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Simultaneous determination of 8 HIV protease inhibitors in human plasma by isocratic high-performance liquid chromatography with combined use of UV and fluorescence detection: Amprenavir, indinavir, atazanavir, ritonavir, lopinavir, saquinavir, nelfinavir and M8-nelfinavir metabolite

R. Verbesselt [a,*], E. Van Wijngaerden [b], J. de Hoon [a]

[a] *Center for Clinical Pharmacology, University Hospital Gasthuisberg (K.U. Leuven), Herestraat 49, B-3000 Leuven, Belgium*
[b] *Department of Internal Medicine, University Hospital Gasthuisberg (K.U. Leuven), Herestraat 49, B-3000 Leuven, Belgium*

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Abstract

A simple, accurate and fast method was developed for determination of the commonly used HIV protease inhibitors (PIs) amprenavir, indinavir, atazanavir, ritonavir, lopinavir, nelfinavir, M8-nelfinavir metabolite and saquinavir in human plasma. Liquid–liquid extraction was used with hexane/ethylacetate from buffered plasma samples with a borate buffer pH 9.0. Isocratic chromatographic separation of all components was performed on an Allsphere hexyl HPLC column with combined UV and fluorescence detection. Calibration curves were constructed in the range of 0.025–10 mg/l. Accuracy and precision of the standards were all below 15% and the lowest limit of quantitation was 0.025 mg/l. Stability of quality control samples at different temperature conditions was found to be below 20% of nominal values. The advantages of this method are: (1) inclusion and determination of the newly approved atazanavir, (2) simultaneous isocratic HPLC separation of all compounds and (3) increased specificity and sensitivity for amprenavir by using fluorescence detection. This method can be used for therapeutic drug monitoring of all PIs currently commercialised and is now part of current clinical practice.

Keywords: Protease inhibitors; Drug monitoring; Chromatography

1. Introduction

Protease inhibitors (PIs) constitute a very important class of drugs in the treatment of patients infected with the human immunodeficiency virus (HIV). Together with the nucleoside reverse transcriptase inhibitors (NRTIs) and the non-nucleoside reverse transcriptase inhibitors (NNRTIs) they are an integral part of the Highly Active Anti-Retroviral Therapy (HAART), standard current treatment of HIV infection.

With the intent of improving daily patient care, there is an increasing need for therapeutic drug monitoring in HIV-positive patients (HIV-TDM) in order to optimise plasma concentrations; in a setting were large interindividual variations in concentrations are present, drug/drug and food/drug interactions are common and medication adherence is frequently suboptimal. Since multidrug therapy is standard and comedication very frequent, there is a need for specific analytical methods for measuring plasma concentrations of individual drugs.

High-performance liquid chromatography in combination with UV detection is the most frequently used technique for measuring plasma concentrations of PIs. Although various methods for simultaneous determination of PIs have been published [1–17], in our method the recently approved protease inhibitor atazanavir was included, allowing separation from the other commonly determined PIs. In addition, the combination of UV

* Corresponding author. Tel.: +32 16 342026; fax: +32 16 342050.
E-mail address: rene.verbesselt@med.kuleuven.ac.be (R. Verbesselt).

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and fluorescence detection, resulted in a more specific determination of amprenavir being the fluorescent compound.

More recent liquid chromatographic–mass spectrometric methods have been developed for simultaneous determination of NNRTIs and PIs including the newest protease inhibitors atazanavir and tipranavir [18–23]; although these methods have good specificity, sensitivity and simple extraction procedures, UV and fluorimetric detectors remain the standard available instruments in most laboratories.

Our validated method is currently being used for HIV-TDM [24] and for studies investigating the adherence of HIV-patients. The validation of the presented method was based on the recommendations published as a Conference Report of the Washington Conference on Analytical Method Validation: Bioavailability, Bioequivalence and Pharmacokinetic Studies [25,26].

2. Experimental

2.1. Chemicals and reagents

Drug standards were generously obtained from several pharmaceutical companies: indinavir sulfate (IDV) and efavirenz (EFV) from Merck Sharp & Dohme (Rahway, NJ, USA); amprenavir (APV) from GlaxoSmithKline (Durham, UK); saquinavir mesylate (SQV) and nelfinavir (NFV) from Roche Diagnostics (Mannheim, Germany); ritonavir (RTV), lopinavir (LPV) and A86093 ((1-benzyl-4-{2-[3-cyclopropyl-3-(2-isopropyl-thiazol-4-ylmethyl)-ureido]-3-methyl-butyrylamino}-3-hydroxy-5-phenyl-pentyl)-carbamic acid thiazol-5-ylmethyl ester) as internal standard from Abbott Laboratories (Abbott Park, IL, USA); atazanavir sulfate (ATV) from Bristol Meyers Squibb (Princeton, NJ, USA); M8 metabolite of nelfinavir (M8-NFV) from Pfizer (Groton, CT, USA); PR25 (2-(4-hydroxy-3-methoxy-phenyl)-2-isopropyl-5-methylamino-pentanenitrile) from Knoll (Ludwigshafen, Germany) and nevirapine (NVP) from Boehringer Ingelheim (Ridgefield, CT, USA).

Chemical structures of PIs and internal standards are shown in Fig. 1a and b.

HPLC grade methanol, acetonitrile, hexane and ethylacetate were purchased from Biosolve (Valkenswaard, The Netherlands). Potassium dihydrogen phosphate, *o*-phosphoric acid (85%), sodium hydroxide, disodium tetraborate were purchased from Merck (Darmstadt, Germany) and were all of analytical grade. Millipore ultrapure water was obtained from a Simplicity System (Millipore, Belgium). Blank drug-free human plasma was obtained from the internal plasma bank (Center for Clinical Pharmacology, Leuven, Belgium).

2.2. Equipment

The HPLC system consisted of a Waters (Milford, MA, USA) 600 E pump with controller, a photodiode array detector 996 (Waters), a fluorescence detector F 1000 (Merck–Hitachi, Darmstadt, Germany), an automatic injector 717plus with cooling module (Waters) and a chromatographic data management system (Millennium^{32}, Waters).

2.3. Chromatographic conditions

Analytes were separated isocratically on a Allsphere hexyl 5 μ column (150 mm × 4.6 mm i.d.) (Alltech Associates Inc., Belgium). The mobile phase was a mixture of acetonitrile, methanol and 15 mM sodiumdihydrogenphosphate buffer pH 4.5 (35/20/45, v/v/v) and the flow rate was 1 ml/min.

The UV detector was set at 215 nm and the fluorescence detector at 280 and 340 nm as excitation and emission wavelengths, respectively.

2.4. Standard solutions, standard curves and quality control samples

Two series of individual weighted stock solutions of IDV, APV, ATV, NFV, M8-NFV, LPV, RTV and SQV were prepared at concentrations of, respectively, 1 and 2.5 mg/ml in methanol for preparation of either the calibration standards or the quality control samples in plasma.

Appropriate dilutions in methanol/water (1/1, v/v) were prepared from these stock solutions to obtain calibration standards in the range of 25–10,000 ng/ml and quality control samples of 200, 1000 and 5000 ng/ml.

Dilutions for calibration standards were prepared daily with each new batch of samples and added to blank plasma, while quality control samples were prepared in plasma, divided in small aliquots and stored at −20 °C until use. A stock solution of the internal standards A86093 and PR25 was prepared at 1 mg/ml in methanol and diluted to 10 μg/ml in methanol/water (1/1, v/v). All stock solutions of protease inhibitors and internal standards were stored at −20 °C up to 6 months.

2.5. Heat deactivation of patient samples

Plasma samples were deactivated for the HIV virus by heating the samples for 1 h at 58 °C.

2.6. Sample preparation

To 0.5 ml of plasma was added 50 μl of the respective standard dilutions in the range of 25–10,000 ng/ml; to the samples and quality control samples was added 50 μl of methanol/water (1/1, v/v); further was added 50 μl of the internal standard solution (A86093 and PR25, 10 μg/ml) to all samples, 500 μl of 0.05 M sodiumtetraborate buffer pH 9.0 and 5 ml of a mixture hexane/ethylacetate (1/1, v/v).

After shaking for 10 min and centrifuging for 5 min at 1286 × *g*, the aqueous layer was frozen in a cooling mixture of acetone and dry ice and the hexane/ethylacetate layer transferred to conical glass tubes (previously washed with methanol).

The organic phase was evaporated to dryness with an airstream in a waterbath at 40 °C.

The dried residues were dissolved in 300 μl of a mixture of acetonitrile and 15 mM potassium phosphate buffer pH 2.4 (+0.05% triethylamine) (45/55, v/v) and washed for 15 s with 2 ml hexane; after centrifugation and freezing the aqueous layer, the hexane layer was discarded.

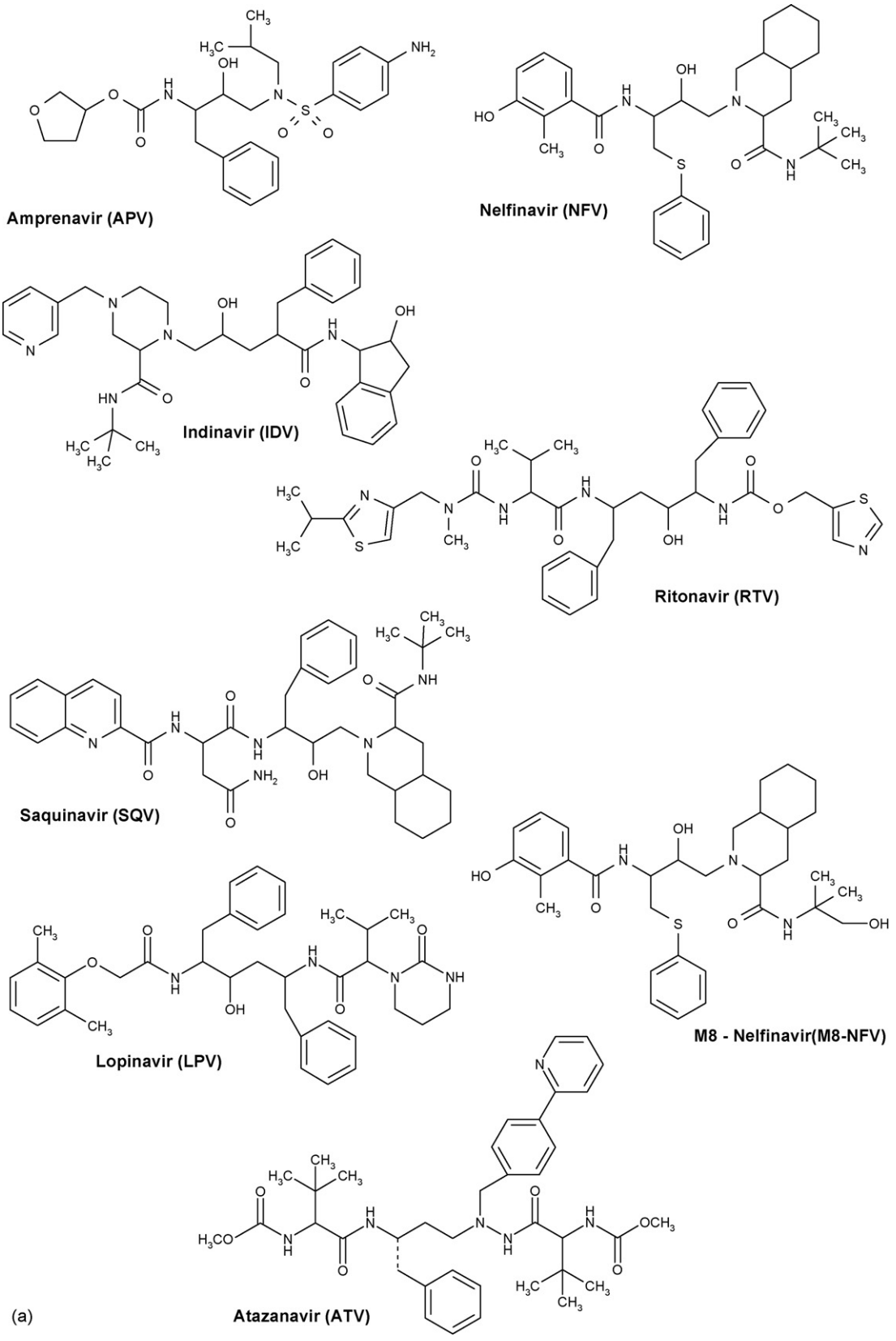
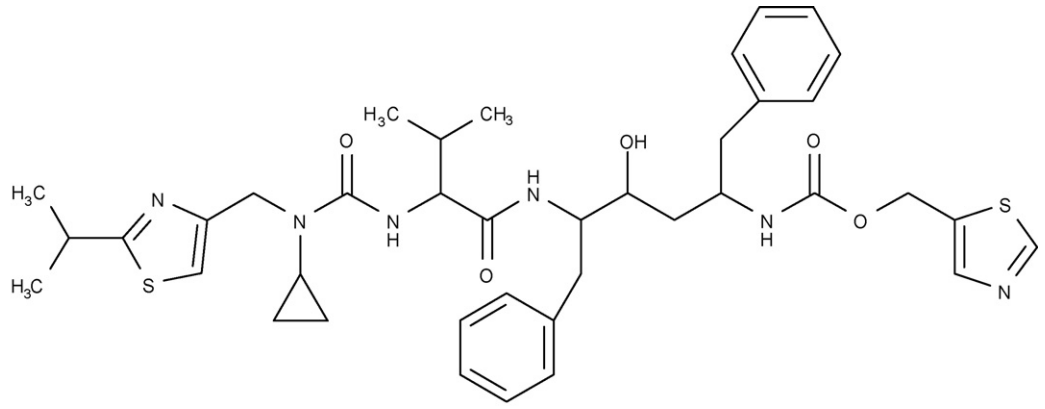
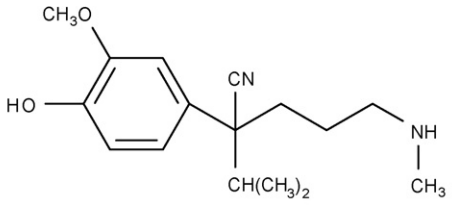


Fig. 1. (a) Chemical structures of protease inhibitors and metabolite M8. (b) Chemical structures of internal standards.





(b)

Fig. 1. (*Continued*).

After thawing and removing possible remaining hexane with a cold airstream for 2 min, 120 μl of the aqueous layer was transferred to injection vials for injection onto the HPLC column.

2.7. Validation procedures

2.7.1. Linearity

Calibration standards were prepared and analysed in fivefold. Calibration curves (peak height ratio with the internal standard versus nominal analyte concentration) were fitted by least-squares linear regression analysis using 1/concentration^2 as weighting factor. Fitting parameters and back-calculated values were obtained by the software program Excel (Microsoft).

To assess linearity, deviations of the mean calculated concentrations should be within ±15% from nominal concentrations for the non-zero calibration standards. At the lower limit of quantification level (LLQ), a deviation of ±20% was allowed.

2.7.2. Precision and accuracy

Accuracy, intra- and inter-assay precision of the method were determined by assaying five replicates of each of the spiked QC samples with analyte concentrations in the low, middle and high concentration range in three separate analytical runs. Accuracy was measured as the percentage deviation from the nominal concentrations. Precision was expressed in terms of relative standard deviation and obtained by one-way analysis of variance (ANOVA) for each test concentration using the analytical run as the grouping variable. The following formulas were used in order to calculate the inter- and intra-assay precision, respectively:

$$\text{Inter-assay}: \frac{[(\text{Day mean square} - \text{error mean square})/n]1/2}{\text{Grand mean}} \times 100\%$$

$$\text{Intra-assay}: \frac{(\text{Error mean square})1/2}{\text{Grand mean}} \times 100\%$$

The day mean square, the error mean square and grand mean are expressions originating from ANOVA, n is the number of replicates within each day (five) for each concentration. If the error mean square is higher than the day mean square, the inter-assay precision is regarded zero. This signifies that no significant additional variation is observed as a result of performing the assay in different runs.

The acceptance criteria applied in the validation were that the intra- and inter-assay precision of the QC samples should not exceed 15% coefficient of variation (CV) except for the LLQ, where it should not exceed 20% CV.

2.7.3. Stability

The stability of all PIs was studied at the following conditions: (1) three freeze-thaw cycles, (2) leaving at room temperature for 24 h and (3) heating at 58 °C for 1 h. For each condition, the quality control samples spiked at 200 and 5000 ng/ml were assayed in fivefold.

The analytes were considered to be stable in the biological matrix when 80–120% of the initial concentration was found.

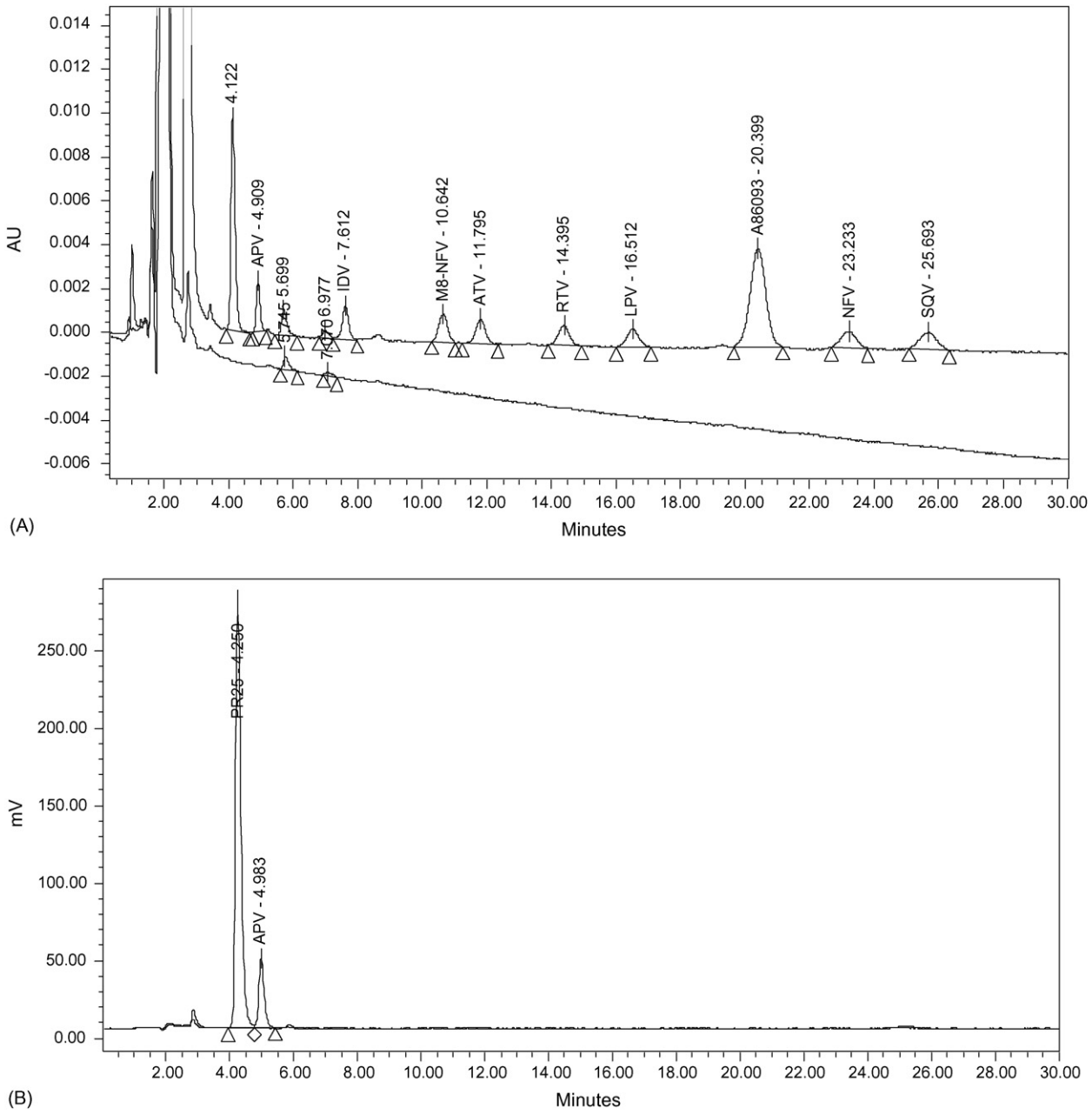


Fig. 2. Chromatograms of (A) blank plasma extract and blank plasma with added 100 ng/ml amprenavir (APV), indinavir (IDV), M8-nelfinavir (M8-NFV), atazanavir (ATV), ritonavir (RTV), lopinavir (LPV), nelfinavir (NFV), saquinavir (SQV) and 1000 ng/ml A86093 (UV detection), (B) blank plasma with added 100 ng/ml amprenavir (APV) and 1000 ng/ml PR25 as internal standard (fluorescence detection).

2.7.4. Recovery

Total recovery of the PIs was determined by comparing the analytical results for extracted samples with those for diluted working solutions. The overall recovery was calculated over the entire range of calibration standards.

Overall recovery of the internal standards was calculated at 500 ng/ml, the concentration added to each tube for the extraction of the calibration curve standards.

2.7.5. Limit of quantification

The lowest limit of quantitation (LLQ) was defined as the concentration for which the compounds could be determined reproducible within 20% of the nominal value.

2.7.6. Specificity and selectivity

Frequently co-administered drugs like the nucleoside reverse transcriptase inhibitors and the non-nucleoside reverse transcriptase inhibitors were investigated in patient samples to see if any interference with the PIs occurred.

Samples from HIV-infected patients treated with protease inhibitors and other comedicated drugs were analysed to look for possible interferences.

2.7.7. Analysis of patient samples

PIs were measured in plasma samples obtained from (1) the Leuven Adherence Project (LAP) in which assessment of adherence to drug regimen by multiple methods was compared and

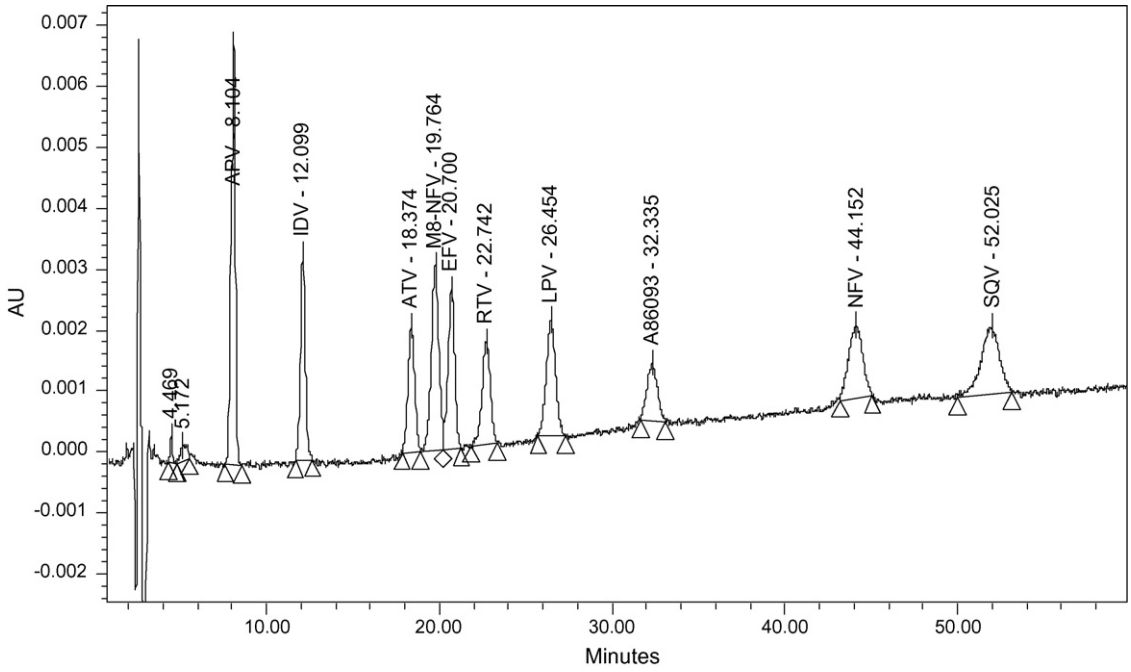


Fig. 3. Chromatogram of injection of stock solution (30 μl of 1 μg/ml) of eight protease inhibitors and efavirenz onto a Allsphere hexyl 5 μ column (250 mm × 4.6 mm i.d.).

from (2) our hospital aids reference center for therapeutic drug monitoring.

3. Results

3.1. Chromatographic separation

Representative chromatograms of the extraction of blank plasma or the standards of IDV, APV, ATV, NFV, M8-NFV, LPV, RTV and SQV together with both internal standards A86093 and PR25, are shown in Fig. 2.

No interference with endogenous substances was seen, especially for APV, which is measured by fluorescence detection. Using UV detection APV is suffering sometimes of interfering compounds, resulting in lower sensitivity.

The retention times were 4.1 min for PR25, 4.8 min for APV, 7.5 min for IDV, 10.5 min for M8-NFV, 11.7 min for ATV, 14.3 min for RTV, 16.3 min for LPV, 20.3 min for A86093, 23.0 min for NFV and 25.2 min for SQV.

3.2. Calibration curves

Calibration curves of IDV, APV, ATV, NFV, M8-NFV, LPV, RTV and SQV were linear in the calibration range of 0.025–10 μg/ml. The best fit was obtained by using a weighting factor of 1/concentration^2. Linear slope and intercept parameters were determined for 10 calibration curves with mean inter-assay slope values (±S.D.) for respective APV: 0.00514 (±0.00020), IDV: 0.00310 (±0.00025), ATV: 0.00222 (±0.00010), M8-NFV: 0.00249 (±0.00028), RTV: 0.00192 (± 0.00005), LPV: 0.00175 (±0.00008), SQV: 0.00472 (±0.00774) and NFV: 0.0013 (±0.00309).

3.3. Selectivity and specificity

In the chromatograms in most cases no interferences with endogenous compounds were seen. APV can sometimes suffer from interferences using UV detection; therefore fluorescence detection was used to obtain a more specific and sensitive determination of APV.

None of the following drugs were shown to interfere with the PIs determination: abacavir, aciclovir, alprazolam, amoxicilline, atenolol, atorvastatin, cetirizine, dapsone, diclofenac, didanosine, diltiazem, fenofibrate, fenprocoumon, fluoxetine, ibuprofen, lamivudine, lisinopril, loperamide, lorazepam, meclozine, methadon, naproxen, nevirapine, olanzapine, omeprazole, paracetamol, pravastatin, ranitidine, rifampicine, sertraline, sildenafil, simvastatin, stavudine, sulfamethoxazole, tenofovir, tramadol, valproic acid, zidovudine and zolpidem.

The latest introduced protease inhibitor tipranavir did not interfere with the other PIs because if its long retentention time (>30 min).

High concentrations of EFV can be a problem for low concentrations of ATV, because of the lower resolution between the two compounds. In that case, we can use a longer HPLC column (250 mm × 4.6 mm i.d.) with the same packing material (Allsphere hexyl 5 μ) and the same mobile phase.to obtain a good separation of all the compounds (Fig. 3).

Coadministration of atazanavir with efavirenz has to be done cautiously because a 70% reduction of the Cmin plasma concentration was found by induction of the CYP3A4 isoenzyme [27].

Table 1
Intra-assay precision and accuracy of standards of PI calibration curves extracted from plasma

Protease inhibitor	Concentration (ng/ml) ($N=5$)	Concentration found (ng/ml) (CV%) ($N=5$)	Accuracy (%) ($N=5$)
Amprenavir	25	24.8 (4.1)	−1.9
	50	50.4 (5.5)	0.8
	250	253.6 (4.2)	1.4
	1000	994.0 (7.6)	−0.6
	5000	4928.8 (3.5)	−1.4
Indinavir	25	25.2 (8.8)	0.0
	50	50.0 (5.7)	0.1
	250	242.9 (5.5)	−2.8
	1000	971.5 (10.0)	−2.8
	5000	5290.6 (3.5)	5.8
M8-nelfinavir	25	25.1 (3.4)	−0.5
	50	49.8 (6.8)	−0.4
	250	249.9 (3.3)	−0.1
	1000	994.3 (6.7)	−0.6
	5000	4963.8 (4.7)	−0.7
Atazanavir	25	25.3 (7.9)	1.6
	50	49.2 (9.2)	−1.6
	250	251.8 (3.3)	0.7
	1000	981.0 (5.1)	−1.9
	5000	5129.2 (2.2)	2.6
Ritonavir	25	24.8 (3.0)	−1.4
	50	50.8 (6.3)	1.6
	250	247.8 (3.1)	−0.9
	1000	978.7 (2.1)	−2.1
	5000	5106.0 (2.2)	−0.7
Lopinavir	25	25.6 (7.0)	1.8
	50	48.2 (4.5)	−3.7
	250	246.6 (2.1)	−1.3
	1000	994.1 (2.8)	−0.6
	5000	5201.2 (3.0)	4.0
Nelfinavir	25	25.2 (5.6)	2.9
	50	50.1 (6.5)	0.2
	250	240.5 (3.7)	−3.8
	1000	1022.6 (7.5)	2.3
	5000	4925.5 (5.5)	−1.5
Saquinavir	25	25.2 (7.0)	2.2
	50	50.1 (3.3)	0.3
	250	242.2 (3.9)	−3.1
	1000	1015.3 (6.9)	1.5
	5000	5058.3 (4.3)	1.2

3.4. Accuracy and precision

Intra-assay precision and accuracy of the calibration standards are shown in Table 1. All values were below 15%. Accuracy, inter-assay and intra-assay precision for the quality control samples are listed in Table 2. Variations in accuracy and coefficient of variation were all below 20%.

3.5. Lower and upper limits of quantitation

The lower limit of quantitation was defined as 25 ng/ml for all PIs, being the lowest concentration of the calibration curves, with a coefficient of variation lower than 20%.

The upper limit of quantitation was set at 10,000 ng/ml. Concentrations higher than this limit are diluted until they fall within the calibration range.

3.6. Recovery

Mean recoveries were calculated for all concentrations of the calibration curve giving following results: 97.2% for APV, 99.8% for IDV, 85.2% for M8-NFV, 101.0% for ATV, 96.2% for RTV, 97.7% for LPV, 98.8% for NFV and 105.1% for SQV. Precision (CV%) of the recovery was lower than 15% for all compounds, except for M8-NFV for which it was less than 20%.

Recoveries for the internal standards A86093 and PR25 averaged 102.9 and 75.9%, respectively, with CV% lower than 15%.

3.7. Stability

QC samples were determined at different temperature conditions: (1) three freeze-thaw cycles, (2) leaving at room temperature during 24 h and (3) heating at 58 °C for 1 h. The percentage

Table 2
Assay precision and accuracy of PI QC samples extracted from plasma

Protease inhibitor	Concentration (ng/ml)	Intra-assay precision (%)	Inter-assay precision (%)	Overall accuracy (%)
Amprenavir	200	3.2	1.0	6.2
	1000	9.8	5.2	5.7
	5000	5.0	6.5	7.0
Indinavir	200	6.6	2.1	−2.1
	1000	10.4	3.6	8.6
	5000	11.6	2.1	8.0
M8-nelfinavir	200	3.9	4.9	1.7
	1000	6.0	4.0	8.9
	5000	8.4	3.5	7.8
Atazanavir	200	5.5	5.4	−5.5
	1000	1.7	6.1	−6.6
	5000	4.5	5.1	−2.2
Ritonavir	200	1.9	2.0	−11.1
	1000	2.8	0.4	−6.6
	5000	4.0	1.3	−3.4
Lopinavir	200	1.0	3.6	9.9
	1000	2.0	2.4	12.7
	5000	2.8	2.1	14.3
Nelfinavir	200	4.6	1.8	1.1
	1000	2.9	2.2	0.4
	5000	2.8	3.1	−2.3
Saquinavir	200	3.7	6.8	−10.6
	1000	4.5	5.7	−5.5
	5000	8.0	3.5	−5.2

deviation from the nominal values were all below 20%, indicating stability of the PIs.

Compared to the other PIs, only the precision for amprenavir and indinavir and the accuracy for NFV and M8-NFV seem more sensitive to room temperature but still within the 20% limit. Table 3 shows the results of all the stability experiments.

3.8. Matrix substitution

Different plasma matrices were used to confirm the selectivity and specificity of our methodology. These plasma samples were obtained from blood samples collected on heparin or EDTA as anticoagulant. No differences were found

Table 3
Stability of plasma PI QC samples at different temperature conditions: freeze-thaw three times, 24 h at room temperature and 60 min at 58 °C for heat deactivation

Protease inhibitor	Concentration (ng/ml)	Freeze-thaw stability ($N=4$)		Room temperature stability ($N=4$)		Heat deactivation stability ($N=4$)	
		Concentration found (ng/ml) (CV%)	Accuracy (%)	Concentration found (ng/ml) (CV%)	Accuracy (%)	Concentration found (ng/ml) (CV%)	Accuracy (%)
APV	200	207.0 (5.2)	3.5	190.5 (18.3)	−4.7	211.8 (9.1)	5.9
	5000	5121.5 (7.1)	2.4	4826.9 (14.3)	−3.5	5413.5 (4.2)	8.3
IDV	200	170.2 (11.3)	−14.9	196.1 (17.9)	2.5	172.2 (2.3)	−13.9
	5000	4898.0 (6.4)	−2.0	5554.3 (15.7)	11.1	4674.5 (5.7)	−6.5
M8-NFV	200	165.4 (3.6)	−17.3	173.6 (14.6)	−13.2	201.3 (5.0)	0.6
	5000	4305.4 (4.2)	−13.9	4623.5 (12.0)	−7.5	4951.1 (1.1)	−1.0
ATV	200	178.1 (3.6)	−11.0	173.7 (9.3)	−13.1	173.7 (5.7)	−13.2
	5000	4477.9 (4.4)	−10.4	4559.8 (7.8)	−8.8	4334.5 (4.3)	−13.3
RTV	200	191.1 (2.2)	−4.4	172.2 (2.6)	−13.9	193.6 (2.1)	−3.2
	5000	4866.6 (3.3)	−2.7	4476.2 (7.7)	−10.5	4582.4 (2.8)	−8.4
LPV	200	197.1 (1.1)	−1.5	214.8 (6.3)	7.4	211.9 (3.0)	6.0
	5000	5594.7 (2.3)	11.9	5310.8 (1.9)	6.2	5578.5 (6.4)	11.6
NFV	200	169.1 (5.4)	−15.4	172.5 (8.9)	−13.8	161.1 (5.2)	−19.5
	5000	4265.5 (3.5)	−14.7	4234.1 (7.3)	−15.3	4110.1 (1.6)	−17.8
SQV	200	191.9 (3.5)	−4.0	189.1 (9.7)	−5.4	171.3 (4.9)	−14.4
	5000	4735.5 (3.4)	−5.3	4816.7 (8.3)	−3.7	4244.6 (1.4)	−15.1

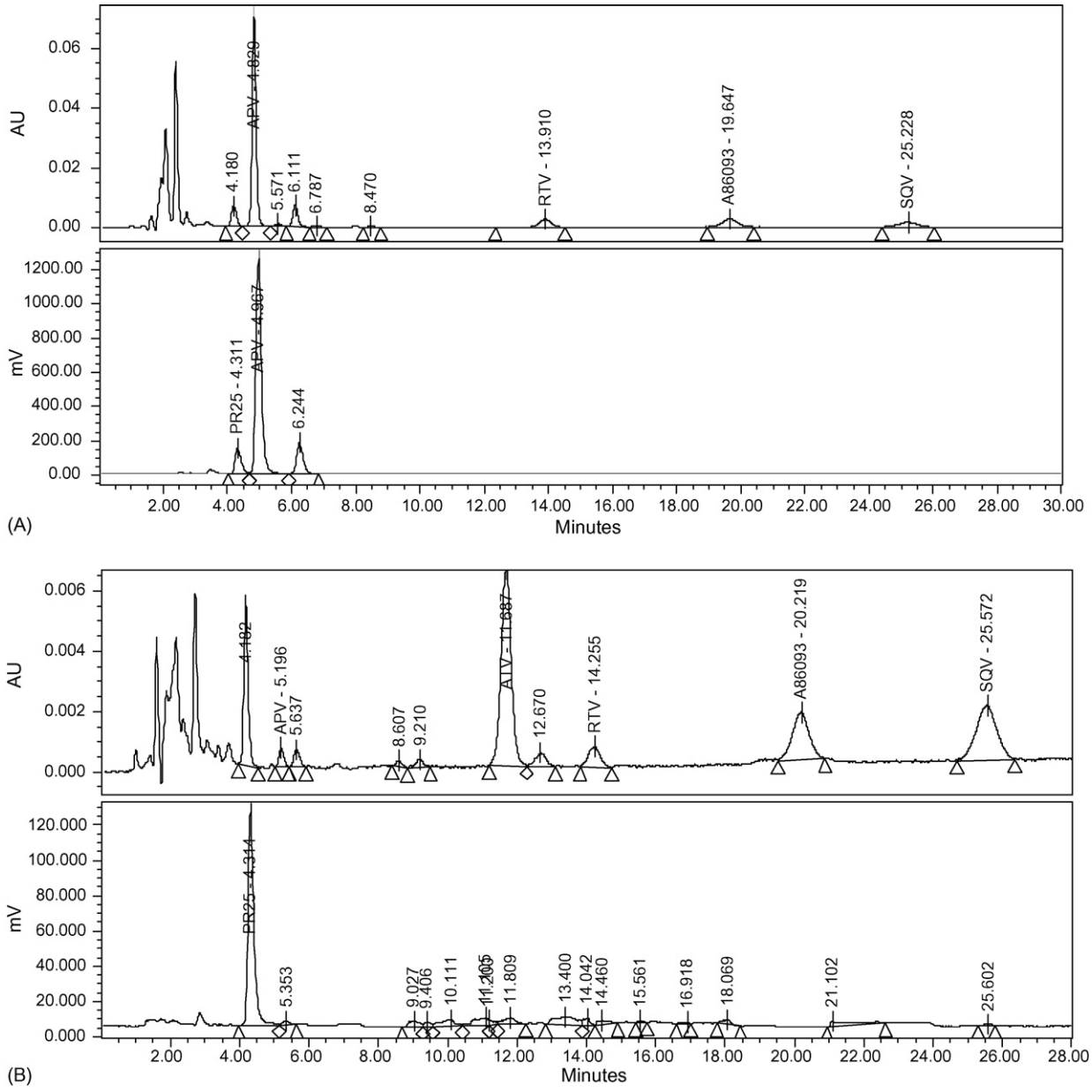


Fig. 4. Chromatograms of patient samples. (A) Chromatogram of patient taking APV 700 mg b.i.d., SQV 1000 mg b.i.d. and RTV 200 mg b.i.d., giving concentrations of 4.49, 0.39 and 0.52 mg/l, respectively; (B) Chromatogram of patient taking ATV 300 mg q.i.d., SQV 1200 mg q.i.d. and RTV 100 mg q.i.d., giving concentrations of 1.62, 0.68 and 0.22 mg/l, respectively. (upper chromatogram with UV detection, lower chromatogram with fluorescence detection).

comparing blank plasma extractions and their chromatograms.

3.9. Proficiency testing

Since 2003, our laboratory participates in the "International Quality Control Program for Therapeutic Drug Monitoring (TDM) in HIV infection" (The Hague, The Netherlands). As all our results are within the ±20% limit around the target values, a good overall precision and accuracy of our analytical procedure has been confirmed.

3.10. Application of the methodology in patient samples.

Plasma samples obtained from patients with antiretroviral therapy were analysed for therapeutic drug monitoring. Chromatograms of plasma extracts of patient samples are shown in Fig. 4.

4. Discussion and conclusions

A robust and sensitive method was developed for bio-analysis of PIs in plasma, suitable for use in patient care. From a practical point of view our method was developed only for the protease

inhibitors including the recently approved atazanavir. The NNRTIs NVP and EFV were analysed by separate methodologies with a higher turnover rate than the simultaneous determination of the protease inhibitors.

Simultaneous determination of NNRTIs and PIs requires the use of a gradient column elution of the compounds, resulting in chromatograms with runtimes of about 1 h.

The latest introduced protease inhibitor tipranavir requires also a separate methodology because of the very long retention times obtained if determined simultaneously with the other PIs.

Optimal extraction parameters were established, such as the pH of extraction, the extraction solvent and the final wash step with hexane. This resulted in a lower limit of quantitation of about 25 ng/ml for all compounds, a chromatogram without interfering peaks and high recovery values.

For APV, we choose measurement in the fluorescent mode with another fluorescent internal standard PR25, because of the higher sensitivity, but also less possible interfering peaks compared to the measurement in UV mode. For higher amprenavir concentrations (above 100 ng/ml), UV detection is still possible.

Intra-assay and inter-assay precision and accuracy were satisfactory as well as the stability of the compounds under different temperature conditions. (i.e. calibration standards below CV of 15%, quality control samples below 20%).

The most important advantages of our analytical method compared to already available methods can be summarized as follows:

(1) isocratic separation of all presently available PIs, including the newly approved ATV on an Allsphere hexyl column packing,
(2) specific and fast determination of all compounds with UV and/or fluorescence detection; especially the specific determination of APV was improved by measuring in the fluorescent mode,
(3) no interferences with comedications or metabolites by using a specific extraction procedure at an optimal pH 9 obtained with a borate buffer and a mixture of hexane and ethylacetate (1/1, v/v), resulting in high sensitivity and high recoveries.

In conclusion, the method proposed in the present paper is reliable and suitable for HIV-TDM and pharmacokinetic studies of all commercially available PIs in the setting of daily patient care in our hospital or clinical studies.

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