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Journal of Chromatography B, 865 (2008) 81–90
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Quantification of seven nucleoside/nucleotide reverse transcriptase inhibitors in human plasma by high-performance liquid chromatography with tandem mass-spectrometry

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Received 15 November 2007; accepted 17 February 2008
Available online 5 March 2008

Abstract

A simple analytical method was developed in 100 μL of plasma for the simultaneous assay of the 7 nucleoside/nucleotide reverse transcriptase inhibitors (abacavir, didanosine, emtricitabine, lamivudine, stavudine, tenofovir, and zidovudine) currently used for the treatment of HIV-infected patients. After adding the internal standard, 6-beta-hydroxy-theophyline, plasma samples were precipitated with 500 μL acetonitrile and the supernatants were evaporated to dryness. The residues were reconstituted with 500 μL of water and 10 μL of the extracts were injected in the chromatographic system. The chromatographic separation was performed with a C-18 column and a gradient mobile phase consisting of a mixture of water and acetonitrile, both containing 0.05% formic acid. Analytes quantification was performed by electrospray ionisation triple quadrupole mass-spectrometry in the positive mode using selected reaction monitoring (SRM). Intra- and inter-assay precision and accuracy were lower than 20% for the limit of quantification, and 15% for higher concentrations. The method has been implemented to assess plasma concentrations of patients infected by HIV and was found suitable for therapeutic drug monitoring.

Keywords: Tandem mass-spectrometry; Antiretroviral drugs; HIV; Liquid chromatography

1. Introduction

Nucleoside/nucleotide reverse transcriptase inhibitors (N(t)RTIs) are the backbone of HIV therapy. They prevent viral replication by inhibiting the synthesis of viral DNA by the reverse transcriptase [1]. These compounds, which are nucleoside or nucleotide analogues, have to be phosphorylated at the intracellular level by endogenous kinases in order to obtain the active triphosphate moieties. These active anabolites then competitively inhibit the incorporation of endogenous nucleoside triphosphate by the viral reverse transcriptase enzyme, and therefore block the synthesis of new viral DNA strands [1].

Despite they are inactive prodrugs, there is a clinical interest in quantifying N(t)RTIs in patients' plasma. Indeed, verifying patients' compliance to treatment is a major concern, as a poor adherence is the main cause of virologic failure and is furthermore associated with the development of resistance mutations to the drugs, which can critically penalize the choice of subsequent treatment strategies [2–4]. Because they are inactive, therapeutic drug monitoring (TDM) of N(t)RTIs is not recom-

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doi:10.1016/j.jchromb.2008.02.008

mended to date. But, TDM could be useful in specific cases corresponding to a drug for which a relationship was evidenced between its plasma concentration and its efficacy or toxicity. For example, zidovudine-induced anaemia are related to high zidovudine plasma concentration [5] and, in children, a zidovudine cut-off concentration of 0.35 mg/L, above which the risk to develop chronic anaemia increased by 3, was identified [6]. Therefore, neonates, and more especially pre-term neonates, could be good candidates for zidovudine TDM since they are particularly exposed to high zidovudine concentrations because of the immaturity of its metabolic pathway [6–8], which can explain the high incidence of anaemia in this population [9,10]. Abacavir, a guanine analogue, could also be a good candidate for TDM as pharmacokinetic/pharmacodynamic relationships were evidenced between its plasma concentration and efficacy (decrease in viral load and increase in CD4 cells) [11]. Tenofovir, a nucleotide analogue, is known to induce a tubular dysfunction that can lead to renal impairment in a limited number of patients [12]. It was recently suggested that a trough plasma concentration greater than 0.2 mg/L was likely associated with such a dysfunction [13], which could also supports the use of TDM for this drug.

Measuring N(t)RTI concentrations in patient's plasma seems therefore useful, at least to check treatment compliance or, in some cases, to prevent adverse events or to ensure a satisfying exposure to the drug.

Seven N(t)RTIs are currently available in France for HIV treatment: 6 nucleoside analogues (abacavir, didanosine, emtricitabine, lamivudine, stavudine, and zidovudine), and 1 nucleotide analogue (tenofovir). As highly active antiretroviral therapy (HAART) always comprises at least 2 or 3 combined N(t)RTIs, it would be therefore useful to simultaneously quantify these 7 compounds in a single run. To date, many methods allowing the concomitant quantification of several N(t)RTIs in human plasma, using liquid chromatography with UV detection [14–20] or tandem mass-spectrometry [21–27] were described. One of these methods used protein precipitation as sample treatment [27], and another used liquid/liquid extraction [26]. All the others previously published methods used solid phase extractions [15–25]. This very high proportion of methods using solid phase extraction is not surprising as this extraction procedure is known to be highly selective, which is more particularly interesting for MS^2 detection since it is the optimal way to avoid matrix effect [28]. Simple protein precipitation is oppositely less selective but is a fast and less expensive procedure, which can be very useful in a clinical practice. Furthermore, despite its low selectivity, it is nonetheless possible to minimize matrix effect via chromatographic conditions allowing the separation from interfering endogenous compounds [29].

To date, no published method described the concomitant assay of all the 7 N(t)RTIs currently available in France. We therefore decided to develop such a method, with an extraction procedure as simple as possible, as it could be useful to routinely monitor the plasma concentrations of N(t)RTIs.

2. Experimental

2.1. Chemicals

Abacavir (ABC), lamivudine (3TC), and zidovudine (AZT) were gifts from GlaxoSmithKline (Greenford, UK). Tenofovir (TDF) and emtricitabine (FTC) were kindly supplied by Gilead (Foster City, USA). Didanosine (DDI) and stavudine (D4T) were given by Bristol-Myers Squibb. The internal standard (i.s.), 6-beta-hydroxy-theophyline, was obtained from Sigma (Saint Quentin Fallavier, France).

Formic acid and Chromanorm® methanol (MeOH) for HPLC were purchased from Prolabo (Fontenay-sous-bois, France). Ultrapure water was obtained from Milli-Q water purification system (Millipore, Molsheim, France).

2.2. Instrumentation

Chromatography was carried out with Accela System liquid chromatograph (Thermo Finnigan, Les Ulis, France) connected to a Thermo-Finnigan TSQ Quantum Discovery Max triple quadrupole mass-spectrometer through Heated-ElectroSpray Ionisation (H-ESI) interface. Instrument control and data collection were handled by computer equipped with Xcalibur software (Version 2.0, Thermo Finnigan).

2.3. Biologic samples

Blank plasmas were obtained from 30 healthy volunteers who gave their blood in our institution after giving written informed consent, and plasmas from HIV-infected patients were samples analyzed in our department for routine TDM.

2.4. Preparation of stock solutions, calibration standards and quality controls

Two 1000 μg/mL stock solutions were prepared for each N(t)RTI, one for the calibration standards and one for the preparation of quality controls (QC). The stock solutions were prepared in methanol for AZT, D4T, 3TC, DDI, and FTC, or in water for ABC and TDF. All these stock solutions were stored in polypropylene tubes at −20 °C and were stable at least 3 months in these storage conditions. Appropriate dilutions in water of the stock solutions were mixed together to produce an intermediate solution containing 40 μg/mL of AZT, D4T, ABC, 20 μg/mL of 3TC, DDI, FTC, and 10 μg/mL of TDF. Calibration standards were obtained by appropriate dilutions of the intermediate solution in water followed by addition of appropriate amounts of those dilutions to blank plasma. The final calibration standards values are displayed in Table 1. The limit of quantification of each compound was set at the value of lowest calibration standard. Four quality control (QC) levels per compound were obtained from appropriate dilutions of the other stock solutions in drug-free human plasma and used for assay validation. QCs were aliquoted by 250 μL in polypropylene tubes and stored at −20 °C until assay. QC values are displayed in Table 1 and the lowest QC was equal to the lowest calibration standard level.

Table 1
Concentration (μg/mL) for the calibration standard (CS) and the quality control (QC) for the seven N(t)RTIs

	D4T	3TC	DDI	FTC	AZT	ABC	TDF
CS1/QC1	0.0200	0.0100	0.0100	0.0100	0.0200	0.0200	0.0050
CS2	0.0500	0.0250	0.0250	0.0250	0.0500	0.0500	0.0125
CS3	0.1000	0.0500	0.0500	0.0500	0.1000	0.1000	0.0250
CS4	0.5000	0.2500	0.2500	0.2500	0.5000	0.5000	0.1250
CS5	1.0000	0.5000	0.5000	0.5000	1.0000	1.0000	0.2500
CS6	2.0000	1.0000	1.0000	1.0000	2.0000	2.0000	0.5000
CS7	4.0000	2.0000	2.0000	2.0000	4.0000	4.0000	1.0000
QC2	0.0300	0.0200	0.0200	0.0750	0.0750	0.0300	0.0300
QC3	0.4000	0.2000	0.2000	0.7500	0.7500	0.4000	0.2000
QC4	3.0000	1.5000	1.5000	1.5000	2.5000	3.0000	0.7500

D4T: stavudine, 3TC: lamivudine, DDI: didanosine, FTC: emtricitabine, AZT: zidovudine, ABC: abacavir, TDF: tenofovir.

A 1000 μg/mL stock solution of the internal standard in water was prepared and stored at 4 °C. An internal standard 2 μg/mL working solution in water was used for the assay.

2.5. Preparation of plasma samples and extraction procedure

Using 5 mL polypropylene tubes, 50 μL of calibration standards was added with 100 μL drug-free human plasma whereas plasma samples (QC and patients) were supplied with 50 μL of water. Fifty microliter of the working solution of the i.s. was then added to all samples. Protein precipitation was performed via the addition of 500 μL of acetonitrile followed by 30 s of vortex-mixing. After being left at ambient temperature for 15 additional minutes in order to optimize protein precipitation, tubes were centrifuged at 2200 × *g* for 10 min at ambient temperature and 600 μL of the supernatant was collected into new polypropylene tubes. The supernatant was next evaporated under a nitrogen stream at 35 °C and the dry residue was dissolved in 500 μL of water. The re-constituted samples were then centrifuged once again (2200 × *g*, 10 min), and 10 μL of each supernatant was injected in the chromatographic system.

2.6. Chromatographic conditions

The chromatographic column consisted of an Atlantis® T3 column (100 mm × 2.1 mm, 3 μm particle diameter, Waters, Saint Quentin en Yvelines, France). The autosampler temperature was set at 10 °C. The analytes were eluted from the column at 40 °C with a mobile phase composed of two different solvents A and B, where A denotes for water containing 0.05% formic acid and B for methanol containing 0.05% formic acid. A gradient was used to optimize the separation of the eight analytes. Initially, the column was equilibrated at 250 μL/min with a mobile phase consisting of 95% A and 5% B. Three minutes after injection, the proportion of B was linearly increased to 40% within 5 min. After this step, composition of the mobile phase was set to initial conditions (95% A and 5% B) and the column was equilibrated for 6 min prior to the next injection. Total analysis time including column equilibration was 14 min.

2.7. MS^2 conditions

After separation, analytes were introduced in the mass-spectrometer through a heated electrospray ionization source (50 °C) operating in the positive mode at constant voltage (5000 V). The temperature of the capillary transfer was set at 270 °C. Nitrogen was obtained by a NM 30 LA generator (Lab Gaz System, Massy, France) and was employed as nebulizing (35 psi) and auxiliary gas (30 arbitrary units). Argon (purchased from Messer, Puteaux, France) was used as collision gas (1.5 mTorr in Q2). The analytes were observed in the selected reaction monitoring (SRM) mode. All the potential settings were optimized by repetitive injections of the analytes in the chromatographic system. Corresponding parameters are given in Table 2.

3. Validation

3.1. Linearity

For all analytes, complete standard curves obtained from spiked blank plasmas (7 levels) were analyzed in triplicate on three separate days. Quality controls (QC) were assayed twice with each standard curve. A linear regression was used to plot the peak height ratio of analyte to i.s. against analyte concentration. For each analyte, the best weighting factor was determined according to the evolution of variance with respect to the concentration. Slope, *y*-intercept and R^2 coefficient were calculated for each standard curve. The mean value for each parameter was calculated. Mean back-calculated standard concentrations had to fulfill the following criteria: no standard outside ± 15% of the theoretical value, except ±20% for the LOQs. A single calibration curve and its corresponding QCs were accepted if 5 out of the 7 standards and 4 out of the 6 QCs satisfied the ±15% criteria (±20% for standards and QCs corresponding to the LOQ).

3.2. Accuracy and precision

Within- and between-assay precision and accuracy of the method were evaluated on the QCs, on the pool of one assay (QCs assayed 6 times in a single assay) and on the QCs assayed twice during 6 different days (i.e. 12 replicates

Table 2
Relevant parameters for the ionization of the analytes

Analyte	Precursor ion mass (*m/z*)	Product ion mass (*m/z*)	Collision energy (V)	Tube lens (V)
D4T	225.1	126.9	12	100
3TC	230.1	112.0	15	100
DDI	237.1	137.0	14	100
FTC	248.0	129.9	14	100
AZT	268.1	127.0	14	130
ABC	287.1	190.0	20	120
TDF	288.1	176.1	24	100
i.s.	225.1	181.3	18	110

D4T: stavudine, 3TC: lamivudine, DDI: didanosine, FTC: emtricitabine, AZT: zidovudine, ABC: abacavir, TDF: tenofovir, i.s.: internal standard.

per QC), respectively. Accuracy was determined as the difference between back calculated concentrations (C_{obs}) and theoretical concentration (C_{theo}) expressed in percent (i.e. $100\times(C_{obs} - C_{theo})/C_{theo}$). Within-assay precision for a given QC level was the coefficient of variation (i.e. 100× standard deviation/mean value) of the 6 back calculated concentrations in a single assay, whereas between-assay precision was obtained by analysis of variance on the calculated values obtained on the 6 different days [30]. The limit of quantification was set at the lowest calibration standard. According to FDA guidance (http://www.fda.gov/cder/guidance/4252fnl.htm), acceptance criteria were ±15% for both precision and accuracy, except for the LOQs for which acceptance criteria were ±20%.

3.3. Specificity

The specificity of the method was verified by injecting in the chromatographic system extracts from plasma of 50 patients treated with other drugs than N(t)RTIs.

3.4. Recovery

The recoveries of the N(t)RTIs were quantified at concentrations corresponding to calibration standards (first, fourth and seventh levels) analyzed in triplicate. The ratios of extracted samples containing both the N(t)RTIs and internal standard were compared to the ratios of samples containing the same amount of IS but no N(t)RTI which were added after the extraction step. The same method was applied for the study of the internal standard recovery with TDF used as reference. Possible differences between recoveries with respect to standards' values were investigated by analysis of variance.

3.5. Stability

Stability of QCs at −20 °C was evaluated monthly by comparing peak heights of QC stored at −20 °C and QC freshly prepared and analyzed in the same assay. An acceptance criterion was ±15% difference between peak heights. Stability of the stock solutions at −20 °C was evaluated every 2 months by comparing the peaks heights obtained from 3 independent 1/1000 dilutions of the stock solutions stored at −20 °C to 3 independent 1/1000 dilutions of stock solutions freshly prepared. Stability of the extracts at ambient temperature was investigated by injecting the same extracts immediately after the extraction procedure and 48 h after being left at ambient temperature. Conservation of stock solutions and extracts were considered satisfying if the peak heights were not different by more than 5%. Freeze–thaw stability was determined by assaying the QC samples (QC2, QC3, and QC4) in triplicate over three freeze–thawing cycles. Acceptance criterion was as follows: ±15% bias from theoretical value.

Table 3
Average parameters of the linear regression obtained from 9 calibration curves of the analytes

Compound	*N*	Slope (±S.D.)	*y*-Intercept (±S.D.)	R^2
D4T	9	0.15 (±0.012)	0.0001 (±0.0003)	0.998 (±0.003)
3TC	9	1.90 (±0.26)	0.006 (±0.002)	0.997 (±0.002)
DDI	9	1.04 (±0.12)	0.0001 (± 0.0004)	0.997 (±0.003)
FTC	9	1.58 (±0.30)	0.0005 (±0.0006)	0.995 (±0.001)
AZT	9	0.34 (±0.03)	0.0002 (±0.0003)	0.991 (±0.002)
ABC	9	6.99 (±0.86)	0.024 (±0.013)	0.986 (±0.006)
TDF	9	0.32 (±0.07)	0.0002 (±0.0004)	0.994 (±0.003)

N: number of values, D4T: stavudine, 3TC: lamivudine, DDI: didanosine, FTC: emtricitabine, AZT: zidovudine, ABC: abacavir, TDF: tenofovir.

3.6. Ion suppression

The ion suppression of the matrix was examined by two ways. First, a continuous and post-column infusion at 5 μL/min of a solution containing all of the N(t)RTIs at the CS4 level and the internal standard into the mass-spectrometer during the chromatographic analysis of a blank extract. A single time window was used to acquire the data and the scan-time was reduced from 0.1 to 0.02 s in order to maintain the number of points acquired for each followed transition. The tune parameters used were identical as described above in the MS^2 conditions paragraph. Thirty different plasmas were used as previously described [31]. Second, the 30 plasmas tested above were submitted to another extraction procedure but were reconstituted finally by an aqueous solution of the eight analytes, at concentrations corresponding to the CS4 level, instead of being reconstituted by water. The peak heights obtained with these spiked blank extracts were compared to the peak heights obtained with the aqueous solution of the eight analytes. Ion suppression was considered negligible if the differences in peak heights obtained with spiked blank extracts and the solution of the analytes were less than 20%.

4. Results and discussion

4.1. LC/MS/MS characteristics

Fig. 1 displays typical chromatograms corresponding to the injection of the lowest calibration standard. According to the retention times, 4 time windows were defined. This segmentation of the acquisition was done in order to increase the scan-time spent by the detector on the analyte, which increased the signal-to-noise ratio, while enough data were acquired for a satisfying definition of the chromatographic peak (about 25 points). The 4 time windows, expressed in minutes, and the corresponding analytes were 0–1.3.5 for TDF and 3TC, 3.5–7.5 for D4T, DDI and FTC, 7.5–8.8 for i.s. and ABC, and 8.8–14 for AZT. As expected, the run time (14 min) was longer than the run times necessary for the quantification of 1or 2 N(t)RTIs [22,23] (about 2–4 min), but was shorter than run times needed for the assay of more than 7 antiretroviral drugs, including protease inhibitors and non-nucleoside reverse transcriptase inhibitors, (17 min for an LC-MS/MS method [26], between 24 and 40 min for HPLC-UV methods [18–20]). The signal-to-noise ratios obtained for the 7 compounds at the lowest calibration standard level were comprised between 30 and 6600. It appears that the limit of quantification of each analyte, except tenofovir (see below), could have been lowered to correspond to a signal-to-noise ratio of 10, which would have allowed some of our LOQs to be similar to the lowest ones previously published for an equivalent plasma volume of 50–100 μL [15,26] (i.e. between 0.001 and 0.01 μg/mL, vs. 0.005 to 0.02 μg/mL for our method). However, we decided to keep the initial calibration ranges as the chosen LOQs allowed the quantification of the clinical concentrations of N(t)RTIs (i.e. concentrations observed for therapeutic doses). It can also be noticed that LOQs as low as 0.0005 μg/mL were previously described [21], however this method needed 500 μL of plasma, versus 100 μL for our method.

4.2. Linearity

All back-calculated concentrations fell within the ±15% acceptance criterion. For all analytes the nine standard curves were linear with minimum R^2 value of 0.986. Average parameters for the nine standard curves are given in Table 3.

4.3. Accuracy and precision

The within- and between-assay precisions and accuracies assessed on four quality control samples are displayed in Table 4. All coefficients of variations and biases were inferior or equal to 20% for the LOQ and inferior to 15% for all other concentrations of all analytes. However, the lowest QC of tenofovir reached the 20% limit value of the acceptance criteria, and this result was confirmed with 6 different blank plasmas. This indicates that this lower value is the true tenofovir LOQ in the conditions of our method. This was confirmed by investigating precision and accuracy for tenofovir concentrations lower than 0.005 μg/mL which led to results >20% (not shown).

Table 4
Within and between day precisions and accuracies, both expressed in percent, assessed on four quality controls

Analyte	QC1		QC2		QC3		QC4	
	Intra $N=6$	Inter $N=12$	Intra $N=6$	Inter $N=12$	Intra $N=6$	Inter $N=12$	Intra $N=6$	Inter $N=12$
D4T								
Bias	−10.00	−16.30	−1.67	+0.28	−0.54	−1.85	+1.11	−3.22
CV	7.90	17.50	6.69	15.00	7.58	6.02	2.08	4.72
3TC								
Bias	−18.30	−16.00	−5.83	+2.00	+5.67	+8.90	−15.00	−14.60
CV	5.00	12.8	4.00	12.5	4.34	8.39	6.63	5.61
DDI								
Bias	0.00	−11.70	−1.67	−2.92	−1.75	−6.50	−1.67	−8.67
CV	6.32	6.54	4.15	11.7	2.55	7.45	2.30	8.10
FTC								
Bias	−8.33	−4.17	+1.56	+4.11	+2.02	+1.30	+1.22	−3.39
CV	4.45	10.4	2.55	9.59	4.42	6.11	2.84	5.50
AZT								
Bias	−2.56	−5.73	+3.43	+0.22	+6.15	+2.45	+2.60	−2.35
CV	2.81	10.40	2.10	9.30	2.57	7.84	1.77	7.63
ABC								
Bias	−11.70	−5.00	−1.67	+5.56	+0.50	+8.75	−9.50	−6.81
CV	4.62	10.00	2.84	8.66	2.25	5.09	2.04	4.14
TDF								
Bias	+18.00	+20.00	−5.56	+5.42	−10.90	−5.31	−3.69	−4.02
CV	12.2	20.00	6.57	8.61	5.64	9.36	2.33	8.67

N: number of values, D4T: stavudine, 3TC: lamivudine, DDI: didanosine, FTC: emtricitabine, AZT: zidovudine, ABC: abacavir, TDF: tenofovir.

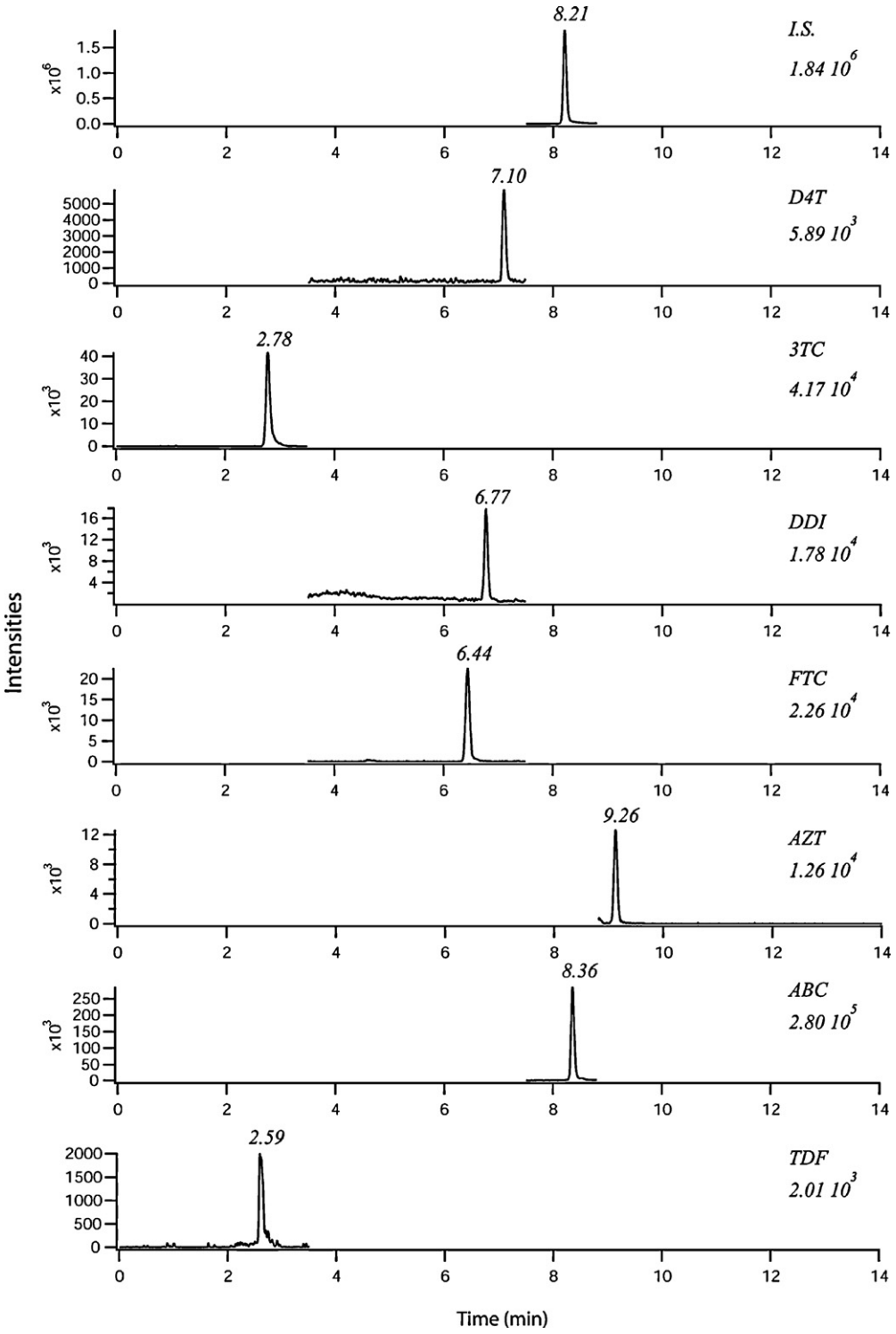

Fig. 1. Typical chromatogram obtained for the lowest calibration standard. Peak intensities are given below the identifications. D4T: stavudine; 3TC: lamivudine; DDI: didanosine; FTC: emtricitabine; AZT: zidovudine; ABC: abacavir; TDF: tenofovir.

4.4. Recovery

Mean recoveries for each concentration of each analyte are given in Table 5. Recoveries ranged from 81.1 to 91.7% for the LOQs and from 77.3 to 94.2% for the other concentrations. Analysis of variance was performed and showed no statistically significant difference between recoveries with respect to concentration.

4.5. Specificity

As an illustration, chromatograms obtained from injection of blank plasma or plasma extract of a patient treated with 3 N(t)RTIs are given in Figs. 2 and 3. No interference has been found between the N(t)RTIs and endogenous substances or other drugs taken by the patients. Drugs taken by the patients were protease inhibitors (tipranavir, daruna-

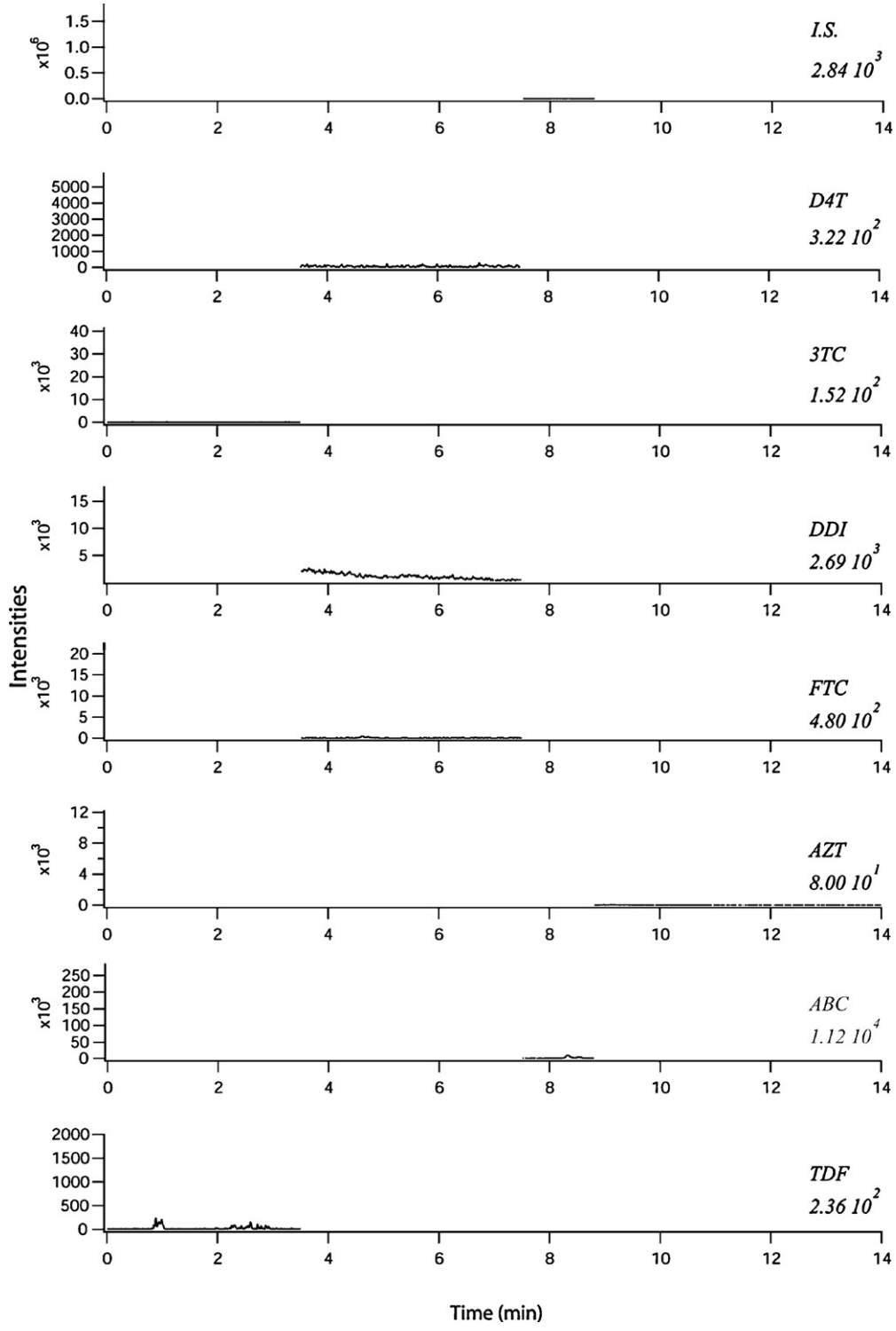

Fig. 2. Chromatograms from extract of blank plasma.

vir, fosamprenavir, ritonavir, lopinavir, saquinavir, atazanavir, indinavir), non-nucleoside reverse transcriptase inhibitors (efavirenz, nevirapine, etravirine), fusion inhibitor (enfivirtide), integrase inhibitor (raltegravir), antifungals (voriconazole, posaconazole, caspofungin, amphotericin B, fluconazole), antibiotics (ceftazidime, ceftriaxone, cefotaxime, ciprofloxacine, pefloxacin, rifampicin, rifabutin, isoniazid). In particular, peaks that were occasionally detected at retention times corresponding to analytes were systematically neglected as peak heights of the lowest calibration standard were at least three times higher.

4.6. Stability

The biases between observed and theoretical concentration ranged from 0.7 to 14.9% for the three quality control levels (QC2, QC3, QC4). Stability of the stock solutions at −20 °C

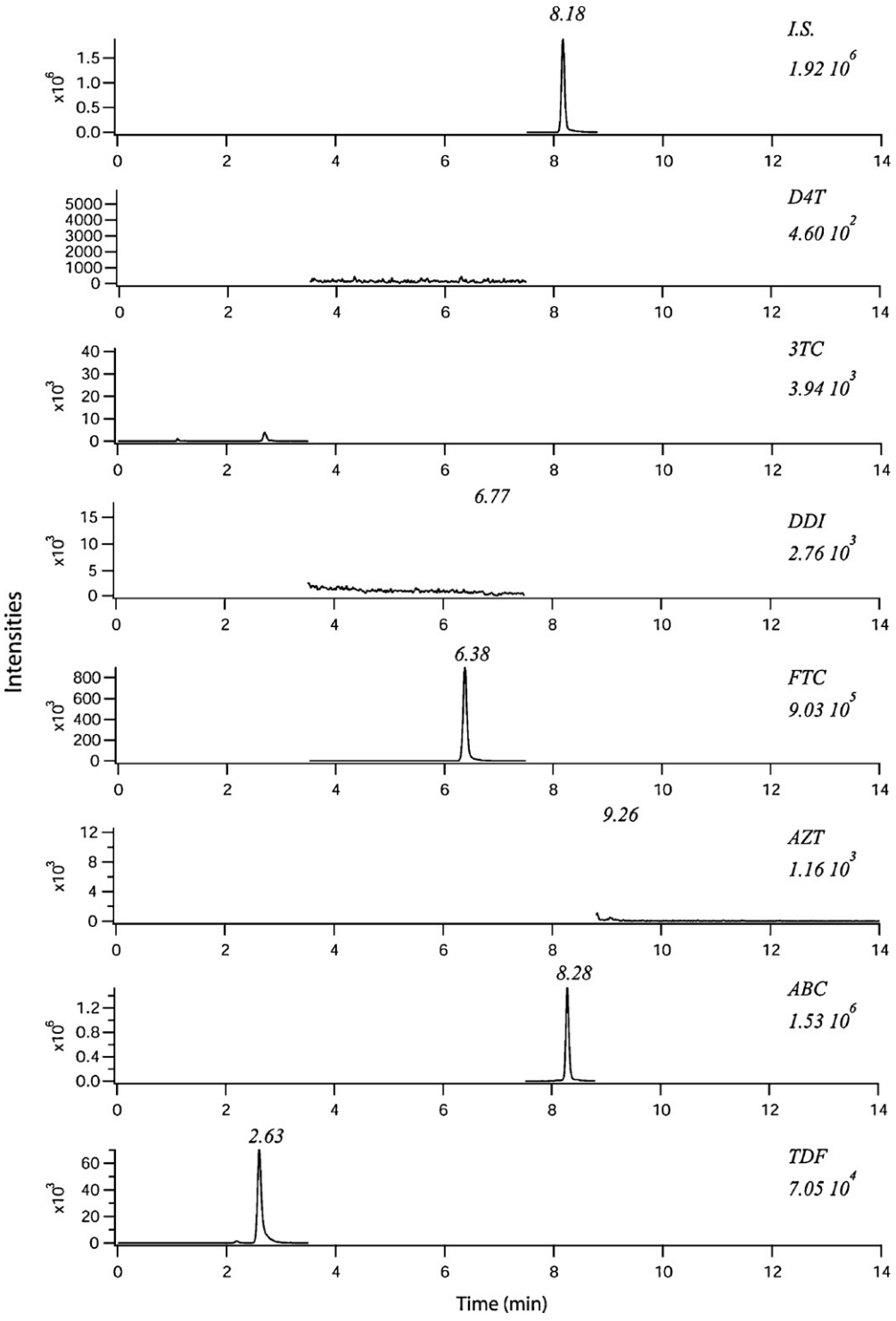

Fig. 3. Chromatograms from extract of patient treated with emtricitabine (FTC), abacavir (ABC), and tenofovir (TDF). The plasma concentrations of the three compounds are 0.604 μg/mL, 0.057 μg/mL, and 0.267 μg/mL, respectively.

for at least 6 months, and stability of the extracts at room temperature for at least 2 days was demonstrated by negligible difference (i.e. less than 5%) between the peak heights obtained from the solution stored at −20 °C or the extracts stored 2 days at room temperature, compared to solutions freshly made or extracts injected immediately after the extraction procedure.

4.7. Ion suppression

Fig. 4 shows typical chromatograms of the observed signals resulting from the injection of a blank plasma extract. Expected elution times of the N(t)RTIs and internal standard are indicated by an arrow. As observed, most ion suppression was observed in the 0.7–1.4 min interval after injection of a plasma extract.

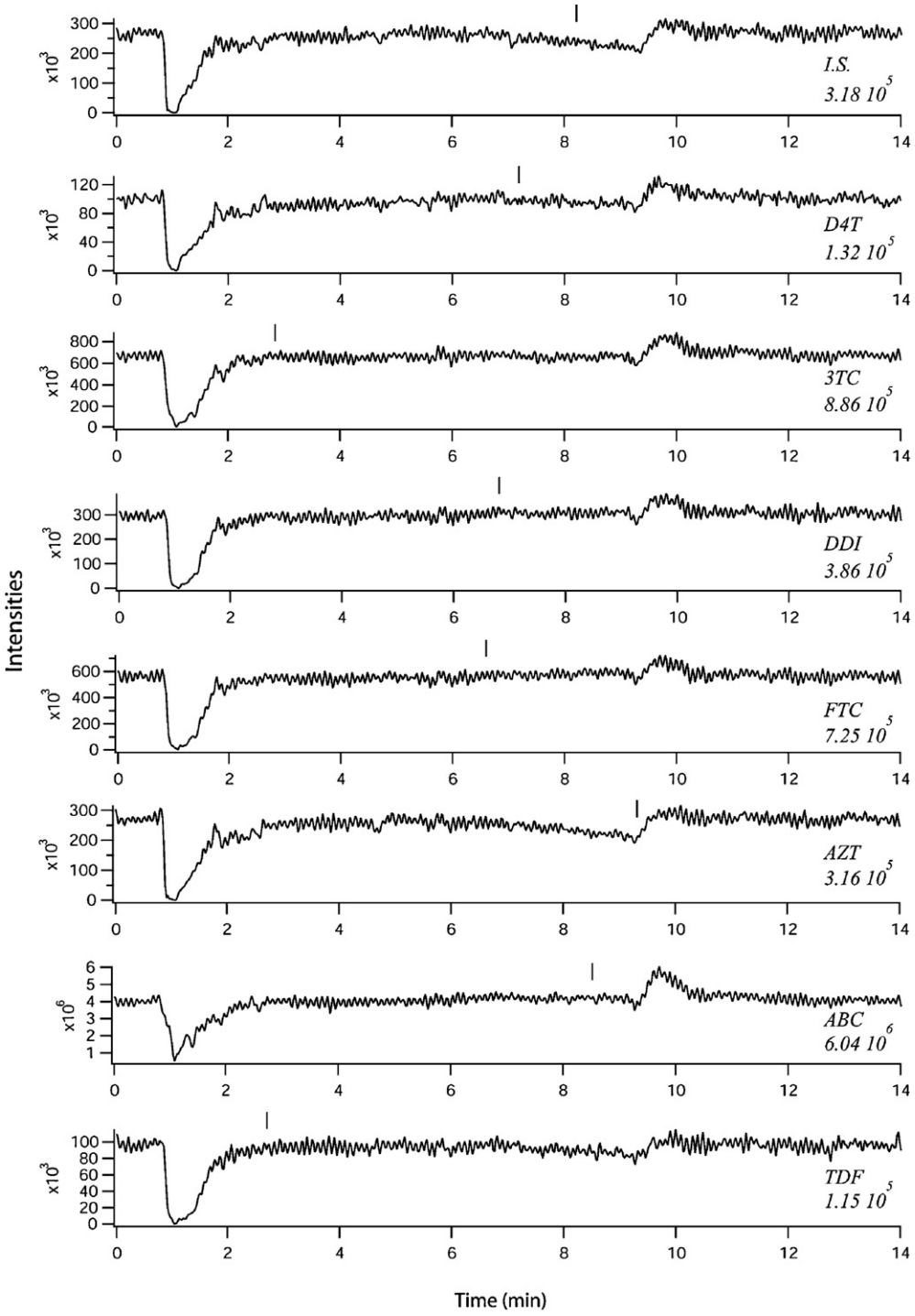

Fig. 4. Observed intensities in chromatographic conditions during continuous and post-column infusion of a solution containing the N(t)RTIs and internal standard after injection of blank plasma extract in the chromatographic system. D4T: stavudine, 3TC: lamivudine, DDI: didanosine, FTC: emtricitabine, AZT: zidovudine, ABC: abacavir, TDF: tenofovir, i.s.: internal standard.

Because of all analytes and the internal standard eluted after this delay, the observed ion suppression did not influence quantitation or sensitivity. This was confirmed by comparing the peak heights of the 30 blank extracts reconstituted with the aqueous solution of the analytes to the peak heights obtained with the aqueous solution as the calculated ion suppression was less than 10% for each analyte and for each plasma extract at the respective elution times of the analytes. It is likely that the dilution of the samples in water and the low injection volume have contributed to this lack of matrix effect.

This is the first method describing the simultaneous assay of the 7 N(t)RTIs currently available in France. Previously published methods comprised indeed 6 N(t)RTIs at best [19,26]. To our knowledge, this is furthermore the first LC-MS/MS method describing the simultaneous assay of tenofovir and emtricitabine, which is useful for a TDM purpose as these 2 compounds

Table 5
Observed recoveries (expressed in percent) for the calibration standards

Analyte	*N*	D4T	3TC	DDI	FTC	AZT	ABC	TDF	i.s.
CS1	3	81.1	87.5	91.7	86.5	87.0	83.8	85.0	
CS4	3	88.0	91.3	90.9	91.8	84.3	89.4	77.3	92.4
CS7	3	80.2	94.2	86.7	89.2	78.0	90.6	80.2	

N: number of values, D4T: stavudine, 3TC: lamivudine, DDI: didanosine, FTC: emtricitabine, AZT: zidovudine, ABC: abacavir, TDF: tenofovir.

are now combined in the same tablet (Truvada®). Another interest of our method, more especially in a routine context, is the sample preparation which consists of a very simple protein precipitation, as all the previously published methods used complex solid phase or liquid/liquid extractions, which is much more expensive and time-consuming. Despite the poor selectivity of this extraction procedure, no ion suppression was evidenced, thanks to the chromatographic separation form interfering endogenous compounds, and to the dilution of the samples by a factor 5 in water combined with a low injection volume.

5. Conclusion

We developed a simple analytical method for the simultaneous assay of the 7 N(t)RTIs used for the treatment of HIV-infected patients. In particular, our method does not use a complicated sample treatment to extract the analytes. The method has been found precise and accurate enough to perform therapeutic drug monitoring of N(t)RTIs.

This validated LC-MS/MS method provides a robust and very simple procedure for determining 7 N(t)RTIs. The small plasma sample volume required is well suited for quantification of zidovudine in premature infants and neonates and the short run time (14 min) may be appreciated in some critical situations where the result is quickly needed, such as over dosage due to mistakes in the administration.

The method has been used routinely for therapeutic drug monitoring in more than a thousand samples without any change in either retention time or sensitivity, evidencing its interest in a routine clinical setting.

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