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Determination and validation of Tenofovir in Human Plasma using LC-MS/MS

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Abstract: In the present study,we developed and approved a particular, explicit and delicate fluid chromatography mass spectrometry (LC-MS/MS) technique for the assurance of tenofovir in human plasma. Entecavir was utilized as an inside norm, and plasma tests were set up by strong stage extraction performed on Phenomenex Strata cartridges (30 mg). The versatile stage comprised of 10mM ammonium acetate in water and methanol (60:40, v/v). The chromatographic partition was performed isocratically on a Phenomenex C18 (4.6 mm×150 mm, 5 μm), and analytes were investigated in multiple response checking (MRM) mode with positive electrospray ionization (ESI) interface utilizing the separate [M+H]+ ions, m/z 288.2→m/z 176.1 for tenofovir and m/z 278.1→m/z 152 for entecavir. The adjustment bend (r2 = 0.9962) of tenofovir was set up inside the scope of 4.096–1000 μg/L. The intra-and between day precisions were under 10%. This approved strategy was effectively applied to a pharmacokinetic concentrate in 12 sound Chinese volunteers after the oral organization of tenofovir disoproxil fumarate.

Keywords: LC-MS/MS, Tenofovir, Pharmacokinetic, Human Plasma.

1. Introduction

Determination and approved a particular, explicit and touchy liquid chromatography mass spectrometry (LC-MS/MS) technique for the assurance of tenofovir in human plasma. Hepatitis B infection (HBV) contamination is a significant medical condition around the world, especially in Asia and Africa.Insome districts of Asia and Africa, the rate of HBV disease comes to almost 9%–12% of the population[1]. Among the antiviral agents, tenofovir disoproxil fumarate (TDF) (Fig. 1A), a nucleotide turn around transcriptase inhibitor, was first evolved by Gilead. As one of the few endorsed drugs utilized against HBV and human immunodeficiency infection (HIV), TDF can be utilized in the treatment of HIV-1 and hepatitis B diseases in blend with other antiretroviral agents[2,3]. As tenofovir isn't well absorbed in the digestive tract, TDF is

synthesized to improve the ingestion of cells and upgrade bioavailability of tenofovir. Tenofovir, chemically 9-[(R)- 2-(phosphonomethoxy)- propyl]adenine (Fig. 1B),is a class of nucleotide analogs. It very well may be intracellularlymetabolized to its dynamic anabolite tenofovir diphosphate. Moreover, tenofovir is a cutthroat inhibitor of HIV-1 reverse transcriptase, and it ends the developing DNA chain because of its absence of the 3′-OH group[4].

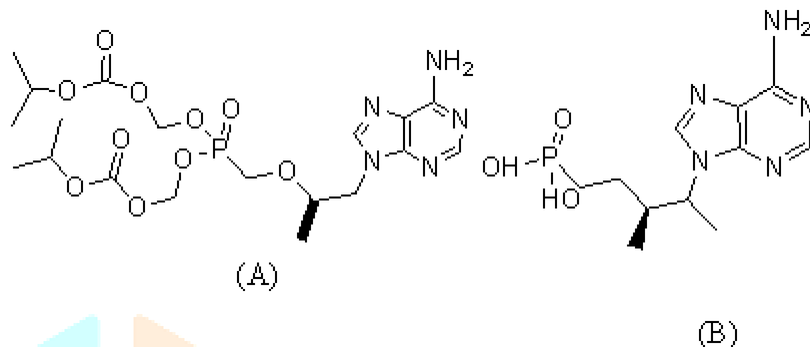


Figure 1. Chemical structures of TDF (A) and tenofovir (B).

Due to the extensive use of tenofovir in anti-HIV therapy, it has become essential to develop a sensitive, specific and selective bioanalytical method for its routine measurement in human samples. Pharmacokinetics of tenofovir *in vivo* have been reported, and methods developed include column derivatization coupled with performance liquid chromatography (HPLC) and fluorescence detection[5,6], HPLC-UV[7], LC-MS[8] and LC-MS/MS[9]. The features of these methods to analyze tenofovir concentration alone are compared and summarized in Table 1.

Table 1. Features of bioanalytical methods developed for tenofovir in human plasma.

S.N0	Extraction procedure(plasma valume)	Elution Mobile Phase, injection volume	Run time(min)	Detection technique	LLOQ(ng/Ml)	Ref No
1	PPwith TCA(0.2ml)	Gradient:10Mm SP and TBAHS buffer Ph7.0+(ACN+7.0pH buffer, 50:50,v/v); 50µL	20.0	HPLC-Fluorescence	20.0	5
2	PPwith MeOH(0.2ml)	Isocratic; phosphate buffer with 5 Mm TBAC-ACN, pH 6.0 (85:15, v/v); 80 µL	11.0	HPLC-fluorescence	5.0	6
3	SPE(1.0 ml)	Gradient; CAN+pH 6 buffer containing 15 mM Na2HPO4 and 10 mM TBAHS;150 µL	12.0	HPLC-UV	10.0	7
4	PPwith CAN(0.5ml)	Gradient; 0.3% TFA+ACN+100 mM AmA; 5 µL	12.0	LC-MS	18.0	8
5	SPEwith Waters OasisMCX cartridge (0.2mL)	Isocratic; HA/AA buffer (pH 6.75)–MeOH (93:7, v/v); 25 µL	10.0	LC-MS/MS	1.0	9
6	SPEwith Phenomenex Strata-X-C (0.5 mL)	Isocratic; 10 mM AmA–MeOH (60:40, v/v); 10 µL	5.0	LC-MS/MS	4.095	Present Method

*PP: protein precipitation; SPE: solid-phase extraction; TCA: trichloroacetic acid; ACN: acetonitrile; TBAC: tetrabutylammonium chloride; TBAHS: tetrabutylammonium hydrogen sulphate; AA: acetic acid; AmA: ammonium acetate; HA: hydroxylamine.

Moreover, there are strategies for assurance tenofovirin blend with other antiretroviral tranquilizes in human or creature plasma[10–14]. A mix of tenofovir and emtriva has been resolved utilizing HPLC after SPE extraction from human plasma[10], and its affectability accomplishes the degree of 10 ng/mL. Gehrig et al.[11] have portrayed 18 antiretroviral drugs, remembering TFV for plasma utilizing triple fourfold mass spectrometry with ESI. Saux et al.[12] have examined seven nucleoside reverse transcriptase inhibitors in human plasma by LC-MS. Lower cutoff of quantification(LLOQ) of tenofovir is 5 ng/mL, and scientific time is 14 min. Manish Yadav et al.[13] have fostered a LC-MS-MS technique for assurance of one nucleotide tenofovir and two nucleosides (emtricitabine and lanivudine) with run-season of 3 min, and LLOQ is 4.0

ng/mL. Jifen Guo et al.[14] havedeveloped and validatedLC-MS-MS method for assurance of tenofovir in monkey plasma. In the current examination, we set up a specific, accurate and touchy fluid chromatography-electrosprayionization mass spectrometry (LC-ESI-MS/MS) method for the assurance of tenofovir in human plasma utilizing entecavir as internal standard (IS). From Table 1, we presumed that among these techniques, our strategy had a more limited run-time and higher sensitivity[9]. The wide direct powerful reach, the little lattice impacts and great recuperation guaranteed pharmacokinetic investigation of TDF tablet in Chinese volunteers, giving significant premise to clinical medication.

2. Experimental

2.1. Chemicals and reagents

Reference standard of tenofovir was given by USP REFERENCE STANDARD, and the IS of entecavir (93.7%) was acquired from China's Food and Drug Verification Research Institute. Methanol of MS grade was bought from Merck Co., Ltd. (Germany). Ammonium acetate and phosphate of insightful evaluation were provided from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Ammonium hydroxide of analytical grade was bought from Kaixin Chemical Reagent Co., Ltd. (Changsha, China). Deionized water was cleansed utilizing a Purelab exemplary framework ELGA Labwater (Shanghai, China). Clear human plasma was got from the solid volunteers. Strong phase extraction section of Strata-X-C (30 mg/lcc) was purchased from Phenomenex. All other chemicals and reagents were of analytical grade from commercial sources unless otherwise stated.

2.2. Instrumentation and conditions

The examples were investigated with a Waters Acquity UPLC framework (Milford, MA) which incorporated a ultra-execution twofold siphon, autosampler, segment chief, and an Acquity TQD triple quadrupole mass spectrometer. Information securing and signal yield were constrained by Empower 3 programming v3471. Prepared examples were set in the autosampler kept up at 10 °C and 30 μL was infused.

The separation was performed on Phenomenex Gemini-C18 analytical segment (4.6 mm×150 mm, 5 μm,Phenomenex, USA), and a Gemini C18 column (4 mm× 3.0 mm, 5 μm, Phenomenex, USA) was utilized as gatekeeper section. The portable stage comprised of 10 mM ammonium acetic acid derivation in water and methanol (60:40, v/v), and its ?ow rate was set at 0.5 mL/min. The infusion volume was 10 μL, and the section temperature was kept up at 35 ºC.

The mass spectrometer was worked in positive ion mode utilizing various response observing (MRM). The antecedent item particle changes observed were m/z 288.2→176.1 for tenofovir and m/z 278.1→152 for entecavir. The other enhanced MS/MS boundaries were as follows: fragmentor voltage 135 V for tenofovir

and 85 V for entecavir, crash energy (CE) 25 eV for tenofovir and 13 eV for entecavir, gas temperature 350 ºC, drying gas (N2) stream 12 L/min, nebulizer pressure 45 psi, and slim voltage 4.0 kV.

2.3. Chromatography

Analytes were isolated on a warmed (40°C) Phenomenex Synergi 4µ Polar-RP 50 × 2 mm segment with a 0.2 µm frit channel. The versatile stages comprised of 0.1% formic corrosive in water (portable stage A) and 0.1% formic corrosive in acetonitrile (portable stage B). The stream rate was 400 µL/min conveying 97% dissolvable An and 3% B for 0–1 min followed by a straight angles to 80% B from 1–2.5 min and to 95% B from 2.5–4 min then, at that point set to the underlying conditions from 4.01 to 5 min.

2.4. Mass spectrometry

Analytes were identified in certain electrospray ionization (ESI+) mode utilizing pair mass spectrometry with chose response checking (SRM). The shower voltage was set to 750V with desolvation gas set at 800 L/hr. The vaporizer temperature and source temperature were set to 400 °C and 150 °C, individually. Crash gas (argon) pressure was set at 1.5 mTorr with a stream pace of 0.5 mL/min. The particle advances, crash energies, and cone voltages are displayed in Table 1 and were resolved utilizing Water's Intellistart programming.

2.5. Assay validation

Every network (plasma and CSF) was freely approved for exactness and accuracy to guarantee solidness dependent on the suggestions of the USFDA Guidelines for Bioanalytical Method Validation.

2.6. Preparation of stock and standard solutions

The stock arrangements of tenofovir (1 g/L) and IS (1 g/L) were both newly set up in methanol and put away in 4 ºC. The IS working arrangement was set up by weakening the IS stock answer for a convergence of 20 µg/L with methanol before use. Seven standard working arrangements of tenofovir were sequentially set up by weakening its stock arrangement with methanol, bringing about the last tenofovir concentrations of 10 000, 4000, 1600, 640, 256, 102.4 and 40.96 µg/L. Standard plasma tests at the centralizations of 1000, 400, 160, 64, 25.6, 10.24, 4.096 µg/L were prepared by spiking 50 µL of the functioning arrangements into 450 µL of clear plasma. Quality control (QC) tests were independently set up similarly at three concentration levels (10.24, 64 and 800 µg/L). All alignment standard examples and QC tests were newly pre-arranged every day.

2.7. Sample preparation

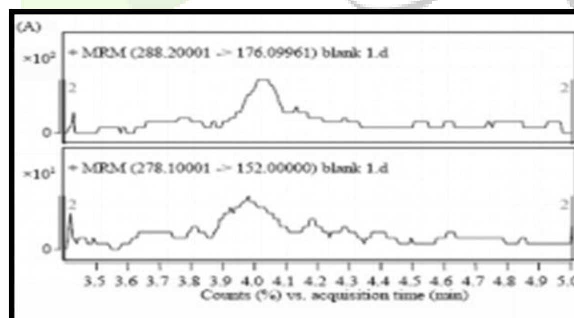
An aliquot of 100 µL standard plasma tests was moved to glass tube, trailed by expansion of 50 µL IS working arrangement (20 µg/L) and 500 µL 2% phosphoric acid arrangement. The blend was vortex-blended for 30 s and then centrifuged at 15 700 ×g for 5 min. SPE columnwas enacted with 1 mL methanol and offset with 1 mL 0.5% phosphoric corrosive arrangement. Accordingly, 1 mL plasma test was extricated by SPE segment, 1 mL 0.5% phosphoric corrosive arrangement was utilized to elute contaminations, and 1 mL 5% smelling salts methanol was utilized for the eluent assortment. The eluent was moved to a spotless cylinder and vanished to dryness under a delicate stream of nitrogen at 45 ºC. The buildup wasdissolved by 300 µL portable stage and centrifuged at 15 700 ×g for 5 min, and afterward 10 µL of the supernatant was injected to the HPLC-MS/MS framework.

3. Results and discussion

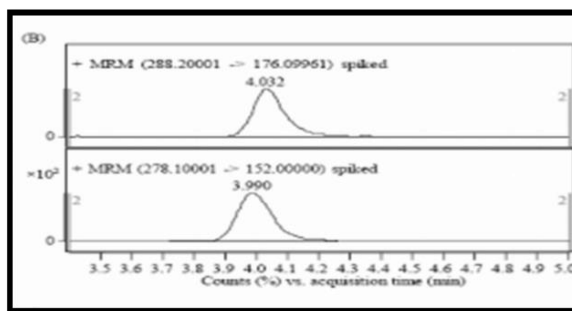
3.1. Specificity

Figure 2 shows the ordinary chromatogram profiles of clear plasma, clear plasma spiked with tenofovir and IS, and plasma got after a solitary oral administration of 300 mg TDF tablet. The maintenance season of tenofovir and IS was around 4.0 min, individually. No undeniable obstructions from endogenous substances were noticed. These outcomes showed that the technique displayed great explicitness and selectivity.

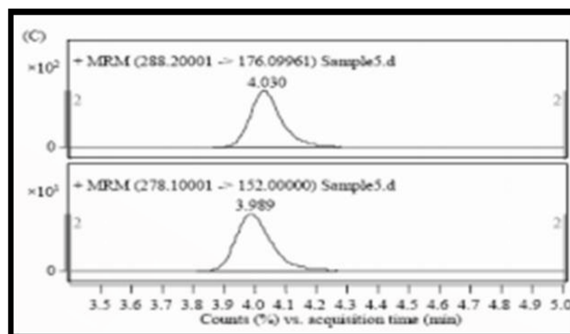
Figure 2. Typical chromatograms of tenofovir in human plasma: (A) blank human plasma; (B) blank plasma spiked tenofovir (4.096 µg/L) and IS; (C) plasma sample from a healthy volunteer 1.5 h after oral administration of 300 mg TDF tablet.



(A) blank human plasma



(B) blank plasma spiked tenofovir (4.096 µg/L) and IS



(C) plasma sample from a healthy volunteer 1.5 h after oral administration of 300 mg TDF tablet.

3.2. Linearity and LLOQ: The calibration curve for tenofovir was linear over a concentration range of 4.096–1000 µg/Lin plasma. The mean regression equation of the calibration curve for tenofovir was Y = 0.0141X + 0.0101 (r^2 = 0.9962). The LLOQ for tenofovir in plasma was 4.096 µg/L on the basis that the accuracy of LLOQ was within ±20% and precision was 8.1%.

3.3. Precision and accuracy

Table 2 lists the intra- and inter-day precision and accuracy values. All the intra- and inter-day precisions were less than 10%. The data demonstratedthat the precision and accuracy of this assay were within the acceptable range and the method could produce satisfactory results.

Nominal concentration (µg/L)		**Intra-day (n = 5)**			**Inter-day (n = 15)**	
	Mean found concentration (µg/L)	Accuracy (%)	Precision(RSD%)	Meanfound concentration (µg/L)	Accuracy(%)	Precision (RSD%)
10.24	10.61	103.8	5.9	10.14	99.0	7.3
64	69.56	107.2	3.9	101.6	101.2	8.2
800	825.7	103.2	9.2	103.2	103.6	8.5

Table 2. Inter- and intra-day accuracy and precision of tenofovir in human plasma.

3.4. Recovery

The recoveries of tenofovir from human plasma were 78.8%, 67.0% and 77.4% at concentrations of 10.24, 64 and 800 μg/L, respectively, while the mean recovery of IS was 75.3%, showing a satisfactory extraction recovery.

3.5. Matrix effect

The matrix effect (%) for tenofovir at 10.24, 64 and 800 μg/L was 101.3%, 97.2% and 101.5% of the nominal concentrations, respectively, while it was 100.7% for the IS. This result indicated that matrix effects were not an issue when using the current method.

3.6. Sample Stability: In terms of stability, the results indicated that tenofovir and IS were stable in human plasma and in processed samples under the conditions described above (Table 3).

Table 3. Stability of tenofovir in human plasma under various conditions(n = 3).

Item	10.24 μg/L			64 μg/L			800 μg/L		
-----	Mean (μg/L)	Accuracy (%)	RSD (%)	Mean (μg/L)	Accuracy (%)	RSD (%)	Mea(μg/L)	Accuracy (%)	RSD (%)
Post-preparative stability	11.27	110.1	3.1	71.43	111.6	6.1	841.4	105.2	8.5
Short-term stability	10.57	103.3	3.4	67.77	105.9	6.3	794.9	99.4	6.8
Freeze-thaw stability	10.12	98.8	10.3	65.13	101.8	2.4	847.4	105.9	11.7
Long-term stability	10.46	102.2	3.9	64.56	100.8	2.9	753.9	94.2	4.3

3.7. Pharmacokinetic study

Figure 3 shows the mean concentration versus time profile of tenofovir under fasting condition. Peak concentration (C_{max}) and time to peak concentration (T_{max}) were directly obtained from experimental observations. The mean of C_{max} and T_{max} was (252.2±108.4) μg/L and (0.82±0.25) h, respectively. Plasma concentration declined with a $T_{1/2}$ of (21.26±6.81) h. The obtained AUC_{0-t} and AUC_{0-inf} values were (1942±681) μg/L·h and (2145±678) μg/L·h, respectively.

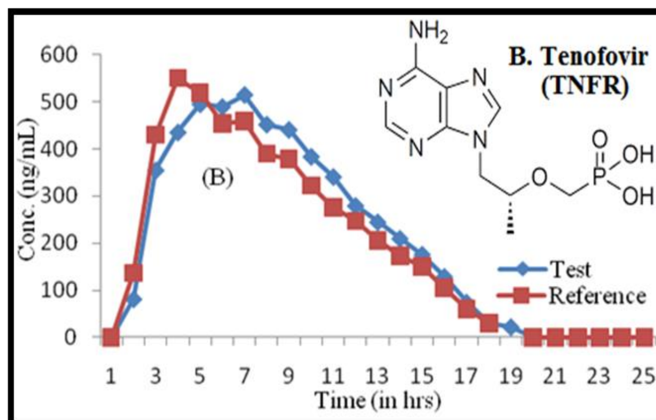


Figure 3. Mean plasma concentration–time curve of tenofovir after oral administration of 300mg tenofovir disoproxil fumarate tablet to 12 healthy Chinese subjects under fasting condition. Each data represents the mean±SD

4. Conclusions

Tenofovir (Fig. 1B) has a place with a remarkable class of nucleotide analogues, wherein a phosphonate bunch bonds to the alkyl side chain of different purines and pyridimines. It is difficult to separate utilizing a typical column due to its high extremity. In the current examination, improvement of SPE measure was done on Phenomenex Strata-X-C, a kind of cation trade section appropriate for partition of soluble mixtures. Expansion of corrosive like phosphate during test planning helped in breaking the medication protein restricting and keeping up the analyte in an ionized structure. In this manner, better maintenance was given on the segment. In addition, utilization of ammoniaand methanol during washing step gave predictable recuperation, particularly at LLOQ level with least network impedance. Contrasted and trifluoroacetic corrosive and methanol protein precipitation[15,16], the present study extraordinarily decreased network impacts and expanded recovery and affect ability.

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