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RESEARCH ARTICLE

Quantitative Analysis of Bictegravir, Emtricitabine, Tenofovir Alafenamide in Human Plasma by HPLC-MS/MS and its Application to Bioequivalence Study in Healthy Subjects

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ABSTRACT

Combination antiretroviral (cARV) treatment is more common in human immunodeficiency virus (HIV) infection. In many instances, treatment regimen includes two or more combination of drugs from six different classes. Some of the antiretroviral combination medications are under study at preclinical and clinical stages. A precise method is required to quantify the drug concentration in biological matrices to study pharmacokinetic behavior and tissue distribution profile in animals and/or humans. We have developed and validated a sensitive and precise liquid chromatography-tandem mass spectrometry method for simultaneous quantification of Bictegravir (BG), Emtricitabine (EC) & Tenofovir Alafenamide (TA) in human plasma using Bictegravir-D2 (BGIS), Emtricitabine 15ND2 (ECIS) and Tenofovir Alafenamide D5 Fumarate (TAIS) as an internal standards (IS). Chromatographic separation was performed on a ZODIAC CN column using an isocratic mobile phase composed of methanol and 5Mm ammonium acetate (75:25, v/v) and delivered at a flow rate of 1.0 mL/min. Analytes were extracted from plasma by a solid phase extraction technique. BG, EC and TA and BGIS, ECIS and TAIS were detected with protonated adducts at m/z 450.30 289.20, 248.30 130.00, 477.40 470.00 and 452.30 289.10, 251.10 130.80, 482.20 470.00 in multiple reaction monitoring (MRM) using AB Sciex API 4500 triple quard mass spectrometer. The method was validated over a linear concentration range of 20.131 to 10015.534 ng/mL, 12.086 to 3006.433 ng/mL and 6.015 to 1496.289 ng/mL for BG, EC and TA, respectively. This method demonstrated acceptable intra and inter-day precision within 3.55 to 5.71 for BG, 0.47 to 7.58 for EC and 1.90 to 6.61 for TA and 3.28 to 5.37 for BG,1.04 to 8.20 fot EC and 1.74 to 7.11 for TA intraday precision, and 5.37 to 3.28 for BG , 1.04 to 8.20 for EC and 1.74 to 7.11 inter-day precision, This method demonstrated acceptable intra and inter-day accuracy within 89.58 to 98.18 for BG,91.25 to 98.04 for EC and 88.16 to100.09 for TA intraday accuracy and 91.04 to 95.68 for BG,93.90 to 98.27 for EC and 92.80 to 99.97 for TA, respectively. The obtained results are according to current U.S Food and Drug Administration (U.S. FDA) Bioanalytical method validation guidelines. This method was successfully applied to a comparative bioavailability/bioequivalence study in 12 healthy human subjects under fed conditions.

Keywords: Bictegravir, Emtricitabine, Tenofovir Alafenamide, LC-MS/MS, Bioequivalence, Human Plasma.

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1. Introduction

Bictegravir (BG) (Fig:1.0) is chemically (1S,11R,13R)-5-hydroxy-3,6-dioxo-N-[(2,4,6-trifluorophenyl)methyl]-12-oxa-2,9-diazatetracyclo[11.2.1.0^{2,11}.0^{4,9}]hexadeca-4,7-diene-7-carboxamide. Bictegravir has a molecular formula of $C_{21}H_{18}F_3N_3O_5$ and a molecular weight of 449.386.Emtricitabine (EC) (Fig:1.0) is chemically 4-amino-5-fluoro-1-(2*R*-hydroxymethyl-1,3- oxathiolan-5*S*-yl)-(1H)-pyrimidin-2-one. Emtricitabine (EC) is the (-)enantio mer of a thio analog of cytidine, which differs from other cytidine analogs in that it has a fluorine in the 5 position. It has a molecular formula of $C_8H_{10}FN_3O_3S$ and a molecular weight of 247.240. Tenofovir Alafenamide (TA) (Fig:1) is chemically propan-2-yl (2S)-2-{[(S)-({[(2R)-1-(6-amino-9H-purin-9-yl)propan-2-yl]oxy} methyl) (phenoxy) phosphoryl]amino}propanoate. Tenofovir alafenamide has an empirical formula of $C_{21}H_{29}N_6O_5P$ and a weight of 476.474.

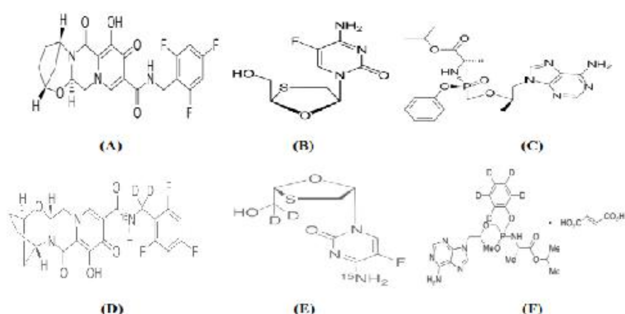

Figure.1.0: Chemical structures of A) Bictegravir (BG) B) Emtricitabine (EC) C) Tenofovir Alafenamide (TA) D) Bictegravir-D2-15N (BGIS) E) Emtricitabine-D2-15N (ECIS) F) Tenofovir Alafenamide-D5 Fumarate (TAIS).

The Bictegravir (BG), Emtricitabine (EC) and Tenofovir Alafenamide (TA) is a oral drug with a fixed dose combination used for the treatment of HIV-1 infection in adults who have no antiretroviral treatment history or to replace the current antiretroviral regimen in those who are virologically suppressed. Bictegravir (BG) is an HIV-1 integrase strand transfer inhibitor and Emtricitabine (EC) and Tenofovir Alafenamide (TA) are both HIV-1 nucleoside analog reverse transcriptase inhibitors [1-3]. In literature many analytical methods have been reported for the determination of BG, EC and TA alone or simultaneously with other durgs by using HPLC [14, 20, 26] in pharmaceutical formulations and LC-MS/MS in biological samples [6-13, 15-19, 21-28]. There is no method by LC-MS/MS for this combination drug in humam plasma as such is available in the litereatur. Hence the proposed LC-MS/MS method for estimation of BG, EC and TA in human plasama is novel.

The most of the reported having some disadvantages like using gradient elution mobile phase, long retention and complex sample preparation. As the range of polarities are broad for the selected drugs of different classes, most of the methods reported involved gradient separation which resulted in long run times or gradient spikes in blank samples. Till date, no analytical method have been reported with isocratic mobile phase using deuterated internal standard for the simultaneous quantification of these selected class of drugs in human plasma by LC-MS/MS . It is important to develop a reliable bio-analytical method with a deuterated internal standard to study the matrix effect and reproducibility. The goal of the present study was to develop a reliable and accurate method for the quantification of BG, EC and TA using Bictegravir-D2 (BGIS), Emtricitabine 15ND2 (ECIS) and Tenofovir Alafenamide D5 Fumarate (TAIS) as an internal standards (IS). Moreover, the sample extraction method should be simple and the analytical method should be highly sensitive and the use of a small amount of plasma. The validated method was succusfully applied to a bio-equivalence study of different formulations in 12 healthy human volunteers.

2. Materials and Methods

Chemicals and Reagents

BG, EC and TA reference standards were purchased from commercial sources. Milli-Q water was obtained from in-house Milli-Q water purification system (Millipore, Bangalore, India). All other chemicals and solvents were purchased from S.D fine chemicals (Mumbai, India). Drug free human plasma was obtained from Deccan Pathological Labs (Hyderabad, India)

Instrumentation: Schimadzu Prominence HPLC system connected with AB Sciex API 4500 triple quard mass

spectrometer was used for the present study. Compound ionization was performed using electro spray ionization (ESI) probe in positive ion mode and the data processing was performed with Analyst 1.6.2 software package (SCIEX). An aliquot of 5µL of the processed samples were chromatographed on a Zodiac CN (100 X 4.6mm, 5 µm) column using methanol and 5mM ammonium acetate (75:25, v/v) as mobile phase with 1.0 mL/min flow-rate in isocratic mode. BG, EC and TA along with their ISs were eluted at 1.80 min, 1.36 min, 1.49 min, respectively allowing the run time of 3 min.

Chromatographic conditions

Chromatography was performed on ZODIAC CN, 5 µ, 100 X 4.6mm. Mobile phase composed of Methanol: 5Mm ammonium acetate (75:25), with 1.0 mL/min. flow-rate in isocratic mode. BGIS, ECIS and TAIS were used as internal standards in terms of chromatography and extractability. The drugs and internal standards were eluted at 1.80 min., 1.36 min., 1.49 min. ± 0.01min. window with 3 min. total run time.

Preparation of Standards and Quality Control (QC) Samples: Standard stock solutions of BG, EC, TA, BGIS, ECIS and TAIS (1 mg/mL) were prepared in methanol and working solutions were prepared in water and methanol (50:50, v/v, diluent). The internal standards spiking solutions (500.0 ng/mL) were prepared in 50% methanol from BGIS, ECIS, TAIS standard stock solutions. Standard stock solutions and Internal standard spiking solutions and all other the solutions were stored in refrigerator conditions (2-8 °C) until analysis.

The working solutions were added to screened drug-free human plasma to obtain calibration standards of 20.131, 40.262, 201.312, 503.281, 1006.561, 2013.122, 4026.245, 6009.320, 8012.427, 10015.534 ng/mL for BG, 12.086, 24.172, 48.343, 120.859, 302.147, 604.293, 1208.586, 1803.860, 2405.147, 3006.433 ng/mL for EC, 6.015, 12.030, 24.060, 60.151, 150.377, 300.754, 601.508, 897.773, 1197.031, 1496.289 ng/mL for TA.

Similarly, the quality control samples namely LLOQ (Lower limit of quality control), LQC (Low quality control), MQC (Medium quality control), HQC (High quality control) were prepared at the concentration of 20.799, 61.174, 5097.802, 7608.659 ng/mL for BG, 12.094, 33.045, 1502.040, 2241.850 ng/mL for EC, 6.034, 16.486, 749.362, 1118.451 ng/mL for TA, respectivley and stored in a -70°C freezer until analysis. The ISs spiking solution (a combined dilution of 500.0 ng/mL of BGIS, ECIS, TAIS) was also prepared in diluent.

Sample preparation

A 100 µL aliquot of plasma sample was mixed with 25 µL of ISs spiking solution and then,vortexed for 30 sec. The discovery C_{18} SPE cartridges were equilibrated with 1mL of methanol followed by 1mL of water and the prepared samples were loaded. After applying maximum pressure, the cartridges were washed two times with each 1 mL of water, followed by drugs eluted with 1 mL of Mobile phase (Methanol : 5 Mm ammonium acetate (75:25)) and 5 µL was injected into the LC-MS/MS instrument.

Method validation

Selectivity and specificity

The selectivity of the method was determined by six different human blank plasma samples, which were pretreated and analyzed to test the potential interferences of endogenous compounds, co-eluting with analytes and ISs. Chromatographic peaks of analyte and IS were identified based on their retention times and MRM responses. The peak areas of BG, EC, TA at the respective retention time in blank samples should not be more than 20% of the mean peak area of drug in LLOQ of BG, EC and TA. Similarly, the peak areas of BGIS, ECIS, TAIS at the respective retention time in blank samples should not be more than 5% of the mean peak area of IS in LLOQ of BGIS, ECIS, TAIS.

Recovery

The extraction recovery of BG, EC, TA and BGIS, ECIS, TAIS from human plasma was determined by analyzing quality control samples. Recovery was determined at three concentrations levels (61.174, 5097.802, 7608.659 ng/mL for BG, 33.045, 1502.040, 2241.850 ng/mL for EC, 16.486, 749.362, 1118.451 ng/mL for TA) by comparing peak areas obtained from the plasma sample and the standard solution spiked with the blank plasma residue. A recovery of more than 50% was considered adequate to obtain required recovery.

Limit of detection (LOD) and Lower limit of quantification (LLOQ): The limit of detection (LOD) is a parameter that provides the lowest concentration in a sample that can be detected from background noise but not quantitated. LOD was determined using the signal-to-noise ratio (s/n) of 3:1 by comparing test results from samples with known concentrations of analytes with blank samples. The Lower limit of quantitation (LLOQ) is defined as the lowest concentration of analyte that can be determined with acceptable precision and accuracy. The LLOQ was found by analyzing six plasma LLOQ standards of BG, ET and TA.

Matrix effect

To predict the variability of matrix effects in samples from individual subjects, matrix effect was quantified by determining the matrix factor, which was calculated as follows: Matrix Factor = Peak response ratio in presence of extracted matrix (post extracted)/ Peak response ratio in aqueous standards. Eight lots of blank biological matrices including lipemic and haemolytic were extracted each in 6 replicates and post spiked with the aqueous standard at the high and low QC level, and compared with aqueous standards of same concentration. The overall precision of the matrix factor is expressed as coefficient of variation (CV %) and %CV should be < 15%.

Calibration curve

The calibration curves were constructed using values ranging from 20.131 to 10015.534 ng/mL of BG, 12.086 to 3006.433 ng/mL of EC, 6.015 to 1496.289 ng/mL of TA in human plasma. Calibration curve was obtained by quadratic model with weighted $1/x^2$ regression analysis. The ratio of BG/BGIS, EC/ECIS and TA/TAIS peak areas were plotted against the ratios of BG/BGIS, EC/ECIS and TA/TAIS concentration in ng/mL. Calibration curve standard samples

and quality control samples were prepared in replicates (n=6) for analysis.

Precission and accuracy

Precision and Accuracy for the back calculated concentrations of the calibration points, should be within 15% and ± 15% of their nominal values. However, for LLOQ the precision and accuracy should be within 20% and ± 20% of their nominal values.

Stability studies:

Bench top stability (9 h), processed samples stability (freeze–thaw stability (six cycles), autosampler stability for 32 h, long-term stability (60 days) were performed at LQC and HQC levels using six replicates at each level. Stability samples were processed and quantified along with the freshly spiked calibration curve standards. Samples were considered to be stable if the assay values were within the acceptable limits of accuracy (±15%), precision (15%).

Analysis of subject samples: A randomized, two-way crossover, single dose bioequivalence study was conducted in healthy human subjects (n=12).The study was conducted at Clinse Labs Private Limited, Hyderabad, India. The reference and test products containing BG, EC and TA at a dose of 50mg, 200mg, 25mg, respectively was used for the study. Study protocol was approved by IEC (Independent Ethics committee) as per ICMR (Indian Council of Medical Research) and each subject was administered with 50mg, 200mg, 25mg of single oral dose of BG, EC and TA with 250mL of drinking water. Blood samples (5mL) were collected at predose (0 h) and at 0.17, 0.33, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.33, 2.67, 3.00, 3.33, 3.67, 4.00, 4.33, 4.67, 5.00, 5.50, 6.00, 8.00, 10.00, 12.00, 16.00, 24.00, 48.00 and 72.00 h after dose in vaccutainers containing K_2EDTA. A total of 56 (28 time points for Reference, 28 time points for Test) time points were collected and centrifuged at 3200 rpm, 10°C, for 10 min to obtain plasma and stored below -70 °C until analysis. Test and Reference tablets were administered to same human volunteers under fed conditions separately with proper washing periods as per protocol.

Pharmacokinetics and statistical analysis

Pharmacokinetics parameters from the human plasma samples were calculated by a noncompartmental statistic model using Win Non-Lin 5.0. Software (Pharsight, USA). Blood samples were taken for a period of 3 to 5 times the terminal elimination half-life (t1/2) and it was considered as the area under the concentration time curve (AUC) ratio higher than 80% as per FDA guidelines. Plasma BG, EC, TA concentration-time profiles were visually inspected, and Cmax and Tmax values were determined.

The AUC0–t was obtained by the trapezoidal method. AUC0– was calculated up to the last measureable concentration and extrapolations were obtained using the last measureable concentration and the terminal elimination rate constant (Kel) was estimated from the slope of the terminal exponential phase of the plasma of the BG, EC and TA concentration-time curve (by means of the linear regression method). The terminal elimination half-life (t1/2), was then calculated as 0.693/Kel. Regarding AUC0–t, AUC0– and Cmax bioequivalence were assessed by means of analysis of variance (ANOVA) and calculating the standard 90% confidence intervals (90% CIs) of the ratio's test/reference (logarithmically transformed data). The bioequivalence was considered when the ratio of averages of log transformed data was within 80-125% for AUC0–t, AUC0– and Cmax.

3. Results and Discussion

Method development and validation

LC-MS/MS instrumentation has been used as one of the most powerful analytical tool in clinical pharmacokinetics due to its selectivity, sensitivity and reproducibility. The aim of the present work is to develop and validate a simple, sensitive, rapid, rugged and reproducible assay method for the quantitative determination of BG, EC and TA simultaneously in human plasma samples. Chromatographic conditions, especially the composition and nature of the mobile phase, usage of different columns, different extraction methods such as solid phase extraction (SPE), protein precipitation (PP), Liquid-liquid extraction (LLE) were optimized through several trials to achieve the best resolution and increase the signal of analytes and ISs. The MS optimization was performed by direct infusion of solutions of both analytes and ISs into the ESI source of the mass spectrometer. The critical parameters in the ESI source include the needle (ESI) voltage, capillary voltage, source temperature and other parameters such as nebulizer gas, heater gas and desolvation gases were optimized to obtain a better spray shape, resulting in better ionization of the protonated ionic analytes and ISs (Fig.2.0). Product ion spectrum for analyte and internal standard yielded high-abundance fragment ions of 289.20, 130.00, 470.00, 289.10, 130.80 and 482.20 for BG, EC, TA, BGIS, ECIS and TAIS, respectively (Fig. 2.0**)**. After mass spectrometer parameters optimized, chromatographic conditions such as mobile phase optimization, column optimization, extraction method optimization was performed to obtain a fast and selective LC method. A good separation and elution were achieved using methanol and 5Mm ammonium acetate (75:25, v/v) combination as the mobile phase, at a flow-rate of 1.0 mL/min and injection volume of 5 µL. Zodiac CN (100 X 4.6mm, 5 µm) column and Solid phase extraction method was optimized by using Orochem panthera deluxe catridges for the best chromatography.

Limit of detection (LOD) and quantification (LLOQ)

The limit of detection was used to determine the instrument detection levels for BG, EC, TA even at low concentrations. An injection volume of 5 µL of concentrations of 20.799, 12.094, 6.034 ng/mL solution gives the estimated LODs of 20.799, 12.094, 6.034 ng/mL of BG, EC, TA with S/N values 5:1. The lower limit of quantification for this method was proved as lowest concentration of the calibration curve which was proved as 20.131, 12.086, 6.015 ng/mL for BG, EC, TA, respectively.

Matrix effect

Eight lots of blank biological matrices (including lipemic and haemolytic) were extracted each in six replicates and post spiked with the aqueous standard at the LQC, HQC level and compared with neat standard of same concentration. The overall IS normalized matrix factor

values obtained was1.056, 1.011 for BG 0.990, 0.999 for EC 0.995,0.999 for TA, respectively, indicating no ion-suppression and enhancement effect on anlaytes.

Calibration curve standards

Calibration curves (Fig.4.0) were plotted as the peak area ratio versus concentration ratios of analytes and ISs. Calibration was found to be linear over the concentration range of 20.131 to 10015.534 ng/mL, 12.086 to 3006.433 ng/mL, 6.015 to 1496.289 ng/mL for BG, EC, TA, respectively. The CV% was less than 4.8 % and the accuracy ranged from 93.20 to 106.60. The determination coefficients (r^2) were greater than 0.99 for all curves (Table 2). Chromatograms obtained from plasma spiked with LLOQs and ULOQs concentrations of 20.131, 12.086, 6.015 ng/mL and 10015.534 ng/mL, 3006.433 ng/mL, 1496.289 ng/mLof BG, EC, TA along with BGIS, ECIS TAIS (500.0 ng/mL) are shown in Fig. 5.0.

Precision and Accuracy

As shown in Table 3, the intra-batch CV% was less than 7.58 %and the overall accuracy ranged from 88.16 to 100.09 %. The inter-batch CV% was also less than 8.20% and the overall accuracy ranged from 91.04 to 99.97 %. These results indicate the adequate reliability and reproducibility of this method within the analytical range.

Recovery

The % recovery was calculated by comparing the peak area of the extracted samples with the neat samples. The % recovery of BG, EC, TA was determined at three different concentrations of 61.174, 5097.802, 7608.659 ng/mL for BG, 33.045, 1502.040, 2241.850 ng/mL for EC, 16.486, 749.362, 1118.451 ng/mL for TA. The overall average recovery of drug (BG, EC, TA) were found as 63.52, 93.87 and 96.63% respectively and internal standard (BGIS, ECIS, TAIS) were found to be 75.03 %, 95.47 % and 97.12 % respectively.

Stability (Freeze - thaw, Auto sampler, Bench top, Long term): The results of various stability studies are listed in Table 4. No significant degradation of the BG, EC, TA was observed even after 30 h storage in the auto sampler and after 9 h storage at room temperature and freezthaw stability established for 6 freezthaw cycles. In addition, the long-term stability of BG, EC, TA samples after 60 days of storage at -70 ^{o}C was also evaluated. The overall %C.V ranged from 0.72 to 2.30% of the theoretical values. These results confirmed the stability of BG, EC, TA in human plasma for at least 60 days at -70°C (Table-3).

Application to biological samples

The above validated method was used in the determination of BG, EC, TA in plasma samples for establishing the bioequivalence of a single dose tablet (one tablet contains 50mg, 200mg, 25mg dose) in 12 healthy volunteers. All the plasma concentrations of BG, EC, TA were within the standard curve region and retained above the LLOQ levels for the entire sampling period. The chromatograms of subject samples and typical plasma concentration versus time profiles of BG, EC, TA were depicted in Fig. 6 and Fig. 7. The mean and Test/Reference ratio of pharmacokinetic parameters of 12 healthy human male volumteers were depicted in Table 5.0 and 6.0.

4. Conclusions

The method described here is novel, fast, robust, sensitive, selective, and rugged with high recovery and has significant advantages over other techniques previously described for measuring Bictegravir (BG), Emtricitabine (EC) & Tenofovir Alafenamide (TA) concentrationsin biological fluids and was quantified by LC-MS/MS. The validated method was successfully applied in Bioequivalence study of two formulations (Test and Reference) by oral administration of single tablet (one tablet contains 50mg, 200mg, 25mg dose) in 12 healthy human volunteers.

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Conflict of Interest: Authors declare that, there is no conflict of interest.

Table 1. Mass parameters and instrument conditions

Parameter	BG	BGIS	EC	ECIS	TA	TAIS
Parenet Ion	450.300	452.300	248.300	451.100	477.400	482.200
Prodcut Ion	298.200	289.100	130.000	130.800	470.000	470.000
Detection mode	Positive	Positive	Positive	Positive	Positive	Positive
Ion Spray Voltage(IS)	5500	5500	5500	5500	5500	5500
Temperature(Temp^{o}C)	500	500	500	500	500	500
Curtain Gas(CUR)	30	10	30	30	30	10
Collision Gas(CAD)	10	10	10	10	10	10
GS1	40	40	40	40	40	40
GS2	45	45	45	45	45	45
DP	130	80	36	36	125	120
CE	25	30	15	15	44	35
CXP	9	9	7	11	14	15
EP	10	10	10	10	10	10

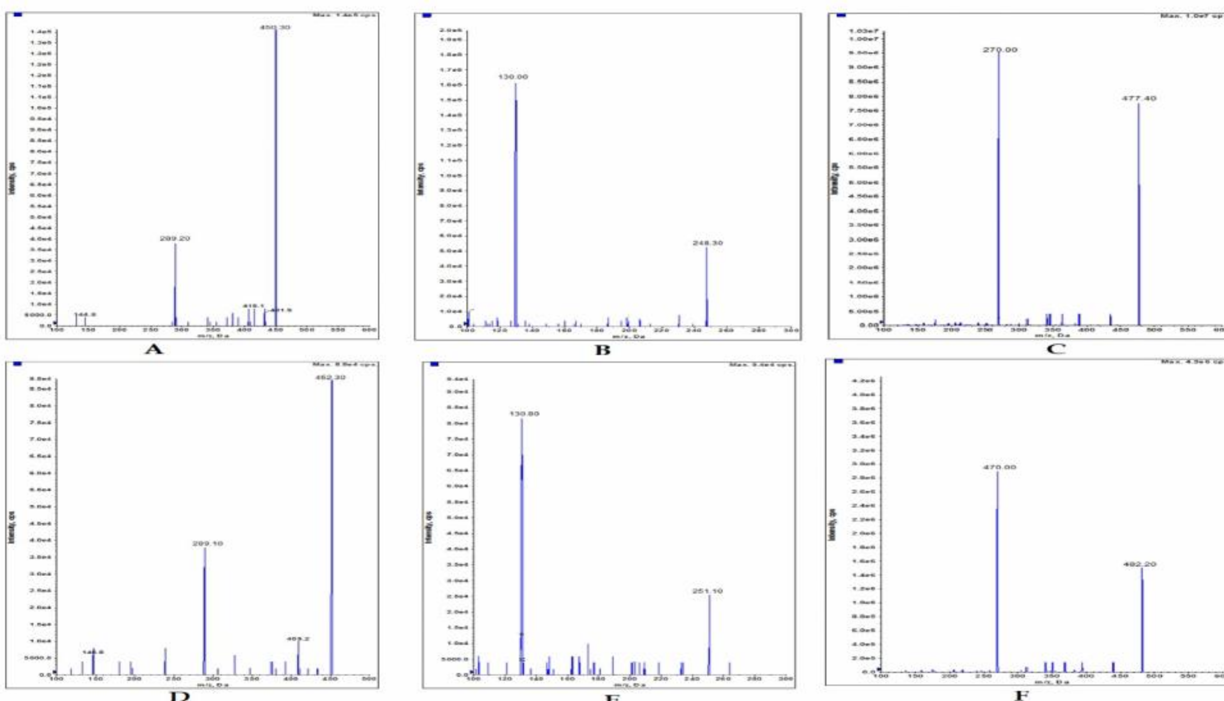


Figure.2.0. Mass Spectra of Q1 Q3 A) BG , B) EC, C) TA, D) BGIS, E) ECIS, F) TAIS.

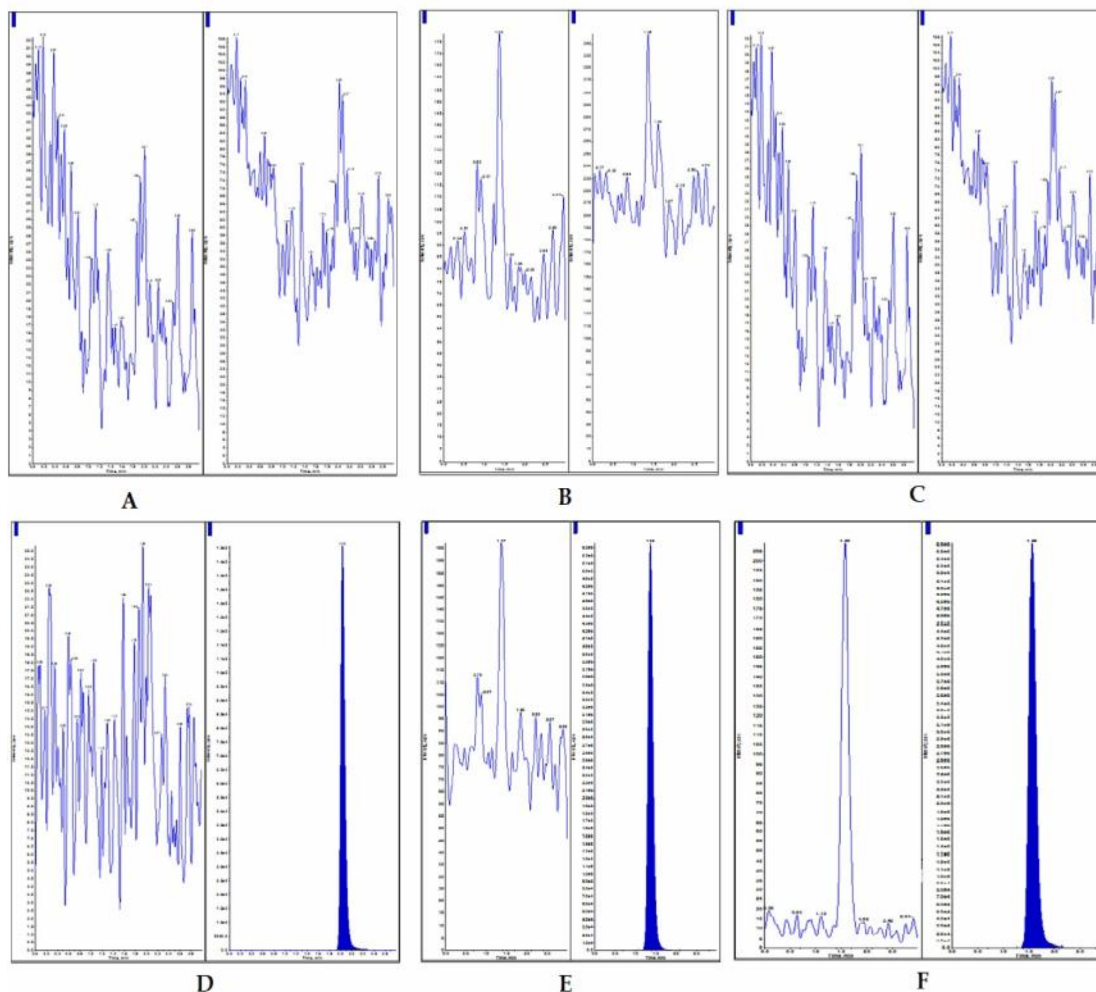
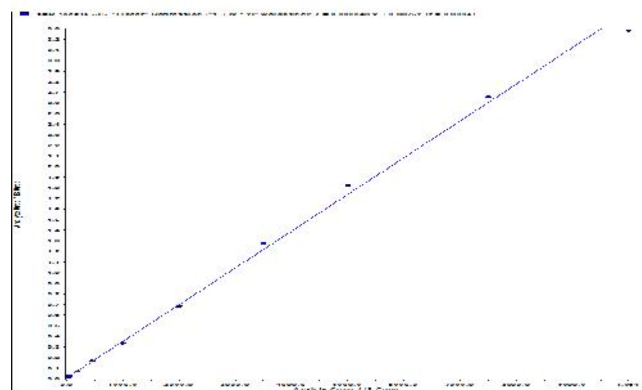
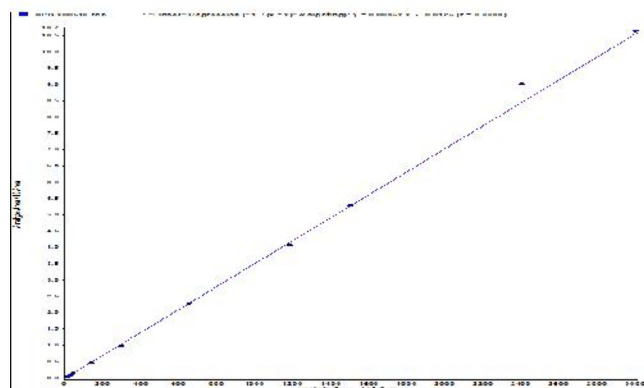


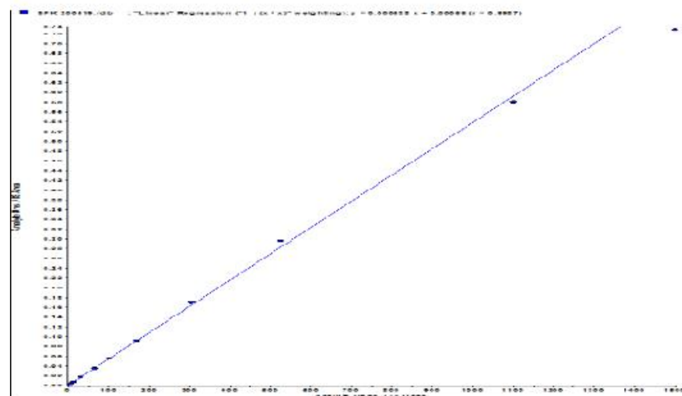
Figure:3.0: Blank plasma chromatograms of A) BG and BGIS, B) EC and ECIS, C) TA and TAIS. Blank plasma spiked internal standard chromatograms of D) BGIS, E) ECIS, F) TAIS.



A



B



C

Figure:4.0: Calibration curve details of A) Bictegravir (BG) B) Emtricitabine (EC) and C) Tenofovir Alafenamide (TA).

Table.2.0: Calibration curve details of Bictegravir (BG), Emtricitabine (EC) and Tenofovir Alafenamide (TA).

STD LEVEL	Bictegravir (BG)			Emtricitabine (EC)			Tenofovir Alafenamide (TA)		
	Conc (ng/mL) (Mean±S.D)	%CV	%Accuracy	Conc (ng/mL) (Mean±S.D)	%CV	%Accuracy	Conc (ng/mL) (Mean±S.D)	%CV	%Accuracy
STD-A	19.5942±0.16	0.79	97.33	11.4568±0.18	1.55	94.79	5.7998±0.22	3.86	96.42
STD-B	42.3436±0.69	1.63	105.17	25.5464±0.29	1.15	105.69	12.4740±0.60	4.77	103.69
STD-C	201.4268±2.93	1.46	100.06	51.5342±2.47	4.79	106.60	25.3976±1.45	5.71	105.56
STD-D	514.9962±7.34	1.43	102.33	128.3052±3.36	2.62	106.16	62.2112±1.50	2.41	103.43

STD-E	1005.0564±19.24	1.91	99.85	315.3986±4.40	1.40	104.39	152.7600±1.29	0.84	101.58
STD-F	1994.4468±40.27	2.02	99.07	605.9256±4.66	0.77	100.27	297.8352±1.44	0.48	99.03
STD-G	4072.2198±87.87	2.16	101.14	1226.9404±23.86	1.94	101.52	615.7012±11.63	1.89	102.36
STD-H	5997.2152±95.98	1.60	99.80	1704.0404±47.71	2.80	94.47	858.2576±23.82	2.77	95.60
STD-I	8005.9218±61.75	0.77	99.92	2234.8926±78.68	3.52	92.92	1147.7410±41.04	3.58	95.88
STD-J	9547.8038±98.82	1.03	95.33	2801.8548±90.81	3.24	93.20	1443.1894±49.24	3.41	96.45

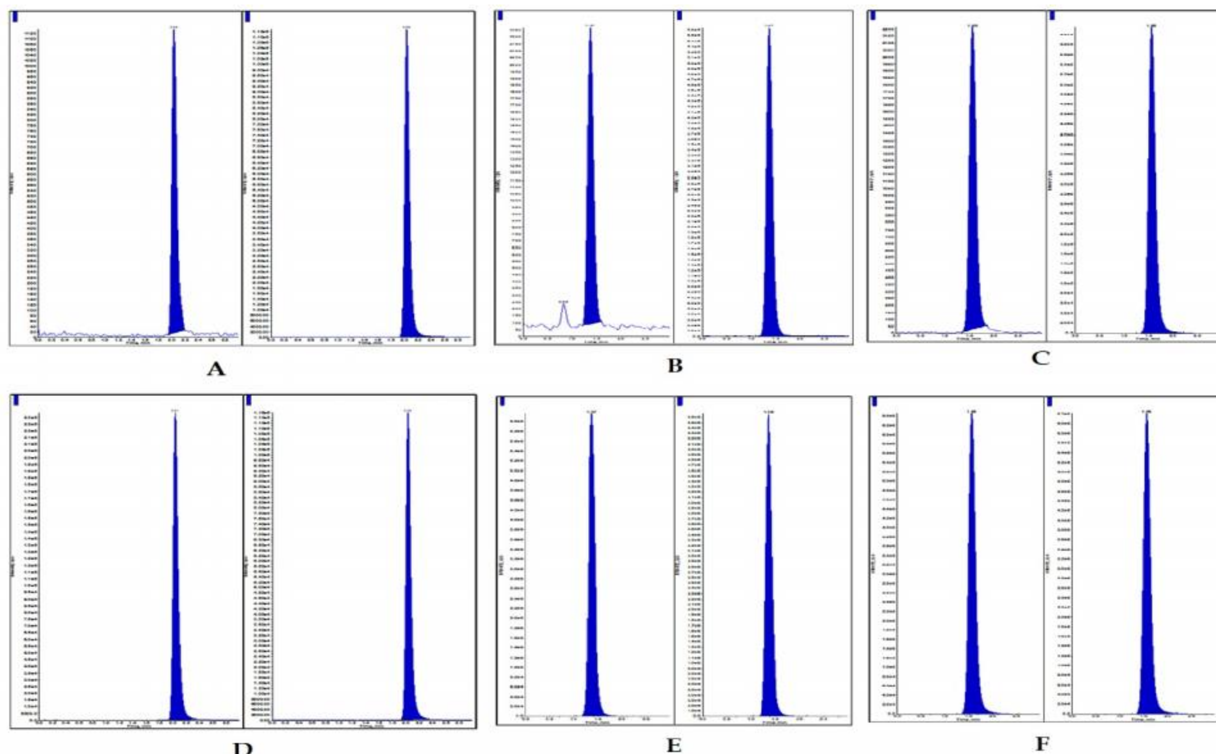


Figure:5.0: Chromatograms of LLOQs A) Bictegravir (BG), B) Emtricitabine (EC) and C) Tenofovir Alafenamide (TA) and ULOQs D) Bictegravir (BG), E) Emtricitabine (EC) and F) Tenofovir Alafenamide (TA).

Table.3.0: Precision and accuracy (Analysis with spiked samples at three different concentrations).

Spiked Plasma Concentration (ng/ml)	**Bictegravir (BG)**					
	Within-run (Intra-day)			**Between-run (Inter-Day)**		
	Concentration measured (n=12; ng/ml; mean±S.D)	**%CV**	**%Accuracy**	**Concentration measured (n=30; ng/ml; mean±S.D)**	**%CV**	**%Accuracy**
LLOQ (20.799)	20.4197±0.72	3.55	98.18	19.7683±1.06	5.37	95.04
LQC (61.174)	54.7971±2.77	5.06	89.58	55.6904±2.12	3.80	91.04
MQC (5097.802)	4605.6442±164.21	3.57	90.35	4743.7216±155.78	3.28	93.05
HQC (7608.659)	6988.7994±399.17	5.71	91.85	7280.1495±357.07	4.90	95.68
Spiked Plasma	**Emtricitabine (EC)**					
	Within-run (Intra-day)			**Between-run (Inter-Day)**		

Concentration (ng/ml)	Concentration measured (n=12; ng/ml; mean±S.D)	%CV	%Accuracy	Concentration measured (n= 30; ng/ml; mean±S.D)	%CV	%Accuracy
LLOQ (12.094)	11.0359±0.84	7.58	91.25	11.4809±0.94	8.20	94.93
LQC (33.045)	30.5712±0.90	2.93	92.51	31.8928±1.85	5.80	96.51
MQC (1502.04)	1393.7085±8.63	0.62	92.79	1410.3435±34.25	2.43	93.90
HQC (2241.850)	2197.8658±10.40	0.47	98.04	2202.9874±22.97	1.04	98.27
Spiked Plasma Concentration (ng/ml)	**Tenofovir Alafenamide (TA)**					
	Within-run (Intra-day)			**Between-run (Inter-Day)**		
	Concentration measured (n=12; ng/ml; mean±S.D)	**%CV**	**%Accuracy**	**Concentration measured (n= 30; ng/ml; mean±S.D)**	**%CV**	**%Accuracy**
LLOQ (6.034)	5.3194±0.35	6.61	88.16	5.5996±0.40	7.11	92.80
LQC (16.486)	14.9337±0.55	3.65	90.58	15.6303±1.00	6.37	94.81
MQC (749.362)	696.3324±13.40	1.92	92.92	705.2746±23.79	3.37	94.12
HQC (1118.451)	1119.4044±21.32	1.90	100.09	1118.1250±19.48	1.74	99.97

Table.4.0: Stability studies of BG, EC, TA in plasma samples.

Bictegravir (BG)										
Spiked Plasma concentration (ng/ml)	**Room temperature Stability**		**Processed sample Stability**		**Long term stability**		**Freeze and thaw stability**		**Auto Sampler Stability**	
	9h		**30 h**		**60 days**		**Cycle 6**		**32h**	
	Concentration measured (n=6;pg/ml; mean±S.D)	**%CV (n=6)**	**Concentration measured (n=6;pg/ml; mean±S.D)**	**%CV (n=6)**	**Concentration measured (n=6;pg/ml; mean±S.D)**	**%CV (n=6)**	**Concentration measured (n=6;pg/ml; mean±S.D)**	**%CV (n=6)**	**Concentration measured (n=6;pg/ml; mean±S.D)**	**%CV (n=6)**
61.174	60.9762±3.12	5.11	59.5135±2.41	4.05	58.5697±1.35	2.30	58.7635±1.10	1.87	58.9070±0.89	1.51
7608.659	7747.6130±48.58	0.63	7736.4142±53.43	0.69	7690.1567±58.90	0.77	7670.9935±30.06	0.39	7764.5567 ± 42.67	0.55
Emtricitabine (EC)										
Spiked Plasma concentration (ng/ml)	**Room temperature Stability**		**Processed sample Stability**		**Long term stability**		**Freeze and thaw stability**		**Auto Sampler Stability**	
	9h		**30 h**		**60 days**		**Cycle 6**		**32h**	
	Concentration measured (n=6;pg/ml; mean±S.D)	**%CV (n=6)**	**Concentration measured (n=6;pg/ml; mean±S.D)**	**%CV (n=6)**	**Concentration measured (n=6;pg/ml; mean±S.D)**	**%CV (n=6)**	**Concentration measured (n=6;pg/ml; mean±S.D)**	**%CV (n=6)**	**Concentration measured (n=6;pg/ml; mean±S.D)**	**%CV (n=6)**
33.045	36.2583±0.9	2.52	34.8025±0.5	1.51	35.4335±0.4	1.24	36.0407±0.5	1.61	35.9007 ±0.52	1.45

	1		2		4		8			
2241.85	2237.9803±18.12	0.81	2245.8812±17.35	0.77	2252.2272±16.19	0.72	2248.2908±14.64	0.65	2221.3463±27.31	1.23
Tenofovir Alafenamide (TA)										
Spiked Plasma concentration (ng/ml)	**Room temperature Stability**		**Processed sample Stability**		**Long term stability**		**Freeze and thaw stability**		**Auto Sampler Stability**	
	9h		**30 h**		**60 days**		**Cycle 6**		**32h**	
	Concentration measured (n=6;pg/ml; mean±S.D)	**%CV (n=6)**	**Concentration measured (n=6;pg/ml; mean±S.D)**	**%CV (n=6)**	**Concentration measured (n=6;pg/ml; mean±S.D)**	**%CV (n=6)**	**Concentration measured (n=6;pg/ml; mean±S.D)**	**%CV (n=6)**	**Concentration measured (n=6;pg/ml; mean±S.D)**	**%CV (n=6)**
16.49	17.9492±0.53	2.97	17.4567±0.43	2.46	17.7087±0.40	2.26	17.9453±0.33	1.85	17.5268±0.63	3.60
1118.45	1149.7470±6.52	0.57	1143.4727±5.06	0.44	1135.7610±9.54	0.84	1150.2942±7.09	0.62	1141.8583±11.09	0.97

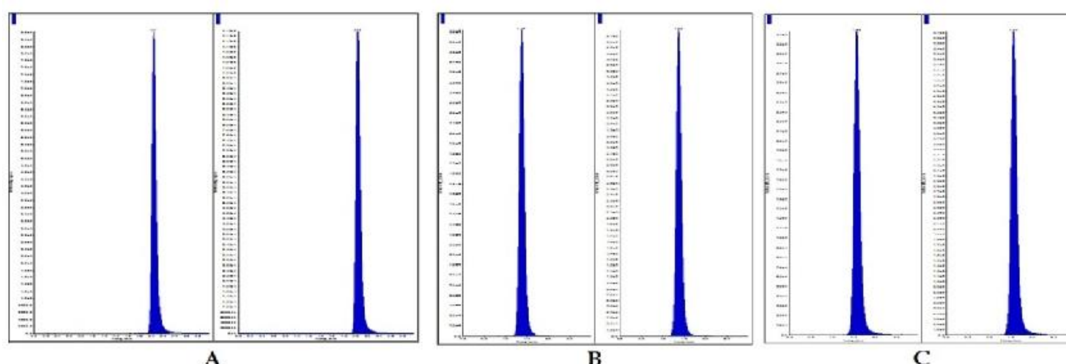


Figure.6.0: Chromatograms of healthy human male Volumteers/Subject samples after administration of 50mg, 200mg, 25mg single oral dose containg A) Bictegravir (BG) and Bictegravir-15N-D2 (BGIS), B) Emtricitabine (EC) and Emtricitabine 15N-D2 (ECIS), C) Tenofovir Alafenamide (TA) and Tenofovir Alafenamide-D5 Fumarate (TAIS).

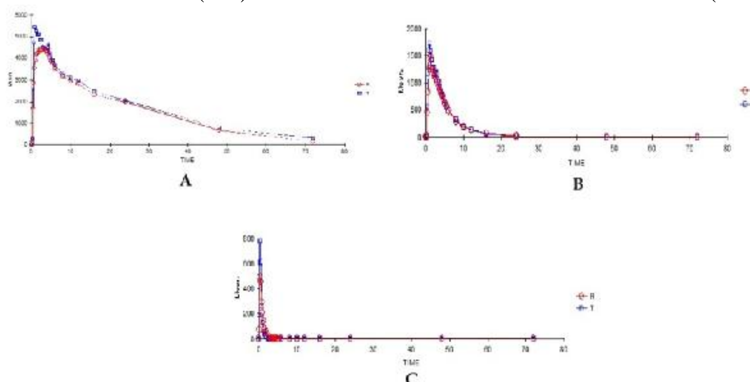


Figure.7.0: Mean plasma concentration of Test Vs Reference after administration of 50mg, 200mg, 25mg single oral dose containg A) Bictegravir (BG), B) Emtricitabine (EC) and C) Tenofovir Alafenamide (TA) in 12 healthy human male volumteers.

Table.5.0: Mean pharmacokinetic parameters of 12 healthy human male volumteers after oral admisntration of 50mg, 200mg, 25mg single dose of Test and Reference product containg Bictegravir (BG), Emtricitabine (EC) and Tenofovir Alafenamide (TA).

Bictegravir (BG)				
Product name	Pharmacokinetic Parameter			
	Cmax (ng/mL)	**Tmax (Hr)**	**AUC0-t (ng/mL)**	**AUC0- t (ng/mL)**

Test	6071.598	1.229	121200.405	132553.582
Reference	5091.801	2.194	104119.156	123663.028
Emtricitabine (EC)				
Product name	Pharmacokinetic Parameter			
	Cmax (ng/mL)	**Tmax (Hr)**	**AUC0-t (ng/mL)**	**AUC0- t (ng/mL)**
Test	1926.984	1.090	8206.175	8476.458
Reference	1821.652	1.278	7658.234	7892.149
Tenofovir Alafenamide (TA)				
Product name	Pharmacokinetic Parameter			
	Cmax (ng/mL)	Tmax (Hr)	AUC0-t (ng/mL)	AUC0- t (ng/mL)
Test	879.009	0.373	428.342	432.013
Reference	686.100	0.679	416.092	419.609

C_{max}: Maximum concentration observed, T_{max}: Time at which the maximum concentration, AUC – Area under the Concentration – time curve, AUC_{0-t}: AUC up to the last measurable concentration, AUC_{0-} : AUC curve to infinite time.

Table.6.0. Pharmacokinetic parameters for Test/Reference of 50mg, 200mg, 25mg single dose after oral administration of of Test and Reference product containg Bictegravir (BG), Emtricitabine (EC) and Tenofovir Alafenamide (TA) in 12 healthy human male volumteers.

	Bictegravir (BG) PK Parameters		
	Cmax	**AUC0-t (ng/mL)**	**AUC0- t (ng/mL)**
Test/Reference	119.24	116.41	107.19
	Emtricitabine (EC) PK Parameters		
Test/Reference	105.78	107.15	107.40
	Tenofovir Alafenamide (TA) PK Parameters		
Test/Reference	128.12	102.94	102.96

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