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LIQUID CHROMATOGRAPHY METABOLITE PROFILING OF TENOFOVIR DISOPROXIL FUMARATE

PROFILISANJE METABOLITA TENOFOVIR-DIZOPROKSIL-FUMARATA TEČNOM HROMATOGRAFIJOM

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Summary: Reverse transcriptase inhibitors are the most frequently prescribed agents for HIV infections. This publication presents a validated, highly sensitive and selective isocratic HPLC method for the quantitative determination of tenofovir disoproxil fumarate and its metabolite tenofovir. Detection was performed on a UV detector. The linearity for the calibration curve in the concentration range of 80–20400 ng/mL for tenofovir disoproxil fumarate (TENDF) and 10–2560 ng/mL for tenofovir (TEN) is presented. Inter- and intra-day precision and accuracy of the proposed method were characterised by relative standard deviation (R.S.D.) and percentage deviation, respectively: with both lower than 4% for all analytes. The limit of detection was 33.46 ng/mL for TENDF and 2.10 ng/mL for TEN.

Keywords: HPLC, metabolite, metabolite profiling, prodrug, tenofovir

Kratak sadržaj: Inhibitori reverzne transkriptaze su agensi koji se najčešće prepisuju za infekcije HIV-om. Ova publikacija predstavlja validirani, veoma senzitivni i selektivni izokratski HPLC metod za kvantitativno određivanje tenofovir-dizoproksil-fumarata i njegovog metabolita tenofovira. Detekcija je obavljena na UV detektoru. Predstavljena je linearnost za kalibracionu krivu u opsegu koncentracije 80–204000 ng/mL za tenofovir-dizoproksil-fumarat (TENDF) i 10–2560 ng/mL za tenofovir (TEN). Preciznost i tačnost predloženih metoda između dana i tokom dana karakterišu relativna standardna devijacija (R.S.D.) i procentualna devijacija: obe su manje od 4% za sve analite. Limit za detekciju bio je 33,46 ng/mL za TENDF i 2,10 ng/mL za TEN.

Ključne reči: HPLC, metabolit, profilisanje metabolita, prolek, tenofovir

Introduction

Ester prodrugs are commonly used to increase the intestinal absorption of drugs with permeability-limited absorption. The high functional activity of esterases in blood and liver allows a rapid bioactivation of the prodrug after reaching the systemic circulation. However, the increased efficiency of prodrugs to pass the intestinal barrier may be decreased by its rapid esterase-mediated hydrolysis at the level of the intestinal mucosa, offsetting the diffusion of the lipophilic, intact esters across the epithelial monolayer. This phenomenon primarily occurs at the level of the mucosa of small intestine, due to its high esterase activity. Tenofovir disoproxil fumarate [(TENDF) is a bis-ester prodrug of the acyclic nucleoside phosphonate tenofovir. The chemical name of TENDF is 9-[(R)-2-[[bis[[(iso propoxy carbonyl) oxy]methoxy] phosphinyl] methoxy] propyl] adenine fumarate (1:1) (*Figure 1*). It has a molecular formula $C_{19}H_{30}N_5O_{10}P$

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List of Abbreviations:

AUC, area under curve; DNA, deoxyribonucleic acid; FTIR, Fourier transform infrared spectroscopy; HIV, human immunodeficiency virus; HPLC, high performance liquid chromatography; IND, Indapamide; LOD, limit of detection; LLOQ, lower limit of quantitation; NMR, nuclear magnetic resonance; ODS, octa decyl silane; RSD, relative standard deviation; TEN, tenofovir; TENDF, tenofovir disoproxil fumarate; UV, ultraviolet.

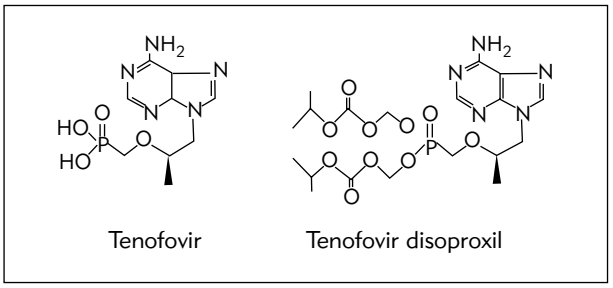


Figure 1 Structure of tenofovir (TEN) and tenofovir disoproxil fumarate (TENDF).

• $C_4H_4O_4$ and a molecular weight of 635.52. Tenofovir (TEN) (*Figure 1*) has a strong activity against human immunodeficiency virus infection in humans (1). However, it is characterized by a permeability-limited oral absorption due to its hydrophilic nature (2). In the prodrug, the two negative charges of TEN are masked by isopropyloxycarbonyloxymethyl moieties, which increase the lipophilicity of the compound and thus its permeation across membranes. Based on its favourable pharmacokinetic profile compared with other ester prodrugs, TENDF was selected as an orally active form of tenofovir (3). It was shown that the degradation of TENDF to its (mono) ester equivalent (mediated by carboxylesterases) and further to TEN (probably mediated by phosphodiesterases) at the level of the intestinal mucosa may be an important barrier limiting the transepithelial transport of TENDF (4). Following oral administration of a single dose of 300 mg to HIV-infected patients in the fasted state, maximum serum concentrations (C_{max}) of TEN are achieved in 1.0±0.4 hours. C_{max} and AUC values are 296±90 ng/mL and 2287±685 ng × h/mL, respectively. The oral bioavailability of TENDF in fasted patients is approximately 25%. TEN is eliminated by a combination of glomerular filtration and active tubular secretion. TENDF requires initial diester hydrolysis (by non-specific esterases in blood and tissues) for conversion to TEN and subsequent phosphorylations by cellular enzymes to form TEN diphosphate, an obligate chain terminator. TEN diphosphate inhibits the activity of HIV reverse transcriptase and HIV polymerase by competing with the natural substrate deoxyadenosine 5′-triphosphate and, after incorporation into DNA, by DNA chain termination. TEN diphosphate is a weak inhibitor of mammalian DNA polymerases α, β, and mitochondrial DNA polymerase γ. Some metabolic side effects associated with TEN have included hyperglycemia, increased urine glucose, fasting triglycerides, fasting cholesterol, creatine kinase, serum amylase, alkaline phosphatase, pancreatic amylase, and serum lipase, altered serum glucose, weight loss, and lactic acidosis. In such cases the health care systems also emphasize the identification and quantitation of the metabolites for comprehensive understanding of biological safety of individual metabolites; thus, revealing the need and scope of bioanalytical research in metabolite and toxicity profiling of drugs. Availability of protocols for qualitative and quantitative characterization of all metabolites will have many applications for therapeutic drug monitoring, bioequivalence, toxicological and all related studies. Identification of metabolites may be done by a variety of chromatographic and spectroscopic techniques, either alone or in combination with other techniques. Rapid and effective ways for the determination of drugs and metabolite in biological fluids are desirable. Conventional liquid chromatography has been exploited widely in the field of metabolite profiling. Metabolite profiling has been established as a multiparallel strategy for relative quantification of a mixture of compounds or compound classes using chromatography and universal detection technologies.

Literature survey reveals that a simple reverse phase high performance liquid chromatographic method for the determination of TENDF in pharmaceutical formulations and human plasma samples is reported (5–6). In this paper we describe a rapid, selective and sensitive HPLC method for simultaneous determination of TENDF and TEN in plasma. The aim of the current study was to quantitate TEN in the presence of TENDF and develop a validated high performance liquid chromatography (HPLC) assay method for the determination of TEN and TENDF from plasma. The method validation was done according to USFDA guidelines (7).

Materials and Methods

Materials

TENDF was supplied as gift sample by Cipla ltd. (Mumbai, Maharashtra, India) and used without further purification. Indapamide (IND) was supplied as gift sample by Glenmark Pharmaceutical ltd. (Goa, India). Potassium hydroxide and hydrochloric acid (both AR grade) were purchased from Loba Chemie Ltd. Chloroform was procured from S.D. Fine Chem Ltd. Methanol (HPLC grade) was purchased from Merck ltd. Double distilled water was obtained from a water distillation unit.

Instrumentation

The HPLC system used was a computer based Jasco series instrument comprising a pump PU-2080 and a UV detector UV-2070. Manual injections were carried out using a Rheodine injector with a fixed 20 μL external loop. The chromatographic separations were performed on a HIQ sil C18 ODS column (250 mm length×4.6 mm internal diameter and 5 μm particle size), operating at ambient temperature, using a mobile phase consisting of methanol:water (70:30v/v), at a flow rate of 1.0 mL/min, and detection was performed at 260 nm using a UV detector. HPLC instrument was controlled by software Borwin. The mobile phase was filtered through a 45 μm nylon membrane filter. A Shimadzu AY 120 analytical balance

was used for weighing. A PCi ultrasonicator was used for sonication. The calibrated glasswares were used throughout the experiment. The mobile phase was used for dilutions of degradation samples throughout the analysis. The characterization was done by using FTIR: KBr (cm^{-1}), 4100 JASCO and NMR: DMSO-d6 (δ); 400 MHz Varian NMR.

Synthesis and characterization of TEN

TEN was synthesized at laboratory scale, and obtained in good yield. 1 gram of TENDF was weighed accurately, to this 10 mL of 0.1 mol/L KOH solution was added and kept at 50 °C for three hours. The reacting solution was cooled at room temperature; to this solution 10 mL of 0.1 mol/L HCl was added. The resulted solution was extracted with 50 mL x 3 times with chloroform. Aqueous layer was evaporated on water bath to get solid TEN. The solid was recrystalized from chloroform (yield 58.30%). After drying it was characterized by IR and NMR.

Preparation of standard solutions

The stock standard solutions of TENDF and TEN were prepared by dissolving standard in mobile phase. The stock standard solutions were then diluted with mobile phase to achieve standard working solutions at concentrations 10, 20, 40, 80, 160, 320, 640, 1280 and 2560 ng/mL for both TENDF and TEN.

Stock internal standard solution was prepared by accurately weighing Indapamide (IND) which was then dissolved in mobile phase. Working internal standard was prepared by accurate dilution of the stock internal standard with mobile phase to a final concentration of 1 μg/mL. Stock internal standard was stored at 40 °C and confirmed to be stable for 5 days.

Preparation of plasma samples

To the 10 mL of blood sample 2 mL of 0.1 mol/L acetic acid was added. RBCs allow settling down. Sample was centrifuged at 5000 r.p.m. for 35 min. The supernatant was collected as plasma. The TENDF and TEN were weighed separately and dissolved together in mobile phase in the same volumetric flask, to this solution two mL of plasma was added. Obtained solution was filtered through a syringe filter the size of 45 μm and final volume was adjusted to 100 mL with mobile phase to get stock solutions containing 100 μg/mL each of TEN and TENDF. The stock standard solutions were then diluted with

Table I Retention time and relative retention times of various peaks.

Peak	Retention time (RT)	Relative retention time (RRT)
TENDF	2.85	1.00
TEN	2.03	0.71
IND	3.49	1.22

TENDF-tenofovir disoproxil fumarate, TEN-tenofovir (metabolite), IND-indapamide (internal standard)

Table II Linearity data for TEN and TENDF.

Regression parameter	TEN	TENDF
Slope	0.0246	0.0155
Intercept	–0.00043	–0.0000
R^2	0.9996	0.9998

TENDF-tenofovir disoproxil fumarate, TEN-tenofovir (metabolite)

Table III Recovery studies using nine different dilutions from plasma samples.

Concentration of TEN (ng/mL)	Concentration of TENDF (ng/mL)	Actual added pure TEN and TENDF concentration (μg/mL)	Measured TEN concentration (μg/mL)	Measured TENDF concentration (μg/mL)	Recovery of TEN (%)	Recovery of TENDF (%)
10	80	20	20.06	19.89	100.30	99.45
20	160	20	19.60	19.78	98.00	98.90
40	320	20	19.87	20.05	99.35	100.25
80	640	20	20.03	19.94	100.15	99.70
160	1280	20	19.89	19.98	99.45	99.90
320	2560	20	20.04	20.03	100.20	100.15
640	5120	20	19.76	19.94	98.80	99.70
1280	10240	20	19.94	20.08	99.70	100.40
2560	20400	20	20.05	19.87	100.25	99.35
Average recovery					99.57	99.75

TENDF-tenofovir disoproxil fumarate, TEN-tenofovir (metabolite)

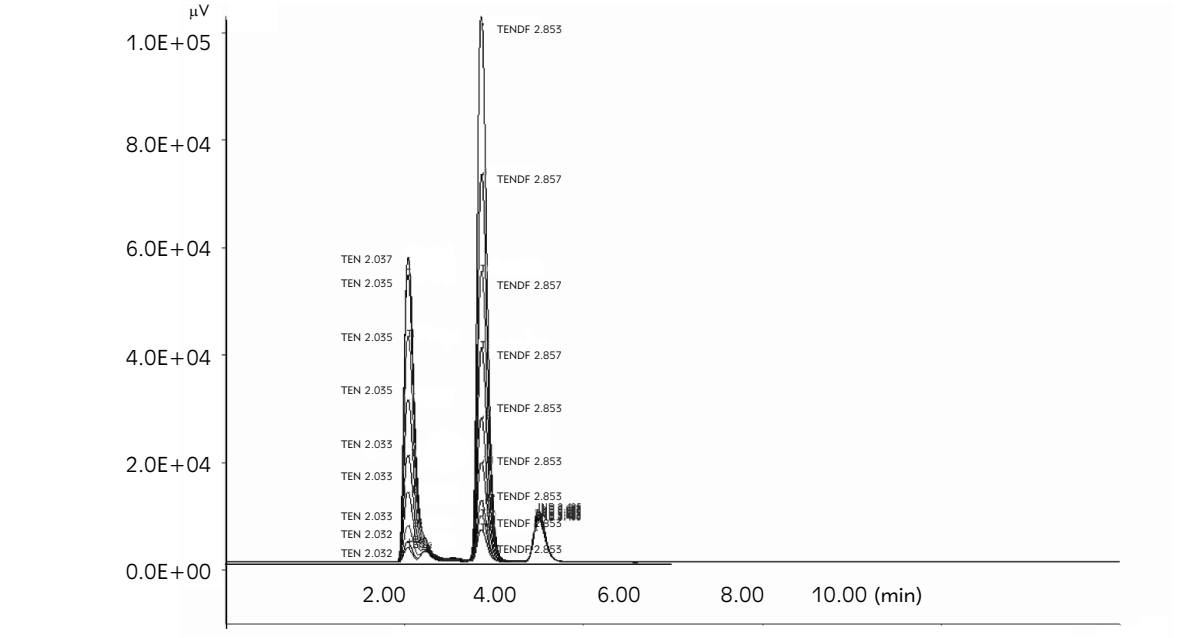
mobile phase to achieve standard working solutions at concentrations 10, 20, 40, 80, 160, 320, 640, 1280 and 2560 ng/mL for TEN and 80, 160, 320, 640, 1280, 2560, 5120, 10240 and 20480 ng/mL for TENDF. To each solution 1 μg/mL of IND as internal standard was added. Each bioanalytical batch consisted of a blank and a blank with internal standard.

Procedure for plotting calibration curve

The above nine mixed standard solutions were used for calibration curve for both the TENDF and TEN. Calibration curves were plotted by response factors against the concentrations of TENDF and TEN separately. The slope, intercept and correlation coefficient values for calibration curves were obtained for both TENDF and TEN. The overlain chromatograms are shown in *Figure 2*.

Results

The metabolite (TEN) was synthesized and characterised by IR and NMR. The separation was achieved with good chromatographic results. The retention times of TENDF and TEN are listed in *Table I*. The



TENDF-tenofovir disoproxil fumarate (2.85±0.04), TEN-tenofovir (2.03± 0.05, metabolite), IND-Indapamide (3.49 ± 0.03, internal standard)

Figure 2 Chromatogram showing overlain of TENDF and TEN.

Table IV Intra-day assay summary.

Drug/ Metabolite	Amount added (ng/mL)	Mean amount found (ng/mL)	%Deviation[a]	%R.S.D.[b]
TENDF	80	79.87	1.7625	7.4625
	320	319.60	3.5325	6.6541
	1280	1279.80	2.2964	4.6254
	10240	10439.76	–0.37524	3.1654
TEN	10	09.89	1.5365	8.6254
	40	39.95	2.7964	7.1654
	320	319.94	2.6846	9.1354
	1280	1279.86	2.8264	3.6547

N=6, [a] accuracy, [b] precision, TENDF-tenofovir disoproxil fumarate, TEN-tenofovir (metabolite)

Table V Inter-day assay summary.

Drug	Amount added (ng/mL)		
	80	1280	10240
TENDF	Mean amount found (ng/mL)/% R.S.D.		
Run 1	79.98/7.3264	1279.95/6.3254	10239.85/5.6248
Run 2	80.02/6.3216	1279.87/8.6254	10239.94/7.1684
Run 3	79.89/8.9615	1279.94/7.9264	10239.76/4.1365
	79.91/7.6219	1279.80/5.9458	10239.84/7.6158
Mean	79.95	1279.89	10239.85
S.D.	0.0605	0.0697	0.0736
% Deviation[a]	0.05	0.05	0.04
% R.S.D.[b]	0.0756	0.0054	0.0007
Metabolite	Amount added (ng/mL)		
	10	160	1280
TEN	Mean amount found (ng/mL)/% R.S.D.		
Run 1	9.70/6.3154	159.84/8.6159	1280.03/3.1654
Run 2	9.93/7.9368	159.72/9.6158	1279.95/8.6157
Run 3	9.95/6.7694	159.67/5.6197	1279.94/8.5947
Run 4	9.92/8.6197	159.74/6.3157	1279.82/6.1354
Mean	9.87	159.74	1279.93
S.D.	0.1173	0.0713	0.0866
% Deviation	0.08	0.04	0.05
% R.S.D.b	1.1884	0.0446	0.0067

[a] Accuracy, [b] precision, TENDF-tenofovir disoproxil fumarate, TEN-tenofovir (metabolite)

Table VI Freeze-thaw stability of TENDF and TEN.

Cycle	Concentration	TENDF mean amount found (ng/mL)	TEN mean amount found (ng/mL)
0	Low	79.95	9.95
	High	10239.96	1179.82
1	Low	79.97	9.87
	High	10240.03	1179.94
2	Low	79.95	9.86
	High	10239.94	1179.89
3	Low	79.96	9.92
	High	10240.02	1179.95

TENDF-tenofovir disoproxil fumarate, TEN-tenofovir (metabolite)

Table VII Long-term stability of TENDF and TEN.

Days	Concentration	TENDF mean amount found (ng/mL)	TEN mean amount found (ng/mL)
0	Low	80.05	9.93
	High	10239.89	1179.87
15	Low	80.05	9.97
	High	10240.13	1180.14
39	Low	79.95	9.96
	High	10239.84	1180.08
91	Low	79.98	10.09
	High	10240.12	1180.95

TENDF-tenofovir disoproxil fumarate, TEN-tenofovir (metabolite)

Table VIII System suitability parameters for TENDF and TEN.

Name of peak	R.T.	Theoretical plates	Selectivity	Capacity	Resolution	Asymmetry
TEN	2.03	2934.61	0.00	202.50	0.00	1.87
TENDF	2.85	4020.20	1.40	284.33	4.49	1.43
IND	3.49	4227.67	1.23	348.50	3.26	1.34

TENDF-tenofovir disoproxil fumarate, TEN-tenofovir (metabolite), IND-Indapamide (internal standard)

slope, intercept and correlation coefficient are summarized in *Table II*. The results of the recovery are summarized in *Table III*. The intra- and inter-day assay results (summarised in *Table IV* and *Table V*) show acceptable precision and accuracy for the proposed method. All results of stability tests presented in *Table VI* and *Table VII* show good stability of TENDF and TEN concentration over all steps of determination; therefore the method is proved to be applicable for routine analyses. The system suitability parameters are shown in *Table VIII*.

Discussion

Characterization of TEN

The solid was recrystalized from chloroform (yield 58.30%). After drying it was characterized by IR and NMR [IR (KBr) cm-1: 3345 (NH stretching); 3320 (OH stretching); 2995 (CH stretching); 2235 (C=N stretching); 1155 (C-O stretching). 1H NMR (DMSO)δ: 1.21 (d, 3H, CH_3); 2.0 (s, 2H, OH); 3.0 (t, 1H, CH_3-CH-O); 3.4 (s, 2H, P-CH_2-O); 3.86 (d, 2H, CH-CH_2-N); 4.0 (s, 2H, NH_2); 8.12 (s, 1H, N-C=N pyrimidine), 8.68 (s, 1H, N-C=N imidazole)].

Performance of liquid chromatography

Several analytical columns (C_8 and C_{18}) were tested to obtain maximal response of TENDF and TEN and reasonable time of analysis. Symmetrical peak shapes of TENDF and TEN and IND could not be obtained easily with the C8 stationary phase. The C18 column (250 nm length x 4.6 internal diameter and 5 μm particle size) was eventually selected for assay because it exhibited excellent peak shape and it had highest response of TENDF and TEN with an acceptable run time.

The selection of mobile phase components was also a critical factor. Finally, two-component mobile phase containing methanol and water was used with satisfying results.

Validation of the TENDF and TEN assay

Specificity and selectivity. Plasma samples were tested for presence of endogenous components, which might interfere with TENDF and TEN or IND. The plasma samples were pretreated according to the sample preparation procedure, apart from addition of internal standard solution. Chromatograms of blank plasma and plasma spiked with TENDF and TEN and IND were compared to show the specificity and selectivity of proposed procedure. No endogenous components interfering with analytes and internal standard were found in the chromatograms of blank plasma samples.

Recovery studies. Accuracy of analysis was determined by performing recovery studies by spiking different concentrations of pure drug in the pre-analysed samples within the analytical concentration range of the proposed method. The added quantities of the individual TENDF and TEN were estimated by the above method.

Limit of detection and quantitation. The limit of detection (LOD) was estimated as the amount of TENDF and TEN. The LOD was calculated by considering LOD=3.3σ/s, where s= slope of calibration curve and σ= standard deviation of blank response. The limit of detection was calculated to be 2.10 ng/mL for TEN and 33.46 ng/mL for TENDF, respectively. The lower limit of quantitation (LLOQ) defined as the lowest concentration analysed with acceptable accuracy and precision was 10 ng/ml for TEN and 80 ng/mL for TENDF, respectively.

Linearity, accuracy and precision. The nine point calibration curve obtained by linear regression showed good linearity over the concentration range 10–2560 ng/mL for TEN and 80–20480 ng/mL for TENDF, respectively. Inter-and intra-day assays were performed to evaluate precision and accuracy. Each assay batch consisted of blank, blank with internal standard, nine calibration standards and plasma samples of the same concentration. Intra-day precision and accuracy were assessed by the analysis of four plasma samples in nine series. Inter-day precision and accuracy were determined by analysing nine series of four plasma samples in four runs within four days.

Stability. Freeze-thaw stability was determined as percent recovery. The test was carried out over four days in four runs. It was concluded that four cycles of freeze-thaw could be carried out with no loss of TENDF and TEN.

The long-term stability test was performed in four runs over three months. The percent recovery was determined. The obtained data showed no loss of analytes.

Room temperature stability was assessed by analyte determination. The samples were left at room temperature for various lengths of time (0, 15, 30, 60 and 120 min) before sample processing. No significant difference was found in TENDF or TEN concentrations. All plasma samples for stability evaluation were prepared as described in the above section. To guarantee the reliability of the method, measured concentrations should not differ more.

Conclusion

The synthesis of TEN from TENDF was achieved in good yields, the synthesized metabolite was characterised by IR and NMR spectroscopy. A method for the simultaneous determination of TENDf and its metabolite (TEN) in plasma was proposed and validated. No interference from endogenous plasma components or other sources was found. TENDF, TEN and internal standard were well separated and their peaks were narrow and symmetric. The assay showed good precision and accuracy. The analytical method presented here has proved to be useful for the simultaneous estimation of TEN with TENDF.

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References

1. Deeks SG, Bardicht CP, Lietman PS, Hwang F, Cundy KC, Rooney JF, et al. Safety, pharmacokinetics and antiretroviral activity of intravenous 9-[2-(R)-(phosphonomethoxy)propyl]adenine, a novel anti-human immunodeficiency virus (HIV) therapy, in HIV-infected adults. Antimicrob Agents Chemother 1998; 42: 2380–4.
2. Shaw JP, Sueoka CM, Oliyai R, Lee WA, Arimilli KM, Kim CU, et al. Metabolism and pharmacokinetics of novel oral prodrugs of 9-[(R)-2-phosphonomethoxypropyl] adenine (PMPA) in dogs. Pharm Res (NY) 1997; 14: 1824–9.
3. Arimilli MN, Kim CU, Dougherty J, Mulato A, Oliyai R, Shaw JP, et al. Synthesis. In vivo biological evaluation and oral bioavailability of 9-(2-(phosphonomethoxy)propyl)adenine (PMPA) prodrugs. Antivir Chem Chemother 1997; 8: 557–64.
4. Naesens L, Bischofberger N, Augustijns P, Annaert P, Van den MG, Arimilli MN, et al. Antiretroviral efficacy and pharmacokinetics of oral bis(isopropyloxycarbonyloxymethyl)- 9-(2-phosphonylmethoxypropyl)adenine in mice. Antimicrob Agents Chemother 1998; 42: 1568–73.
5. Hood CB, Mary C, Long C, Acosta EP, et al. Development of a sensitive and specific liquid chromatography/mass spectrometry method for the determination of tenofovir in human plasma. Rap Commun Mass Spectro; 2007: 21,13: 2087.
6. Kandagal PB, Manjunatha DH, Seetharamappa J, Kalanur SS, et al. RP-HPLC Method for the Determination of Tenofovir in Pharmaceutical Formulations and Spiked Human Plasma. Anal Lett; 2008: 41,4: 561.
7. Guidance for industry. Bioanalytical Method Validation. May 2001, http://www.fda.gov/cder/guidance/index.htm.

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