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Sensitive assay for determining plasma tenofovir concentrations by LC/MS/MS

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Abstract

An LC/MS/MS assay for the determination of tenofovir (TNF) was developed and validated for use with the EDTA anticoagulated human plasma matrix. Heparin-treated plasma and serum matrices were also validated. After addition of adefovir as an internal standard, trifluoroacetic acid was used to produce a protein-free extract. Chromatographic separation was achieved with a Polar-RP Synergi, 2.0 mm × 150 mm, reversed-phase analytical column. The mobile phase was 3% acetonitrile/1% acetic acid, aq. Detection of TNF and the internal standard was achieved by ESI MS/MS in the positive ion mode using 288/176 and 274/162 transitions, respectively. The method was linear from 10 to 750 ng/ml with a minimum quantifiable limit of 10 ng/ml when 250 μl aliquots were analyzed. The usefulness of this LC/MS/MS method to routinely monitor plasma concentrations of TNF was demonstrated along with its ability to assist in the performance of pharmacokinetic studies.

Keywords: Tenofovir (TNF); Adefovir (PMEA); LC/MS/MS; Plasma

1. Introduction

Tenofovir (TNF), also known as (phosphorylmethoxy) propyl-adenine [PMPA], is a relatively new anti-HIV drug which is a nucleotide reverse transcriptase inhibitor [1]. The pro-drug, tenofovir disoproxil fumarate, is used for oral administration to improve intestinal absorption [2]. The absorption, circulation and intracellular activation of the free TNF occurs subsequent to hydrolysis of the pro-drug. Chemically, TNF is a monophosphonate adenosine analogue (Fig. 1) that is diphosphorylated to its active moiety, tenofovir diphosphate, within the cell where it functions as a competitive inhibitor of reverse transcriptase and as a HIV-DNA chain terminator [3].

The occasional monitoring of TNF concentrations in plasma from HIV-infected patients may be useful if poor compliance is suspected, or if an underlying clinical condition such as renal insufficiency exists which might result in toxic drug concentrations (TNF is cleared by the kidney [1]). Monitoring may also be useful in the setting of drug–drug interactions, such as those with protease inhibitors lopinavir/ritonavir, atazanavir, and atazanavir/ritonavir that have been shown to increase TNF plasma concentrations [4,5]. Finally, pharmacokinetic studies requiring plasma concentration data may be used to assist in the determination of the most suitable dosing regimen for each patient.

Although there are several reports describing the determination of TNF in plasma using HPLC coupled with fluorescence and UV detection [6,7], the specificity of these techniques for accurately quantitating the drug in the presence of other medications and endogenous compounds is of concern. A pharmacokinetic study of TNF in children that used LC–MS has recently been reported, but performance characteristics of the method were not described [8]. Furthermore, the sample preparation method used by these authors was time-consuming.

This report describes an LC/MS/MS method to determine TNF concentrations in plasma from patients not concomitantly

Abbreviations: DI, deionized; QC, quality control; IDV, indinavir; DLV, delavirdine; APV, amprenavir; NFV, nevirapine; M8, nelfinavir metabolite; SQV, saquinavir; EFV, efavirenz; RTV, ritonavir; LPV, lopinavir; ATV, atazanavir; 3TC, lamivudine; d4T, stavudine; AZT, zidovudine; ABC, abacavir; DDI, didanosine; ACTG, aids clinical trials group; LLOQ, lowest Level of Quantitation; ACD-Plasma, acid citrate dextrose-plasma; FWHM, full width of ion peak at half maximum height

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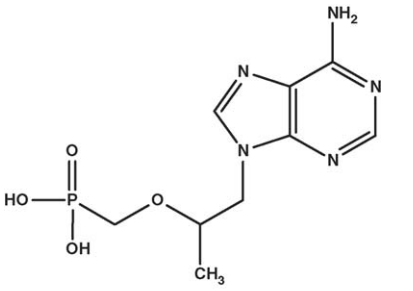
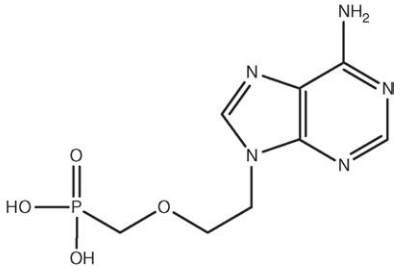


Tenofovir MW: 288



Adefovir MW: 274

Fig. 1. The chemical structures of tenofovir and adefovir (IS).

treated with adefovir, which is rapid, precise, sensitive, accurate and rugged. The ability of tandem MS to specifically quantify TNF using a unique precursor/product combination offers considerable advantages over conventional detection methods in regard to sensitivity and specificity. The results obtained from the analysis of plasma from TNF-treated patients are also described herein.

2. Experimental

2.1. Chemicals

TNF (99.9% pure) and the internal standard adefovir (PMEA, 99.8% pure) were obtained from Moravek Biochemicals Inc., Brea, CA. Trifluoroacetic acid was obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO). Methanol and acetonitrile, both HPLC grade and concentrated ammonium hydroxide, A.C.S. certified, were obtained from Fisher Scientific (Fairlawn, NJ). The deionized/distilled (DI) water was prepared from tap water with a Barnstead Mega-Pure System. Human EDTA-plasma was purchased from Biological Specialty Corporation (Colmar, PA).

2.2. Instruments

The HPLC consisted of a Waters 2690 Alliance Separations Module (Waters Corporation, Milford, MA). The analytical column was a Synergi 4 μ Polar RP HPLC Column 80A, 2.0 mm × 150 mm, protected by a Polar RP guard cartridge, both obtained from Phenomenex, Torrance, CA. The life time of the column under the conditions described herein exceeded 3000 injections. The mobile phase consisted of 3% acetonitrile/1% acetic acid in DI water flowing at 0.2 ml/min. A second external LC pump was used to rinse the ion source of the MS with 100% methanol flowing at 0.5 ml/min (Model LC 1120, GBC Scientific Equipment Ltd., Adelaide, Australia). The MS/MS detector was a TSQ Quantum, operating in the ESI, positive polarity mode (Thermo-Electron, San Jose, CA) and the data system was Xcaliber, version 1.3, also from Thermo Electron. A bench top high speed microtube centrifuge (Microfuge Lite™) was obtained from Beckman Instruments, Fullerton, CA.

2.3. TNF standard and QC solutions

Two separate 1 mg/ml stock solutions of TNF (#1 and #2) were prepared by dissolving approximately 1 mg aliquots of the accurately weighed drug in the appropriate volumes of DI water. TNF stocks #1 and #2 were used for the preparation of working standards and quality controls (QCs), respectively. Eight TNF working standard solutions in 50:50 methanol/water (v/v) were prepared from the stock solution #1. These solutions were stable for at least 1 year at −20 °C. The calibrators were prepared fresh daily as follows: 20 μl aliquots of each working TNF standard were separately added to eight 250 μl aliquots of human plasma resulting in calibration concentrations of 10–750 ng/ml for the calibration curve. The stock internal standard (IS) solution was prepared by dissolving 1 mg of adefovir in 1 ml of 50:50 methanol/water (v/v). This solution was stable for at least one yr when stored at −20 °C. The working IS solution (5 μg/ml) was prepared by the appropriate dilution of the stock IS solution in water. TNF QCs: high QC (600 ng/ml), medium QC (300 ng/ml) and low QC (30 ng/ml) were prepared from the TNF stock 1 mg/ml solution #2 and blank human EDTA plasma using volumetric flasks. Aliquots of one ml were stored in screw-top Cryovial™ tubes at −20 °C.

2.4. Sample preparation

After thawing, 250 μl of patient and QC plasmas were vortexed briefly with 10 μl of working IS in 1.5 ml labeled microcentrifuge tubes. The freshly prepared plasma-based standards were similarly prepared. Trifluoroacetic acid (25 μl) was added under the fume hood to each tube followed by prompt capping and vortexing. After 15 min standing at room temperature, all tubes were ultra-centrifuged in the Beckman Microfuge at 13,000 rpm for 15 min. The clear protein-free extracts were transferred to labeled vials containing 500 μl inserts and vortex-mixed with 20 μl concentrated ammonium hydroxide (14.8 M) to partially neutralize the acid before capping and loading onto the autosampler tray maintained at 4 °C.

2.5. LC–MS procedure

Ten microliter aliquots of sample were injected into the HPLC with the mobile phase flowing isocratically at 0.2 ml/min with 7 min sample run times. No carryover was found when the

TNF concentrations were within the range of the calibrators. The analytical column was maintained at ambient temperature (22–25 °C). The detector settings of the TSQ Quantum were: ESI with the stainless steel spray needle, positive polarity ionization; spray voltage, 3200 V; capillary temperature, 300 °C; argon collision gas pressure, 1.8 mTorr (0.24 Pa); collision energy, 28 V; unit resolution for Q1 and Q3, 0.7 u (FWHM); and ions detected (*m*/*z*), adefovir (IS), precursor 274, product 162; tenofovir precursor 288, product 176. The positive ion mode was chosen for this assay since initial studies indicated a more sensitive detector response than the negative ion mode, possibly due to the acidity of the mobile phase. The low pH was necessary for adequate analyte retention.

2.6. Divert valve program

The divert valve feature of the TSQ Quantum was utilized to limit unwanted compounds in the eluate from entering the ESI MS source and potentially clogging the transfer tube. Methanol flowing at 0.5 ml/min from an external secondary pump rinsed the source while the HPLC eluate was diverted to waste. The HPLC eluate flow destination was switched alternatively between the detector and waste as follows: 0–2 min: waste; 2–5 min: detector; 5–7 min: waste.

2.7. Validation

Validation of the method included the assessment of calibration curve performance, accuracy and precision of the method (determined by QC performance), stability of the analytes at various test conditions, evaluation of specificity and matrix effect. Finally, the assay performance was assessed by inclusion in the ACTG proficiency testing program.

Inter- and intra-day accuracy and precision for the assay were characterized by the performance of four levels of QCs run on 5 separate days in five replicates each day. The lowest limit of quantitation (LLOQ) was 10 ng/ml (equivalent to the assay limit of quantitation), the low QC was 30 ng/ml (equivalent to three times the LLOQ), the mid-range QC was 300 ng/ml, and the high QC was 600 ng/ml (between our two high standards and within 80% of the top standard). Accuracy was assessed by calculating the percent deviation from the theoretical concentration. Precision was determined by calculating the coefficient of variation for the inter- and intra-day replicates.

The accuracy and precision of measuring TNF in diluted samples was determined by measuring the concentration of a highly concentrated TNF sample, diluted as follows: blank plasma was spiked with a TNF stock standard to a concentration of 3000 ng/ml. Aliquots of this solution were diluted 1:10 (300 ng/ml) and 1:5 (600 ng/ml) with blank plasma and analysed in triplicate with the eight calibrators and two sets of QCs.

The stability of TNF in the plasma matrix was also investigated using the low (30 ng/ml) and medium (300 ng/ml) QCs in triplicate. The medium QC was chosen because this level closely corresponded to the maximum concentrations (C_{max}) expected in an actual patient's plasma during routine pharmacokinetic monitoring. Test conditions included three freeze–thaw cycles and room temperature stability (4 and 72 h). Stability was checked by extracting the appropriate QC which had been maintained at ambient temperature for the specified time and analyzing the extracts for TNF concentration. A comparison was made with a control at the same level which had been stored at −20 °C and analysed in the same analytical run. Additionally, extracted sample stability in the autosampler (4 °C) was tested by comparing the initial results from QC extracts with that determined after 8 days of autosampler storage. Deterioration of TNF was defined as greater than a 10% difference of tested sample versus control at the same nominal concentration.

Assay specificity was tested by analyzing plasma spiked with several antiviral drugs likely to be present in the plasma of HIV-positive patients and checking for false TNF positives. Blank plasma aliquots were spiked using mid-range standards (1000 ng/ml) having the following composition: solution A: IDV, DLV, APV, NFV, M8, SQV, EFV, RTV and LPV. Solution B: ATV. Solution C: 3TC, d4T, AZT, ABC and DDI. Solution D: NVP. (Definitions of these drug abbreviations can be found in Section abbreviations.) Furthermore, the possibility of these drugs adversely affecting the TNF assay was checked by similarly spiking a medium TNF QC and analysing for TNF.

Matrix effects were assessed by using a method similar to that reported by Matuszewski et al. [9]. Five different EDTA plasma lots from individuals whose plasmas were free of anti-viral drugs were checked for possible co-eluting compounds which could potentially interfere with the TNF assay. Each lot was spiked with TNF working standards at the 50, 100 and 500 ng/ml levels (IS was added to each) and analyzed. The potential for differing matrix effects was assessed by comparing the resulting regression line slopes and the peak area ratio variation for each level from the five different plasma lots (see below).

The assay performance was also assessed by participation in the ACTG proficiency testing program. Samples at six different concentration levels (blinded) and two blank samples were analyzed by the method, submitted to the testing agency and results evaluated.

2.8. Clinical application

Plasma samples from HIV-infected patients who were receiving a dose of 300 mg tenofovir disoproxil fumarate once daily were analyzed for TNF using the method described herein. The time elapsed from the morning dose to the blood sample collection varied from 3 to 6 h for these random visits. Pharmacokinetic studies were also performed on eight HIV-infected patients who underwent frequent blood sampling after tenofovir disoproxil fumarate administration.

3. Results and discussion

The chemical structures of TNF and adefovir are shown in Fig. 1. As can be seen, adefovir is a demethylated analog of TNF and therefore a logical choice as an internal standard. Although an isotopic analog of TNF (containing, for example, ^{13}C instead

of ^{12}C) would be an ideal internal standard for this assay, it was not available at the time of validation and furthermore would have to be custom synthesized and thus would not be readily available in most laboratories. Therefore, adefovir is a reasonable choice even though it could theoretically be co-administered with TNF. Although adefovir is used to treat Hepatitis B virus infection, it is unlikely to be co-administered with TNF to a patient with both HBV and HIV infection since TNF is also effective against the former infection [10]. Additionally, adefovir was used as an IS for TNF assays by Hazra et al. [8], Sparidans et al. [6] and Cundy et al. [11].

Fig. 2(A) depicts a typical chromatogram showing the peak corresponding to the TNF present in the low QC (TNF = 30 ng/ml) and Fig. 2(B) depicts the peak corresponding to the IS. The respective precursors (*m*/*z*; 288 and 274) and products (*m*/*z*; 176 and 162) in the positive ion mode were monitored. A clear chromatographic separation of TNF from the IS can be seen from a comparison of Fig. 2(A and B). Fig. 2(C) depicts the chromatogram obtained when a patient's plasma extract was analyzed (TNF = 130 ng/ml). Fig. 2(D) depicts the chromatogram obtained when a blank plasma was analyzed; the lack of a peak corresponding to TNF is noted. The IS chromatogram depicted in Fig. 2(B) is typical for all the chromatograms generated.

3.1. Calibration curve performance

The calibration curves were created by plotting the peak area ratios of TNF relative to the IS against the various TNF concentrations in the spiked plasma standards (analytical range: 10–750 ng/ml). A 1/*X* weighted linear regression of the type $y = bx + a$ was used. Five consecutive calibration analyses were performed on different days with freshly aliquoted standards and the back-calculated values for each level recorded (Table 1). The % CV at each level varied from 1.01 to 8.19 and the % deviation from the theoretical value varied from −3.79 to 6.9. The % CV of the five slopes was 11.1 and the lowest coefficient of determination (r^2) among the five calibration curves was 0.9971 (mean = 0.9985). Thus, the calibration curves did not exhibit any non-linearity within the chosen range. Since the back calculated results showed good day-to-day accuracy and precision, it was concluded that the TNF standard curve produced by this method could be used to reliably determine plasma drug concentrations in a consistent fashion.

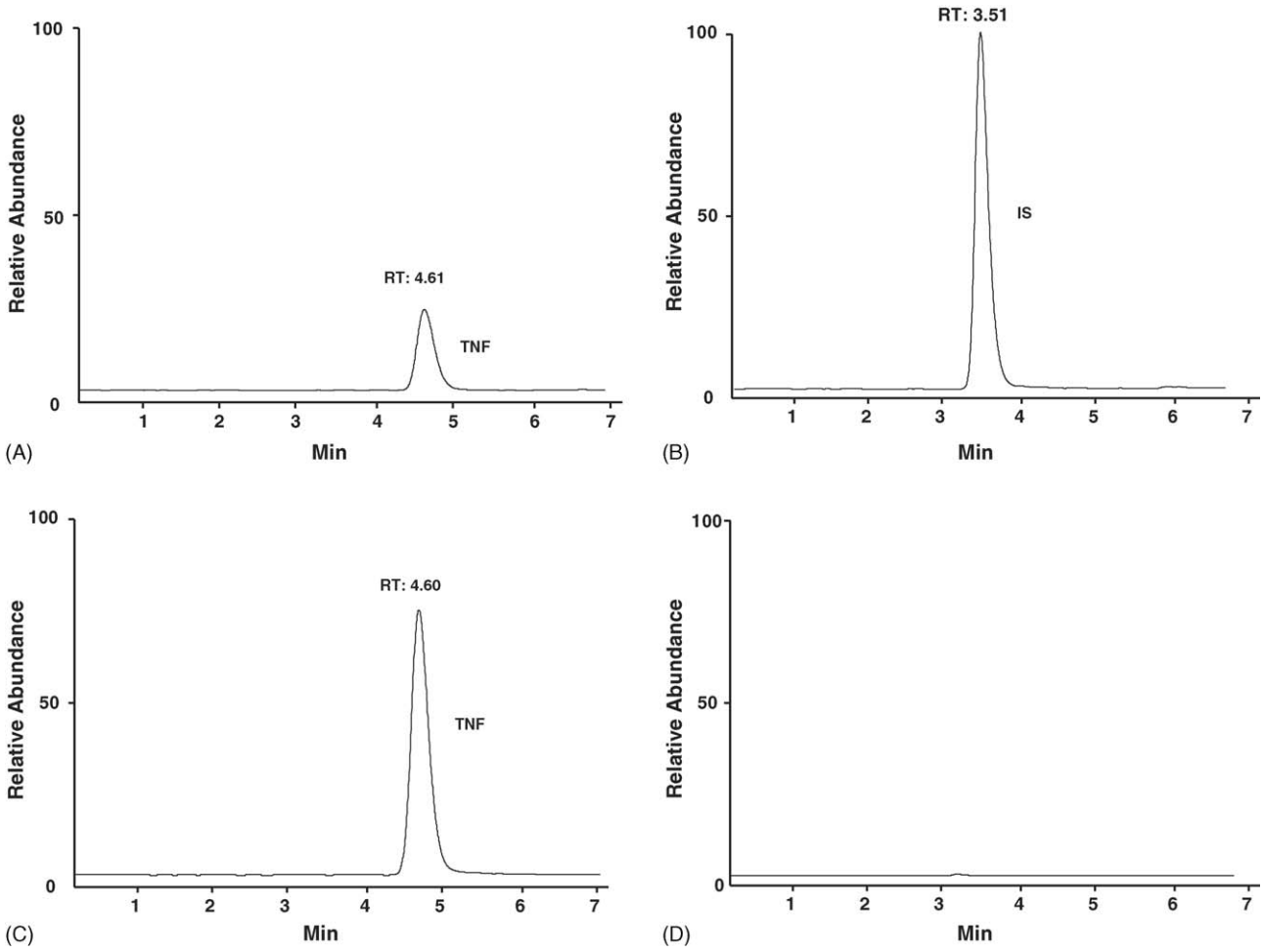


Fig. 2. (A) LC/MS/MS chromatogram of TNF low QC (30 ng/ml). (B) LC/MS/MS chromatogram of IS (200 ng/ml). (C) LC/MS/MS chromatogram of patient's plasma (130 ng/ml). (D) LC/MS/MS chromatogram of blank plasma.

Table 1
Mean inter-day back-calculated standard and standard curve results

	Mean inter-day back calculated standard results								Standard curve results		
	Standard								Slope	*Y*-intercept	r^2
	STD 1 10 ng/ml[a]	STD 2 25 ng/ml[a]	STD 3 50 ng/ml[a]	STD 4 75 ng/ml[a]	STD 5 100 ng/ml[a]	STD 6 250 ng/ml[a]	STD 7 500 ng/ml[a]	STD 8 750 ng/ml[a]			
Run ID											
I	11.4	25.7	48.8	64.9	101	237	416	771	0.00869	−0.01168	0.9971
II	10.1	24.8	48.9	73.8	103	254	498	748	0.00696	0.00104	0.9999
III	10.5	22.7	48.0	73.9	109	256	492	748	0.00810	−0.00036	0.9990
IV	11.0	24.0	49.3	76.8	92.5	251	496	760	0.00788	−0.01286	0.9993
V	10.4	23.0	49.2	75.7	106	241	520	735	0.00661	−0.01410	0.9986
Mean	10.7	24.1	48.9	73.0	102	248	484	752	0.00765	−0.00759	0.9988
S.D.	0.53	1.25	0.49	4.69	6.35	8.25	39.7	13.8	0.00085	0.00731	0.0011
%CV	4.95	5.2	1.01	6.43	6.21	3.33	8.19	1.83	11.1	NA	0.11
%Dev	6.9	−3.79	−2.28	−2.66	2.27	−0.9	−3.15	0.31			
n	5	5	5	5	5	5	5	5	5	5	5

Note: Calibration curves were weighted 1/conc. NA, not applicable; conc., concentration; STD, standard; TNF, tenofovir.
[a] TNF conc.

3.2. Accuracy and precision

The inter- and intra-day accuracy and precision results are summarized in Table 2. As can be seen, the assay was both accurate and precise between runs and within individual runs for each level. The greatest mean inter-day percent deviation for TNF was 11.6% for the LLOQ (10 ng/ml). All non-LLOQ QC levels had inter-day percent deviations less than 7%. The within-run precision for the non-LLOQ QCs was less than 6% on each of the 5 days of the study and the LLOQ accuracy and precision results were within ±20%. When the accuracy of measuring TNF in diluted samples was assessed as described above, the mean% deviation from the theoretical values for the 1:10 and 1:5 diluted samples were −7 and −3%, respectively, suggesting that should a sample have a concentration exceeding the upper limit of the calibration curve, the remaining plasma sample could be diluted up to 10-fold and reanalyzed to fit within the established parameters.

3.3. Stability in plasma

The stability of TNF in the plasma matrix was also investigated as described above. Any deterioration of TNF during freeze–thaw cycles or extended time on the counter-top was monitored. Three freeze–thaw cycles (where the samples were completely thawed and either re-frozen or prepared for analysis) and one 4 h stability check at ambient temperature were performed. There was no deterioration in TNF at either QC level (30 or 300 ng/ml) for the various freeze–thaw cycles, suggesting that drug concentrations can be confidently determined in samples that had been previously thawed up to three times prior to the analysis or that have been thawed and kept at ambient temperature for up to 4 h. The 4 h stability test at ambient temperature was performed since the plasma sample could conceivably stand on the bench for up to 4 h after thawing or before freezing.

Additionally, a high QC (600 ng/ml) was kept at ambient temperature for 72 h before analysis; no deterioration in the TNF concentration was noted, suggesting that TNF is stable for several days at room temperature in plasma. Even though samples are routinely shipped to analytical laboratories on dry ice, transportation delays could theoretically occur which might result in the samples being inadvertently brought to ambient temperature for an extended period.

3.4. Stability in extract

The stability of TNF in the extract was also tested after 8 days storage at 4 °C to allow for sample re-injection should a chromatographic or instrument malfunction occur during the initial analysis. The % difference between the two sets of results was 10% or less, except for the LLOQ, which was 15% (an acceptable variation for the LLOQ), suggesting that there was no significant decline in the response for TNF plasma-based QCs during the 8 days of extract storage at 4 °C. The extract stability at ambient temperature was not assessed.

3.5. Assay specificity

Several antiviral drugs likely to be present in the plasma of HIV-positive patients were utilized to assess the specificity of the method. When solutions A–C or D were included in a typical TNF analytical run as described above, no TNF was detected in any of the spiked samples, indicating that none of the drugs listed are likely to give a false positive TNF result. The unlikely possibility that the above antiviral drugs might negatively affect the TNF assay was checked by spiking in triplicate the TNF medium QC (300 ng/ml) with 1000 ng/ml of each antiviral drug and then analyzing for TNF. The MS/MS response was not significantly different from that obtained with the unspiked QC (% difference from theoretical = 1.5, $p > 0.2$, d.f. = 4), confirming that the

Table 2
Inter- and intra-assay accuracy and precision of tenofovir in plasma

	LLOQ 10 ng/ml[a]	Low 30 ng/ml[a]	Middle 300 ng/ml[a]	High 600 ng/ml[a]
Inter-assay accuracy and precision				
Mean	10.0	29.8	286	588
S.D.	1.16	1.72	9.58	40.9
%CV	11.6	5.78	3.35	6.96
%Dev	0.03	−0.63	−4.62	−1.94
n	25	25	25	25
Intra-assay accuracy and precision				
Run ID 1				
Mean	9.86	29.5	286	581
S.D.	1.64	1.41	5.62	16.8
%CV	16.6	4.77	1.97	2.9
%Dev	−1.36	−1.74	−4.69	−3.24
n	5	5	5	5
Run ID II				
Mean	9.54	31.1	285	651
S.D.	1.32	1.12	11.6	15.2
%CV	13.8	3.59	4.08	2.34
%Dev	−4.57	3.53	−4.92	8.47
n	5	5	5	5
Run ID III				
Mean	9.41	28.1	279	546
S.D.	0.41	1.31	4.25	15.6
%CV	4.41	4.65	1.52	2.86
%Dev	−5.94	−6.2	−7.06	−8.97
n	5	5	5	5
Run ID IV				
Mean	10.4	28.9	287	560
S.D.	0.77	1.63	11.6	13
%CV	7.41	5.63	4.03	2.33
%Dev	4.4	−3.52	−4.18	−6.71
n	5	5	5	5
Run ID V				
Mean	10.8	31.4	293	604
S.D.	1.09	0.62	9.9	24.7
%CV	10.1	1.98	3.37	4.08
%Dev	7.64	4.79	−2.23	0.74
n	5	5	5	5

[a] TNF conc.

presence of other antiviral drugs would not negatively affect the TNF assay.

3.6. Matrix effects

The approach used to assess possible matrix differences among different plasmas was a modification of that described by Matuszewski et al. [9]. When analyzed as described above, the five plasma lots were each free of any TNF response, demonstrating the selectivity of the TNF/IS precursor/product transition. In addition, these five lots were spiked with TNF concentrations of 50, 100, and 500 ng/ml (IS was also added to each) as described above and analyzed. The possibility of matrix differences between the various lots was assessed by comparing the TNF/IS area ratios corresponding to the different lots at each concentration level. The %CV between the area ratios for the different TNF concentrations was acceptable (5.63, 5.51 and 8.41, respectively) and the regression line slopes for the five plasma matrices had a CV of 9.64%. Furthermore, the slopes of the three-point calibration curves were not significantly different for the different plasma lots (0.007–0.0086) and was comparable to that of the calibration curves found in Table 1. This study suggests that EDTA plasma obtained from different individuals will not adversely affect the TNF assay.

EDTA-plasma, heparin-plasma, ACD-plasma and serum lots ($n = 2$ each) were spiked with TNF as described above and compared for analytical performance. No change in the ratio was noted when EDTA-plasma, heparin-plasma and serum was used, suggesting that these matrices were acceptable. However, the ACD-based plasma was unacceptable due to a selective ion enhancement of the IS which was not apparent with TNF (with ACD the ratio was decreased by 22.1% in comparison to that obtained with EDTA plasma). Thus, this method is selective and specific for TNF analysis using EDTA and heparin-plasma and serum. However, ACD plasma is not an acceptable plasma matrix and is a limitation of this method.

Participation in the ACTG proficiency testing program was also used to evaluate the method. The method passed proficiency for all levels of TNF tested. This included the necessary dilution of two levels tested, since the original result was greater than the upper limit of quantitation for the method (750 ng/ml).

3.7. Clinical application

Plasma samples from eight HIV-infected patients receiving a dose of 300 mg tenofovir disoproxil fumarate were analyzed as described above. The TNF concentrations ranged from 80.9 to 511 ng/ml ($n = 22$) and the mean concentration was 240.7 ng/ml. Pharmacokinetic studies were also performed on eight HIV-infected patients by sampling blood at frequent times after tenofovir disoproxil fumarate administration. As can be seen in a typical TNF pharmacokinetic curve (Fig. 3), a time to maximum concentrations (T_{max}) of approximately 1 h was obtained. Trough TNF plasma concentrations (24 h following the previous dose) from patients receiving 300 mg/day, were 88.4 ± 18.5 ng/ml (mean ± S.D.). In addition, none of the trough or C_{min} concentrations were below the LLOQ (10 ng/ml).

Although Hazra et al. did not illustrate the chromatograms generated in their pharmacokinetic study [8], the brief description of the method which they published would suggest similarities to the TNF method reported herein. However, the Hazra et al. method involved the use of solid phase extraction cartridges to prepare the samples for analysis resulting in a more

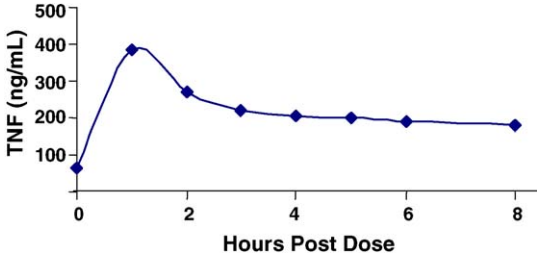


Fig. 3. A typical TNF pharmacokinetic profile.

time-consuming assay than the simple trifluoroacetic acid precipitation technique used in our method. In view of the Sentenac et al. extraction procedure which used C-18 cartridges to prepare samples for TNF analysis [7], we tried C-18 cartridges (albeit from a different manufacturer) but encountered considerable variation in results and a time consuming, laborious extraction. Furthermore, the use of EDTA-plasma may be preferable to serum (used in the Hazra et al. assay) since plasma samples are more efficiently processed than serum samples and TNF losses could conceivably occur during the coagulation step at ambient temperature. However, this potential loss of TNF has not been investigated in the current study.

In conclusion, we have developed an LC/MS/MS assay for TNF quantitation that is rapid, specific, sensitive and rugged. Potentially interfering plasma proteins are removed by a simple acid precipitation/centrifugation technique before the extracts are directly injected into the isocratic mobile phase stream without prior derivatization (a necessary cumbersome step in the fluorometric assay [6]). Since the unique precursor and product ions are monitored in the MS detector, a high degree of specificity is achieved. This method permits laboratory scientists with access to the appropriate instrumentation to rapidly perform TNF therapeutic drug monitoring or pharmacokinetic studies.

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References

[1] B.P. Kearney, J.F. Flaherty, J. Shah, Clin. Pharmacokinet. 43 (2004) 595.
[2] 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.
[3] Z. Suo, K.A. Johnson, J. Biol. Chem. 273 (1998) 27280.
[4] B.P. Kearney, A. Mittan, J. Sayre, J.F. Flaherty, L. Zhong, J.J. Toole, A.K. Cheng, in: Program and Abstracts of the 43rd Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, Illinois, Abstract A-1617, 14–17 September 2003.
[5] S. Agarwala, T. Eley, C. Villegas, Y. Wang, E. Hughes, D. Grasela, in: Program and Abstracts of the 6th International Workshop on the Clinical Pharmacology of HIV Therapy, Que., Canada, Abstract 16, 28–30 April 2005.
[6] R.W. Sparidens, K.M. Crommentuyn, J.H. Schellens, J.H. Beijnen, J. Chromatogr. B 791 (2003) 227.
[7] S. Sentenac, C. Fernandez, A. Thuillier, P. Lechat, G. Aymard, J. Chromatogr. B 793 (2003) 317.
[8] R. Hazra, F.M. Balis, A.N. Tullio, E. DeCarlo, C.J. Worrell, S.M. Steinberg, J.F. Flaherty, K. Yale, M. Poblenz, B.P. Kearney, L. Zhong, D.F. Coakley, S. Blanche, J.L. Bresson, J.A. Zuckerman, S.L. Zeichner, Antimicrob. Agents Chemother. 48 (2004) 124.
[9] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019.
[10] H.B. Fung, E.A. Stone, F.J. Piacenti, Clin. Therapeutics 24 (2002) 1515.
[11] K.C. Cundy, C. Sueoka, G.R. Lynch, L. Griffen, W.A. Lee, J.P. Shaw, Antimicrob. Agents Chemother. 42 (1998) 687.