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Simultaneous determination of the HIV-protease inhibitors indinavir, nelfinavir, saquinavir and ritonavir in human plasma by reversed-phase high-performance liquid chromatography

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Abstract

A sensitive high-performance liquid chromatographic method has been developed for the simultaneous determination of the four licensed HIV-protease inhibitors indinavir, nelfinavir, saquinavir and ritonavir. An aliquot of 500 μl plasma, spiked with internal standard, was extracted with 0.5 ml 0.1 *M* NH_4OH and 5 ml methyl *tert*.-butyl ether. After evaporating, the residue was dissolved in eluent consisting of acetonitrile–50 m*M* phosphate buffer, pH 5.63 (40:60, v/v). Subsequently, the eluent was washed with hexane. Chromatography was performed using a C_{18} reversed-phase column and gradient elution with a linear increase of acetonitrile from 36 to 66%. Ultraviolet detection at 215 nm was used. Linearity of the method was obtained in the concentration range of 45–30 000 ng/ml for all four analytes. The method was validated extensively and stability tests under various conditions were performed. The assay is now in use to analyse plasma samples from patients treated with (combinations of) HIV-protease inhibitors.

Keywords: Indinavir; Nelfinavir; Saquinavir; Ritonavir

1. Introduction

A breakthrough in the treatment of HIV-positive patients was the introduction of the HIV-protease inhibitors in 1996, to be used in combination with HIV-reverse transcriptase inhibitors. The first protease inhibitors registered were indinavir, saquinavir and ritonavir and recently, the fourth, nelfinavir, became available. First, single protease inhibitors were used in this combination; however, new regimes that are being evaluated now often consist of combinations with two protease inhibitors. The reasons for this development are the limited bioavailability of some of the protease inhibitors, the rapid development of resistance as a result of low plasma levels or irregular ingestion and the possibility to reduce dosing frequency when protease inhibitors are combined.

For all protease inhibitors, separately, a method of analysis has already been described, but we developed a method to analyse all protease inhibitors in one analytical run [1–6]. With this all-in-one method

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it is possible to save time and costs in comparison with the need of four different assays. Also, it augments the quality of care because we can check on incorrect filling in of the sampling forms in the hospitals. This reduces the amount of false negative results when the wrong protease inhibitor has been marked. The method, which has been validated according to standard operating procedures used at our laboratory for the validation of bioanalytical methods, enables us to routinely monitor the plasma concentrations of all patients who use protease inhibitors for the treatment of their HIV infection.

2. Experimental

2.1. Chemicals

Indinavir·1H_2O was obtained from Merck (Rahway, NJ, USA), ritonavir and the internal standard (I.S.) A 86093.0 ((5*S*,8*S*,10*S*,11*S*)-9-hydroxy-2-cyclopropyl-5-(1-methylethyl)-1-[2-(1-methylethyl)-4-thiazolyl]-3,6-dioxo-8,11-bis(phenylmethyl)-2,4,7,12-tetraazatridecan-13-oic acid, 5-thiazolylmethyl-ester) from Abbott (North Chicago, IL, USA), saquinavir mesylate from Roche (Basel, Switzerland) and nelfinavir was isolated from the commercial product (Agouron, San Diego, USA) (Fig. 1). The isolation of nelfinavir was performed by adding acetonitrile to the tablets, and after shaking and filtrating 200 ml of demineralised water was added to the filtrate. By doing so the nelfinavir precipitated. After that the nelfinavir was filtered and the same process was repeated. After washing the residue with water two times, the nelfinavir was freeze-dried and the purity was established by comparing the residue with nelfinavir reference substance by mass-spectrometry. Super gradient acetonitrile, HPLC quality methanol and *n*-hexane were purchased from Lab-Scan Analytical Sciences (Dublin, Ireland). HPLC quality water was obtained from Baker (Deventer, The Netherlands) and ammonia water from OPG (Utrecht, The Netherlands). All other reagents were purchased from Merck (Darmstadt, Germany). The drugs that were investigated for possible interference with the assay were obtained from Sigma (St. Louis, MO, USA) or were extracted from commercial products.

2.2. Standard preparation

Stock solutions of all four analytes and the I.S. were prepared by dissolving the appropriate amount of drug, accurately weighed, in methanol. The stock solutions were kept at 4°C.

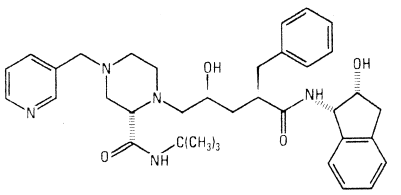
For the preparation of the standard and quality control (QC) samples, first the stock solutions of indinavir, saquinavir, ritonavir and nelfinavir were diluted with blank plasma. To achieve calibration concentrations of 45 to 30 000 ng/ml, appropriate amounts of this diluted stock solution were added to blank plasma. The I.S. was diluted to 50 μg/ml in CH_3OH–water, 1:1. For the QCs concentrations of 75, 1500 and 7500 ng/ml were prepared from a different stock solution. The standard samples and QCs were kept at −18°C.

2.3. Equipment

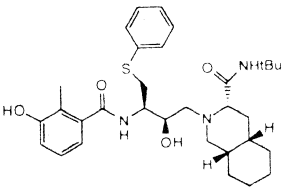
The HPLC system consisted of the following components: a Spectra Physics (SP) 8800 ternary gradient pump; a Separations DG 153 degasser; a Kratos Analytical Spectroflow 757 UV detector; a SP 8780 autosampler; a SP 4290 integrator. The analytical column was a Chrompack Inertsil ODS-2 C_{18} column (5 μm particle size; 150×4.6 mm). The guard column was a Chrompack SS C_8 reversed-phase column (30–40 μm particle size, spherical; 10×3 mm).

2.4. Sample preparation

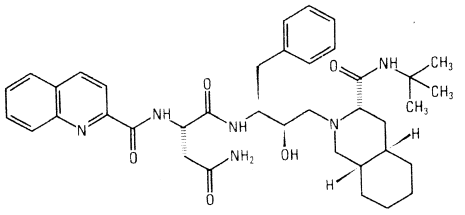
Before use, standard, QC and patient samples were thawed, vortexed and centrifuged for 5 min at 3000 *g*. An aliquot of 500 μl plasma was transferred to a 10-ml glass tube and spiked with 50 μl of the I.S. solution. Second, 0.5 ml NH_4OH and 5 ml methyl *tert*.-butyl ether were added. The tube was closed with a plastic cap. After vortexing for 1 min the samples were subsequently centrifuged for 5 min at 3000 *g*. After freezing at −40°C for 5 min the organic supernatant was transferred to a clean 10-ml glass tube and evaporated to dryness under a nitrogen stream at 37°C. Next, the residue was dissolved in 300 μl eluent (acetonitrile–phosphate buffer, 40:60%, v/v) and 3 ml hexane. The samples were vortexed for 5 min and also centrifuged for 5 min at



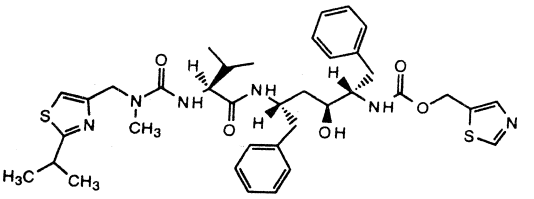
A Indinavir



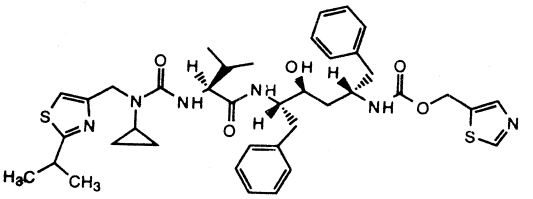
B Nelfinavir



C Saquinavir



D Ritonavir



E Internal Standard (A-86093)

Fig. 1. Chemical structures of indinavir (A), nelfinavir (B), saquinavir (C), ritonavir (D) and the internal standard (E).

3000 *g*. The eluent was transferred to an autosampler vial with inserted for injection into the HPLC system.

2.5. Chromatography

Chromatographic analysis was performed at ambient temperature with gradient elution at a wavelength of 215 nm. The mobile phase consisted of acetonitrile and 50 m*M* KH_2PO_4 adjusted to pH 5.63 with 50 m*M* Na_2HPO_4. In the gradient elution the acetonitrile content of the mobile phase was increased linearly from 36 to 66% during the first 25 min at a flow of 1.5 ml/min. During 2 min the acetonitrile content was returned to 36% and semiequilibrated during 3 min before the next injection. Aliquots of 50 μl were injected into the chromatograph.

2.6. Validation procedures

The specificity of the assay was investigated by analysing blank plasma samples of six different individuals who did not use a protease inhibitor. No interfering peaks with the peaks of nelfinavir, indinavir, saquinavir or ritonavir or the peak of the I.S. were allowed. In addition, a large number of drugs

which are frequently combined with protease inhibitors in HIV-positive patients were chromatographed.

Interference with metabolites was investigated only in samples of patients who used the protease inhibitors, because these substances were not available.

The limit of detection was defined as the lowest quantity that reliably could be differentiated from background level. The difference between a spiked sample and a background sample was tested with a paired *t*-test. Significance was considered if *P* was less than 0.05. Quantities that gave a signal-to-noise ratio of three were first selected to determine the limit of detection.

The lower limit of quantitation was determined as the concentration for which the relative standard deviation and the percentual deviation from the nominal concentration were both less than 20%. Samples were assayed as five replicates.

Accuracy and precision were determined in three separate runs. Five replicates of three different concentrations of indinavir, nelfinavir, saquinavir and ritonavir (75, 1500 and 7500 ng/ml) were determined. The accuracy was calculated as the average percentage of the nominal concentration. Analysis of variance (ANOVA) was used to calculate the inter- and intra-assay variation.

The inter-assay precision was calculated as follows:

$$\frac{\{(\text{day mean square} - \text{error mean square})/n\}^{0.5}}{\text{grand mean}} \cdot 100\%$$

The intra-assay precision was calculated as follows:

$$\frac{(\text{error mean square})^{0.5}}{\text{grand mean}} \cdot 100\%$$

in which day mean square, error mean square and grand mean are terms form ANOVA and *n* is the number of replicates within each day (five) per concentration.

Recovery was determined by comparing the peak areas of seven concentrations in human plasma to the peak areas of identical concentrations prepared in the mobile phase without extraction. This comparison was made for three different runs and subsequently averaged.

2.7. Stability

The stability of all four HIV-protease inhibitors was investigated for a number of conditions that are relevant for clinical pharmacokinetic research. Plasma was spiked with four concentrations of nelfinavir, ritonavir, saquinavir and indinavir (0.3, 1, 3 and 10 μg/ml) and subsequently stored at 20°C for 8 days. Blank blood was also spiked with the four protease inhibitors in two concentrations (0.3 and 3 μg/ml) and kept at 20°C for 5 days.

To determine the freeze–thaw stability of the protease inhibitors, a set of controls was compared with samples of the same batch that were frozen and thawed and stored at room temperature for 6 h. This freeze–thaw cycle was repeated three times and after that the samples were analysed together with the freshly thawed controls. Also the stability of the QCs at −20°C was determined.

Besides the stability in blood and plasma, the stability in eluent after extraction at room temperature, the stability after evaporation to dryness at 4°C and the stability of the stock solution in methanol at −20°C were also determined.

2.8. Pharmacokinetic data

In our hospital HIV-positive patients are asked to participate in a clinical pharmacokinetic experiment for all registered HIV-protease inhibitors, for which the local Ethics Committee has given approval. In this experiment plasma is collected for 0–8 h after ingestion of the standard dose of one or two of the protease inhibitors. For indinavir (Crixivan®) this dose is 800 mg, for nelfinavir (Viracept®) 750 mg, for ritonavir (Norvir®) 600 mg, for saquinavir (Invirase®) 1200 mg (standard dose in the Netherlands) and for the combination of ritonavir and saquinavir 400 mg for both drugs. With these samples the pharmacokinetic profile in these patients was established. Pharmacokinetic analysis was performed by noncompartmental analysis.

3. Results

3.1. Development phase

In the development phase a method was sought to combine steps of several described methods for the individual HIV-protease inhibitors [1–6], but because of the large differences between all these methods a completely new method had to be developed.

The protease inhibitors were dissolved in methanol and chromatography was performed with fixed amounts of acetonitrile in the mobile phase. For a good separation with acceptable retention times a switch to gradient elution was made.

During the development of the extraction phase, several internal standards with structures like the protease inhibitors were tested. A 86093.0 (Abbott) showed a good retention time and a recovery of approximately 100% in the final extraction method. It was therefore chosen as the I.S. for the analysis.

In the gradient elution a pH of 5.1 appeared to be the best starting point. Because of interfering metabolites of nelfinavir and ritonavir, the pH was changed to 5.6. A slightly lower pH may result in a plasma peak under the indinavir peak. Also the peak of one of the metabolites of nelfinavir may interfere with the saquinavir peak. At a higher pH level the separation between saquinavir and ritonavir diminishes. This stresses the importance to exactly set the pH at the desired level.

Different wavelengths were examined. After all, 215 nm still seemed to be the best wavelength and noise reduction had to be accomplished by other methods.

In search for an extraction method, different extractions described in the published methods were tried. They all were not good enough. The best extraction appeared to be with methyl *tert.*-butyl ether containing a small proportion of methanol. NH_4OH was added to obtain a higher recovery of indinavir during the extraction. Because of disturbing peaks in the next injection and a varying baseline, a hexane washing step was added.

Only glass tubes can be used in this method, because the organic solvents will dissolve components of polypropylene tubes.

Impurities in the applied water resulted in disturbing peaks, so a change to HPLC quality water (Baker) was made, which solved this problem.

Peroxides in the methyl *tert.*-butyl ether cause decomposition of nelfinavir, so extra pure ether (Merck) is used in our method.

Fig. 2 gives representative chromatograms of a spiked plasma sample with and without 1050 ng/ml of all four protease inhibitors.

3.2. Validation phase

Blank plasma of six different individuals who did not use a HIV-protease inhibitor, did not contain interfering compounds that co-eluted with the protease inhibitors or the I.S. Samples of patients using a protease inhibitor were analysed to assess the absence of disturbance of metabolites with the other protease inhibitors. The drugs listed in Table 1, which are frequently used by HIV-positive patients, did not show interference with the analytical method.

At 40 ng/ml for all four protease inhibitors the percentual deviation of the nominal concentration and the relative standard deviation (of five replicates) were both less than 20%. Therefore, this concentration was considered to be the lower limit of quantitation, while the detection limit was 15 ng.

Table 2 shows the results of the determination of the accuracy and the precision of the assay.

The average recoveries (determined in three separate runs) were 91.8% (SD 5.7%) for indinavir, 73.4% (SD 5.5%) for nelfinavir, 93.3% (SD 4.4%) for saquinavir and 94.1% (SD 6.1%) for ritonavir.

The calculations of protease inhibitor concentrations from the calibration curve were performed with linear regression after logarithmic transformation of the data, because of the large range of concentrations (45 to 30 000 ng/ml).

The stability experiments demonstrated that the protease inhibitors are stable under the investigated conditions, as shown in Table 3.

3.3. Pharmacokinetic data

This paper reports an example of the pharmacokinetic profile of the protease inhibitors for three HIV-infected patients using indinavir, nelfinavir or saquinavir plus ritonavir in their antiretroviral

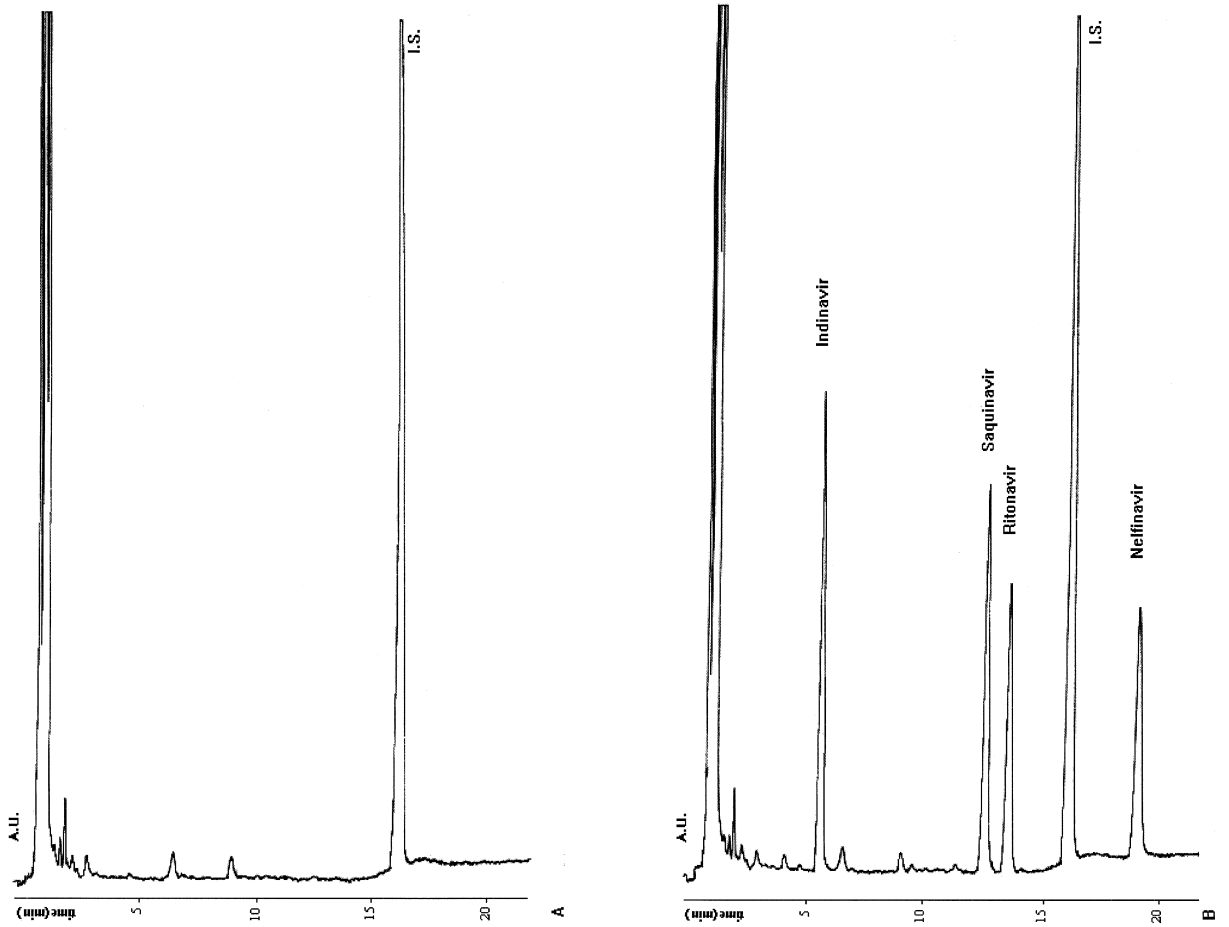


Fig. 2. (A) Chromatogram of a blank plasma sample containing 5000 ng/ml of I.S. (B) Chromatogram of a spiked plasma sample containing 5000 ng/ml of I.S. and 1050 ng/ml of indinavir, nelfinavir, saquinavir and ritonavir.

Table 1
Comedicated drugs that did not interfere with the analytical method

Acetaminophen	Didanosine	Ketoconazole	Rifampin
Acyclovir	Erythromycin	Lamivudine	Stavudine
Amphotericin B	Ethambutol	Methadone[b]	Sulfamethoxazole
Amoxycillin	Famotidine	Nevirapine	Sulfametrol[a]
Atovaquone	Fluconazol	Oxazepam	Trimethoprim
Azithromycine	Folic acid	Pentamidine	Zalcitabine
Clarithromycin	Folinic acid	Phenytoin	Zidovudine
Clindamycin	Ganciclovir	Pyrazinamide	
Caffeine	Isoniazid	Pyrimethamine	
Dapsone	Iitraconazole	Rifabutin[b]	

[a] Metabolites interfered only before extraction.
[b] Peak after extraction, but no interference.

Table 2
Accuracy and precision data

Protease inhibitor	Concentration (ng/ml)	Accuracy (%)	Intra-assay precision (%)	Inter-assay precision (%)
Indinavir	76	104	7.48	3.46
	1520	108	2.12	0.43
	7570	107	2.61	2.03
Nelfinavir	79	96	5.35	0.00[a]
	1580	100	3.24	0.00[a]
	7860	100	4.52	2.43
Ritonavir	82	102	8.11	3.53
	1640	108	2.00	1.56
	8180	105	2.52	0.43
Saquinavir	66	101	9.67	5.36
	1320	103	1.59	0.98
	6600	100	3.78	0.25

[a] In cases where the error mean square is greater than the day mean square, the resulting variance estimate is negative. In these cases no significant additional variation is observed as a result of performing the assay at different days.

Table 3
Stability experiments

Protease inhibitor	Concentration (ng/ml)	Condition	Matrix	Time interval	Concentration found (mean% (SD))	
Indinavir	300–10 500	20°C	Plasma	8 days	98.3	(2.8)
Nelfinavir	300–10 500	20°C	Plasma	8 days	110.0	(2.9)
Saquinavir	300–10 500	20°C	Plasma	8 days	97.8	(3.9)
Ritonavir	300–10 500	20°C	Plasma	8 days	98.5	(2.4)
Indinavir	300, 3000	20°C	Blood	5 days	109.0	(5.7)
Nelfinavir	300, 3000	20°C	Blood	5 days	121.5	(26.2)
Saquinavir	300, 3000	20°C	Blood	5 days	114.0	(15.6)
Ritonavir	300, 3000	20°C	Blood	5 days	106.0	(4.2)
Indinavir	75–7500	Freeze–thaw	Plasma	3 cycles	99.3	(9.3)
Nelfinavir	75–7500	Freeze–thaw	Plasma	3 cycles	113.7	(19.7)
Saquinavir	75–7500	Freeze–thaw	Plasma	3 cycles	110.3	(18.8)
Ritonavir	75–7500	Freeze–thaw	Plasma	3 cycles	102.0	(9.5)
Indinavir	75–7500	−20°C	Plasma	3 months	101.8	(6.8)
Nelfinavir	75–7500	−20°C	Plasma	3 months	98.8	(9.5)
Saquinavir	75–7500	−20°C	Plasma	3 months	95.3	(7.8)
Ritonavir	75–7500	−20°C	Plasma	3 months	107.5	(2.6)
Indinavir	300–30 000	20°C	Eluent	20 days	98.3	(1.2)
Nelfinavir	300–30 000	20°C	Eluent	20 days	99.3	(2.5)
Saquinavir	300–30 000	20°C	Eluent	20 days	98.7	(0.6)
Ritonavir	300–30 000	20°C	Eluent	20 days	98.0	(0.0)
Indinavir	300–30 000	4°C	Dry	20 days	97.7	(5.5)
Nelfinavir	300–30 000	4°C	Dry	20 days	97.3	(3.5)
Saquinavir	300–30 000	4°C	Dry	20 days	95.0	(1.7)
Ritonavir	300–30 000	4°C	Dry	20 days	100.3	(2.1)
Indinavir	1260	−20°C	Methanol	7 months	107.5	
Nelfinavir	1310	−20°C	Methanol	7 months	109.3	
Saquinavir	1120	−20°C	Methanol	7 months	102.6	
Ritonavir	1360	−20°C	Methanol	7 months	106.0	

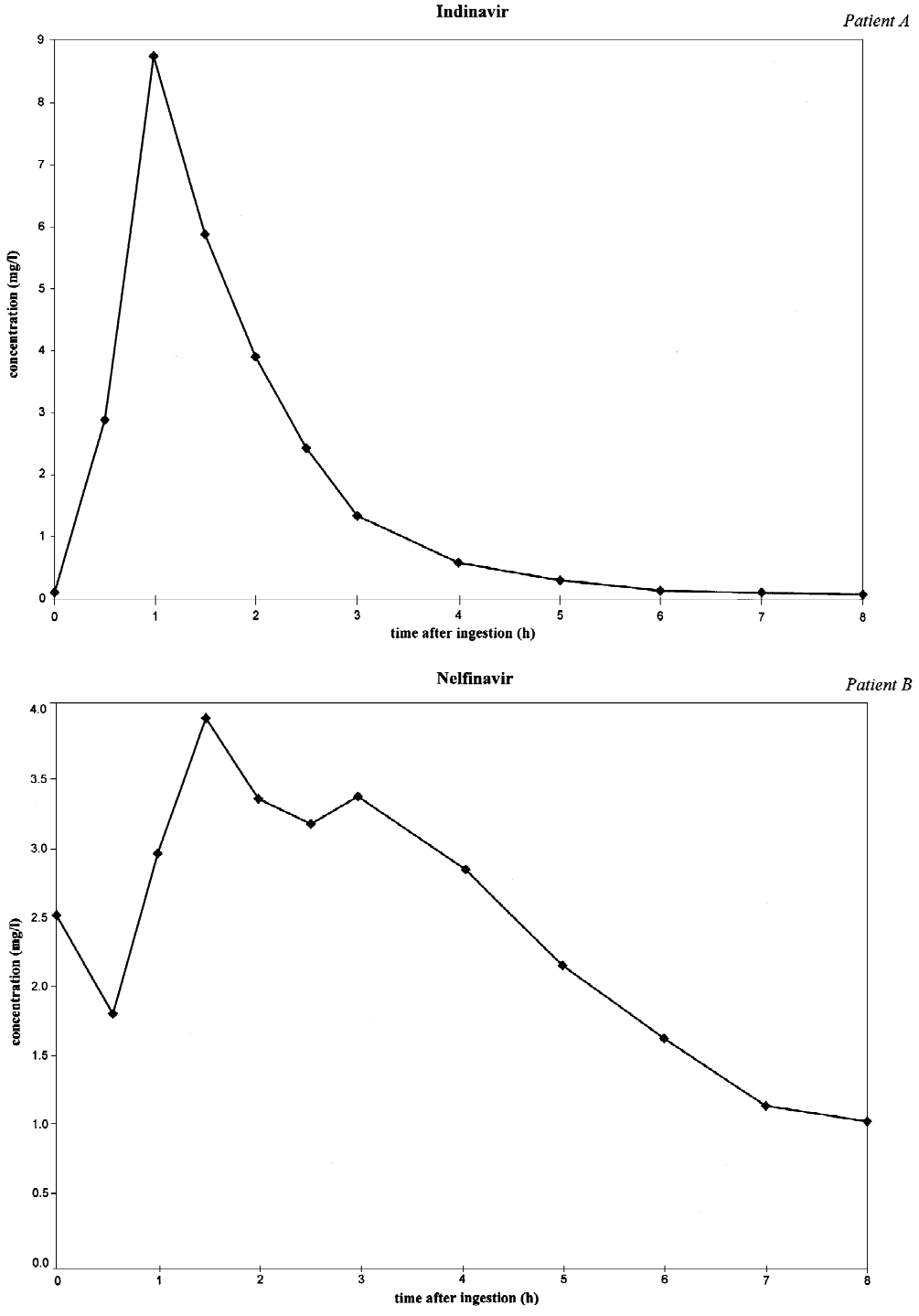
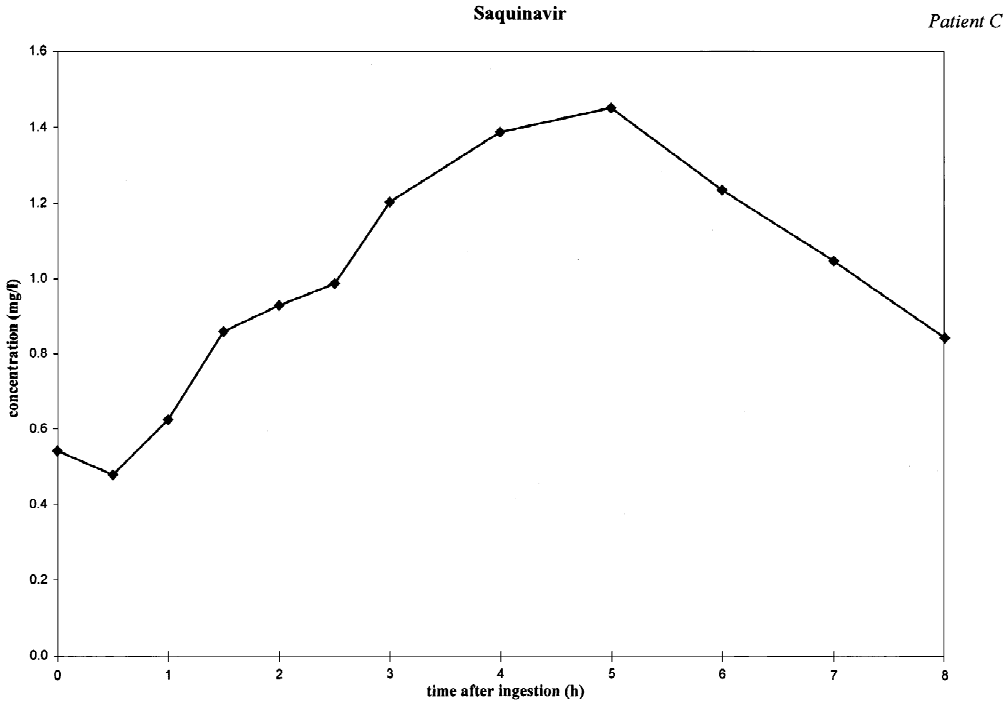


Fig. 3. Plasma concentration vs. time curves for HIV-infected patients using 800 mg of indinavir tid (A), 750 mg of nelfinavir tid (B) or 400 mg of saquinavir (Fortovase®) plus 400 mg of ritonavir bid (C).



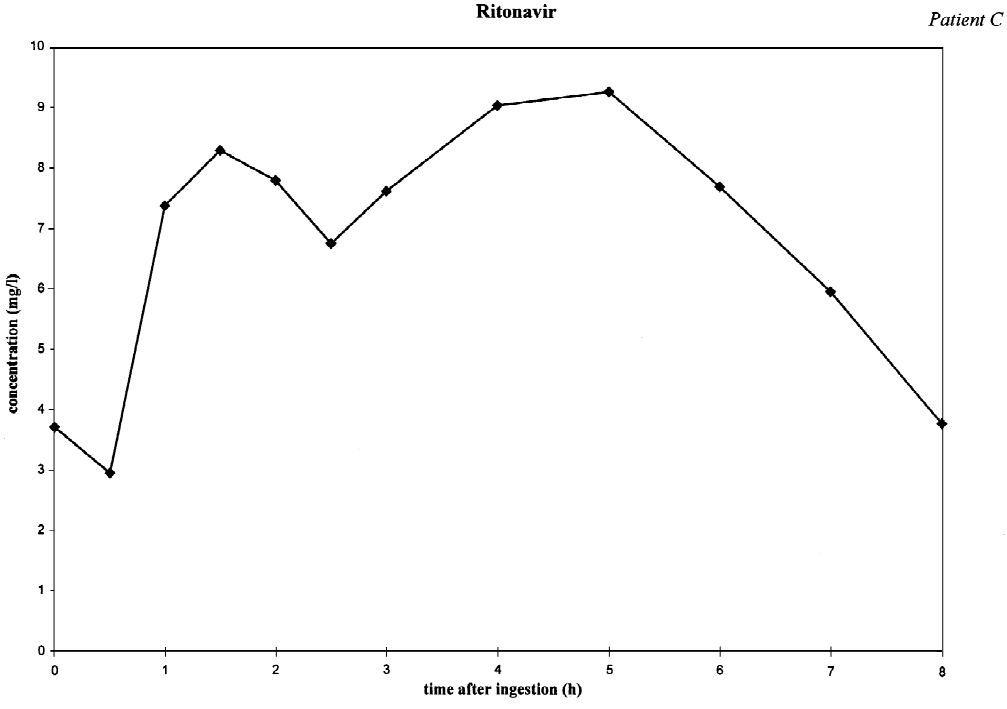


Fig. 3. (*continued*)

Table 4
Pharmacokinetic data

Patient code	A	B	C	
Patient characteristics				
Gender (M/F)	M	M	M	
Age (year)	33	36	32	
Length (cm)	178	180	184	
Weight (kg)	84	80	86	
Protease inhibitor	Indinavir	Nelfinavir	Saquinavir + ritonavir	
Dose (mg)	800 mg tid	750 mg tid	400 mg bid	
Co-medication	Stavudine Zalcitabine	Saquinavir Nevirapine Zalcitabine	Cotrimoxazole	
Pharmacokinetic parameters	Indinavir	Nelfinavir	Saquinavir	Ritonavir
C_{max} (mg/l)	8.7	3.9	1.5	9.3
T_{max} (h)	1.0	1.5	5.0	5.0
C_{min} (mg/l)	0.08	1.0	0.48	3.8
AUC_{0-8} (h·mg/l)	14.0	14.9	8.5	57.0
Cl/F (l/h)	55.6	39.7	30.9	5.9
Vd/F (l)	271	154	163	17
$T_{1/2}$ (h)	3.4	2.7	3.7	2.0

combination therapy. Fig. 3 shows the curves as measured by our method and Table 4 lists the characteristics and the pharmacokinetic parameters of these patients. Only for indinavir and nelfinavir data are available from the literature. For indinavir the C_{max} is 7.7 mg/l after 0.8 h and the C_{min} is 0.15 mg/l [7]. The elimination half life is 2 h. According to the product monograph for nelfinavir the C_{max} is 3–4 mg/l after 2–4 h and the C_{min} is 1–1.5 mg/l. The elimination half life is between 3.5 and 5 h [8].

For the three patients discussed here the bioanalytical method appeared to be suitable for the determination of HIV-protease inhibitor concentrations, also if they use two protease inhibitors at the same time.

4. Discussion

This paper describes the development, validation and application of a bioanalytical method for the determination of the four registered HIV-protease inhibitors. For all protease inhibitors a method to determine them in plasma or serum already has been published [1–6]. However, these were all methods to determine one single protease inhibitor at a time. The method we describe is a sensitive method to analyse all four protease inhibitors simultaneously. In the era of combined use of protease inhibitors this is a real advantage. With 500 μl of plasma all protease inhibitors can be analysed in one injection, after a simple extraction procedure. A hexane washing is needed to obtain a clean chromatogram, but after that it is possible to measure at a low wavelength (215 nm). Gradient elution is necessary to obtain a good separation and good k' values. The purity of the applied water and methyl *tert*.-butyl ether is important, just like the setting of the desired pH and the use of glass instead of polypropylene. If these conditions are controlled closely, this method is sensitive enough to determine a wide range of concentrations for all four protease inhibitors.

The method appears to be applicable to determine plasma concentrations of all protease inhibitors in the patients described in this article and is currently being used to analyse samples of patients treated with one or more of the approved protease inhibitors. Because interference with other medication is rare, the method is usable even in this sometimes heavily comedicated patient group.

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