Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 793 (2003) 317–324
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Sensitive determination of tenofovir in human plasma samples using reversed-phase liquid chromatography

S. Sentenac[a], C. Fernandez[b,c,*], A. Thuillier[b], P. Lechat[a], G. Aymard[a]

[a]*Clinical Pharmacology and Drug Monitoring Unit, Pitie-Salpetriere Hospital, Paris, France*
[b]*Pharmacy Department, Pitie-Salpetriere Hospital, Paris, France*
[c]*Faculty of Pharmacy, Clinical Pharmacy Department, UPRES 2706, Chatenay-Malabry, France*

Received 7 January 2003; received in revised form 14 April 2003; accepted 14 April 2003

Abstract

A new high-performance liquid chromatography assay was developed for the determination of tenofovir, a nucleotide analogue, in plasma. A solid–liquid extraction procedure was coupled with a reversed-phase HPLC system. The system requires a mobile phase containing Na_2HPO_4 buffer, tetrabutylammonium hydrogen sulfate and acetonitrile for different elution through a C_{18} column with UV detection. The method proved to be accurate, precise and linear between 10 and 4000 ng/ml. The method was applied to determine trough levels of tenofovir in 11 HIV-infected patients with virologic failure under multiple antiretroviral therapy. This method was also successfully applied to a pharmacokinetic study in an HIV infected patient with renal failure.

Keywords: Tenofovir

1. Introduction

Tenofovir {(2′*R*)-9-[2′-phosphorylmethoxy)-propyl]adenine}, formerly known as PMPA (Fig. 1) is a new nucleotide analog belonging to the class of acyclic nucleoside phosphonates. Tenofovir showed low oral bioavailability in animal studies. A prodrug of tenofovir, tenofovir disoproxil fumarate (tenofovir DF; Bis-POC PMPA) is indicated in combination with other antiretroviral drugs for the treatment of patients above 18 years of age infected with the human immunodeficiency virus (HIV) who failed or are intolerant to nucleoside analog therapy or are not controlled by their current antiretroviral regimen.

Tenofovir monitoring is based on the determination of residual concentrations in plasma. As HIV-infected patients usually receive multiple antiretroviral therapy, a selective method of quantification is required.

Analytical parameters, for the determination of plasmatic concentrations of adenine acyclic derivatives (9-[2′-phosphorylmethoxy)ethyl]adenine) (PMEA), {(2′*S*)-9-[2′-(phosphorylmethoxy)-3′-hydroxypropyl]adenine} (HPMPA), {(2′*S*)-9-[2′-(phosphorylmethoxy)-3′-fluoropropyl]adenine} (FMPA), were optimized by Naesens et al. [1]. Since a specific

*Corresponding author. Groupe Hospitalier Pitie-Salpetriere, Service Pharmacie, 47 Boulevard de l'Hôpital, 75013 Paris, France. Fax: +33-1-4216-2010.
E-mail address: christine.fernandez@psl.ap-hop-paris.fr (C. Fernandez).

1570-0232/03/$ – see front matter
doi:10.1016/S1570-0232(03)00333-7

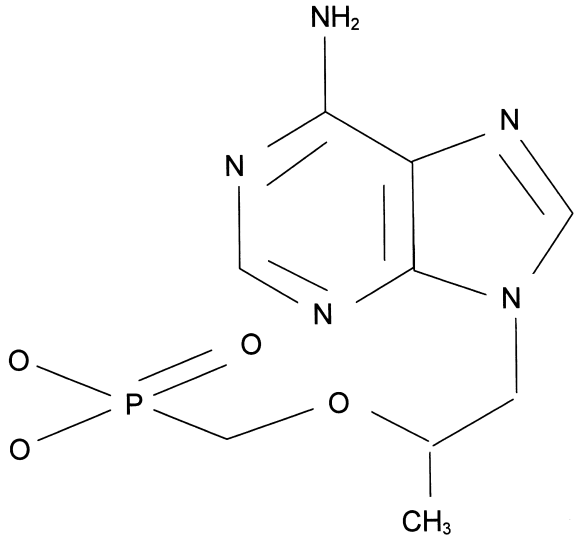

Fig. 1. Chemical structure of tenofovir.

method for the determination of PMPA in plasma was critically important in preclinical and clinical studies, Cundy et al. [2], and Barditch-Crovo et al. [3] modified Naesens et al.'s method for the selective quantification of this new substance.

In the validated method of Cundy et al., the concentration of PMPA in plasma was determined by derivatization and high-performance liquid chromatography (HPLC) analysis with fluorescence detection. The plasma samples were deproteinised by addition of 0.1% trifluoroacetic acid and no specific extraction was done. The limit of quantification (LOQ) for 1 ml sample was 25 ng/ml [2].

Another method was described by Lynch et al. [4], using liquid chromatography (LC) coupled with mass spectrometry (MS). LC–MS allows improved sensitivity and simultaneous determination of intracellular and plasma concentrations of tenofovir, of its active metabolite PMPA diphosphate (PMPApp) and of its prodrug (Bis-POC PMPA). However, there is no need for metabolite and prodrug determination in routine drug monitoring. Hence, this work is restrictively done in pharmacokinetic/pharmacodynamic relation studies for dose adaptation in clinical research.

Since the methods described in the literature for the quantification of PMPA in plasma are time consuming (derivatization) for Cundy et al. [2], and Barditch-Crovo et al. [3], or require specific material (MS) for Lynch et al. [4], we have developed a sensitive method for the quantification of tenofovir in plasma applicable to drug monitoring. This method was validated and applied to the analysis of plasma samples obtained from 11 HIV-infected patients with normal renal function.

As tenofovir is extensively and rapidly excreted in urine, the follow up of treated patients with renal failure is critical. However, there is no available data on tenofovir pharmacokinetics in patients with renal failure. We report in this paper the drug monitoring of one patient with renal renal failure treated with tenofovir.

2. Materials and methods

2.1. Chemicals

Tenofovir (TNF) monohydrate was supplied by Gilead Sciences (Foster City, CA, USA). Its molecular structure is shown in Fig. 1 (molecular mass of TNF monohydrate: 305.23 g). Stock solutions of TNF (1 mg/ml) were prepared by dissolving 10,6 mg of equivalent free and pure base in 10 ml of distilled water (containing 50 μl of 10 *M* sodium hydroxide). Stock solutions stored at +4 °C were stable for at least 6 months. Stock solutions of TNF were extemporaneously diluted with water to achieve 100 μg/ml working solutions.

Acetonitrile, chloroform (Carlo Erba, Milan, Italy) and methanol (Chromanorm, HPLC, Prolabo, Fontenay sous Bois, France) were HPLC grade. Disodium hydrogenphosphate dihydrate (Na_2HPO_4) (Merck, Darmstadt, Germany), tetrabutylammonium hydrogen sulfate (TBA) (Sigma–Aldrich, Steinheim, Germany), trifluoroacetic acid (TFA) (Sigma–Aldrich) and distilled water (Fresenius France Pharma, Louviers, France) were analytical-reagent grade. Blank drug-free plasmas were obtained from the Pitie-Salpetriere Hospital blood bank.

2.2. Instrumentation and chromatographic conditions

The HPLC system consisted of a 600E multisolvent delivery module, connected on line to an in-line

degasser AF, a Wisp 717 Plus autosample injector set at 25 min/sample for the run time, a 2996 AF photodiode array detector set at 259 nm (wavelength corresponding to the second maximum absorbance of TNF). Millenium[32] software was used for the acquisition and the treatment of spectra data and chromatograms. All the material was from Waters (Milford, MA, USA). Separation was achieved at room temperature on a reversed-phase Symmetry Shield 5 μm RP_{18} column (250×4.6 mm I.D.) (Waters) equipped with a Symmetry Shield RP_{18} (5 μm) precolumn. The mobile phase was a mixture of pH 6 buffer (15 m*M* Na_2HPO_4 and 10 m*M* TBA (A) and acetonitrile (B). The buffer was filtered through a 0.22 μm filter Durapore GVWP 047 (Millipore, Bedford, MA, USA) before use. The mobile phase was degassed ultrasonically for 5 min before use. An isocratic mobile phase consisting of A–B (94:6) at a flow-rate of 1 ml/min was used during the first 10 min of the run, followed by a linear gradient elution of 1 to 1.3 ml/min consisting of A–B (30:70) for the next 6 min. Then, the system was reequilibrated for an additional 9 min with the initial conditions The analytical mobile phase was not recycled during the chromatographic session. It was delivered with an average operating pressure of 1.6 kp.s.i. (1 p.s.i.= 6894.76 Pa). At the end of each chromatographic session, the column was washed with water (for about 20 min) and then with acetonitrile for another 20 min at 1 ml/min.

2.3. Extraction procedure

Spiked plasma samples were used for the preparation of the calibration and quality control (QC) samples. The calibration samples and the QC samples were prepared extemporaneously before each chromatographic session. Calibration curve samples, QC samples and patient samples were extracted simultaneously.

Solid-phase extractions (SPEs) were performed with a silica-bonded reversed-phase sorbent: Supelclean TM LC-18 SPE cartridges (500 mg, 3 ml, Sigma–Aldrich). Before use, the SPE cartridge was conditioned with 3 ml of methanol and 2.5 ml of 0.6% (v/v) TFA. To 1 ml of 0.6% TFA, 1 ml of plasma sample was added in the conditioned column. The cartridge was subsequently washed with 2 ml of 0.6% TFA followed by vacuum suction for 30 s. Elution was performed twice with 500 μl methanol. The eluted methanolic solution was mixed well and evaporated to dryness under a gentle stream of nitrogen in a water bath at 40 °C. The residues were redissolved in 250 μl of 20 m*M* Na_2HPO_4, pH 7 and agitated in a Buchner for 5 min.

In order to eliminate silica residues from the sample and preserve the stationary phase, chloroform (2.5 ml) was added, tubes were then capped, shaken horizontally for 20 min and centrifuged for 10 min at 2500 *g*. Carefully, 150 μl of the upper phase was taken and injected into the chromatographic system.

2.4. Preparation of calibration curves

A calibration curve based on the area-surface ratio was constructed for each assay by adding known amounts of TNF to drug-free plasma. Nine different spiked plasma samples covering a concentration range from 0 to 1000 ng/ml were assayed (0, 10, 20, 50, 100, 400, 600, 800, 1000 ng/ml). Linearity of the calibration curves was assessed by unweighted least-square regression analysis. A second calibration curve with eight different spiked samples covering a concentration range of 100 to 4000 ng/ml was constructed, under the same conditions, for the pharmacokinetic study of the HIV-infected patient with renal insufficiency.

2.5. Specificity and selectivity

The concomitant therapeutic agents most likely to be encountered in the plasma of HIV positive patients were screened under the HPLC assay conditions. Drugs were checked as pure solutions in mobile phase or distilled water to reach 500 to 1000 μg/ml, injected in the system and compared to the peak retention time of TNF. An ''interfering drug'' is defined as a molecule which exhibits a retention time close to 0.3 min from TNF.

2.6. Accuracy, precision, limit of quantification and recovery

Six replicate spiked plasmas were assayed between-day and within-day at three different concentrations (20, 400 and 1000 ng/ml). The con-

centrations were calculated using calibration curves prepared with plasma and analysed in the same run. Accuracy was calculated as percent deviation from the nominal concentration. Within-day and between-day precision were expressed as the relative standard deviation (RSD, %=100×SD/mean) of each calculated concentration. For the concentration to be accepted as the lowest limit of quantification (LOQ), the percent deviation from the nominal concentration (mean accuracy) and the RSD had to be within the range ±20% and less than 20%, respectively. The maximum linearity range (upper part of the calibration curves) was tested by analysis of spiked plasma containing TNF at 4000 ng/ml. Average recovery (n=9) was determined by comparing the area-surface ratios of the extracts at three concentrations (100, 400 and 800 ng/ml) with those obtained by direct injection of the same amount of drug in mobile phase (500, 2000 and 4000 ng/ml, three samples for each level).

2.7. Analysis of patient samples

Blood samples were obtained from HIV-1 infected patients before administration of tenofovir. Plasmas from 11 patients aged 45.5±11.8 years (mean±SD), weighing 65.2±6.6 kg (mean±SD) were analysed. All patients were infected by the HIV and were treated with concomitant antiretroviral therapy in the department of Infectious Diseases (Pitie-Salpetriere Hospital, Paris, France). At the time of analyses, patients were required to have a calculated creatinine clearance rate ≥50 ml/min (according to the Cockroft–Gault formula) [5]. Each patient received orally one tablet of 300 mg tenofovir DF (VIREAD) once daily with a light breakfast. Blood samples for determination of plasma concentrations of tenofovir were drawn prior to drug administration. Blood samples were collected on lithium heparinate (7 ml) tubes, centrifuged for 10 min at 2000 g and plasmas were immediately frozen (−20 °C) until assay. We had previously checked that TNF was stable in plasma for at least 6 months if stored at −20 °C.

The pharmacokinetic profile of tenofovir (300 mg×1/day in the morning with a light breakfast) was determined for one patient aged 46 years, weighing 30.3 kg, with renal failure (creatinine clearance rate 10 ml/min). Blood samples were drawn before drug administration and at various times (t, h) $t_{0.5}$, t_1, $t_{1.5}$, t_2, $t_{2.5}$, t_3, $t_{3.5}$, t_4, t_5, t_7, t_9, t_{17}, t_{21}, and t_{24} after administration.

3. Results and discussion

3.1. Extraction and recoveries

With our SPE procedure, the mean recovery of TNF was 63.7% (RSD=10.6%) for the three different concentrations (100, 400 and 800 ng/ml). The very low pH (~1.5) keeps TNF in a non-ionised form which will be better retained by the stationary phase. In the methods described in the literature, plasma samples were deproteinised by the addition of 0.1% trifluoroacetic acid and no specific extraction was done. Even if this procedure is more simple, it is impossible to compare it to our method as no recovery results were reported by the authors [2,3].

3.2. Specificity and selectivity

Under the described conditions, the mean retention time of TNF was 7.55±0.08 min (RSD=1.01%). Fig. 2 shows chromatograms obtained from (A) a blank drug-free human plasma, (B) a QC plasma sample spiked with 400 ng/ml, (C) a t_0 plasma sample of a patient with normal renal function receiving TNF orally, and (D) a t_{12} plasma sample of the patient with renal failure receiving TNF orally. Unlike the methods described in the literature [2,3], we have studied the selectivity on several plasmas: blank drug-free human plasma, blank plasma samples of multitreated HIV-infected patients with normal renal function and blank plasma samples of multitreated HIV-infected patients with renal failure.

Neither the drugs used for treatment of HIV infection (zidovudine, didanosine, zalcitabine, stavudine, lamivudine, efavirenz, abacavir, ritonavir, amprenavir, saquinavir, indinavir, lopinavir, nelfinavir, delavirdine) nor HIV associated infections (acyclovir, gancyclovir and valacyclovir) interfered with TNF: didanosine was degraded under our extraction conditions (very low pH), acyclovir, gancyclovir and valacyclovir had retention times of 3 min, lamivudine and stavudine had retention times of 5.5 min and the other antiretrovirals were not eluted

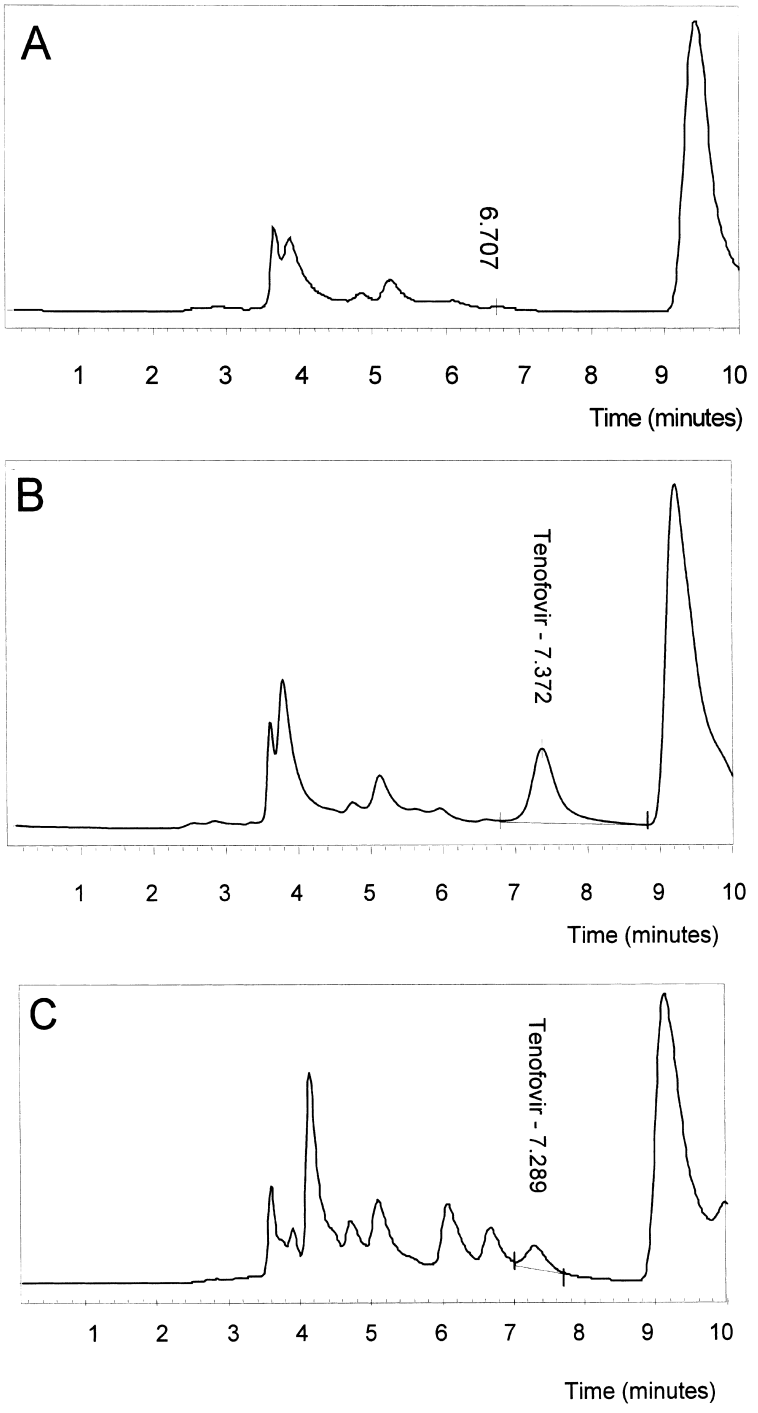

Fig. 2. Chromatograms obtained under described conditions. The retention time of tenofovir was 7.55±0.08 (RSD=1.01%). (A) Blank chromatogram obtained from a drug-free human plasma. (B) Plasma sample spiked with 400 ng/ml of TNF. (C) t_0 plasma sample of a patient with normal renal function treated orally with 300 mg of tenofovir DF (VIREAD), containing 174 ng/ml of TNF. (D) t_{12} plasma sample of the patient with renal failure treated orally with 300 mg of VIREAD, containing 2153 ng/ml of TNF.

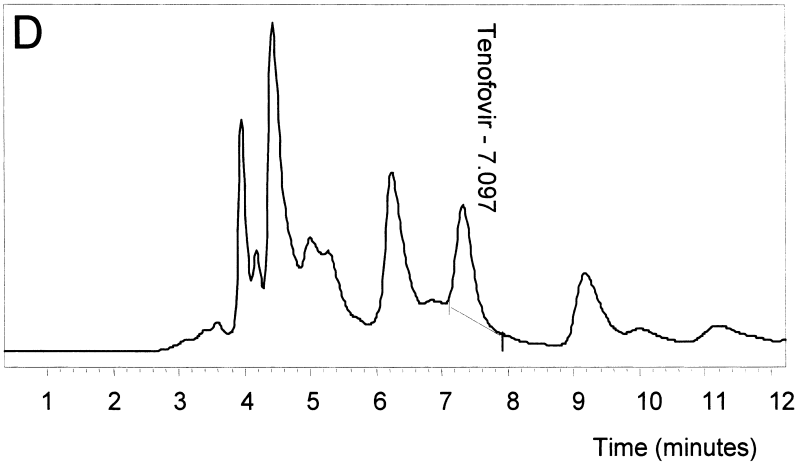

Fig. 2. (*continued*)

at all (zidovudine, zalcitabine, efavirenz, abacavir, ritonavir, amprenavir, saquinavir, indinavir, nefinavir, lopinavir, delavirdine).

3.3. Accuracy, precision and linearity

The results from the validation of the method in human plasma are listed in Table 1. The method proved to be accurate and precise: mean deviations between concentrations added (20, 400,and 1000 ng/ml) and found ranged from −8.5 to +1.9%; the within-day and between-day precision (RSD) ranged from 1.8 to 11%. Our results are close from those of Cundy et al. [2] (the deviations from nominal for the within-day precision and accuracy were <9.4 and <6.2%, respectively). Correlation coefficients (r) of calibration curves (determined by least-squares regression) were higher than 0.995. The deviations from the nominal concentrations (tested in the pharmacokinetic study of the HIV-infected patient with renal failure) are listed in Table 2. The calibration curve of TNF was linear up to 4000 ng/ml and allowed us to determine the different plasma concentrations observed in the pharmacokinetic study of TNF in the HIV-infected patient with renal failure.

The LOQ was 10 ng/ml. For a sample of 1 ml, the LOQ obtained by our method was 2.5-times lower than that obtained by Cundy et al. [2].

3.4. Analysis of patient samples

Antiretroviral drug monitoring is undertaken when the efficacy decreases, when non compliance or malabsorption is suspected, when drug interactions occur and for patients with renal failure. The TNF residual concentrations in the plasmas of 11 HIV-

Table 1
Accuracy, within-day and between-day [mean (RSD, %)] precision for the analysis of tenofovir (TNF) in plasma samples

Theoretical concentration of TNF (ng/ml) in quality control samples	Mean calculated concentration (RSD, %)	
	Within-day (n=6)	Between-day (n=6)
20	19.3 (6.7)	18.3 (11)
400	407.4 (1.9)	400.9 (2.4)
1000	999.3 (3.9)	1019.3 (1.8)

Table 2
Linearity of the method for the measurement of TNF in plasma

Theoretical concentration of TNF (ng/ml)	Deviation from the nominal concentration (%)
0	0
100	−12.7
200	0.3
400	−8.0
1000	−3.1
2000	−4.0
3000	4.9
4000	−1.4

Table 3
Plasmatic residual concentrations of TNF from 11 HIV-infected patients treated with concomitant antiretroviral therapy and who have received orally one tablet of 300 mg of VIREAD with a light breakfast

Patient	Plasmatic residual concentration of TNF (ng/ml)
BOU	90
DON	22
JAR	103
FUS	162
AGU	54
PER	174
HUP	145
DUC	99
BRO	25
BAZ	55
MAN	155

infected patients with normal renal function are listed in Table 3. The mean residual concentration calculated was 98.5±55.0 ng/ml (min–max: 22–174 ng/ml) and was comparable with the median residual concentration of 70 ng/ml reported by Barditch-Crovo et al. [3]. The lowest residual concentration measured in these patients was 22 ng/ml, increased twofold if compared to the LOQ. The pharmacokinetic profile of TNF from one HIV-infected patient with renal insufficiency is presented in Fig. 3. In this study, conducted when the patient had a creatinine clearance of 10 ml/min, C_{max} (peak plasma concentration) was 2836 ng/ml and $AUC_{0\rightarrow 24\ h}$ (area under the curve, calculated by the trapezoidal rule) was 45 808 ng h/ml. These parameters were drastically increased (9.3- and 15.6-fold, respectively) as compared to the values found in patients with normal renal function [3]. The terminal half-life ($t_{1/2}$) was increased by a 2.5-fold (from 13.7 to 35 h). It was suggested that this increase in C_{max} and AUC was the result of an accumulation of TNF due to renal failure.

4. Conclusion

This paper describes a new, sensitive, specific and validated assay for the determination of TNF in plasma. This method is simple, cheap (relatively low price of the materials) and can be easily applied in hospital laboratories for TNF monitoring in plasma, and for pharmacokinetic studies in HIV-infected patients with renal dysfunction. TNF monitoring is important to check drug compliance, prevent virus resistance and follow up patients with renal failure. Due to the lack of interference with other medications, the method can be applied to multidrug-treated patients. From only 1 ml of plasma sample, the method appears to be applicable to determine residual plasma concentrations of TNF and is currently being used to analyze samples of patients treated with approved combination therapy regimens.

The pharmacokinetic pilot study, conducted on a

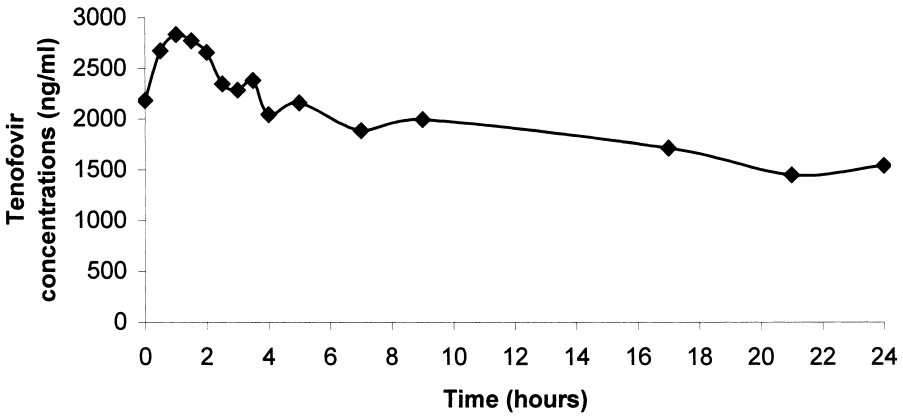

Fig. 3. Pharmacokinetic profile of tenofovir in an HIV-infected patient with renal failure after oral administration of 300 mg of VIREAD.

patient with renal failure, showed an accumulation of TNF in blood, requiring dose adjustment.

References

[1] L. Naesens, J. Balzarini, E.D. Clercq, Clin. Chem. 38 (1992) 480.
[2] K.C. Cundy, C. Sueoka, G.R. Lynch, L. Griffin, W.A. Lee, J.P. Shaw, Antimicrob. Agents Chemother. 42 (1998) 687.
[3] P. Barditch-Crovo, S.G. Deeks, A. Collier, S. Safrin, D.F. Coakley, M. Miller, B.P. Kearney, R.L. Coleman, P.D. Lamy, J.O. Kahn, I. McGowan, P.S. Lietman, Antimicrob. Agents Chemother. 45 (2001) 2733.
[4] T. Lynch, G. Eisenberg, M. Kernan, Nucleosides Nucleotides Nucleic Acids 20 (2001) 1415.
[5] D.W. Cockroft, M.H. Gault, Nephron 16 (1976) 31.