Journal of Pharmaceutical and Biomedical Analysis 54 (2011) 779–788

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

A HPLC–MS method for the simultaneous quantification of fourteen antiretroviral agents in peripheral blood mononuclear cell of HIV infected patients optimized using medium corpuscular volume evaluation

Antonio D'Avolio*, Marco Simiele, Marco Siccardi, Lorena Baietto, Mauro Sciandra, Valentina Oddone, Francesca Romana Stefani, Silvia Agati, Jessica Cusato, Stefano Bonora, Giovanni Di Perri

Laboratory of Clinical Pharmacology and Pharmacogenetics[1,2], Department of Infectious Diseases, University of Torino, Amedeo di Savoia Hospital, Corso Svizzera 164, 10149 Turin, Italy

ARTICLE INFO

Article history:
Received 7 May 2010
Received in revised form 12 October 2010
Accepted 15 October 2010
Available online 10 November 2010

Keywords:
HPLC–MS
Quantification
Intracellular
Mean corpuscular volume
Antiretrovirals

ABSTRACT

A sensitive and accurate high performance liquid chromatography–mass spectrometric (HPLC–MS) method for the intracellular determination of 14 antiretroviral drugs in peripheral blood mononuclear cells (PBMCs) for HIV+ patients was validated. PBMCs are isolated by Ficoll density gradient centrifugation and cells count and the relative mean volume is performed with a Coulter® instrument. Extraction of drugs from PBMCs pellets was obtained with methanol:water (70:30, v/v), with quinoxaline added as internal standard, after a sonication step. Supernatant was dried and then dissolved in water/acetonitrile (60/40, v/v), before injection into a 2.1 mm × 150 mm Atlantis® T3 3μ column. Chromatographic separations were performed using a gradient program with a mixture of water (0.05% formic acid), as mobile phase A and acetonitrile (0.05% formic acid), as mobile phase B. Analytes quantification was performed by electrospray ionisation–single quadrupole mass spectrometry using the selected ion recording (SIR) detection mode. The positive ionization was used for the HIV protease inhibitors (PIs) indinavir, saquinavir, nelfinavir, nelfinavir M8 metabolite, amprenavir, darunavir, atazanavir, ritonavir, lopinavir, tipranavir, the integrase inhibitor (II) raltegravir and the non-nucleoside reverse transcriptase inhibitors (NNRTIs) nevirapine and etravirine, while the negative ionization is applied for efavirenz. The calibration curves were built using blank PBMCs spiked with antiretroviral drugs at concentrations ranging from 0.1 to 32 ng/mL (1–320 ng/mL for tipranavir) and fitted to a quadratic regression model weighted by $1/X$. The mean extraction recovery for all PIs, II and NNRTIs was always above 82%. The method was precise, with a range of intra/inter-day percent standard deviation within 2.6–14.8%, and accurate with mean of percent coefficient of variation (CV%) from nominal values −7.85 to +9.7%. Each drug concentration evaluated was expressed in ng/mL and optimized using each patient medium corpuscolar volume and cell number. This analytical method is routinely used in our clinical research center for the assessment of intracellular levels of all PIs, raltegravir and NNRTIs commercially available at present.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

Highly Active Antiretroviral Therapy (HAART) is based on the co-administration of drugs from two o three different classes, aiming to inhibit multiple viral targets. In recent years, the new drugs have been introduced for and major advances have been made towards the treatment of human immunodeficiency virus type 1 (HIV-1) infected patients. Even though HAART has markedly improved the clinical outcome of HIV-infected patients, virological treatments often fail within the first year of therapy [1,2].

Drug resistance due to mutations of the viral genome accounts for a large proportion of treatment failures. Sub-inhibitory antiretroviral drug concentrations promote the selection of resistance mutations in the HIV genome during viral replication. Poor penetration of drugs into several profound compartments of the body (sanctuary sites), inadequate treatment adherence, and variability in drug pharmacokinetics may contribute to the occurrence of sub-therapeutic drug level in vivo [3].

Therapeutic drug monitoring (TDM) of HIV protease inhibitors (PIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) considers the adjustment of total plasma concentrations to

* Corresponding author. Tel.: +39 011 4393979; fax: +39 011 4393882.
E-mail address: antonio.davolio@unito.it (A. D'Avolio).

[1] UNI EN ISO 9001:2000 Certified Laboratory; Certificate No. IT-64386.

[2] Certification for: "Design, development and application of determination methods for anti-infective drugs", www.tdm-torino.org.

0731-7085/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2010.10.011

optimise response to antiretroviral therapy [1]. However, only the fraction reaching the intracellular compartment is reasonably expected to exert an antiviral action. The mechanisms by which antiretroviral drugs accumulate within cells remain generally unknown.

Previous in vitro [4] and in vivo [5–9] studies have shown striking differences in the intracellular kinetics of PIs and NNRTIs.

Moreover, a number of transmembrane transport proteins, such as P-glycoprotein, the gene product of ABCB1 (MDR1), and related ABC (ATP binding cassette) transporters are known to actively mediate the efflux of drugs from cells, and have been shown to play an important role in the intracellular antiviral drug concentration [10–13]. The genes coding for these transport and regulatory proteins are polymorphic in humans, with consequences on the expression and function, potentially influencing the intracellular levels of antiretroviral drugs.

Thus, as intracellular concentrations of antiretroviral drugs are influenced by both their physico-chemical properties and host genetic factors, an assay enabling the monitoring of IIs, PIs and NNRTIs levels at the site of their pharmacological action appears to be an essential tool for the ongoing investigations aimed at preventing antiretroviral therapy failure or toxicity.

Liquid chromatography coupled with mass spectrometry (LC–MS or MS/MS), owing to its sensitivity and selectivity, is particularly attractive for the measurement of intracellular analytes. LC–MS/MS technology has been applied for measuring the active triphosphate anabolites of several nucleosidic reverse transcriptase inhibitors (NRTIs) in peripheral blood mononuclear cells (PBMCs) [14–20].

PIs and NNRTIs are directly acting pharmacological species requiring no bioactivation. However although several LC–MS and MS/MS assays have been published for the quantitative determination of PIs in plasma [21–32], validated methods for their determination in PBMCs have rarely been described [9,33–41] and only one method has been published for quantifying IIs [42].

Other enzyme immunoassays for a few PIs and NNRTIs plasma and intracellular level measurements have been published [43–46], including, recently, an assay using MALDI technology [47].

In most published papers mean corpuscular volume (MCV) is commonly estimated to be 400 fL, on the basis of a paper published in 1993 [48], and cell count is often manually performed through microscope observation at the Burker/Malassez counting chamber [49,50]. Thus, intracellular concentration measurement could be potentially biased by error in the PBMC count and by inter-individual variability of corpuscular volume evaluation [49].

In this paper, we describe the development and validation of an analytical method for the simultaneous quantification of 14 antiretroviral drugs (PIs: indinavir, saquinavir, nelfinavir and its metabolite M-8, amprenavir, darunavir, atazanavir, ritonavir, lopinavir, tipranavir; NNRTIs: efavirenz, nevirapine, etravirine; II: raltegravir) in PBMCs by liquid chromatography coupled with single-mass spectrometry detection. Moreover, this method is characterized by a novel approach to measure the mean cell volume and number with the introduction of a cell counter instrumentation. This instrument allows us to accurately calculate the cell number and the mean corpuscular volume (MCV) [51,52] for each patient/sample, during PBMC separation.

2. Material and methods

2.1. Chemicals

Compounds were kindly provided by the following pharmaceutical companies: Nevirapine (NVP) and Tipranavir (TPV) from Boehringer Ingelheim Pharmaceuticals, Inc. (Ridgefield, CT, USA); Efavirenz (EFV) and Atazanavir (ATV) from Bristol Myers Squibb Company (Princeton, NJ, USA); Indinavir (IDV) and Raltegravir (RGV) from Merck Sharp & Dohme-Chibret Labs. (West Point, PA, USA); Amprenavir (APV) from GlaxoSmithKline (Brentford, UK); Darunavir (DRV) and Etravirine (ETV) from Tibotec (Mechelen, Belgium); Saquinavir (SQV) from Roche (Mannheim, Germany); Nelfinavir (NFV) and M-8 from Pfizer Inc (Groton, CT, USA); Lopinavir (LPV) and Ritonavir (RTV) from Abbott Laboratories (Chicago, IL, USA).

Acetonitrile HPLC grade and Methanol HPLC grade were purchased from J.T. Baker (Deventer, Holland). HPLC grade water was produced with Milli-DI system coupled with a Synergy 185 system by Millipore (Milan, Italy). Quinoxaline (QX), phosphate buffered saline (PBS) pH 7.4 (10 mM) and formic acid were obtained from Sigma–Aldrich (Milan, Italy). Lymphoprep was purchased from Sentinel Diagnostics (Milan, Italy).

Blank cells (PBMCs) were isolated from the blood of healthy donors, kindly supplied by the Blood Bank of the Maria Vittoria Hospital (Turin, Italy).

2.2. PBMC isolation

Clinical samples were collected, after obtaining written informed consent according to local Ethics Committee indications, from HIV infected patients treated with various combinations of antiretrovirals. Blood samples were collected in two EDTA tubes (2×7 mL).

PBMCs were isolated from 12 to 14 mL of blood using lymphoprep density gradient centrifugation ($700 \times g$, 25 min, 4 °C with a Jouan Centrifuge [Model BR4i, Saint-Herblain, France]) at each sampling, as described previously [8,12,35,53]. PBMCs were then fast washed twice in 40 mL cold-ice phosphate-buffered saline and centrifuged ($750 \times g$, 6 min, 4 °C).

Cell number and MCV were determined by a Bekman Coulter Z2 (Instrumentation Laboratory, Milan, Italy), and managed by Z2 AccuComp Software (Version 3.01), without the use of the trypan blue exclusion. The resulting pellet of washed PBMCs was dissolved with 1 mL extraction solution (methanol:water, 70:30, v/v), switched in two criovials (500 μl each) and then stored at −80 °C until analyses, and for no longer than three months.

The time taken to process PBMCs from phlebotomy to methanol extraction solution was less than 1 h, ensuring that sampling conditions were ice cold to prevent drug loss [8,35]. Blank PBMCs isolated from the blood of healthy donors, as previously described, were stored in aliquots of around 5×10^6 cells.

2.3. Stock solutions, standards (STDs) and quality controls (QCs)

DRV, NVP, IDV, APV, SQV, M-8, NFV, ATV and RGV, stock solutions were made in a solution of methanol and HPLC grade water (90:10) and EFV, ETV, RTV and LPV were made in a solution of methanol and HPLC grade water (95:5) to obtain a final concentration of 1 mg/mL. TPV stock solution was made with methanol to obtain a concentration of 10 mg/mL. All stock solutions were then refrigerated at 4 °C until use, within 3 months.

The Internal Standards (IS) working solution was prepared with QX [2 μg/mL] in methanol and HPLC grade water (50:50, v/v) and stored at 4 °C until use. The eight calibration standards and three quality controls (QCs) were prepared adding a determined volume of stock solutions, or diluted stock solution, to each blank PBMCs aliquots before storage at –80 °C, during no more than three months, in a manner similar to that described in other publications [6,10,33,39,40,42,47,54,55].

Calibration ranges, from STD 8 to STD 1, and QC concentrations for all drugs are listed in Table 1. Under these conditions, ETV, RGV, DRV, APV, NVP, IDV, SQV, M-8, NFV, ATV, EFV, RTV, LPV and TPV

Table 1
Detected mass (Da), cone voltage used (V) and retention time (RT, in min) used to quantify Internal Standard and each drug, and relative concentrations from STD8 to STD1 (LOQ), QCs (QC high, QC medium and QC low) and LOD. At the end of the table the mass spectrometer parameter.

Drugs Data				Concentrations (ng/ml)											
Drugs	RT (min)	Mass (Da)	Cone voltage (V)	STD 8	STD 7	STD 6	STD 5	STD 4	STD 3	STD 2	LOQ/STD 1	QC high	QC medium	QC low	LOD
IDV	3.3	614.20	42	32	16	8	4	2	0.5	0.25	0.125	15	5	1	0.06
NVP	3.5	267.15	30	32	16	8	4	2	0.5	0.25	0.125	15	5	1	0.06
M-8	5.4	584.15	35	32	16	8	4	2	0.5	0.25	0.125	15	5	1	0.06
NFV	8.1	568.18	32	32	16	8	4	2	0.5	0.25	0.125	15	5	1	0.06
RGV	8.2	445.00	30	32	16	8	4	2	0.5	0.25	0.125	15	5	1	0.06
SQV	8.3	671.25	37	32	16	8	4	2	0.5	0.25	0.125	15	5	1	0.06
APV	10.2	506.10	18	32	16	8	4	2	0.5	0.25	0.125	15	5	1	0.06
DRV	10.2	548.10	19	32	16	8	4	2	0.5	0.25	0.125	15	5	1	0.06
ATV	10.3	705.22	33	32	16	8	4	2	0.5	0.25	0.125	15	5	1	0.06
RTV	13.5	580.20	25	32	16	8	4	2	0.5	0.25	0.125	15	5	1	0.06
EFV*	13.9	313.85	30	32	16	8	4	2	0.5	0.25	0.125	15	5	1	0.06
LPV	14.0	629.20	25	32	16	8	4	2	0.5	0.25	0.125	15	5	1	0.06
ETV	15.2	434.90	60	32	16	8	4	2	0.5	0.25	0.125	15	5	1	0.06
TPV	16.2	603.10	28	320	160	80	40	20	5	2.5	1.250	150	50	10	0.15
IS	8.6	313.30	50	–	–	–	–	–	–	–	–	–	–	–	–

Detector settings were: ESI, positive polarity ionization *(except for EFV which was detected by negative polarity ionization, in the same run simultaneously using instantaneous switching); capillary voltage, 3.5 kV; source temperature, 110 °C; desolvation temperature 350 °C; nitrogen desolvation flow, 400 l/h; nitrogen cone flow, 40 l/h.

have been proven to be stable [22–24,39,40,42,56–59]. Therefore no further stability evaluation was carried out.

2.4. Chromatographic conditions

The chromatographic run was optimised for this assay by modifying our previously published plasma methods [23,24]. The HPLC–MS instrument used 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.

The chromatographic separation was performed at 35 °C using a column oven, on Atlantis T3 C-18 3 μ column (150 mm × 2.1 mm I.D.) (Waters; Milan, Italy), protected by a Security Guard with C18 (4.0 mm × 3.0 mm I.D.) pre-column (Phenomenex, CA, USA). The chromatographic run was performed with a gradient (Table 2), and the mobile phase was composed by HPLC grade water containing 0.05% formic acid, for mobile phase A, and HPLC grade acetonitrile containing 0.05% formic acid, for mobile phase B.

Detector settings, ions detected and cone voltages for each drug are listed in Table 1.

2.5. STD, QC and samples preparation for injection

The stored aliquots of STDs, QCs and patient samples PBMCs were defrosted with HIV inactivation thermal treatment (58 °C for 35 min). Fifty μl of IS working solution were added to each tube and the samples were vortexed for 10 s. STDs, QCs and patient tube samples were sonicated in an ice–water bath three time (cycle 0.75; amplitude 80%), to fully lyse PBMCs, using a sonicator UP-50 H (Dr Hielscher GmbH, Teltow, Germany). After a centrifugation at (7000 × g, 10 min at 4 °C), supernatant were collected into glass tubes, and remaining pellets were washed by vortex for 10 s with 200 μl of acetonitrile:methanol solution (50:50, v/v), centrifuged (7000 × g,10 min at 4 °C) and each supernatant was collected in the indicated glass tubes to be treated by vortex–vacuum evaporation to dryness at 60 °C.

Each extract was reconstituted with 60 μl of HPLC-grade water and acetonitrile solution (60:40, v/v) and 20 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, excluding that of sonication.

Table 2
Chromatographic condition (gradient): mobile phase: Buffer A (HPLC grade water + 0.05% formic acid) and Buffer B (HPLC grade acetonitrile + 0.05% formic acid). The flow was 0.3 ml/min.

TIME (min)	% Buffer A	% Buffer B
0.0	59	41
0.1	58	42
2.0	45	55
4.0	40	60
9.0	30	70
9.5	25	75
9.6	5	95
14.0	5	95
14.1	95	5
15.0	95	5
15.1	70	30
25.0	60	40

2.6. Specificity and selectivity

Interference from endogenous compounds was investigated through the analysis of six different blank PBMC samples. Potential interference by antiretroviral drugs concomitantly administered to the patients was also evaluated by spiking blank PBMCs with them. These included: ribavirin (RBV), zidovudine (AZT), didanosine (ddI), stavudine (d4T), lamivudine (3TC), abacavir (ABC), tenofovir (TDF), emtricitabine (FTC) and enfuvirtide (T-20). No other concomitant drugs were investigated.

An "interfering drug" was considered a molecule which exhibits a retention time close to 0.3 min from the analytes, and with the potential capability to cause ion suppression. Co-elution between DRV and APV, on the basis of their very similar chemical and physic characteristics, was expected [23,57].

2.7. Matrix effect

The "matrix effect" was investigated on six replicates. Peak areas from blank extracts spiked with all analytes at three concentrations was compared with peak areas from standard solutions (water and acetonitrile, 60:40, v/v) spiked with the same amount of analytes, as described by Taylor [60]. The possible "matrix effect" was calculated, as deviation %, comparing the peak area obtained from the PBMCs extract with the peak area obtained from the standard solution.

2.8. Accuracy, precision, calibration and limit of quantification

Intra-day and inter-day accuracy and precision were determined by assaying 6 spiked PBMC samples at three different concentrations (QCs) for all drugs. Accuracy was calculated as the percentage of deviation from the nominal concentration. Inter-day and intra-day precision were expressed as the relative standard deviation (RSD%) at each QC concentration reported in Table 1.

Each calibration curve was obtained using 8 calibration points in duplicate. The ranges are listed in Table 1. Calibration curves were created by plotting the peak area ratios of each drug relative to the IS against the various drug concentrations in the spiked plasma standards. A $1/X$ weighted quadratic regression was used for all curves.

The limit of detection (LOD) was defined as the concentration that yields a signal-to-noise ratio of 3/1. The percentage of deviation from the nominal concentration (measure of accuracy) and relative standard deviation (measure of precision) of the concentration considered as the limit of quantification (LOQ) had to be <20%, and this was considered as the lowest calibration standard, as requested by FDA guidelines [61].

2.9. Recovery

Recovery from PBMC, using the extraction procedures, was assessed by comparing the peak area obtained from multiple analyses of spiked samples (QCs) with the peak area from the standard solution of all analytes in HPLC-grade water and acetonitrile (60:40) solution at the same concentrations.

2.10. Stability

The stability of all drugs considered at different conditions has been previously assayed in various papers [22–24,39,40,42,56–59]. For this reason stability assays were not performed.

3. Results

Retention times are summarized in Table 1. APV and DRV eluted at the same retention time, due to their similar chemical structure and physiochemical properties, but this co-elution did not affect the quantification of the two drugs, as shown in the following sections.

Representative chromatograms of a blank PBMCs extracted and STD1 are shown in Fig. 1 , and an example of chemical mix chromatogram is reported in Fig. 2. Mean regression coefficient (r^2) of all calibration curves was more than 0.998. A $1/X$ weighted quadratic regression was chosen due to its r^2 higher than other equations. Moreover this regression allows to place more weight on low calibration points, a curve region where the greater number of C_{trough} intracellular concentrations were expected.

3.1. Specificity and selectivity

The assay did not show any significant interferences from other antiretrovirals drugs taken at therapeutic dosage by patients. Moreover, the six blank PBMC test did not show any interference peaks for the specified ions detected and in the retention time analyte windows (Fig. 1).

APV has a very similar retention time to DRV, as also reported by other authors [23,24,57,62–64], due to their chemical analogy. Similarly, NFV, RGV and SQV, APV, DRV and ATV, LPV and EFV, respectively, have a very close retention time. These co-elutions did not affect quantification, as has been fully proven for APV–DRV–ATV and LPV–EFV in our previous study [23,24], and in "matrix effect" section.

3.2. Accuracy, precision, limit of quantification

The results of the validation of the methods are listed in Table 3 for all analytes. All observed data (Intraday and Interday precision [RSD%]), including LOQ, were below 15.0%, according to FDA guidelines [61]. Analytes coelution did not alter the quantification of the drugs, as described above. LOQ and LOD are listed in Table 1.

3.3. Recovery

Multiple aliquots ($n = 6$) at each of the three QCs concentration were assayed and mean recovery of all drugs ranged from 76% to 98% (mean CV 6.7%). Mean IS recovery was 89% (mean CV 2.9%).

3.4. Matrix effect

The deviation % of the peak area at the three concentrations for all analytes is comparable, ranging from −17.0% to +8.8% (mean −3.1%), showing the absence of the "matrix effect". The lowest data (−17%) was related to indinavir, which has the shortest retention time and could be potentially affected by poor column retained analytes.

4. Discussion and conclusion

Excluding the fusion inhibitor enfuvirtide and the CCR5 inhibitor maraviroc, all antiretrovirals such as nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, protease inhibitors and integrase inhibitor raltegravir act on intracellular viral targets.

Intracellular concentrations of these agents may be an important determinant of antiviral activity, and some research has been performed establishing the relationship between plasma and intracellular concentrations for several antiretroviral drugs [33,35,49,53,56,65]. Moreover, the pharmacokinetics of intracellular drug accumulation (including binding to cytosolic proteins, intracellular-free fraction, influx and efflux kinetics and intracellular drug metabolism) are likely to impact on efficacy and toxicity. To date, intracellular drug levels has been poorly studied in vivo, due to methodological difficulties and the relatively large volumes of blood required.

The study of the intracellular pharmacokinetics of HIV drugs is central to investigating putative sanctuary sites where HIV may replicate with little selective pressure. However, stringent methodological procedures need to be applied, and there is no a standard or reference technique for measuring intracellular drug.

Our first aim was to develop an easy and reliable method of PBMC separation and extraction of drugs from isolated cells, overcoming the potential problems during these steps [49].

PBMCs can be isolated using either conventional Ficoll gradient centrifugation or Vacutainer cell preparation tubes. These two procedures were compared by Becher et al. [16], using phosphorylated anabolites of two NRTIs, and were shown to give identical results. However, the use of cell preparation tubes was found to be easier, less time consuming and therefore quicker which, in the case of stavudine triphosphate, was the most important, as the drug was shown to be unstable in the cell ring of the Ficoll gradient (40% loss within 40 min), and this led the authors to collect the ring in <10 min [16,49]. However, before this isolation step, the stability of the phosphorylated anabolites and that of NNRTIs, raltegravir and PIs in blood should be considered. The phosphorylated anabolites, as consequence of their low molecular size and their chemical properties, have a different behavior respect to non NRTIs drugs. Moreover, to maximize PBMC recovery with Vacutainer cell preparation tubes, as indicated in the brochure, the centrifugation should be conducted at room temperature, with a potential higher loss of

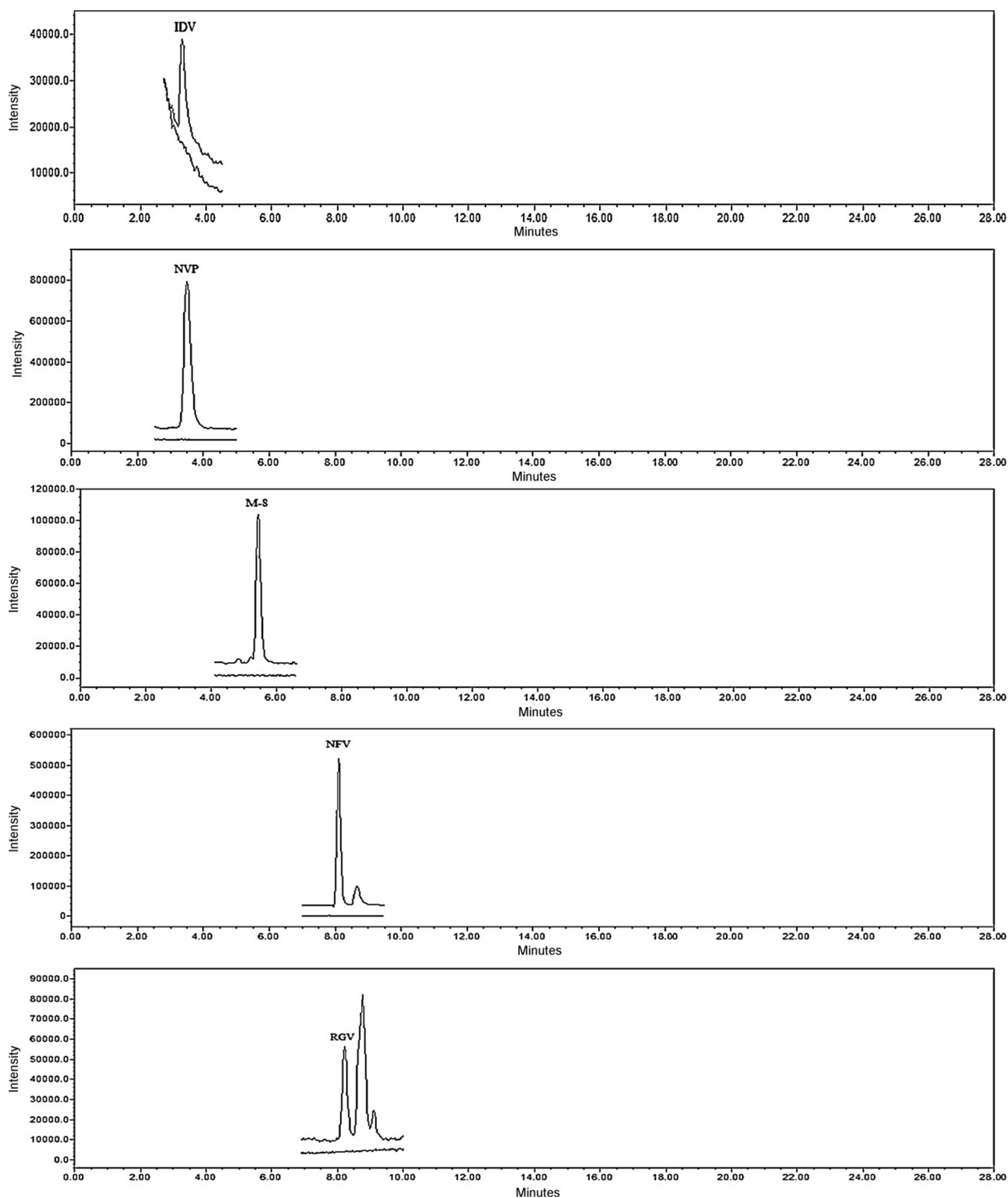

Fig. 1. Overlapping of STD1 and blanks PBMC extracted ions detection.

drugs. For this reason, we have chosen the Ficoll gradient centrifugation method to isolate PBMC, and all procedure was performed at 4 °C to inhibit enzymatic activity and passive drug efflux. The full-procedure from blood sampling to storaging takes always less than 2 h to prevent active drug efflux [6,33,49].

Calibration standards and QC validation samples for the determination of drugs in cell lysate were prepared similarly to many other paper [6,10,33,39,40,42,47,54,55].

Moreover, to increase the recovery and sensitivity of our extraction, we have added two additional step to the extraction procedure: namely, the PBMC sonication and a 100% organic extraction treatment. In fact, the storage at −80 °C in water:methanol (30:70) lead to a first PBMC lyses passage and the sonication step allowed to fully break PBMC membranes.

The remaining pellets from sonication and centrifugation were washed by vortex for 10 s with 200 μl of acetonitrile:metanol

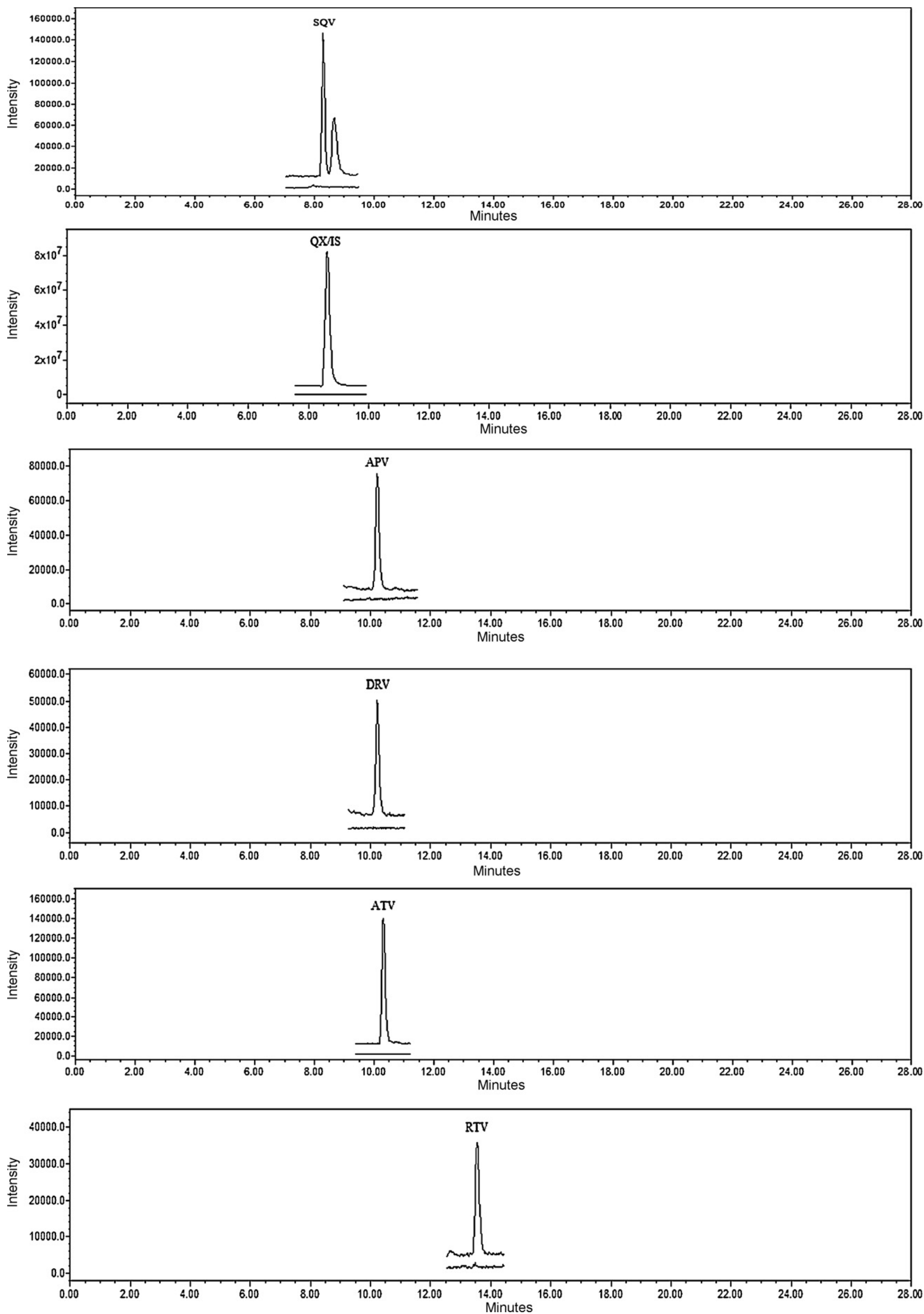

Fig. 1. (*Continued*).

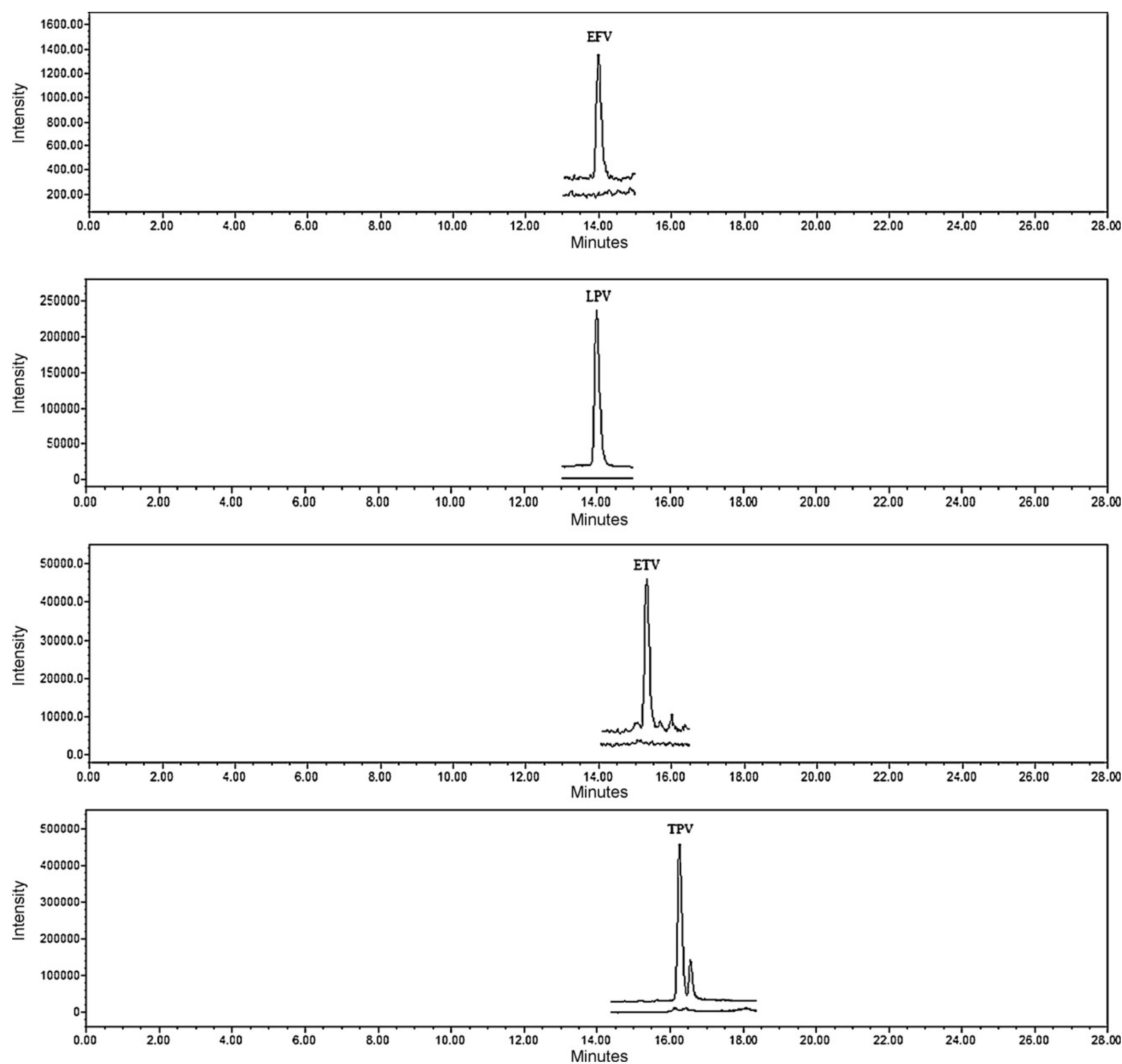

Fig. 1. (*Continued*).

Table 3
Accuracy (CV%), intraday and interday precision, as relative standard deviation (RSD%), assayed for all drugs ($n = 6$).

Drugs	QC high			QC medium			QC low		
	Accuracy CV%	Precision RSD%		Accuracy CV%	Precision RSD%		Accuracy CV%	Precision RSD%	
		Intra-day	Inter-day		Intra-day	Inter-day		Intra-day	Inter-day
IDV	4.80	5.29	9.14	8.94	9.19	9.48	−2.76	9.70	14.89
NVP	−3.44	6.25	10.30	9.70	6.36	10.80	5.81	7.79	13.96
M-8	2.67	4.55	7.82	9.54	6.66	7.94	5.79	7.48	11.48
NFV	1.73	6.29	7.24	6.29	8.22	10.35	9.43	12.92	11.21
SQV	7.41	4.39	8.50	8.39	11.97	10.91	5.55	7.57	8.81
RGV	8.19	2.60	7.27	8.35	3.42	5.92	−0.87	14.02	14.46
APV	3.08	5.22	6.37	4.42	5.74	10.59	−2.22	9.52	13.48
DRV	−2.18	6.04	11.71	−1.33	5.04	10.97	−1.64	11.14	14.70
ATV	3.88	3.41	6.42	1.57	5.40	8.85	−3.44	9.69	13.76
RTV	2.49	4.43	4.84	3.68	6.04	6.89	−7.85	11.63	10.56
LPV	−3.41	4.00	10.11	1.70	8.77	10.36	7.42	10.75	10.21
EFV	0.17	7.13	10.25	0.93	7.72	9.69	−6.24	8.65	12.22
ETV	0.26	3.84	3.99	3.74	7.12	10.01	−1.13	9.82	13.13
TPV	3.04	3.88	9.41	8.38	11.09	11.80	8.38	11.09	11.80

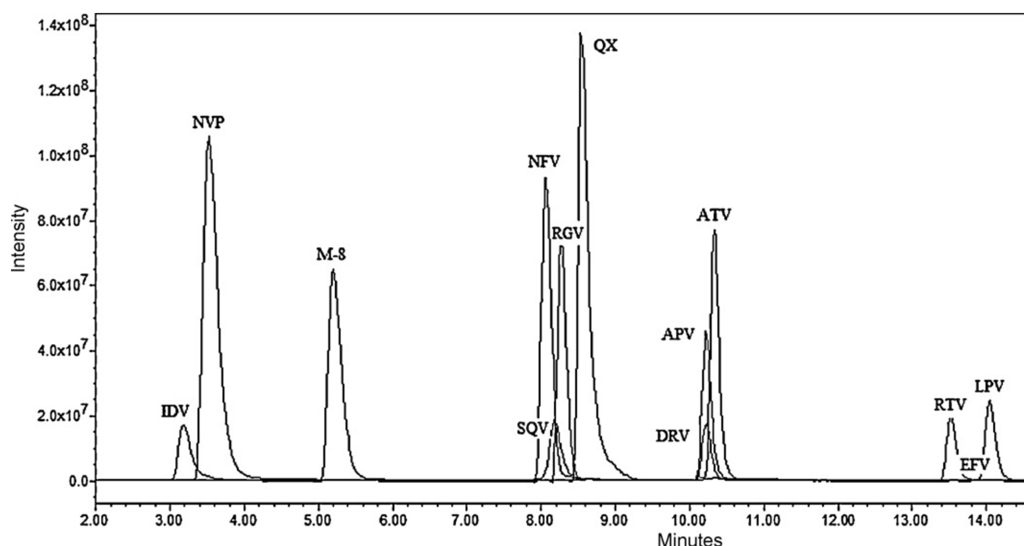

Fig. 2. Chemical mix chromatogram.

organic solution (50:50). This step allowed us to remove all remaining drugs from cellular membrane residue, particularly for the most lipophilic drugs such as lopinavir, etravirine and tipranavir. All these extraction steps allowed us to reach low limit of quantification for all the drugs, below the clinically relevant range of concentration encountered in patients PBMCs contained in a 12–14 mL-blood [33–37,39–42,53,56,65–67].

Our additional aim was to develop and indicate a potentially standard methodology for antiretrovirals drugs intracellular measurement. For this purpose we have chosen as our internal standard quinoxaline, as it is easy to purchase, inexpensive (compared with deuterated drug isotopes) and reliable in its chemical behavior. This xenobiotic was fully used as IS in our validated method [23,24,57,68], with UV and mass detectors. Furthermore, our IS is not deuterated and then cheaper than other analogues used in previous publication [41]. Moreover it is not a potential coadministred drug as was the case in other studies [33,39,40,42].

The extraction procedure and the HPLC–MS method here described allows for the accurate and reproducible simultaneous quantification of fourteen antiretroviral agents in PBMC by a single assay. A low blood volume, a good extraction efficiency and a low limit of quantification make this a suitable method for use in clinical trials and for the study of intracellular concentration of RGV, PIs and NNRTIs, including ETV.

This method, developed and validated following FDA guidelines [61] and according to UNI EN ISO 9001:2000 certification planning rules, is currently being used in our certified laboratory [52], is now being successfully applied for our intracellular routine analysis, coupled with plasmatic therapeutic drug monitoring and pharmacokinetic studies in HIV-infected patients.

Moreover, from our previous data, the observed mean MVC (±CV%) from 20 patients was 281.2 fL (±18.9 min 255.6–max 325.5) [CV% = 6.7] [52], and the mean MVC (±CV%) from 86 patients [200 samples] was 282.5 fL (±22.4 min 232.5–max 341.2) [CV% = 7.9] (see also Fig. 3) [51], respectively. These mean MVC values were around 30% lower than the 400 fL [48], used in the most methodological and clinical publications [33–37,39–42,53,56,65–67]. Use of the latter as a presumptive standard value could significantly bias the methods of quantification, and consequently previous reports could have potentially underestimated intracellular drug exposure. Our latest data (data submitted) confirm previous results [51,52]. Bazzoli et al. highlight in their review that the accuracy of 0.4 pL, as PBMC volume is questionable. The PBMC volume varies according

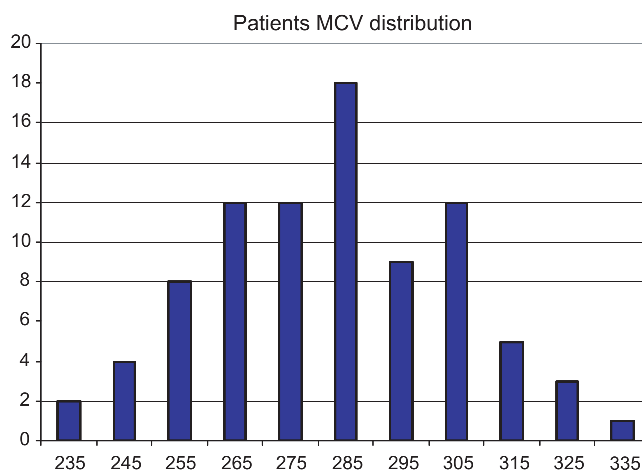

Fig. 3. Distribution of patients MCV. 86 samples were clustered in 11 classes in according to MCV value and their frequencies plotted.
[Data from Simiele et al. 2010. Poster n.62, 11th International Workshop on Clinical Pharmacology of HIV Therapy, Sorrento, Italy].

to the state of the cells (quiescent or stimulated) or the nature of the cells (cell volume of human lymphoblast: around 2.1 pL)[49].

We therefore suggest the calculation of individual MCV as a standard for a more accurate and reliable tool way to quantify intracellular antiretroviral drugs concentrations.

In conclusion, we have fully validated a quantitative assay for the routinely determination of PIs, NNRTIs and RGV in PBMC using a LC-MS system, coupled with individual MCV, which can drive to more reliable understanding of antiretroviral intracellular pharmacokinetics.

References

[1] R.E. Aarnoutse, J.M. Schapiro, C.A.B. Boucher, Y.A. Hekster, D.M. Burger, Therapeutic drug monitoring—an aid to optimising response to antiretroviral drugs? Drugs 63 (2003) 741–753.

[2] R.P.G. van Heeswijk, Critical issues in therapeutic drug monitoring of antiretroviral drugs, Ther. Drug Monit. 24 (2002) 323–331.

[3] C.F. Perno, G. Moyle, C. Tsoukas, W. Ratanasuwan, J. Gatell, M. Schechter, Overcoming resistance to existing therapies in HIV-infected patients: the role of new antiretroviral drugs, J. Med. Virol. 80 (2008) 565–576.

[4] M. Nascimbeni, C. Lamotte, G. Peytavin, R. Farinotti, F. Clavel, Kinetics of antiviral activity and intracellular pharmacokinetics of human immunodefi-

ciency virus type 1 protease inhibitors in tissue culture, Antimicrob. Agents Chemother. 43 (1999) 2629–2634.

[5] L.M. Almond, P.G. Hoggard, D. Edirisinghe, S.H. Khoo, D.J. Back, Intracellular and plasma pharmacokinetics of efavirenz in HIV-infected individuals, J. Antimicrob. Chemother. 56 (2005) 738–744.

[6] L.M. Almond, D. Edirisinghe, M. Dalton, A. Bonington, D.J. Back, S.H. Khoo, Intracellular and plasma pharmacokinetics of nevirapine in human immunodeficiency virus-infected individuals, Clin. Pharm. Ther. 78 (2005) 132–142.

[7] J. Ford, S.H. Khoo, D.J. Back, The intracellular pharmacology of antiretroviral protease inhibitors, J. Antimicrob. Chemother. 54 (2004) 982–990.

[8] S.H. Khoo, P.G. Hoggard, I. Williams, E.R. Meaden, P. Newton, E.G. Wilkins, A. Smith, J.F. Tjia, J. Lloyd, K. Jones, N. Beeching, P. Carey, B. Peters, D.J. Back, Intracellular accumulation of human immunodeficiency virus protease inhibitors, Antimicrob. Agents Chemother. 46 (2002) 3228–3235.

[9] R. Tanaka, H. Hanabusa, E. Kinai, N. Hasegawa, M. Negishi, S. Kato, Intracellular efavirenz levels in peripheral blood mononuclear cells from human immunodeficiency virus-infected individuals, Antimicrob. Agents Chemother. 52 (2008) 782–785.

[10] S. Chaillou, J. Durant, R. Garraffo, P.M. Roger, P. Dellamonica, Intracellular penetration of antiproteases and MDR-1 expression in patients infected with HIV, Medecine et Maladies Infectieuses 32 (2002) 103S–104S.

[11] K. Jones, P.G. Hoggard, S.D. Sales, S. Khoo, R. Davey, D.J. Back, Differences in the intracellular accumulation of HIV protease inhibitors in vitro and the effect of active transport, AIDS 15 (2001) 675–681.

[12] E.R. Meaden, P.G. Hoggard, P. Newton, J.F. Tjia, D. Aldam, D. Cornforth, J. Lloyd, I. Williams, D.J. Back, S.H. Khoo, P-glycoprotein, MRP1 expression and reduced ritonavir and saquinavir accumulation in HIV-infected individuals, J. Antimicrob. Chemother. 50 (2002) 583–588.

[13] J. Wijnholds, C.A.A.M. Mol, L. van Deemter, M. de Haas, G.L. Scheffer, F. Baas, J.H. Beijnen, R.J. Scheper, S. Hatse, E. De Clercq, J. Balzarini, P. Borst, Multidrug-resistance protein 5 is a multispecific organic anion transporter able to transport nucleotide analogs, Proc. Nat. Acad. Sci. U.S.A. 97 (2000) 7476–7481.

[14] F. Becher, A. Pruvost, J. Gale, P. Couerbe, C. Goujard, V. Boutet, E. Ezan, J. Grassi, H. Benech, A strategy for liquid chromatography/tandem mass spectrometric assays of intracellular drugs: application to the validation of the triphosphorylated anabolite of antiretrovirals in peripheral blood mononuclear cells, J. Mass Spectrom. 38 (2003) 879–890.

[15] F. Becher, R. Landman, S. Mboup, C.N.T. Kane, A. Canestri, F. Liegeois, M. Vray, M.H. Prevot, G. Leleu, H. Benech, Monitoring of didanosine and stavudine intracellular trisphosphorylated anabolite concentrations in HIV-infected patients, AIDS 18 (2004) 181–187.

[16] F. Becher, A. Pruvost, C. Goujard, C. Guerreiro, J.F. Delfraissy, J. Grassi, H. Benech, Improved method for the simultaneous determination of d4T, 3TC and ddl intracellular phosphorylated anabolites in human peripheral-blood mononuclear cells using high-performance liquid chromatography/tandem mass spectrometry (vol 16, pg 555–565, 2002), Rapid Commun. Mass Spectrom. 21 (2007) 2168.

[17] J.D. Chi, A. Jayewardene, J. Stone, J.G. Gambertoglio, F.T. Aweeka, A direct determination of thymidine triphosphate concentrations without dephosphorylation in peripheral blood mononuclear cells by LC/MS/MS, J. Pharm. Biomed. Anal. 26 (2001) 829–836.

[18] Y. Huang, E. Zurlinden, E. Lin, X.H. Li, J. Tokumoto, J. Golden, A. Murr, J. Engstrom, J. Conte, Liquid chromatographic-tandem mass spectrometric assay for the simultaneous determination of didanosine and stavudine in human plasma, bronchoalveolar lavage fluid, alveolar cells, peripheral blood mononuclear cells, seminal plasma, cerebrospinal fluid and tonsil tissue, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 799 (2004) 51–61.

[19] J.D Moore, G. Valette, A. Darque, X.J. Zhou, J.P. Sommadossi, Simultaneous quantitation of the 5'-triphosphate metabolites of zidovudine, lamivudine, and stavudine in peripheral mononuclear blood cells of HIV infected patients by high-performance liquid chromatography tandem mass spectrometry, J. Am. Soc. Mass Spectrom. 11 (2000) 1134–1143.

[20] A. Pruvost, F. Theodoro, L. Agrofoglio, E. Negred, H. Benech, Specificity enhancement with LC-positive ESI-MS/MS for the measurement of nucleotides: application to the quantitative determination of carbovir triphosphate, lamivudine triphosphate and tenofovir diphosphate in human peripheral blood mononuclear cells, J. Mass Spectrom. 43 (2008) 224–233.

[21] J.D Chi, A.L. Jayewardene, J.A. Stone, T. Motoya, F.T. Aweeka, Simultaneous determination of five HIV protease inhibitors nelfinavir, indinavir, ritonavir, saquinavir and amprenavir in human plasma by LC/MS/MS, J. Pharm. Biomed. Anal. 30 (2002) 675–684.

[22] K.M.L. Crommentuyn, H. Rosing, L.G.A.H. Nan-Offeringa, M.J.X. Hillebrand, A.D.R. Huitema, J.H. Beijnen, Rapid quantification of HIV protease inhibitors in human plasma by high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry, J. Mass Spectrom. 38 (2003) 157–166.

[23] A. D'Avolio, M. Siccardi, M. Sciandra, B. Lorena, S. Bonora, L. Trentini, G. Di Perri, HPLC–MS method for the simultaneous quantification of the new HIV protease inhibitor darunavir, and 11 other antiretroviral agents in plasma of HIV-infected patients, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 859 (2007) 234–240.

[24] A D'Avolio, M. Simiele, M. Siccardi, L. Baietto, M. Sciandra, S. Bonora, P.G. Di, HPLC–MS method for the quantification of nine anti-HIV drugs from dry plasma spot on glass filter and their long term stability in different conditions, J. Pharm. Biomed. Anal. (2010).

[25] A. Fayet, A. Beguin, B. Zanolari, S. Cruchon, N. Guignard, A. Telenti, M. Cavassini, H.F. Gunthard, T. Buclin, J. Biollaz, B. Rochat, L.A. Decosterd, A LC-tandem MS assay for the simultaneous measurement of new antiretroviral agents: Raltegravir, maraviroc, darunavir, and etravirine, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 877 (2009) 1057–1069.

[26] V.A. Frerichs, R. DiFrancesco, G.D. Morse, Determination of protease inhibitors using liquid chromatography-tandem mass spectrometry, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 787 (2003) 393–403.

[27] S. Gunawan, M.P. Griswold, D.G. Kahn, Liquid chromatographic-tandem mass spectrometric determination of amprenavir (agenerase) in serum/plasma of human immunodeficiency virus type-1 infected patients receiving combination antiretroviral therapy, J. Chromatogr. A 914 (2001) 1–4.

[28] T. Koal, H. Burhenne, R. Romling, M. Svoboda, K. Resch, V. Kaever, Quantification of antiretroviral drugs in dried blood spot samples by means of liquid chromatography/tandem mass spectrometry, Rapid Commun. Mass Spectrom. 19 (2005) 2995–3001.

[29] S. Quaranta, C. Woloch, A. Paccou, M. Giocanti, C. Solas, B. Lacarelle, Validation of an electrospray ionization LC-MS/MS method for quantitative analysis of raltegravir, etravirine, and 9 other antiretroviral agents in human plasma samples, Ther. Drug Monit. 31 (2009) 695–702.

[30] K.M. Rentsch, Sensitive and specific determination of eight antiretroviral agents in plasma by high-performance liquid chromatography-mass spectrometry, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 788 (2003) 339–350.

[31] A. Schuster, S. Burzawa, M. Jemal, E. Loizillon, P. Couerbe, D. Whigan, Quantitative determination of the HIV protease inhibitor atazanavir (BMS-232632) in human plasma by liquid chromatography-tandem mass spectrometry following automated solid-phase extraction, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 788 (2003) 377–386.

[32] P.G. Wang, J.S. Wei, G. Kim, M. Chang, T. El-Shourbagy, Validation and application of a high-performance liquid chromatography-tandem mass spectrometric method for simultaneous quantification of lopinavir and ritonavir in human plasma using semi-automated 96-well liquid–liquid extraction, J. Chromatogr. A 1130 (2006) 302–307.

[33] S. Colombo, A. Beguin, A. Telenti, J. Biollaz, T. Buclin, B. Rochat, L.A. Decosterd, Intracellular measurements of anti-HIV drugs indinavir, amprenavir, saquinavir, ritonavir, nelfinavir, lopinavir, atazanavir, efavirenz and nevirapine in peripheral blood mononuclear cells by liquid chromatography coupled to tandem mass spectrometry, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 819 (2005) 259–276.

[34] S Djabarouti, D. Breilh, I. Pellegrin, M. Lavit, F. Camou, O. Caubet, H. Fleury, M.C. Saux, J.L. Pellegrin, Intracellular and plasma efavirenz concentrations in HIV-infected patients switching from successful protease inhibitor-based highly active antiretroviral therapy (HAART) to efavirenz-based HAART (SUSTIPHAR Study), J. Antimicrob. Chemother. 58 (2006) 1090–1093.

[35] J. Ford, M. Boffito, A. Wildfire, A. Hill, D. Back, S. Khoo, M. Nelson, G. Moyle, B. Gazzard, A. Pozniak, Intracellular and plasma pharmacokinetics of saquinavir-ritonavir, administered at 1600/100 milligrams once daily in human immunodeficiency virus-infected patients, Antimicrob. Agents Chemother. 48 (2004) 2388–2393.

[36] M. Hennessy, S. Clarke, J.P. Spiers, F. Mulcahy, D. Kelleher, E. Megdon, B. Maher, C. Bergin, S. Khoo, J. Tjia, P. Hoggard, D. Back, M. Barry, Intracellular indinavir pharmacokinetics in HIV-infected patients: comparison with plasma pharmacokinetics, Antiviral Ther. 8 (2003) 191–198.

[37] M. Hennessy, S. Clarke, J.P. Spiers, D. Kelleher, F. Mulcahy, P. Hoggard, D. Back, M. Barry, Intracellular accumulation of nelfinavir and its relationship to P-glycoprotein expression and function in HIV-infected patients, Antiviral Ther. 9 (2004) 115–122.

[38] M. Jemal, S. Rao, M. Gatz, D. Whigan, Liquid chromatography-tandem mass spectrometric quantitative determination of the HIV protease inhibitor atazanavir (BMS-232632) in human peripheral blood mononuclear cells (PBMC): practical approaches to PBMC preparation and PBMC assay design for high-throughput analysis, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 795 (2003) 273–289.

[39] H.R. Ter, M. Davids, H. Rosing, E.C. van Gorp, J.W. Mulder, Y.T. van der Heide, J.H. Beijnen, A.D. Huitema, Quantification of HIV protease inhibitors and non-nucleoside reverse transcriptase inhibitors in peripheral blood mononuclear cell lysate using liquid chromatography coupled with tandem mass spectrometry, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 877 (2009) 575–580.

[40] H.R. Ter, H. Rosing, E.C. van Gorp, J.W. Mulder, J.H. Beijnen, A.D. Huitema, Quantification of etravirine (TMC125) in plasma, dried blood spots and peripheral blood mononuclear cell lysate by liquid chromatography tandem mass spectrometry, J. Pharm. Biomed. Anal. 49 (2009) 393–400.

[41] L. Elens, S. Veriter, J.C. Yombi, V. Di Fazio, R. Vanbinst, D. Lison, P. Wallemacq, B. Vandercam, V. Haufroid, Validation and clinical application of a high performance liquid chromatography tandem mass spectrometry (LC-MS/MS) method for the quantitative determination of 10 anti-retrovirals in human peripheral blood mononuclear cells, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 877 (2009) 1805–1814.

[42] H.R. Ter, M.J. Hillebrand, H. Rosing, E.C. van Gorp, J.W. Mulder, J.H. Beijnen, A.D. Huitema, Quantification of the HIV-integrase inhibitor raltegravir and detection of its main metabolite in human plasma, dried blood spots and peripheral blood mononuclear cell lysate by means of high-performance liquid chromatography tandem mass spectrometry, J. Pharm. Biomed. Anal. 49 (2009) 451–458.

[43] S. Azoulay, M.C. Nevers, C. Creminon, L. Heripret, J. Durant, P. Dellamonica, J. Grassi, R. Guedj, D. Duval, Sensitive enzyme immunoassay for measuring

plasma and intracellular nevirapine levels in human immunodeficiency virus-infected patients, Antimicrob. Agents Chemother. 48 (2004) 104–109.
[44] AzoulayF S., M.C. Nevers, C. Creminon, L. Heripret, R. Garraffo, J. Durant, P. Dellamonica, J. Grassi, R. Guedj, D. Duval, An enzyme immunoassay for the quantification of plasma and intracellular lopinavir in HIV-infected patients, J. Immunol. Methods 295 (2004) 37–48.
[45] C. Roucairol, S. Azoulay, M.C. Nevers, C. Creminon, T. Lavrut, R. Garraffo, J. Grassi, A. Burger, D. Duval, Quantitative immunoassay to measure plasma and intracellular atazanavir levels: Analysis of drug accumulation in cultured T cells, Antimicrob. Agents Chemother. 51 (2007) 405–411.
[46] F. Akeb, B. Ferrua, C. Creminon, C. Roptin, J. Grassi, M.C. Nevers, R. Guedj, R. Garraffo, D. Duval, Quantification of plasma and intracellular levels of the HIV protease inhibitor ritonavir by competitive ELISA, J. Immunol. Methods 263 (2002) 1–9.
[47] J.J.A. van Kampen, P.C. Burgers, R.A. Gruters, A.D.M.E. Osterhaus, R. de Groot, T.M. Luider, D.A. Volmer, Quantitative analysis of antiretroviral drugs in lysates of peripheral blood mononuclear cells using MALDI-triple quadrupole mass spectrometry, Anal. Chem. 80 (2008) 4969–4975.
[48] W.Y. Gao, A. Cara, R.C. Gallo, F. Lori, Low levels of deoxynucleotides in peripheral blood lymphocytes: a strategy to inhibit human immunodeficiency virus type 1 replication, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 8925–8928.
[49] C. Bazzoli, V. Jullien, T.C. Le, E. Rey, F. Mentre, A.M. Taburet, Intracellular pharmacokinetics of antiretroviral drugs in HIV-infected patients, and their correlation with drug action, Clin. Pharmacokinet. 49 (2010) 17–45.
[50] H. Benech, F. Theodoro, A. Herbet, N. Page, D. Schlemmer, A. Pruvost, J. Grassi, J.R. Deverre, Peripheral blood mononuclear cell counting using a DNA-detection-based method, Anal. Biochem. 330 (2004) 172–174.
[51] M. Simiele, A. D'Avolio, M. Siccardi, L. Baietto, F.R. Stefani, S. Agati, J. Cusato, M. Sciandra, S. Bonora, G. Di Perri, Are intracellular drug concentrations understimated? The role of medium corpuscolar volume (MCV) for a correct determination, 11th International Workshop on Clinical Pharmacology of HIV Therapy, Rev. Antiviral Ther. (2010).
[52] TDM Torino Web-www.tdm-torino.org. THERAPEUTIC DRUG MONITORING - WEB SITE; Laboratory of Clinical Pharmacology and Pharmacogenetics. University of Turin/ASL TO2. 30-4-2010.
[53] J. Ford, D. Cornforth, P.G. Hoggard, Z. Cuthbertson, E.R. Meaden, I. Williams, M. Johnson, E. Daniels, P. Hsyu, D.J. Back, S.H. Khoo, Intracellular and plasma pharmacokinetics of nelfinavir and M8 in HIV-infected patients: relationship with P-glycoprotein expression, Antiviral Ther. 9 (2004) 77–84.
[54] M. Ehrhardt, M. Mock, W.E. Haefeli, G. Mikus, J. Burhenne, Monitoring of lopinavir and ritonavir in peripheral blood mononuclear cells plasma, and ultrafiltrate using a selective and highly sensitive LC/MS/MS assay, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 850 (2007) 249–258.
[55] J.J.A. van Kampen, P.C. Burgers, R. de Groot, T.M. Luider, Qualitative and quantitative analysis of pharmaceutical compounds by MALDI-TOF mass spectrometry, Anal. Chem. 78 (2006) 5403–5411.
[56] K.M.L. Crommentuyn, J.W. Mulder, A.T.A. Mairuhu, E.C.M. van Gorp, P.L. Meenhorst, A.D.R. Huitema, J.H. Beijnen, The plasma and intracellular steady-state pharmacokinetics of lopinavir/ritonavir in HIV-1-infected patients, Antiviral Ther. 9 (2004) 779–785.
[57] A. D'Avolio, L. Baietto, M. Siccardi, M. Sciandra, M. Simiele, V. Oddone, S. Bonora, P.G. Di, An HPLC-PDA method for the simultaneous quantification of the HIV integrase inhibitor raltegravir, the new nonnucleoside reverse transcriptase inhibitor etravirine, and 11 other antiretroviral agents in the plasma of HIV-infected patients, Ther. Drug Monit. 30 (2008) 662–669.
[58] C. Marzolini, A. Beguin, A. Telenti, A. Schreyer, T. Buclin, J. Biollaz, L.A. Decosterd, Determination of lopinavir and nevirapine by high-performance liquid chromatography after solid-phase extraction: application for the assessment of their transplacental passage at delivery, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 774 (2002) 127–140.
[59] M.L. Turner, K. Reed-Walker, J.R. King, E.P. Acosta, Simultaneous determination of nine antiretroviral compounds in human plasma using liquid chromatography, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 784 (2003) 331–341.
[60] P.J. Taylor, Matrix effects: The Achilles heel of quantitative high-performance liquid chromatography-electrospray-tandem mass spectrometry, Clin. Biochem. 38 (2005) 328–334.
[61] Center for Drug Evaluation and Research of the U.S. Department of Health and Human Services Food and Drug Administration, Guidance for Industry Bioanalytical Method Validation. FDA. www.fda.gov. 2001.
[62] H.R Ter, C.G. rden-Los, H. Rosing, M.J. Hillebrand, E.C. van Gorp, A.D. Huitema, J.H. Beijnen, Fast and simultaneous determination of darunavir and eleven other antiretroviral drugs for therapeutic drug monitoring: method development and validation for the determination of all currently approved HIV protease inhibitors and non-nucleoside reverse transcriptase inhibitors in human plasma by liquid chromatography coupled with electrospray ionization tandem mass spectrometry, Rapid Commun. Mass Spectrom. 21 (2007) 2505–2514.
[63] L. Goldwirt, S. Chhun, E. Rey, O. Launay, J.P. Viard, G. Pons, V. Jullien, Quantification of darunavir (TMC114) in human plasma by high-performance liquid chromatography with ultra-violet detection, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 857 (2007) 327–331.
[64] M. Takahashi, Y. Kudaka, N. Okumura, A. Hirano, K. Banno, T. Kaneda, The validation of plasma darunavir concentrations determined by the HPLC method for protease inhibitors, Biol. Pharm. Bull. 30 (2007) 1947–1949.
[65] J. Ford, M. Boffito, D. Maitland, A. Hill, D. Back, S. Khoo, M. Nelson, G. Moyle, B. Gazzard, A. Pozniak, Influence of atazanavir 200 mg on the intracellular and plasma pharmacokinetics of saquinavir and ritonavir 1600/100 mg administered once daily in HIV-infected patients, J. Antimicrob. Chemother. 58 (2006) 1009–1016.
[66] D. Breilh, I. Pellegrin, A. Rouzes, K. Berthoin, F. Xuereb, H. Budzinski, M. Munck, H.J.A. Fleury, M.C. Saux, J.L. Pellegrin, Virological, intracellular and plasma pharmacological parameters predicting response to lopinavir/ritonavir (KALEPHAR Study), AIDS 18 (2004) 1305–1310.
[67] S. Colombo, A. Telenti, T. Buclin, H. Furrer, B.L. Lee, J. Biollaz, L.A. Decosterd, Are plasma levels valid surrogates for cellular concentrations of antiretroviral drugs in HIV-infected patients? Ther. Drug Monit. 28 (2006) 332–338.
[68] A. D'Avolio, M. Sciandra, M. Siccardi, L. Baietto, R.D. Gonzalez de, S. Bonora, P.G. Di, A new assay based on solid-phase extraction procedure with LC-MS to measure plasmatic concentrations of tenofovir and emtricitabine in HIV infected patients, J. Chromatogr. Sci. 46 (2008) 524–528.