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Journal of Chromatography B, 740 (2000) 43–58

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Simultaneous determination of the HIV protease inhibitors indinavir, amprenavir, saquinavir, ritonavir, nelfinavir and the non-nucleoside reverse transcriptase inhibitor efavirenz by high-performance liquid chromatography after solid-phase extraction

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Received 15 September 1999; received in revised form 7 December 1999; accepted 17 December 1999

Abstract

As part of an on-going study on the suitability of a formal therapeutic drug monitoring (TDM) of antiviral drugs for improving the management of HIV infection, a high-performance liquid chromatography method has been developed to quantify simultaneously in plasma five HIV protease inhibitors (PIs) (i.e., indinavir, amprenavir, saquinavir, ritonavir, nelfinavir) and the novel non-nucleoside reverse transcriptase inhibitor efavirenz. After viral inactivation by heat (60°C for 60 min), plasma (600 μl), with clozapine added as internal standard, is diluted 1:1 with phosphate buffer, pH 7 and subjected to a solid-phase extraction on a C_{18} cartridge. Matrix components are eliminated with 2×500 μl of a solution of 0.1% H_3PO_4 neutralised with NaOH to pH 7. PIs and efavirenz are eluted with 3×500 μl MeOH. The resulting eluate is evaporated under nitrogen at room temperature and is reconstituted in 100 μl 50% MeOH. A 40-μl volume is subjected to HPLC analysis onto a Nucleosil 100, 5 μm C_{18} AB column, using a gradient elution of MeCN and phosphate buffer adjusted to pH 5.15 and containing 0.02% sodium heptanesulfonate: 15:85 at 0 min→30:70 at 2 min→32:68 at 8 min→42:58 at 18 min→46:54 at 34 min, followed by column cleaning with MeCN–buffer, pH 5.15 (90:10), onto which 0.3% AcOH is added. Clozapine, indinavir, amprenavir, saquinavir, ritonavir, efavirenz and nelfinavir are detected by UV at 201 nm at a retention time of 8.2, 13.0, 16.3, 21.5, 26.5, 28.7 and 31.9 min, respectively. The total run time for a single analysis is 47 min, including the washing-out and reequilibration steps. The calibration curves are linear over the range 100–10 000 ng/ml. The absolute recovery of PIs/efavirenz is always higher than 88%. The method is precise with mean inter-day relative standard deviations within 2.5–9.8% and accurate (range of inter-day deviations −4.6 to +4.3%). The in vitro stability of plasma spiked with PIs/efavirenz at 750, 3000 and 9000 ng/ml has been studied at room temperature, −20°C and +60°C. The method has been validated and is currently applied to the monitoring of PIs and efavirenz in HIV patients. This HPLC assay may help clinicians confronted to questionable compliance, side effects or treatment failure in elucidating whether patients are exposed to adequate circulating drug levels. The availability of such an assay represents an essential step in elucidating the utility of a formal TDM for the optimal follow-up of HIV patients.

Keywords: Protease inhibitors; Indinavir; Amprenavir; Saquinavir; Ritonavir; Nelfinavir; Efavirenz

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0378-4347/00/$ – see front matter
PII: S0378-4347(99)00573-3

1. Introduction

HIV protease inhibitors (PIs) in combination with nucleoside reverse transcriptase inhibitors (NRTIs) and recently non-nucleoside reverse transcriptase inhibitors (NNRTIs) have transformed the short-term prognosis of HIV patients [1]. However, the infection progressively escapes control in a significant number of patients. Among other factors, variability in drug intake (compliance) and drug disposition influence the maintenance of adequate circulating drug levels, which may affect the treatment effectiveness and patient's outcome. Moreover, the long term side effects of PIs on metabolism, including altered body fat distribution, increased blood lipids levels and glucose intolerance, could be concentration-dependent, suggesting that monitoring of PI levels could be useful in the clinical management of HIV disease [2]. The introduction of the NNRTI efavirenz to the antiviral regimen appears promising in PI, NNRTI, lamivudine-naive patients [3,4]. Only limited information is available on the influence of efavirenz and PIs on their mutual disposition [1]. Therefore, an analytical method for their determination in blood on a routine basis may represent a useful clinical tool, enabling the study of the relationship between plasma levels, metabolic disorders and virological treatment failure, and treatment fine-tuning. Moreover, it may contribute to ameliorate patient management, in particular in evaluating drug–drug interactions and assessing compliance to treatment.

Several high-performance liquid chromatography (HPLC) methods have been reported for separately quantifying indinavir [5–9], ritonavir [10,11], saquinavir [12,13], nelfinavir [14] in animal and human biological fluids. All these methods aimed at individually determining one protease inhibitor and some of them used sophisticated and expensive HPLC instruments, with either column switching techniques or mass spectroscopy, not commonly accessible in conventional hospital laboratory settings. Up to now, only a few analytical methods have appeared for the simultaneous assay of PIs. One unpublished report was issued by the laboratory of the Mayo Clinic, in which liquid–liquid extraction was used for the clean-up sample procedure [15]. The recovery of the PIs with this procedure was not reported. A simultaneous assay of five protease inhibitors (including the new PI amprenavir) has been recently published but requires an automatic switchable UV detector at 210 and 239 nm [16]. This is an important contribution, although the selectivity of the measurements of indinavir and amprenavir, with a retention time of 2 min and 2.9 min (at a time very close to the early eluting endogenous peaks and the delarvidine peak at 2.6 min), was questionable. Moreover, the non-symmetric shape of the last nelfinavir peak suggested that the reported method may not be sensitive enough to quantitate trough concentrations. Furthermore, whereas the stability of the PIs in biological medium was studied, the actual processing of patients' plasma samples for HIV inactivation by heat was not clearly described. Additional HPLC methods for the simultaneous assay of PIs were also proposed, without considering however the assay of amprenavir [17–20] or efavirenz [34]. To the best of our knowledge, only two groups have reported, very recently [21–23], an HPLC assay of the NNRTI efavirenz after liquid–liquid extraction from biological fluids.

A detailed method for the assay of the five clinically used PIs plus the NNRTI efavirenz, with UV detection at one single wavelength, with a gradient elution program rendering the method amenable to automation, is therefore highly desirable.

We report the setting up and validation of an HPLC method with single-wavelength UV detection at 201 nm for the simultaneous separation and quantitation of indinavir, amprenavir, saquinavir, ritonavir, nelfinavir and efavirenz in plasma from HIV-positive patients, after plasma thermisation and solid-phase extraction on C_{18} cartridges.

2. Materials and methods

2.1. Chemicals

Ritonavir (RTV), indinavir (IDV), saquinavir (SQV), nelfinavir (NFV), amprenavir (APV) pure substances were kindly provided by Abbott (Baar, Switzerland), Merck Sharp Dohme Chibret (Glattbrugg, Switzerland), Roche Discovery Welwyn (Welwyn Garden City, UK), Agouron (La Jolla, CA, USA) and Glaxo Wellcome R&D (Stevenhage, UK), respectively. Stock standard solutions of efavirenz (EFV) were prepared by extracting with methanol

(MeOH) one 200 mg (Stocrin, Sustiva) tablet reduced to powder, in a sonicator prior to filtration on a paper filter and dilution into a volumetric flask. Clozapine (internal standard, I.S.) stock solution (250 μg/ml) in MeOH was obtained by extraction with MeOH of a Leponex tablet. This solution was diluted down to 45 μg/ml before use. Acetonitrile (MeCN) and MeOH for chromatography LiChrosolv, 100% acetic acid (AcOH) and 85% phosphoric acid (H_3PO_4) were from E. Merck (Darmstadt, Germany). Sodium hydroxide puriss. pellets and sodium heptanesulfonate (both analytical reagent grade) 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).

2.2. Chromatographic system

The chromatographic system consisted of a Hewlett-Packard 1090 (serie II) (Hewlett-Packard, Germany) equipped with a spectrophotometric UV–Vis photo-diode array detection (DAD) system set at 201 nm. An 8-nm slit was used for the maximum detection of the less UV absorbent IDV, APV and NFV. The peak width and sampling interval was set at 0.053 min and 0.32 s, respectively. The separations were performed at room temperature (RT) on a ChromCart cartridge column (125×4 mm I.D.) filled with Nucleosil 100, 5 μm C_{18} AB (Macherey–Nagel, Düren, Germany) equipped with a guard column (8×4 mm I.D.) filled with the same packing material. The injection volume was 40 μl.

The mobile phase was delivered at 1 ml/min and the gradient program conditions are given in Table 1.

The HP ChemStation A.06.03 software loaded on a Compaq DeskPro EP 6/400c was used to pilot the HPLC instrument and to process the data (area integration, calculation and plotting of chromatograms) throughout the method validation and sample analysis. Baselines were visually inspected and were manually adjusted using peak start and end features of the HP ChemStation software.

2.3. Mobile phase solutions

Solvent A consisted of pure MeCN. Solution B was prepared by adding 11.8 ml 8.5% H_3PO_4 and 0.2 g sodium heptanesulfonate to 988.2 ml water with pH carefully adjusted to 5.15±0.02 with 10 *M* NaOH. The solvent C was 0.3% AcOH in MeCN. Solutions B and C were prepared regularly and stored in the dark at +4°C prior to use. All solvents were degassed by sparging with helium.

Table 1
Gradient elution program

Time (min)	Solvent A (%)	Solvent B (%)	Solvent C (%)
0	15	85	0
2	30	70	0
8	32	68	0
18	42	58	0
34	46	54	0
35[a]	33	34	33
36[a]	0	10	90
37[a]	0	10	90
38[a]	33	34	33
39[a]	46	54	0
40	15	85	0
47	15	85	0

[a] Rinsing/washing programme.

2.4. Buffer solutions for sample processing

The buffer used for the dilution of plasma samples was prepared by mixing 413 ml of 1/15 *M* KH_2PO_4 (9.97 g/l) and 587 ml of 1/15 *M* $Na_2HPO_4 \cdot 2H_2O$ (11.9 g/l) (=buffer D). The solid-phase extraction (SPE) cartridge conditioning (see below) was performed with a solution of 0.1% H_3PO_4 neutralized with NaOH to pH 7 (=buffer E).

2.5. Stock solution, working solution, plasma calibration and control samples

Stock solutions of indinavir sulfate (substance, solvent: IDV, MeOH–water, 50:50), amprenavir base (APV, MeOH), saquinavir mesylate, (SQV, MeOH), ritonavir base (RTV, absolute EtOH), efavirenz (EFV, MeOH), nelfinavir mesylate (NFV, MeOH) were prepared at a concentration of 1 mg/ml (calculated as base) and where appropriately diluted with MeOH–water (50:50) for the preparation of working solutions at concentrations of 1000–100 000 ng/ml. Plasma calibration samples at 100, 250, 1000, 5000, 8000, 10 000 ng/ml of IDV, APV, SQV, RTV, EFV, NFV together with plasma control samples at 750, 3000, 9000 ng/ml were prepared by 1:10 dilution of the respective working solution to blank plasma from

outdated transfusion bags (total added volume≤10% of the biological sample volume), in accordance with the recommendations on bioanalytical method validation [28]. The six calibration standards and control samples were thermised at 60°C for 60 min (see below), stored at −20°C as 1500-μl aliquots in polypropylene Eppendorf tubes, and thawed on the day of analysis.

2.6. Sample collection and thermisation (HIV inactivation)

According to a protocol previously approved by the Ethics Committee of the Hospital and with the written informed consent of the patients, blood samples were taken approximately every 3 months, from patients of the HIV visit at the University Hospital of Lausanne. Patients were instructed not to take their morning pills prior to the consultation.

Blood samples (5 ml) were collected in Monovettes (Sarstedt, Nümbrecht, Germany) with Li heparinate as anticoagulant prior to (trough level), and 1–4 h (peak level) after an oral dose of the prescribed drugs taken at the hospital with a light standardized breakfast. Samples were immediately taken to the laboratory and were processed under a protection hood wearing nitrile gloves and long-sleeve laboratory coats. The Monovettes were centrifuged at 3000 rpm (1850 *g*) for 10 min at 4°C (Beckmann Centrifuge, Model J6B) and the plasma was separated and transferred into polypropylene test tubes before being heated at 60°C for 60 min in a thermostated water bath (Memmert WB 7, Schwabach, Germany). This treatment has been shown to effectively inactivate HIV particles present in the samples [24–27]. The stability of PIs and EFV is reported in detail in the method validation (see Section 2.9 below). The processed plasma were stored at −20°C up to the time of analysis.

2.7. Sample preparation

On the day of analysis, calibration, quality control and patient samples were thawed and allowed to equilibrate at RT.

A 600-μl volume of plasma (calibration, control, patients) sample was diluted with 500 μl of solution D and 100 μl of I.S. solution in an Eppendorf microvial. The resulting solution was vortexed and centrifuged for exactly 5.0 min on a benchtop centrifuge at 14 000 rpm (20 000 *g*) (Hettich Benchtop Universal 16R centrifuge, Bäch, Switzerland).

The clean-up procedure of diluted plasma samples was performed by SPE using a 24-tube vacuum manifold Macherey–Nagel. The C_{18} cartridges (Supelclean LC_{18} tubes, Supelco) were conditioned with 2×1 ml MeOH followed by 2×1 ml buffer E. An aliquot (1000 μl) of the diluted plasma sample was applied onto the cartridge and drawn through completely under vacuum (typically 1–2 mmHg; 1 mmHg=133.322 Pa).

The cartridge was washed twice with 500 μl of buffer E and light vacuum (6–7 mmHg) applied for 5 min. PIs and EFV were subsequently desorbed with three times 500 μl of MeOH, with a final drying step (6–7 mmHg vacuum) after the third elution. The eluted solutions were evaporated under nitrogen steam at room temperature for approximately 30 min and the residue reconstituted in 100 μl of MeOH–water (50:50). The resulting solutions were carefully vortexed and centrifuged at 20 000 *g* for 5 min. The supernatants were introduced into 200-μl HPLC microvials (Hewlett-Packard) and a 40-μl volume was used for HPLC analysis.

2.8. Calibration curves

Quantitative analysis of PIs and EFV was performed using the internal standard method.

The calibration curves were obtained by unweighted least-squares linear regression of the peak-area ratio of IDV, APV, SQV, RTV, EFV, NFV to clozapine (I.S.) versus the ratio of the injected amount of the respective PIs/EFV to I.S., in each standard samples. The calibration was established over the range 250–10 000 ng/ml for IDV, RTV, EFV, NFV, and 100–10 000 ng/ml for APV and SQV, where the response ratio/amount ratio was linear.

2.9. Analytical method validation

The validation of the method was based on the recommendations published as a Conference Report of the Washington Conference on Analytical methods

Validation: Bioavailability, Bioequivalence and Pharmacokinetic Studies [28].

Each level of the calibration curve was measured with two sets of calibrators: one set at the beginning and a second set at the end of the HPLC run. Throughout patient sample analysis, control samples at three concentrations levels (low, medium and high, respectively, 750, 3000 and 9000 ng/ml) were assayed every five samples.

Quality control samples were used for the precision and accuracy determination, the precision being calculated as the relative standard deviation (RSD) 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.

The limit of quantitation (LOQ) for PIs and EFV in plasma was experimentally chosen as the minimal concentration in plasma samples which could be confidently determined in accordance with the Conference Report on Analytical Method Validation [28] recommending that the deviation between measured and nominal concentration should not deviate more than ±20%. The LOQ values were used for the low concentration level of the standard curve.

2.10. Stability of PIs and EFV

The stability of the drugs (PIs/EFV) in plasma under the thermisation process (60°C for 60 min) was assessed as follows:

Two series of calibration samples at the six concentrations reported above (100 up to 10 000 ng/ml) were analyzed in parallel. One was heated at 60°C for 60 min, while the thermisation procedure was omitted in the second. Aliquots (600 μl) of both series were submitted simultaneously to SPE according to the procedure described above. The slope of the resulting calibration (response ratio/concentration ratio) curves established with each series was compared.

Further stability studies included: (a) stability of plasma samples after multiple freeze–thaw cycles: aliquots of plasma spiked with 750, 3000 and 9000 ng/ml of PIs/EFV underwent four freeze–thaw cycles: frozen samples were allowed to thaw at ambient temperature for 2–3 h and were subsequently refrozen. The PIs/EFV levels were measured in aliquots after each of the four freeze–thaw cycles. The variations of PIs/EFV concentrations were expressed in percentage of the starting levels. (b) Stability of plasma extracts into HPLC vials: processed samples (i.e., reconstituted in 50% MeOH) containing PIs/EFV at 750, 3000 and 9000 ng/ml were analyzed in duplicate immediately after preparation, after being left 24 h at room temperature, in the autosampler rack, kept refrigerated at 4°C for 48 h, or kept frozen at −20°C.

2.11. Recovery

The efficiency of the SPE was determined with control samples at 750, 3000 and 9000 ng/ml. The absolute recovery [29] of PIs/EFV from plasma was obtained as the peak-area response of the processed samples, expressed as a percentage of the response of the substances contained in the 40-μl injection volume and not subjected to SPE.

2.12. Selectivity

The selectivity was determined by injecting onto the HPLC column all currently prescribed anti-HIV drugs and/or employed in the treatment/prophylaxis of opportunistic infections (see below).

3. Results

3.1. Chromatograms

This HPLC method provides a simple procedure to measure simultaneously IDV, APV, SQV, RTV, EFV and NFV in plasma in a single run with single-wavelength UV detection at 201 nm. Fig. 1a shows the chromatogram of pure standard mixture of each PI and EFV, Fig. 1b a blank plasma spiked with 100 μl of I.S. solution (clozapine), and Fig. 1c a plasma with I.S. and 3000 ng/ml of each PI and EFV. With the gradient program used (Table 1), the retention times for clozapine, IDV, APV, SQV, RTV, EFV, NFV are 8.2, 13.0, 16.3, 21.5, 26.5, 28.7 and 31.9 min, respectively. Interestingly, the gradient elution program yields sharp peaks for PIs/EFV without producing any significant drift of the baseline, even at a such low wavelength. Fig. 2 shows the plasma

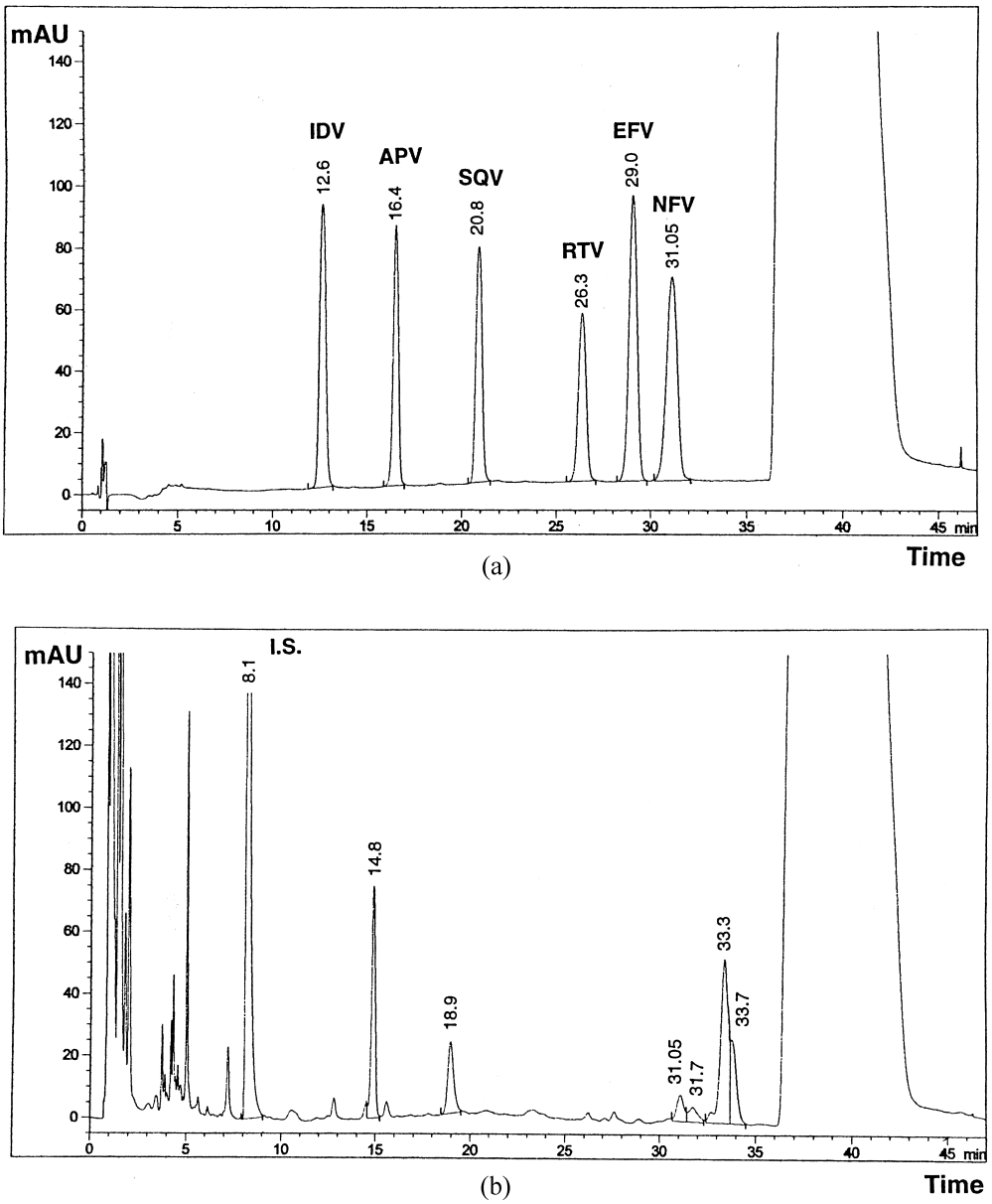

Fig. 1. (a) Chromatogram of a pure standard mixture of PIs and EFV. (b) Chromatographic profile of a blank plasma. (c) Chromatographic profile of a plasma control at 3000 ng/ml of PIs/EFV spiked with I.S.

chromatogram obtained from a HIV-patient 14 h and 12 h after efavirenz and nelfinavir intake, respectively. This patient received abacavir 300 mg bid, efavirenz 600 mg od and nelfinavir 1250 mg bid. Trough levels are 3450 and 1510 ng/ml for efavirenz and nelfinavir, respectively. Fig. 3 shows the chromatographic profile of a plasma sample from a patient receiving a regimen of stavudine 40 mg bid, lamivudine 150 mg bid, saquinavir 1000 mg bid and nelfinavir 1250 mg bid. Trough concentrations of SQV and NFV measured 10 h after dosing were 1070 and 8705 ng/ml, respectively. Fig. 4 shows the

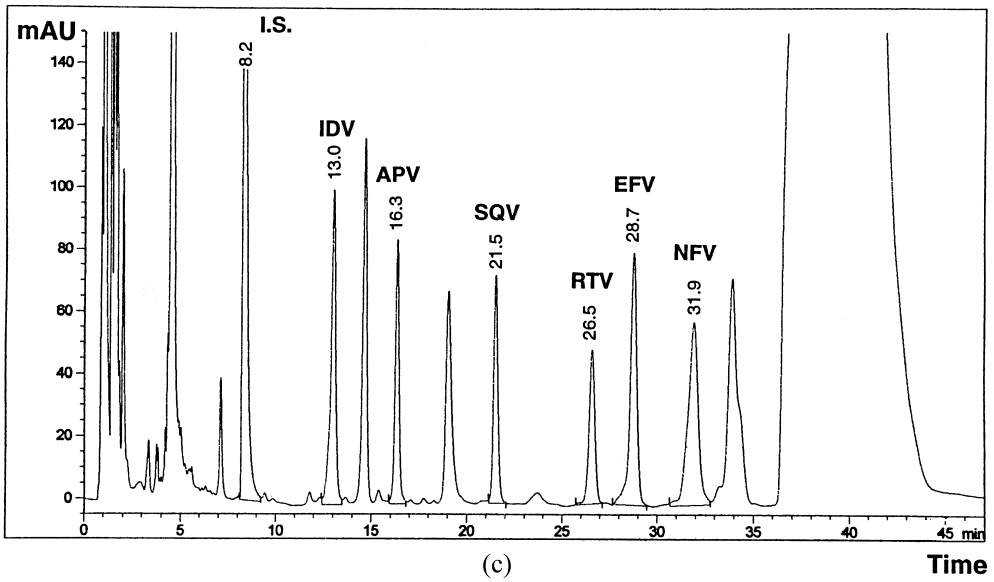

Fig. 1 (*continued*).

chromatogram of a plasma sample obtained from a patient under saquinavir 400 mg bid, ritonavir oral solution 400 mg bid, and efavirenz 600 mg od. Levels measured 13 h and 10 h after the SQV/RTV and EFV dosing were 1150, 7315 and 3150 ng/ml, respectively. Fig. 5 shows the plasma chromatogram obtained from a HIV-patient 13 h after receiving zidovudine 300 mg bid, lamivudine 150 mg bid,

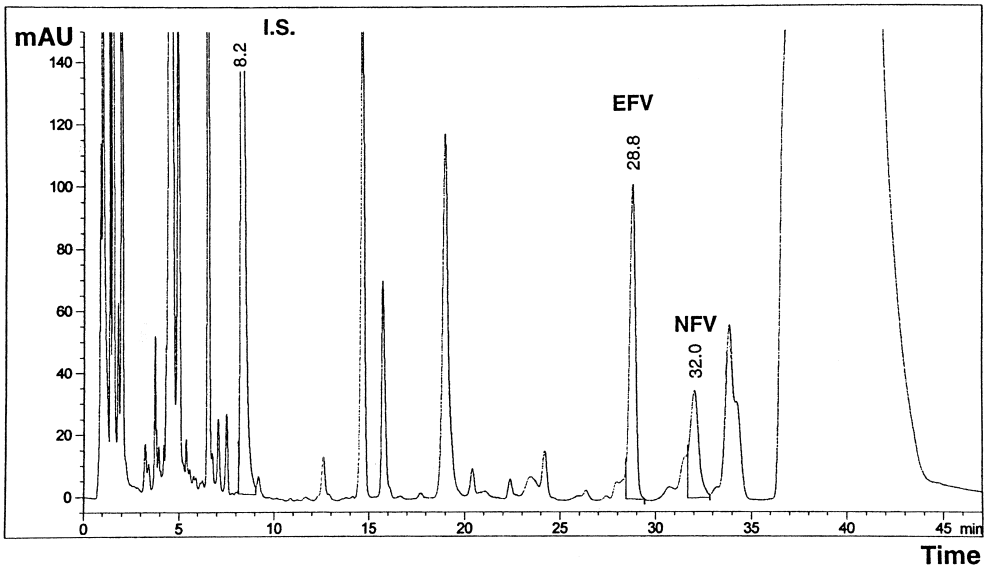

Fig. 2. Plasma chromatogram of a HIV-patient receiving abacavir, efavirenz and nelfinavir (details in the text). Trough levels of EFV and NFV were 3450 and 1510 ng/ml, respectively.

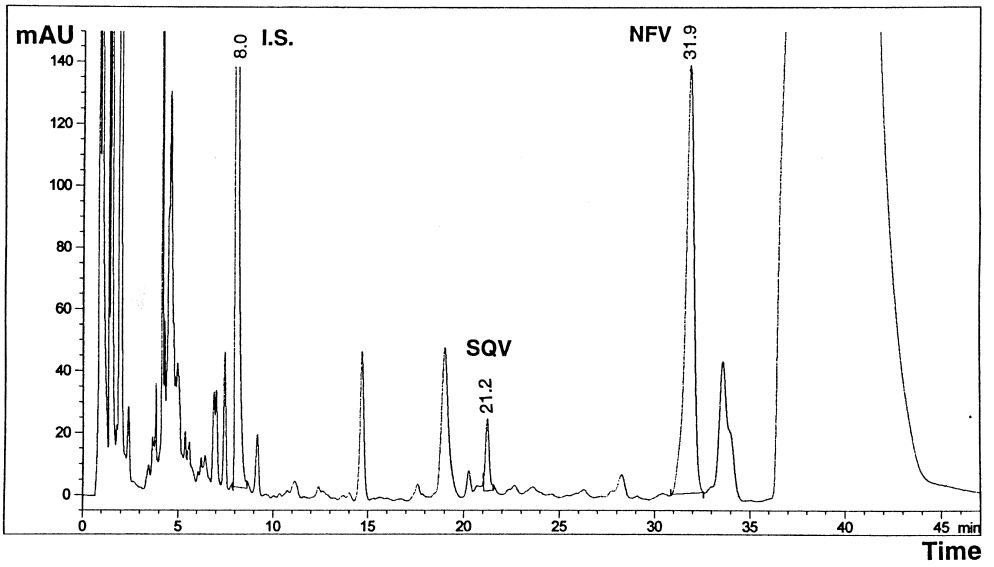

Fig. 3. Plasma chromatogram of a HIV-patient receiving stavudine, lamivudine, saquinavir and nelfinavir (explanations in the text). Trough levels of SQV and NFV were 1070 and 8705 ng/ml, respectively.

indinavir 800 mg bid and ritonavir 100 mg bid. Trough levels are 2240 and 1970 ng/ml for IDV and RTV, respectively.

3.2. HPLC solvents composition

Careful control of the pH of solution B at

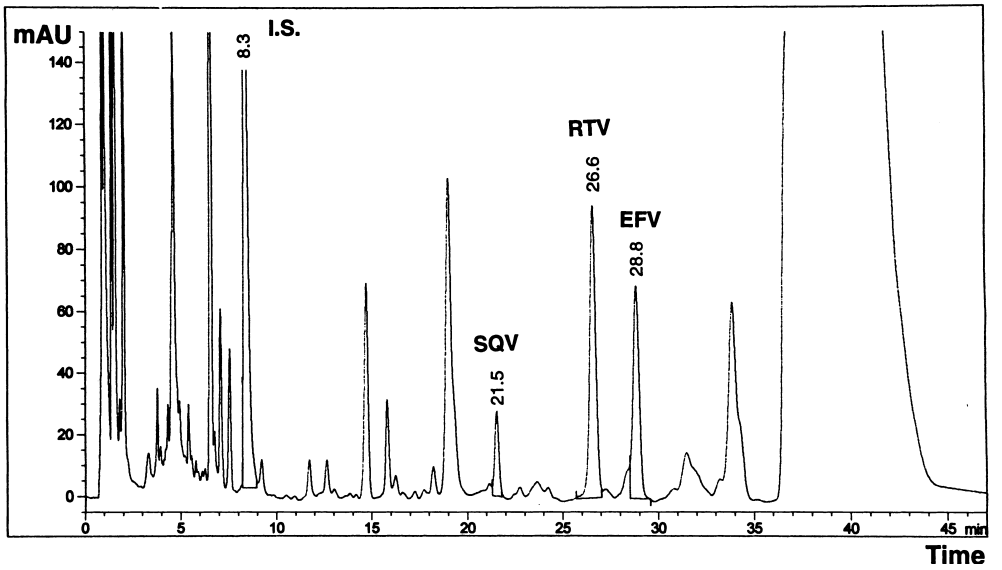

Fig. 4. Plasma chromatogram of a HIV-patient receiving saquinavir, ritonavir and efavirenz (explanations in the text). Trough levels of SQV, RTV and EFV were 1150, 7315 and 3150 ng/ml, respectively.

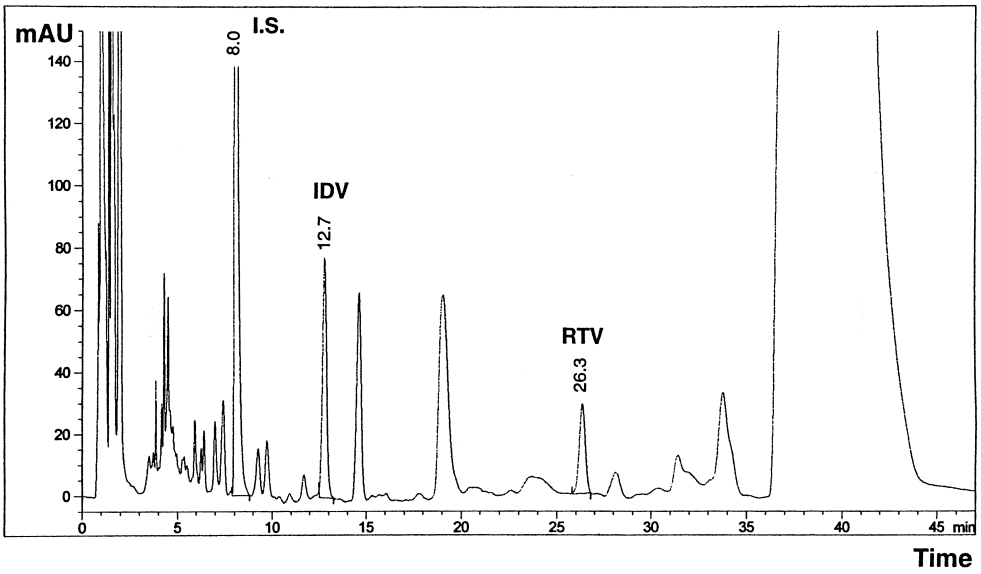

Fig. 5. Plasma chromatogram of a HIV-patient receiving indinavir and ritonavir (explanations in the text). Trough levels of IDV and RTV were 2240 and 1970 ng/ml, respectively.

5.15±0.02, of the composition and of the gradient elution program of the mobile phase is mandatory for standardizing peak shape and elution order of the various PIs and EFV, with satisfactory separation from minor nearby peaks.

A rinsing step with a mixture of solvent B–solvent C (10:90), and a 7 min re-equilibration with solvent A–solvent B (15:85), is also required for the next analysis not to be perturbed by late eluting peaks. This rinsing, between 36 and 45 min, (see gradient program conditions, Table 1), generates a large UV absorbing signal at the end of the run (see Figs. 1–5).

3.3. Calibration curves

The standard curves for IDV, APV, SQV, RTV, EFV, NFV are satisfactorily described by unweighted least-squares linear regression. The slope of the calibration curves obtained throughout method validation and initial analysis of patient samples ($n=7$) is stable, with values averaging 1.02 (±2.4%), 0.77 (±2.8%), 0.72 (±3.9%), 0.67 (±4.5%), 1.11 (±2.2%), 1.08 (±3.5%) for IDV, APV, SQV, RTV, EFV, NFV, respectively. Over the concentration range 100–10 000 ng/ml for APV and SQV, and 250–10 000 ng/ml for IDV, RTV, EFV, NFV, the regression coefficient r^2 of the calibration curves remained excellent and always greater than 0.998.

3.4. Validation of the HPLC method: precision, accuracy and LOQ

Precision and accuracy of the control samples are given in Table 2. The concentration levels of control samples of IDV, APV, SQV, RTV, EFV, NFV (750, 3000 and 9000 ng/ml) were selected to encompass the range of concentrations expected in plasma samples.

Throughout these concentration ranges, the mean intra-assay precision was always lower than 6.0, 2.9, 10.2, 6.7, 5.4, 4.5% for IDV, APV, SQV, RTV, EFV, NFV, respectively. Overall, the mean inter-day precision for PIs/EFV was similar, with mean RSDs within 2.5–9.8%. The intra-assay deviation from the nominal concentrations of PIs/EFV were always <9.2% and the range of inter-day deviation was between −4.6 and +4.3%.

By analyzing plasma form outdated transfusion bags spiked with decreasing concentrations of PIs/

Table 2
Precision and accuracy of the assay for PIs/EFV in plasma

Nominal concentration (ng/ml)	Indinavir			Amprenavir			Saquinavir			Ritonavir			Efavirenz			Nelfinavir		
	Concentration found (ng/ml)	Precision RSD (%)	Accuracy[a] deviation (%)	Concentration found (ng/ml)	Precision RSD (%)	Accuracy[a] deviation (%)	Concentration found (ng/ml)	Precision RSD (%)	Accuracy[a] deviation (%)	Concentration found (ng/ml)	Precision RSD (%)	Accuracy[a] deviation (%)	Concentration found (ng/ml)	Precision RSD (%)	Accuracy[a] deviation (%)	Concentration found (ng/ml)	Precision RSD (%)	Accuracy[a] deviation (%)
(A) Intra-assay (n=5)																		
9000	8895±175	2.0	1.1	8850±189	2.1	1.7	9175±194.5	2.1	−1.9	8995±181	2.0	0.1	8980±230	2.6	0.2	8820±121.5	1.4	2.0
3000	2985±71.5	2.4	0.6	2990±85.5	2.9	0.4	3015±61.5	2.0	−0.5	3020±43	1.4	−0.7	2970±114.5	3.9	1.0	3000±131.5	4.4	0.1
750	720±43	6.0	3.9	760±21.5	2.8	−1.6	725±74	10.2	3.2	760±51	6.7	−1.3	680±36.5	5.4	9.2	800±36	4.5	−6.5
(B) Inter-assay (n=7)																		
9000	9090±361	4.0	−1.0	9145±323	3.5	−1.6	9225±362	3.9	−2.5	9185±399	4.3	−2.0	9205±355	3.9	−2.3	9120±353	3.9	−1.3
3000	2980±149	5.0	0.6	2995±77	2.6	0.1	3065±96	3.1	−2.1	3090±76	2.5	−3.0	3050±88	2.9	−1.6	3080±100	3.2	−2.6
750	718±63	8.8	4.3	769±29	3.7	−2.5	764±29	3.8	−1.9	785±77	9.8	−4.6	750±66	8.8	0.0	748±40	5.3	0.3

[a] Found−nominal/nominal×100.

Table 3
Precision and accuracy of the assay for PIs/EFV at their LOQ[a]

	Nominal concentration at the LOQ (ng/ml)	Intra-assay		Inter-assay (n=5)	
		Precision RSD (%)	Accuracy (deviation, %)	Precision RSD (%)	Accuracy (deviation, %)
Indinavir	250	2.7	4	5.6	3.5
Amprenavir	100	3.3	7.5	4	2.8
Saquinavir	100	7.4	−5	7.2	3.8
Ritonavir	250	4	7	13.5	−0.7
Efavirenz	250	4.1	3	10	−2.9
Nelfinavir	250	1.4	3	11.3	−2.1

[a] Concentrations of the lowest calibration levels.

EFV, the LOQ was experimentally found to be 100 ng/ml for SQV and APV, and 250 ng/ml for IDV, RTV, EFV and NFV. The intra- and inter-assay precisions and accuracies (deviation between nominal and measured values) achieved for samples at these low concentrations are reported in Table 3. As clearly shown, such values are well below the recommended allowances [28] that the accuracy and precision of sample at the LOQ must not exceed ±20%. However, most of the samples collected during the implementation of the PI monitoring had concentrations values above the LOQ. Decreasing these values, though desirable, may not be necessary when considering the requirements of our study.

Table 4
Absolute recovery of PIs/EFV after SPE from plasma

	Recovery (%)		
	750 ng/ml	3000 ng/ml	9000 ng/ml
Indinavir	131	111	103
Amprenavir	101	105	101
Saquinavir	98	97	88
Ritonavir	108	101	92
Efavirenz	98	97	89
Nelfinavir	114	115	94

3.5. Recovery

The absolute recovery of PIs/EFV from plasma after SPE is shown in Table 4. The clean-up procedure by SPE was found a reliable way of eliminating interfering material from plasma, with satisfactory recovery (>88%), except for indinavir at 750 ng/ml.

3.6. Sample stability

3.6.1. Stability during thermisation (HIV inactivation)

The slope of the calibration curves of PIs/EFV in samples submitted to the thermisation procedure (60°C for 60 min) was very close to that obtained with non-heated samples, as shown in Table 5. Taking into account the experimental variability (Table 2), these results indicate that such a procedure does not affect at all, or only slightly, EFV and PI concentrations (in agreement with previous results on EFV and PI stability [16,17,23]). Nevertheless, to standardize at best the analytical method, all calibration and control samples were similarly treated and heated at 60°C for 60 min in a similar manner.

Table 5
Parameters of the calibration curves for PIs and EVF before and after plasma thermisation at 60°C for 60 min

	Indinavir			Amprenavir			Saquinavir			Ritonavir			Efavirenz			Nelfinavir		
	m	b	r^2	m	b	r^2	m	b	r^2	m	b	r^2	m	b	r^2	m	b	r^2
No thermisation	1.02	0.017	0.9998	0.744	−0.0094	0.9998	0.771	−0.0093	0.9997	0.72	−0.0047	0.9999	1.16	0.0043	0.9998	1.15	0.01	0.9999
Thermisation for 60 min at 60°C	1.04	0.023	0.9992	0.77	−0.0095	0.999	0.72	−0.0057	0.9994	0.66	0.006	0.9992	1.12	0.0094	0.9993	1.05	0.045	0.9992
Variation (%)	2			3.5			−6.6			−8.3			−3.5			−8.7		

Table 6
Stability of plasma samples left at room temperature for 24 h (concentration changes expressed in % of the starting level)

	750 ng/ml	3000 ng/ml	9000 ng/ml
Indinavir	10.0	4.3	3.6
Amprenavir	11.0	6.7	4.7
Saquinavir	10.3	7.7	4.4
Ritonavir	13.0	8.6	5.0
Efavirenz	13.7	8.9	4.8
Nelfinavir	−7.3	9.6	7.6

3.6.2. Stability of plasma samples at room temperature

The variations of PIs/EFV levels in control plasma samples left at room temperature for 24 h are shown in Table 6 showing, with the exception of nelfinavir at the low level, an apparent increase of concentration. This increase was concentration-dependent (i.e., was more marked in controls at 750 ng/ml) suggesting that it arises from evaporation, even if plasma are contained in tight propylene tubes. When shipment of plasma samples is required such as in multicentric studies, plasma samples should therefore be kept frozen or, if this is not possible, the time of transportation should be kept to a minimum to reduce the likelihood of spuriously elevated concentrations.

3.6.3. Stability of plasma samples kept frozen at −20°C

No evidence of PIs/EFV decomposition was found during plasma samples storage in the freezer at −20°C. Calibration and quality control samples are prepared in batches intended to be used up to 1 month in our routine PI monitoring facility. The concentrations of the control samples remained stable at least up to 1 month, and the slope of the calibrations curves constant, as previously stated, confirming stability studies performed up to 3 months, reported in details elsewhere [16,17,23,34].

3.6.4. Stability of plasma samples after four freeze–thaw cycles

The variations of PIs/EFV concentrations when submitting control plasma at 750, 3000 and 9000 ng/ml to successive freeze–thaw cycles are reported in Table 7. This indicates that no significant loss of PIs/EFV is to be expected after up to four freeze–thaw cycles. This may be of importance when confirmatory analysis are required.

3.6.5. Stability of extracts samples into HPLC vials (i.e., ready for HPLC analysis) at room temperature and at +4°C

The stability of plasma extracts (i.e., reconstituted in MeOH–water, 50:50) submitted to HPLC analysis was checked at room temperature for 24 h and at +4°C for 48 h. The variations over time of PIs/EFV, expressed in percentage of the starting levels, were within the −7.1 to +7.8% range, except for the somewhat higher value obtained with IDV low control in samples left at room temperature for 24 h (Table 8). Again, with this exception, the extracts samples for HPLC appear relatively stable (variation$<-13.4\%$) when stored up to 48 h in the refrigerator. These results indicate therefore that processed plasma samples stability is acceptable throughout HPLC analysis performed within 1 day.

3.6.6. Stability of extract samples into HPLC vials after freeze–thaw cycles

The variations of PIs/EFV levels in processed

Table 7
Stability of plasma samples after one and four freeze–thaw cycles (concentration change expressed in % of the nominal concentration)

	750 ng/ml		3000 ng/ml		9000 ng/ml	
	1 freeze–thaw cycle	4 freeze–thaw cycles	1 freeze–thaw cycle	4 freeze–thaw cycles	1 freeze–thaw cycle	4 freeze–thaw cycles
Indinavir	−5.3	8.7	1.1	−4.5	−0.1	−3.8
Amprenavir	0.7	11.0	−0.5	−5.6	−0.4	−3.5
Saquinavir	−4.0	0.0	1.5	−3.8	0.3	−3.1
Ritonavir	3.7	5.0	4.4	−1.6	0.9	−2.7
Efavirenz	−3.3	1.0	1.8	−4.7	−1.0	−1.6
Nelfinavir	3.3	−3.3	5.3	−7.9	−1.0	−0.9

Table 8
Stability of PIs/EFV in extracts samples left at room temperature (RT) for 24 h and at +4°C for 48 h (% change versus initial concentration)

	750 ng/ml		3000 ng/ml		9000 ng/ml	
	RT/24 h	+4°C/48 h	RT/24 h	+4°C/48 h	RT/24 h	+4°C/48 h
Indinavir	28.6	−15.1	7.8	−1.3	3.5	−0.2
Amprenavir	3.4	−0.3	0.5	−3.4	0.2	−1.1
Saquinavir	−2.0	−10.5	−2.5	−3.2	−0.8	−2.8
Ritonavir	−4.9	−10.7	−3.6	−4.7	−1.1	−3.2
Efavirenz	−2.3	−6.0	−4.9	−3.6	−3.3	−3.3
Nelfinavir	2.5	−13.4	−7.1	−8.1	−2.1	−0.2

Table 9
Stability of extracts samples ready for HPLC analyses subjected to one and two freeze–thaw cycles (change % versus initial concentration)

	750 ng/ml		3000 ng/ml		9000 ng/ml	
	1 freeze–thaw cycle	2 freeze–thaw cycles	1 freeze–thaw cycle	2 freeze–thaw cycles	1 freeze–thaw cycle	2 freeze–thaw cycles
Indinavir	−6.1	8.1	−5.3	−0.3	−3.6	−0.8
Amprenavir	0.6	4.2	−0.3	−4.4	−1.9	−0.7
Saquinavir	2.3	3.6	−3.7	−5.2	−3.1	−2.5
Ritonavir	8.2	−5.5	−3.8	−3.0	−1.7	0.1
Efavirenz	5.9	9.8	−1.8	−2.5	−1.7	−0.1
Nelfinavir	−14.9	−14.2	−4.6	0.4	−0.9	2.1

samples (i.e., reconstituted in MeOH–water, 50:50) containing PIs/EFV at 750, 3000 and 9000 ng/ml are shown in Table 9. Except the somewhat larger decay of NFV at 750 ng/ml, the extracts were not notably affected by at least two freeze–thaw cycles. From a practical point of view, this indicates that plasma samples may be processed (i.e., subjected to SPE) and reconstituted simultaneously in batches, before being stored as extract samples in HPLC vials at −20°C, and thawed on the day of analysis.

3.7. Selectivity

No significant interfering peaks were observed at the retention time of clozapine, APV, SQV, RTV and EFV. In a few samples however, a baseline sepa-

Table 10
Retention time of drugs commonly prescribed to HIV-infected patients

Drug (DCI)	Registered mark in Switzerland	Retention time (min)
Zidovudine/lamivudine	Combivir	0.8 and 1.2
Pancreatine	Créon	0.8
Calcium folinate	Leucovorin	0.9
Sulfadiazine	Sulfadiazine	1.1
Didanosine	Videx	1.2
Stavudine	Zérit	1.2
Nevirapine	Viramune	3.4
Trimethoprim/sulfamethoxazole	Bactrim	4.4 and 5.0
Clindamycine	Dalacin	5.1
Pyrimethamine	Daraprim	6.1
Atorvastatine	Sortis	18.9
Loperamide	Imodium	18.9

ration between NFV (retention time=31.9 min) and two minor peaks at 31.05 and 31.7 min present in blank plasma (see Fig. 1b) could not always be achieved, without precluding however an acceptable determination of NFV levels. In addition, a very small endogenous peak eluting nearby IDV (retention time=12.9 min) could affect the precise determination of trough level in a few cases, while having no significant influence on IDV peak levels.

The method selectivity was confirmed by analyzing all the currently prescribed anti-HIV drugs – part notably of various potent antiretroviral therapy regimens – and/or employed in the treatment/prophylaxis of opportunistic infections in the studied HIV+ patients cohort. The retention time of each individual compound are listed in Table 10. Most drugs are eluted early and do not perturb the analyses. Loperamide and atorvastatine have a retention time of 18.9 min, which is however unlikely to interfere with any of the considered PIs/EFV.

4. Discussion and conclusion

This optimized HPLC method provides a fairly simple procedure for determining simultaneously in a single run the five currently used PIs and efavirenz in plasma from HIV-infected patients. Sodium heptanesulfonate was chosen as ion-pairing agent to retain the five PIs on the reversed-phase column long enough to achieve an efficient separation. As expected with charged molecules, careful control of pH (5.15 ± 0.02) of solvent B of the mobile phase was crucial for obtaining reproducible retention times for PIs. The pH control had also a profound influence on the separation efficiency of nelfivavir from small nearby peaks at 31.1, 31.7 and 33.3 min (see Fig. 1b).

After each HPLC run, rinsing the column for 1 min with a mixture of solvent B–acetonitrile (1:9), with the addition of 0.3% AcOH, enabled an adequate cleaning of adsorbed contaminants and guaranteed a stable baseline UV signal at 201 nm for the next run.

The previously proposed viral inactivation by Triton X-100 [30,31] was found unsatisfactory in our hands. This detergent added to samples strongly perturbed UV detection at 201 nm even after SPE and influenced the peak shape of PIs. Plasma thermisation at 60°C for 60 min for the inactivation of HIV [24–27] was therefore chosen. Thermised plasma samples have to be diluted with buffered solution D and to be centrifuged at 20 000 *g* for precisely 5 min before being applied onto the SPE cartridge, to prevent the clogging of the cartridge column.

Both precision and accuracy of the method were always $<\pm15\%$, in accordance with published recommendations [28].

When considering the lower LOQ achieved for SQV and APV, the 750 ng/ml concentration for the low level quality control samples of these two PIs could be slightly reduced. This concentration however did not depart much from those actually encountered in most of the samples collected in our study which were in the 400–1000 ng/ml range for SQV.

The LOQ achieved with this method (100 ng/ml for APV, SQV and 250 ng/ml for IDV, RTV, EFV, NFV with a 40-μl injection volume), are somewhat higher than the values reported by other groups with LOQ values as low as 25 ng/ml and 50 ng/ml for IDV, APV, SQV and NFV, RTV, respectively [16]. Loading a larger amount of samples may enable to increase the sensitivity of our assay. As mentioned, the achieved precision and accuracy of our assay at the reported LOQ values are actually well below the $\pm20\%$ recommended allowances [28], and we may have been too conservative in this case. We were however concerned that lowering these limits could increase the risks of spurious results, considering the complexity of samples from such a heavily treated population of patients (see Figs. 2–5). Such a high sensitivity is furthermore not required for the routine analysis of most trough and all peak levels of PIs/EFV in treated patients. We found that the chosen range of concentration of 100–10 000 ng/ml encompasses almost all concentrations of PIs/EFV encountered so far in the studied cohort. In some cases however, unexpectedly high peak or trough levels of NLV or RTV were observed, requiring samples dilution. Conversely, very low trough levels of IDV (probably due to its rapid elimination) in a few patients could not always be quantitated with enough precision.

The occurrence of PIs metabolites, notably for nelfinavir [32] and for ritonavir [33], have been reported. There is some concern that they could interfere with the assay. A number of patients in our

cohort receive only one PI in their triple therapy regimen. Chromatographic profiles of plasma from these patients do not show additional signal peaks eluted at retention times corresponding to the other parent PIs, indicating that PI metabolites, if any, should not reciprocally interfere with the assay of other PIs taken simultaneously.

The HPLC run may be considered lengthy (more than 45 min for the analysis of the six drugs), but since plasma extract samples are stable at room temperature in the autosampler rack, the assay can be fully automated and requires no tedious technical supervision. In our studies, batches of samples have been analyzed in a row over 24 h without any problems. Using one devoted HPLC apparatus and a technician employed at half time, it is possible to analyze approximately 30 samples per day in our laboratory.

The proposed method, relatively time consuming, has been developed using instruments usually available in conventional hospital laboratories. Its performance could certainly be enhanced by HPLC–MS. The specificity achieved with the UV–DAD system could be enhanced by specific ion monitoring of each individual drug, resulting in a shortening of the gradient program used.

This assay is currently applied to the monitoring of the five PIs and efavirenz in HIV patients under triple therapy. Combination therapy is complicated by interactions frequently involving the inhibition of metabolism. Coadministration of usual doses of two protease inhibitors may increase the plasma levels of one of the agents by two- to eight-fold, and even up to 20-fold for saquinavir with ritonavir. With interactions of such magnitude, there is a formal interest in the individualization of dosage, ideally through a formal therapeutic drug monitoring [34]. Evaluation of adherence to treatment is also of value to assure long-term efficacy of antiretroviral regimens. Our method, enabling such a concentration-oriented approach, represents a useful complement to the current monitoring of anti-HIV treatment based on CD4 count and viremia.

5. Note added in proof

During the routine assay of plasma from patients treated with efavirenz, an additional peak with UV–DAD features similar to efavirenz was observed at 16.0 min, at a retention time very close to that of amprenavir (16.3 min). This peak, which was separated from that of amprenavir, arises presumably from an as yet unidentified metabolite of efavirenz. However, the chromatograms of samples from patients receiving amprenavir in association with efavirenz should be cautiously examined, especially at low amprenavir levels, to avoid confounding it with efavirenz metabolite.

Furthermore, the peak appearing at 20.5 min in samples from patients receiving nelfinavir corresponds to a metabolite of nelfinavir (though not yet identified) but does not interfere with the dosage of any other measured drugs.

Acknowledgements

The Fonds OFSP/Programme National sur le SIDA 97-7339 provided initial support for this research project. Thanks to T. Moyer, Mayo Clinic, for initial input into the project. We are indebted to Natacha Bélaz for technical assistance during the routine implementation of the assay.

References

[1] D.V. Havlir, J.M.A. Lange, AIDS 12 (Suppl. A) (1998) S165–S174, and references cited therein.
[2] E.P. Acosta, Medscape HIV/AIDS 5 (4) (1999).
[3] J.C. Adkins, S. Noble, Drugs 56 (1998) 1055–1064, and references cited therein.
[4] B.G. Gazzard, Int. J. Clin. Pract. 53 (1999) 60–64, and references cited therein.
[5] T. Woolf, H. Haddix, B. Matuszewski, J. Chromatogr. B 692 (1995) 45–52.
[6] D.M. Burger, M. de Graaff, E.W. Wuis, P.P. Koopmans, Y.A. Hekster, J. Chromatogr. B 703 (1997) 235–241.
[7] A.L. Jayewardene, F. Zhu, F.T. Aweeka, J.G. Gambertoglio, J. Chromatogr. B 707 (1998) 203–211.
[8] I. Fu, E.J. Woolf, B.K. Matuszewski, J. Pharm. Biomed. Anal. 18 (1998) 347–357.
[9] E.J. Woolf, B.K. Matuszewski, J. Pharm. Sci. 86 (1997) 193–198.
[10] K.C. Marsh, E. Eiden, E. McDonald, J. Chromatogr. B 704 (1997) 307–313.
[11] R. Hoetelmans, M. van Essenberg, M. Profijt, P.L. Meenhorst, J.W. Mulder, J.H. Beijnen, J. Chromatogr. B 705 (1998) 119–126.

[12] H.R. Ha, F. Follath, Y. Bloemhard, S. Krähenbühl, J. Chromatogr. B 694 (1997) 427–433.
[13] R. Hoetelmans, M. van Essenberg, P.L. Meenhorst, J.W. Mulder, J.H. Beijnen, J. Chromatogr. B 698 (1997) 235–241.
[14] E.Y. Wu, J.M. Wilkinson, D.G. Naret, V.L. Daniels, L.J. Williams, D.A. Khalil, B.V. Shetty, J. Chromatogr. B 695 (1997) 373–380.
[15] T.P. Moyer, L.E. Ebnet, J.B. Pruett, in: Toxicology-Methods-Procedure: Delavirdine, Indinavir, Ritonavir, Saquinavir and Nelfinavir in Plasma High Performance Liquid Chromatography, Mayo Clinic, unpublished.
[16] R.P. van Heeswijk, R. Hoetelmans, R. Harms, P.L. Meenhorst, J.W. Mulder, J.M. Lange, J.H. Beijnen, J. Chromatogr. B 719 (1998) 159–168.
[17] P.W.H. Hugen, C.P.W.G.M. Verweij-van Wissen, D.M. Burger, E.W. Wuis, P.P. Koopmans, Y.A. Hekster, J. Chromatogr. B 727 (1999) 139–149.
[18] I. Utkin, S. Cox, N. Gerber, P.D. Watson, L. Crim, M. Brady, Ther. Drug Monit. 21 (1999) 456, Abstract 114.
[19] K. Gallicano, G. Carignan, L. Oliveras, Y. Khaliq, B. Foster, Ther. Drug Monit. 21 (1999) 453, Abstract 100.
[20] S. Frappier, D. Breilh, E. Diarte, B. Ba, D. Ducint, J.L. Pellegrin, M.C. Saux, J. Chromatogr. B 714 (1999) 384–389.
[21] P. Villani, M. Pregnolato, S. Banfo, M. Rettani, D. Burroni, E. Seminari, R. Maserati, M. Regazzi, Ther. Drug Monit. 21 (1999) 346–350.
[22] M.B. Regazzi, P. Villani, A. Bartoli, R. Maserati, E. Seminari, A. Pan, S. Locaputo, Ther. Drug Monit. 21 (1999) 453, Abstract 100.
[23] A.I. Veldkamp, R.P.G. van Heeswijk, P.L. Meenhorst, J.W. Mulder, J.M.A. Lange, J.H. Beijnen, R.M.W. Hoetelmans, J. Chromatogr. B 734 (1999) 55–61.
[24] J.S. McDougal, L.S. Martin, S.P. Cort, M. Mozen, C.M. Heldebrant, B.L. Evatt, J. Clin. Invest. 76 (1985) 875–877.
[25] S.S. Good, D.J. Reynolds, P. De Miranda, J. Chromatogr. B 431 (1988) 123–133.
[26] G.V. Quinnan Jr., M.A. Wells, A.E. Wittek, M.A. Phelan, R.E. Mayner, S. Feinstone, R.H. Purcell, J. S Epstein, Transfusion 26 (1986) 481–483.
[27] C.H.W. Koks, H. Rosing, P.L. Meenhorst, A. Bult, J.H. Beijnen, J. Chromatogr. B 663 (1995) 345–351.
[28] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman, S. Spector, Eur. J. Drug Metab. Pharmacokin. 16 (1991) 249–255.
[29] H.T. Karnes, G. Shiu, V.P. Shah, Pharm. Res. 8 (1991) 421–426.
[30] J.S. Roth, H. Ford, M. Tanaka, H. Mitsuya, J.A. Kelley, J. Chromatogr. B 712 (1998) 199–210.
[31] E.L. White, B.J. Bowdon, W.M. Shannon, J. Virol. Methods 70 (1998) 113–115.
[32] J.F. Delfraissy, in: Prise en Charge Thérapeutique des Personnes Infectées Par Le VIH, Recommandations du Groupe d'Experts, Médecine-Sciences, Flammarion, 1999, p. 112.
[33] K.C. Marsh, E. Eiden, E. McDonald, J. Chromatogr. B 704 (1999) 307–313.
[34] T.P. Moyer, Z. Temesgen, R. Enger, L. Estes, J. Charlson, L. Olivier, A. Wright, Clin. Chem. 45 (1999) 1465–1467.