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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

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Abstract

A sensitive and accurate liquid chromatography–tandem mass spectrometric (LC–MS/MS) method for the intracellular determination of nine antiretroviral drugs in human peripheral blood mononuclear cells (PBMCs) is proposed. PBMCs are isolated by density gradient centrifugation using Vacutainer™ CPT tubes and cell count is performed with a Coulter® instrument. Single-step extraction of drugs from PBMCs pellets is performed with MeOH 50% (with clozapine added as internal standard, I.S.) and the supernatant is injected onto a 2.1 mm × 30 mm SymmetryShield™ 3.5 μm-RP18 column equipped with a 2.1 × 10 mm guard column. Chromatographic separations are performed using a gradient program with a mixture of 2 mM ammonium acetate containing 0.1% formic acid and acetonitrile with 0.1% formic acid. Analytes quantification is performed by electro-spray ionisation–triple quadrupole mass spectrometry using the selected reaction monitoring (SRM) detection mode. The positive mode is used for the HIV protease inhibitors (PIs) indinavir, amprenavir, saquinavir, ritonavir, nelfinavir, lopinavir, atazanavir and the non-nucleoside reverse transcriptase inhibitors (NNRTIs) nevirapine, and the negative mode is applied for efavirenz. The calibration curves are prepared using blank PBMCs spiked with antiretroviral drugs at concentrations ranging from 0.5 to 100 ng/ml of cell extracts and fitted to a quadratic regression model weighted by 1/(concentration)^2. The lower limit of quantification is less than 0.5 ng/ml. The mean extraction recovery for all PIs/NNRTIs is always above 88%. The method is precise, with mean inter-day CV% within 0.6–10.2%, and accurate (range of inter-day deviation from nominal values −7.2 to +8.3%). This analytical method can be conveniently used in clinical research for the assessment of intracellular levels of all PIs/NNRTIs commercially available at present using a simple one-step cell extraction of PBMCs followed by liquid chromatography coupled with tandem triple quadripole mass detection.

Keywords: Protease inhibitors; Non-nucleoside reverse transcriptase inhibitors; PBMCs; Peripheral blood mononuclear cells; HPLC–MS/MS; Indinavir; Amprenavir; Saquinavir; Ritonavir; Nelfinavir; Lopinavir; Atazanavir; Efavirenz; Nevirapine

1. Introduction

Major advances have been accomplished in recent years for the treatment of human immunodeficiency virus type 1 (HIV-1) infected patients. However, many patients experience treatment failure within one year after initiation of antiretroviral therapy [1]. Drug resistance due to mutations of

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the viral genome accounts for a large proportion of treatment failures [2–4]. The emergence of genetic changes in the genome of HIV-1 is fostered by ongoing viral replication in the presence of sub-inhibitory concentrations of antiretroviral drugs. Poor penetration of drugs into various 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.

Therapeutic drug monitoring (TDM) of HIV protease inhibitors (PIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) considers the adjustment of total plasma concentrations as a mean to optimise response to antiretroviral therapy [5]. However, only the fraction reaching the intracellular compartment is reasonably expected to exert an antiviral action [6,7]. Whereas PIs and NNRTIs (Figs. 1 and 2) are two therapeutic class of drugs showing distinct physicochemical properties, both are mostly lipophilic in nature and are assumed to enter cells by passive diffusion through the cellular phospholipidic bilayer membrane. However, the intimate mechanisms by which antiretroviral drugs accumulate within cells remains generally unknown. Previous in vitro [7] and in vivo [8] studies have shown striking differences in the intracellular kinetics of PIs. Similarly, a marked variability has been observed in their accumulation in lymphoblastoid cell lines in vitro, and in peripheral blood mononuclear cells (PBMCs) in vivo [9–11]. Moreover, a number of transmembrane transport proteins, such as P-glycoprotein, the gene product of *ABCB*1 (*MDR*1), and related ABC (ATP binding cassette) transporters are known to actively expel drugs out of cells, and were shown to play an important role in the intracellular antiviral drugs concentration [12–17]. The genes coding for these transport proteins are polymorphic in humans, with consequences in term of 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 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. Owing to its sensitivity and selectivity, liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) is particularly attractive for the measurement of intracellular analytes [18]. LC–MS/MS technology has been applied for measuring the active triphosphate anabolites of several nucleosidic reverse transcriptase inhibitors (NRTIs) in PBMCs [19–22]. PIs and NNRTIs are directly acting pharmacological species requiring no bioactivation but, although several LC–MS/MS assays have been published for the quantitative determination of PIs in plasma [23–28], validated methods for their determination in PBMCs have rarely been described. Except two reports recently published for atazanavir [29] and for four PIs simultaneously measured with efavirenz [30], the analytical methods for the intracellular measurements of PIs and NNRTIs have been published up to now only in excerpt form, as part of *Materials and methods* section [31–33], with limited information on method validation procedures. An enzyme immunoassay for nevirapine plasma and intracellular levels measurement has been published recently [34].

In this report, we describe the development and validation of an analytical method for the quantification in PBMCs of nine antiretroviral drugs (indinavir, amprenavir, saquinavir, ritonavir, nelfinavir, lopinavir, atazanavir, efavirenz, nevirapine), by liquid chromatography coupled with tandem triple quadrupoles mass spectrometry detection. This method is characterized by a very low limit of quantification ($\leq$0.5 ng/ml), well below the clinically relevant range of concentration encountered in patients PBMCs contained in a 8 ml-blood cell preparation tube (CPT, Vacutainer®). The stringent workup for PBMCs isolation and careful washing is counterbalanced by a simplified extraction step. Since experience in handling PBMCs samples for intracellular drug measurements are rather limited, special attention has been given to describe in detail the methodology we used.

2. Experimental

2.1. Chemicals and reagents

Ritonavir (RTV), lopinavir (LPV), saquinavir (SQV), nelfinavir (NFV), amprenavir (APV), indinavir (IDV), atazanavir (ATV), efavirenz (EFV) and nevirapine (NVP) (Figs. 1 and 2) were kindly provided by Abbott (Baar, Switzerland), Roche discovery Welwyn (Welwyn Garden City, UK), Agouron (La Jolla, CA, USA), Glaxo Wellcome R&D (Stevenhage, UK), Bristol Myers Squibb (Baar, Switzerland), Merck Sharp Dohme Chibret (Glattburg, Switzerland) and Boehringer Ingelheim (Ridgefield, CT, USA), respectively. Clozapine (internal standard, I.S.) stock solution (250 μg/ml) in MeOH was obtained by extraction with MeOH of Leponex® (Novartis, Basel, Switzerland) tablet. Solvent used for chromatography such as acetonitrile (MeCN) and methanol (MeOH), both of LiChrosolv® grade, and 100% formic acid were purchased from Fluka (Buchs, Switzerland). All other chemicals were of analytical grade and used as received. Ultrapure water was obtained from a Milli-Q® UF-Plus apparatus (Millipore Corp., Burlington, MA, USA). The phosphate buffered saline (PBS) solution used for the preparation of PBMCs matrix was obtained from Sigma-Aldrich (St. Louis, MO, USA) and the heat inactivated foetal bovine serum (FBS) from Invitrogen (Basel, Switzerland).

Blank PBMCs used for the assessment of matrix effect and for the preparation of calibration and control samples were isolated from leucocytes collected in filters (ASAHI Sepacell RZ 2000, Baxter, La Châtre, France) routinely employed for the leukapheresis of blood donation units in the Hospital Blood Transfusion Centre (CHUV, Lausanne, Switzerland). Isolation of PBMCs from leucocytes was carried out using a Ficoll-Hypaque density gradient centrifugation solu-

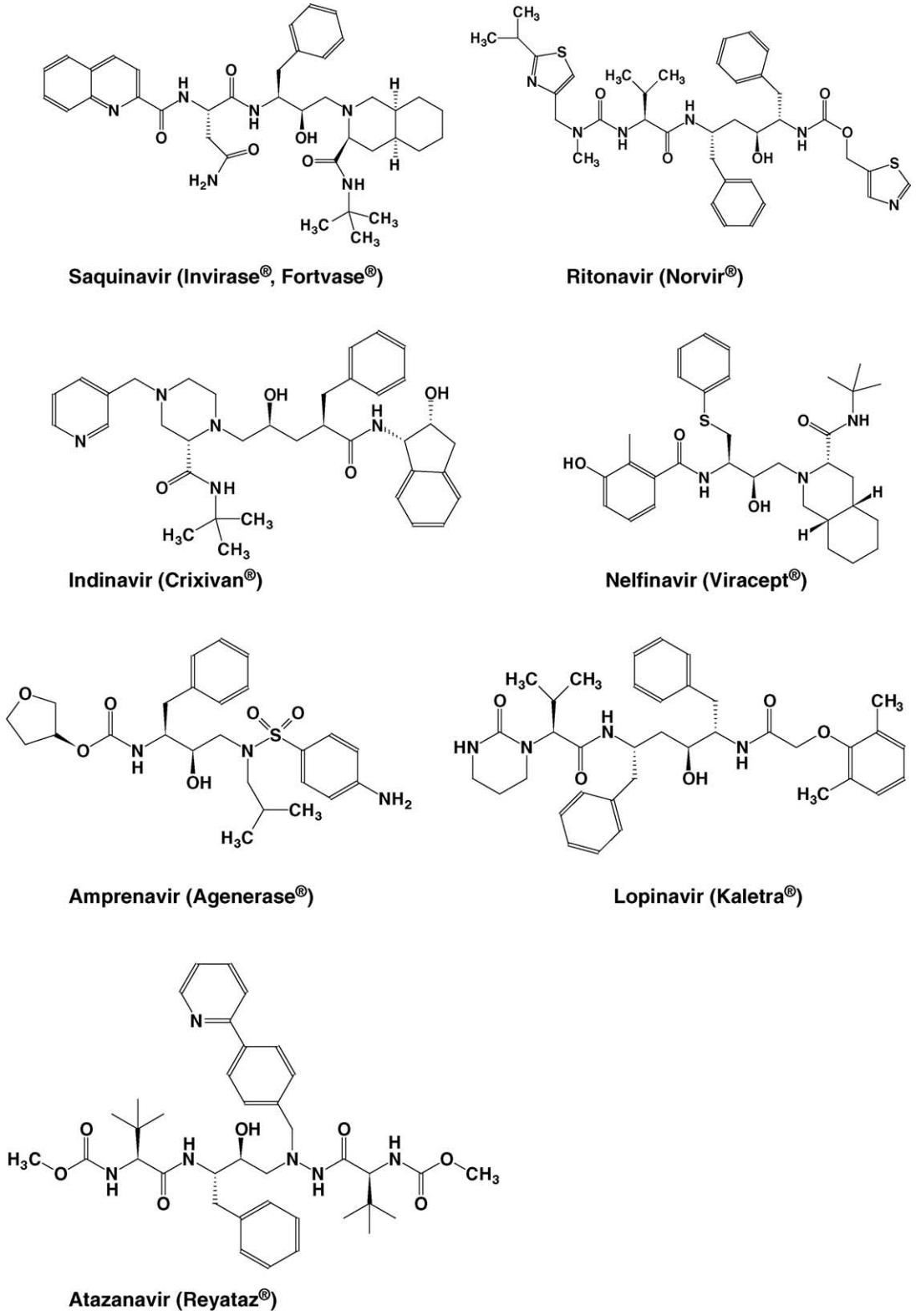

Fig. 1. Chemical structures of protease inhibitors.

tion (Biocoll Separating Solution, Seromed, Biochrom KG, Berlin, Germany). Patient's PBMCs were isolated using Vacutainer™ CPT tubes (Becton Dickinson Vacutainer™ System, Franklin Lakes, NJ, USA).

2.2. Equipment

The HPLC system consisted of an Agilent HP1100 binary pump equipped with an online degasser and a

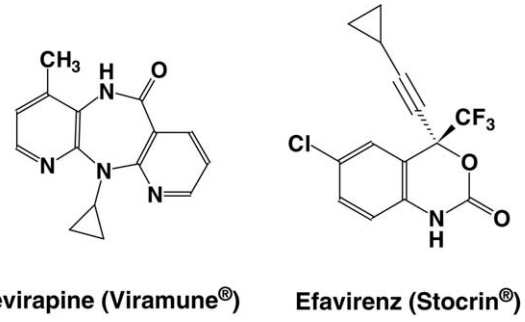

Fig. 2. Chemical structures of nonnucleoside reverse transcriptase inhibitors.

100-vials autosampler (Agilent Technologies, Palo Alto, CA, USA). Separations were done on a 2.1 mm × 30 mm SymmetryShield™ 3.5 μm-RP18 (Waters, Milford, MA, USA) analytical column equipped with a 2.1 mm × 10 mm guard column containing the same packing material, and placed in a thermostated column oven CH5-A100 (Bon Technologies SA, Lausanne, Switzerland) maintained at 30 °C. The chromatographic system was coupled to a triple stage quadrupole mass spectrometer (TSQ Quantum) from Thermo Electron Corporation (San Jose, CA, USA), equipped with an electrospray ionisation (ESI) interface and operated with the Xcalibur 1.0.0.1 software (Thermo Electron Corporation, San Jose, CA, USA).

Cell count was performed using a Cell-dyn® apparatus (Cell-dyn® 3500R, Abbott AG, Baar, Switzerland). For the drug extraction procedure, a HS 250 control horizontal shaker (IKA-Works Inc., Staufen, Germany) was set at a rate of 300 cycles per minute. PBMCs samples were sonicated using an ultrasonic bath (Kleiner AG, Wohlen, Switzerland). An Hettich® benchtop centrifuge (Benchtop Universal 16R centrifuge, Bäch, Switzerland) was used for centrifugation.

2.3. Solutions

2.3.1. Mobile phase solutions

Solution A consisted of 2 mM ammonium acetate in bidistillated water containing 0.1% formic acid (pH 2.85). Solution B consisted of 0.1% formic acid in MeCN. Solvents were regularly prepared for each series of analysis and stored in the dark at +4 °C prior use.

2.3.2. Internal standard, calibration standards and quality controls (QCs) solutions

A stock solution of clozapine (CLZ) at 250 μg/ml was diluted with MeOH/H_2O 50/50 (vol/vol) to obtain a working solution at 2 ng/ml. This solution was used as extracting solvent (internal standard already present) as well as solvent for the preparation of calibration and quality control solutions in order to have an identical amount of internal standard in each sample.

An aliquot (0.500 ml) of each stock solutions of IDV, APV, SQV, RTV, NFV, LPV, ATV, EFV and NVP at 1 mg/ml (calculated as base) in MeOH was further diluted with the CLZ working solution in a volumetric flask (50.0 ml) for the preparation of a solution for each analyte at a concentration of 0.01 mg/ml. This solution was further diluted with the same CLZ working solution to prepare seven different calibration concentrations (0.5, 1.0, 5.0, 10.0, 50.0, 75.0, 100.0 ng/ml), and three QC concentrations (2.0, 20.0, 80.0 ng/ml). The calibration standard and control solutions were stored at 4 °C in glass flasks, with caps tightly wrapped with Parafilm®, for no more than one month. Duplicate calibration and quality control samples were prepared prior to each series of analyses.

2.4. LC–MS/MS conditions

The mobile phase was delivered using the following stepwise gradient elution program: 2% of B at 0 min, 25% of B at 2 min, 100% of B at 10 min with a flow rate of 0.3 ml/min. The second part of the run includes an intensive rinsing (100% B at 10.10 with 0.5 ml/min) and a re-equilibration step to the initial solvent composition up to 20 min (at 15.10 with 0.4 ml/min and at 18 min with 0.3 ml/min). The thermostated column oven was set at 30 °C and the autosampler was maintained at 20 °C. The mass spectometer was operated with the electrospray ionisation source in the positive and negative mode for PIs/NVP and EFV, respectively. Samples were analysed via selected reaction monitoring (SRM) detection mode, employing the transition of the $(M + H)^+$ and $(M - H)^-$ precursor ions to product ions, for PIs/NVP and EFV, respectively. The selected *m/z* transitions and the collision energy for each analyte are reported in the Table 1. The determination of optimal potential settings and MS/MS transitions were chosen by direct infusion into the MS/MS detector of a MeOH:H_2O 50:50 solution of each drug at a concentration of 1 μg/ml. The first (Q1) and third (Q3) quadrupoles were set at 1 amu mass resolution (full-width half-maximum = 0.7). Scan time and scan width were 1 s and 0.5 *m/z*, respectively, and each chromatographic peak was the result of at least 15 scans. Two distinct segments of data acquisition were programmed in the positive mode: a first acquisition segment from 0 to 8.55 min, and a second segment from 8.55 to 15.25 min, for the analyse of NFV, IDV, SQV, NVP, CLZ, and LPV, APV, RTV, ATV, respectively.

The ionisation conditions were as follows: the capillary temperature was set at 350 °C. The ESI spray voltage was set at 4 kV, the source collision induced dissociation (CID in source) was set at 10 V. The sheath and auxiliary gas (nitrogen) flow-rate was set at 60 psi and 5 (arbitrary units), respectively. The tubes lens voltages range from 120 to 178 V and the Q2 collision gas (argon) pressure was 1 mTorr.

Chromatographic data acquisition, peak integration and quantification were performed using the Xcalibur LC-Quan software package.

Table 1
Instrument method for the LC–MS/MS analysis for PIs and NNRTIs with clozapine as internal standard

Drug	Parent (*m/z*)	Product (*m/z*)	CE (eV)	Tube lens (V)	Mean RT	Segment
Clozapine (IS)	327.10	269.90	29	120	6.2	1
Indinavir	614.50	420.95	40	164	7.0	
Nevirapine	267.25	226.05	35	123	7.2	
Saquinavir	671.50	569.90	38	178	8.0	
Nelfinavir	568.50	329.75	38	164	8.1	
Atazanavir	705.30	168.10	48	146	9.1	2
Amprenavir	506.40	245.00	24	98	9.2	
Ritonavir	721.40	295.95	26	123	9.7	
Lopinavir	629.40	447.00	20	122	9.8	
Efavirenz	314.00	243.85	18	43	9.4	

CE, collision energy; mean RT, retention time; MS acquire time (min), 15.25; first segment time, 8.55 min; second segment time, 6.7 min; Q2 collision gas pressure, 1.00 mTorr.

2.5. PBMCs isolation

2.5.1. Preparation of blank control PBMCs from healthy blood donor

Filters containing leucocytes removed from blood donation were provided to our Laboratory shortly (within 60 min) after blood filtration by the Hospital Blood Transfusion Centre.

The leucocyte suspension was obtained by washing these filters with a syringe filled with 60 ml of PBS solution containing 2% FBS. In two 50 ml-Falcon® tubes containing each 15 ml of Ficoll solution equilibrated at room temperature (RT), a 30 ml-aliquot of leucocyte suspension was added being careful not to mix the two solutions. The biphasic solution was centrifuged 30 min at 300 × *g* (RT), without brake. The monocytes/lymphocytes, separated as a distinct layer at the interface, were collected with a thin polypropylene pipette, and pooled in a 50 ml Falcon tube. The volume was completed to 50 ml with PBS solution and inverted gently several times, for washing. After centrifugation (650 × *g*, RT, 10 min) the supernatant was discarded and the washing step was repeated twice. The pellet was suspended and carefully homogenised in approximately 30 ml PBS. This suspension was distributed as 1 ml-aliquots in 1.5 ml polypropylene Eppendorf vials ($n \sim 30$ vials). One aliquot was used for cell counting and the remaining samples were centrifuged (350 × *g*, RT, 5 min) and the supernatant discarded. The number of PBMCs in each pellet was typically between 4 and 10×10^6 cells per Eppendorf vial. The PBMCs pellets were immediately frozen in polypropylene Eppendorf vials and stored at −20 °C until the day of analyses. For each series of cells, a PBMCs pellet aliquot was analysed (i.e. extraction and LC–MS/MS analysis, see below) for ascertaining the absence of interfering peaks at the time of retention of antiviral drugs. These blank PBMCs were used for the assessment of PBMCs matrix effect (see below) and for the preparation of calibration and QC samples.

Cell counting of PBMCs aliquots by Coulter® was done to ensure that simultaneously prepared series of blank aliquots contain a cell number similar to those generally obtained from patient in a 8 ml-blood Vacutainer tube collection. The Celldyn® instrument provides also graphical information on the identity and percentage of respective blood cells present in the pellet, enabling the correct separation of PBMCs to be confirmed.

2.5.2. Collection for intracellular drug determination

Blood samples were obtained from HIV-1 infected patients from Lausanne and Berne University Hospital Centers, during their regular medical visit, as part of a Swiss HIV Cohort Study protocol (SHCS #383, http://www.shcs.ch) approved by the Institutional Ethics Committee. Written informed consent was obtained from all patients. Samples were immediately (within 5 min) taken to the laboratory and processed under a Class II Biohazard Protection Hood wearing nitrile gloves and long-sleeve lab coats. PBMCs were isolated using Vacutainer™ CPT tubes according to the manufacturer recommended procedures [35]. The CPT tube contains citrate as anticoagulant, a polyester gel layer, and a Ficoll solution enabling the direct separation of mononuclear cells from other blood components. One tube per patient (approximately 8 ml of blood) was collected, gently inverted 8–10 times for the careful homogenisation with the anticoagulant, and centrifuged within 5 min at 1650 × *g* for 20 min at RT, without brake. The thin mononuclear cell layer was collected at the interface with a pipette and transferred into a polypropylene tube (15 ml) pre-chilled at 4 °C on an ice bath. The entire cell washing procedure was performed using pre-chilled PBS solutions kept at +4 °C and the centrifuge was maintained at +4 °C to inhibit enzymatic activity and to prevent active drug efflux out of cells.

The collected cells were washed with 10 ml of cold PBS solution containing 2% FBS, and, after careful cell homogenisation, the tube was centrifuged for 10 min at 650 × *g* (+4 °C) and the supernatant discarded. The washing procedure was repeated twice. The third (final) supernatant wash solution was completely aspirated off and an exact volume of 1.0 ml of PBS was added to the cell pellet. Cells were carefully homogenised and put in suspension, from which an aliquot (20 μl) was taken out and diluted 1:10 with 180 μl of PBS in a 0.5 ml Eppendorf tube. This aliquot was homogenised for cell counting within 10 min,

enabling the total number of PBMCs contained in each patient's pellet sample to be calculated, and used for normalizing the intracellular drug concentration determined by LC–MS/MS.

The remaining 980 μl cell suspension was centrifuged at 650 × *g* for 10 min at 4 °C. The supernatant was aspirated off, leaving the PBMCs pellet at the bottom of the vial which was immediately frozen and stored at −20 °C up to the day of the analysis. At the initial stage of the method validation, each PBS solution wash was retained and subsequently checked by LC–MS/MS for the presence of antiretroviral drugs. These analyses confirmed that no drug was detectable in the third wash, indicating not only that the washing procedure was efficient in removing any extracellular medium possibly associated with the cells, but also that the washing procedure at 4 °C prevented drug efflux from the cell.

2.5.3. Selection of the extraction/lysing solvent

During the initial development of the method, the following solvents were evaluated for the extraction of antiretroviral drugs spiked at a concentration of 10, 100 and 1000 ng/ml into blank PBMCs: MeOH 100%; MeOH 75% (i.e. MeOH/H_2O 75/25); MeOH 50%; MeOH 50% + diethylamine (DEA) 0.1%; MeOH 75% + DEA 0.1%; MeOH 100% + DEA 0.1%; MeOH 50% + DEA 0.25%; MeOH 50% + HCl 0.01N; MeOH 75% + HCl 0.01 N. As a first approximation, the extraction recovery of the solvent mixture, assessed by peak-area values of antiretroviral drugs, were determined. Among the solvent mixtures tested, MeOH 50% provides the best recovery overall for the nine antiretroviral drugs, at each concentration, and was consequently used thereafter throughout the method validation and subsequent application to patient samples.

2.5.4. PBMCs extraction procedure

On the day of the analysis, calibration standard and quality control solutions, and the extracting solvent MeOH 50% were allowed to equilibrate at room temperature. Blank PBMCs (4–10 × 10^6 cell per pellet) and patient's cell pellets were similarly thawed. An aliquot (500 μl) of calibration and quality control solutions of antiretroviral drugs at the previously reported concentrations (see § 2.3.2) were added to blank PBMCs pellets. Similarly, 400 μl of the extracting solvent were added to patient's cell pellets. The resulting suspensions were vortex-mixed to ensure adequate mixing and subsequently sonicated 5 min in an ultrasound bath for cell lysis. All samples were extracted during 3 h, using a horizontal backward/forward shaker set at 300 cycles/min, and were centrifuged for 10 min at 20 °C on a Hettich benchtop centrifuge at 20,000 × *g* (14,000 rpm).

The supernatants were introduced into 200 μl HPLC microvials (Agilent, Germany) and a volume of 10 μl (20 μl for efavirenz) was used for LC–MS/MS analysis.

2.6. Quantification

2.6.1. Calibration curves

Quantitative analysis of PIs and NNRTIs in PBMCs was performed using the internal standard method. Each level of the calibration curve was measured with two sets of calibrators: one at the beginning and the second at the end of the run. Calibration curves were established with calibration standards prepared with PBMCs pellets isolated from different batches of cell collection.

A seven-point calibration standard curves has been calculated and fitted by quadratic least-square regression of the peak area ratios (drug peak area/IS peak area) versus concentrations, using 1/concentration^2 ($1/x^2$) as weighting factor for PIs and NNRTIs. To determine the best weighting factor, concentrations were back-calculated and the residual plot examined. The model with the lowest bias and the most constant variance across the concentration range was considered the best suited. The calibration was established over the range 0.5–100 ng/ml for all the drugs so as to cover the range of concentrations expected in patients.

2.6.2. Drug quantification in PBMCs

Calibration curves yield drug concentrations expressed in ng/ml of extracting solvent (i.e. 50% MeOH cell extract supernatants). However, absolute, not relative, amount of drug in the whole sample must be known for the calculation of drug levels in PBMCs. Thus, the total amount of drug in PBMCs pellet (in ng) was obtained by multiplying the drug concentration, by 0.4 (i.e. 0. 4 ml being the volume of extracting solvent added to patient cell pellet).

Finally, drug levels in PBMCs may be expressed either in ng per 10^6 cells, taking into account the number of cells determined in the sample, or alternately, in ng/ml, as the so-called "intracellular" drug concentration, assuming a 0.4 pL PBMCs volume [36].

2.7. Analytical methods validation

The validation of the method was based on the recommendations published on-line by the FDA [37] as well as on the recommendations of the Conference Report of the Washington Conference on "Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic Studies" [38] and of the Arlington Workshop "Bioanalytical Methods Validation—A revisit with a Decade of Progress" [39]. Recent SFSTP (*Société Française des Sciences et Techniques Pharmaceutiques*) recommendations were also considered [40].

2.7.1. Accuracy and precision

Replicate analysis ($n = 6$) of QC samples at the three concentrations (§ 2.3.2) were used for the intra-assay precision and accuracy determination. Inter-assay accuracy and precision were determined by repeated analysis performed on six different days. The concentration in each sample was de-

termined using calibration standards prepared on the same day. The precision being calculated as the coefficient of variation (C.V.%) within a single run (intra-assay) and between different assays (inter-assays), and the accuracy as the percentage of deviation between nominal and measured concentration.

During the routine analysis of patient samples, duplicate control samples at each QC concentration levels were assayed. The analytical series were considered valid and accepted only if the percentage of deviation (bias) between theoretical and back-calculated (experimental) concentrations for each calibration level and quality control samples were less than ±15%, and less than 20% at the limit of quantification (defined as the lowest calibrator).

2.7.2. Limit of quantitation and limit of detection

The lowest limit of quantification (LLOQ) for each drug analysed was experimentally chosen as the minimal concentration in PBMCs samples which could be confidently determined in accordance with the documents mentioned above [37–39], recommending that the deviation between measured and nominal concentration should not deviate more than ±20%. Calibration curves were established with standard solutions starting at a concentration of 0.1, 0.2, 0.4 and 0.5 ng/ml, for the lowest calibration level, up to 100 ng/ml, all analysed in triplicates. Back-calculated values of the lowest calibration samples with a bias and CV% below ±20%, enables to determine the LLOQ values. The limit of detection (LOD) was defined as the concentration that produced a signal three times above the noise level of a blank preparation.

2.7.3. Stability of PIs and NNRTIs

Stability studies of PIs and NNRTIs included:

a) Stability of PIs and NNRTIs standard solutions mixed with PBMCs and kept at room temperature: the concentration of PIs and NNRTIs in QC cell extract samples were measured immediately after preparation and after being left at room temperature for 72 h.
b) Stability of cell extract samples after multiple freeze-thaw cycles: processed samples (i.e. 50% MeOH cell extract supernatents) of PIs and NNRTIs at 2, 20 and 80 ng/ml underwent three freeze-thaw cycles. Frozen samples were allowed to thaw at ambient temperature for 2 h and were subsequently refrozen during approximately 24 h. PIs and NNRTIs level were measured in aliquots from the three consecutive freeze-thaw cycles. The variations of PIs and NNRTIs concentrations were expressed in percentage of the starting levels measured at the beginning of the stability study.
c) Stability of cell extracts into LC–MS/MS vials at room temperature (20 °C, autosampler rack temperature): processed samples (50% MeOH cell extract supernatents) of PIs and NNRTIs at 2, 20 and 80 ng/ml were analysed immediately after preparation and after being left 24 h at room temperature.

2.7.4. Matrix effect and recovery

In the initial step of method validation, the matrix effect was examined qualitatively by the simultaneous post-column infusion of PIs/NNRTIs and I.S. into the MS/MS detector during the chromatographic analysis of a blank PBMCs extract. The standard solution of all analytes at 75 ng/ml and I.S. at 2 ng/ml was infused at a flow-rate of 10 μl/min during the chromatographic analysis of blank PBMCs extracts from three different sources. The chromatographic signals of each selected MS/MS transition were examined to ascertain that no signal perturbation (drift or shift) of the MS/MS signal was present at the analyte's retention time.

Subsequently, the quantitative determination of the matrix effect was also assessed. Three series of QC samples at 2, 20 and 80 ng/ml in triplicates were prepared as followed:

- pure standard solutions samples directly injected onto column;
- PBMCs extract samples from three different sources, spiked which drugs and I.S *after* extraction;
- PBMCs samples from three different sources (same as in B) spiked with drug standards solution and I.S. *before* extraction.

The recovery and ion suppression/enhancement of the MS/MS signal of drugs in the presence of PBMCs matrix was assessed by comparing the peak areas of analytes either in 50% MeOH solution, or added before and after extraction of three different batches of PBMCs, as well as the corresponding peak areas ratios of analytes to I.S., according to the recommendation recently proposed by Matuszeski et al. [41].

The *extraction recoveries* of PIs/NNRTIs and I.S. were calculated as the absolute peak-area response in processed PBMCs samples spiked with drugs *before* extraction, expressed as the percentage of the response of the same amount of drugs (prepared in 50% MeOH) added into blank PBMCs extracts *after* the extraction procedure. Similarly, to correct for the possible presence of residual liquid in PBMCs pellet and possible variations over time of the MS/MS detector performance, the recoveries were also calculated taking into account the I.S. response; and the drug/I.S. signal ratio in PBMCs samples prior to- and after the extraction processing were compared. Recovery studies were done in triplicate QC samples.

2.7.5. Influence of cell number variability in PBMCs pellet

Following PBMCs harvesting from blood, the cell content finally obtained in pellet samples may vary from one sample to another, even with the same blood volume, depending on the overall cell recoveries and actual cell count in patient blood samples, often not known a priori. Whether the variability in cell content of pellets used in calibration, control and patient samples (within the $4–10 \times 10^6$ cells range) is likely to significantly influence the accuracy of the method was verified with the following experiments: blank PBMCs

isolated from the same batch of leukocytes (i.e. same filter) were distributed into Eppendorf vials for the preparation of two series of pellet samples containing either a low (1.4×10^6 cells) or a high (9.6×10^6 cells) cellular content. After sample centrifugation and supernatant removal, the two series of cell pellets were spiked with calibrations (and quality control solutions of all antiretroviral drugs (§ 2.3.2). The two series of samples were analysed in parallel. Cross-validation was performed using the three QC samples with the low and high cellular content which were back-calculated using calibration curves established with either the same, or the alternate calibration standards, respectively. The percentage of deviation (bias) from nominal values were compared.

2.7.6. Dilution effect

Some patient samples were found to contain drug concentrations in PBMCs exceeding the highest level of the calibration curve (100 ng/ml). It was therefore necessary to ascertain whether the dilution of these samples prior to a subsequent analysis would affect the accuracy of the drug determination. Six blanks PBMCs pellets were spiked with antiretroviral drugs at a concentration of 150 ng/ml (i.e. above the highest calibration level of 100 ng/ml) and the I.S. After extraction, samples were analysed in triplicate either directly, or after a two- and three-fold dilution. Dilution was carried out with a blank PBMCs extract supernatant (in 50% MeOH containing the I.S. at 2 ng/ml).

2.7.7. Selectivity

The assay selectivity was assessed by analysing extracts from 10 batches of PBMCs extracts from different sources.

3. Results

3.1. Chromatograms

The proposed method enables the quantification of all PIs and NNRTIs commercially available at present in PBMCs extracts by liquid chromatography coupled with tandem MS/MS. A typical chromatographic profile of a calibration sample containing PIs/NVP and EFV at a concentration of 75 ng/ml is shown in Fig. 3a and b, in the positive (two segments) and negative mode respectively, using the selected monitoring reaction mode and the proposed gradient program (§ 2.4). The respective retention times of antiretroviral drugs and the I.S. clozapine are reported in Table 1.

Even if all antiretroviral drugs and I.S. were eluted within 11 min, a relatively prolonged rinsing step of 5 min with 100% of solvent B at a flow rate of 0.5 ml/min was introduced to efficiently eliminate the *memory effect* observed in the initial setting-up of the analytical method. This rinsing step was followed by the column conditioning step with the initial solvent composition (98/2 solvent A/solvent B) at a flow-rate of 0.4 ml/min (2.9 min) and 0.3 ml/min (2 min). A divert valve was programmed during the chromatographic run to direct the eluent flow to the waste from 0 to 4 min and from 12 to 15 min, to avoid contamination of the electrospray of the MS detector part.

The Fig. 4 shows the signals at the transitions selected for the antiretroviral drugs and I.S. when a PIs/NNRTIs/I.S. solution was continuously infused post column directly into the MS/MS detector during the chromatographic analysis of a blank PBMCs extract. Even though no noticeable matrix effect (no drifts or shifts of the signals) could be observed at the respective retention time of the antiretroviral drugs and I.S. in this experiment, some matrix effect were however found as shown in the experiments reported below (see below § 3.5).

The chromatograms in Fig. 5, were obtained from a patient under APV (750 mg BID), RTV (133 mg BID) and LPV (533 mg BID). The blood sample was taken 3.6 h after drugs intake. The intracellular levels measured were 1089, 1000 and 5644 ng/ml for APV, RTV and LPV, respectively.

A careful control of the pH of the solution A at 2.85, and of the gradient elution program of the mobile phase is mandatory for standardizing the peak shape and retention time of the drugs. Retention time reproducibility is particularly important, as two segments of data acquisition are programmed in the positive mode detection, for the simultaneous analysis of PIs/NVP in a same run. An increase in the number of MS/MS transitions recorded simultaneously results in a sensitivity fall because of the time required for data acquisition at each of the selected MS/MS transition channel. To circumvent this limitation, two consecutive sets of MS/MS transitions in the positive mode were programmed during the analytical run, as given in the LC–MS/MS condition (§ 2.4).

3.2. Internal standard and calibration curve

The choice of the I.S. is a critical aspect of the method development, because it influences repeatability, reproducibility and accuracy, particularly important aspects when using electrospray mass spectrometry as compared to HPLC/UV [20]. Ideally, deuterated analogues of the antiretroviral drugs would be the first-choice standards, but these were not available to us. Thus, a number of peptidomimetic compounds, structurally related to PIs, were evaluated as potential internal standards. None were found suitable, due to a marked memory effect with this type of column. Conversely, the compound clozapine, though not structurally related to any antiretroviral drugs, but previously used in our HPLC–UV method for determination of PIs and NNRTIs in plasma [42–44], was found to have a satisfactory chromatographic profile, with a negligible memory effect. The I.S. was present in the extraction solvent added at the beginning of the PBMCs pellet sample processing, correcting for possible discrete residues from the wash solution. This is an advantage over the method proposed previously for the assay of ATV in PBMCs, where the I.S. is not part of the lysis/extracting solution [29].

For all antiretroviral drugs, the model with the lowest bias and the most constant variance across the concentration

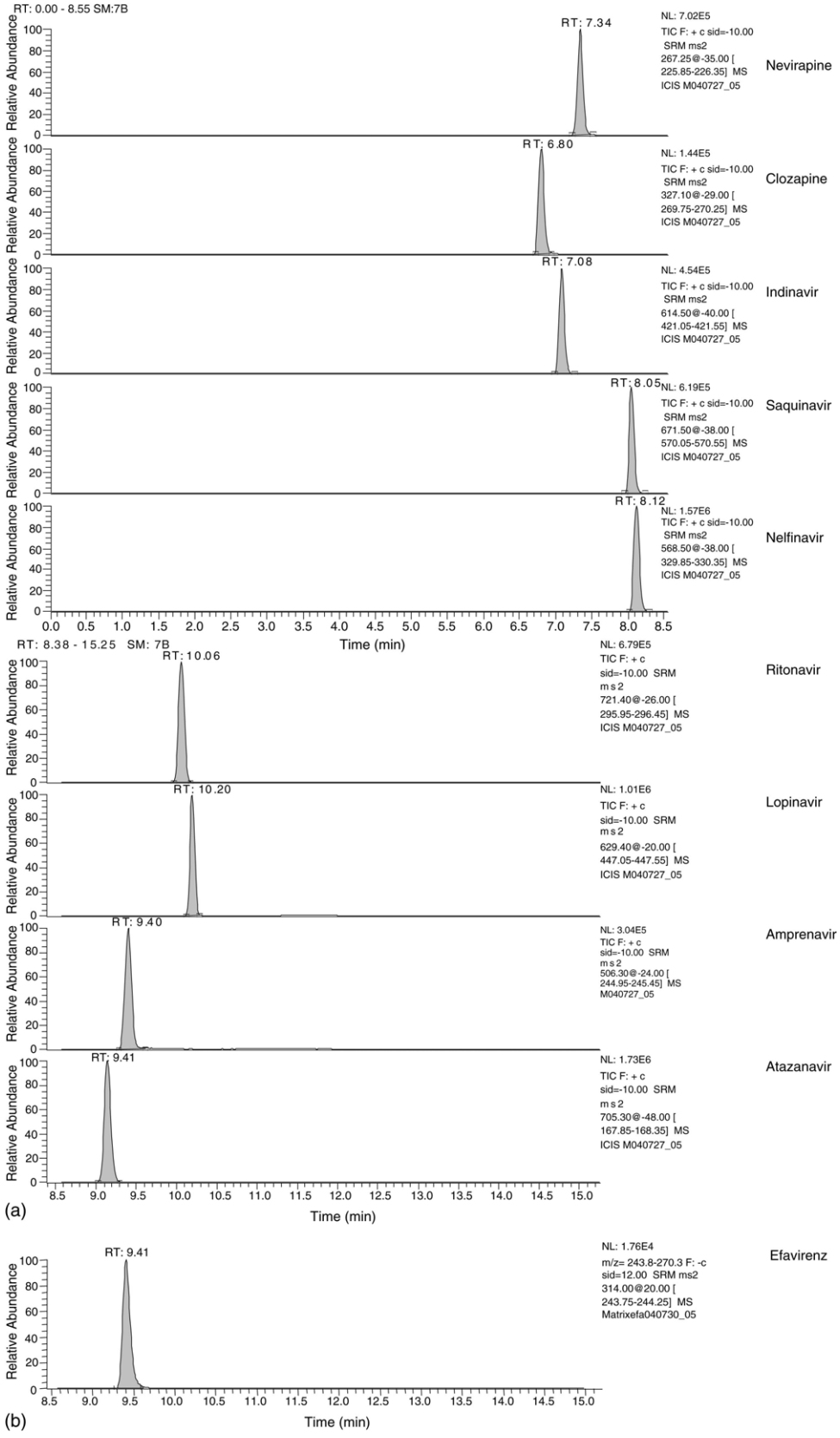

Fig. 3. Chromatogram of a calibration sample containing 75 ng/ml of each antiviral drug (2 ng/ml of internal standard), showing the first and second segments, at 0–8.55 and 8.55–15.25 min, respectively, for the positive mode (a), as well as the negative mode for the EFV (b).

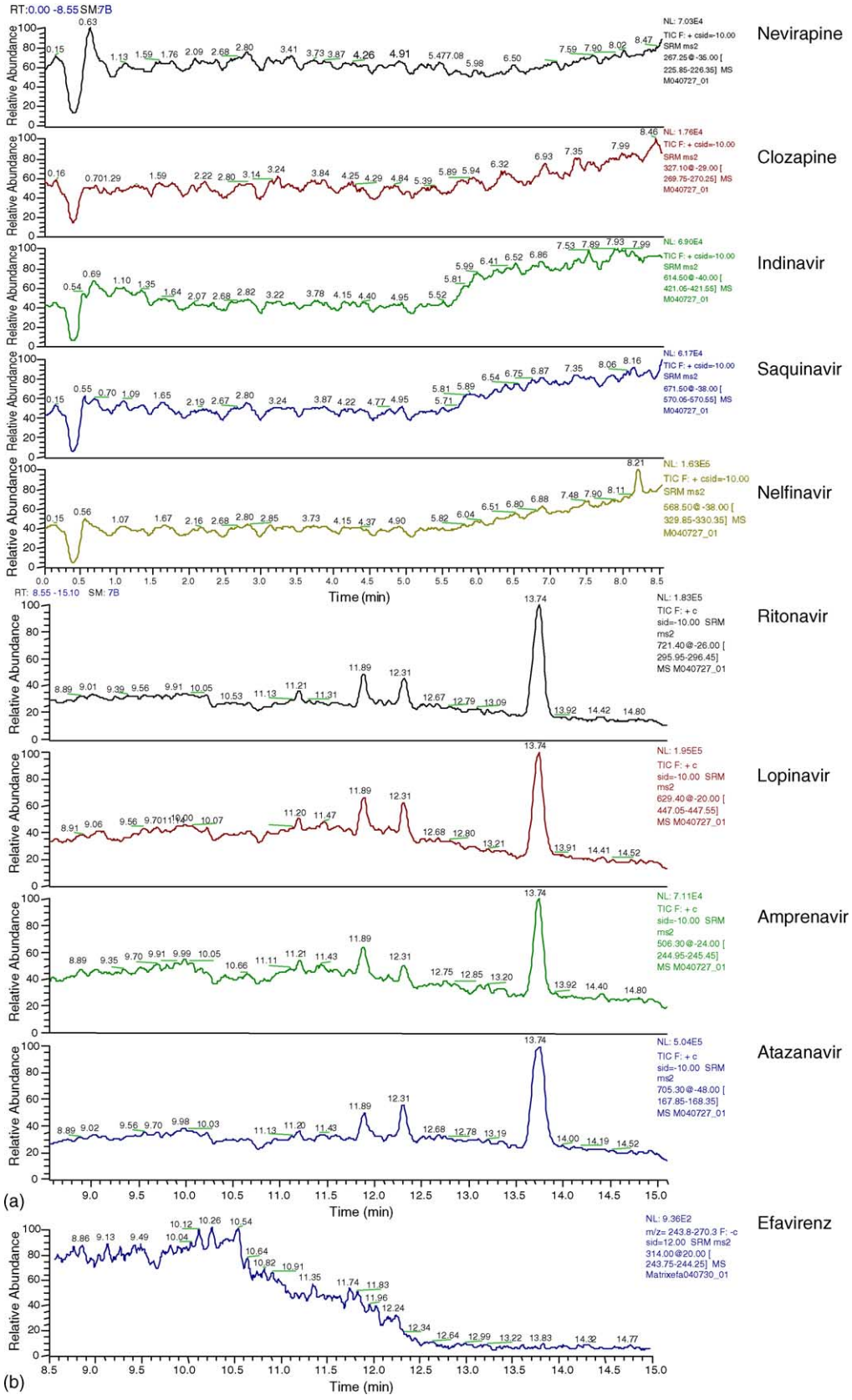

Fig. 4. Chromatogram of a blank extract with post column infusion of a calibration sample at 75 ng/ml of each analysed drug and 2 ng/ml of internal standard. Positive mode (first and second segments at 0–8.55 and 8.55–15.25 min, respectively) as well as the negative mode are shown.

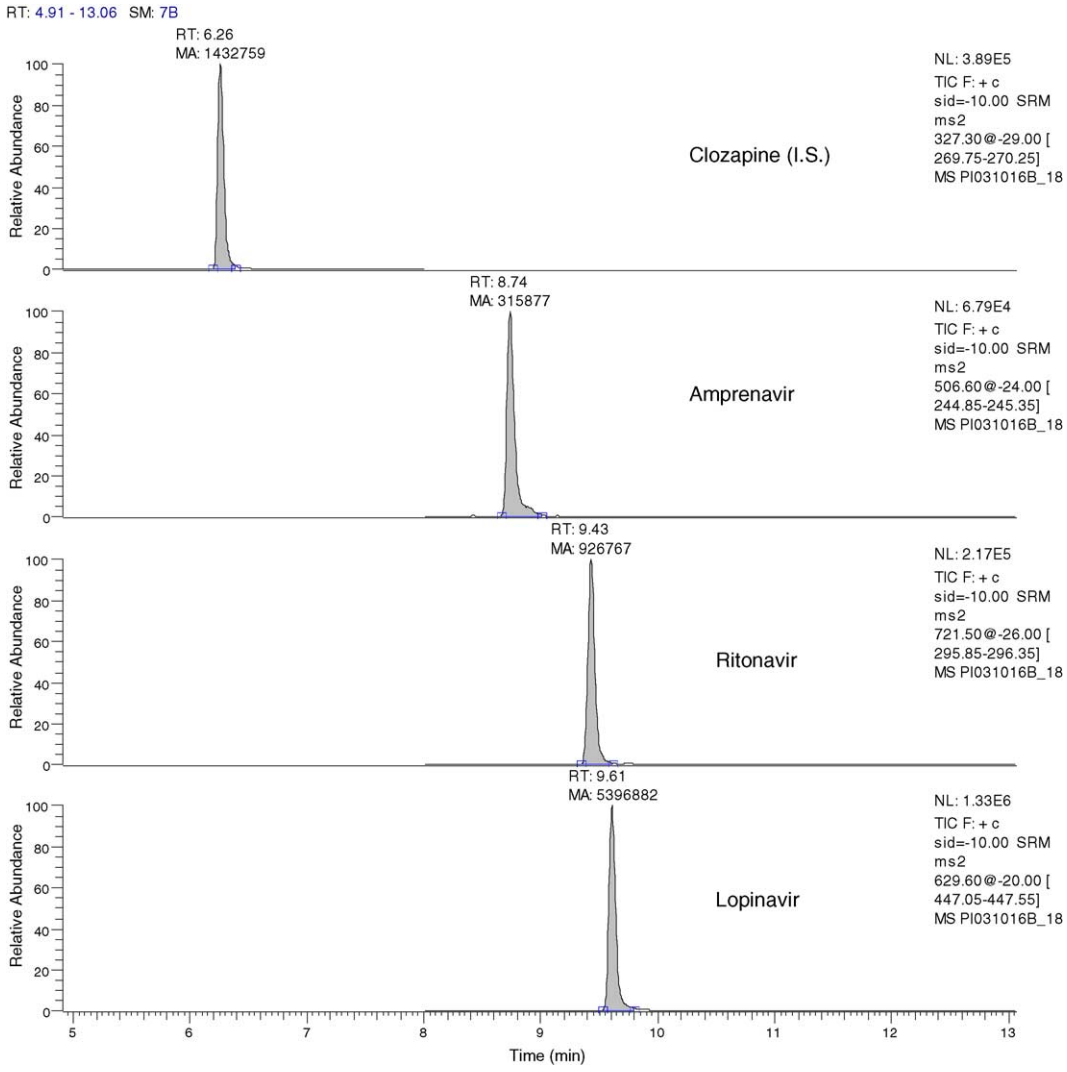

Fig. 5. Chromatogram of PBMCs of a HIV-1 infected patient receiving APV, LPV and a baby dose of RTV. The blood sample was taken 3.6 h after drugs intake. The intracellular levels measured were 1089, 5644 and 1000 ng/ml for APV, LPV and RTV, respectively.

range was considered to be the best fit. Calibration curves from 0.5 to 100 ng/ml were satisfactory described by $1/x^2$ weighted quadratic regression, of the peak-area ratio of PIs / NNRTIs to I.S., versus the ratio of the concentrations of the respective PIs and NNRTIs to I.S. in each standard samples. With this model, visual inspection of the plot of residuals (difference between experimental and calculated values using the established regression) versus concentration levels indicates that there are no trend in variability throughout the delineated range of concentrations (Fig. 6). Moreover, the homogeneity of variances have been statistically verified according to Levene's test, previously described in [44].

Over the concentration range from 0.5 to 100 ng/ml regression coefficient r^2 of the calibration curves were always greater than 0.99. Back calculated values of calibration samples prepared with different batches of cell pellet collection were analysed in duplicate at the beginning and end of analytical series and were always within ±15% of their nominal concentration (±20% at the LLOQ) as reported in Table 2 (intra-assay/inter-assay).

3.3. Precision, accuracy, LLOQ and LOD

Precision and accuracy were determined with the QC samples and are given in Table 2. The levels of control samples were selected to reflect low, medium and high range of the calibration curves chosen for encompassing the range of concentrations found in patients' PBMCs. The magnitude of intracellular levels in PBMCs was a priori largely unknown at the beginning of our study, and a few exploratory analyses were therefore required. The mean intra-assay precision was similar over the entire concentration range and always less than 8.5%. Overall, the mean inter-day precision was good, with average CVs within 0.6–10.2%. The intra-assay deviation (bias) from the nominal concentrations of each analysed antiretroviral drug was between −6.1 and 12.2% and the range of inter-day deviations always lower than 8.5%.

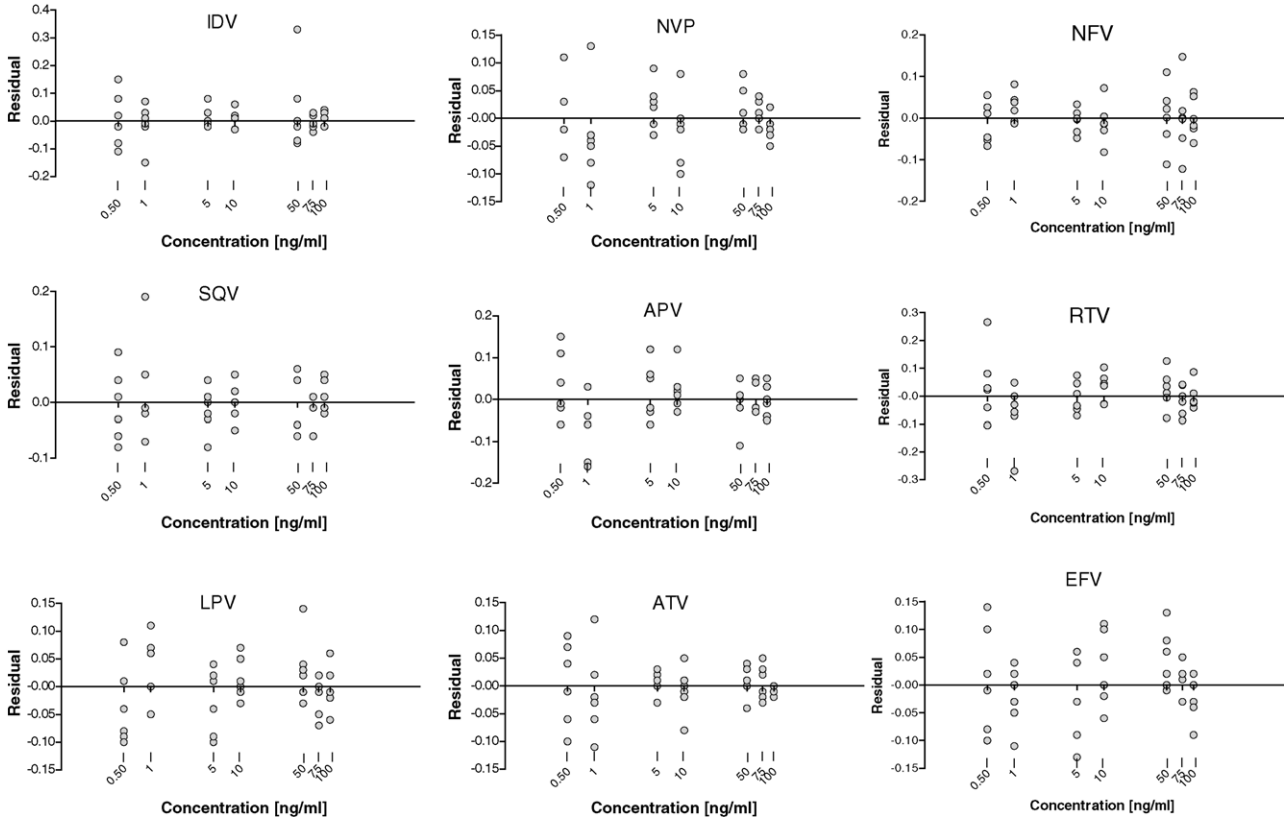

Fig. 6. Residual plot of PIs and NNRTIs plasma calibration samples back-calculated with a $1/x^2$ weighted quadratic regression ($n = 6$).

The chosen levels of calibration samples between 0.5 and 100 ng/ml were selected initially to encompass the relevant range of concentrations expected to be present in the PBMCs samples. During the course of the method validation however, it was observed a posteriori that the performance of the Thermo Quantum tandem MS/MS detector enables us to attain lower detection and quantification limits, well below the clinically relevant range of concentrations of 0.5–100 ng/ml.

The results of the determination of LLOQ and LOD of antiretroviral drugs in PBMCs (as described in the Section 2.7.2). are shown in Table 3. By analysing PBMCs extracts, spiked with decreasing concentrations of antiretroviral drugs, the lowest achievable LOD obtained for SQV and NFV was 1 pg/ml. The lowest LOQ obtained for ATV and APV was 0.2 ng/ml, corresponding to an amount of 2 pg of drug in the 10 μl-injection volume (i.e. injected onto column). Overall, with a calibration established between 0.5 and 100 ng/ml, the precision and the accuracy of the calibration sample at 0.5 ng/ml were, for all antiretroviral drugs, comprised within the ±20% limit recommended by the FDA [37] and the Washington and Arlington Conference Report [39]. More importantly, this method has the level of sensitivity required for the analysis of PBMCs collected during clinical studies. Of note, the LLOQ and LOD attained were found to differ depending on the drug considered, as reported in Table 3. However, for the sake of standardisation and for the simplification of standard solution preparation, it was decided to keep the same range of calibration of 0.5–100 ng/ml for *all* drugs, regardless of their LLOQ. In the particular context of bioanalytical method validation, it would be generally recommended to reduce the concentration of the lowest calibration levels to the respective LLOQ concentration of each drug considered.

3.4. Stability of PIs and NNRTIs

The stability of PIs and NNRTIs standard solutions left at room temperature was ascertained up to 72 h. At the concentrations of 2, 20 and 80 ng/ml, the variation over time of each drug level was always less than 10% (Table 4), with the exception of the quality control low concentration of indinavir and nelfinavir with a deviation of −16.7% and 13.3%, respectively. In our experiments, PIs and NNRTIs standard solutions were never allowed to stay more than 24 h at room temperature, indicating that the stability of PIs and NNRTIs in standard solutions at room temperature is such that the accuracy is not likely to be notably affected.

Table 4 shows the variation of PIs and NNRTIs concentrations after one, two and three freeze-thaw cycles. For all PIs and for NVP the variation was always less than 10%, indicating no significant loss of drug after up to three freeze-thaw cycles. For EFV, the variations at the three concentrations tested were higher but remained always less than 20%.

The stability study of PBMCs extracts into HPLC vials, kept for 24 h in the autosampler rack at room temperature

Table 2
Precision and accuracy of the assay for PIs and NNRTIs with isolated PBMCs (2, 25 and 80 ng/ml)

	Nominal cone (ng/ml)	Intra-assay ($n = 6$)				Inter-assay ($n = 6$)			
		Concentration found (ng/ml)	SD±	Precision C.V.%	Accuracy bias %	Concentration found (ng/ml)	SD±	Precision C.V.%	Accuracy bias %
	2	2.0	0.1	7.3	−0.9	2.0	0.1	3.0	−0.7
Indinavir	20	20.2	0.5	2.3	0.9	20.6	1.1	5.6	3.0
	80	79.8	1.7	2.1	−0.2	82.6	5.8	7.0	3.3
Nevirapine	2	2.1	0.1	3.9	3.3	2.0	0.2	8.6	−2.2
	20	20.4	0.7	3.6	2.0	21.3	0.7	3.1	6.7
	80	78.2	2.9	3.7	−2.3	77.2	4.0	5.2	−3.4
Nelfinavir	2	2.1	0.1	6.1	5.1	1.9	0.1	6.1	−4.3
	20	20.6	0.9	4.3	3.1	21.7	1.0	4.8	8.3
	80	78.2	3.1	3.9	−2.3	78.5	5.2	6.7	−1.9
Saquinavir	2	2.1	0.1	5.6	6.6	1.9	0.2	10.2	−5.5
	20	21.2	1.2	5.5	6.1	20.9	0.1	0.6	4.6
	80	80.0	1.2	1.5	0.0	78.0	7.0	8.9	−2.5
Amprenavir	2	2.0	0.1	6.9	2.1	1.9	0.1	3.9	−4.3
	20	20.0	0.8	3.9	0.2	19.6	1.0	4.9	−1.8
	80	76.3	2.4	3.1	−4.6	82.8	4.9	5.9	3.4
Ritonavir	2	2.0	0.1	5.8	0.2	1.9	0.1	6.3	−7.2
	20	19.0	0.6	3.0	−4.9	19.6	1.8	9.1	−2.0
	80	75.1	2.6	3.4	−6.1	81.9	6.7	8.2	2.4
Lopinavir	2	2.2	0.1	4.6	12.2	2.1	0.1	5.1	3.0
	20	19.6	0.8	3.9	−1.8	19.0	1.7	9.0	−4.8
	80	75.9	3.0	3.9	−5.1	80.7	6.3	7.8	0.9
Atazanavir	2	2.0	0.2	8.4	2.4	2.1	0.1	2.9	6.0
	20	20.7	0.6	3.0	3.5	21.0	1.3	6.2	5.0
	80	84.7	3.6	4.3	5.9	84.6	4.7	5.6	5.8
Efavirenz	2	2.1	0.2	8.1	3.6	2.0	0.1	2.7	−0.4
	20	20.9	1.0	4.6	4.7	20.8	0.3	1.6	3.8
	80	77.0	1.3	1.6	−3.7	78.3	2.1	2.6	−2.1

(Table 4), indicates a variation less than 15% for all drugs at each concentration. Taking into account the analytical variability, the processed samples are thus stable throughout the LC–MS/MS analysis, always completed within 24 h.

3.5. Matrix effect and recovery

In the absence of PBMCs samples with known amounts of *intracellular* antiretroviral drugs, the true extraction efficiency of a given extracting solvent cannot be ascertained. The extraction recovery obtained using spiked samples is not necessarily an accurate indicator of the true recovery; some solvent mixture may be more appropriate for denaturing cellular proteins, maximizing the analytes extraction at a more favourable pH, resulting in more reproducible analytical results. Among the solvent mixtures tested (mentioned in § 2.5.3), the best overall recovery for the nine antiretroviral drugs was 50% MeOH. This is in accordance with the 60% MeOH proposed in the literature [29,45,46], without discus-

Table 3
Limit of detection and lower limit of quantitation of PIs and NNRTIs

	LOD [pg/ml]	LOQ [ng/ml]	Accuracy at LOQ (% error ± SD)	Minimum quantifiable drug on column (pg)
Indinavir	6	0.4	−12.2 ± 8.1	4
Nevirapine	25	0.4	6.7 ± 3.1	4
Saquinavir	1	0.4	−7.6 ± 3.8	4
Nelfinavir	1	0.5	12 ± 8.5	5
Atazanavir	6	0.2	−13.6 ± 4.1	2
Amprenavir	6	0.2	−9.8 ± 14.6	2
Ritonavir	3	0.4	−12.3 ± 0.3	4
Lopinavir	13	0.4	−11.4 ± 12.6	4
Efavirenz	100	0.4	6.4 ± 13.0	8

LOD, limit of detection; LOQ, limit of quantification; SD, standard deviation.

Table 4
Stability of PIs and NNRTIs in standard solutions and in cells extracts

	Indinavir[a]			Nevirapine[a]			Saquinavir[a]		
	2[b]	20[b]	80[b]	2[b]	20[b]	80[b]	2[b]	20[b]	80[b]
Standard solutions									
Room temperature (72 h)	−16.7	−2.0	−6.1	1.9	−9.1	−3.2	−4.2	−6.6	5.3
Human PBMCs extracts									
Free-thaw									
Cycle 1	−2.7	−0.5	1.7	−6.0	−1.7	−1.6	−2.5	−0.6	−1.2
Cycle 2	2.4	−1.0	2.9	−6.0	0.6	−2.7	1.9	−1.9	−1.1
Cycle 3	1.3	1.0	−5.2	2.2	0.5	−5.0	−0.2	2.0	−4.8
Autosampler stability (24 h, 20 °C)	−2.9	0.0	2.6	0.2	−0.9	−5.1	9.2	−2.2	2.3
	Nelfinavir[a]			Atazanavir[a]			Amprenavir[a]		
Standard solutions									
Room temperature (72 h)	13.3	0.1	8.8	0.5	1.0	−0.3	−6.4	−1.1	0.5
Human PBMCs extracts									
Free-thaw									
Cycle 1	0.5	0.6	−1.9	1.4	−4.4	−6.0	−0.7	1.9	−3.1
Cycle 2	1.8	0.7	−1.7	6.5	−3.6	−4.0	0.3	−2.8	−1.0
Cycle 3	−3.4	4.3	−5.1	0.5	−1.9	−7.1	−7.1	−2.2	−1.9
Autosampler stability (24h, 20 °C)	14.0	−3.5	−3.1	6.2	0.1	−1.2	5.4	4.0	−1.1
	Ritonavir[a]			Lopinavir[a]			Efavirenz[a]		
Standard solutions									
Room temperature (72 h)	−4.3	1.1	−2.9	2.8	−1.9	−1.1	−1.3	−0.4	2.3
Human PBMCs extracts									
Free-thaw									
Cycle 1	1.1	−6.0	−1.4	−0.7	−0.7	−5.7	−12.1	−6.9	−4.5
Cycle 2	−0.6	−2.5	−0.3	3.6	−6.8	−8.6	−14.2	−13.5	−5.8
Cycle 3	−6.2	−6.5	−1.0	−3.6	0.3	−4.9	−11.9	−5.9	−6.0
Autosampler stability (24h, 20 °C)	4.9	5.2	1.7	11.7	6.4	1.0	6.0	4.9	2.3

[a] Drug.
[b] Nominal concentration (ng/ml).

sion as to why this particular medium and concentration was chosen.

Matrix effect was examined by the simultaneous post-column infusion of PIs/NNRTIs and I.S. into the MS/MS detector during the chromatographic analysis of three different batches of blank PBMCs extracts from healthy volunteers. As exemplified in Fig. 4, no drifts or shifts of the selected transition signals were apparent during the chromatography of a typical blank PBMCs matrix, at the retention time of the ten drugs (Table 1) shown in Fig. 3.

Co-eluting undetected matrix components may nevertheless reduce or enhance the ion intensity of analytes and affect the reproducibility and accuracy of the assay. Consequently, for the sake of validation, all calibration standards and QC samples were prepared by spiking drugs in the same biological matrix (i.e. a similar amount of PBMCs isolated from leucocytes removed from blood unit).

The assessment of the *matrix effect* (Table 5) was quantitated as the peak-area response of analytes added to blank PBMCs extracts (i.e. drugs added *after* extraction), expressed as a percentage of the response of standard solution of drugs directly injected onto the column (ratio B/A, in Table 5). A value above or below 100% for the matrix effect indicates an ionization enhancement or suppression, respectively. The results indicate that co-eluting PBMCs matrix components appear to reduce the ion intensity for NFV (mean 85%) and NVP (mean 83%), and by contrast enhance the IDV ion intensity (mean 138%). Matrix effects have already been reported to influence this latter protease inhibitor [47].

In addition, the recovery of the *extraction procedure* and the *analytical recovery* were also determined and are given in Table 5, column named *ext RE* and *analysis RE*, respectively. The recovery of the *extraction procedure* for all drugs and I.S., expressed in %, is calculated as the ratio of the peak area of analytes spiked before (C) and after (B) the extraction procedure (ratio C/B, in Table 5). The *analytical recovery* was calculated similarly, considering though drugs response/I.S. ratio instead, before (C2) and after (B2) the extraction procedure (ratio C2/B2, in Table 5). Taking into account I.S response in the calculation enables to correct for the possible presence of variable volume of residual wash solution in cell pellets and for the occurrence of any variation over time in the MS/MS spectrometer performance. As indicated in Table 5, the extraction recovery for the I.S. was essentially 100%. Both the *ext RE* and *analysis RE* achieved were always higher than 90%, except for efavirenz (79%).

Table 5
Matrix effect, extraction recovery, analysis recovery and process efficiency of PIs and NNRTIs

Drug	Nominal cone (ng/ml)	Mean peak area ($n = 3$)			Mean peak area ratio ($n = 3$)		ME (%)	ext RE (%)	Analysis RE (%)	Mean	SD	PE (%)
		A	B	C	B2	C2						
Indinavir	2	1.08E+05	1.58E+05	1.49E+05	0.158	0.147	146	94.2	93.0	95.6	2.3	137
	20	1.12E+06	1.56E+06	1.52E+06	1.550	1.507	140	97.1	97.2			136
	80	4.37E+06	5.59E+06	5.53E+06	5.689	5.495	128	99.1	96.6			127
Nevirapine	2	1.27E+05	1.03E+05	9.88E+04	0.103	0.098	81	96.0	94.8	95.3	1.5	78
	20	1.23E+06	1.01E+06	9.73E+05	0.996	0.966	82	96.7	97.0			79
	80	4.54E+06	3.87E+06	3.73E+06	3.933	3.701	85	96.3	94.1			82
Saquinavir	2	1.61E+05	1.75E+05	1.59E+05	0.193	0.171	109	90.6	88.8	90.0	1.3	99
	20	1.58E+06	1.57E+06	1.43E+06	1.557	1.423	99	91.2	91.4			91
	80	5.78E+06	6.02E+06	5.53E+06	6.108	5.493	104	92.0	89.9			96
Nelfinavir	2	1.09E+06	9.58E+05	8.79E+05	0.962	0.872	88	91.8	90.7	91.1	2.6	80
	20	4.58E+06	3.82E+06	3.58E+06	3.779	3.552	83	93.5	94.0			78
	80	1.54E+07	1.30E+07	1.18E+07	13.143	11.668	84	90.7	88.8			76
Atazanavir	2	4.93E+05	5.16E+05	4.95E+05	0.518	0.492	104	96.0	94.9	93.2	1.5	100
	20	4.74E+06	4.92E+06	4.55E+06	4.876	4.521	104	92.5	92.7			96
	80	1.84E+07	1.86E+07	1.75E+07	18.907	17.382	101	94.1	91.9			95
Amprenavi	2	6.97E+04	7.32E+04	7.08E+04	0.074	0.070	105	96.8	95.5	92.8	3.4	102
	20	7.02E+05	7.34E+05	6.86E+05	0.727	0.682	104	93.6	93.8			98
	80	2.80E+06	2.83E+06	2.58E+06	2.878	2.561	101	91.1	89.0			92
Ritonavir	2	1.91E+05	2.06E+05	1.90E+05	0.207	0.188	108	92.2	91.0	91.7	2.4	100
	20	1.88E+06	2.06E+06	1.94E+06	2.046	1.932	110	94.3	94.4			103
	80	7.33E+06	8.14E+06	7.49E+06	8.272	7.432	111	92.0	89.8			102
Lopinavir	2	2.82E+05	3.05E+05	2.75E+05	0.306	0.274	108	90.4	89.5	95.3	5.1	98
	20	2.77E+06	2.81E+06	2.77E+06	2.778	2.751	101	98.7	99.1			100
	80	1.01E+07	1.05E+07	1.05E+07	10.647	10.376	104	99.8	97.5			104
Efavirenz	2	2.56E+03	2.48E+03	2.19E+03	0.002	0.001	96.8	88.4	73.5	79.8	8.3	86
	20	3.24E+04	2.89E+04	2.80E+04	0.018	0.016	89.0	96.8	89.2			86
	80	1.19E+05	1.23E+05	1.16E+05	0.086	0.066	103.0	94.9	76.6			98
Clozapine ($n = 9$)	2	1.13E+06	9.97E+05	1.01E+06			89	101.1				90

A, peak area of standard solutions without matrix and without extraction (MeOH/H2O 50/50); B, peak area of analytes spiked after extraction; C, peak area of analytes spiked before extraction; B2, ratio of the peak area of the analyte and the I.S. spiked *p* after extraction; C2, ratio of the peak area of the analyte and the I.S. spiked before extraction; ME, matrix effect expressed as the ratio of the mean peak area of the analytes spiked after extraction (B) to the mean peak area of the same standard solution without matrix (A) multiplied by 100. A value of >100% indicates ionization enhancement, and a value of <100% indicates ionization suppression; ext RE, extraction procedure recovery calculated as the ration of the mean peak area of the analytes spiked before extraction (C) to the mean peak area of the analytes spiked after extraction (B) multiplied by 100; analysis RE, analysis recovery calculated as the ration of the mean peak area ratio of the analytes spiked before extraction (C2) to the mean peak area ratio of the analytes spiked after extraction (B2) multiplied by 100; PE, process efficiency expressed as the ratio of the mean peak area of the analyte spiked before extraction (C) to the mean area of the same analyte standards (A) multiplied by 100.

Finally, the overall *process efficiency* is given in Table 5 (column named *PE*) and was obtained as the peak-area response of analytes spiked into PBMCs pellet samples *before* the extraction procedure (C)—such as calibration and control samples—expressed as the percentage of the peak area of pure drug standard solution (A) directly injected into the column. This *process efficiency* takes into account the recovery and the matrix effect: indinavir for example, has a mean matrix-mediated ionization enhancement close to 140% (Table 5, column ME), which combined with an extraction recovery of 97% (Table 5, column ext RE) gives finally a process efficiency around 130%. Overall, these results indicate that even though no apparent matrix effect could be observed in the infusion experiment (Fig. 4), matrix components do influence the overall process efficiency requiring therefore the preparation of calibration and control samples in a PBMCs matrix that would reflect at best the composition of the samples to be analyzed.

3.6. Influence of PBMCs cell count on the accuracy of the method

Given the PBMCs numbers generally encountered in our study, the 400 μL-volume of MeOH:H_2O 50:50 solvent is expected to provide good lysis and extraction efficiency of cell pellets, whose own volume can be considered negligible. The volume of extracting solvent is higher than the volume previously proposed for the extraction of antiretroviral drug from PBMCs [29,48]. The use of a larger volume of solvent for extraction increases sample dilution, resulting in an easier sample handling, and is made possible by the analytical sensitivity provided by the TSQ Quantum instrument.

Calibration samples were so prepared as to contain PBMCs count ranging from 4 to 10×10^6 cells, close to the cell counts usually encountered in patients. However, it is difficult to prepare calibration samples that always match perfectly the cell counts encountered in patients. In fact, cell count may vary up to ca. nine-fold from one sample to another, particularly in HIV patients, even when a same volume of blood is collected. The median cell recovery obtained from a CPT tube (8 ml of blood) in our group of HIV infected patients ($n = 80$) was 8.0×10^6 cells with an interquartile range from 6.2 to 10.2×10^6 cells, in accordance with previous reports [18].

Thus, the possible influence of cells number on the accuracy of drug measurements was verified by a cross-validation study performed with QCs samples with a seven-fold difference (1.4×10^6 cells versus 9.6×10^6) in PBMCs content. The obtained signals were quantified using calibration samples containing the same, or the alternate cell number. A slight increase (+7%) in deviation could be noticed when QCs samples with low cell content were quantified using calibration established with high cell content samples. Similarly, the bias was slightly increased (−6%) when QCs samples with high cell content were quantified using calibration established with low cell content samples. However, these deviations were small, always resulting in a bias less than the ±15% recommended ranges [37–40]. Considering the experimental variability (Table 2), these analytical results indicate that within the range of 1.4–9.6×10^6 cells per vial and with the proposed volume of extraction solvent, cells number does not affect, or only slightly, the accuracy of antiretroviral drugs determination over the considered concentrations range. Thus, though PBMCs are necessary for the matrix effect correction, calibration can be established within the 4–10×10^6 cells range without individualized standardisation of the cell number in calibration samples. Confirming this, calibration curves could be established routinely with satisfactory accuracy and precision (Table 2) [37–40], irrespective of the PBMCs batch and cell count in calibration level or QCs samples. This is an advantage over a method proposed previously for the ATV assay in PBMCs [29], as it eliminates the burden of adding a variable volume of extraction solvent to each patient pellet samples according to its cell count, a tedious procedure for large series of analyses. Nevertheless, the latter approach can be used, though it will not add much to the performance of the proposed analytical method.

3.7. Dilution effect

The mean effect of dilution on precision was always less than 8%, for all antiretroviral drugs at the two dilutions (1:2 and 1:3) tested. After dilution, the deviation (bias) from the expected concentrations was less than 9.5%. This indicates that PBMCs extract samples containing antiretroviral drugs above the high level of calibration can be adequately diluted with blank PBMCs extract (containing I.S. at 2 ng/ml) prior to the LC–MS/MS analysis.

3.8. Selectivity

No peaks from endogenous compounds were observed at the drugs selected transition and retention time in any of 10 different blank PMBC extracts evaluated. The product ion monitoring was chosen, given its relative abundance, while avoiding possible structural analogies with the other drugs or fragments analysed. Every channel was simultaneously observed, and we never saw any selectivity problem as well as any *crosstalk* signal between acquisition channels.

3.9. Clinical applications

LC–MS/MS is the method of choice for intracellular drug measurements, offering the best sensitivity, selectivity and high throughput capability. This analytical method has proved to be reliable, sensitive enough for monitoring concentrations of PIs and NNRTIs in PBMCs isolated from HIV infected patients, and is being applied in research studies done under the auspices of the Swiss HIV Cohort Study. On-going studies are addressing the influence of genetic factors on the intracellular concentrations of antiretroviral drugs, and their consequences on clinical responses and treatment toxicities. This method was applied to the measurement of intracellular levels of efavirenz, found much higher in HIV patients with the 516 G > T (Gln172His) polymorphim of cytochrome P450 2B6 (CYP2B6) associated with a diminished activity of this isoenzyme [49]. Of interest, high efavirenz levels in PBMCs, but not in plasma, were associated with an increased incidence and a higher grade of severity of central nervous system adverse drug reactions (mood disorder and fatigue). In addition, this analytical method has also enabled to determine the range of concentration found in PBMCs from HIV patients and their relation with total plasma concentration measured simultaneously.

4. Conclusion and discussion

This validated LC–MS/MS method provides a robust procedure for determining PIs and NNRTIs concentrations in PBMCs from HIV patients, thereby enabling drug monitoring and enhancing our understanding of antiretroviral drugs efficacy and toxicity. Of note, the intracellular concentration of antiretroviral drugs should be regarded as a *cell-associated amount*, as the drug in its cellular environment may be embedded in membrane lipid bilayers, complexed to cytoplasmic proteins or sequestrated through intracellular protein binding [20,50], so that a fraction of the so-called *intracellular amount* of drug is available to exert the actual antiviral action. In the field of HIV pharmacology, there is at present a high interest for the intracellular measurement of antiretroviral drugs, even though detailed validation reports for their assay in PBMCs samples is still infrequent [29]. Early analytical efforts have been done for the determination of the phosphorylated nucleoside reverse transcriptase inhibitors in

PBMCs from HIV patients, and have also described PBMCs handling and practical difficulties to ensure the validity of the results [19–22].

The efflux of antiretroviral drugs out of cells has been reported to be rapid ex vivo, and critically time and temperature dependent [8,34]. Thus, for minimizing drug efflux, blood samples were placed in ice-cold conditions within 5 min. The processing time of PBMCs from phlebotomy to their storage at −20 °C was less than one hour. The use of ice-cold PBS solution and centrifugation at 4 °C during the wash procedure was reported to result in the retention of more than 80% of the intracellular protease inhibitors content at 60 min [8]. The high sensitivity and specificity attained with LC–MS/MS enable to quantify as little as 2 to 8 pg on-column, depending on the drug analysed (Table 3), in PBMCs isolated from a 8 ml-volume of blood sample. Compared to previously published methods using a combination of liquid–liquid and solid-phase extraction [29], or two consecutive liquid–liquid extractions [30], the procedure is simplified, involving only a single-step liquid–liquid extraction. However, as some matrix effect is notable for some of the drugs (IDV, NFV and NVP), calibration curves have to be established with standards prepared using blank PBMCs. Filtration of blood transfusion units represents a convenient method to get a substantial provision of blank PBMCs. We have shown that the analytical method maintains satisfactory accuracy within the PBMCs range of $1.4–9.6 \times 10^6$ cells.

This is, to the best of our knowledge, the first validation report of a LC–MS/MS method for the assay in PBMCs after a single-step liquid extraction, of all antiretroviral drugs commercially available at present. The PIs and nevirapine can be measured simultaneously in the same analytical run by ESI in the positive ionisation mode, while a separate run is necessary for efavirenz in the negative mode, for optimal sensitivity. While programming alternately positive and negative mode during the analytical run is feasible, this results in our hand in a notable reduction of the overall sensitivity. In turn, sequential segments in positive and negative mode for the consecutive assay of PIs/NVP and EFV were unfeasible with our gradient program, as EFV retention time is very close to the late eluting PIs, making it difficult to switch from the positive to the negative mode during the short time interval between peaks elution.

This analytical method provides a sensitive method for the intracellular measurement of antiretroviral drugs in PBMCs. The limiting step for its routine application is certainly the labor-intensive procedure for the timely PBMCs isolation. The methodology should enable a better understanding of the intracellular pharmacokinetics of antiretroviral drugs in vivo and of their clinical efficacy and toxicities. It is too early to appreciate whether antiretroviral drug measurement in PBMCs may add clinically relevant information to the current TDM performed in plasma for the routine follow-up of HIV patients. Further studies will determine if it can contribute to minimize the risk of major adverse reactions and to increase the probability of efficient, long lasting, therapeutic response.

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