A New Assay Based on Solid-Phase Extraction Procedure with LC–MS to Measure Plasmatic Concentrations of Tenofovir and Emtricitabine in HIV Infected Patients

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Abstract

A new solid-phase extraction (SPE) method has been developed and validated on a liquid chromatography (LC) coupled with a mass spectrometer for the determination of plasma concentrations of tenofovir (TNF) and emtricitabine (FTC) in HIV infected patients. Chromatographic separation was achieved with a gradient (acetonitrile and water with formic acid 0.05%) on an Atlantis 4.6 mm × 150 mm, reversed phase analytical column. Detection of TNF, FTC, and internal standard (IS) was achieved by electrospray ionization mass spectrometry (ESI-MS) in the positive ion mode. Calibration ranged from 15.6 to 4000 ng/mL for TNF and 11.7 to 3000 ng/mL for FTC. Plasma was analyzed, and the limit of quantitation was 15.6 ng/mL for TNF and 11.7 ng/mL for FTC; limit of detection was 2 ng/mL for TNF and 1.5 ng/mL for FTC. Mean recovery of TNF, FTC, and IS were 46.5% [relative standard deviation (RSD): 8.8%] and 88.8% (RSD: 1.0%), and 81.7% (RSD: 3.1%), respectively. The method did not show any significant interference with antiretrovirals or other concomitant drugs administered to patients, and no significant "matrix effects" were observed. The method was applied for the determination of antiretroviral plasma concentration of HIV-positive patients treated with FTC and/or TNF, in combination with various other antiretrovirals.

Introduction

Highly Active Antiretroviral Therapy (HAART) has improved the treatment of HIV infection, dramatically reducing HIV-related morbidity, and mortality (1,2). HAART is based on the administration of at least 3 drugs of different classes: generally 2 compounds belonging to nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs/N(t)RTIs) plus 1 or 2 drugs among non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), or fusion inhibitors (FIs).

NRTIs/N(t)RTIs mimic natural nucleosides and interact with the HIV reverse transcriptase (HIV-RT), causing the termination of newly forming viral DNA. Eight different NRTIs/N(t)RTIs have been approved by the US Food and Drug Administration (FDA) for the treatment of HIV infection (3,4), of which emtricitabine (FTC) is the most recently (2003) licensed. FTC is active against HIV types I and II and hepatitis B virus.

Similarly to the others NRTIs, FTC is phosphorylated by intracellular kinase and is converted to FTC-TP, which is the active form of the drug.

Tenofovir (TNF) one of the most widely used antiretrovirals, is the only N(t)RTI currently available. TNF is intracellularly metabolized to active form, TNF diphosphate, and pro-drug tenofovir-disoproxil-fumarate (TDF) is used to improve oral absorption.

TDF and FTC are the active principles of Viread and Emtriva (Gilead Sciences Intl. Ltd.; Cambridge, UK), respectively, but they are also co-formulated in one fixed dose tablet Truvada (Gilead Sciences Intl. Ltd.; Cambridge, UK) (5). Moreover, FDA has recently approved Atripla, a fixed dose combination of an NNRTI (efavirenz), FTC, and TDF, to be taken as one a once-a-day tablet.

Therapeutic drug monitoring has become an essential tool for the management of HIV-positive patients. Measurement of antiretroviral plasma concentrations can be useful in several clinical setting, such as management of side effects, optimization of efficacy, metabolic impairment, and drug–drug interaction (6,7).

TNF is an inhibitor of cytochrome P450 (8), which suggests a potential for clinically important drug–drug interactions with compounds that are substrate or inducers/inhibitors of this pathway. Moreover, TNF has been shown to significantly affect pharmacokinetic of didanosine and atazanavir (9,10), and its plasma concentrations have been described to be increased by concomitant administration of lopinavir/ritonavir (11–13). Furthermore, as TNF has a renal elimination mainly by tubular secretion, dose-interval adjustments TDM-guided are needed in subjects with renal impairment, considering also that has per se a potential nephrotoxicity probably concentration-related (14–18). Therefore, evaluation of the TNF plasma levels shows to have a clinical usefulness in different settings (6,19,20).

FTC is not a substrate or an inhibitor of cytochrome P450, and it is mainly eliminated by renal tubular secretion; to date FTC has shown a lack of significant interactions with other NRTI (21,22) and TNF (23). Clinical usefulness of TDM of FTC has not been yet fully evaluated; however, measurement of FTC plasma levels could be a tool for management of special populations (children, patients with renal impairment), and for pharmacokinetic studies.

Therefore, due to the increased use of mentioned co-formulations, simultaneous measurement of TNF and FTC drugs levels could be a useful tool for pharmacokinetic evaluation.

Other previous high-performance liquid chromatographic methods have been developed for the quantitation of TNF or FTC and TNF. Jullien et al. (24) and Sparidans et al. (25) determined TNF using a liquid–liquid extraction, a derivatization step, and the spectrofluorimetric detection. While Sentenac et al. (26) applied an solid-phase extraction method and the UV detection, El Barkil et al. (27) used an SPE method for TNF coupled with UV and MS; afterwards Rezk et al. (28) developed an super method with UV detection for the quantitation of both TNF and FTC, and Delahunty et al. (29) quantitated TNF using a electrospray ionization tandem mass spectrometry method after a protein precipitation step.

The aim of our study was to develop and validate a novel method for the simultaneous quantitation of FTC and TNF in LC coupled with ESI-MS.

Experimental

Materials and Chemicals

TNF and FTC were kindly donated by Gilead Sciences Intl. Ltd. (Cambridge, UK), trifluoroacetic acid (TFA), acetonitrile (HPLC grade), and methanol (HPLC grade) were purchased from J.T. Baker (Deventer, Holland). HPLC–MS-grade water was produced with Milli-DI system coupled with a Synergy 185 system by Millipore (Milan, Italy). Thymidine and formic acid were obtained from Sigma-Aldrich (Milan, Italy). Blank plasma from healthy donors was kindly supplied by the Blood Bank of Maria Vittoria Hospital (Turin, Italy). SPE cartridges C-18 (100 mg, 40-63 μm particle size) were obtained from VWR (Milan, Italy).

Stock solutions, standards, and quality controls

TNF and FTC stock solutions were prepared to a final concentration of 1 mg/mL in HPLC-grade water, then were refrigerated at 4°C until use, and discarded after 1 month.

Working solutions of internal standard (IS) were made with thymidine (50 μg/mL) in methanol and HPLC-grade water (50:50 v/v) and stored at 4°C until use and never more than a week.

TNF and FTC stock solutions were used to prepare the highest calibration standard (STD 9) and quality controls (QCs). Highest concentration point of the calibration curve (STD 9), containing 4000 ng/mL of TNF and 3000 ng/mL of FTC, was prepared by addition of 40 μL of the TNF stock solution and 30 μl of the FTC stock solution in 10 mL of drug-free plasma. Calibration curves included a wide range of TNF and FTC concentrations, and each range was based on the values reported in the clinical reports (30–32).

QCs were prepared in the same way, with final concentrations of 40 ng/mL (QC 1), 400 ng/mL (QC 2), and 1600 ng/mL (QC 3) for TNF and 37.5 ng/mL (QC 1), 375 ng/mL (QC 2), and 1500 ng/mL (QC 3) for FTC.

STDs were prepared by serial dilution from STD 9 to STD 1 (final concentrations for TNF and for FTC were 15.6 ng/mL and 11.7 ng/mL, respectively) with drug-free plasma, to obtain 9 different spiked concentrations plus a blank sample (STD 0).

STDs, QCs, and patient samples underwent heat inactivation procedure for HIV (35 min at 58°C), and then stored at –20°C until analyzes, avoiding more than three freeze-thaw cycles.

In this condition, TNF and FTC have been demonstrated to be stable (24,25,27,28), therefore no further stability evaluation was carried out.

Chromatographic and MS conditions

The LC–MS system used to assay TNF and FTC was a Waters system (Milan, Italy), with binary pump model 1525, AF degaser, 717-plus autosampler, and Micromass ZQ mass detector. LC–MS Empower Pro software (version year 2002, Waters; Milan, Italy) was used.

Chromatographic separation was performed at 35°C using a column oven, on an Atlantis dC-18 3 μ column (150 × 4.6 mm i.d.) (Waters, Milan, Italy), protected by a SecurityGuard with C18 (4.0 × 3.0 mm i.d.) pre-column (Phenomenex, CA).

Run was performed with a gradient (Table I), and the mobile phase was composed of Eluent A (HPLC grade water + 0.05% formic acid) and Eluent B (HPLC grade acetonitrile + 0.05% formic acid).

With a "T" switch tube, only 200 μL/min of total flow (1000 μL/min) was introduced into the MS detector. The detector settings were: ESI, positive polarity ionization; capillary voltage, 3500 V; source temperature, 110°C; desolvation temperature, 350°C; nitrogen desolvation flow, 600 l/h; nitrogen cone flow 60 L/h.

Ions detected were m/z 288.20 with a cone voltage of 20 V for TNF, m/z 248.20 with a cone voltage of 15 V for FTC, and m/z 243.15 with a cone voltage of 15 V for thymidine (IS).

STD, QC, and sample preparation

Plasma samples from patients administered with regimens including TNF and FTC were analyzed. Patients who were receiving standard dosing of Truvada, Viread, or Emtriva as a component of their antiretroviral association, and who needed therapeutic drug monitoring (TDM) underwent blood sampling. The patients gave their informed consent for the measurement of plasma TNF and FTC concentrations. Blood samples were collected in lithium heparin tube (7 mL), plasma was obtained after centrifugation at 1400 g (3000 rpm) for 10 min at + 4°C (Jouan Centrifuge, Model BR4i, Saint-Herblain, France) and then underwent heat inactivation, as described earlier. To avoid

Table I. Chromatographic Condition (Gradient): Mobile Phase: Eluent A (HPLC Grade Water + 0.05% Formic Acid) and Eluent B (HPLC Grade Acetonitrile + 0.05% Formic Acid)

Time (min)	% Eluent A	% Eluent B	Flow (mL/min)
0.0	99	1	1.0
0.5	99	1	1.0
2.0	95	5	1.0
5.0	85	15	1.0
8.0	20	80	1.0
8.1	0	100	1.0
9.5	0	100	1.0
10.0	99	1	1.0
15.0	99	1	1.0

thawing cycles, each patient plasma sample was separated into two 300 μL aliquots.

For SPE extraction, 100 μL of plasma were diluted with 100 μL of a solution of HPLC-grade water with 0.6% of trifluoroacetic acid (TFA). Fifty μL of IS working solution were added to each tube, and the samples were vortexed for 10 s. SPE cartridges (C-18) were placed on a vacuum elution manifold WAT 200677 (Waters, Milford, MA) and activated with 1 mL of methanol, followed by 1 mL of HPLC-grade water with 0.6% of TFA before loading of the samples. Loading was carried out under gravity. Then the cartridges were washed with 250 μL of HPLC-grade water with 0.6% of TFA, and elution was carried out using of 500 μL of methanol and acetonitrile solution (90:10, v/v). Eluted solutions were collected into glass tubes, and treated by vortex-vacuum evaporation to dryness at 50°C. Each extract was reconstituted with 200 μL of water–acetonitrile (95:5) solution and 30 μL were injected into the column.

For validation purposes, all samples were extracted and analyzed in duplicate; all procedure steps were carried out at room temperature.

Specificity and selectivity

Interference from endogenous compounds was investigated by analysis of five different blank plasma samples. Potential interference by antiretroviral drugs concomitantly administered to the patients was also evaluated by spiking blank plasma samples. These included: NRTIs [zidovudine (AZT), didanosine (ddI), stavudine (d4T), lamivudine (3TC), abacavir (ABV)], NNRTIs [nevirapine (NVP), efavirenz (EFV)], FI [enfuvirtide (T-20)], and PI [saquinavir (SQV), nelfinavir (NFV), and its active metabolite (M-8), indinavir (IDV), amprenavir (APV), atazanavir (ATV), ritonavir (RTV), lopinavir (LPV), tipranavir (TPV)]. Likewise for antiretroviral drugs, other concomitant drugs were also investigated: acetylsalicylic acid, amoxicillin, atorvastatin, clavulanic acid, enalapril meleate, furosemide, insulin, interferon (PEG), levofloxacin, methadone, nimesulide, omeprazole, paracetamol, pravastatin, and ribavirin.

An "interfering drug" was considered as a compound that exhibited a retention time within to 0.3 min from TNF, FTC, and IS and with the potential capability to cause ion suppression.

We investigated the "matrix effect" using five different blank plasma and compared the peaks areas obtained from standard solutions of water and acetonitrile (95:5) containing TNF, FTC, and IS at three different concentration and the peaks areas obtained from a post extraction solution with the same amount of analytes as described by Taylor (33) and FDA guideline (34).

Accuracy, precision, calibration, and limit of quantitation

Intra-day and inter-day accuracy and precision were determined by assaying 10 spiked plasma samples at three different concentrations (QCs) for each drug. Accuracy was calculated as the percent deviation from the nominal concentration. Inter-day and intra-day precision were expressed as the standard deviation at each QC concentration.

Each calibration curve was obtained using 9 calibration points in duplicate, ranging, for TNF, from 15.6 to 4000 ng/mL and, for FTC, 11.7 to 3000 ng/mL.

Calibration curves were created by plotting the peak area ratios of TNF and FTC relative to the IS against the various TNF and FTC concentrations in the spiked plasma standards. A 1/Y weighted quadratic regression was used for both.

The limit of detection (LOD) in plasma was defined as the concentration that yields a signal-to-noise ratio of 3/1. Percent deviation from the nominal concentration (measure of accuracy) and relative standard deviation (measure of precision) of the concentration considered as the limit of quantitation (LOQ) had to be < 20%, and it was considered the lowest calibration standard, as requested by FDA (34).

Recovery

Recovery of TNF and FTC from plasma using the extraction procedures was assessed by comparing the peak area obtained from multiple analyzes of spiked samples (1600, 400, and 40 ng/mL for TNF and 1500, 375, and 37.5 ng/mL for FTC) with the peak area from standard solution of TNF and FTC in a solution of water–acetonitrile (95:5) at the same concentrations.

Results

Mean retention times (n = 10) for TNF, IS (Thimydine), and FTC were 7.00 (± 0.30), 8.20 (± 0.20), and 8.7 (± 0.20) min, respectively. Representative chromatogram of the blank human plasma is shown in Figure 1. Figure 2 shows chromatograms of a patient sample. Mean regression coefficient (r^2) of all calibration curves was 0.999. Choice of a 1/Y weighted quadratic regression was made to give more weight to low calibration points (curve region where the greatest number of C_{trough} concentrations was expected).

Specificity and selectivity

The method did not show any significant interference with antiretrovirals or other concomitant drugs taken at therapeutic dosage by patients. Blank plasma samples presented no interference peaks at the TNF, IS, and FTC retention times at specified ions detected (Figure 1). No significant (less than 15%) (33) "matrix effects" was observed.
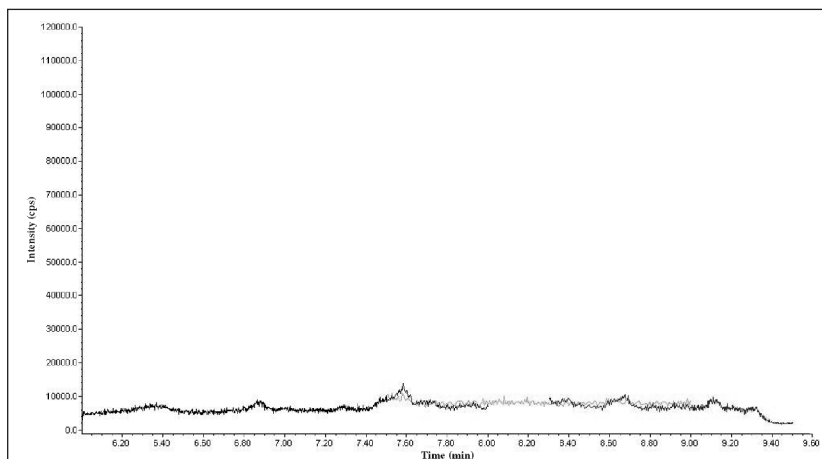

Figure 1. Chromatograms of a typical extracted blank human plasma.

Accuracy, precision, limit of quantitation

Results of the validation of the method are listed in Table II. All observed data [intraday and interday precision (RSD%)] were below 15.0, according to FDA guidelines (34).

The LOQ for TNF and FTC were 15.6 and 11.7, respectively (Figure 3), and the intraday and interday precisions (RSD%) are listed in Table II. The LOD for TNF and FTC were 2.0 and 1.5, respectively.

Table II. Intraday and Interday Precision for the Analysis of Tenofovir and Emtricitabine, QCs and LOQ (*n* = 10) [mean (RSD%) and Concentrations]

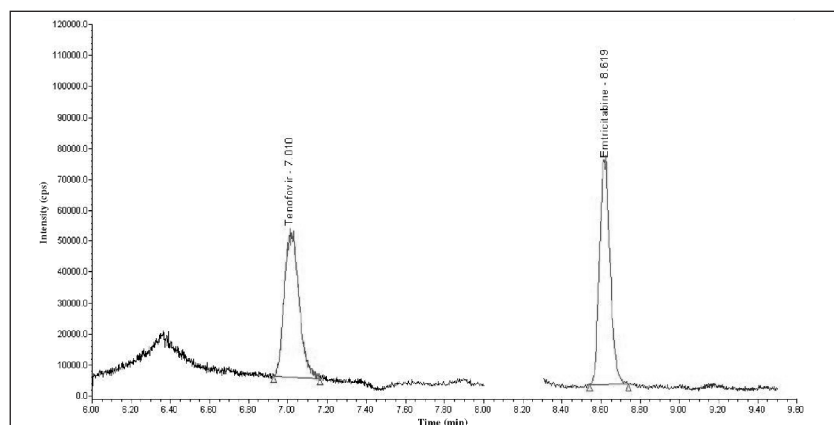| Theoretical concentration (ng/mL)	Observed concentration (ng/mL)	Accuracy (%)	Intraday precision (RSD%)	Interday Precision (RDS%)
Tenofovir				
1600	1558.6	–2.59	2.58	4.60
400	388.6	–2.84	2.37	8.71
40	35.8	–10.50	3.35	8.33
15.6	14.5	–7.05	5.16	11.01
Emtricitabine				
1500	1387.8	–7.48	3.12	3.03
375	349.0	–6.94	4.20	8.26
37.5	36.0	–4.04	3.57	3.96
11.7	10.8	–7.69	5.65	9.87

Figure 3. Chromatogram of LOQ (15.6 ng/mL of Tenofovir; 11.7 ng/mL of Emtricitabine).
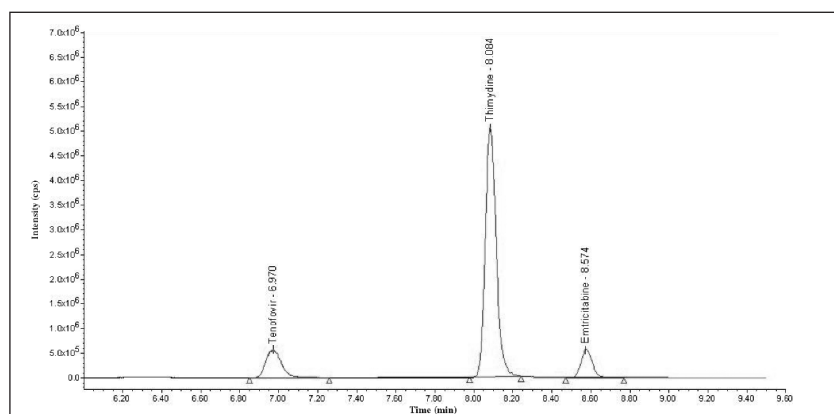

Figure 2. Chromatograms of the same patient sample, treated with Truvada (275 ng/mL of Tenofovir; 252 ng/mL of Emtricitabine).

Recovery

Multiple aliquots (*n* = 5) at each different concentration were assayed. Mean recovery of TNF and FTC were 46.5% (RSD: 8.8%) and 88.8% (RSD: 1.0%), respectively. Mean recovery of IS was 81.7% (RSD: 3.1%).

Analysis of samples from treated patients

Our method was used to assay 16 samples obtained from 13 patients treated with FTC; the lowest C_{trough} observed was 76.5 ng/mL (no C_{max} samples were obtained). On the other hand, 645 samples from 147 TNF treated patients were analyzed, and the lowest observed C_{trough} was 16 ng/mL, while the highest C_{max} was 3274 ng/mL.

Discussion

Reliability of our methods has been demonstrated for TNF concentrations between 15.6 and 4000 ng/mL, an optimal range for the quantitation of this drug, considering that the lowest C-trough and the highest C_{max} values measured were 16 ng/mL and 3274 ng/mL, respectively. In the same way, the calibration range for FTC (from and 11.7 to 3000 ng/mL) fully covered the expected range in patients, allowing correct drug quantitation between previously reported C_{max} (32) and measured C_{trough} (76.5 ng/mL).

The use of thymidine as IS is an essential tool of the procedure, allowing us to improve the reproducibility of the analysis.

The SPE extraction produced a clean extract, and in particularly the wash step with 250 µL of water with 0.6% of TFA. This removed salts from the extract and to prevent thymidine-sodium adduct.

TNF, IS, and FTC retention times were 7.00 (± 0.30), 8.20 (± 0.20), and 8.7 (± 0.20) min, respectively.

Moreover, LOQ and LOD of our procedure and the amount of plasma requested by our extraction were significantly lower as compared to other methods for quantitation of TNF only or TNF and FTC simultaneously (24,25,28,29), with the exception of El Barkil's LOQ, which used 1000 µL of plasma (27).

In the SPE method reported by Rezk et al., recovery of TNF and FTC was nearly 100% (28). Our SPE method shows relatively poor recovery when compared to Rezk's method; however we chose these SPE columns for their suitability and the presence of many other applications involving these cartridges in our laboratory. The low SPE recoveries were compensated for by low RSD for all analytes, and by the high sensitivity and specificity of the chromatographic instrumentation. Moreover, our chromatographic runtimes are faster (5 min/run) than any previously published method.

Finally, relative error at QC and LOQ concentrations, intra-day and inter-day precision (Table II) supported the accuracy and precision of our procedure.

Conclusion

A new LC–MS method for the quantitation of TNF and FTC plasma level, based on an SPE extraction, has been validated.

The method required only 100 µL of plasma, exhibited a good reproducibility and precision, and was shown to be reliable and rugged. The recovery results were adequate and reproducible. Moreover, the absence of interference peaks at the retention time of TNF, FTC, and IS allowed accurate measurement of drugs plasma levels, also in patients administered with several concomitant drugs as listed in specificity and selectivity paragraph.

The use of thymidine as IS and our mass settings are optimized for a reliable identification and quantitation of TNF and FTC.

In conclusion, this SPE method coupled with LC–MS was shown to be suitable for clinical application (TDM), and for PK studies of TNF and FTC.

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