untill analysis.

Sample Extraction Procedure: The stored plasma samples were retrieved from freezer and thawed un-assisted at room temperature and are subjected to the sample preparation procedure as given below.

A 300 µL aliquot of each sample was transferred to a 5 mL polypropylene tube, followed by addition of 50 µL of ISTD solution (containing 2000 ng/mL DDI, 500 ng/mL D4T and 250 ng/mL of ABC) and 100 µL of formic acid (0.1% formic acid in water). The contents were briefly mixed by vortex and were subjected to solid phase extraction. On the positive pressure SPE unit, HLB 30 mg/1 mL cartridges were placed and conditioned with 1 mL each of methanol and Milli-Q water. After dispensing of plasma samples, washing of the cartridges was performed with 1 mL of Milli-Q water followed by 1 mL of methanol: water (85:15, v/v). Finally the cartridges were eluted with 1 mL of acetonitrile and the eluates were evaporated to dryness under stream of nitrogen in a 50°C water bath. The residue of each sample was reconstituted in 600 µL mobile phase, and a 3 µL aliquot was injected on to the LC-MS/MS system.

Preparation of Mobile Phase and System Suitability Test: The composition of the mobile phase was determined during method development. In order to get consistent results throughout the validation and study analysis, a large volume of mobile phase was prepared by adding 850 mL of methanol to 150 mL of Milli Q water. System suitability solution was prepared in mobile phase at ULOQ concentration (i.e., 1500ng/mL for BTGR and TNFR and 3000ng/mL for EMTB along with ISTDs). A System Suitability Test (SST) was performed at the beginning of each validation or study analytical run to verify the suitability of system for analysis. As a part of the test, six replicate injections of system suitability solution was made prior to each analytical run and the system performance was considered acceptable if the %CV for reponse ratios for each analyte was ≤ 4.0%.

Method development: Optimization of mass spectral conditions: Method development is initiated with scanning of the analyte solutions for the parent and fragment ions using 200ng/mL solution of BTGR, TNFR, EMTB as analytes and DDI, D4T and ABC were used as internal standards. Solution of analyte was injected using the syringe pump and scanned for the parent mass of the analyte. The parent ion was further scanned for product ions using MS/MS mode. Based on their ability to accept the protons, analytes and ISTDs were tuned in positive mode using electro spray ionization technique. Mass scanning was done in the range of 100 to 600 amu. Atmospheric pressure chemical ionization (APCI) has showed less sensitivity over ESI for analytes of interest. ESI negative ionization mode exhibited very weak signals for the parent and product ions. Antiviral drugs have high sensitivity in electrospray ionization mass spectrometric analysis compared to non- polar and ionizable organic compounds, due to their polar nature and presence of highly ionizable function groups such as amine or carboxylic acid in their structures. Turbo ion spray interface (TIS) operated in positive ionization mode was used for the detection. A highly stable and intense product ion was formed at MRM transitions monitored were m/z 450.1 → 289.1 (BTGR), m/z 237.1→ 137.1 (DDI), m/z 287.90 →176.05 (TNFR), m/z 225.2 → 127 (D4T), m/z 248.1 → 130.0 (EMTB) and m/z 287.2 → 191.2 (ABC) with a dwell time of 200 ms per transition and the quadruples 1 and 3 were set at unit resolution. In the optimization of compound parameters, comparatively high collision energy was used for Emtricitabine to get appropriate response. Increase of source temperature beyond 450°C has shown as negative impact on the signal of BTGR and TNFR.

Optimization of extraction procedure: In the optimization of extraction procedure liquid-liquid extraction and precipitation techniques were not used due to polar nature of the analytes. Solid phase extraction was selected to get consistent and reproducible results with low matrix effects for the intended mass spectrophotometric analysis. An offline solid-phase extraction procedure was carried out using HLB 30mg/ICC cartridges. HLB is a polymeric based sorbent with both hydrophilic and lipophilic sites and which can provide consistant results for polar as well as non-polar compounds. During the initial optimization of the extraction procedure a basic protocol was followed by using 1mL Methanol and water in conditioning step and 1mL of water and 5% methanol in washing step. The final elution of compounds was made using Methanol. However, high matrix effect was observed in TNFR during the optimization process, which was subsequently eliminated using Methanol: water mixture 85:15, v/v in washing step and acetonitrile in elution step.

Method Validation: Validation runs were conducted on six separate days. Each precision and accuracy validation run organized with a set of spiked calibration standard samples distributed at eight concentration levels over the dynamic range, a blank (without ISTD), a zero sample (blank with ISTD) and QC samples (n=6 at each of four concentration levels; LLOQ, low, medium and high). Standard samples were analyzed at the beginning of each validation run and other samples were distributed randomly throughout the run. Results of the QC samples from four inter & intraday runs were used to evaluate the accuracy and precision of the method. Sensitivity (at lower limit of quantification), dilution integrity (two fold and four fold), and ruggedness of the method were also determined.

Selectivity: Selectivity is defined as 'the ability of the bio-analytical method to measure unequivocally and to differentiate the analyte in the presence of endogenous components, which may be expected to be present, typically the endogenous components might include metabolites, impurities, matrix components, etc'. Method selectivity is established by proof of the lack of response by analyzing human K_2 EDTA plasma blank matrices from eight different individual matrix lots along with one lipemic and one hemolytic plasma lot. Peak responses in blank lots were compared against the mean response of spiked LLOQ samples (n = 6) and negligible interferences were observed in all the screened lots, at the retention time of analytes and ISTDs. Figure 3, 4 and 5 demonstrates the selectivity of the method, with the chromatograms of blank plasma, blank plasma with internal standards and LLOQ sample respectively.

Sensitivity: The lower limit of quantification (LLOQ) is defined as the lowest amount of an analyte in a sample that can be quantitatively determined with suitable precision and accuracy (bias). This was performed by injecting six replicates of extracted LLOQ samples against a calibration curve. The lower limit of quantification (LLOQ) was found to be 5 ng/mL for both BTGR and TNFR and 10 ng/mL for EMTB. At LLOQ accuracy (%RE) for all the three analytes was ranged from -6.0 to 6.0%, with a %CV of < 8.6%. The mean signal to noise ratio at LLOQ (n=6) was found to be 150:1, 123:1 and 118:1 for TNFR, BTGR and EMTB respectively. All the results were found to be within the acceptable limits.

Linearity: The relationship between the concentration analyte in the sample versus ratios of analyte and ISTD must be investigated. Calibrators must cover the whole calibration range and should be matrix based. A minimum of five to eight concentration levels should cover the dynamic linear range, excluding the blank and zero samples. The linearity of each calibration

curve was determined by plotting the peak-area ratio (y) of analytes to ISTDs versus the nominal concentration (x) of analytes. Calibration curves were linear from 5 to 1500 ng/mL for BTGR, TNFR and 10 to 3000 ng/mL for EMTB with coefficient of correlation (r^2) values more than 0.9926. The r^2 values, slopes and intercepts were calculated using weighted ($1/X^2$) linear regression analysis with four intraday and inter day calibration curves. The observed mean back calculated concentrations with accuracy (%) and precision (%CV) from 4 linearity curves were presented in Table 2.7.

Precision and Accuracy: The precision of a method is defined as the closeness of agreement between independent test results obtained under prescribed conditions. Each precision and accuracy run consisting of QC samples (6 replicates each of the LLOQC, LQC MQC and HQC) were analysed and back calculated against a set of calibration curve standards. Inter and intra batch accuracy and precision evaluation was done using four different 4 batches analysed on different days. Results of inter and intra batch accuracy and precision were presented in *table 2.8.*

Table 4: Summery of Calibration Standard solutions.

Analyte	Nominal Conc. ng/mL	Mean found Conc. ng/mL	% CV	% Re
Bictegravir	5.01	5 .15	3.3	2.8
	10.02	9.74	5.7	-2.8
	25.04	21.80	6.1	-12.9
	100.17	101.32	1.4	1.1
	400.67	396.67	7.1	-1.0
	801.34	822.97	4.0	2.7
	1203.21	1201.31	1.8	-0.2
	1504.01	1582.25	4.4	5.2
Tenofovir	5.00	5 .05	1.2	1.0
	10.00	9.71	5.7	-2.9
	25.01	24.82	6.1	-0.8
	100.03	94.98	1.4	-5.0
	400.11	399.15	7.1	-0.2
	800.21	758.04	4.0	-5.3
	1201.52	1253.30	1.8	4.3
	1501.90	1561.05	4.4	3.9
Emtricitabine	10.03	10.10	0.8	0.7
	20.04	19.38	1.6	.3.3
	50.10	50.68	2.4	1.2
	200.51	211.50	1.2	5.5
	801.84	505.13	2.0	0.5
	1603.75	1533.34	1.4	-4.4
	2407.97	2371.28	1.9	-1.5

	3009.98	3028.51	10	0.6
Mean of 4 Replicates of each concentrations % CV- Coefficient of Variation % RE- Percent Relative Error				

Table 5: Summery of Intra- and Inter batch Precision and accuracy study.

Analyte	QC Level	Nominal Conc. ng/mL	Intra batch			Inter batch		
			Mean found Conc. ng/mL	% CV	% Re	Mean found Conc. ng/mL	% CV	% Re
Bictegravir	LLOQQC	5.03	5.56	10.5	62	5.33	6.0	8.6
	LQC	14.79	15.10	2.1	2.3	14.74	-0.3	6.5
	MQC	799.21	848. 07	6.1	2.1	814.36	1..9	4.0
	HQC	1200.03	1329.62	10.8	2.7	1239.23	3.3	6..2
Tenofovir	LLOQQC	5.02	5.32	6.0	7.4	5.18	3.2	5.8
	LQC	14.77	14.80	0.2	1.8	14.68	-0.6	3.9
	MQC	798.58	811.60	1.6	3.7	815.13	2.1	3.0
	HQC	1199.06	1234.46	3.0	3.8	1243.02	3.7	3.2
Emtricitabine	LLOQQC	10.12	9.25	-8.6	6.1	9.51	-6.0	7.7
	LQC	29.64	28.73	-3.1	1.8	29316	-1.6	5.1
	MQC	1600.26	1631.14	1.9	3.8	1659.85	3.7	2.0
	HQC	2402.82	2365.53	-1.6	3.8	2396.09	-0.3	2.3
Mean of 6 Replicates of each concentrations Mean of 24 Replicates of each concentrations % CV- Coefficient of Variation % RE- Percent Relative Error								

Matrix effect: Co-eluting matrix components can suppress or enhance ionization but might not result in a detectable response in matrix blanks due to the selectivity of MS detection, however precision and accuracy of the method may get affected. The matrix effect was investigated by extracting blank plasma from six different sources, including one hemolytic and one lipemic lot. After extraction, residue from each blank lot was reconstituted with mobile phase having known amount of analyte (LQC level along with ISTD; post extracted samples) and analyzed along with equivalent aqueous samples. Compared the response ratio obtained from each of the post extracted matrix lot, against the mean response ratio of aqueous samples. Matrix Factor for analyte / ISTD were calculated by comparing the peak response in presence of matrix ions to that of peak response in absence of matrix ions.

The potential for variable matrix related ion suppression or enhancement was evaluated in six independent sources (containing one hemolytic and one lipemic lot) of human plasma, by calculating the IS normalized matrix factor. The mean IS normalized matrix factor for all the three analytes was ranges between 0.93 to 1.02 with a % CV of ≤ 8.5 as shown in Table-2.9.

Table-6: Matrix Effect results.

Analyte	Bictegravir			Tenofovir			Emtricitabine		
	MF for Analyte	MF for ISTD	IS Normalized MF	MF for Analyte	MF for ISTD	IS Normalized MF	MF for Analyte	MF for ISTD	IS Normalized MF
Lot 1	1.2510	1.2253	1.0210	0.9911	1.0187	0.9729	1.3413	1.2581	1.0661
Lot 2	1.1497	1.1486	1.0010	0.9035	0.9569	0.9442	1.2205	1.1652	1.0475
Lot 3	1.0836	1.2441	0.8710	0.9362	0.9377	0.9984	1.1571	1.2012	0.9633
Lot 4	1.1232	1.1381	0.9869	0.9375	1.0455	0.8967	1.1811	1.1704	1.0091
Lot 5	1.0626	1.2891	0.8243	0.9220	0.9335	0.9877	1.1824	1.1315	1.0450
Lot 6	1.0325	1.1406	0.9052	0.9261	0.9787	0.9463	1.0458	1.0458	1.0000
Mean			0.9349			0.9577			1.0218
% CV			8.5			3.9			3.7
MF: Matrix Factor									

Extraction Recovery: Recovery is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the pure authentic standard. Recovery of the analyte need not to be 100%, but the extent of recovery of an analyte and of the internal standard should be consistent, precise and reproducible. The extraction recovery of BTGR, TNFR and EMTB was determined (at low, medium and high concentration) by comparing the responses from plasma samples spiked before extraction (n=6) with those from plasma samples extracted and spiked after extraction. The mean recovery of BTGR, TNFR and EMTB was found to be 77.6%, 50.2% and 78.5% respectively with %CV across the three levels ranging between 1.2 to 13.9%, as shown in Table 2.10.

Table-7: Extraction recovery for Bictegravir Tenofovir and Emtricitabine.

Analyte	QC Level	A	B	% Recovery	% Mean Recovery	% CV
Bictegravir	LQC	4597	5153	89.2	77.6	13.5
	MQC	242249	351159	69.0		
	HQC	332274	445773	74.5		
Tenofovir	LQC	10988	18986	57.9	50.2	13.9
	MQC	594371	1341563	44.3		
	HQC	828202	1709535	48.4		
Emtriicitabine	LQC	7934	9978	79.5	78.5	1.2
	MQC	414395	533309	77.7		
	HQC	550633	704197	78.2		
Extraction recovery for internal standard at MQC Level						
Internal STD		A	B	% Recovery		
Didanosine		287265	409778	70.1		
Emtricitabine		237827	312639	76.1		
Abacavir		255118	310574	82.1		

A -Mean response in extraction samples
B – Mean response in post extraction spiked samples

Dilution Integrity: Dilution integrity of the method was evaluated after ½ and ¼ dilution. The mean back calculated concentrations for ½ and ¼ dilution samples were within 85-115% of their nominal concentration with a %CV of ≤ 3.6.

Stability: Stability in Aqueous Solutions: Stability of analytes and ISTDs in stock solutions and in working solutions was evaluated at both room temperature and at 1-10°C. The stock solutions were prepared in Methanol and the working solutions were prepared in methanol: water mixture (50:50, v/v). At the time of stability evaluation, dilutions (n=6) from both stability solutions and comparision solutions were prepared in mobile phase and analyzed with alternate injections from stability and comparision solutions. Analytes and ISTDs are found stable in aqueous solutions with % stability ranging between 98.4 to 101.2%.

Stability in Biological Matrix: Demonstration of analyte stability in matrix should mimic the conditions, as best as possible, under which the study samples were collected, stored, processed and analyzed. These establishments include Bench top stability, freeze thaw stability, Long-term stability to assess the processing and storage conditions. In addition the stability in processed samples was also demonstrated using Dry extract and In-injector stability evaluations. Matrix stability evaluations were performed using freshly prepared calibration standards and quality control samples.

Freeze and Thaw Stability: From a practical standpoint, it is often necessary to expose study samples for multiple freeze-thaw cycles before final analytical results are obtained. Freeze thaw (FT) stability was evaluated at LQC and HQC Levels. During freeze cycles the stability samples were maintained at -70 ± 15°C and the thaw cycles were made unassisted at room temperature. The first freeze cycle was made for 24 hours followed 12 hours to the subsequent cycles. Stability samples were analyzed against freshly prepared calibration standards comparison quality control samples. After subjecting to 4 freeze-thaw cycles, six replicates each of low and high quality control stability samples were processed and analyzed along with freshly spiked calibration standards and quality control samples (comparison samples). The concentrations of stability and comparison samples were back calculated and the % stability was computed by comparing the mean concentration of stability samples against the mean concentration of comparison samples.

Bench-top Stability: Bench top (BT) stability was evaluated to confirm that analyte degradation does not occur during preparation or extraction of validation and study samples prior to their analysis. Six replicates each of low and high quality control samples were retrieved from the freezer and are placed un-assisted at room temperature (at ~25°C). After a period of 18 hours the stability samples were processed and analyzed along with freshly spiked calibration standards and quality control samples (comparison samples). The concentration of stability and comparison samples were back calculated and the % stability was computed by comparing the mean concentration of stability samples against the mean concentration of comparison samples.

Long-term Stability: Long-term storage stability was assessed to confirm analyte stability in the test system matrix covering the length of time from study sample collection to sample analysis. After 75 days of storage at -20 ± 5°C and / -70 ± 15°C, six replicates each of low and high stability samples were processed and analyzed with freshly spiked calibration standards and quality control samples (comparison samples). The concentration of stability and comparison samples were back calculated and the % stability was computed by comparing the mean concentration of stability samples against the mean concentration of comparison samples.

Dry-extract stability*:* Dry-extract stability was carried out whenever the sample processing involves evaporation before injecting in to chromatographic system to anticipate the sample exposure to room temperature after evaporation. Six replicates each of low and high quality control samples (stability samples) were processed as per the established extraction procedure and are stored in a refrigerator (at 1 - 10°C) without reconstitution. After 41 hours of storage, the stability samples were retrieved from the freezer and analyzed along with freshly spiked calibration standards and quality control samples (comparison samples). The concentration of stability and comparison samples were back calculated and the % stability was computed by comparing the mean concentration of stability samples against the mean concentration of comparison samples.

In-injector Stability*:* Stability of processed samples in the instrument over the anticipated run time needs to be assessed as in case of instrument failure. Six replicates each of low and high quality control samples (stability samples) were processed and are stored in Auto sampler at 10°C. After 41 hours of storage, the samples were analyzed along with freshly spiked calibration standards and quality control samples (comparison samples). The

concentration of stability and comparison samples were back calculated and the % stability was computed by comparing the mean concentration of stability samples against the mean concentration of comparison samples. All the stability establishments have comfortably met the specifications for % stability demonstrating insignificant degradation of BTGR, TNFR and EMTB over the specified durations and conditions. The stability dats are listed in table

Table-8: Stability result for Bictegravir Lamivudine and Emtricitabine.

Stability	Analyte	QC Level	A	% CV	B	% CV	% Stability
Bench-top (18hrs at-25®C)	Bictegravir	LQC	14.67	6.9	14.86	6.9	98.7
		HQC	1207.82	6.2	1241.99	7.2	97.2
	Tenofovir	LQC	14.93	8.4	14.79	4.8	100.9
		HQC	1233.59	3.1	1221.06	2.7	101.0
	Emtricitabine	LQC	30.57	2.8	28.95	5.1	105.6
		HQC	2414.42	1.5	2397.83	4.0	100.7
Freeze-thaw (after 4th cycle)	Bictegravir	LQC	15.66	8.4	14.86	6.9	105.4
		HQC	1221.48	2.9	1241.99	7.2	98.3
	Tenofovir	LQC	15.07	4.2	14.79	4.8	101.9
		HQC	1221.77	3.3	1221.06	2.7	100.1
	Emtricitabine	LQC	30.02	3.5	28.95	5.1	103.7
		HQC	2399.55	3.0	2397.83	4.0	100.1
In-injector(at 10® C for 41 hrs)	Bictegravir	LQC	15.38	8.2	15.43	8.2	99.7
		HQC	1269.39	6.5	1285.20	5.3	98.8
	Tenofovir	LQC	14.57	7.6	15.09	8.2	96.6
		HQC	1191.51	2.7	1195.45	3.0	99.7
	Emtricitabine	LQC	30.12	5.7	29.65	5.1	101.6
		HQC	2411.63	2.3	2406.67	3.9	100.2
Dry Extract (at 1-10®C for 41hrs	Bictegravir	LQC	15.21	3.8	15.43	8.2	98.6
		HQC	1245.18	6.1	1285.20	5.3	96.9
	Tenofovir	LQC	15.42	6.3	15.09	8.2	102.2
		HQC	1215.31	2.4	1195.45	3.0	101.7
	Emtricitabine	LQC	30.93	4.0	29.65	5.1	104.3
		HQC	2376.97	2.1	2406.67	3.9	98.8
Long-term stability (at-70® C for 75 Days)	Bictegravir	LQC	15.03	4.0	15.06	6.7	99.8
		HQC	1166.80	4.4	1136.71	5.4	102.6
	Tenofovir	LQC	13.99	3.0	13.86	5.9	100.9
		HQC	1205.94	0.5	1214.97	5.4	99.3
	Emtricitabine	LQC	30.86	5.7	31.37	5.0	98.4
		HQC	2363.61	2.5	2500.65	2.0	94.5
Long-term stability (at-20® C for 75 Days)	Bictegravir	LQC	15.60	2.2	15.06	6.7	103.6
		HQC	1135.32	4.8	1136.71	5.4	99.9
	Tenofovir	LQC	13.83	6.7	13.86	5.9	99.8
		HQC	1188.06	3.2	1217.97	5.4	97.8
	Emtricitabine	LQC	30.19	4.9	31.37	5.0	96.2
		HQC	2431.38	2.3	2500.65	2.0	97.2

A- Mean Concentration (ng/ML) of stability Samples
B- Mean Concentration (ng/ML) of comparison Samples

Application: The validated method was successfully applied to 'An open label, randomized, two, treatment, two sequence, two period, cross-over, single-dose comparative oral bioavailability study of fixed dose combination Biktarvy® (bictegravir, emtricitabine, and tenofovir alafenamide) is a fixed dose combination tablet containing bictegravir, emtricitabine, and tenofovir alafenamide for oral administration. Each tablet contains 50 mg of BIC (equivalent to 52.5 mg of bictegravir sodium), 200 mg of FTC, and 25 mg of TAF (equivalent to 28 mg of tenofovir alafenamide fumarate) and the following inactive ingredients: croscarmellose sodium, magnesium stearate, and microcrystalline cellulose. The tablets are film-coated with a coating material containing iron oxide black, iron oxide red, polyethylene glycol, polyvinyl alcohol, talc, and titanium dioxide. conducted over 36 healthy male human volunteers.

The study was conducted according ethics committee approved protocol in compliance to the current GCP guidelines after obtaining the duly signed informed consent from the study participants. The test and reference products were administered according to the randomization schedule generated using SAS software version 9.1.3. A total of 27 blood samples were withdrawn (5 mL each, including pre-dose) till 288 hours post dose in each period. The blood samples were collected in K_2 EDTA anticoagulant vacutainers and are centrifuged immediately using a refrigerated centrifuge at 5°C and 3500rpm for 15minutes to separate the plasma. The separated plasma was stored frozen below at -70 ± 15ºC until analysis. A 0.3 mL aliquot of each sample was extracted using the same procedure described above for the Calibration standards and Quality control samples. Study samples from each subject were analysed against a calibration curve, after interspersing with two replicates each of low, middle and high quality control samples. Results of these quality control samples (accuracy for 67% of low, middle and high QC samples should be within 85-115%, including 50% at each level) were made basis for accepting or rejecting an analytical run. The global precision and accuracy of calibration standards and quality control samples analyzed along with study samples were within 85-115% with an precision of ≤ 15%. Chromatograms of BTGR, TNFR and EMTB in real subject samples are presented in Figure.

The pharmacokinetic parameters i.e., Cmax (maximum observed drug concentration), AUC_{0-t} (area under the plasma concentration versus time curve from time zero to the last measurable concentration, calculated by linear trapezoidal method), $AUC_{0-\alpha}$ (area under the plasma concentration versus time curve from time zero to infinity), Tmax (time of maximum

measured plasma concentration), and $T_{1/2}$ (terminal half-life as determined by quotient 0.693/Kel) were computed using WinNonlin software version 5.0.1 and 90% confidence interval was estimated using SAS software version 9.1.3. The mean plasma concentrations versus time profiles for BTGR, TNFR and EMTB under fasted condition are presented in Figure 2.5.

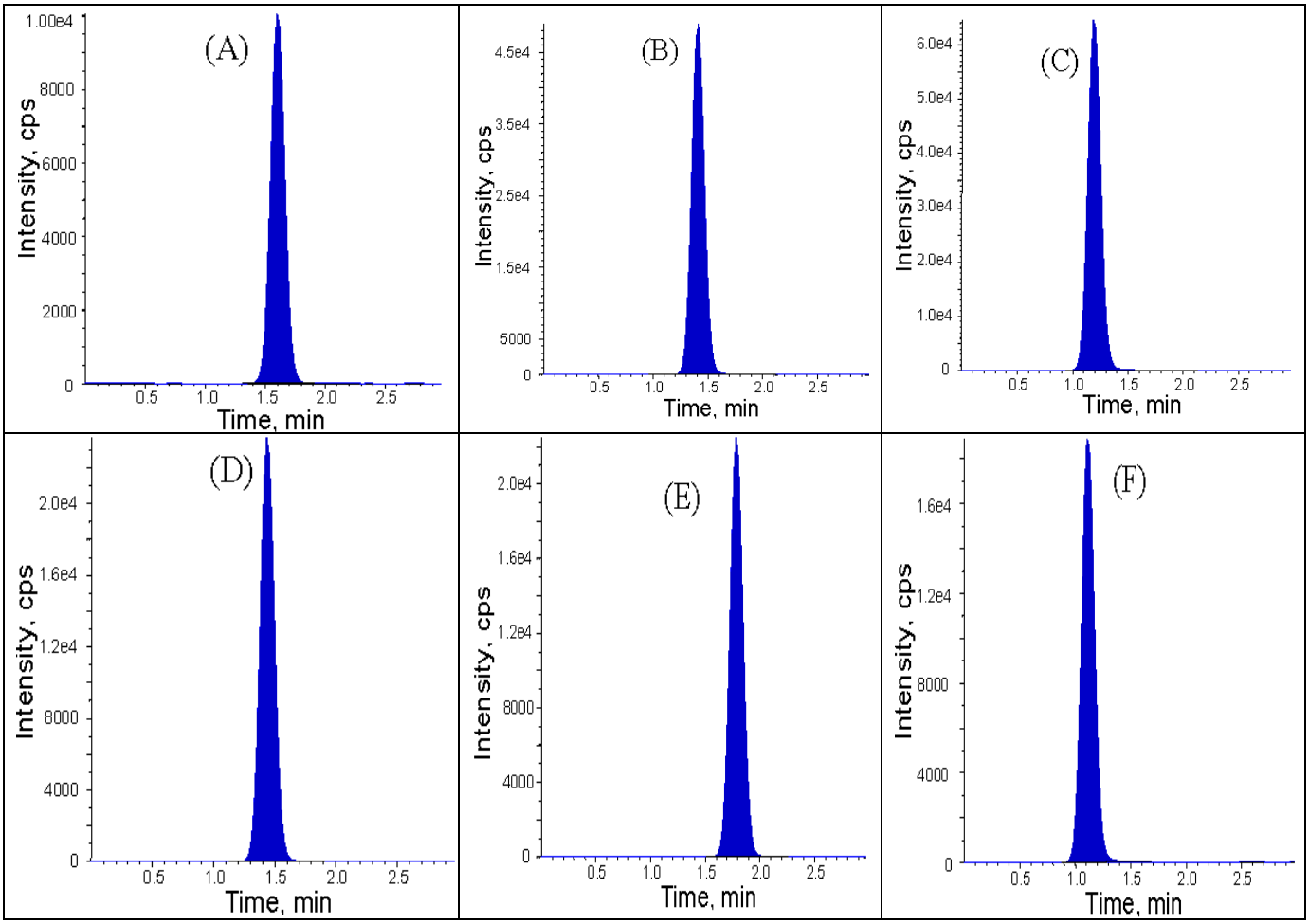


Figure 2.5: Representative chromatograms of (A) BTGR, (B) DDI (C) TNFR, (D) D4T (E) EMTB and ABC (F) in real subject Samples.

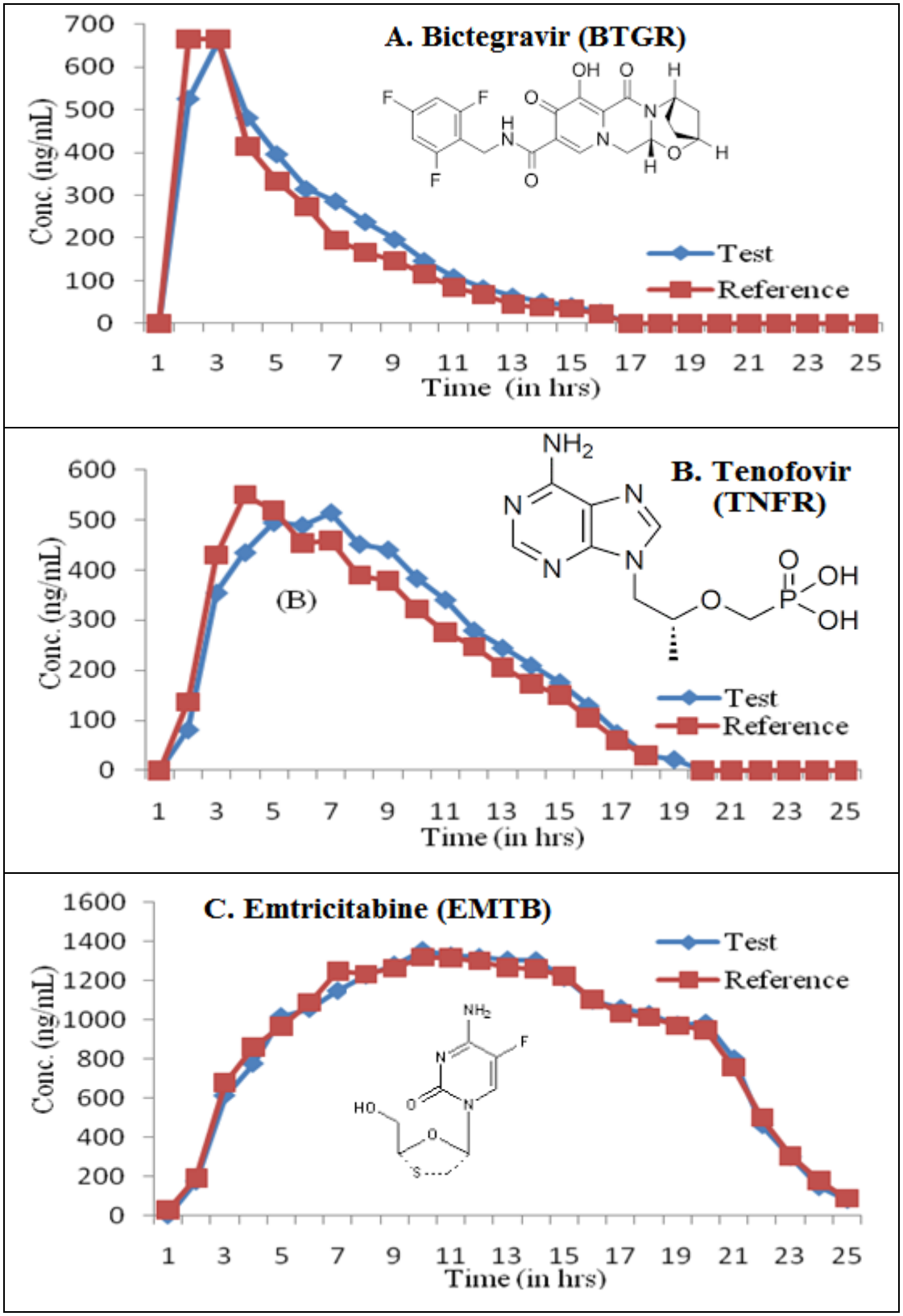


Figure2.6-: Mean plasma concentration versus time profile of BTGR (120 mg) (A), TNFR (60 mg) (B) and EMTB (100 mg) (C) after oral administration to 36 healthy Indian human volunteers under fasting conditions.

CONCLUSION

Antiviral drugs are most frequently prescribed drugs for HAART effects. Systemic use of antiviral drugs is associated with significant side effects and low doses may provide a favourable benefit / risk ratio than higher doses for the therapeutic applications. This calls for quantification of the drug at very low concentrations in body fluids. Developing a method for the simultaneous estimation of several synthetic antiviral agents is a challenging task, owing to different ionization efficiencies of the analytes, matrix effect and recovery.

A simple, rapid and rugged LC–MS/MS method was developed and validated for the determination of Bictegravir (BTGR), Tenofovir (TNFR), and Emtricitabine (EMTB) in human K_2EDTA plasma. The method was validated in accordance with USFDA guidelines "Guidance for the Industry: Bioanalytical Method Validation, 2001". The mass spectra for each analyte were obtained individually, and the most abundant product ions were selected for SRM of each analyte. The signal intensities were found to be better in positive ionization mode when compared to the negative ionization mode because of better electrospray ionization of positively charged antiviral agents. The MRM transitions monitored were monitored were m/z 450.1 → 289.1 (BTGR), m/z 237.1→ 137.1 (DDI), m/z 287.90 →176.05 (TNFR), m/z 225.2 → 127 (D4T), m/z 248.1 → 130.0 (EMTB) and m/z 287.2 → 191.2 (ABC) with a dwell time of 200 ms per transition and the quadruples 1 and 3 were set at unit resolution. The MS/MS conditions for each transition were optimized in order to achieve the maximum signal-to-noise-ratio (S/N), and to avoid endogenous contamination of the chromatogram. The position of the spray needle was adjusted to achieve the optimal S/N for all compounds. Based on the physicochemical properties and compatibility with the mobile phase stavudine (d4T), abacavir and didanosine were selected as internal standards. The analytes were extracted by solid phase extraction technique and chromatographed using Zorbax C18 (150 X 4.6, 5 µm). The HPLC mobile phase consisted of methanol and 0.1% formic acid in water (85:15 v/v). 5µ column showed good peak shape when compared 3µ and 1µcolumns. No significant interferences were observed in the blank plasma samples of each analyte. A weighting factor of 1/x2 was used to construct the calibration curve. The specificity/selectivity has been determined by using different sources of plasma including hemolyzed and lipemic plasma.

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