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HPLC and Tandem MS Device for the Recognition of Emtricitabine and Tenofovir Alafenamide in K_2EDTA Human Plasma Utilizing Emtricitabine $15ND_2$ Tenofovir D5 as an IS

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Abstract

*Developed very impressionable, discriminating, and exact extreme-efficiency liquid chromatography-tandem bulk spectrometry form for measurement of Emtricitabine and Tenofovir Alafenamide in human plasma. Emtricitabine15ND2 Tenofovir D5 was used as a within the standard (IS). The samples were divided on Phenomenex Kinetex C18 5 µm 100A, 100*4.6 mm procession utilizing Acetonitrile: 0.2% formic acid, 60:40 V/V as movable state, the retention period of Emtricitabine: 1.36 minutes (±0.5 minutes), Emtricitabine 15ND2: 1.36 minutes (±0.5 minutes), Tenofovir Alafenamide: 1.41 minutes (±0.5 minutes) and Tenofovir Alafenamide D5: 1.41 minutes (±0.5 minutes) individually. The precursor to product ion evolutions of Emtricitabine 130.200/248.200 m/z and Tenofovir 176.200/477.250 m/z were used to discover and compute Emtricitabine and Tenofovir Alafenamide and Emtricitabine 15ND2 Tenofovir D5 individually. A Shimadzu LC-MS-8045 whole-equipped accompanying vehicle propelled by ejection of pressurized gas or liquid ion spray (TIS) source and conducted in multiple reactions monitoring (MRM) style was pre-owned for the study.*

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INTRODUCTION

Emtricitabine (FTC) and Tenofovir alafenamide (TAF) two together are HIV nucleoside natural converse transcriptase inhibitors (NRTIs). They are explained accompanying other antiretroviral specialists for the situation of HIV-1 ailment in mature-ascends and pediatric victims, also in blend accompanying additional antiretroviral technicians apart from protease inhibitors that demand a CYP3A prevention for the situation of HIV-1 adulteration in pediatric. To highest in the rank of our information, earlier, skilled is no examining order stated for concurrent perseverance of the alliance drug Emtricitabine and Tenofovir Alafenamide. Therefore, an attempt has existed fashioned to cultivate a natural, correct, swift and reproducible LC-MS/MS order for concurrent perseverance of Penfluridol in human plasma, following international instructions

Analytical Methodology

Emtricitabine and Tenofovir Alafenamide in plasma will be analyzed using the validated LC-MS/MS method at Synergen Bio Pvt. Ltd.

Pharmacokinetic Parameters

Employing the estimated plasma concentration-time profiles of Emtricitabine and Tenofovir Alafenamide, the following pharmacokinetic parameters will be calculated using SAS (SAS Institute Inc., USA) version 9.4 or higher. Primary pharmacokinetic parameters: Cmax, AUC0-t and AUC0-∞Secondary pharmacokinetic parameters: Tmax, t1/2, Keland extrapolated AUC.

Statistical Analysis

Statistical study of the pharmacokinetic limits will be acted utilizing SAS (SAS Institute Inc., USA) variant 9.4 or greater. Descriptive enumerations will be computed and stated for the pharmacokinetic limits. The Ln-transformed pharmacokinetic limits Cmax, AUC0-t and AUC0-∞will bring upon oneself Analysis of Variance (ANOVA) for bioequivalence appraisal. The model will contain order, subject (series), period and expression belongings as established effects determinants.

Bioequivalence Criteria

Bioequivalence assessment will be done based on the 90% confidence intervals of the differences of least squares treatment means for Ln-transformed Cmax, AUC0-t and AUC0-∞ of Emtricitabine and Tenofovir Alafenamide obtained after single oral dose administration under fasting conditions. The acceptance criteria for bioequivalence are that the entire confidence intervals for the difference of means of Ln-transformed Cmax, AUC0-t and AUC0-∞should fall within 80.00–125.00%.

Investigational Medicinal Product Summary

This is an established-bulk blend pellet property held emtricitabine (FTC) and tenofovir alafenamide (TAF) for the expressed administration. Each 200/25 mg pill holds 200 mg of FTC and 25 mg of TAF (equivalent to 28 mg of Tenofovir alafenamide fumarate) and the following passive preservatives: croscarmellose sodium, magnesium stearate, and microcrystalline hydrogen. The tablets are film-glassy, following a top material assets a earth's atmosphere glowing aluminium pool, polyethene glycol, polyvinyl exciting, easy grains fashioned by beating a hard, and titanium dioxide. Emtricitabine: The artificial name of FTC is 4-amino-5-fluoro-1-(2R-hydroxymethyl-1, 3-oxathiolan-5S-yl)-(1H)-pyrimidine-2-individual. F.T.C. is the (-) enantiomer of a thio circle cytidine that clashes accompanying supplementary cytidine analogues cause it has a fluorine in the five positions. FTC has a atomic formula of C8H10FN3O3S and a negligible pressure of 247.24 and has the following synthetic recipe composition: FTC is a sterling to polychromatic powder following a solubility of almost 112 mg per mL in water at 25°C. Tenofovir Alafenamide: The artificial name of Tenofovir alafenamide fumarate drug aim is L-alanine, N-[(S)-[[(1R)-2-(6-amino-9H-purine-9-yl)-1-methylethoxy]methyl] phenoxyphosphinyl]-, 1-methyl ethyl ester, (2E)-2-butenedioate (2:1). Tenofovir alafenamide fumarate has a realistic rule of $C_{21}H_{29}O_5N_6P \cdot ½(C_4H_4O_4)$ *and a rule pressure of 534.50 and has the following synthetic recipe composition: Tenofovir alafenamide fumarate is a bright to sandy color colour or tan powder following a solubility of 4.7 mg per mL in water at 20°C.*

Clinical Pharmacology

Pharmacodynamic Properties: Mechanism of Action

Emtricitabine

FTC, a manufactured nucleoside simple of cytidine, is phosphorylated by cell catalysts to shape emtricitabine 5'- triphosphate. Emtricitabine 5'- triphosphate restrains the action of the HIV-1 opposite transcriptase by rivalling the normal substrate deoxycytidine 5'- triphosphate and by being joined into nascent viral DNA [5], which brings about chain end. Emtricitabine 5′-triphosphate is a powerless inhibitor of mammalian DNA polymerases α, β, Ɛ, and mitochondrial DNA polymerase γ.

Tenofovir Alafenamide

TAF is a phosphorodiamidate prodrug of Tenofovir (2'- deoxyadenosine monophosphate natural). Plasma openness to TAF considers seepage into containers; following, TAF is intracellularly altered over to Tenofovir through hydrolysis by cathepsin A. Tenofovir is phosphorylated by container kinases to the active metabolite tenofovir diphosphate. Tenofovir diphosphate restrains HIV-1 copy through melding into vigorous DNA for one HIV switch transcriptase, that causes success the DNA chain-end. Tenofovir has an operation against HIV-1. Cell breeding considers proved that two together Tenofovir and FTC maybe entirely phosphorylated when combined in containers. Tenofovir diphosphate is a weak prevention of carnal DNA polymerases that combine mitochondrial DNA polymerase γ, and skilled is no evidence of malevolence to mitochondria in container idea.

Pharmacokinetics and Statistical Evaluation

Assessment of Pharmacokinetic Parameters

Focus information of subjects versus time, which are gotten after investigation of tests, will be remembered for the last information examination. If pharmacokinetic boundaries can be assessed using the remaining information focuses, information from subjects with missing fixation (missed blood tests, lost examples) might be utilized. In any case, information from these subjects will be rejected from the last investigation. If the subject was dropout or removed throughout the investigation, plasma focuses acquired from the bioanalytical research facility won't be utilized to estimate pharmacokinetic boundaries and measurable examination. Be that as it may, these fixations will be arranged on a different table. All fixation esteems underneath the constraint of measurement (BLOQ) will be set to zero for all pharmacokinetic and synopsis computations. Any missing examples will be accounted for as 'Missing' and won't be incorporated for pharmacokinetic and rundown estimations. The following pharmacokinetic bounds will be recorded taking advantage of SAS real compute interpretation 9.4 or bigger. Essential Pharmacokinetic Parameters:

- *Cmax:* Maximum noticed drug aggregation in red body fluid.
- *AUC0-t:* Area under the body tissue aggregation-period curve calculated to the last determinable aggregation, utilizing the Linear Trapezoidal rule.
- *AUC0-∞:* AUC0-tplus supplementary extent inferred to endlessness, deliberate utilizing ability AUC0-t+Ct/Kel, place Ctis the last determinable drug aggregation and Kelis is the removal rate determined.

Secondary Pharmacokinetic Parameters

- *Tmax:* Time to the noticed maximum drug aggregation in body tissue. If this worth is noticed at in addition individual point, the first point be going to pass away as Tmax
- *Kel:* Apparent first-order terminal removal rate neverending planned from a to a certain extent-record plot of the ancestry aggregation against opportunity curve, utilizing the smallest square reversion procedure.
- *t1/2:* Terminal half-history as persistent by outcome 0.693/Kel [skin aggregation half-history]
- *Extrapolated AUC:* The leftover region in portion will be contingent on ability, [(AUC0-∞-AUC0-t)/AUC0-∞] × 100.For all the same computations, the actual time for action or event of the sample accumulations will be secondhand. No profit of Tmax, t1/2, Kel and inferred AUC will make public for cases that do not exhibit a terminal record-undeviating step in the concentrations against occasion characterization.

Statistical Analysis of Pharmacokinetic Parameters

Statistical pharmacokinetic parameters will be analyzed using SAS version 9.4 or higher [8]. Descriptive statistics (geometric mean, arithmetic mean, median, standard deviation, coefficient of variation, minimum and maximum) will be computed and reported for primary and secondary pharmacokinetic parameters for Emtricitabine and Tenofovir Alafenamide. Statistical analysis will be

performed on the data obtained from subjects completing both the treatment periods with no significant protocol deviations. Any such deviations are to be documented before the bio-analysis of the sample(s). In case of significant inclusion or exclusion criteria violation, the subject(s) will be excluded from the statistical analysis and considered as 'dropouts'. As far as possible, all subjects excluded from the statistical analysis will be identified before starting the study samples' bioanalysis. Suppose in any subject. The pre-dose concentration appears to be >5% of the Cmax. In that case, the concentration data of that subject will be excluded from the pharmacokinetic and statistical analyses.

Analysis of Variance

The Ln-reconstructed pharmacokinetic limits Cmax, AUC0-t and AUC0-∞of Emtricitabine and Tenofovir Alafenamide will bring upon oneself study of difference (ANOVA) for distinct-measure bioequivalence appraisal. The ANOVA model will contain order [9], subject (series), ending and formulations as established effect determinants. Differences 'tween the situation periods and drug sequences will be thought-out statistically meaningful if the expectation principles of the specific belongings (p-principles are ≤0.05. The importance of the series effect will be proven utilizing the subject's reside inside sequences mean square as the mistake term and stated. Intra-subject instability of the pharmacokinetic limits Cmax, AUC0-t and AUC0-∞will be supposed utilizing the root mean square wrong got afterwards resolving difference for bioequivalence estimate. Individual uninterrupted and tractor trailer-mathematical graphs of red body fluid aggregation dossier will be given in addition to the mean.

Outlier Detection Method

Anomalies in an informational index are characterized as perceptions that seem, by all accounts, to be conflicting in Test/Reference proportions with the remainder of the information. They can be distinguished as the perceptions (values), contort elucidating measurements. Subjects who display amazingly high or low perceptions (values) are recognized using factual techniques, specifically the Lunds test (utilizing measurable bundle SAS® programming. The subject referred to will be distinguished as an anomaly for one test or reference item for Cmax, AUC0-t and AUC0-∞. After that, bioequivalence choice is to be based. Other pharmacokinetic boundaries might be utilized whenever required to pass judgment on the legitimate clinical or physiological explanation. In any case, a measurable examination will be performed to avoid bias in the outcomes, including just barring the anomalies. Exception testing will be performed before the aftereffects of the examination are summed up into certainty spans (i.e., whether or not outcomes satisfy the guideline, the anomaly sop will be followed).

Ratio Analysis

Ratio calculations using the least squares mean of the Ln-transformed Cmax, AUC0-t, and AUC0-∞ will be performed for bioequivalence assessment. The comparison of interest is T vs R, so the ratios determined will be of the form T/R where T = Test Product and R = Reference Product.

Ethics Committee

This convention and relating educated assent report (ICD) (containing data about the investigation to be given to the subjects) to be utilized to acquire composed educated assent regarding study subjects will be checked by the EC Subjects won't be selected for the examination until the EC has supported the convention, and the ICD and some other legitimately required endorsement have been gotten. The examination will be led according to the ICMR Guidelines for Biomedical Research on Human Subjects, ICH-GCP Guidelines and the Declaration of Helsinki.

MATERIALS AND METHODS

For the biography-proportion inquiry of test part Emtricitabine 200 mg and Tenofovir Alafenamide 25 mg Tablets of Emcure Pharmaceuticals Ltd., India and remark article DESCOVY® (emtricitabine and tenofovir alafenamide) Tablets 200 mg/25 mg of Gilead Sciences, Inc. Cultivate city, CA 94404 in continuous volunteer's The aim of the test study acted search out examine the rate and standard of

adjustment of Emtricitabine and Tenofovir Alafenamide from Emtricitabine 200 mg and Tenofovir Alafenamide 25 mg Tablets of Emcure Pharmaceuticals Ltd., India (Test Product) and DESCOVY® (emtricitabine and tenofovir alafenamide) Tablets 200 mg/25 mg of Gilead Sciences, Inc. Cultivate city, CA 94404 (Reference article) in well-being enlists. The body tissue was therefore examined employing the certified LC-MS/MS procedure at Synergen Bio Pvt. Ltd. The determined red body fluid focus opportunity descriptions of emtricitabine, and Tenofovir Alafenamide were persistent accompanying the following pharmacokinetic borders appropriating (SAS Institute Inc., USA) form 9.4 or greater.

Chromatographic System and Conditions

The LC-MS/MS framework comprised a Shimadzu 8045 instrument with a parallel siphon, an Autosampler injector with 10ul infusion volume and an MS locator. The product utilized was lab arrangement 5.99 and Insight 3.7. A detachment was accomplished on a converse stage Kinetix C18 5 µm 100*4.6 mm segment.

The portable stage utilized was an Isocratic strategy wherein Acetonitrile: 0.2% formic corrosive, 60:40 v/V with stream pace of 0.600 ml/minute. The maintenance of Emtricitabine 1.36 min, Emtricitabine 15ND2 1.36 min, Tenofovir alafenamide 1.41 min, Tenofovir alafenamide D5 1.41 min All the tests were performed at encompassing temperature.

Standard Solutions and Calibrator Preparation for Chromatographic Measurement

Stock standard arrangements were ready by dissolving 1 mg Emtricitabine 15ND2 inside norm with Methanol and moving into a 1.000 mL volumetric bottle and made up the volume with the equivalent to create an answer of around 1 mg/mL of the grouping of Emtricitabine 15ND2. Put away the stock arrangement at 2–8°C condition. Additionally, pre-arranged the Calibrators by utilizing stock arrangements at the following fixation level, as shown in Table 1.

Table 1. Stock solutions.

	STD 1	STD 2	STD 3	STD4	STD 5	STD 6	STD 7	STD 8
Emtricitabine	25.000	50.000	250.000	1000.000	2000.000	3000.000	4000.000	5000.000
Tenofovir Alafenamide	2.000	4.000	25.000	100.000	200.000	300.000	400.000	500.000

Solvent Solutions Preparation

For the preparation of 4 reagents and solvents used in the LC-MS/MS method detailed protocol was used as mentioned below:

0.2% Formic Acid

Take 02 ml of formic acid Transfer into 1000 ml of volumetric carafe and discontinue in HPLC grade water, capacity flatter the mark accompanying HPLC grade water. Transfer into acid-base indicator container join well and degas in sonicator and continue range hotness. Used this solution inside 05 days of arrangement.

Rinsing Solution (Acetonitrile: Water: 60: 40, v/v)

Take 600 ml of Acetonitrile and 400 ml of water using a measuring cylinder, transfer into a reagent bottle, mix well and degas in a sonicator and keep at room temperature. Used this solution within 05 days of preparation.

Diluent Solution (Methanol: Water: 50: 50, v/v

Take 500 ml of Methanol and 500 ml of water using a measuring cylinder, transfer into reagent bottle, mix well and degas in sonicator and keep at room temperature. Used freshly prepared diluent.

Mobile Phase (Acetonitrile: 0.2% Formic Acid 60: 40, v/v)

Take 600 ml of Acetonitrile and 400 ml of 0.2% formic acid by using a measuring cylinder and

transfer into a 1000 ml reagent bottle, mix well and degas in sonicator and keep at room temperature. Used this solution within 05 days of preparation [2].

Method Validation

The created strategy was approved by the USFDA and EMEA rules. Six repeat analytes of emtricitabine assessed the framework reasonableness with arranged stock fixation 118001.625 ng/ml and Tenofovir Alafenamide with arranged stock focus 11152.145 ng/ml and Matrix spiked focus 2360.033 ng/ml for Emtricitabine and 223.043 for Tenofovir [4].

Standard adjustment bends were ready in the portable stage with eight fixations going from 25.000 ng/mL to 5000.00 ng/mL for Emtricitabine and From 2.000 ng/ml to 500.000 ng/ml for Tenofovir Alafenamide into the LC-MS/MS framework keeping the infusion volume stead [3]. The pinnacle regions were plotted against the relating focus' to get the adjustment charts. To examine the unwavering quality and reasonableness of the created strategy, recuperation tests were done at 53.094, 53.163and 59.105% [6–8].

Extraction Procedure/Protein Precipitation Extraction (PPE) method

1. Aliquot 200 µl of Plasma sample into pre-labelled RIA vial.
2. Aliquot 384 µl Blank Plasma add 08 µl of each Analyte, spiking solution of CC/QC samples except Blank and STD 0, add 16 µl diluent in Blank and STD 0, vortex for complete mixing.
3. Add 50 µl of internal standard dilution solution (~ 2000.000 ng/ml for Emtricitabine 15ND2 + 500.000 ng/ml for Tenofovir Alafenamide D5) to each pre-labelled RIA vial except for blank sample and add 50 µl of diluent solution in blank sample vortex for few second.
4. Add 2.000 ml of Acetonitrile and cap all the samples.
5. Vortex all the samples on Multitube Vortexer for 10 minutes at 2500 rpm.
6. Keep all samples for Centrifugation for 5 minutes at 4.0°C and 4500 rpm in a refrigerated centrifuge.
7. Transfer the samples into pre-labelled Autosampler vials.

Chromatographic Method

Emtricitabine - Tenofovir Alafenamide Quantification is done in Human K2EDTA (K2EDTA is the anticoagulant used for the study) plasma by using SHIMADZU LCMS/MS-8045 Separation of Emtricitabine-Tenofovir Alafenamide was done by using the column Kinetex Phenomenex -C18 100*4.6 mm 5µ and mobile phase used as an isocratic method in which Acetonitrile (60%) and 0.2% Formic acid 60:40 v/v with a flow rate of 0.600 ml/minute [9–10, 1, 2–5]. The retention of Emtricitabine 1.36 min. Tenofovir Alafenamide was observed at Retention time 1.41 min and Emtricitabine 15ND2 1.36 min and Tenofovir D5 at Retention time 1.41 min.

RESULTS AND DISCUSSION

The method was validated for linearity ranging from 25.047 ng/mL to 5009.472 ng/mL for Emtricitabine and From 2.004 ng/ml to 504.731 ng/ml for Tenofovir Alafenamide. Chromatographic separation was achieved on LC-MS/MS method with Kinetex Phenomenex -C18 100*4.6 mm 5µ column using 0.2% Formic acid and Acetonitrile (40:60, v/v) as mobile phase. The retention of Emtricitabine 1.36 min. Tenofovir Alafenamide was observed at a Retention time of 1.41 min and Emtricitabine 15ND2 at 1.36 min, and Tenofovir D5 at a Retention time of 1.41 min, respectively. M.S. parameters of Emtricitabine-Tenofovir Alafenamide analyte and Emtricitabine 15ND2 Tenofovir D5 were used as the internal standard in the experiment [7]. The results are presented in Table 2.

Intra-day and Inter-day precision for the determination of Emtricitabine-Tenofovir Alafenamide results are presented in Tables 3 & 4. Emtricitabine-Tenofovir Alafenamide was tested and found stable in human plasma under different conditions. Results are presented in Table 5. % Recovery of Analyte and internal standard was found to be consistent at all three levels. Results are presented in

Table 6.

The method was selective at 25.000 ng/ml for emtricitabine and 2.000 ng/ml for Tenofovir Alafenamide in 8 different plasma lots. Sensitivity was reproducible at concentration levels of 25.000 ng/ml for emtricitabine and 2.000 ng/ml with % a nominal of 98.9752 for emtricitabine and 96.1615 for Tenofovir Alafenamide and %CV 3.31. For Emtricitabine and 3.23 for Tenofovir Alafenamide.

Table 2. MS parameters of Emtricitabine-Tenofovir Alafenamide

Analyte/IS.	Q1 Mass	Q3 Mass	Dwell (m/sec)
Emtricitabine (Analyte)	248.20	130.20	100.00
Tenofovir (Analyte)	477.25	176.20	100.00
Emtricitabine 15ND2 (IS)	251.00	131.00	100.00
Tenofovir D5 (IS)	482.20	176.20	100.00

Table 3. Intra-Day Precision for Determination of Emtricitabine

	LLOQC	LQC	M1QC	MQC	HQC
AVG.	24.5687	68.8873	518.6217	2012.8975	3918.6702
SD	0.59835	0.88861	2.95158	14.84725	58.53262
% CV	2.44	1.29	0.57	0.74	1.49
% NOMINAL	98.028	92.134	96.623	93.754	94.910

Table 4. Inter-Day Precision for Determination of Emtricitabine

	LLOQC	LQC	M1QC	MQC	HQC
AVG.	24.5608	71.6933	526.3328	2098.8198	4076.2827
SD	1.79476	4.51369	24.36261	135.92161	288.25558
% CV	7.31	6.30	4.63	6.48	7.07
% NOMINAL	97.996	95.886	98.060	97.756	98.727

Table 5. Stability of Emtricitabine-Tenofovir Alafenamide

Name of Stability	% Stability	Condition
Benchtop (21 Hours 47 Minutes)	Q.C. % Change value –3.794% for L.Q.C. and -5.941% for H.Q.C.	Ambient temperature
Freeze-thaw (-70°C ± 10°C after 5th Cycle)	Q.C. % Change value –5.041% for L.Q.C. and -6.004% for H.Q.C.	-70°C ± 10°C and room temp.
Auto-sampler/Wet extracted (70 Hours 15 Minutes)	Q.C. % Change value –5.978% for L.Q.C. and -5.865% for H.Q.C.	Ten °C
Whole blood (02 Hours 17 Minutes)	QC Mean % Peak Area Ratio is 102.7812% for LQC and 98.4854% for HQC.	Ambient temperature
Stock solution (after 04 days)	Mean % Stability is 91.626% for L.Q.C. and 99.046% for H.Q.C.	2–8°C

Table 6. % Recovery

	Analyte	Internal standard
Recovery	55.121%	54.867%

Mass Spectrums

We prepared the tuning solutions of Emtricitabine-Tenofovir Alafenamide containing the concentrations of 100 ng/mL solution and injected them into the mass spectrometer (SHIMADZU 8045). Which showed the full mass range of the targeted Emtricitabine-Tenofovir Alafenamide analyte m/z Q1 248.20Dalton and Q3 130.20Dalton.

DISCUSSION

Acquire chromatograms utilizing Shimadzu's calculating-located Lab answers operating system (tale 5.99) (Šafranko et al., 2019). Process the dossier by peak district percentage utilizing intuitiveness spreadsheet 3.7 or taller interpretation(Okahashi et al., 2022). The unknown aggregation is deliberate from the following equating utilizing reversion study of the barbed body tissue measurement standard accompanying the alternate of the square of the drug aggregation balancing determinant (1/aggregation* Concentration, that is 1/C∧2) for Analyte. Acquired dossier will be inspected utilizing Insight 3.7 or greater story.

Y = mx+b

x = concentration of Analyte

m = slope of the calibration curve

Y = peak area ratio of Analyte to internal standard

b = y-axis intercept of the calibration curve

CONCLUSION

The system was used in a pharmacokinetic study on 24 active Indian cases. Evaluated prime pharmacokinetic limits Cmax, Tmax, AUC0t and AUC0-∞ for remark and test commodity. The study dossier was corroborated by attending earned sample reanalysis (ISR). This grown plan accompanying a chromatographic run period of 3.00 brief period was favourably used in a bioequivalence study of 200 mg/25 Emtricitabine-Tenofovir Alafenamide capsule.

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