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HPLC–MS method for the simultaneous quantification of the new HIV protease inhibitor darunavir, and 11 other antiretroviral agents in plasma of HIV-infected patients

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Abstract

A new method using high performance liquid chromatography coupled with electrospray mass spectrometry (HPLC–MS) was developed and validated, for the quantification of plasma concentration of the new protease inhibitors darunavir (DRV) and other 11 antiretroviral agents (ritonavir, amprenavir, atazanavir, lopinavir, saquinavir, indinavir, nelfinavir and its metabolite M-8, nevirapine, efavirenz and tipranavir). A simple protein precipitation extraction procedure was applied on 50 μl of plasma aliquots and chromatographic separation of drugs and Internal Standard (quinoxaline) was achieved with a gradient (acetonitrile and water with formic acid 0.05%) on an C-18 reverse phase analytical column with 25 min of analytical run. Calibration curves were optimised according to expected ranges of drug concentrations in patients, and correlation coefficient (r^2) was higher than 0.998 for all analytes. Mean intra- and inter-day precision (relative standard deviation %) for all compounds were 8.4 and 8.3%, respectively, and mean accuracy (% of deviation from nominal level) was 3.9%. Extraction recovery ranged within 93 and 105% for all drugs analysed. This novel HPLC–MS methodology allows a specific, sensitive and reliable determination of DRV and 11 other antiretrovirals. In our hand, it was used to measure DRV and ritonavir plasma concentration in HIV-positive patients, and it is now successfully applied for routine therapeutic drug monitoring and pharmacokinetics studies.

Keywords: TMC114; DRV; Protease inhibitors; NNRTI; Protein precipitation extraction; HPLC–MS; Quantification

1. Introduction

Treatment of HIV infection relies on an association of antiretrovirals drugs, including at least one protease inhibitor (PI) or a non-nucleoside transcriptase inhibitor (NNRTI) and/or one or more nucleoside or nucleotide transcriptase inhibitor (NRTIs-NtRTI) and/or a fusion inhibitor (FI).

Selection of mutations in HIV genome conferring cross-resistance to different compounds of each class is unfortunately a not infrequent occurrence. Therefore, there is the need of a new therapeutic tools able to overcome the extensive class resistances observed in multi-treated patients [1].

Darunavir (Prezista™), formerly known as TMC114, is a new and promising PI active against HIV strains resistant to other currently available PIs [2–5]. It is administered 600 mg two times a day in association with 100 mg of ritonavir (RTV, Norvir™) as a booster. Darunavir (DRV) is a key component of many salvage therapies in multiexperienced patients. This compound was licensed in June 2006 in the United States and in February 2007 in European Union.

Accurate measurement of PIs and NNRTIs plasmatic levels is crucial for pharmacokinetic/pharmacodynamic analyses, drug–drug interaction studies, and therapeutic drug monitoring (TDM). The latter is currently considered a useful tool for the optimisation of antiretroviral therapy in most international guidelines [6–9].

Knowledge of pharmacokinetics (PK) of DRV in the clinical setting, namely pharmacokinetic/pharmacodynamic properties and drug interaction profile, is still limited due to the recent availability of this compound. Therefore, PK studies of DRV are requested in order to define the possible role of TDM of this drug in the clinical context.

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Aim of our study was to develop and validate an HPLC–MS analytical tool for the simultaneous quantification of DRV and most used NNRTIs and PIs, including RTV and other 11 compounds.

2. Experimental

2.1. Chemicals

The compounds were kindly obtained from the following pharmaceutical companies: nevirapine (NVP) and tipranavir (TPV) from Boehringer Ingelheim Pharmaceuticals, Inc. (Ridgefield, CT, USA); efavirenz (EFV) and atazanavir (ATV) from Bristol Myers Squibb Company (Princeton, NJ, USA); indinavir (IDV) from Merck Sharp & Dohme-Chibret Labs. (West Point, PA, USA); amprenavir (APV) from GlaxoSmithKline (Brentford, UK); darunavir (DRV) from Tibotec (Mechelen, Belgium); saquinavir (SQV) from Roche (Mannheim, Germany); nelfinavir (NFV) and M-8 from Pfizer Inc. (Groton, CT, USA); lopinavir (LPV) and ritonavir (RTV) from Abbott Laboratories (IL, USA).

Acetonitrile HPLC grade and methanol HPLC grade were purchased from J.T. Baker (Deventer, Holland). HPLC grade water was produced with Milli-DI system coupled with a Synergy 185 system by Millipore (Milan, Italy). Quinoxaline (QX) and formic acid were obtained from Sigma–Aldrich (Milan, Italy). Blank plasma from healthy donors was kindly supplied by the Blood Bank of Maria Vittoria Hospital (Turin, Italy).

2.2. Stock solutions, standards (STD) and quality controls (QC)

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

Working solution of Internal Standard (IS) was made with QX (0.625 μg/ml) in methanol and HPLC grade water (50:50, v/v) and stored at 4 °C until use.

The highest calibration standard (STD 9) and three quality controls (QCs) were prepared adding a determined volume of stock solutions to blank plasma; the others STDs were prepared by serial dilution from STD 9 to STD 1 with blank plasma, to obtain nine different spiked concentrations plus a blank sample (STD 0).

Calibration ranges, from STD 9 to STD 1, and QCs concentrations for all drugs are listed in Table 1.

STDs, QCs and patient samples underwent to heat inactivation procedure for HIV (35 min at 58 °C), and then stored at −20 °C until analyses, avoiding more then three freeze–thaw cycles, and not more than 3 months.

In this condition, NVP, IDV, APV, SQV, M-8, NFV, ATV, EFV, RTV, LPV and TPV have been demonstrated to be stable [10–13], therefore no further stability evaluation was carried out. Stability assay was performed only for DRV.

Table 1
Concentrations of STD 9, STD 1 (LOQ), QCs (QC high, QC medium and QC low) and LOD

Drugs	Concentrations (ng/ml)					
	STD 9	STD 1/LOQ	QC high	QC medium	QC low	LOD
IDV	8,000	31.2	6,000	1500	150	15.6
NVP	8,000	31.2	6,000	1500	150	15.6
M-8	4,000	15.6	2,000	500	50	7.8
NFV	8,000	31.2	4,000	1000	100	15.6
SQV	7,000	27.3	4,000	1000	100	13.6
ATV	6,000	23.4	4,000	1000	100	11.7
APV	10,000	39.1	8,000	2000	200	19.5
DRV	10,000	39.1	8,000	2000	200	19.5
RTV	2,500	9.8	2,000	500	50	4.9
LPV	15,000	58.6	10,000	2500	250	29.3
EFV	8,000	31.2	6,000	1500	150	15.6
TPV	45,000	175.8	25,000	6250	625	87.9

2.3. Chromatographic and MS conditions

The HPLC–MS instrument used was a Waters system (Milan, Italy), with binary pump model 1525, AF degasser, 717-plus autosampler, and Micromass ZQ mass detector. LC–MS Empower Pro software (version year 2002, Waters; Milan, Italy) was used.

Chromatographic separation was performed at 35 °C using a column oven, on Atlantis dC-18 3μ column (150 mm × 2.1 mm i.d.) (Waters; Milan, Italy), protected by a Security Guard with C-18 (4.0 mm × 3.0 mm i.d.) pre-column (Phenomenex; CA, USA).

Chromatographic run was performed with a gradient (Table 2), and the mobile phase was composed by Buffer A (HPLC grade water + 0.05% formic acid) and Buffer B (HPLC grade acetonitrile + 0.05% formic acid).

Detector settings were ESI, positive polarity ionization (except for EFV which was detected by negative polarity ionization, in the same run simultaneously using instantaneous switching); capillary voltage 3.5 kV; source temperature

Table 2
Chromatographic condition (gradient): mobile phase: Buffer A (HPLC grade water + 0.05% formic acid) and Buffer B (HPLC grade acetonitrile + 0.05% formic acid)

Time (min)	% Buffer A	% Buffer B	Flow (ml/min)
0.0	59	41	0.25
0.1	59	41	0.25
4.0	40	60	0.25
9.0	30	70	0.25
9.5	25	75	0.25
9.6	5	95	0.25
14.0	5	95	0.25
14.1	100	0	0.25
15.0	100	0	0.25
15.1	60	40	0.25
25.0	60	40	0.25

Table 3
Drugs retention times, ions detected, cone voltages and ionization typologies

Drugs	Retention time (min)	Ions detected (*m/z*)	Cone voltage (V)	Ionization
IDV	3.4 ± 0.1	614.40	33	+
NVP	3.6 ± 0.1	267.20	33	+
M-8	5.3 ± 0.1	584.35	33	+
IS	8.9 ± 0.1	313.30	50	+
NFV	10.6 ± 0.1	568.30	32	+
SQV	10.9 ± 0.1	671.35	37	+
ATV	11.9 ± 0.1	705.40	33	+
APV	12.4 ± 0.1	506.30	18	+
DRV	12.4 ± 0.1	548.30	18	+
RTV	15.9 ± 0.1	580.35	23	+
LPV	16.5 ± 0.1	629.40	25	+
EFV	16.7 ± 0.1	314.40	25	−
TPV	18.5 ± 0.1	603.22	28	+

110 °C; desolvation temperature 350 °C; nitrogen desolvation flow 800 l/h; nitrogen cone flow 100 l/h.

Ions detected, cone voltages and ionization typologies for each drug are listed in Table 3.

2.4. STD, QC and samples preparation

Patients receiving standard dosing of Prezista™ and Norvir™, 600 + 100 mg bis in die (BID), underwent blood sampling after obtaining their informed consent for the measurement of plasma DRV and RTV concentrations. Blood samples were collected in lithium heparin tube (7 ml), plasma was obtained after centrifugation at 1400 × *g* (3000 rpm) for 10 min at +4 °C (Jouan Centrifuge, Model BR4i, Saint-Herblain, France) and then underwent to heat inactivation, as described above. To avoid thawing cycles each patient plasma sample was aliquoted into two 300 μl criovials.

Extraction method developed was based on protein precipitation: 600 μl of protein precipitation solution (methanol:acetonitrile [50:50]) was added to 50 μl of plasma sample and 40 μl of Internal Standard Working Solution in a PTFE (polytetrafluoroethylene; Teflon) microfuge tube, then the tube was vortexed for 30 s and centrifuged at 13,000 × *g* (12,000 rpm) for 10 min at 4 °C.

Two hundred microliters of supernatant were then transferred to a glass vial, diluted with 200 μl of HPLC grade water and briefly vortexed; 40 μl of this solution was injected into the column.

All analyses were performed in duplicate, only for our validation assay, and all procedure steps were carried out at room temperature.

2.5. Specificity and selectivity

Interference from endogenous compounds was investigated by analysis of five different blank plasma samples. Potential interference by antiretroviral drugs concomitantly administered to the patients was also evaluated by spiking blank plasma with them. These included: zidovudine (AZT), didanosine (ddI), stavudine (d4T), lamivudine (3TC), abacavir (ABV), tenofovir (TDF), emtricitabine (FTC) and enfuvirtide (T-20); other concomitant drugs were also investigated: acetylsalicylic acid, amodiaquine, amoxicillin, atorvastatin, caspofungin, ceftazidime, ciprofloxacin, clavulanic acid, enalapril maleate, furosemide, insulin, levofloxacin, nimesulide, omeprazole, paracetamol, pravastatin and ribavirin.

An "interfering drug" has been considered as a molecule which exhibits a retention time close to 0.3 min from the analytes, and with the potential capability to cause ion suppression.

2.6. Matrix effect

"Matrix effect" was investigated using five different blank plasma and comparing peak areas obtained from standard solutions of solution of water, methanol and acetonitrile (50:25:25), containing all our analytes at three different concentrations, and peak areas obtained from blanks post-extraction solution with the same amount of analytes, as described by Taylor [14].

Possible "matrix effect" was calculated, as deviation %, comparing the peak area obtained from the plasma extract with the peak area obtained from the standard solution.

2.7. Accuracy, precision, calibration and limit of quantification

Intra-day and inter-day accuracy and precision were determined by assaying six spiked plasma samples at three different concentrations (QCs) for each drug. Accuracy was calculated as the percent deviation from the nominal concentration. Inter-day and intra-day precision were expressed as the standard deviation at each QC concentration.

Each calibration curve was obtained using nine calibration points in duplicate, and the ranges are listed in Table 1.

Calibration curves were created by plotting the peak area ratios of each drugs relative to the IS against the various drugs concentrations in the spiked plasma standards. A 1/*X* weighted quadratic regression was used for all curves.

The limit of detection (LOD) in plasma was defined as the concentration that yields a signal-to-noise ratio of 3/1. Percent deviation from the nominal concentration (measure of accuracy) and relative standard deviation (measure of precision) of the concentration considered as the limit of quantification (LOQ) had to be <20%, and it was considered the lowest calibration standard, as requested by FDA [15].

2.8. Recovery

Recovery from plasma, using the extraction procedures, was assessed by comparing the peak area obtained from multiple analyses of spiked samples (QCs) with the peak area from standard solution of all analytes in a solution of water, methanol and acetonitrile (50:25:25) at the same concentrations.

2.9. Stability

The stability of NNRTIs and PIs at different conditions has been previously assayed in many articles, but data about DRV stability were not fully available [10–13,16].

For this reason, stability assays were conducted only for DRV in different matrixes: stock solution, plasma and the post-extracted solution.

For stock solution, peak area of each drug obtained from fresh stock solution was compared to peak area obtained after 3 months storing at 4 °C. For plasma, peak area obtained from extraction at three different concentrations (fresh QCs) was compared to those obtained after 3 months storing at −20 °C, and after heat inactivation. For post-extracted solution, peak area obtained from extracted QCs at three different concentrations was compared to those obtained after 24 h storing at room temperature.

3. Results

Our analyte retention times are shown in Table 3. APV and DRV eluted at the same retention time, due to their similar chemical structure and physiochemical properties, but this coelution did not affect the quantification of two drugs.

Representative chromatograms of a blank plasma extracted and STD 1 are shown in Fig. 1. Mean regression coefficient (r^2) of all calibration curves was more than 0.998. A $1/X$ weighted quadratic regression was chosen due to its r^2 higher than other equations, and to the fact that it gave more weight to low calibration points, curve region where the greater number of C_{trough} concentrations were expected.

3.1. Specificity and selectivity

The assay did not show any significant interference with antiretrovirals or other concomitant drugs taken at therapeutic dosage by patients (see Section 2.5).

The tested five blank plasma did not show any interference in the retention times analytes windows for each specified ion detected (Fig. 1).

3.2. Accuracy, precision, limit of quantification

Results of the validation of the methods are listed in Table 4 for all analytes. All observed data (intra-day and inter-day precision [R.S.D.%]) were below 15.0%, according to FDA guidelines [15].

Coelution of DRV and APV did not alter the quantification of two drugs. Performance of the assay was tested in triplicates and similar results were obtained using calibrators and QCs containing both of them or DRV only (data not shown).

LOQ and LOD are listed in Table 1.

3.3. Recovery

Multiple aliquots ($n = 6$) at each of the three QCs concentration were assayed and mean recovery of all drugs ranged from 93 to 110%.

Mean recovery of DRV and APV (107.4 and 103.5%) was not affected by their coelution and it was tested in triplicates using one set of QCs and standards containing both drugs and other two sets with DRV only, as described above.

3.4. Analysis of samples from treated patients

Our method was applied for assaying of 81 samples, corresponding to C_{trough}, C_{max}, and other points of time–concentration curve, obtained from 30 patients administered with DRV and RTV.

Mean C_{trough} observed for DRV was 3705 ng/ml and the lowest C_{trough} value for DRV and RTV was 40 and 44 ng/ml, respectively. Mean C_{max} observed for DRV was 6371 ng/ml and the highest C_{max} for DRV and RTV was 7721 and 1175 ng/ml, respectively. Such values resulted in the expected range of concentrations according to previous phase II reports [17].

3.5. Matrix effect

The deviation % of the peak area at the three concentrations for all analytes is comparable, ranging from −15.1 to −2.7%, showing absence of "matrix effect". Moreover, "matrix effect" for DRV is smaller than 7.7% at all three concentrations.

3.6. Stability

DRV showed to be stable in all tested condition; relative standard deviation (R.S.D.) % of peak area was 5.9% for stock solution at 4 °C for 3 months, 3.9% for plasma at −20 °C for 3 months, 5.3% in plasma after heat inactivation and 2.7% in the extraction solution at room temperature for 24 h.

4. Discussion

The use of inhibitors of HIV-1 protease as a component of highly active antiretroviral therapy in patients with HIV infection has been shown to achieve durable virological suppression as well as to decrease the considerable morbidity and mortality associated with HIV disease [18–20]. As a result, PIs have

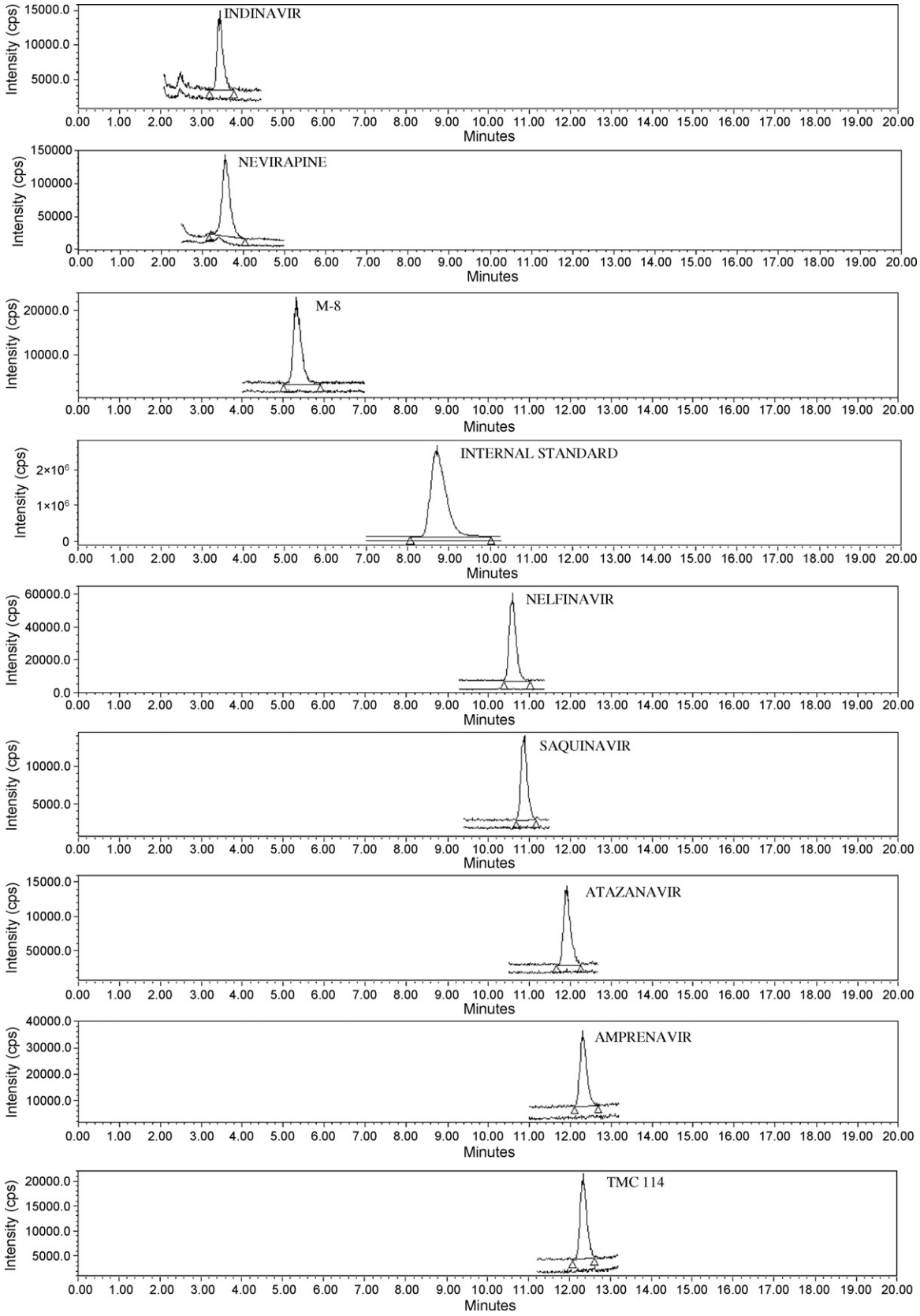

Fig. 1. Overlapping of SDT1 and blank plasma extracted ions detection.

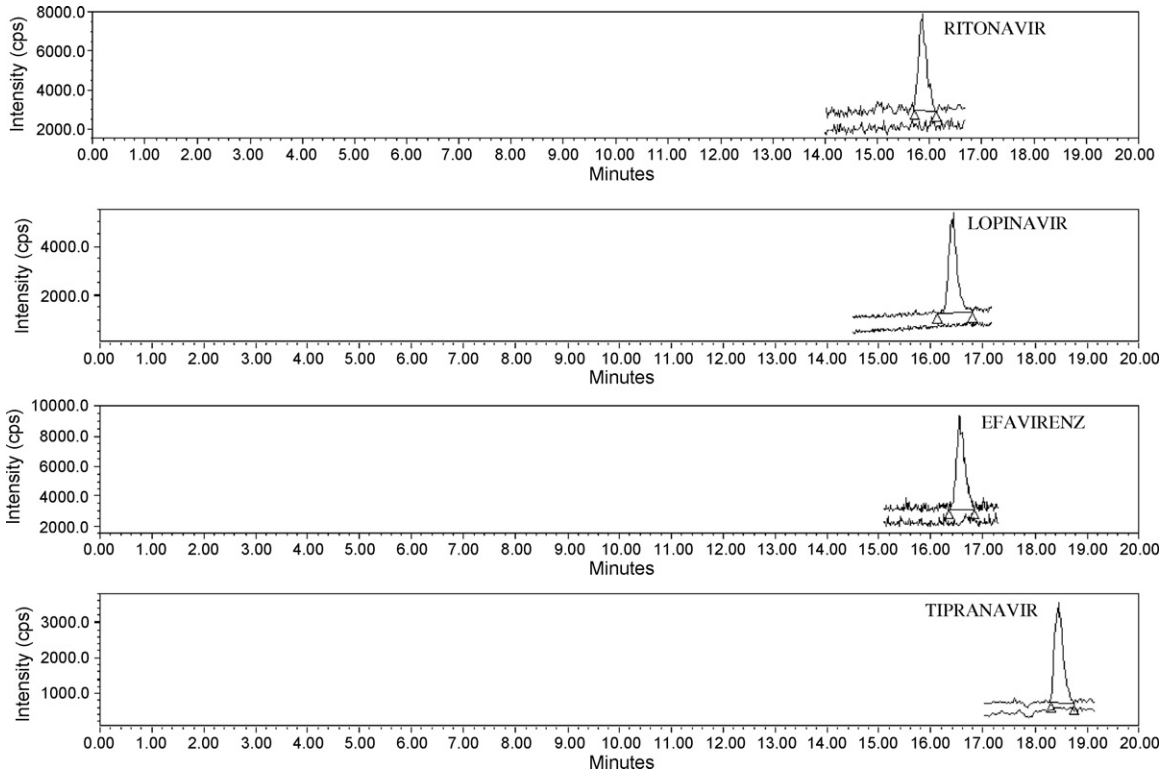

Fig. 1. (*Continued*).

become cornerstones in the treatment of HIV infection, particularly in patients with a long history of antiretroviral therapy. However, the emergence of drug-resistant HIV-1 strains continues to jeopardize the efficacy of existing anti-HIV medications, and the need to develop novel therapies with activity against resistant virus is of increasing importance.

TDM has become an essential tool for the management of HIV-positive patients. Measurement of antiretroviral plasma concentrations can be useful in several clinical setting, such as management of side effects, optimisation of efficacy, metabolic impairment and drug–drug interaction [6–9].

Clinical usefulness of TDM of DRV has not been yet fully evaluated, however measurement of DRV plasma levels could be a tool for management of special populations and for pharmacokinetic studies.

For the screening and quantification of DRV and many other antiretroviral agents in HIV-infected patients, a novel analytical method was developed using liquid chromatography with mass spectrometry technology. Other UV, LC–MS and LC–MS/MS methods for simultaneously measuring of PIs and NNRTIs antiretroviral agents have been reported [10–13,21–29], and only one is able to assaying DRV [16].

Table 4
Accuracy (%), intra-day and inter-day precision (R.S.D.%) assayed for all drugs ($n = 6$)

Drugs	QC high			QC medium			QC low		
	Accuracy (%)	Precision (R.S.D.%)		Accuracy (%)	Precision (R.S.D.%)		Accuracy (%)	Precision (R.S.D.%)	
		Intra-day	Inter-day		Intra-day	Inter-day		Intra-day	Inter-day
IDV	−0.8	8.0	11.0	3.0	6.3	11.5	3.4	7.8	7.9
NVP	−1.6	8.3	7.7	0.6	3.4	5.1	10.3	6.7	7.4
M-8	1.0	5.3	7.2	1.5	4.7	9.8	7.2	5.7	11.6
NFV	2.4	4.2	5.7	1.8	3.9	13.6	8.3	9.2	12.4
SQV	7.6	6.6	6.3	6.6	2.7	9.9	11.6	3.5	6.8
ATV	1.2	7.2	8.2	1.9	5.2	10.3	12.4	8.9	11.4
APV	−1.7	6.5	5.0	−1.2	3.4	5.9	10.0	2.8	3.9
DRV	2.5	4.8	7.0	1.1	3.9	5.5	8.0	4.9	7.4
RTV	7.4	5.2	8.9	1.4	4.3	8.5	5.9	7.4.	8.9
LPV	−2.5	6.7	7.5	7.3	5.9	13.5	12.9	0.9	2.4
EFV	0.6	9.1	8.9	2.0	13.7	12.7	4.6	5.9	5.8
TPV	−6.2	6.7	8.7	3.0	4.6	8.7	9.5	8.7	8.7

In our method, all analytes, detected with positive polarity ionization, are precursor ions $M+H^+$, resulted from the addiction of a proton to form the charged molecular ion, except for RTV, which has a molecular weight of 721 *m/z* but it has been detected at 580.35 *m/z*, at RTV retention time (ion son) with the best sensitivity. A explanation for the ion formation has been described by Notari et al. [26].

Calibration curves included a wide range of PI and NNRTI concentrations. Choice of limits of these ranges was based on the highest values reported in the clinical reports and pharmacokinetic studies, except for TPV, for which that was monitored only for C_{trough} range [17,30–32].

Reliability, costs, difficulty of performance and reproducibility are key points of measurement of drug plasma concentrations. Our protein precipitation/HPLC–MS method showed to be simple, reliable, sensitive, low cost and rapid to perform. Reliability of our method has been demonstrated for all analyte concentrations analysed.

Relative error at QC concentrations, intra-day and inter-day precision (Table 4) support accuracy and precision of our procedure.

Absence of interference peaks at the analyte retention times, with the overlapping of DRV and APV without "matrix effect", allowed accurate measurement of drugs plasma levels, also in patients administered with several concomitant drugs.

The protein precipitation method showed to be easy to perform and poor expensive, showing reliability and ruggedness.

As compared to recently published assay of Ter et al., our method, despite a longest chromatographic run to achieve analytes separation, showed to require lowest plasma volume (50 μl versus 100 μl); a single, easy to buy and cheap, I.S. (quinoxaline versus D5-saquinavir, D6-indinavir, 13C6-efavirenz and dibenzepine); and a less expensive instrumentation (single mass versus tandem mass). Moreover, only in our work long-term stability of DRV was evaluated and measurements of plasmatic concentrations in clinical samples were done.

5. Conclusion

The HPLC–MS method described allowed accurate and reproducible simultaneous quantification of DRV and other 11 antiretroviral agents in small volumes (50 μl) of plasma by a single assay. High extraction efficiency and low limit of quantification make this a suitable method for use in clinical trials and for TDM of PIs and NNRTIs. This method is now successfully applied for our routine therapeutic drug monitoring and pharmacokinetics studies in HIV-infected patients.

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