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Simultaneous determination of the antiretroviral agents: amprenavir, lopinavir, ritonavir, saquinavir and efavirenz in human peripheral blood mononuclear cells by high-performance liquid chromatography–mass spectrometry

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Abstract

A selective and accurate assay for the simultaneous quantitation of four protease inhibitors (PIs) (amprenavir (APV), lopinavir (LPV), ritonavir (RTV) and saquinavir (SQV)) and a non-nucleoside reverse transcriptase inhibitor (NNRTI) (efavirenz, EFV) in human peripheral blood mononuclear cells using high-performance liquid chromatography–mass chromatography (LC/MS) has been developed and validated. After liquid–liquid extraction, the antiretroviral agents were separated within 15 min. The calibration curves of each drug showed a good linearity in a range of concentration between 2 and 200 ng/3 × 10^6 cells for amprenavir, lopinavir, efavirenz, 1.60 and 128 ng/3 × 10^6 cells for ritonavir and saquinavir. Mean intra- and inter-assay coefficients of variation over the ranges of the standard curves were less than 15% and mean extraction recoveries ranged 88.7–112.1%. The limits of quantification were 2 ng/3 × 10^6 cells for amprenavir, lopinavir, efavirenz, 1 ng/3 × 10^6 cells for ritonavir and 1.6 ng/3 × 10^6 cells for saquinavir. This novel LC/MS assay, which provides an excellent method for simultaneous intra-cellular determination of amprenavir, lopinavir, ritonavir, saquinavir and efavirenz in human peripheral blood mononuclear cells, could be successfully applied for therapeutic drug monitoring and pharmacokinetic studies.

Keywords: Intra-cellular quantitation; Protease inhibitors (PIs); Non-nucleoside reverse transcriptase inhibitor (NNRTI); LC/MS

1. Introduction

In recent years, the development of highly active antiretroviral therapy (HAART) has improved the treatment of human immunodeficiency virus (HIV) infection. This standard treatment included at least three drugs from two different antiretroviral classes among nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), and protease inhibitors (PIs). The NRTIs were intra-cellularly phosphorylated to their corresponding triphosphorylated derivatives, which competed with the corresponding natural nucleotide for binding to HIV reverse transcriptase (RT) and inhibited it. The NNRTIs acted as non-competitive inhibitors of the HIV-1 RT. The PIs

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interfered with viral replication by inhibiting the HIV protease enzyme, preventing maturation of the HIV virus and causing the formation of non-infectious virions.

Although, triple drug combination decreased plasma HIV-1 RNA levels below the limit of detection (<50 copies/ml) in most cases, some patients became non-responsive to HAART because of the rapid development of drug-resistant variants of HIV-1. Among other factors, inter-patient pharmacokinetic variability, non-compliance to drug therapy or drug–drug interactions influenced the maintenance of adequate drug levels, which affected the treatment effectiveness.

Recently, data have emerged showing a possible link between intra-cellular drug concentrations and antiviral effect [1–3]. Whereas, several high-performance liquid chromatography methods using ultraviolet [4–7] or mass spectrometric detection [8–11] for quantitation of antiretrovirals in plasma have been previously reported, only a few study described the intra-cellular quantitation of antiretroviral agents [12–14], mostly for NRTIs whose activity involves intra-cellular conversion. Moreover, to date no study has reported the simultaneous intra-cellular quantitation of several PIs or NNRTIs. As HIV replicated within the cells, antiretroviral agents must penetrate intra-cellularly at concentrations sufficient to inhibit viral replication. Consequently, monitoring intracellular drug concentrations was useful to ensure that efficacious levels were achieved in target cells, particularly for patients in virological failure despite effective plasma concentrations.

Consequently, we developed and validated a simple, sensitive and selective assay for the simultaneous determination of four PIs (amprenavir, lopinavir, ritonavir and saquinavir) and a NNRTI (efavirenz) in human peripheral blood mononuclear cells (PBMCs) using high-performance liquid chromatography–mass spectrometry (LC/MS) with atmospheric pressure chemical ionization (APCI).

2. Experimental

2.1. Reagents and materials

Analyte drugs (Fig. 1) were kindly provided by the following pharmaceutical companies: amprenavir (APV) by GlaxoSmithKline (Research Triangle Park, NC, USA), efavirenz (EFV) by Dupont Pharmaceuticals (Wilmington, DE, USA), ritonavir (RTV), lopinavir (LPV) and the internal standard (IS) A-86093 by Abbott Laboratories (Abbott Park, IL, USA), and saquinavir mesylate (SQV) by Roche Products, Research and Development (Welwyn Garden City, UK).

All chemicals were of analytical reagent grade and all solvents were of HPLC grade. Acetonitrile, methanol and *n*-pentane were purchased from Scharlau (Barcelona, Spain). Sterile distilled water Versol® was obtained from Aguettant (Lyon, France). Ammonium hydroxide, sodium carbonate, sodium azide, ethyl acetate and formic acid were provided from Prolabo (Fontenay-sous-Bois, France).

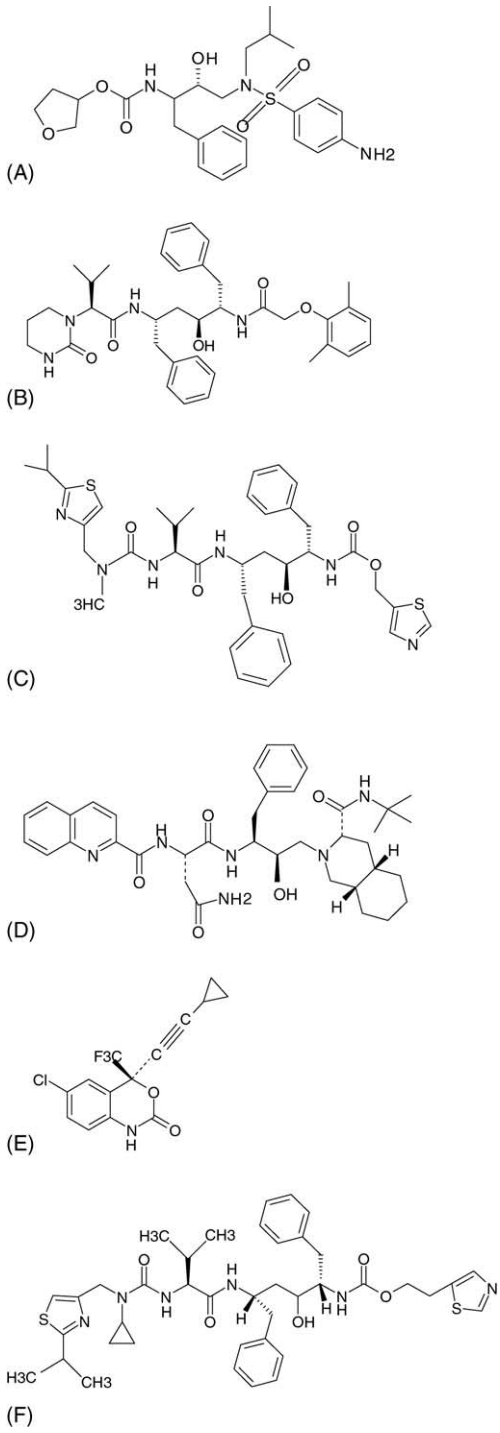

Fig. 1. Chemical structures of (A) APV, (B) LPV, (C) RTV, (D) SQV, (E) EFV and (F) the internal standard. Molecular weights were 506 g/mol for APV, 629 g/mol for LPV, 721 g/mol for RTV, 671 g/mol for SQV, 316 g/mol for EFV and 747 g/mol for the internal standard, respectively.

Foetal calf serum (FCS), phosphate-buffered saline Dulbecco's GIBCO™ (PBS) and hepes buffer solution 1 M GIBCO™ were from Invitrogen Corporation (Grand

Island, NY, USA). RPMI-1640 cell culture medium, L-glutamine and bovine α1-acid glycoprotein were obtained from Sigma–Aldrich (Saint-Quentin-Fallavier, France).

Vacutainer® CPT™ tubes (Becton Dickinson, Le Pont-de-Claix, France) were used for the separation of PBMCs from whole blood. KOVA® glasstic® slides were provided from Hycor Biomedical Inc. (Garden Grove, CA, USA).

2.2. Apparatus

A Jouan GR 4-22 centrifuge (Saint Herblain, France) was used for centrifugation during the sample preparation.

Agilent (Palo Alto, CA, USA) supplied the 1100 Series HPLC system connected to a quadrupole 1100 mass spectrometer. The ionization mode was positive atmospheric pressure chemical ionization for all analytical compounds. Data were processed by a Hewlett-Packard Chemstation software (Palo Alto, CA, USA).

2.3. Separation conditions

2.3.1. Chromatographic conditions

Analytes were separated on a X-TERRA™ MS C_{18} column (Waters, Saint Quentin Yvelines, France), which was 4.6 mm × 100 mm i.d. packed with 5 μm particles.

The separation of the analytes was achieved with an isocratic solvent delivery system at a flow-rate of 1 ml/min at 20 °C. The mobile phase consisted of acetonitrile and a buffer (50:50, v/v). The buffer was prepared by dissolving 400 μl of formic acid in 1 l HPLC-grade water, adjusting pH to 3 with ammonium hydroxide. A millipore vacuum filtration system equipped with a 0.45 μm filtration disk membrane was used for filtration and the mobile phase was degassed by using ultra sonic. The sample injection volume was 10 μl and the run time of the assay was 15 min.

2.3.2. Mass spectrometric conditions

Mass spectral analyses were accomplished on an Agilent quadrupole MSD 1100 mass spectrometer, fitted with an APCI source and operated in the positive ionization mode.

The vaporizer was operated at 450 °C; the discharge current fixed at 8 μA and the capillary voltage set at 2500 V. Amprenavir, lopinavir, ritonavir, saquinavir, efavirenz and the internal standard were detected by their monocation (*m*/*z*, respectively, 506.20, 629.30, 721.00, 671.00, 272.00 and 747.30).

2.4. Preparation of stock solutions

Two stock solutions of each analyte and internal standard were prepared independently at a concentration of 1 mg/ml for APV, LPV, SQV, EFV, IS and 0.32 mg/ml for RTV in methanol. One solution was used to prepare the calibration samples; the quality control samples were made from the other solution. Stocks solutions were kept at −20 °C.

2.5. Preparation of calibration standards and quality control samples

2.5.1. Cell culture and lysis

The matrix chosen for validation of the assay was Jurkat cells. Before using this human T–cell line, we compared Jurkat cells with PBMC fraction obtained from blood specimen collected from healthy subjects into Vacutainer® CPT™ tubes. Quality controls at low, medium and high concentrations for all analytes were prepared with cell lysates obtained from either Jurkat cells ($n = 18$) or human PBMC fractions ($n = 18$). Precision, stability and extraction recovery of each matrix were studied and there was no statistical difference between Jurkat cells and human PBMC fraction collected from Vacutainer® CPT™ tubes. Jurkat cells were maintained in RPMI 1640 medium supplemented with heat-inactivated FCS (10%, v/v), L-glutamine 2 mM (2.5%, v/v), hepes 1 mM (1%, v/v) in a humidified incubator containing 5% CO_2 in air at 37 °C. The cells were passaged every three days. Cells were washed with PBS to obtain a cell suspension density at 3×10^6 cells/ml; 1 ml of cell suspension was centrifuged at $600 \times g$ for 10 min at 4 °C. Cell pellet was suspended in 200 μl of a solution of α1-acid glycoprotein 1 mg/ml in sodium azide (0.1%, w/v). Cells lysates were stored at −80 °C until standards and controls preparation.

2.5.2. Preparation of standards and controls

Both stock solutions were diluted with methanol to obtain working solutions with concentrations of 10 μg/ml for APV, EFV and LPV, 4.8 μg/ml for SQV and 3.2 μg/ml for RTV. Calibration standards (five concentrations) and quality control samples (low, medium, high and very high concentrations) were prepared by first diluting various volumes of the working solutions in methanol, and adding 10 μl of each diluted solution to cell lysates. The following ranges were validated: 2–200 ng/3×10^6 cells for APV, LPV and EFV, 1.6–128 ng/3×10^6 cells for RTV and SQV.

2.6. Preparation of samples

2.6.1. Isolation of PBMCs from patients

Peripheral blood samples were drawn from patients under therapy including one or more following antiretrovirals APV, LPV, RTV, SQV and EFV, after informed consent had been obtained. Approximately 8 ml of peripheral venous blood was collected by venipuncture into Vacutainer® CPT™ tubes containing sodium citrate as anticoagulant and PMBCs were isolated by density gradient separation. PBMCs fraction was worked in 30 min maximum after collection in order to measure the real quantity of PIs at sampling time. Vacutainer® CPT™ tubes were centrifuged at $1650 \times g$ for 20 min at 20 °C. Cellular fraction was transferred to a fresh tube and centrifuged at $600 \times g$ for 10 min at 4 °C. The pelleted PBMC were washed with 2 ml ice-cold PBS, put on KOVA® slide for cell account and centrifuged as described above. Cell pellet was suspended in 200 μl of a solution of α1-acid

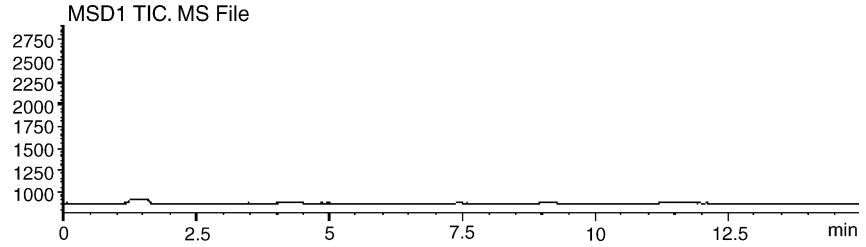

Fig. 2. Total ion chromatogram (TIC) from a blank PBMCs sample.

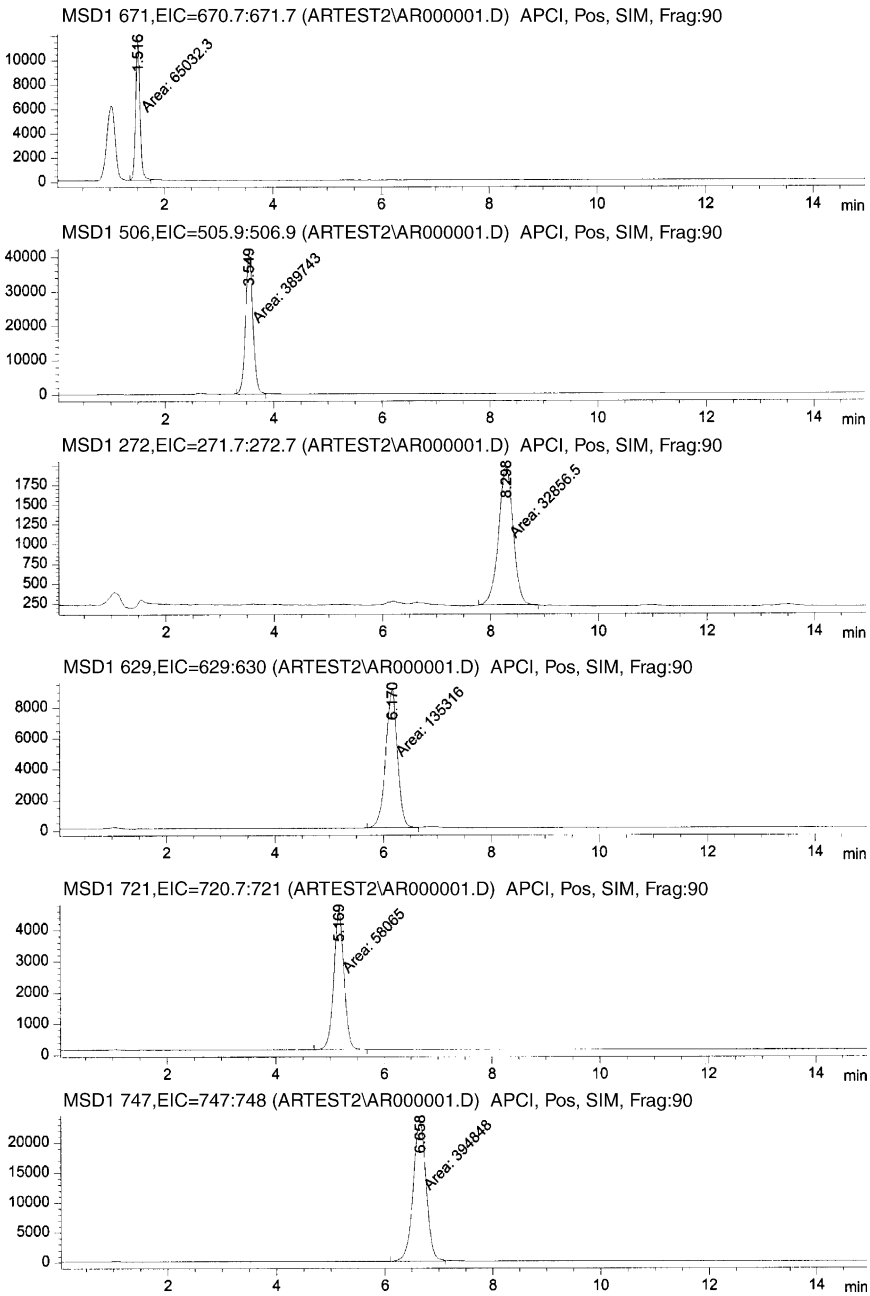

Fig. 3. Extracted ion chromatogram from a standard sample with SQV, APV, EFV, LPV, RTV and the internal standard from the first (upper) to the last chromatogram, respectively.

glycoprotein 1 mg/ml in sodium azide (0.1%, w/v). Cells lysates were stored at −80 °C until analysis by LC/MS assay.

2.6.2. Liquid–liquid extraction procedure

On the day of analysis, PBMC lysates of patients, calibration standards and controls were thawed at room temperature. The same procedure was followed for all samples. Samples were mixed with 200 μl of 0.05 M Na_2CO_3, 1.2 ml of a mixture of *n*-pentane and ethyl acetate (50:50, v/v) and 10 μl of I.S. (1 μg/ml). After the samples were centrifuged at 2360 × *g* for 10 min, the organic layer was removed and evaporated to dryness under a stream of nitrogen at 45 °C. A second *n*-pentane and ethyl acetate extraction was performed and after centrifugation, the organic layer was added to the first dried extract and evaporated as described above. The resulting residue was reconstituted by vortexing for 2 min by 25 μl of a mixture of methanol and distilled water (80/20, v/v) in an auto sampler vial for injection into the HPLC system.

2.7. Assessment of performance characteristics

2.7.1. Linearity

Calibration standards were prepared and analyzed in triplicate in six independent runs. Daily standard curves were constructed for each drug using the ratio of the observed peak area for each antiretroviral to the I.S. Unknown concentrations were computed from the linear regression equation of the peak area ratio against the concentration of each antiretroviral. An equal weighted regression was used. To assess linearity, deviations of the mean calculated concentrations over three runs should be within ±15% from nominal concentrations for the non-zero calibration standards.

2.7.2. Precision and accuracy

Accuracy, intra- and inter-assay precision of the method were determined by assaying six replicates of each of the spiked QC samples with analyte concentrations around the limit of quantification (LOQ) and the low, medium and high concentration ranges in three separate analytical runs. Accuracy was measured as the percentage deviation from the nominal concentrations. The intra- and inter-assay precision should not exceed 15% coefficient of variation (CV).

2.7.3. Recovery

Recovery was calculated as the extraction yield. It was performed by comparing the analytical results for extracted standards at four concentrations (low, medium, high and very high concentrations) with unextracted standards that represent 100% recovery.

2.7.4. Limit of quantitation (LOQ) and limit of detection (LOD)

The LOQ was defined as the lowest concentration such as the deviation between the measured and nominal concentration was less than 20% CV, as determined in three separate analytical runs.

Table 1
Intra- and inter-assay accuracy and precision for the determination of APV, LPV, RTV, SQV and EFV in human PBMCs

Antiretroviral agent	Nominal amount (ng/3 × 10^6 cells)	Intra-assay (n = 6)			Inter-assay (n = 18)		
		Mean measured (ng/3 × 10^6 cells)	Accuracy (%)	CV (%)	Mean measured (ng/3 × 10^6 cells)	Accuracy (%)	CV (%)
PIs							
APV	3	2.96	98.80	10.51	3.02	100.7	5.26
	15	13.79	91.93	6.64	14.47	96.52	9.22
	35	33.65	96.15	8.38	34.40	98.37	3.10
	100	97.22	97.22	7.45	98.81	98.81	6.44
LPV	3	2.81	93.68	9.44	3.12	104.1	6.83
	15	14.69	97.96	5.03	14.20	94.65	5.58
	35	32.14	92.03	5.48	32.6	93.11	5.34
	100	98.90	98.90	6.88	99.10	99.10	6.02
RTV	1.5	1.51	101.10	8.76	1.49	97.78	11.81
	4.8	5.11	106.40	5.72	4.84	100.90	12.90
	11.6	10.80	96.40	7.96	11.10	95.90	12.30
	64	63.26	98.84	8.22	63.81	99.70	11.47
SQV	3.6	3.53	98.09	5.14	3.34	93.89	5.06
	7.2	7.50	104.13	3.42	6.72	93.33	4.75
	16.8	18.47	109.94	7.04	16.87	100.40	3.08
	48	46.40	96.67	8.21	47.25	98.44	3.28
NNRTI							
EFV	3	3.30	109.40	10.78	2.91	97	12.23
	15	15.55	103.70	5.99	14.90	99.27	9.58
	35	34.55	98.70	7.24	34.50	98.62	5.98
	100	99.20	99.20	6.74	98.97	98.97	6.94

The LOD was the lowest concentration that the bioanalytical procedure can reliably differentiate from background noise (×3).

3. Results

3.1. Chromatographic separation

Representative chromatograms of a blank sample and a standard solution extracted from a PBMCs matrix were, respectively, illustrated in Figs. 2 and 3. Under the specified chromatographic conditions, typical retention times of APV, LPV, RTV, SQV, EFV and IS were 3.55, 6.17, 5.17, 1.52, 8.30 and 6.66 min, respectively.

All peaks were symmetrical and well resolved.

3.2. Performance characteristics

3.2.1. Linearity

The correlation coefficients (r^2) of the calibration curves were >0.997 for all antiretroviral agents except for SQV ($r^2 = 0.984$) as determined by least-squares analysis over a concentration range of 2–200 ng/3 × 10^6 cells for APV, LPV, EFV, 1.6–128 ng/3 × 10^6 cells for RTV and SQV. The standard curves for each antiretroviral were linear in the calibration ranges.

Table 2
Recovery of the determination of APV, LPV, RTV, SQV and EFV in human PBMCs

Concentration	Nominal amount (ng/3 × 10^6 cells)	Recovery (%)	Standard deviation (%)
PIs			
APV			
Low	3	88.2	11.84
Medium	15	89.2	13.43
High	35	88.0	14.07
Very high	100	89.7	12.31
LPV			
Low	3	93.8	6.43
Medium	15	86.2	11.77
High	35	87.4	8.13
Very high	100	88.2	9.63
RTV			
Low	1.5	92.2	10.41
Medium	4.8	87.3	10.34
High	11.6	88.6	12.89
Very high	64	89.4	10.25
SQV			
Low	3.6	136.9	10.33
Medium	7.2	103.5	14.95
High	16.8	109.6	11.43
Very high	48	98.4	10.24
NNRTI			
EFV			
Low	3	85.3	11.49
Medium	15	92.7	9.28
High	35	87.8	11.62
Very high	100	88.8	10.92

Table 3
Limits of detection and quantitation of APV, LPV, RTV, SQV and EFV in human PBMCs

	LOD (ng/3 × 10^6 cells)	LOQ (ng/3 × 10^6 cells)	Precision at LOQ (CV%)
PIs			
APV	0.92	2.00	2.69
LPV	0.49	2.00	15.97
RTV	0.48	1.00	7.86
SQV	0.97	1.60	14.95
NNRTI			
EFV	0.31	2.00	11.65

3.2.2. Precision and accuracy

The results of the precision and accuracy experiments were given in Table 1. Intra- and inter-assay accuracy varied from 91.93 to 109.94%, and from 93.11 to 104.10%, respectively. Within-day precision varied from 3.42 to 10.78% and between-day from 3.08 to 12.90%. The results indicated that

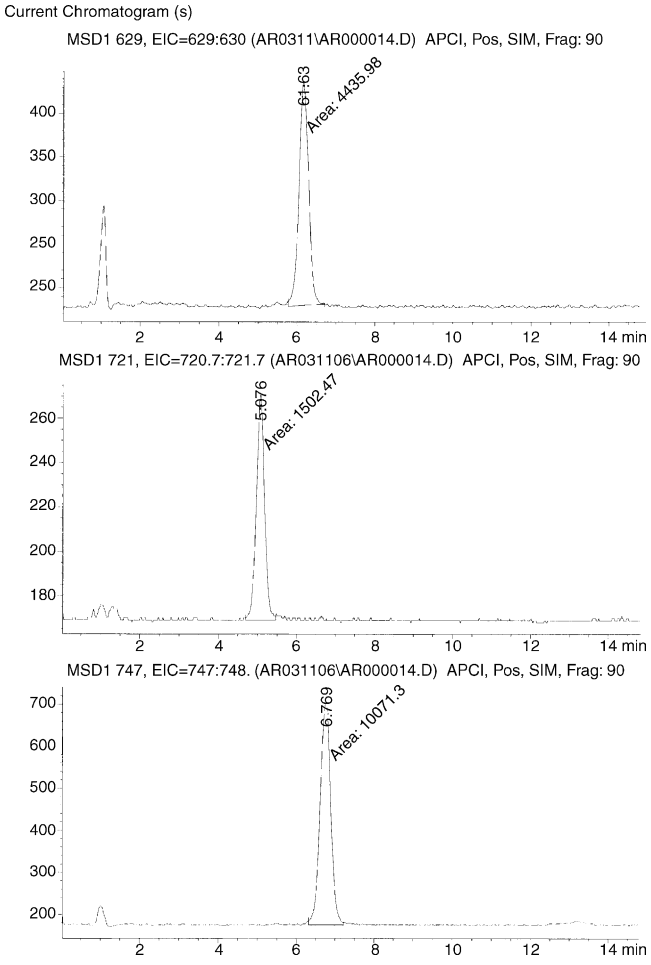

Fig. 4. Extracted ion chromatogram from a PBMCs sample of a patient under LPV and RTV. Concentrations of LPV and RTV were determined to be 11 ng/3 × 10^6 cells and 2.09 ng/3 × 10^6 cells, respectively.

the method was accurate and precise in the calibration range of each compound.

3.2.3. Recovery

Multiple aliquots ($n = 6$) at each of four different concentrations (low, medium, high and very high concentrations) were assayed. The recoveries were shown in Table 2. Mean extraction recoveries were 88.50% for APV, 89.10% for LPV, 89.40% for RTV, 116.65% for SQV and 88.60% for EFV. Precision was within 15% for all analytes.

3.2.4. Limits of detection and quantitation

Results for the limits of detection and quantitation were shown in Table 3. At a concentration of 2 ng/3 $\times 10^6$ cells for APV, LPV and EFV, 1 ng/3 $\times 10^6$ cells for RTV and 1.6 ng/3 $\times 10^6$ cells for SQV, the relative standard deviation was less than 20%. Therefore, these concentrations were considered to be the LOQ for each antiretroviral.

4. Discussion and conclusion

This study described the development and validation of a bioanalytical method for the determination of four PIs and a NNRTI in PBMCs from HIV-infected patients.

Monitoring intra-cellular drug concentrations may be useful to ensure efficacious antiretroviral levels were achieved in target cells, especially for non-responsive patients to HAART despite effective plasma concentrations. Moreover, pharmacokinetics of intra-cellular antiretroviral drugs could enhance knowledge of efficacy and toxicity. In this way, the determination of intra-cellular antiretroviral concentrations may improve the management of adult and paediatric patients infected with HIV.

The initial approach was to develop an accurate assay in human HIV-1 target cells for simultaneous determination of several antiretroviral agents commonly used in HAART (APV, LPV, RTV, SQV, EFV), while maintaining suitable sensitivity and selectivity. The small working PBMCs volume used for the quantification of intra-cellular PIs required a high sensitive and specific assay such as LC/MS, which offered major benefits over UV detection and seemed adapted to this kind of samples.

PBMCs isolation and collection were achieved with Vacutainer® CPT™ tubes. This system was considered to be easier, less time-consuming, and with almost identical cellular recovery than classical Ficoll treatment [14].

Separation of all antiretroviral agents was simultaneously achieved under isocratic conditions in a short runtime (15 min). Moreover, through validation, the LC/MS method described in this study proved to be accurate and precise for the quantitative determination of APV, LPV, RTV, SQV and EFV in human PBMCs; precision was always $<\pm 15\%$ CV and accuracy was between 80 and 120% in accordance with published recommendations [15]. The standard curves exhibited good linearity ($r^2 > 0.98$ for SQV and $r^2 > 0.99$ for the other antiretroviral agents analyzed over the concentration ranges chosen. Analysis and comparison of Jurkat cells and PBMC fraction demonstrated equivalence of the two matrices for the assay. As PBMC matrix required an important amount of blood (a minimum of 6 ml of blood was necessary) and as cell culture was more manageable, Jurkat cells were used as a matrix in the assay.

Representative chromatogram of a patient sample was shown in Fig. 4. More than 500 patients samples have been analyzed using this LC/MS methodology. Although these patients have been treated with different comedications (e.g. sulfamethoxazole/trimethoprime, pravastatin, gemfibrozil, tenofovir, lamivudine, abacavir, . . .), the absence of peak at the same retention time and with the same m/z of one of the antiretroviral drugs demonstrated the absence of interfering substances.

In this study, we reported concentrations as ng/3 $\times 10^6$ cells because of similar units of measurement reported in the literature when analyzing PBMCs samples [16,17]. These units underlined the importance to get an accurate cell count in the samples. Nevertheless in clinical practice expressing intra-cellular concentrations in μg/ml appeared to be most convenient. As classical described, the concentrations (ng/ml) were obtained by dividing the amount obtained from regression equation by the volume corresponding to cell count.

The concentration ranges reported in this study were 2–200 ng/3 $\times 10^6$ cells for APV, LPV, EFV, 1.6–128 ng/3 $\times 10^6$ cells for RTV and SQV. The choice of these ranges was based on the intra-cellular concentrations achieved in the daily practice and they were considered adequate for therapeutic drug monitoring [1,18] The LOQs were 2 ng/3 $\times 10^6$ cells for APV, LPV and EFV, 1 ng/3 $\times 10^6$ cells for RTV and 1.6 ng/3 $\times 10^6$ cells for SQV. According to these values (equal or less than the low quality control concentration), observed coefficient of variations were adequate and waited.

The method was applied to determine intra-cellular levels of lopinavir (PI coformulated with ritonavir) and of efavirenz (NNRTI) in HIV-1-infected, antiretroviral-experienced patients. A total of 112 blood samples were drawn for intracellular dosages at the pharmacokinetic steady state: 32 patients received lopinavir/ritonavir (400/100 mg twice a day) and 80 patients were under efavirenz (600 mg once a day). Blood samples were drawn prior to drug administration (antiretroviral residual concentration C_{min}). The median C_{min} [25th; 75th] were calculated, respectively, for lopinavir and efavirenz: 2.96 μg/ml (1.65; 5.83) and 7.72 μg/ml (4.00; 12.33).

As a conclusion, a simple, accurate and specific method for simultaneous quantification of APV, LPV, RTV, SQV and EFV has been developed and validated. To date, this was the first time that simultaneous intra-cellular quantitation have been obtained for APV, LPV, RTV, SQV and EFV, providing the opportunity to understand better the in vivo intra-cellular pharmacokinetic of PIs and NNRTI. This could be useful for pharmacokinetic studies and therapeutic drug monitoring,

which improves the clinical management of HIV-infected patients.

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