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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

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ABSTRACT

This paper reports the validation of a liquid chromatography tandem mass spectrometry (LC-MS/MS) method that allows the quantification of 10 antiretroviral (ARV) drugs in peripheral blood mononuclear cells (PBMCs) using 6 different isotopic internal standards (IS) and its clinical application. PBMCs are isolated from blood by density gradient centrifugation and drugs are extracted with a 60% methanol (MeOH) solution containing the 6 IS. The cell extract is then injected in the HPLC system and analytes are separated on a Symmetry Shield RP18 2.1 mm × 50 mm column. The different molecules are then detected by MS/MS in electrospray positive or negative ionisation modes and data are recorded using the multiple reaction monitoring (MRM) mode. Calibration curves are constructed in the range of 0.25–125 ng/ml of cell extract by a $1/x^2$ weighted quadratic regression. The regression coefficients obtained are always greater than 0.99 and back calculated values always comprised in the range of ±15% from their nominal concentration. Mean extraction recoveries are greater than 80% for all analytes and the method is accurate and precise with CV and bias lower than 9.4%. The lower limits of quantification (LLOQ) of the different drugs range from 0.0125 to 0.2 ng/ml of cell extract. This method was successfully applied to a cohort of 98 HIV-infected patients treated with Kaletra® (400/100 mg of lopinavir/ritonavir (LPV/RTV) twice a day, $n = 48$) or with Stocrin® (600 mg once a day, $n = 50$) and has been tested for cellular quantification of tipranavir (TPV) in 2 patients treated with Aptivus® (500 mg twice a day). The patients treated by Kaletra® showed mean cell-associated concentrations (CC) of 1819.0 and 917.2 ng/ml, for LPV and RTV, respectively. Patients treated with Stocrin® showed mean CC of 2388.11 ng/ml while both patients under Aptivus® showed TPV CC of 4322.7 and 1078.0 ng/ml, respectively. This method can be used to analyze ARV drug concentrations within the target tissue.

1. Introduction

Analytical tools for monitoring the plasma concentrations of anti-HIV drugs are largely available in clinical settings and have already contributed to improve anti-HIV therapy through the means of therapeutic drug monitoring (TDM). Despite these advances, failures of anti-HIV therapy are still frequently encountered in daily clinical practice and the reasons for these failures are not always understood. A possible explanation could be that measuring plasma drug concentration is of limited clinical value because the major target of these drugs is within the infected cells and only the fraction reaching this intracellular compartment is expected to be active against HIV replication. In this respect, several studies have, however, shown a good correlation between intracellular and plasma drug concentrations for several protease inhibitors (PIs) [1–3] but not for NNRTIs [3,4], suggesting that plasma concentration could constitute a valid parameter for the TDM of PIs but not, or at least less accurately, for NNRTIs. Direct measurement of intracellular drug concentrations may therefore contribute to improve and adjust antiretroviral therapy. A method to quantify the active fraction of these drugs would allow to better characterize the pharmacokinetics (PK) and the accumulation profile of these drugs in the cellular compartment and, would possibly give a better insight into understanding the reasons for some therapeutic

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failures. Also, it would allow to investigate the determinants of the variability in intracellular concentrations and the potential discrepancies between plasma and intracellular concentrations.

To date, only few LC-MS/MS methods are fully validated for the intracellular quantification of PIs and/or NNRTIs [5–9], in comparison to the number of validated LC-MS/MS methods available for the quantitative determination of PIs and NNRTIs in plasma [10–21]. Most intracellular quantification methods are only partially described in clinical reports [1,2,22–27]. Furthermore, among fully validated methods, only two allow the simultaneous intracellular quantification of all commercially available PIs and NNRTIs, except tipranavir [5] and atazanavir [8].

The present paper reports an improved LC-MS/MS method using isotopic internal standards allowing the intracellular quantification of the relatively new PI, tipranavir (TPV).

2. Experimental

2.1. Chemicals, biologicals and reagents

The drugs investigated were amprenavir (APV), atazanavir (ATZ), efavirenz (EFV), indinavir (IDV), lopinavir (LPV), nelfinavir (NFV), nevirapine (NVP), ritonavir (RTV), saquinavir (SQV) and tipranavir (TPV). APV and ATZ were kindly provided by GlaxoSmith Kline Research and development (Middelsex, UK) and Bristol-Myers and Squibb (New Brunswick, NJ, USA), respectively. NFV and SQV were kindly provided by Roche diagnostics (Mannheim, Deutschland), and IDV and EFV by Merck (NJ, USA). LPV and RTV were kindly provided by Abbott Laboratories (Chicago, USA). NVP and TPV were kindly provided by Boehringer and Ingelheim (Ridgefield, USA). IDV-d6 and SQV-d5 were kindly provided by Merck and Roche diagnostics, respectively. Other isotopic internal standards (EFV-d4, LPV-d8, ATZ-d5 and RTV-^{13}C13) were purchased from Toronto Research Chemicals (North York, Ontario, Canada).

HPLC grade acetonitrile (ACN) and methanol (MeOH) were purchased from Biosolve (Valkenswaard, The Netherlands). Ammonium acetate and acetic acid were obtained from Merck (Germany), formic acid from Sigma–Aldrich (Germany) and ficoll-Paque™ Plus solution from Amersham Biosciences AB (Uppsala, Sweden). The Dulbecco's phosphate buffered saline (DPBS) solution and foetal bovine serum (FBS) were obtained from Invitrogen.

Blank PBMCs used for calibration curves and quality control (QC) samples and for the assessment of matrix effect were isolated from leucodepletion filters (Leucoflex LCR, Macopharma) kindly provided by the blood transfusion centre of Namur (Belgium).

PBMCs count was performed on a Sysmex K-1000 haematology analyzer (Norderstedt, Germany).

2.2. LC-MS/MS system and chromatographic conditions

The HPLC system consisted of a Waters 2795 Alliance High Throughput HPLC system with an integrated autosampler (Waters, Mildford, MA, USA) thermostated at 10 °C. The chromatographic separation was performed on a Symmetry shield RP18, 2.1 mm × 50 mm column (Waters) applied in an oven maintained at 25 °C. The chromatographic system was coupled with a Quattro *micro*™ tandem mass spectrometer (Micromass UK Ltd., Manchester, UK) fitted with a Z-Spray™ ion source. The instrument was operated in both electrospray positive and negative ionisation modes. All aspects of system operation and data acquisition were controlled by a *MassLynx NT*™ v3.5 software (Micromass, Manchester, UK) and data processing was performed with the *QuanLynx*™ Application Manager (Micromass). Data were recorded in the multiple reaction monitoring (MRM) mode.

The mobile phase was delivered in the column with a start flow rate of 0.3 ml/min. Eluent A consisted of 10 mM ammonium acetate/10 mM formic acid and eluent B was ACN/10 mM formic acid. From 0 to 2 min, the mobile phase consisted of 95% of eluent A and 5% of B. A linear elution gradient was set to reach 10% of A and 90% of B at 12 min followed by an accelerated rinsing with 100% of B at a flow rate of 0.5 ml/min. from 12 to 15 min and of 0.4 ml/min till 18 min. Initial conditions were restored in 2 min and maintained during 5 min for re-equilibration.

The mass spectrometer was operated in electrospray positive and negative ionisation modes for PI/NVP and EFV, respectively. Consequently, the mass of the precursor ions corresponded to M + 1 and M − 1 for PI/NVP and EFV, respectively. Tuning of the MS/MS detector was performed by direct injection of compounds with a syringe at concentration of 1000 ng/ml in 50:50 ACN/H_2O (10 mM formic Acid). Optimized detection parameters are reported in Table 1. Three distinct windows of acquisition were programmed in the positive mode and one in the negative mode. The source and capillary temperature were maintained at 120 and 300 °C, respectively, while the capillary voltage was set at 3.5 kV. The collision gas was argon, and pressure was monitored at 2.5×10^{-6} bar in the collision cell. The nebulising, and cone gas was nitrogen and set at flow rates of 550 and 20 l/h, respectively.

2.3. Stock solutions, calibrators and quality controls (QC)

All drugs and internal standards were solubilised in methanol at a concentration of 1 mg/ml (stock solution) and kept at −80 °C. A working solution of the six internal standards at a concentration of 4000 ng/ml was prepared in 60:40 MeOH:H_2O. Similarly, a working solution containing all drugs at a concentration of 1,250 ng/ml was prepared in 60:40 MeOH:H_2O by appropriately diluting the stock solutions.

These working solutions were then diluted in 60:40 MeOH:H_2O to obtain 8 calibrators (0.25; 0.625; 1.25; 6.25; 12.5; 62.5; 93.75; 125 ng/ml for all drugs) and 3 QC solutions (2.5, 25 and 75 ng/ml) containing all 6 IS (IDV-d6, SQV-d5, EFV-d4, LPV-d8, ATZ-d5 and RTV-^{13}C13) at a concentration of 20 ng/ml. The extraction solvent consisted of a 60:40 MeOH:H_2O solution containing the 6 IS at 20 ng/ml.

2.4. Blank and patients PBMCs preparation and drug extraction procedure

2.4.1. Preparation of blank PBMCs isolated from leucodepletion filters

This method was adapted from [5] with slight adaptations. Leucodepletion filters were washed with 30 ml DPBS supplemented with 2% FBS. PBMCs were then isolated from total leucocytes population by Ficoll density gradient separation (Ficoll-Paque™ Plus). Three washing steps were performed with DPBS solution. PBMCs were quantified by cell counting on a burker cell with Trypan blue coloration and then, aliquoted in 1.5 ml Eppendorf to obtain typically between 3 and 8×10^6 cells/vial. Finally, the vials were centrifuged at 650 × *g*, the supernatant was discarded and PBMCs were immediately stored at −20 °C.

2.4.2. Collection of PBMCs from patients

The patients were recruited at the Saint-Luc Hospital (Brussels) and the samples were drawn together with those collected for routine clinical follow-up. The protocol of the present study has been approved by the local ethical committee and written informed consent was obtained from each patient. 48 patients receiving Kaletra® (LPV/RTV 400/100 mg twice daily) associated with 2 NRTIs (3TC + AZT *n* = 7; 3TC + ABC *n* = 23; 3TC + d4T *n* = 1) or tenofovir plus one NRTI (3TC *n* = 15; emtricitabine *n* = 1) or 3NRTIs (3TC + AZT + d4T *n* = 1) were recruited for the present study between July 2007 and January 2008. These patients did not receive another

Table 1
Optimized detection parameters, acquisition windows and retention times of all analytes.

Compound	Selected IS	Precursor ion	Product ion	Collision energy (eV)	Cone voltage (V)	Ionisation mode	Acquisition window	Retention time (min)
NVP	ATZ-d5	267.14	226.24	25	40	+	1	6.97
IDV	IDV-d6	614.29	421.38	35	40	+	1	8.11
IDV-d6	–	620.32	421.46	35	40	+	1	8.11
SQV	SQV-d5	671.40	570.42	30	45	+	2	8.88
SQV-d5	–	676.34	575.47	35	45	+	2	8.88
NFV	IDV-d6	568.12	330.26	30	40	+	2	9.46
APV	ATZ-d5	506.10	245.36	15	20	+	3	9.53
ATZ	ATZ-d5	705.39	168.15	45	40	+	3	10.11
ATZ-d5	–	710.31	168.16	45	40	+	3	10.11
RTV	RTV-^{13}C13	721.31	296.25	20	25	+	3	10.31
RTV-^{13}C13	–	724.28	296.30	20	25	+	3	10.31
LPV	LPV-d8	629.32	447.32	15	20	+	3	10.50
LPV-d8	–	637.14	447.49	15	20	+	3	10.50
EFV	EFV-d4	313.92	244.16	15	30	–	–	10.90
EFV-d4	–	317.91	248.14	15	30	–	–	10.90
TPV	ATZ-d5	602.94	411.18	20	25	+	3	11.88

PI or a NNRTI. 50 patients receiving Stocrin® associated with 2 NRTIs (3TC + AZT $n = 5$; 3TC + ABC $n = 22$; 3TC + ddI $n = 2$) or 3NRTIs (3TC + AZT + d4T $n = 1$) or tenofovir plus one NRTI (3TC $n = 14$) or plus 2 NRTIs (3TC + ABC $n = 4$), or receiving tenofovir associated with 3TC and 1 PI (atazanavir $n = 1$ or LPV/r $n = 1$) were recruited for the present study between July 2007 and January 2009. The general characteristics of the patients receiving Kaletra® and Stocrin® are listed in Table 2. The mean post-intake delay for both cohorts was 13h40 and 16h30, respectively. The present population study also included 2 patients receiving Aptivus® (TPV 500 mg twice daily) associated with RTV (200 mg twice daily), maraviroc (2 × 150 mg twice daily) and a NRTI (3TC, 150 mg twice daily) or tenofovir (245 mg once daily). One of the two patients was receiving raltegravir (400 mg twice daily). The post-intake delays for both patients were 3h10 and 12h50, respectively.

Approximately 8 ml of blood from HIV-infected patients were collected in heparin vacutainer Cell Preparation Tubes (CPT, Becton Dickinson) and PBMCs were separated according to manufacturer instructions. The thin mononuclear layer was collected with a polypropylene Pasteur pipette and transferred to a 15 ml polypropylene tube pre-chilled on ice. The volume was completed to 15 ml with cold DPBS to block enzymatic activity and to avoid active transport out of the cells. The PBMCs were homogeneously re-suspended and then centrifuged at 650 × g for 10 min at 4 °C. The supernatant was discarded and these steps were repeated twice. The final cell pellet was re-suspended in 1 ml of DPBS and transferred in a 1.5 ml Eppendorf. A 20-μl aliquot was diluted in 180 μl of DPBS and brought within 10 min to the Sysmex apparatus for cell counting. The remaining 980 μl was centrifuged at 650 × g for 10 min at 4 °C, the supernatant was discarded, and the pellet was stored immediately at −80 °C until analysis.

2.4.3. Drug extraction procedure

Before drug extraction, all solutions were equilibrated at room temperature. Patient and blank PBMCs for the calibration curves were thawed in parallel. The extraction solvent consisted of 60:40 MeOH:H_2O as proposed in the literature [1,2,7,25,26,28,29]. 400 μl of extraction solvent was added to the patient's PBMCs while 400 μl of calibrators at the concentrations reported above was added to blank PBMCs, for the calibration curve. After vortex-mixing during few seconds, the cells were placed during 5 min in an ultrasound bath for cell lysis and subsequently disposed on a horizontal shaker at 300 rpm for overnight extraction. Finally, samples were centrifuged (20,000 × g, 10 min), the supernatant was transferred in a HPLC vial and 10 μl (20 μl for EFV) were injected on the column.

2.5. Analytical validation

The analytical validation was based on the recommendations of the Food and Drug Administration (FDA) [30].

2.5.1. Calibration curves

A total of 15 calibration curves (three calibration curves on five separate days) were run during the validation procedure. Each calibration curve was obtained using eight calibration points (see above). The calibration curves were fitted with a quadratic least square regression of the peak area ratio for each compound to IS versus the nominal concentration of the sample with a weighting factor of 1/concentration^2 ($1/x^2$). The selection of the weighting factor has been made by assessing the back calculated values of each standard and through the examination of the residual plots.

2.5.2. Precision, accuracy and lower limit of quantification

Accuracy was evaluated by the within and between day deviation of the calculated concentration from the nominal concentration of the sample and precision was estimated by the within and between day coefficient of variation (CV). Precision and accuracy were determined by multiple replicate ($n = 5$) at the three QC concentrations.

The lower limit of quantification (LLOQ) was determined by quantifying blank PBMCs spiked with decreasing concentrations of drugs and defined as the lowest concentration for which the

Table 2
General characteristics of the study population.

	Kaletra®	Stocrin®
Mean duration of therapy (range)	26 months (3–58)	51 months (2–113)
Age (mean ± SD)	44.0 ± 1.4 years	48.5 ± 1.5
Weight (Kg) (mean ± SD)	73.7 ± 1.6	69.3 ± 1.8
Gender	♀ = 14 ♂ = 34	♀ = 12 ♂ = 38
CD4 cell count (cells/mm^3) (mean ± SD)	479.6 ± 30.2	534.6 ± 30.1
Ethnic origin	Afr = 25 Cau = 21 Mag = 2	Afr = 14 Cau = 31 Mag = 2 As = 3

Afr = African; Cau = Caucasian; Mag = Maghrebi; As = Asiatic.

deviation from the nominal concentration and the imprecision (CV) did not exceed 20%.

2.5.3. Recovery and matrix effect

The matrix effect was examined first by post-column infusion. A blank PBMCs extract was injected in the LC-MS/MS system simultaneously with an infusion of PI/NNRTI or SI at 2 different concentrations (20 and 100 ng/ml) directly in the mass spectrometer at a flow rate of 10 μl/min. The chromatogram for each MS/MS transition was then recorded and examined to check for potential perturbation of the signal at the retention time of the analyte.

The quantitative assessment of the matrix effect has been performed at the 3 QC levels (2.5, 25 and 75 ng/ml). It was estimated as the ratio of the signal provided by a blank PBMCs extract spiked with drugs after extraction on the signal provided by a pure QC solution.

Extraction recovery (ER) was calculated as the ratio of the peak area of a processed QC sample on the peak area of a processed blank PBMCs spiked with drugs after extraction. These analyses were performed in triplicate and blank PBMC were obtained from different batches for each replicate.

2.5.4. Selectivity

The selectivity has been assessed according to the FDA guidelines by analyses of blank PBMCs from 7 different batches.

2.6. Determination of cell-associated concentration (CC)

Once the concentration in the cell extract was determined on calibration curves, the absolute amount of drug (ng) present in extracted cells was determined. This amount was then reported on the number of cells (in million) and the CC (ng/ml) was determined assuming that the mean volume of a PBMC is 0.4 pl [31].

2.7. Determination of plasma drug concentrations

Blood samples were obtained from HIV-infected patients on heparinised Starstedt tubes simultaneously with the CPT tubes. These samples were centrifuged at 4 °C at 1125 × *g* for 10 min and plasma was collected. 1 ml of plasma was used for drug extraction and quantification according to our previously validated method using very high-pressure liquid chromatography coupled with diode array detection (DAD) [32].

2.8. Statistical analysis

Statistical analyses were performed using the SPSS package, version 16.0 for Windows (Chicago, Illinois, USA).

To assess the possible influence of gender and ethnicity on the different PK parameters, the unpaired Student *t*-test and one-way analyses of variance on continuous (accumulation ratio) or log transformed (TPC and CC) values were used. In all cases, difference between groups was assumed if $p < 0.05$.

Multiple linear regressions were performed to assess the contribution of ethnicity and gender on the studied PK parameters. For these multiple linear regression analyses, each variable was coded as a distinct "dummy variable". When appropriate, significant covariates of PK parameters were traced by a stepwise regression procedure with $p < 0.05$ being considered statistically significant for entry and $p < 0.10$ for staying in the model.

3. Results and discussion

The method described in the present paper enables the quantification of 8 PI and 2 NNRTI in PBMCs. A typical chromatogram of a calibration sample (62.5 ng/ml) is shown in Fig. 1. The retention times of the drugs ranged from 6.97 to 11.88 min (Table 1).

The standard curves covered a range from 0.25 to 125 ng/ml of cell extract for all drugs. The regression coefficients were always greater than 0.99 and the back calculated value of each point of the calibration curve was always comprised in the range of ±15% from the nominal concentration.

Within and between days accuracy and imprecision assessed with the QC samples are reported in Table 3. The method is accurate and precise at low (2.5 ng/ml), medium (25 ng/ml) and high concentration (75 ng/ml) of drug with bias (deviation from the nominal concentration) and CV always lower than 9.4%.

LLOQ are shown in Table 4. These values are at least 5 times lower than the minimal concentrations reported in previous studies, except for IDV for which the lower reported concentration was equal to our LLOQ (Table 4). The method allows to quantify very low amounts of drugs (comprised between 0.005 (LPV and ATZ) and 0.08 ng/cell pellet (EFV)) without modifying the range of the calibration curve (0.25–125 ng/ml of cell extract), with good accuracy (ranging from 90.7 to 115.8%) and imprecision (ranging from 3.3 to 12.2%). As previously stated, the LLOQ values obtained with our method are generally lower than the values obtained in previous reports (Table 4). For some of these earlier methods, the LLOQ was higher than the minimal concentration reported in other publications assessing CC in HIV-infected patients. Therefore, such methods could not be sensitive enough to be applied in extended clinical practice and/or to all dosage schedules, especially for drugs characterized by low CC (e.g. NVP, IDV, NFV and ATZ).

The choice of the internal standard is a critical aspect of an analytical method development as it influences repeatability and accuracy, especially with electrospray mass spectrometry [36]. Ideally, isotopic analogues are the best alternative. The method reported here is the first allowing the cellular quantification of a large array of PIs/NNRTIs using matched isotopic IS for most of the drugs administrated in clinical practice. The use of matched isotopic IS for LPV, IDV, ATZ, EFV, SQV and RTV allows very precise and accurate quantification even at very low concentrations. Other matched isotopic compounds (Nevirapine-d5, Nelfinavir-d3, Amprenavir-d4 and Tipranavir-d6 from Toronto Chemicals Research (North York, Ontario, Canada)) were not available when the method was developed. For these compounds (NVP, NFV, APV and TPV), the signal variations for each drug were carefully monitored and compared to those of the available IS. The IS which allowed to obtain the lowest variability in the area ratio and the best back calculated values was selected so that accuracy, precision and LLOQ values for these drugs appear satisfactory and still improved in comparison to other methods [5–9]. Others have used as IS commercialised drugs like clozapine [5] or ketoconazol [8]. This might, however, limit the clinical application of these methods as HIV-infected patients are generally polymedicated and these drugs may be present in some samples.

The matrix effect was assessed by the simultaneous post-column infusion of a solution of PI/NNRTI or SI at 2 different concentrations (20 and 100 ng/ml) during the acquisition of the chromatogram of a blank processed sample. No perturbation of the signal was noticed at the retention times of all the substances, at both tested concentrations (data not shown). This qualitative analysis was complemented by the quantitative determination of the ion suppression or enhancement effect of the matrix on the ionisation of the molecules at the 3 QC concentrations. Results are shown in Table 5 and indicate a slight enhancement of the ionisation for IDV (109.2%), NFV (116.3%) and RTV (106.3%). A similar effect has been already reported for IDV [5,37] although a suppression of ionisation has also been reported for NFV [5].

Because PBMCs with certified amount of drugs are not available yet, the true extraction recovery of the extraction step cannot be

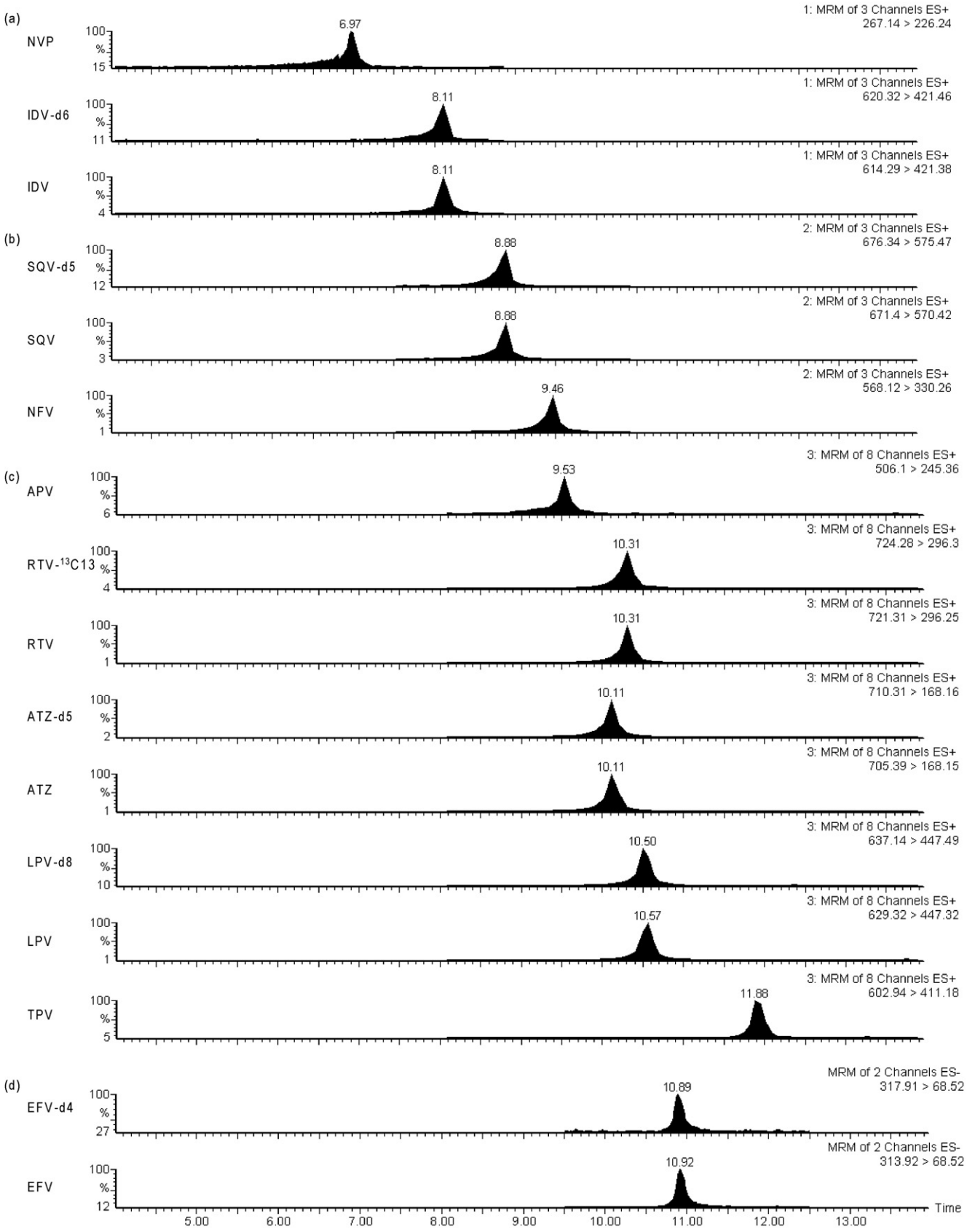

Fig. 1. Chromatogram of a calibration sample containing all anti-HIV drugs (62.5 ng/ml) and the 6 IS (20 ng/ml). The 4 acquisition windows are represented: (a) NVP, IDV and IDV-d6, (b) SQV, SQV-d5 and NFV, (c) APV, RTV, RTV-^{13}C13, ATZ, ATZ-d5, LPV, LPV-d8 and TPV, (d) EFV and EFV-d4.

Table 3
Within and between day imprecision and accuracy for all anti-HIV drugs.

Anti-HIV drug	Within day ($n=5$)				Between day ($n=5$)			
	NC[a]	MC[b,c]	CV (%)	Accuracy (%)	NC[a]	MC[b,c]	CV (%)	Accuracy (%)
NVP	2.5	2.37 ± 0.04	1.7	−5.1	2.5	2.51 ± 0.11	4.5	0.4
	25	24.89 ± 0.98	4.0	−0.5	25	25.29 ± 0.87	3.4	1.2
	75	76.35 ± 0.78	1.0	1.8	75	77.13 ± 1.75	2.3	2.8
IDV	2.5	2.47 ± 0.06	2.5	−1.1	2.5	2.50 ± 0.05	2.0	0.2
	25	24.48 ± 0.31	1.3	−2.1	25	24.38 ± 1.02	4.2	−2.5
	75	73.96 ± 4.81	6.5	−1.4	75	74.42 ± 3.39	4.6	−0.8
SQV	2.5	2.50 ± 0.08	3.3	0.0	2.5	2.53 ± 0.05	1.8	1.2
	25	25.10 ± 1.20	4.8	0.4	25	24.58 ± 0.68	2.8	−1.7
	75	78.88 ± 2.01	2.6	5.2	75	75.97 ± 2.14	2.8	1.3
NFV	2.5	2.60 ± 0.04	1.6	3.8	2.5	2.60 ± 0.21	8.2	4.2
	25	26.38 ± 1.01	3.8	5.5	25	25.28 ± 1.95	7.7	1.1
	75	77.07 ± 1.11	1.4	2.8	75	78.99 ± 2.28	2.9	5.3
APV	2.5	2.40 ± 0.03	1.1	−4.0	2.5	2.48 ± 0.15	6.0	−0.8
	25	24.88 ± 2.09	8.4	−0.5	25	22.70 ± 1.40	6.2	−9.2
	75	76.47 ± 3.18	4.2	2.0	75	73.37 ± 2.20	3.0	−2.2
ATZ	2.5	2.59 ± 0.14	5.3	3.5	2.5	2.47 ± 0.08	3.3	−1.4
	25	25.31 ± 0.89	3.5	1.3	25	23.83 ± 0.96	4.0	−4.7
	75	76.19 ± 1.25	1.6	1.6	75	74.38 ± 1.43	1.9	−0.8
RTV	2.5	2.68 ± 0.11	4.0	7.3	2.5	2.51 ± 0.11	4.2	0.3
	25	24.81 ± 1.00	4.0	−0.7	25	23.45 ± 0.83	3.5	−6.2
	75	75.64 ± 0.76	1.0	0.9	75	74.11 ± 1.37	1.8	−1.2
LPV	2.5	2.63 ± 0.08	3.1	5.3	2.5	2.50 ± 0.11	4.3	0.1
	25	24.43 ± 0.57	2.3	−2.3	25	23.23 ± 0.80	3.5	−7.1
	75	73.68 ± 0.56	0.8	−1.8	75	73.16 ± 0.75	1.0	−2.5
EFV	2.5	2.62 ± 0.01	0.2	4.7	2.5	2.54 ± 0.07	2.9	1.4
	25	25.76 ± 1.32	5.1	3.0	25	24.96 ± 1.06	4.3	−0.2
	75	77.39 ± 3.68	4.7	3.2	75	75.54 ± 2.66	3.5	0.7
TPV	2.5	2.54 ± 0.23	9.0	1.4	2.5	2.68 ± 0.16	5.8	7.1
	25	22.64 ± 0.94	4.1	−9.4	25	24.25 ± 1.38	5.7	−3.0
	75	77.06 ± 5.44	7.1	2.8	75	78.26 ± 4.68	6.0	4.3

[a] Nominal concentration (ng/ml).
[b] Measured concentration (ng/ml).
[c] Mean ± SD.

assessed properly. The extraction recovery was assessed by calculating the peak area ratio of a processed QC on the peak area of a processed blank spiked with drugs after extraction [5]. The recovery was also assessed by taking into account the response of the IS to correct for possible interference from remaining washing solution in the cell pellet and for potential change in the MS/MS performance over the time [5,38]. Finally, the overall process efficiency was estimated by the peak area of a processed QC expressed in percentage of the response obtained with a QC solution. Analysis recoveries were satisfactory for all drugs with values near 100% (always >90%) except for NFV for which both extraction and analysis recovery were around 80% with an overall process efficiency of about 97%, probably because of the ionisation enhancement observed for this compound.

The selectivity of the method has been assessed by injecting processed blank samples from 7 different batches of PBMCs. No matrix interference was found in the LC-MS/MS chromatograms recorded for these samples.

Table 4
LLOQ values and comparison with other reported methods.

Compound	LLOQ (ng/ml of cell extract)	Precision at LLOQ (%)	Accuracy at LLOQ (%)	LLOQ (ng/cell pellet)	LLOQ (ng/cell pellet) of other reported methods	Clinical values (ng/ml)[a]	Clinical values (ng/5 × 10^6 cells)[b]
NVP	0.200	8.8	96.8	0.080	0.16 [5]; 0.5 [8]	(81–2045) [33]	0.41–10.23
IDV	0.050	8.3	90.7	0.020	0.16 [5]; 0.5 [8]	(10–6,750) [25]	0.020–13.50
SQV	0.100	10.4	103.3	0.040	0.16 [5]; 0.5 [8]; 1.6 [9]	(200–15,300) [2]	0.40–30.60
NFV	0.100	3.3	94.4	0.040	0.2 [5]; 0.5 [8]	(40–52,900) [1]	0.08–105.80
APV	0.100	7.5	92.2	0.040	0.08 [5]; 0.5 [8]; 2 [9]	1,089 [5]	2.18
ATZ	0.0125	12.2	115.8	0.005	0.08 [5]; 0.018 [7]	(30–1,800) [34]	0.06–3.60
RTV	0.050	14.4	104.4	0.020	0.16 [5]; 0.1 [6]; 0.5 [8]; 1 [9]	(1,090–1,990) [24]	2.18–3.98
LPV	0.0125	11.1	104.4	0.005	0.16 [5]; 0.1 [6]; 0.5 [8]; 2 [9]	(3,700–16,100) [35]	7.40–32.20
EFV	0.200	10.4	99.0	0.080	0.16 [5]; 0.5 [8]; 2 [9]	(2,830–11,530) [4]	5.66–23.06
TPV	0.200	5.0	113.5	0.080	–	–	–

[a] Minimal and maximal concentrations reported in [ref].
[b] Assuming that the volume of a cell is 0.4 pl except for [33] were PBMC volume was assumed to be 1 pl.

Table 5
Matrix effect, extraction recovery and overall process efficiency for all anti-HIV drugs.

Anti-HIV drug	NC	Matrix effect	Extraction recovery	Analysis extraction recovery (ER)[a]	Mean analysis ER	SD	Process efficiency
NVP	2.5	100.0	87.6	90.0			87.6
	25	103.7	88.5	97.5	93.3	3.8	91.8
	75	98.3	93.8	92.5			92.2
IDV	2.5	110.4	96.7	101.9			106.8
	25	109.4	95.5	96.9	98.1	3.4	104.6
	75	107.7	109.0	95.4			117.4
SQV	2.5	104.8	95.7	100.5	99.4		100.3
	25	103.8	98.4	101.0		2.3	102.2
	75	102.6	99.3	96.7			101.9
NFV	2.5	115.7	77.8	81.1			90.0
	25	120.2	80.9	82.0	79.3	4.0	97.2
	75	112.9	91.9	74.7			103.8
APV	2.5	100.7	82.6	90.0			83.2
	25	100.1	88.7	97.8	92.4	4.6	88.8
	75	101.6	90.6	89.5			92.1
ATZ	2.5	101.7	82.5	94.5			83.9
	25	101.1	88.7	97.7	96.3	1.6	89.6
	75	93.6	97.8	96.6			91.5
RTV	2.5	109.0	87.7	98.4			95.6
	25	105.3	85.6	94.0	96.1	2.2	90.1
	75	104.3	94.9	95.8			99.0
LPV	2.5	98.8	95.1	102.9			94.0
	25	92.2	95.8	107.4	103.9	3.2	88.3
	75	94.6	102.4	101.3			96.9
EFV	2.5	101.1	96.3	96.7			97.3
	25	99.3	93.4	100.4	97.5	2.6	92.8
	75	100.9	93.2	95.5			94.0
TPV	2.5	100.9	85.5	101.3			86.3
	25	99.0	82.6	91.0	95.3	5.4	81.7
	75	102.2	91.8	93.6			93.7

NC = nominal concentration; SD = standard deviation.
[a] Including IS.

Stability of drugs in frozen PBMCs cannot be assessed formally because, as mentioned above, certified PBMCs are not available. The stability of drugs in cell extract has, however, been demonstrated [5].

For the clinical application of the method, total plasma concentrations (TPC) were determined in 48 HIV-infected patients treated with Kaletra® and 50 patients treated with Stocrin®. While LPV TPC ranged from 101.2 to 9548.3 ng/ml and RTV TPC from 25.0 to 942.7 ng/ml for patients treated with Kaletra®, EFV TPC were comprised between 56.0 and 21001.0 ng/ml for patients treated with Stocrin® (Table 6, Fig. 2a). The mean TPCs were respectively 5063.0 and 238.1 ng/ml for LPV and RTV and 2887.3 ng/mL for EFV, which is consistent with previously reported trough concentrations of these drugs [39–41].

Typical chromatograms of a PBMC extract obtained from patients under LPV/RTV or EFV are shown in Fig. 3a and b respectively. For the total population, cell-associated concentrations ranged from 64.8 to 6346.5 ng/mL for LPV, from 96.1 to 2506.7 ng/mL for RTV and from 55.8 to 13634.3 for EFV with means of 1619.0, 917.2 and 2388.1 ng/mL, for LPV, RTV and EFV, respectively (Table 6, Fig. 2a). The CC/TPC ratios were 0.42, 5.2 and 1.0 for LPV, RTV and EFV, respectively (Table 6, Fig. 2b–d) which is consistent with the literature data [3,24,42]. There was no influence of the sampling time on the ratio, which is consistent with other data [3]. There was a high inter-individual variability in the CC/TPC ratio with CV of 84, 61 and 78% for LPV, RTV and EFV, respectively.

A good correlation between cell-associated and plasma concentrations was observed for LPV, RTV and EFV. Fig. 2e–g shows the scatter plots of the log/log linear correlations between plasma and cell-associated concentrations. The regression coefficients were 0.58, 0.69 and 0.65 for LPV, RTV and EFV, respectively ($p < 0.0001$, Table 6). This correlation was already reported for LPV [3] with a regression coefficient of 0.63 while for EFV, the published data are sometimes contradictory. Indeed, two studies reported a good correlation between CC and TPC with regression coefficients ranging between 0.77 and 0.58 [3,22], while another study failed to show such an association between intracellular and plasma concentrations [4].

Based on these associations between CC and TPC, we calculated theoretical therapeutic CC by injecting the consensus therapeutic concentrations in the regression model. For LPV, it has been proposed a TPC_{min} efficacy threshold of 4000 μg/l in HAART-experienced patients [43]. Considering the relationship between LPV, TPC and CC, it would correspond to a $[LPV]_{CC}$ of approximately 1500 ng/ml. In total, among the 48 patients treated with Kaletra®, 31 patients had TPC concentrations above 4000 μg/l and 28 patients had CC concentrations above the calculated threshold of 1500 ng/l. Among the 31 patients that had $[LPV]_{TPC}$ above 4000 μg/l, only 23 reached theoretical therapeutic $[LPV]_{CC}$. On the other hand, 5 patients reached therapeutic $[LPV]_{CC}$ despite subtherapeutic $[LPV]_{TPC}$ illustrating some possible discrepancies between plasma and cellular concentrations. For EFV, the minimum efficacy threshold has been previously fixed at a TPC of 1000 μg/l [43]. When this value is injected in the log-linear regression model, an $[EFV]_{CC}$ of approximately 955 ng/ml is calculated. Among the 50 patients treated with Stocrin®, only 6 patients had subtherapeutic EFV plasma concentrations. All these 6 patients had $[EFV]_{CC}$ lower than 955 ng/ml. Four more patients had $[EFV]_{CC}$ below the theoretical threshold, maybe reflecting the fact that these patients

Table 6
Pharmacokinetic parameters of the study population.

Parameters	Kaletra®		Stocrin®
	LPV	RTV	EFV
TPC[a] (ng/ml)	5063.0 ± 382.8 [2845.9; 7315.8]	238.1 ± 25.3 [107.1; 301.8]	2887.3 ± 454.7 [1476.5; 3223.8]
CC[a] (ng/ml)	1819.0 ± 191.6 [848.3; 2480.3]	917.2 ± 66.3 [655.8; 1113.2]	2388.11 ± 339.7 [1084.2; 2750.3]
CC/TPC[a]	0.42 ± 0.05 [0.23; 0.51]	5.2 ± 0.5 [3.2; 6.3]	1.01 ± 0.11 [0.52; 1.13]
Log CC versus log TPC	Slope = 0.66, intercept = 0.78, $r = 0.58$, $p < 0.0001$	Slope = 0.50, intercept = 1.78, $r = 0.69$, $p < 0.0001$	Slope = 0.74, intercept = 0.76, $r = 0.65$, $p < 0.0001$

TPC = total plasma concentrations; CC = cell-associated concentrations.
[a] Mean ± SD [interquartile range].

require higher plasma concentrations to achieve sufficient intracellular amount of drug.

TPV TPC were 24029.0 and 7265.0 ng/ml for the patients treated by Aptivus® with a post-intake delay of 3h20 and 12h50, respectively. RTV TPC were 546.5 and 152.8 ng/ml. These values are consistent with literature data [44,45]. TPV CC were 4322.7 and 1078.0 ng/ml and RTV CC were 155.5 and 92.3 ng/ml for each patients, respectively. The CC/TPC ratios were 0.18 and 0.15 for TPV and 0.60 and 0.28 for RTV, respectively. These results suggest that RTV accumulation is less important when co-administered with TPV than with LPV, maybe because of the induction of P-glycoprotein activity observed with the TPV/RTV regimen [46].

Finally, we assessed the possible influence of ethnicity and gender on the plasma and cellular concentrations as well as on the accumulation ratio of LPV, RTV and EFV. There was no significant difference on TPC, CC and the accumulation ratio according to the gender for LPV, RTV and EFV (data not shown). Similarly, there was no significant influence of the ethnicity on TPC, CC and the accumulation ratio for LPV and RTV as well as on CC and accumulation ratio for EFV (data not shown). However, we observed

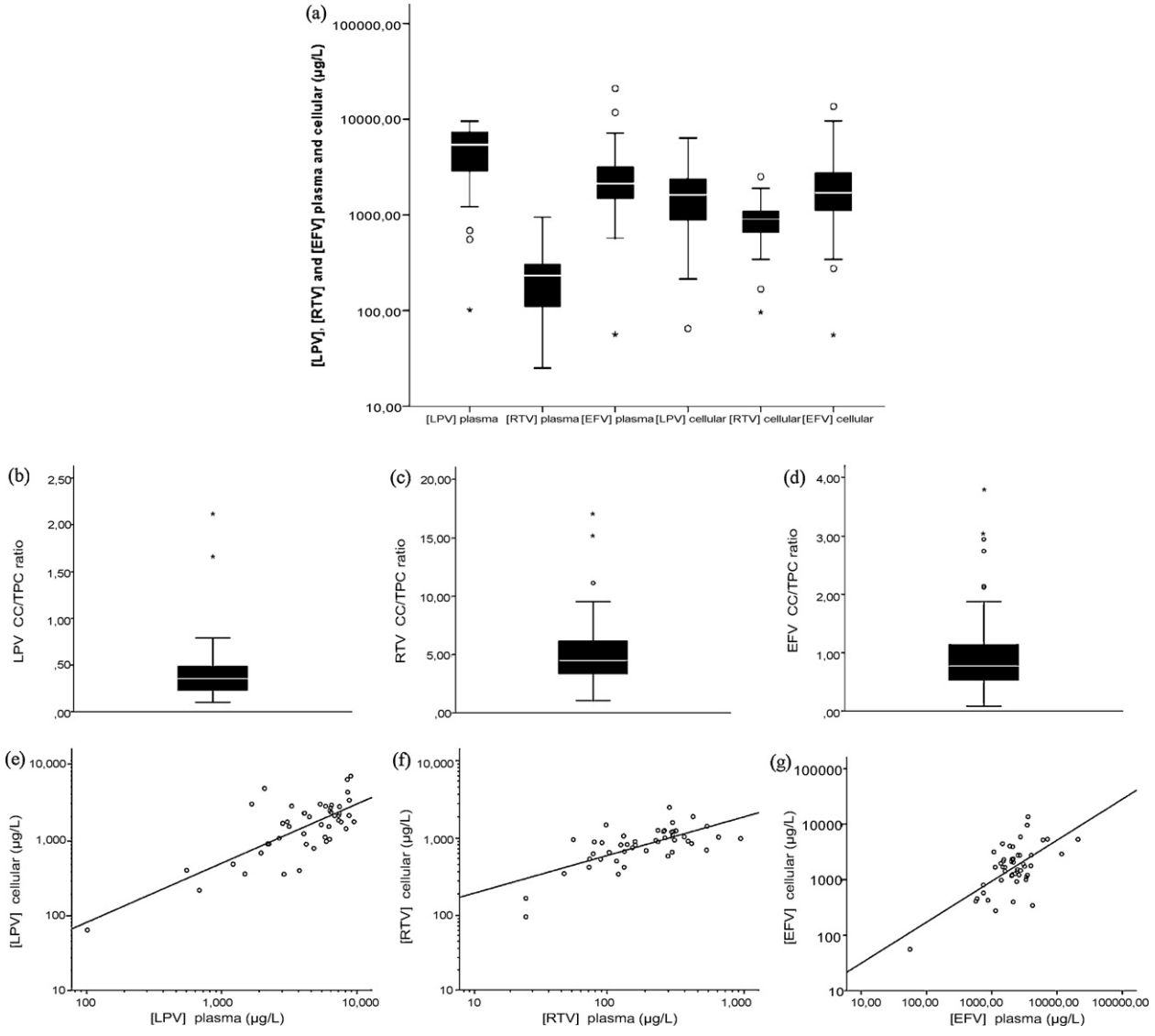

Fig. 2. (a) TPC and CC concentrations for LPV, RTV and EFV, (b–d) CC/TPC ratio for LPV, RTV and EFV, respectively, (e–g) scatter plots of CC versus TPC concentrations for LPV, RTV and EFV, respectively.

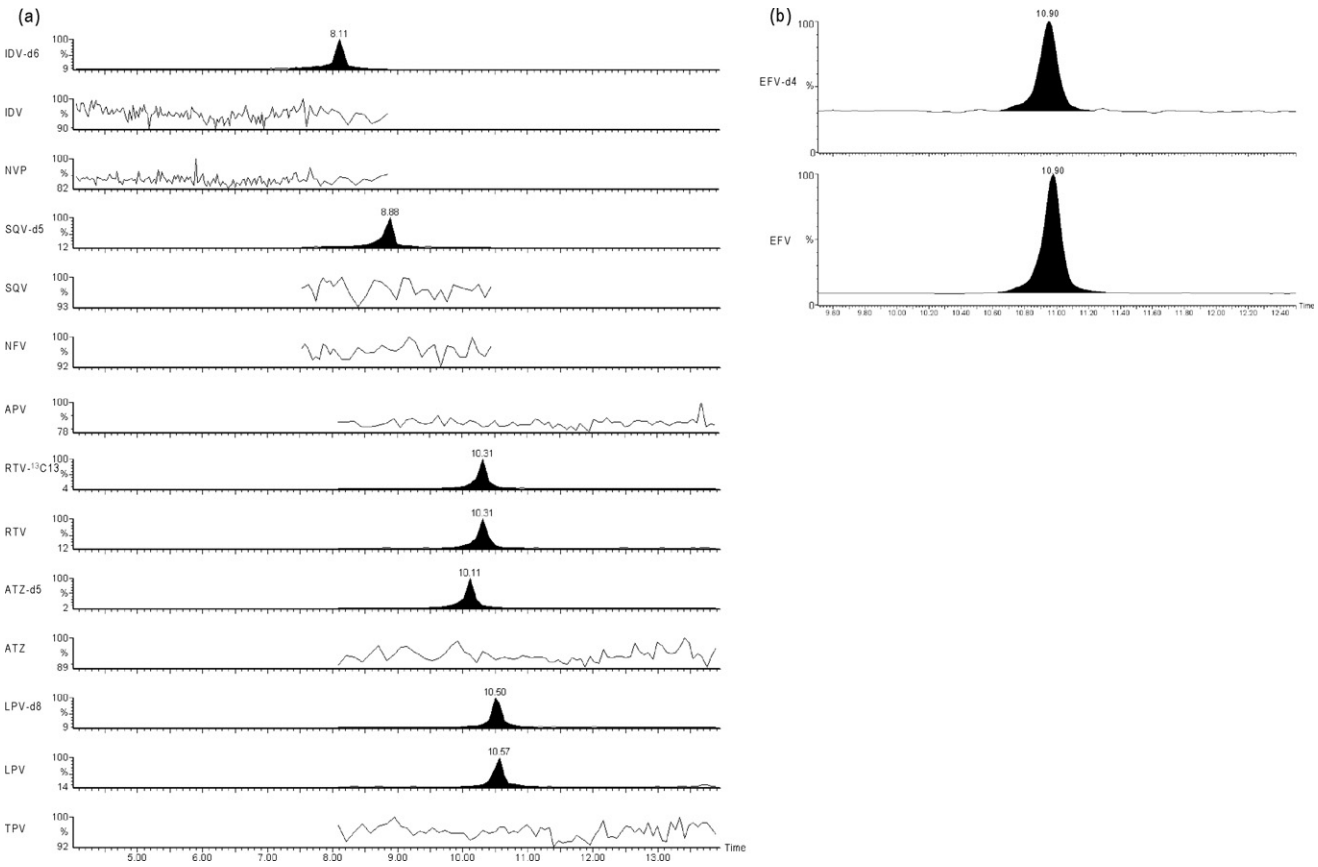

Fig. 3. Chromatograms of cell extracts of PBMCs from 2 patient treated (a) with Kaletra® (LPV = 847.8 ng/ml, RTV = 630.7 ng/ml) and (b) with Stocrin® (EFV = 5337.8 ng/ml).

a significant influence of the ethnicity on the EFV TPC (Fig. 4, $p = 0.008$). This observation probably reflects the difference in the allelic frequencies of some genetic polymorphisms within different populations. Indeed, it has been shown that CYP2B6, the main enzyme responsible for EFV oxidative metabolism, is characterized by a wide inter-individual variability in expression and/or activity which is partly explained by genetic polymorphisms [47]. In line with that hypothesis, we observed that EFV TPC was higher among group of black origin for which the *CYP2B6*6* allele is more frequent [48]. Indeed, the defective *CYP2B6*6* allele has been previously associated with significant loss of enzyme function leading to lower EFV clearance and higher EFV plasma concentrations [49,50]. When considering multiple linear regression models, EFV TPC were significantly dependent on the African and Asian dummy variables ($r = 0.45$, $p = 0.004$). The relationship was positive for Africans ($b = 0.222$), i.e. TPC increased when the patient is of black origin, in line with a hypothetical decreased CYP2B6 activity for Africans. By contrast, the relation was inverse for Asians ($b = -0.499$). Also, we observed a positive relationship between the accumulation ratio and the Asian dummy variable ($b = 1.144$, $r = 0.35$, $p = 0.014$) which could potentially reflect the fact that EFV accumulate more extensively in PBMCs from patients of Asian origin. Indeed, it was shown that the functional *CYP2B6*1* allele was more frequent in that particular population [48]. However, only 3 patients were Asian in the present population.

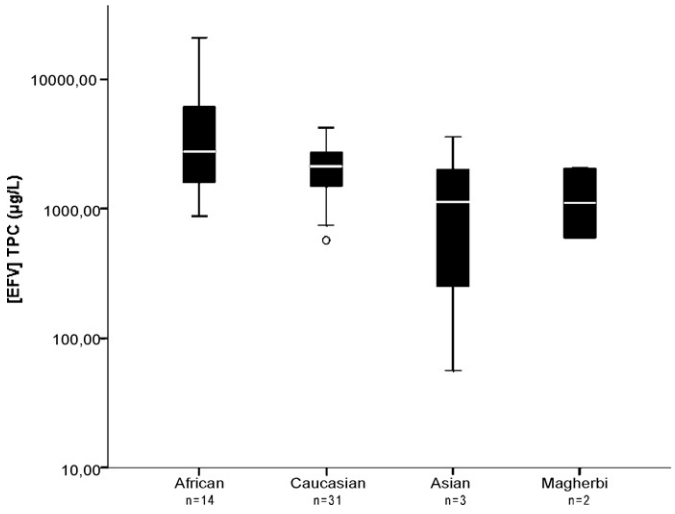

Fig. 4. [EFV] TPC (µg/L) according to the ethnicity of the patients. Data are shown by a box and whisker plot.

4. Conclusion

The original method of Colombo et al. [5] has been improved for the cellular quantification of EFV, NVP and all currently prescribed PIs. Our method provides the advantages of (1) the use of isotopic IS for 6 of the investigated drugs, (2) the selection of the best non isotopic analogue IS for the 4 remaining investigated ARV, both advantages contributing to obtain lower LLOQs and (3) the cellular quantification of TPV, a relatively new PI. The application of this method for TPV cellular quantification suggests that TPV intracellular accumulation is less important than for other PI.

Furthermore, the clinical data reported in the present study provide evidence that for LPV, RTV and EFV, a quite good correlation between plasma and cellular concentrations exists confirming that

plasma concentrations can be considered as appropriate surrogate for routine therapeutic drug monitoring when considering practical difficulties to implement CC measurement in daily clinical practice. However, we have also shown that it may exist some discrepancies between cellular and plasma concentrations which could potentially explain therapeutic failures despite appropriate plasma levels in some patients. In that way, the method proposed in the present paper could be used to further investigate the potential causes of such discrepancies.

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