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Development of an assay method for the detection and quantification of protease and non-nucleoside reverse transcriptase inhibitors in plasma and in peripherical blood mononuclear cells by liquid chromatography coupled with ultraviolet or tandem mass spectrometry detection

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Abstract

We present a simple chromatographic method to detect and quantify protease inhibitors (PI), metabolites and non-nucleoside reverse transcriptase inhibitors (NNRTIs) in human plasma of HIV-1 infected patients and in peripheral blood mononuclear cells (PBMCs) using either liquid chromatography coupled with ultraviolet (LC–UV) or liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS). A solid–liquid extraction was carried out on 500 μl of plasma as pre-treatment. Calibration curve ranges were from 50 (100) to 5000 ng/ml (indinavir). PBMC pellets from 7 ml of blood were lysed with methanol/tris with a calibration curve ranging from 0.25 to 250 ng/pellet. Simple modifications in the mobile phase composition (slight increase of ammonium acetate concentration and addition of methanol for LC–UV) easily linked the two analytical systems.

Keywords: PI; NNRTI; LC–UV; LC–MS/MS; Plasma; Intracellular

1. Introduction

Pharmacological studies of antiretroviral drugs against HIV can be used to develop new treatment optimisation strategies. Current treatments combine HIV protease inhibitors (PIs), HIV non-nucleoside reverse transcriptase inhibitors (NNRTIs) and HIV nucleoside reverse transcriptase inhibitors (NRTIs). The use of highly active antiretroviral therapy (HAART), the combination of at least three or four different antiretroviral drugs in the clinical management of HIV-1 infection, has improved the prognosis for HIV-1-infected patients. Knowledge of plasma (PIs and NNRTIs) or intracellular mainly NRTIs but also PIs and NNRTIs levels is required to ensure efficacy and to prevent treatment resistance [1]. There is increasing evidence that virological treatment failure is sometimes due to pharmacological reasons [2,3]: (i) poor drug diffusion within the site of viral replication (blood compartment, tissue and cellular compartment, viral reservoir compartment), (ii) inter-patient variability in drug disposition, especially variations in activity of metabolic enzymes, (iii) drug interactions, (iv) and finally genetic context. The relationship between plasma drug concentration and drug activity has led to the development of numerous analytical methods designed to study the kinetic parameters of drugs used in HIV-1-infected patients [4].

Several methods have been published for the simultaneous determination of PIs [5–8] and for two classes of antiretrovi-

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ral compounds [9–16]. However, all of these analytical methods were developed to quantify drugs only in human plasma.

All in all, there was still a need for a more general method of simultaneously detecting and quantifying PIs and NNRTIs in different biological matrices for pharmacological purposes, including the possibility of carrying out in vitro and in vivo analysis both for quantification and metabolic studies. In the present work, we report the development and validation of a simple liquid chromatography coupled with ultraviolet (LC–UV) method easily switched to liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) for the simultaneous determination of numerous PIs including the M8 metabolite of nelfinavir, and NNRTIs both in plasma and peripheral blood mononuclear cells (PBMCs) from HIV-1 positive patients.

2. Experimental

2.1. Chemicals

Ritonavir (RTV) was from Morarek (USA), amprenavir (APV) was a generous gift from Glaxo Wellcome (Hertfordshire, UK), nelfinavir (NFV) was from Agouron pharmaceuticals (San Diego, CA, USA), and its metabolite M8 from was a gift of Dr. F. Gimenez (Chatenay-Malabry University, France), efavirenz (EFV) from DuPont Pharmaceuticals (Wilmington, DE, USA), delavirdine (DLV) from Pharmacia Upjohn (Saint Quentin, France), nevirapine (NVP) from Boehringer Ingelheim Pharmaceuticals, Inc, indinavir (IDV) from MERCK, saquinavir (SQV) from Roche products (Welwyn Garden City, UK), lopinavir (LPV) from Abbot Laboratories (North California, IL, USA). Naphthalene acid and ketoconazole as internal standards (ISTDs) for LC/UV and LC/MS/MS, respectively, were from Sigma-Aldrich (France). Acetonitrile (HPLC gradient) was from SDS (Peypin, France), methanol (Lysochrosolv) from Merck (Darmstadt, Germany), ammonium acetate from Sigma-Aldrich (France), acetic acid from Merck (Germany), water from Millipore.

2.2. Chromatographic LC/UV system (system 1)

2.2.1. Equipment

HPLC was performed using a Waters (Milford, MA, USA) liquid chromatography system with a Model 600 controller, a Model 717 plus Autosampler, a Model 2487 Dual Wavelength UV detector, and Millennium software (Version 3.05).

2.2.2. Separation conditions

The analytical column was a Nova Pak C1860 Å, 4 μm particle size, 150 mm × 3.9 mm (Waters) with a (C18) guard column, cartridge modulocart 10 mm × 2 mm (Interchim, France).

The sample injection volume was 50 μl. Chromatographic analyses were performed at 40 °C. Detection was performed at 215 nm. The flow-rate was 1 ml/min. Separation of molecules was facilitated by gradient elution. All solutions were filtered through a 0.45 μm membrane filter (Millipore, VWR, France) before use. The mobile phase consisted of (A) acetonitrile/25 mM ammonium acetate buffer (0.1% acetic acid) (10/90, v/v), (B) acetonitrile/25 mM ammonium acetate buffer (0.1% acetic acid) (90/10, v/v), and (C) methanol with linear adjustments in a 20 min run time (Table 1A).

Table 1
Gradient elution

(A) In LC–UV system

Time	%A[a]	%B[b]	%C[c]
Initial	84	15	1
At 8 min	20	10	70
At 15 min	30	40	30
At 20 min	84	15	1

(B) In LC/MS/MS system

Time	%A[d]	%B[e]
Initial	85	15
At 8 min	20	80
At 15 min	50	50
At 20 min	85	15

[a] A = 10% ACN + 90% ammonium acetate at 25 mM with 0.1% acetic acid.
[b] B = 90% ACN + 10% ammonium acetate at 25 mM with 0.1% acetic acid.
[c] C = 100% MeOH.
[d] A = 10% ACN + 90% ammonium acetate at 10 mM with 0.1% acetic acid.
[e] B = 90% ACN + 10% ammonium acetate at 10 mM with 0.1% acetic acid.

2.3. LC–MS/MS system (system 2)

2.3.1. Equipment

The LC–MS/MS system consisted of an HPLC system 1100 (Agilent Technology, Les Ulis, France) connected to an API 3000 tandem mass-spectrometer equipped with electrospray ionisation source (ESI) (SCIEX, Applied Biosystem, USA). Data processing was performed on Analyst 1.1 software package (Perkin-Elmer, USA) and two ionisation modes (positive and negative) were used.

2.3.2. Separation conditions

The analytical guard columns, sample injection volume, column temperature and flow rate were identical as for system 1. Prior to entering the electrospray source housing, the flow rate was split 1.5:1 using a PEEK tubing splitter (0.13 mm internal diameter, Interchim, France) with the one split line directed to waste and the other to the Turbo-ionspray source.

All solutions were filtered through a 0.45 μm membrane filter (Millipore, VWR, France) before use. The mobile phase consisted of (A) acetonitrile–10 mM ammonium acetate buffer (0.1% acetic acid) (10/90, v/v), (B) acetonitrile–10 mM ammonium acetate buffer (0.1% acetic acid) (90/10, v/v), with linear adjustments in a 20 min run time (Table 1B).

Table 2
Optimised mass spectrometric parameters of each protease inhibitor and non-nucleoside reverse transcriptase inhibitor

Drug class	Molecular name	Retention time (min)	Ionisation mode	Parent ion	Fragment ion	Declustering potential (DP)	Focusing potential (FP)	Entrance potential (EP)	Collision energy (CE)	Collision cell energy potential (CXP)
NNRTI	Neviparine, NVP	3.4	+	267.0	226.1	61	260	−8.5	32	13
NNRTI	Delavidine, DLV	6.6	+	457.6	221.1	70	290	−10.5	33	15
PI	Amprenavir, APV	6.7	+	506.3	245.1	35	160	−5	25	17
PI	Indinavir, IDV	6.7	+	614.9	421.1	67	260	−9	45	14
ISTD	Ketoconazole	7.8	+	530.6	489.2	55	240	−10	42	25
PI	M8 (NFV metabolite)	7.9	+	584.2	467.5	65	250	−9	40	17
PI	Ritonavir, RNV	8.3	+	721.2	295.9	50	220	−4	27	17
PI	Lopinavir, LPV	8.6	+	629.8	447.5	50	190	−8	21	15
NNRTI	Efavirenz, EFV	8.7	−	314.1	69.2	−75	−230	10.5	−48	−11.5
PI	Saquinavir, SQV	9.2	+	671.0	570.5	70	320	−7	45	30
PI	Nelfinavir, NFV	9.6	+	568.6	330.3	70	290	−6	42	18

2.3.3. MS/MS conditions

To observe how settings affected primary and fragment ions, analytes were directly injected into the instrument using a syringe pump (300 μl/min) at the concentration 1 μg/ml prepared in a mixture of acetonitrile/water (50:50, v/v). Sequentially ramping each potential allowed for identification of the appropriate settings for the precursor and product ions. For one NNRTI (EFV), negative ionisation proved to be the best in terms of detectability. For the internal standard and the other drugs, positive mode was the best.

The pairs of ions (monitored by *m*/*z*) used for LC–MS/MS in positive mode for each PI were 506.3 → 245.1 (APV), 614.9 → 421.1 (IDV), 721.2 → 295.6 (RTV), 671.0 → 570.5 (SQV), 629.8 → 447.5 (LPV), 568.6 → 330.3 (NFV), 584.2 → 467.5 (M8).

The pairs of ions used (monitored by *m*/*z*) for LC–MS/MS for NNRTIs and internal standard (ketoconazole) were in positive mode 267.0 → 226.1 (NVP), 457.6 → 221.1 (DLV), 531.1 → 489.3 (ketoconazole) in negative mode the transition was 314.1 → 69.2 for EFV. Table 2 shows the resulting optimised detection parameters.

2.3.4. Preparation of stock solutions

Individual stock solutions of each drug (PIs and NNRTIs) were prepared at 5 mg/ml in methanol and were stored at −20 °C. No degradation was observed over 1 year of storage at −20 °C. Naphthalene acid and ketoconazole used as ISTD in systems 1 and 2, respectively, were prepared at 1 mg/ml in methanol and stored at −20 °C.

2.3.5. Clinical samples

Blood samples were collected from HIV-1-infected patients within the framework of normal laboratory monitoring. Approximately 7 ml of blood were collected in Vacutainer CPT tubes (Cell Preparation Tube, Becton Dickinson, Le Pont de Claix, France). All blood samples were centrifuged at 20 °C and 1000 × *g* for 30 min. Plasma and PBMCs were separated and stored frozen at −80 °C until analysis.

2.4. Preparation and extraction of plasma samples (standards, quality controls)

Calibration standards at final concentrations of 50, 100, 500, 1000, 2000, 3000, and 5000 ng/ml (except for IDV starting from 100 ng/ml) were prepared by adding appropriate volumes of diluted stock solutions (methanol/water, 50:50, v/v) to 500 μl of drug-free human plasma. Quality controls (QCs) were also prepared at four concentrations for each drug: 50, 150, 2500 and 4000 ng/ml (except for IDV: 100, 300, 2500, 4000 ng/ml) in the same way. All calibration and QC samples were frozen at −20 °C until assay.

Frozen clinical samples, QCs and calibration standards were thawed at room temperature. Twelve microliters of ISTD (2 μg/ml in methanol/water, 50:50, v/v) were added to 500 μl of each clinical sample QC and calibration standard, which were then completed to 600 μl with methanol/water, 50:50, v/v. After rapid vortex mixing, 75 μl of 1/10 diluted (water) phosphoric acid were added to each, clinical sample, QC and calibration standard and the tube were vortexed, centrifuged (16,000 × *g* over 5 min), and analytes were extracted as follows: Solid-phase extraction cartridges (OASIS®, 1 cc, 30 mg, Waters) were conditioned successively with 1 ml of methanol and 1 ml of water. A 600 μl aliquot of the plasma sample was applied to the cartridge. The cartridges were washed with 1 ml methanol/water (30:70, v/v). The drugs were eluted with 0.5 ml of methanol. The methanol was evaporated with a TurboVap evaporator (Zymark, France) at 37 °C to dryness. The residue was reconstituted with 130 μl of mobile phase A and was centrifuged (5 min, 16,000 × *g*) and 50 μl were injected into the LC–UV system (system 1).

2.5. Preparation of PBMC samples (standards, quality controls and clinical samples) and extraction

2.5.1. Standards and quality controls

Blank frozen PBMCs were spiked with 500 μl of standard solutions at concentration of 1, 2, 10, 50, 100, 300

Table 3
Intra-day accuracy and precision of the assay of antiretroviral drugs in LC–UV system, in human plasma sample

Assay	Theoretical concentration (ng/ml)	*n*	Mean measured concentration (ng/ml)	CV (%)	Inaccuracy (%)
Intra-day					
Nevirapine	50	5	51.01	1.28	−2.03
	150		150.0	1.68	−0.03
	2500		2477	1.98	0.92
	4000		4049	1.64	−1.23
Delavirdine	50	5	50.40	1.88	−0.79
	150		149.3	2.17	0.45
	2500		2596	6.72	−3.84
	4000		4075	3.16	−1.88
Amprenavir	50	5	53.14	5.22	6.27
	150		163.4	16.3	8.92
	2500		2316	8.14	−7.35
	4000		4452	6.37	11.3
Indinavir	100	5	92.44	16.8	7.56
	300		340.7	0.97	−13.6
	2500		2476	3.63	0.93
	4000		3928	4.60	1.79
Ritonavir	50	5	54.08	6.22	8.16
	150		146.0	10.72	−2.74
	2500		2862	1.63	14.5
	4000		3882	9.38	−2.95
Lopinavir	50	5	52.61	4.79	−5.21
	150		159.5	6.49	−6.30
	2500		2545	11.8	−1.84
	4000		4211	6.40	−5.3
Efavirenz	50	5	53.30	4.22	−6.6
	150		164.7	4.72	−9.81
	2500		2618	5.33	−4.75
	4000		4505	10.1	−12.6
Saquinavir	50	5	51.07	8.64	2.14
	150		140.5	9.04	−6.35
	2500		2401	2.68	−3.93
	4000		3508	7.09	−12.3
Nelfinavir	50	5	49.75	9.43	0.50
	150		156.7	7.99	−4.50
	2500		2195	5.33	12.2
	4000		4080	8.14	−2

and 500 ng/ml in methanol for calibration standards, or 5, 75 and 250 ng/ml in methanol for quality controls, with ketoconazole as internal standard (10 μl, 50 μg/ml in methanol), and 220 μl of Tris–HCl (0.05 M, pH 7.4) were added. Final amounts were 0.5, 1, 5, 25, 50, 150 and 250 ng of each analyte per sample containing around 10×10^6 cells for calibration standards and 2.5, 37.5 and 125 ng of each analyte per sample containing around 10×10^6 cells for quality controls.

2.5.2. Clinical samples

Clinical frozen PBMC samples were spiked with internal standard (10 μl, 50 μg/ml in methanol), then 500 μl of methanol and 220 μl of Tris solution (0.05 M, pH 7.4) were added.

2.5.3. Analyte extraction

Analytes were extracted from PBMCs (standards, quality controls and clinical samples) as previously described by Pruvost et al. [17]. After cell lysis and vortex mixing, tubes were centrifuged (18,000 × *g* for 30 min at +4 °C). The supernatants were transferred into polypropylene tubes and evaporated to dryness with a TurboVap evaporator (Zymark, France) at 37 °C. The residue was reconstituted in 200 μl of Tris solution (0.05 M, pH 7.4). The resulting solutions were vortexed and transferred to 1.5 ml vials with 200 μl

Table 4
Inter-day accuracy and precision of the assay of antiretroviral drugs in LC–UV system, in human plasma sample

Assay	Theoretical concentration (ng/ml)	*n*	Mean measured concentration (ng/ml)	CV (%)	Inaccuracy (%)
Inter-day					
Nevirapine	50	5	50.58	8.09	−1.36
	150		146.4	3.36	2.37
	2500		2748	11.8	−9.94
	4000		4372	14.7	−9.30
Delavirdine	50	5	48.68	3.98	2.65
	150		157.9	14.0	−5.26
	2500		2544	2.20	−1.77
	4000		4253	4.99	−6.33
Amprenavir	50	5	56.83	3.55	13.6
	150		134.7	7.85	−10.2
	2500		2227	6.55	−10.9
	4000		3779	7.99	−5.50
Indinavir	100	5	89.47	19.8	10.5
	300		315.7	6.42	−5.23
	2500		2025	9.97	11.5
	4000		3507	10.7	12.3
Ritonavir	50	5	53.44	10.1	6.88
	150		129.0	15.3	−14.0
	2500		2601	7.42	4.06
	4000		4081	6.35	2.04
Lopinavir	50	5	52.62	7.04	−5.25
	150		155.8	4.51	−3.90
	2500		2701	12.2	−8.03
	4000		3949	18.3	1.27
Efavirenz	50	5	52.92	5.61	−5.84
	150		161.6	8.06	−7.72
	2500		2537	16.8	−1.49
	4000		4245	9.45	−6.13
Saquinavir	50	5	44.54	11.2	−10.9
	150		136.4	15.4	−9.10
	2500		2211	7.46	−11.6
	4000		3535	12.9	−11.6
Nelfinavir	50	5	50.38	6.34	−0.75
	150		149.7	6.63	0.18
	2500		2213	17.5	11.5
	4000		3698	15.6	7.56

polypropylene insert, and 80 μl were injected into the chromatographic system.

2.6. Validation of LC/UV chromatography (system 1) in plasma

The assay in plasma using the LC/UV system was validated using three by three drugs. This assay for metabolite M8 of NFV was not validated in this system.

2.6.1. Specificity and selectivity

Interference by endogenous compounds was investigated by analysis of nine different drug-free plasma samples. To check specificity against co-medications, 13 plasma samples from HIV-infected patients on highly active antiretroviral therapy were analysed in system 1. Solutions of nucleoside reverse transcriptase inhibitors lamivudine (3TC), stavudine (d4T), zidovudine (AZT), didanosine (ddI) were also analysed in the chromatographic system for possible interference.

2.6.2. Precision and accuracy

The intra-day and inter-day precision and accuracy of the assay were determined by assaying five replicate QC plasma samples spiked at four different concentrations (50, 150, 2500, 4000 ng/ml) except for IDV (150, 300, 2500, 4000 ng/ml) during five analytical runs. Accuracy was expressed as the absolute percent deviation from the theoretically determined concentration (% difference).

2.6.3. Lower limit of quantification (LLOQ)

LLOQ was established for the lowest QCs (the smallest quantity of analyte likely to be quantified), which exhibited precision and inaccuracy lower than 20% during intra- and inter-day precision experiments.

2.6.4. Recovery

Extraction recovery of each analyte was determined by comparing detector response of plasma extracts spiked with standard solutions of equivalent concentration, across the range of calibration standard concentrations. Multiple aliquots ($n = 5$) at each of four different concentrations, including LLOQ, were assayed.

2.7. Validation of LC–MS/MS chromatography (system 2) in PBMCs

The validation of the LC–MS/MS system was performed for all the drugs simultaneously. This assay for metabolite M8 of NFV was validated in this system.

2.7.1. Precision and accuracy

The intra-day and inter-day precision and accuracy of the assay were determined by assaying five replicate QC PBMC samples spiked with the analytes at four different concentrations (0.5, 2.5, 37.5 and 125 ng/sample) during five analytical runs. Inter-day precision was assessed using different sources of matrices. Accuracy was expressed as the absolute percent deviation from the theoretically determined concentration (% difference). The precision was evaluated as the relative standard deviation of the mean expressed as a percent (%CV: coefficient of variation) for each concentration.

2.7.2. Lower limit of quantification

Lower limit of quantification was assumed to be the smallest quantity of analyte likely to be quantified accurately and precisely in the ±20% during intra- and inter-day experiments. LOQ was assessed both in the intra- and inter-day experiments using different sources of matrices.

2.7.3. Specificity and selectivity

Interference by endogenous compounds was investigated by analysis of five different human PBMCs blank samples and we used different human PBMCs samples per day during the inter-day assay to verify the influence of the endogenous compounds on quantitation. Solutions of nucleoside reverse transcriptase inhibitors were also analysed in the chromatographic system for possible interference.

2.7.4. Extraction recovery and matrix effect

Extraction recovery of the analytes from PBMCs was assessed in replicate of four by comparing the response of drugs added after extraction of blank samples with response of drugs added before extraction. Matrix effect was assessed in replicate of five and four (for APV, IDV, LPV, SQV) by comparing the response of drugs added after extraction of blank samples with response of standards in solutions extraction recovery and matrix effect were both assessed at three concentrations (5; 37.5 and 125 ng/pellet).

3. Results and discussion

We have developed a method applied to protease inhibitors, metabolites and non-nucleoside reverse transcriptase inhibitors easily applicable both to plasma and intracellular media from HIV-infected patients using LC–UV and LC–MS/MS. We analysed six PIs and one active metabolite and three NNRTIs using the two systems.

The extraction of analytes was different for the two biological matrices (described above), but there were only two minor differences between the two analytical methods. Firstly, concentrations of ammonium acetate were 10 and 25 mM for LC–MS/MS and LC–UV system, respectively. A low concentration of ammonium salt allowed good performance and sensitivity in mass spectrometry [18]. However, at 10 mM, in LC–UV, chromatographic peaks were broader than at 25 mM ammonium acetate and the overall chromatographic separation needed in the detection UV mode was improved using 25 mM ammonium acetate. Secondly, the gradient elution mode in LC–MS/MS system was simplified (without methanol). Methanol was only needed for better separation between analytes in LC–UV system. The order of elution was identical in the two systems, thus facilitating peak identification (if necessary). The run time was the same for both systems.

3.1. LC–UV system 1

3.1.1. Chromatographic characteristics

For a good separation with reduced retention time we used a gradient which separated all these compounds in a single run in less than 20 min. This run time is shorter than that of Simon et al. [14] (50 min) and other authors (30–35 min) [10,15]. The chromatographic separation of the PIs and NNRTIs mentioned above is shown in Fig. 1.

The mobile phase initially consisted of buffer/acetonitrile, but some drugs were not separated (APV, DLV and IDV). Methanol was therefore used to replace part of the acetonitrile. As the solvent strength of methanol is lower than that of acetonitrile, the retention times of compounds were

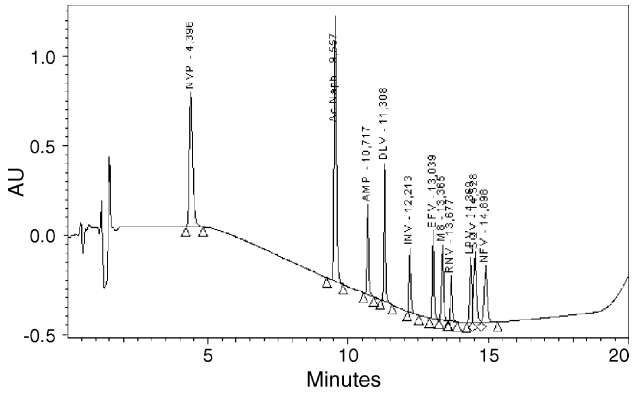

Fig. 1. LC–UV chromatogram of extracted human plasma spiked with all PIs and NNRTIs.

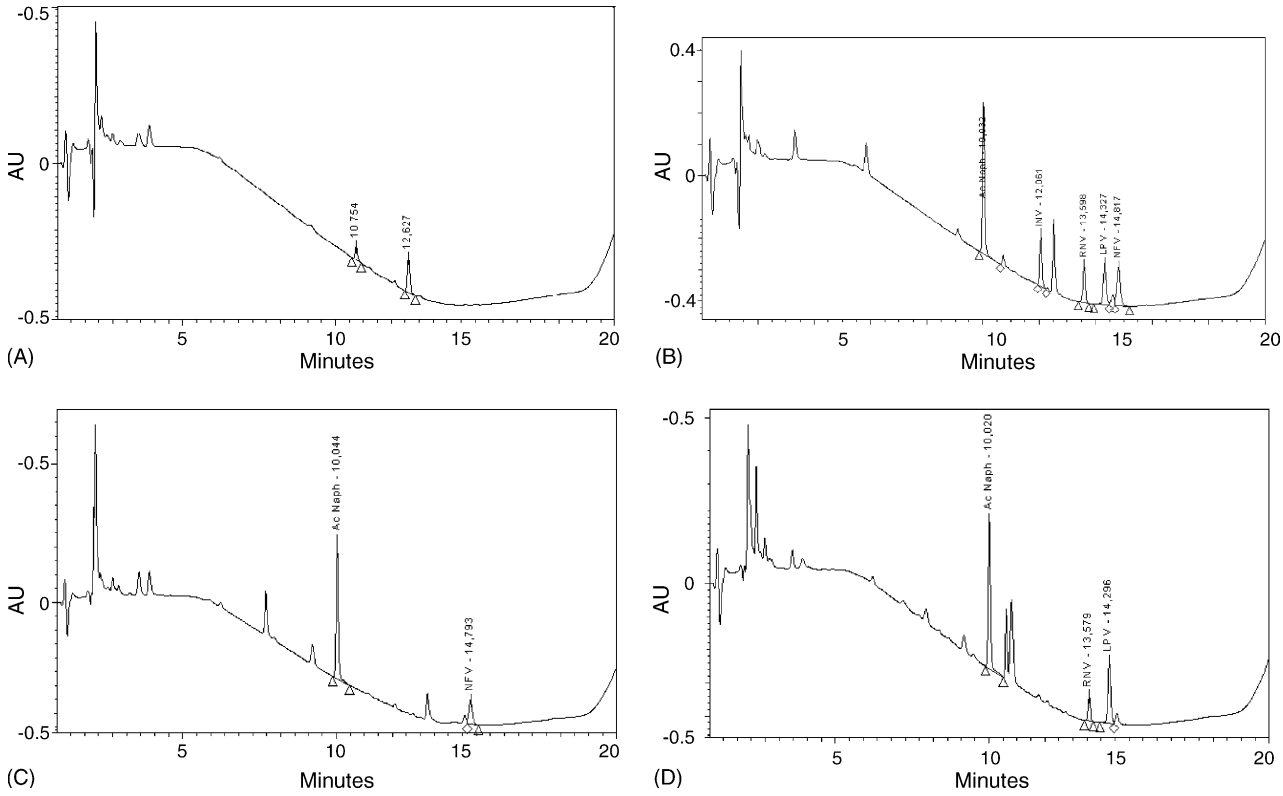

Fig. 2. Chromatograms obtained after extraction from blank plasma (A), (B) plasma samples loaded with 3000 ng/ml of IDV, RTV, LPV, and NFV to assay, (C) adult patient, NFV containing regimen (1815 ng/ml); and (D) adult patient, LPV/RTV-containing regimen (3922 ng/ml for LPV and 2102 ng/ml for RTV).

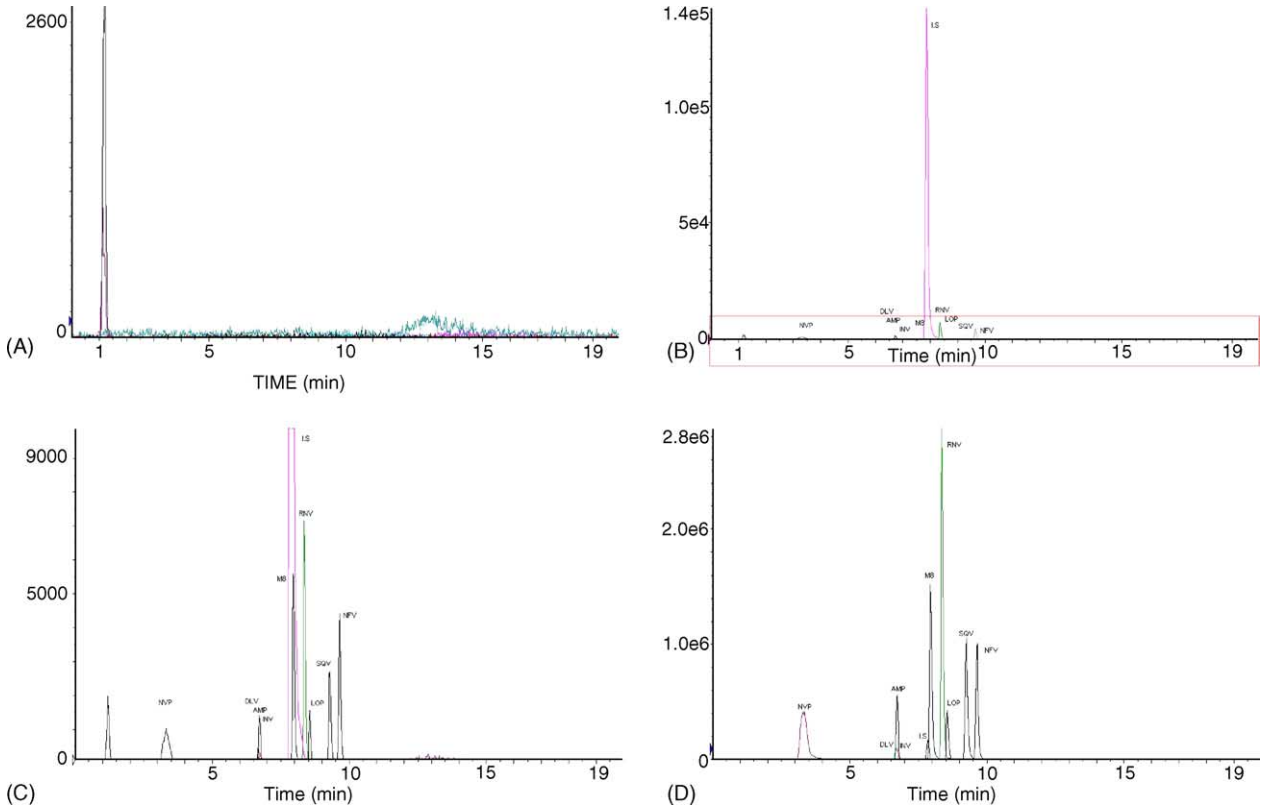

Fig. 3. (A) Chromatogram of blank intracellular medium. (B) Chromatographic profile of calibrator at 0.5 ng/pellet (LLOQ). (C) Chromatographic enlargement profile of calibrator at 0.5 ng/pellet (LLOQ). (D) Chromatographic profile of calibrator at 125 ng/pellet (upper calibration standards). Note that DLV, APV and IDV have the same retention time.

increased, and consequently analytes were also separated. Methanol thus improved resolution and assay selectivity. Thus, the column, mobile phase, temperature and use of a multiple steps gradient allowed the complete separation of most of the compounds within 20 min. Unfortunately, the resolution between saquinavir and lopinavir was below 1, but they did not overlap chromatographically and could be detected. Quantification was possible if these drugs were given alone. For simultaneous co-administration, quantification was possible by LC–MS/MS.

Several wavelengths were examined (i.e., 260, 215 and 280 nm) during the development of this method. At 280 nm, many endogenous peaks interfered with PIs and NNRTIs, so detection of PIs and NNRTIs was difficult at LOQ. At 260 nm, the sensitivity and signal-to-noise ratio were lower than at 215 nm so 215 nm was selected.

3.1.2. Calibration curve and lower limit of quantification

The standard curve ranged from 50 to 5000 ng/ml for all compounds except INV (100 to 5000 ng/ml). Linear regres-

Table 5
Intra-day accuracy and precision of the assay of antiretroviral drugs in LC–MS/MS system in human PBMCs

	Intra precision ($n = 5$)				
	Concentration (ng/ml)	*N*	Mean (ng/pellet)	Inaccuracy (%)	CV (%)
NVP	0.5	5	0.46	−7.8	8.4
	2.5		2.30	−8.0	3.7
	37.5		37.9	1.1	13
	125		114	−8.5	1.6
DLV	0.5	5	0.57	15	15
	2.5		2.20	−12	6.0
	37.5		37.5	−0.1	12
	125		138	11	4.5
APV	0.5	5	0.45	−10	3.6
	2.5		2.70	8.0	13
	37.5		43.0	15	7.9
	125		144	15	7.2
IDV	0.5	5	0.41	−18	4.3
	2.5		2.74	9.6	11
	37.5		40.7	8.5	8.8
	125		124	−0.8	3.8
M8	0.5	5	0.48	−7.0	10
	2.5		2.81	13	4.6
	37.5		40.9	9.1	8.9
	125		142	14	6.2
RTV	0.5	5	0.51	1.2	4.6
	2.5		2.54	1.6	11
	37.5		39.9	6.5	12.8
	125		125	0.2	14
LPV	0.5	5	0.45	−9.6	15
	2.5		2.30	−8.1	12
	37.5		41.5	11	7.2
	125		127	1.6	5.4
EFV	0.5	5	0.52	4.6	4.5
	2.5		2.30	−8.0	11
	37.5		41.9	12	9.8
	125		135	7.6	8.4
SQV	0.5	5	0.53	6.0	4.0
	2.5		2.67	6.8	10
	37.5		42.2	13	8.3
	125		132	5.6	4.2
NFV	0.5	5	0.49	−1.5	11
	2.5		2.18	−13	8.0
	37.5		37.2	−0.7	7.7
	125		123	−1.3	5.5

Table 6
Inter-day accuracy and precision of the assay of antiretroviral drugs in LC–MS/MS system in human PBMCs

	Inter precision ($n = 5$)				
	Concentration (ng/ml)	*N*	Mean (ng/pellet)	Inaccuracy (%)	CV (%)
NVP	0.5	5	0.52	3.0	13
	2.5		2.49	−0.3	12
	37.5		35.7	−4.8	15
	125		141	12	2.3
DLV	0.5	5	0.47	−5.1	14
	2.5		2.25	−9.8	9.5
	37.5		38.6	3.0	7.3
	125		124	−1.1	9.8
APV	0.5	5	0.42	−1.0	12
	2.5		2.31	−7.8	12
	37.5		38.9	4.0	13
	125		118	−6.0	12
IDV	0.5	5	0.45	−10	7.7
	2.5		2.65	6.0	10
	37.5		39.1	4.0	10
	125		129	3.0	7.4
M8	0.5	5	0.46	−7.1	10
	2.5		2.69	8.0	11
	37.5		38.6	3.0	13
	125		121	−3.0	8.5
RTV	0.5	5	0.52	5.0	7.6
	2.5		2.39	−4.2	12
	37.5		39.0	4.0	9.4
	125		116	−6.9	11
LPV	0.5	5	0.46	−7.3	11
	2.5		2.29	8.5	8.5
	37.5		39.2	5.0	11
	125		120	4.4	11
EFV	0.5	5	0.52	5.0	3.9
	2.5		2.67	7.0	9.9
	37.5		41.0	9.0	7.1
	125		139	11	6.8
SQV	0.5	5	0.47	−6.2	12
	2.5		2.76	10	9.7
	37.5		38.5	3.0	6.6
	125		123	1.6	12
NFV	0.5	5	0.46	−8.3	7.8
	2.5		2.45	1.8	14
	37.5		39.0	4.0	4.6
	125		128	2.0	11

sion ($y = Ax + B$) was performed between the ratios of the peak area of analyte to that of the internal standard versus corresponding theoretical spiked concentration (X). A $1/X$ weighted regression was used. The LLOQ was 50 ng/ml for all compounds except indinavir where the LLOQ was 100 ng/ml.

3.1.3. Recovery, accuracy and precision

These antiretroviral drugs have been previously extracted from plasma using either liquid–liquid extraction (LLE) or solid-phase extraction (SPE). We chose SPE, from a 500 μl aliquot of plasma, for more convenience (rapid) than LLE and did not use ter-butyl-ether solvent despite the higher cost of this method. To improve sample clean-up and peak efficiency plasma deproteinisation step was carried out with phosphoric acid. The mean recoveries for the assayed antiretroviral drugs were greater than 60% for all analytes except EFV (47%).

The accuracy and precision of the antiretroviral LC-UV assay (system 1) in human plasma are listed in Tables 3 and 4.

3.1.4. Selectivity and stability

No significant matrix interferences were found in the LC/UV chromatograms at the retention times for all tested drugs. No NRTI tested interfered with the assay (all eluted in the solvent front). In addition, plasma taken from several patients treated with HAART did not show any interfering peak at the retention time of any analyte. We did not assess the stability of the solutions or the frozen plasma samples as data on this point have been reported previously [10,13,16,19]. All analytes except NFV and its metabolite M8 were stable over 6 months in plasma.

3.1.5. Patient sample testing

Fig. 2 illustrates the application of the LC–UV method to LC–UV chromatogram of blank plasma (Fig. 2A), plasma spiked with IDV, RTV, IDV and NFV (Fig. 2B), plasma sample of patient treated with NFV (Fig. 2C), and Fig. 2D shows a LC–UV chromatogram of plasma sample of patient treated with LPV and RTV.

3.2. LC–MS/MS system 2

3.2.1. LC–MS/MS characteristics

The retention times of each PI, NNRTI and internal standard were 3.4, 6.6, 6.7, 6.7, 7.8, 7.9, 8.3, 8.6, 8.7, 9.2, and 9.6 min for NVP, DLV, APV, IDV, ketoconazole (ISTD), NFV metabolite (M8), RTV, LOP, EFV, SQV and NFV, respectively. The chromatograms of all PIs and NNRTIs in spiked intracellular medium were shown in Fig. 3. As explained below, without methanol, APV, IDV, DLV were not separated. However, the selectivity of mass spectrometry in multiple reactions monitoring (MRM) mode allowed the quantitation of

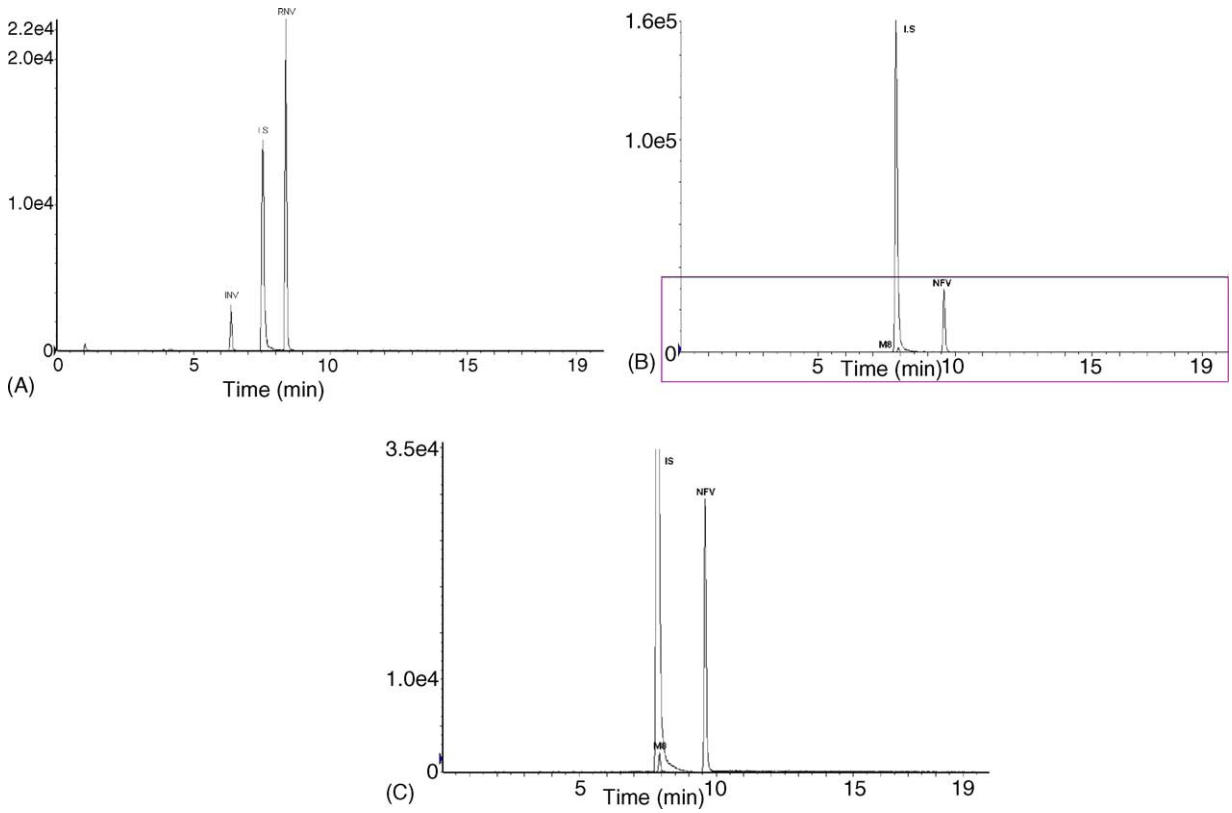

Fig. 4. (A) Chromatogram for an HIV-infected patient (IDV measured at 40.4 pmol/pellet and RTV measured at 46.8 pmol/pellet), (B) chromatogram for an HIV-infected patient (NFV measured at 18.9 pmol/pellet and M8 its metabolite measured at 3.15 pmol/pellet), (C) chromatographic enlargement profile for an HIV-infected patient (NFV and M8 its metabolite). A pellet corresponds approximately to 10^7 cells.

the chromatographic peaks even if analytes were co-eluted. A representative MRM chromatogram of blank intracellular medium sample is illustrated in Fig. 3A. No endogenous substances interfered with any of the analytes in the intracellular medium.

3.2.2. Calibration curve and limit of quantification

The standard curve range was from 0.5 to 250 ng/sample for all compounds. The LLOQ was set at the lowest standard concentration. Chromatograms showing blank intracellular medium, LLOQ, and high concentration standard are shown in Fig. 3 and data reported in Tables 5 and 6.

3.2.3. Intra- and inter-day inaccuracy and precision

Mean results are summarised in Table 5 for the intra-day assay.

For inter-day assay, the inaccuracy and precision were lower than 15% for all analytes (data shown in Table 6). Fig. 4 illustrates the application of the method to patient sample analyses. Fig. 4A shows a sample analysed for INV and RTV. Fig. 4B and C relate to a patient sample containing NFV and its metabolite M8.

3.2.4. Selectivity

No significant matrix interferencewas found in the LC/MS/MS chromatograms at the retention times for all tested drugs (cf. Fig. 3A, an example of MRM chromatogram of extracted human blank PBMCs sample was represented). The inter-day assay data illustrated that no endogenous compounds in human PBMCs sample interfered with PIs or NNRTIs quantitation (cf. Table 6, accuracy and precision were ±20%). No NRTIs tested interfered with the assay (data not shown).

3.2.5. Extraction recovery and matrix effect

Mean extraction recoveries of PIs and NNRTIs were found to be upper than 96% except for NFV (92.7%) and LPV (85%). Matrix effect are given in Table 7. Mean matrix effect was between 100 (SQV) and 170% (EFV), indicating either no effect or an enhancement of the ionisation due to endogenous compounds. There is no major difference among concentrations for all analytes tested. A slight trend toward a matrix effect lower in NNRTIs against PIs was evidenced. In addition, a second experiment was also performed with another source of matrix for APV, IDV, LPV and SQV. It is shown in Table 7 that the mean matrix effect recovery is

Table 7
Mean matrix effect of the assay of antiretroviral drugs in LC–MS/MS system in human PBMCs

Concentration (ng/pellet)	*N*	Mean matrix effect (ionisation recovery %): ratio area analyte/internal standard (mean) (CV%)	Mean matrix effect (ionisation recovery %) for first; second matrix	Overall mean matrix effect (ionisation recovery %)
2.5	5	103 (10)		
37.5	5	140 (14)	–	126
125	5	135 (15)		
2.5	5	157 (11)		
37.5	5	178 (7)	–	161
125	5	149 (7)		
2.5	5; 4	111 (9); 117 (9)		
37.5	5; 4	102 (12); 130 (15)	107; 112	110
125	5; 4	108 (6); 89 (12)		
2.5	5; 4	101 (6); 98 (10)		
37.5	5; 4	114 (10); 124 (11)	101; 110	105
125	5; 4	87 (3); 107 (9)		
2.5	5	170 (23)		
37.5	5	141 (10)	–	161
125	5	173 (11)		
2.5	5; 4	87 (6); 78 (14)		
37.5	5; 4	115 (9); no result	111; 89	102
125	5; 4	131 (4); 99 (9)		
2.5	5	No result		
37.5	5	148 (7)	–	170
125	5	192 (10)		
2.5	5; 4	101 (6); 97 (12)		
37.5	5; 4	133 (10); 92 (15)	106; 95	100
125	5; 4	83 (5); 95 (21)		
2.5	5	147 (13)		
37.5	5	145 (6)	–	144
125	5	139 (12)		
2.5	5	105 (14)		
37.5	5	103 (14)	–	107
125	5	113 (9)		

For APV, IDV, LPV and SQV, two sets of experiments were performed using two origins of matrices. Results are reported as mean result first matrix (CV%); mean result second matrix (CV%).

quite similar between the two sets of experiments, indicating that the matrix effect is well taken into account by the internal standard ketoconazole. This is not quite surprising, since the retention times of all analytes are closed (from 6.6 to 9.6 min, except 3.4 min for NVP, the internal standard eluting in the middle range: t_R: 7.8 min. Moreover, the inter-day precision and accuracy, even at the LOQ were also assessed with different origins of matrices. As shown in the Table 6, the CV% for precision as well as the inaccuracy fulfilled the criteria of 15% (20% at the LOQ), confirming that the matrix effect has not impact on the drugs quantification, even at the LOQ.

4. Conclusion

We have developed a rapid, simple and specific method using LC–UV and LC–MS/MS for simultaneous determination of several antiretroviral drugs (PIs and NNRTIs) against HIV in different biological matrices. This assay compares favourably with other assays since the LC–UV and the LC–MS/MS match. Most of the previous assays for PIs or NNRTIs involved a mobile phase not compatible with mass spectrometry. Our assay allows the use of one or the other method according to the availability, the instrumentation or the expected concentrations, with the great advantage of giving a very similar chromatographic profile with both LC–UV and LC–MS/MS. The assay spans the concentration range of clinical interest and this assay can be used easily for the monitoring of antiretroviral drugs concentrations in plasma or PBMCs from HIV-1 infected individuals.

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