ELSEVIER

Journal of Chromatography B, 774 (2002) 127–140

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Determination of lopinavir and nevirapine by high-performance liquid chromatography after solid-phase extraction: application for the assessment of their transplacental passage at delivery

C. Marzolini[a], A. Béguin[a], A. Telenti[b], A. Schreyer[c], T. Buclin[a], J. Biollaz[a], L.A. Decosterd[a,*]

[a]*Division de Pharmacologie Clinique, Laboratoire BH 18-218, Département de Médecine, Centre Hospitalier Universitaire Vaudois, 1011 Lausanne CHUV, Switzerland*

[b]*Division des Maladies Infectieuses, Médecine 2, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland*

[c]*Division de Gynécologie et d'Obstétrique, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland*

Received 9 November 2001; received in revised form 7 March 2002; accepted 7 March 2002

Abstract

An adaptation of the HPLC method previously described for the simultaneous assay of amprenavir, ritonavir, indinavir, saquinavir, nelfinavir and efavirenz after solid-phase extraction is proposed here for the separate analysis of the newer PI lopinavir (LPV) and the NNRTI nevirapine (NVP). 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. LPV and NVP are eluted with 3×500 μl MeOH. The resulting eluate is evaporated under nitrogen at room temperature and is reconstituted in 100 μl MeOH 50%. A 40-μl volume is injected onto a Nucleosil 100, 5 μm C_{18} AB column. LPV and NVP are analysed separately using a gradient elution program with solvents constituted of MeCN and phosphate buffer adjusted to pH 5.07 and containing 0.02% sodium heptanesulfonate. LPV and NVP are detected by UV at 201 and 282 nm, respectively. The calibration curves are linear up to 10 μg/ml. The mean absolute recovery of LPV and NVP is 91% and 88%, respectively. The method is precise with mean inter-day C.V.s within 2.1–6.6% and 0.9–1.7% for LPV and NVP, and accurate (range of inter-day deviations −1.1 to +2.4%, and −1.9 to +0.8%, for LPV and NVP, respectively). The method has been validated and is currently applied to the monitoring of LPV and NVP in HIV patients, and has been notably applied in a study aimed at assessing the extent of transplacental passage of nevirapine and PIs, notably lopinavir, at the time of delivery in pregnant HIV-infected women.

Keywords: Lopinavir; Nevirapine

1. Introduction

The therapeutic drug monitoring (TDM) of HIV protease inhibitors (PIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) is increasingly recognised as useful for the optimal clinical manage-

*Corresponding author. Tel.: +41-21-314-4272; fax: +41-21-314-4288.

E-mail address: laurentarthur.decosterd@chuv.hospvd.ch (L.A. Decosterd).

1570-0232/02/$ – see front matter
PII: S1570-0232(02)00169-1

ment of HIV-positive patients. This is particularly the case for treatment failure, drug-related toxicity [1], drug–drug interactions, compliance assessment and evaluation of drug exposure [2]. In association with the assessment of HIV resistance virtual phenotyping [3], the TDM of PIs and NNRTIs may optimise the follow-up of HIV-patients. To that endeavour, a high-performance liquid chromatography method has been recently proposed to quantify simultaneously in plasma five HIV protease inhibitors (PIs) (i.e. indinavir, amprenavir, saquinavir, ritonavir, nelfinavir) and the non-nucleoside reverse transcriptase inhibitor efavirenz [4]. The appearance of resistant HIV strains continuously necessitates alternative regimens. A newer PI, lopinavir [5,20] has been recently marketed, and multiple combinations of HIV drugs include nevirapine.

Since pharmacokinetic–pharmacodynamic relationships were demonstrated for nevirapine [6], several high-performance liquid chromatography methods have been reported for separately quantifying nevirapine in plasma [7–13] or saliva [14] as well as its metabolites [15–17], or nevirapine together with PIs and NNRTI [18,19] in human biological fluids.

Lopinavir (formerly known as ABT-378) is a novel peptidomimetic HIV protease inhibitor with ~10-fold greater in vitro potency compared to ritonavir [20]. Lopinavir is essentially metabolized by the CYP450 3A [21], so that it is given under the form of Kaletra®, in combination with a small dose (33 mg) of ritonavir used as pharmacokinetic enhancer. Ritonavir inhibits lopinavir first pass and hepatic metabolism, thereby increasing lopinavir circulating levels. One unpublished report was issued for the quantitation of lopinavir with ritonavir in biological samples by HPLC, using either UV or tandem mass spectrometry detection, after liquid–liquid extraction at alkaline pH with hexane–AcOEt [22]. To the best of our knowledge, only one report has recently been published describing a HPLC method for the assay of lopinavir, together with other PIs, after liquid–liquid extraction from human plasma [23].

We report here a method for the separate analysis of lopinavir and nevirapine in biological fluids by solid-phase extraction, using an adaptation of the methodology proposed for the five first marketed PIs and efavirenz [4]. This method is currently used as a part of routine TDM of antiretrovirals for HIV-positive patients [25], and has also been applied in a study aimed at assessing the extent of transplacental passage of nevirapine and PIs, among others lopinavir, at delivery in pregnant HIV-1 infected women [24].

2. Materials and methods

2.1. Chemicals

Nevirapine (NVP, lot # RM-1439) and lopinavir (LPV, ABT-378, lot # 638634-AX) pure substances were kindly provided by Boehringer Ingelheim (Ridgefield, CT, USA) and Abbott AG (Baar, Switzerland), respectively. Clozapine (internal standard, I.S.) stock solution (250 μg/ml) in MeOH was obtained by extraction with MeOH of Leponex® tablet. This solution was diluted down to 45 μg/ml before use. Acetonitrile (MeCN) for chromatography was from J.T. Baker (Deventer, The Netherlands). Methanol (MeOH) for chromatography LiChrosolv®, 100% acetic acid (AcOH) and 85% phosphoric acid (H_3PO_4) were from E. Merck (Darmstadt, Germany). Sodium hydroxide puriss. p.a. pellets and sodium heptanesulfonate p.a. 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 1050 (Agilent, formerly Hewlett-Packard, Germany) equipped with a spectrophotometric UV–Vis variable wavelength detector set at 282 and 201 nm, for nevirapine and lopinavir, 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 1a and b, for LPV and NVP, respectively.

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 H_3PO_4 8.5% and 0.2 g sodium heptanesulfonate to 988.2 ml H_2O with pH carefully adjusted to 5.07±0.03 with NaOH 10 N. The solvent C (used for lopinavir) 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 for (a) nevirapine and (b) lopinavir

Time (min)	Solvent A (%)	Solvent B (%)	Solvent C (%)
(a) Nevirapine			
0	5	95	
11	14.7	85.3	
20	14.7	85.3	
30	45	55	
31[a]	100	0	
35[a]	100	0	
36[a]	5	95	
42[a]	5	95	
(b) Lopinavir			
0	15	85	–
2	30	70	–
8	32	68	–
18	42	58	–
20	46	54	–
34	46	54	
35[a]	33	34	33
36[a]	–	10	90
37[a]	–	10	90
38[a]	33	34	33
39[a]	46	54	–
40[a]	15	85	–
47[a]	15	85	–

[a] Rinsing/re-equilibration program.

2.4. Buffer solutions for sample processing

The buffer used for the dilution of plasma samples was prepared by mixing 413 ml of KH_2PO_4 1/15 *M* (9.07 g/l) and 587 ml of $Na_2HPO_4.2H_2O$ 1/15 *M* (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 solution of lopinavir base was prepared at a concentration of 1 mg/ml and was appropriately diluted with MeOH–H_2O 50:50 for the preparation of working solutions at concentrations of 1–100 μg/ml. Stock solution of nevirapine base was prepared at a concentration of 400 μg/ml in MeOH–H_2O 50:50 and further diluted for the preparation of the working solution of 20–200 μg/ml. Plasma calibration samples at 0.1, 0.25, 1.0, 5.0, 8.0, 10.0 μg/ml for LPV, and 0.5, 1.0, 3.0, 5.0 and 10.0 μg/ml for NVP together with plasma control samples at 0.75, 3.0, 9.0 μg/ml and 0.7, 2.0 and 8.0 μg/ml, for LPV and NVP, respectively, 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 [26].

Of note, the proposed method for the assay of lopinavir may also be used for analysing simultaneously amprenavir and efavirenz in the same HPLC run. In this case, calibration samples should accordingly be prepared by adding amprenavir and efavirenz to plasma at the concentrations ranging from 0.1 μg/ml to 10.0 μg/ml, in addition to lopinavir. The HPLC analysis of amprenavir and efavirenz, not discussed here, has been previously reported [4].

The calibration standards and control samples were thermised at 60 °C for 60 min (see below), stored at −20 °C as 1.5-ml aliquots in polypropylene Eppendorf tubes, and thawed on the day of analysis.

2.6. Sample collection and thermisation (HIV inactivation)

According to a study protocol previously approved by the local Ethics Committee, blood samples were taken from HIV patients during their regular visit (approximately every 3 months) at the University Hospital of Lausanne.

Blood samples (5 ml) were collected in Monovettes® (Sarstedt, Nümbrecht, Germany) with K-EDTA or Li heparinate as anticoagulant. Samples were immediately taken to the laboratory and were processed under a protection hood wearing nitrile gloves and long-sleeve lab coats. The Monovettes were centrifuged at 1850 *g* (3000 rev./min) 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 [27–30]. The stability of lopinavir and nevirapine under these conditions is reported in the method validation (see below). The processed plasma samples 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 room temperature (RT).

The sample preparation procedure is identical for samples containing either nevirapine or lopinavir (with or without amprenavir/efavirenz).

Six hundred microlitres of plasma (calibration, control, patients) samples were 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 20,000 *g* (14,000 rev./min) (Hettich® Benchtop Universal 16R centrifuge, Bäch, Switzerland).

The clean-up procedure of diluted plasma samples was performed by solid-phase extraction (SPE) using a 24-tube vacuum manifold Macherey-Nagel (Düren, Germany). 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).

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 ~30 min and the residue reconstituted in 100 μl of MeOH–H_2O 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, Germany) and a volume of 40 μl was used for HPLC analysis.

2.8. Calibration curves

Quantitative analysis of lopinavir and nevirapine was performed using the internal standard method.

The calibration curves were obtained by unweighted least-squares linear regression of the peak-area ratio of LPV and NVP to clozapine (I.S.) versus the ratio of the injected amount of the respective LPV and NVP to I.S., in each standard sample. The calibration was established over the range 0.5–10.0 μg/ml for NVP, and 0.1–10.0 μg/ml for LPV (and APV and EFV if applicable), 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 [26].

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 concentration levels (low, medium and high; i.e. 0.75, 3.0 and 9.0 μg/ml, and 0.7, 2.0 and 8.0 μg/ml for LPV and NVP, respectively) were assayed every five samples.

Quality control samples were used for the preci-

sion and accuracy determination, the precision being calculated as the coefficient of variation (C.V.%) within a single run (intra-assay) and between different assays (inter-assays), and the accuracy as the percentage of deviation between nominal and measured concentration.

The limit of quantitation (LOQ) for LPV and NVP 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 [26] recommending that the deviation between measured and nominal concentration should not deviate more than ±20%.

2.10. Stability of LPV and NVP

The stability of the drugs (LPV and NVP) 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 (0.1 up to 10.0 μg/ml and 0.5 up to 10.0 μg/ml for LPV and NVP, respectively) 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 solid-phase extraction 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) the stability of plasma samples after multiple freeze–thaw cycles: aliquots of plasma spiked with 0.75, 3.0 and 9.0 μg/ml, and 0.7, 2.0 and 8.0 μg/ml of LPV and NVP, respectively, underwent two freeze–thaw cycles: frozen samples were allowed to thaw at ambient temperature for 2 h and were subsequently refrozen. The LPV/NVP levels were measured in aliquots after each of the two freeze–thaw cycles. The variations of LPV/NVP concentrations were expressed in percentage of the starting levels measured immediately.
(b) Stability of plasma extracts into HPLC vials: processed samples (i.e. reconstituted in MeOH 50%) containing LPV or NVP at low, medium and high concentration were analyzed in duplicate immediately after preparation, after being left 24 h at room temperature—in the autosampler rack—or kept refrigerated at +4 °C for 24 h.

2.11. Recovery

The efficiency of the solid-phase extraction was determined with control samples at 0.75, 3.0 and 9.0, and 0.7, 2.0 and 8.0 μg/ml for LPV and NVP, respectively. The absolute recovery of LPV and NVP 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 of both analytical methods was determined by injecting onto the HPLC column all currently prescribed anti-HIV drugs and/or employed in the treatment/prophylaxis of opportunistic infections, which included: stavudine (Zerit®), zidovudine/lamivudine (Combivir®), didanosine (Videx®), calcium folinate (Leucovorin®), atorvastatine (Sortis®), pancreatine (Créon®), sulfadiazine (Sulfadiazine®), loperamide (Imodium®), trimethoprim/sulfamethoxazole (Bactrim®), pyrimethamine (Daraprim®), azithromycine (Zithromax®), clindamycine (Dalacin®), abacavir (Ziagen®), lopinavir/r (Kaletra®), indinavir (Crixivan®), amprenavir (Agenerase®), saquinavir (Invirase®, Fortovase®), ritonavir (Norvir®), nelfinavir (Viracept®) and efavirenz (Stocrin®, Sustiva®).

2.13. Applications

This method is currently used as a part of our routine TDM of lopinavir [25] and nevirapine for HIV-positive patients, in addition to the previously reported assay for the other PIs/NNRTI [4]. It has also been more specifically applied in a study, reported in detail elsewhere [24], aimed at assessing the extent of transplacental passage of nevirapine and PIs, notably lopinavir, at delivery in pregnant HIV-1 infected women. In brief, pregnant women from the

Swiss Mother+Child HIV Cohort Study treated with antiretrovirals including PIs and/or nevirapine were eligible for the study which was approved by the local Ethics Committee. Placental transfer was determined by comparison of drug concentrations in blood samples collected simultaneously from a peripheral maternal vein and the umbilical cord.

Blood samples (10 ml) were collected into lithium heparin Monovettes (Sarstedt), drawn from both the mother within 15 min of delivery and from umbilical cord (artery or vein) after cord section at delivery. Plasma was isolated by centrifugation, viro-inactivated in a water-bath at 60 °C for 60 min and stored at −20 °C prior to the HPLC analysis.

3. Results

3.1. Chromatograms

The proposed HPLC method enables the measurement of LPV in plasma with UV detection at 201 nm. With the gradient program used for the analysis of LPV (Table 1b), the retention times for clozapine and LPV are 7.0 and 24.9 min, respectively (Fig. 1b). The gradient elution program for LPV yields sharp peaks without producing any significant drift of the baseline, even at such a low wavelength. Interestingly, the gradient program applied for the assay of LPV, is also suitable for the simultaneous quantitation of APV and efavirenz which are eluted at 15.1 and 25.7 min, respectively (Fig. 1c). The detailed validation for the assay for APV and EFV has already been previously reported [4].

Fig. 1a shows the chromatogram of a blank plasma, using the gradient program for LPV reported in Table 1b. Fig. 1b shows the chromatographic profile of a plasma (onto which the internal standard has been added) obtained from a patient #931 receiving a regimen of Videx 200 mg bid, nevirapine 200 mg bid, and Kaletra® (LPV 400 mg +ritonavir 100 mg) bid. The level of LPV measured 7 h 50 min after the Kaletra® intake is 4.99 μg/ml. The quantification of ritonavir, solely used as a pharmacokinetic enhancer, is not considered here.

Fig. 1c shows the plasma chromatogram obtained from a HIV-patient #861 under a regimen of Kaletra® (lopinavir 533 mg+ritonavir 133 mg) bid, Agenerase® (amprenavir) 750 mg bid, and Stocrin® (efavirenz) 600 mg qd. Levels measured 2 h 50 and 14 h 50 min after LPV/APV and efavirenz intake, respectively, are 8.52, 1.34 and 2.26 μg/ml, respectively. Of note, the peak which is eluted at 14.5 min just prior to amprenavir arises presumably from an as yet unidentified metabolite of efavirenz.

By using the same column type and solvent mixture, but with the gradient program given in Table 1a, NVP can be assayed in plasma at 282 nm with a high sensitivity and selectivity. Such a selectivity is of importance because nevirapine is more polar and tends to be eluted at a time close to numerous potential co-administered drugs (notably antibiotics) which may perturb the analysis at this wavelength (see infra). Fig. 2a shows the chromatogram of a blank plasma using the gradient program in Table 1a. NVP and clozapine are eluted with this more polar gradient elution at 16.0±0.4 min and 29.0±0.4 min, respectively, as shown in Fig. 2b. Fig. 2b represents the chromatographic profile of the same plasma obtained from the patient #931 (previously assayed for LPV in Fig. 1b) who has a nevirapine plasma level of 4.64 μg/ml, 7 h 50 min after his nevirapine 200 mg dose.

As part of our study on the transplacental passage of antiretrovirals at delivery, Figs. 3a and 4a show the chromatographic profiles of a plasma sample taken 5 min prior to delivery from a pregnant woman receiving a regimen of 3TC® 150 mg bid, Ziagen® 300 mg bid, Kaletra® (533 mg+133 mg ritonavir) bid and Viramune® (nevirapine) 200 mg bid. The plasma concentrations of LPV and NVP in the mother at delivery, 12 h 15 min after drug intake, are 3.11 and 2.66 μg/ml, respectively. Figs. 3b and 4b show the chromatograms of the corresponding umbilical cord sample taken after cord section at delivery. In this cord sample, the level of nevirapine is 2.16 μg/ml, while the concentration of lopinavir is below the quantitation limit of the assay.

3.2. HPLC solvent compositions

Careful control of the pH of the solution B at 5.07±0.03, of the composition and gradient elution program of the mobile phase is mandatory for standardizing the peak shape and retention time of LPV, and obtaining satisfactory separation from

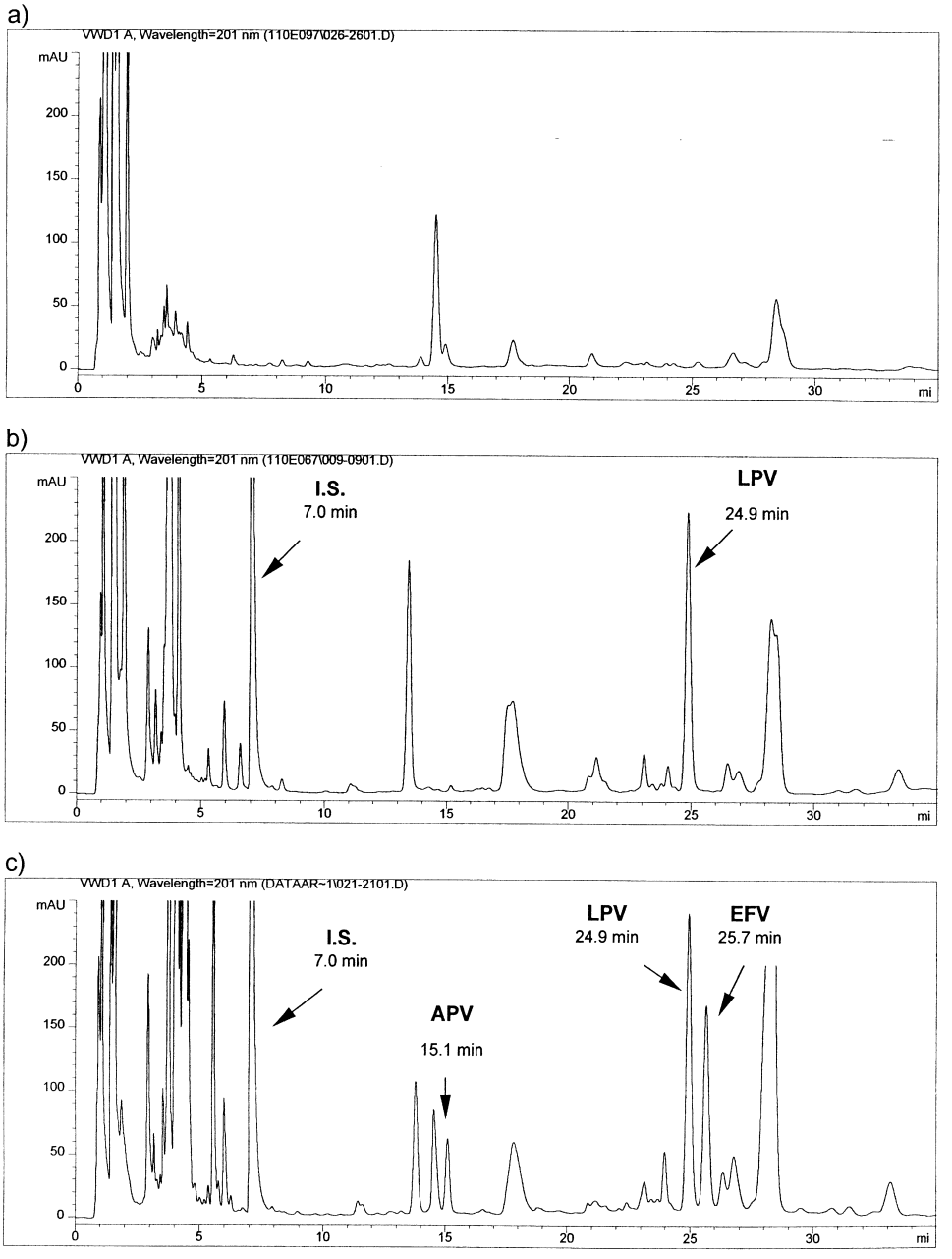

Fig. 1. (a) Chromatographic profile of a blank plasma, using the gradient elution program for LPV (Table 1a). (b) Plasma chromatogram of a HIV-patient receiving didanosine, nevirapine and Kaletra® (LPV/RTV) (details in the text). Level of LPV was 4.99 μg/ml 7 h 50 min after Kaletra® dose intake. (c) Plasma chromatogram of a HIV-patient receiving Kaletra® LPV/RTV), amprenavir (APV) and efavirenz (EFV) (explanations in the text). Levels measured 2 h 50 min and 14 h 50 min after LPV/APV and EFV intake were 8.52, 1.34 and 2.26 μg/ml, respectively.

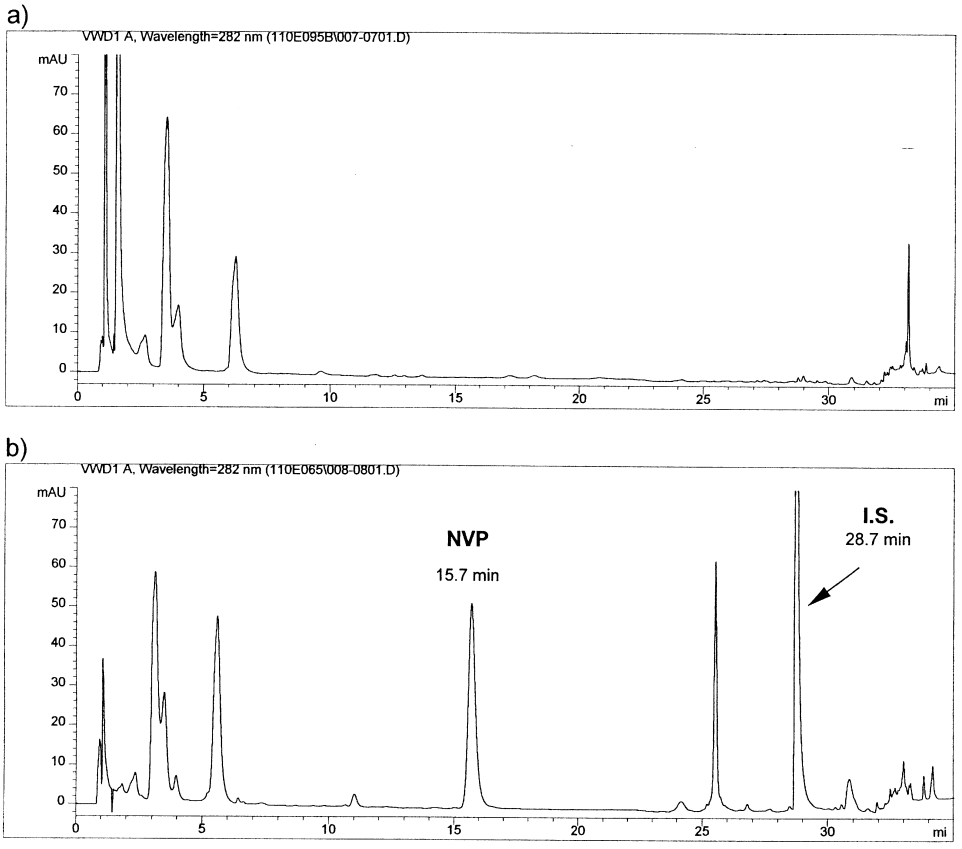

Fig. 2. (a) Chromatographic profile of a blank plasma, using the gradient elution program for NVP (Table 1b). (b) Chromatographic profile of the plasma of the same HIV-patient receiving didanosine, nevirapine and Kaletra® (LPV/RTV) (shown in Fig. 1b). Level of NVP was 4.64 μg/ml 7 h 50 min after the nevirapine 200 mg dose.

nearby peaks, notably efavirenz eluted at 25.7 min, a retention time close to that of LPV (Fig. 1c).

For the LPV analysis at 201 nm, a rinsing step with a 10/90 mixture of solvent B and the additional solvent C, followed by a re-equilibration with 15/85 solvent A–solvent B is recommended for avoiding perturbation of the next analysis by late eluting peaks.

3.3. Selectivity

No significant interfering peaks were observed at the retention time of LPV and NVP at 201 and 282 nm, respectively.

The method selectivity was confirmed by analysing all the currently prescribed anti-HIV drugs and/or drugs employed in the treatment/prophylaxis of opportunistic infections in the studied HIV+ patients cohort (listed in Materials and methods). All drugs are eluted at different times and do not interfere with the analyses of either LPV or NVP. Of note, with the gradient program used for NVP, trimethoprim/sulfamethoxazole and azithromycine were eluted ~1 min before NVP, however a sufficient separation between peaks makes an interference unlikely.

3.4. Calibration curves

The standard curves for LPV and NVP are satisfactorily described by unweighted least-squares linear regression. The slope of the calibration curves obtained throughout method validation and initial

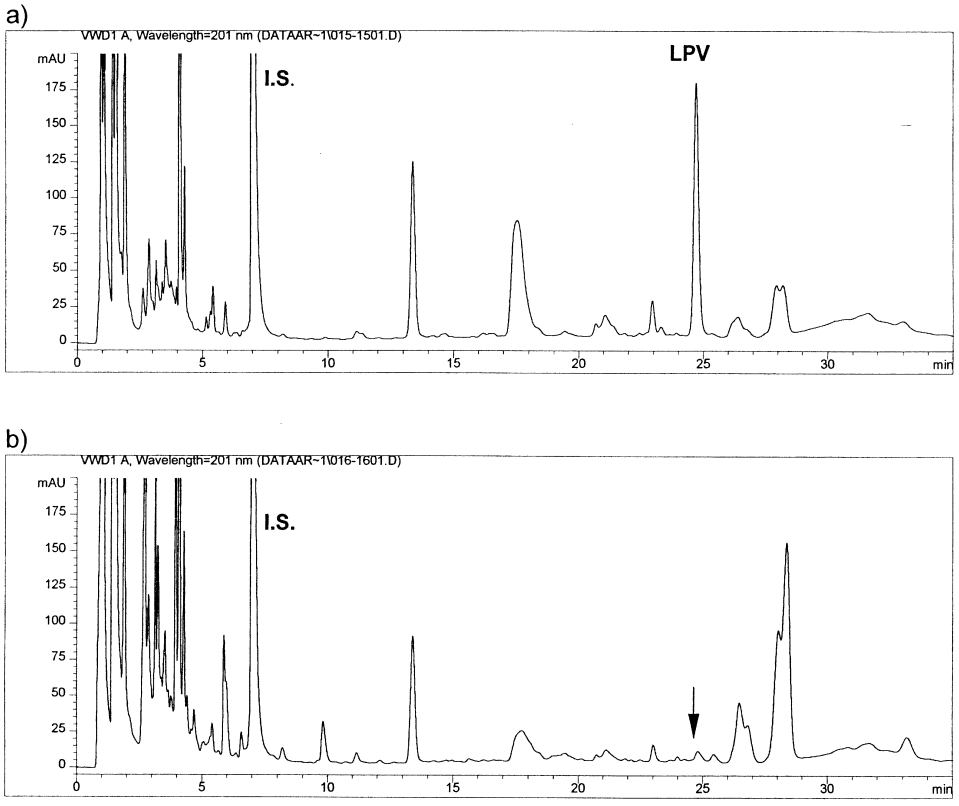

Fig. 3. (a) Chromatogram of a plasma taken 5 min prior to delivery from a pregnant HIV woman receiving 3TC, abacavir, nevirapine (NVP) and Kaletra (LPV/RTV). Levels of LPV, measured 12 h 15 min after drug intake, were 3.11 μg/ml. (b) Chromatographic profile of the corresponding umbilical cord sample taken after cord section at delivery. The concentration of lopinavir was below the quantitation limit of the assay.

analysis of patient samples is stable, with values averaging 1.05 (±4.7%) and 0.21 (±4.9%) for LPV ($n=12$) and NVP ($n=10$), respectively. Over the concentration range 0.1–10.0 μg/ml for LPV, and 0.5–10.0 μg/ml for LPV and NVP, the regression coefficient r^2 of the calibration curves remained excellent and always greater than 0.999.

3.5. Validation of the HPLC method: precision, accuracy and LOQ/LOD

Precision and accuracy of the control samples are given in Table 2. The concentration levels of control samples of LPV (0.75, 3.0 and 9.0 μg/ml) and NVP (0.7, 2.0 and 8.0 μg/ml) were selected to encompass the range of concentrations expected in plasma samples.

Throughout these concentration ranges, the mean intra-assay precision was similar, always lower than 2.5 and 2.8% for LPV and NVP, respectively. Overall, the mean inter-day precision for LPV and NVP was good with mean C.V.s within 0.9–6.6%. The intra-assay deviation (bias) from the nominal concentrations of LPV and NVP was between −5.8 and +6.3% and the range of inter-day deviation was always <2.4%.

By analysing plasma from outdated transfusion bags spiked with decreasing concentrations of LPV and NVP, the limit of quantification (LOQ) was experimentally found to be 0.10 μg/ml for LPV, and 0.06 μg/ml for NVP. Since the minimal concen-

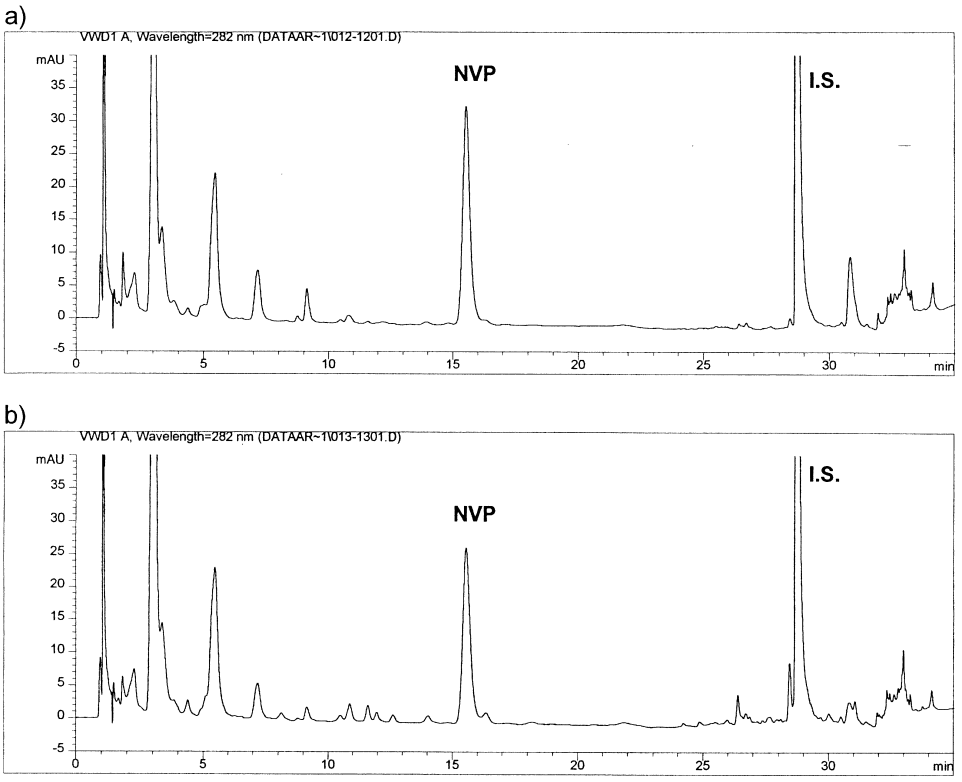

Fig. 4. (a) Chromatographic profile of the same plasma from the pregnant HIV woman taken 5 min prior to delivery. Levels of NPV in the mother measured 12 h 15 min after drug intake was 2.66 μg/ml. (b) Chromatographic profile of the corresponding umbilical cord sample taken after cord section at delivery. The concentration of NVP in the cord sample was 2.16 μg/ml.

tration of NVP and LVP reported to exert an antiviral activity are much higher than those values, decreasing LOQ values, makes little sense.

The limit of detection (LOD) of LPV and NVP with this assay was 0.05 and 0.015 μg/ml, respectively.

Table 2
Precision and accuracy of the assay for lopinavir and nevirapine in plasma

Lopinavir					Nevirapine				
Nominal conc. (μg/ml)	Concentration found (μg/ml)		Precision C.V. %	Accuracy[a] Bias %	Nominal conc. (μg/ml)	Concentration found (μg/ml)		Precision C.V. %	Accuracy[a] Bias %
A. Intra-assay (n=6)		±			A. Intra-assay (n=6)		±		
9.000	8.858	0.222	2.5	−1.6	8.000	8.116	0.101	1.3	1.5
3.000	3.038	0.034	1.1	1.3	2.000	1.971	0.019	0.9	−1.4
0.750	0.798	0.017	2.3	6.3	0.700	0.659	0.020	2.8	−5.8
B. Inter-assay (n=6)		±			B. Inter-assay (n=5)		±		
9.000	8.898	0.193	2.1	−1.1	8.000	8.063	0.074	0.9	0.8
3.000	3.072	0.072	2.4	2.4	2.000	1.962	0.035	1.7	−1.9
0.750	0.762	0.049	6.6	1.6	0.700	0.698	0.009	1.3	−0.3

[a] (Found−nominal)/nominal×100.

Table 3
Absolute recovery of LPV and NVP after SPE from plasma

	0.75 μg/ml		3.0 μg/ml		9.0 μg/ml	
	%	C.V. (%)		C.V. (%)		C.V. (%)
Lopinavir	93.6	0.5	91.8	2.6	87.8	3.0
	0.70 μg/ml		2.0 μg/ml		8.0 μg/ml	
	%	C.V. (%)		C.V. (%)		C.V. (%)
Nevirapine	90.4	2.6	89.3	1.1	83.7	1.1

3.6. Recovery

The mean absolute recovery of LVP and NVP measured with the high, medium and low QC controls were 91.1±3.0% and 87.8±3.6%, respectively. The clean-up procedure by SPE was found to be a reliable way of eliminating interfering material from plasma, with low recovery variability for both analytes (Table 3). The slightly lower recovery of nevirapine had no negative effects on the assay performance and does not preclude its applicability, since the reported therapeutic ranges of nevirapine are much higher than its limit of quantitation.

3.7. Sample stability

3.7.1. Stability during thermisation (HIV inactivation)

The slope of the calibration curves of LPV and NVP in samples submitted to the thermisation procedure (60 °C for 60 min) was essentially identical to that obtained with non-heated samples, as shown in Table 4. Taking into account the experimental variability (Table 2), these results indicate that such a procedure does not affect NVP and LPV concentrations. This is in agreement with previous results on NPV [9] and lopinavir stability [22]. Nevertheless, to standardize at best the analytical method, all calibration and control samples were similarly treated and heated at 60 °C for 60 min.

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

No evidence of LPV and NVP decomposition was found during plasma sample 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 PIs/NNRTI 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.

3.7.3. Stability of plasma samples left at room temperature

The stability of plasma samples left at room temperature for 24 h was checked. The variations of the levels of LPV and NVP, at concentrations of 5.0 and 3.0 μg/ml, respectively, was always lower than ±5%, a value comprised within the assay variability, indicating that at room temperature, the plasma samples appear to be stable over at least 24 h.

3.7.4. Stability of plasma samples after one, two and three freeze–thaw cycles

The variations of NVP/LPV concentrations when submitting control plasma to successive freeze–thaw

Table 4
Parameters of the calibration curves for LPV and NVP before and after plasma thermisation at 60 °C for 60 min

	Lopinavir			Nevirapine		
	m	*b*	r^2	*m*	*b*	r^2
No thermisation	1.106	0.01	0.99964	0.198	−1.05E−02	0.9995
Thermisation 60 min at 60 °C	1.067	0.002	0.99995	0.201	−4.48E−03	1.0
Variation (%)	−3.5			+1.5		

Table 5
Stability of plasma after multiple freeze–thaw cycles (concentration changes expressed as the % of the starting concentration)

	0.75 μg/ml			3.0 μg/ml			9.0 μg/ml		
	1	2	3	1	2	3	1	2	3
Lopinavir	2.7	2.7	1.3	4.7	7.8	4.4	2.9	−1.1	−3.0
	0.70 μg/ml			2.0 μg/ml			8.0 μg/ml		
	1	2	3	1	2	3	1	2	3
Nevirapine	1.6	7.1	3.7	−1.2	1.7	2.0	0.7	4.5	3.1

cycles are reported in Table 5. This indicates that no significant loss of LPV/NVP is to be expected after up to three freeze–thaw cycles.

3.7.5. Stability of extract 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–H_2O 50:50) submitted to HPLC analysis was checked at RT and at +4 °C for 24 h and is reported in Table 6. The variations over time of LVP and NVP, expressed in percentage of the starting levels (i.e. after immediate analysis), were within the −8 to +7% range in samples left at room temperature for 24 h (Table 6). These results indicate therefore that, taking account of the analytical variability, the processed sample stability is acceptable throughout HPLC analysis performed within 1 day. Interestingly, the extract samples for HPLC appear also relatively stable (variation comprised within −9 to +4%) when stored 24 h in the refrigerator.

3.8. Clinical applications

Besides being used as part of the routine TDM in HIV-positive patients [25], this HPLC assay has also been specifically applied to the analysis of samples collected for assessing the extent of transplacental passage of nevirapine and PIs, notably lopinavir, at delivery in pregnant HIV infected women [24].

Table 6
Stability of LPV/NVP in extract samples left at RT for 24 h and at +4 °C for 24 h (concentration variations expressed as the % of the starting levels)

	0.75 μg/ml		3.0 μg/ml		9.0 μg/ml	
	RT	+4 °C	RT	+4 °C	RT	+4 °C
Lopinavir	7	4	1	4	0	5
	0.70 μg/ml		2.0 μg/ml		8.0 μg/ml	
	RT	+4 °C	RT	+4 °C	RT	+4 °C
Nevirapine	−8	−9	0	−2	0	−1

The chromatographic profiles (Figs. 3a, b, and 4a, b) of samples taken in one patient exemplify the marked difference of placental transfer between lopinavir and nevirapine. The transfer of lopinavir was essentially null (Fig. 3b) even though lopinavir concentration measured simultaneously in the maternal circulation was 3.11 μg/ml (Fig. 3a). On the contrary, nevirapine's transfer was quite extensive (Fig. 4a vs. Fig. 4b), with a concentration of 2.66 μg/ml in the mother plasma and 2.16 μg/ml in the cord, giving a transplacental ratio of nevirapine of 0.8, a value comparable to the value reported after a single dose of nevirapine [31]. The clinical implications of such observations are discussed in detail elsewhere [24].

4. Discussion and conclusion

This optimised HPLC method provides a fairly robust procedure for determining the new PI lopinavir and the NNRTI nevirapine in plasma from HIV-infected patients. It uses an adaptation of the previous gradient elution program initially proposed for the assay of the first series of antiretrovirals and run routinely in our laboratory on a HPLC 1090 Agilent instrument [4]. Organisational constraints in our laboratory led us to perform the assay of LPV and NVP on a separate HPLC 1050 Agilent system. These adjustments were in fact necessary for the assay of lopinavir, since efavirenz co-elutes with lopinavir with the gradient program originally proposed. With this adaptation, a baseline separation of

efavirenz and lopinavir was achieved, as shown in Fig. 1c. Being able to monitor these antiretrovirals is important. Indeed lopinavir (as well as amprenavir) is administered most often in combination with efavirenz to heavily pretreated patients having experienced treatment failure, wherein exposure, tolerance and adherence assessments are critical issues.

Of note, the sample processing, solvent composition and column type are identical to those previously proposed for the first marketed antiretrovirals [4], a clear advantage for a laboratory where large numbers of samples containing multiple analytes are to be analysed on a routine basis.

To the best of our knowledge, this is the first report describing an assay of lopinavir after solid-phase extraction from plasma. Alternately, a number of assays have already been published for nevirapine, but only a few [9,11–14] addressed the selectivity issues, by assessing the retention time of potential co-administered drugs. By comparison with previously published methods, nevirapine is eluted at a relatively prolonged retention time of 16 min with the proposed gradient system, but this increases the selectivity of the method, reducing the likelihood of interferences when analysing samples from patients receiving multiple drugs therapy.

As previously mentioned, lopinavir is given under the form of Kaletra®, that contains a small dose (33 mg) of ritonavir which inhibits lopinavir systemic and presystemic metabolism. At such a low dose, the plasma levels of ritonavir are less than 7% of those achieved with 600 mg bid ritonavir alone, so that the antiviral activity is primarily due to lopinavir [22]. The quantification of ritonavir, solely used here as a pharmacokinetic enhancer, has therefore not been considered in this study. If requested, it can be done using the proposed gradient elution [4].

The next generation of PIs (atazanavir, tipranavir, mozenavir, etc) are expected in the near future [32], and if well-designed clinical studies demonstrate unambiguously the usefulness of the TDM of antiretrovirals, clinical laboratories should be prepared to face the challenging task of analysing an increasing number of antiviral drugs. This could certainly be facilitated by having the selectivity provided by the LC–MS–MS technology, which is however not available in all hospital laboratories. This will require therefore to optimize or adjust current analytical methods, using widely available technology such as the UV detection. Our method, though relatively time consuming, has been developed using instruments available in conventional hospital laboratories and enables to analyse the six protease inhibitors marketed to date (saquinavir, ritonavir, indinavir, nelfinavir, amprenavir and lopinavir) and two NNRTIs (nevirapine and efavirenz). It can be done conveniently on a routine basis, with minor adjustments, using one single sample processing procedure followed by a chromatographic step on an identical column type, using the same solvent mixture, and changing only the elution gradient program. Our approach represents therefore a useful and convenient tool for an optimal follow-up of HIV patients including TDM.

Acknowledgements

Support for this work was provided by the Swiss National Science Foundation (grant 3345-062092.99)

References

[1] C. Marzolini, A. Telenti, L.A. Decosterd, G. Greub, J. Biollaz, T. Buclin, AIDS 15 (2001) 71.
[2] C. Marzolini, T. Buclin, L.A. Decosterd, J. Biollaz, A. Telenti, Ther. Drug Monit. 23 (2001) 394.
[3] D. Kempf, A. Hsu, P. Jiang, R. Rode, K. Hertogs, B. Larder, A. Zolopa, N. Shulman, D. Havlir, J. Gallant, E. Race, S. Boller, J. Swerdlow, O. Jasinsky, C. Renz, E. Sun, 8th Conference on Retroviruses and Opportunic Infections, February 2001, Chicago, Abstract 523.
[4] C. Marzolini, A. Telenti, T. Buclin, J. Biollaz, L.A. Decosterd, J. Chromatogr. B (Biomed. Sci. Appl.) 740 (2000) 43.
[5] M. Hurst, D. Faulds, Drugs 60 (2000) 1371.
[6] A.I. Veldkamp, G.J. Weverling, J.M.A. Lange, J.S.G. Montaner, P. Reiss, D.A. Cooper, S. Vella, D. Hall, J.H. Beijnen, R.M.W. Hoetelmans, AIDS 15 (2001) 1089.
[7] A. Jajaray, J. Alexander, C. Price, D. Daly, J. Pav, S. Hattox, J. Keirns, Pharm. Res. 9 (1992) S334.
[8] S.H. Cheeseman, S.E. Hattox, M.M. McLaughlin, R.A. Koup, C. Andrews, C.A. Bova, J.W. Pav, T. Roy, J.L. Sullivan, J.J. Keirns, Antimicrob. Agents Chemother. 37 (1993) 178.
[9] R.P.G. van Heeswijk, R.M.W. Hoetelmans, P.L. Meehorst, J.W. Mulder, J.H. Beijnen, J. Chromatogr. B (Biomed. Sci. Appl.) 713 (1998) 395.

[10] C. Merry, M.G. Barry, F. Mulcahy, M. Ryan, J.F. Tjia, K.L. Halifax, A.M. Breckenridge, D.J. Back, AIDS 12 (1998) 1163.
[11] J.W. Pav, L.S. Rowland, D.J. Korpalski, J. Pharm. Biomed. Anal. 20 (1999) 91.
[12] R.M.F. Hollanders, E.W.J. van Ewijk-Beneken Kolmer, D.M. Burger, E.W. Wuis, P.P. Koopmans, Y.A. Hekster, J. Chromatogr. B (Biomed. Sci. Appl.) 744 (2000) 65.
[13] R.M. Lopez, L. Pou, M.R. Gomez, I. Ruiz, J. Monterde, J. Chromatogr. B (Biomed. Sci. Appl.) 751 (2001) 371.
[14] R.P.G. van Heeswijk, A.I. Veldkamp, J.-W. Mulder, P.L. Meenhorst, J.H. Beijnen, J.M.A. Lange, R.M.W. Hoetelmans, Ther. Drug Monit. 23 (2001) 255.
[15] P. Riska, M. Lamson, T. McGregor, J. Sabo, S. Hattox, J. Pav, J. Keirns, Drug Metab. Dispos. 27 (1999) 895.
[16] P. Riska, D.P. Joseph, R.M. Dinallo, W.C. Davidson, J.J. Keirns, S.E. Hattox, Drug Metab. Dispos. 27 (1999) 1434.
[17] D.A. Erickson, G. Mather, W.F. Trager, R.H. Levy, J.J. Keirns, Drug Metab. Dispos. 27 (1999) 1488.
[18] E. Dailly, L. Thomas, M.F. Kergueris, P. Jolliet, M. Bourin, J. Chromatogr. B (Biomed. Sci. Appl.) 758 (2001) 129.
[19] P. Villani, M. Feroggio, L. Gianelli, A. Bartoli, M. Montagna, R. Maserati, M.B. Regazzi, Ther. Drug Monit. 23 (2001) 380.
[20] H.L. Sham, D.J. Kempf, A. Molla, K.C. Marsh, G.N. Kumar, C.-M. Chen, W. Kati, K. Stewart, R. Lal, A. Hsu, D. Betebenner, M. Korneyeva, S. Vasavanonda, E. McDonald, A. Saldivar, N. Wideburg, X. Chen, P. Niu, C. Park, V. Jayanti, B. Grabowski, G.R. Granneman, E. Sun, A.J. Japour, J.M. Leonard, J.J. Plattner, D.W. Norberck, Antimicrob. Agents Chemother. 42 (1998) 3218.
[21] G.N. Kumar, V. Jayanti, R.D. Lee, D.N. Whittern, J. Uchic, S. Thomas, P. Johnson, B. Grabowski, H. Sham, D. Betebenner, D.J. Kempf, J.F. Denissen, Drug Metab. Dispos. 27 (1999) 86.
[22] R. Wieboldt, E. McDonald, J.M. McVey, L. Hernandez, T. El-Shourbagy, K.C. Marsh, Data on file, Abbott Laboratories, Abbott Park, IL, USA.
[23] D. Kuschak, S. Mauss, G. Schmutz, B. Gantke, Clin. Lab. 47 (2001) 471.
[24] C. Marzolini, C. Rudin, L.A. Decosterd, A. Telenti, A. Schreyer, J. Biollaz, T. Buclin, AIDS 16 (2002) 889–893.
[25] C. Marzolini, A. Telenti, L.A. Decosterd, G. Greub, J. Biollaz, T. Buclin, submitted.
[26] 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. Pharmacokinet. 16 (1991) 249.
[27] J.S. McDougal, L.S. Martin, S.P. Cort, M. Mozen, C.M. Heldebrant, B.L. Evatt, J. Clin. Invest. 76 (1985) 875.
[28] S.S. Good, D.J. Reynolds, P. De Miranda, J. Chromatogr. B 431 (1988) 123.
[29] 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.
[30] C.H.W. Koks, H. Rosing, P.L. Meenhorst, A. Bult, J.H. Beijnen, J. Chromatogr. B 663 (1995) 345.
[31] P. Musoke, L.A. Guay, D. Bagenda, M. Mirochnick, C. Nakabiito, T. Fleming, T. Elliott, S. Horton, K. Dransfield, J.W. Pav, A. Murarka, M. Allen, M.G. Fowler, L. Mofenson, D. Hom, F. Mmiro, J.B. Jackson, AIDS 13 (1999) 479.
[32] M. Youle, Investigational antiretrovirals in existing classes, in: 1st IAS Conference on HIV Pathogenesis and Treatment, Buenos Aires, July 2–9, 2001, http://www.medscape.com/Medscape.