Simultaneous quantitative determination of the HIV protease inhibitors amprenavir, indinavir, nelfinavir, ritonavir and saquinavir in human plasma by ion-pair high-performance liquid chromatography with ultraviolet detection

R.P.G. van Heeswijk[a],*, R.M.W. Hoetelmans[a], R. Harms[a], P.L. Meenhorst[b], J.W. Mulder[b], J.M.A. Lange[c], J.H. Beijnen[a]

[a]Department of Pharmacy and Pharmacology, Slotervaart Hospital, Amsterdam, The Netherlands
[b]Department of Internal Medicine, Slotervaart Hospital, Amsterdam, The Netherlands
[c]The Netherlands AIDS Therapy Evaluation Center (NATEC), Department of Internal Medicine, Academic Medical Center, Amsterdam, The Netherlands

Abstract

A reversed-phase high-performance liquid chromatographic assay for the simultaneous quantitative determination of five HIV protease inhibitors (i.e. amprenavir, indinavir, nelfinavir, ritonavir, and saquinavir) in human plasma is described. Sample pretreatment consisted of solid-phase extraction prior to ion-pair, reversed-phase high-performance liquid chromatography with ultraviolet detection at 210 nm (amprenavir, indinavir and nelfinavir) and 239 nm (saquinavir and ritonavir). For amprenavir, indinavir and saquinavir the method has been validated over the range of 25 ng/ml to 25 µg/ml using a 0.6 ml sample volume. For nelfinavir and ritonavir the method has been validated over the range of 50 ng/ml to 25 µg/ml. The method proved to be accurate, with an average accuracy at four concentrations ranging from 90.6 to 109.2%, and precise, with the within-day and between-day precision ranging from 1.8 to 6.7%, and 0.7 to 7.6%, respectively. The protease inhibitors which can be quantified by using this assay proved to be stable under various conditions. This assay can readily be used in a hospital laboratory for the routine monitoring of plasma concentrations of these protease inhibitors. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Amprenavir; Indinavir; Nelfinavir; Ritonavir; Saquinavir

1. Introduction

Human immunodeficiency virus (HIV) protease inhibitors are new and potent antiretroviral drugs that have changed the treatment of infection with HIV dramatically. Inhibition of HIV protease activity by representatives of this class of antiretroviral drugs leads to production of non-infectious virions, which have the morphological features of immature particles [1]. To date, four protease inhibitors have been approved by the FDA under its accelerated approval regulations (i.e. indinavir, nelfinavir, ritonavir, and saquinavir) and others are currently being investi-

*Corresponding author.

gated in phase III clinical trials (such as amprenavir, formerly known as 141W94/VX-478).

Current treatment regimens of HIV infection consist of a combination of antiretroviral drugs. Because of the increasing number of antiretroviral drugs available, the number of different combinations is rapidly growing. Some very promising combination regimens contain two protease inhibitors [2,3]. Because of inhibition of cytochrome P450 enzymes by protease inhibitors, combinations of these drugs may lead to favourable pharmacokinetic interactions, and to increased plasma concentrations and antiviral effect of concurrently administered protease inhibitors [4,5]. Furthermore, the protease inhibitors show non-overlapping toxicities and, possibly, divergent resistance patterns [6], making combination of these drugs attractive.

Because of substantial inter-individual variation in exposure to these drugs and the increasing evidence for pharmacokinetic–pharmacodynamic relationships, therapeutic drug monitoring may be warranted to prevent or delay the occurrence of viral resistance for protease inhibitors, and to ensure optimal therapy for HIV infected patients [7,8]. Therefore, there is an urgent need for relatively simple bioanalytical assays exploitable with standard hospital laboratory equipment, to quantitate plasma concentrations of various protease inhibitors in one analytical run. We have developed and validated such a methodology for the simultaneous, quantitative determination of amprenavir, indinavir, nelfinavir, ritonavir, and saquinavir in human plasma (Fig. 1).

2. Experimental

2.1. Equipment

The HPLC system consisted of a Model 6000A solvent delivery pump (Waters Associates, Milford, MA, USA), a Model AS300 automatic sample injection device (Spectra Physics, San Jose, CA, USA), a Spectra 200 programmable wavelength detector (Spectra Physics), and a Chromjet® integrator (Spectra Physics). The analytical column was a Zorbax® SB-C$_{18}$ column (75×4.6 mm I.D./particle size 3.5 μm; Rockland Technologies, Newport, DE, USA) protected by a Chromguard® C$_{18}$ column

(10×3 mm I.D./Chrompack Nederland, Middelburg, The Netherlands). Analytical runs were processed by the PC 1000 system software (version 3.01, Spectra Physics). UV spectra of protease inhibitor solutions in mobile phase were recorded with a Model 918 UV–VIS spectrophotometer (GBC Scientific Equipment, Dandenons, Australia).

2.2. Chemicals

Amprenavir was a kind gift of Vertex Pharmaceuticals Inc. (Cambridge, MA, USA), indinavir–H$_2$O was a kind gift of Merck Sharp and Dohme (Haarlem, The Netherlands), nelfinavir mesylate and saquinavir mesylate were supplied by Roche Products, Research and Development (Welwyn Garden City, UK), ritonavir was provided by Abbott Laboratories (Abbott Park, IL, USA). Acetonitrile (HPLC supra-gradient) and methanol (supra-gradient) were purchased from Biosolve (Valkenswaard, The Netherlands). Hydrochloric acid 37% p.a., ammonium acetate p.a., sodium acetate p.a., and hexane-1-sulfonic acid (sodium salt) p.a. were purchased from Merck (Darmstadt, Germany). Distilled water was used throughout. Blank, drug-free plasma was obtained from the Central Laboratory of Blood Transfusion Service (Amsterdam, The Netherlands).

2.3. Preparation of standards

Separate stock solutions of amprenavir, indinavir and nelfinavir were prepared by dissolving the appropriate amount of drug, accurately weighed, in methanol to yield a final drug concentration (as base) of 400 μg/ml. Separate stock solutions of ritonavir and saquinavir were prepared by dissolving the appropriate amount of drug, accurately weighed, in 50% (v/v) methanol to yield a final drug concentration (as base) of 400 μg/ml. Each of these stock solutions was used for preparation of a solution containing the five protease inhibitors in a similar concentration (i.e. 92 μg/ml). For the preparation of calibration curves this solution was used. For the preparation of quality control samples, five independently prepared stock solutions of the protease inhibitors were made, as well as a working solution

A. Amprenavir

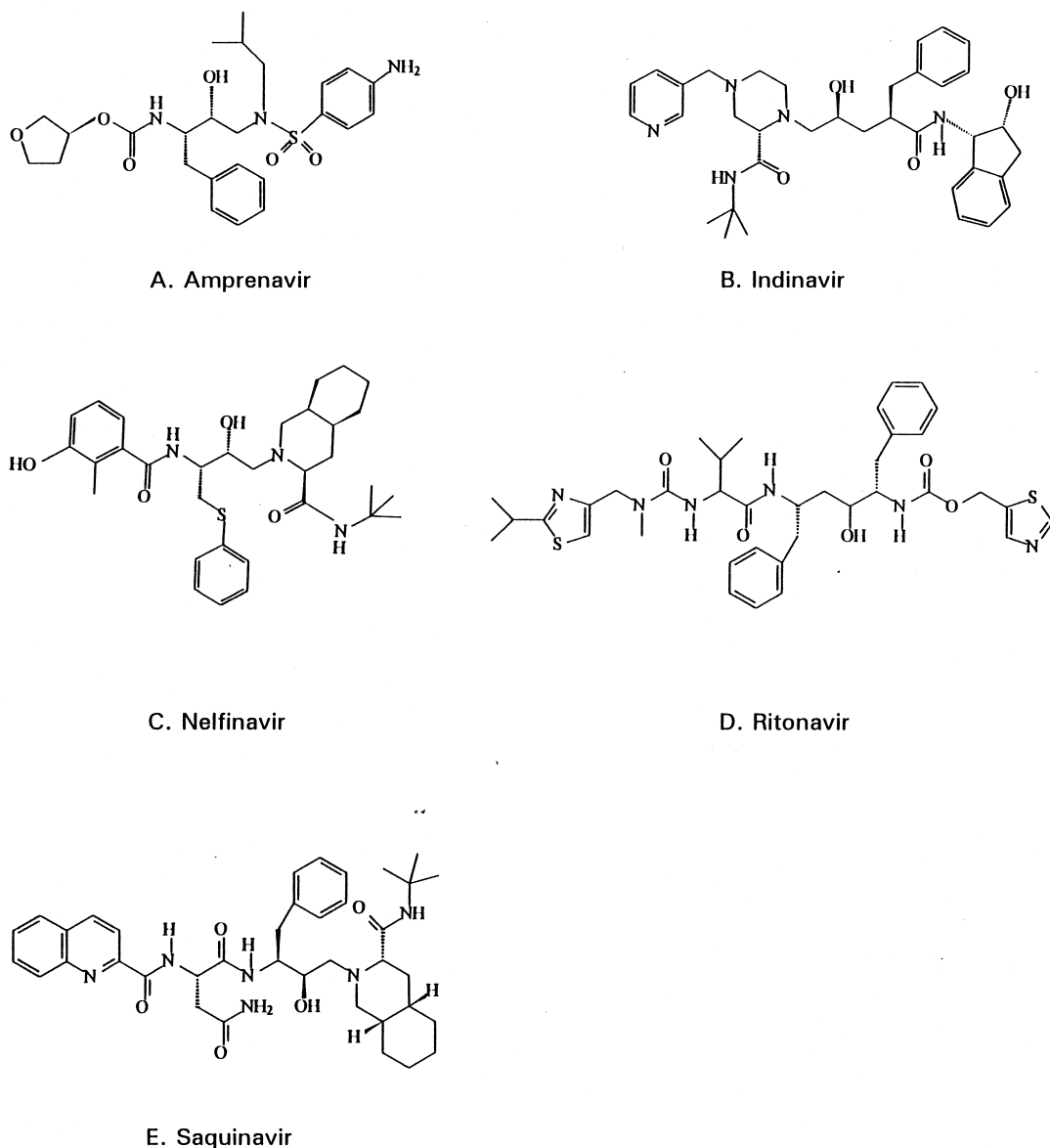B. Indinavir

C. Nelfinavir

D. Ritonavir

E. Saquinavir

Fig. 1. Molecular structures of amprenavir (A), indinavir (B), nelfinavir (C), ritonavir (D), and saquinavir (E).

containing all protease inhibitors in a concentration of 75 µg/ml. Drugs for interference analysis were obtained from the hospital pharmacy (Slotervaart Hospital, Amsterdam, The Netherlands), either as solutions for injection or after dissolving solid reference material in 50% (v/v) methanol (final concentration 500 µg/ml in 50% (v/v) methanol).

2.4. Sample pretreatment

For the preparation of standard samples the solution containing all protease inhibitors was diluted with an appropriate amount of 50% (v/v) methanol. To achieve calibration concentrations of 25 ng/ml to 25 µg/ml, appropriate quantities of the various

diluted solutions were added to blank plasma in Eppendorf tubes (Merck). The solutions were mixed on a vortex mixer for 10 s. Next, 600 µl of plasma was mixed on a vortex mixer for 10 s with 600 µl of a 0.1 M ammonium acetate solution. The tubes were then centrifuged for 3 min at 10 500×g, and 1.00 ml of the diluted samples was subjected to solid-phase extraction. Prior to solid-phase extraction, C_2 extraction columns (100 mg, 1 ml; Varian, Harbor City, CA, USA) were placed on a vacuum elution manifold (Baker 12-SPE System; J.T. Baker, Phillipsburg, NJ, USA), and rinsed with 1.0 ml of acetonitrile, followed by 1.0 ml of 0.1 M ammonium acetate solution in distilled water. Care was taken that the columns did not run dry. Next, 1.0 ml of the diluted plasma samples was transferred onto the columns and drawn into them by applying reduced pressure. The columns were then washed with 1.0 ml of a mixture of acetonitrile and 0.1 M ammonium acetate solution (3:7, v/v), followed by vacuum suction for 1 min. Elution of the absorbed analyte was performed with 400 µl of a mixture of acetonitrile and 2.5 mM ammonium acetate solution (8:2, v/v) into Eppendorf tubes, and evaporated to dryness under a gentle stream of nitrogen at 40 C. The residues were redissolved in 100 µl of mobile phase, mixed on a vortex mixer for 60 s and centrifuged for 3 min at 10 500×g. The clear supernatants were brought into autosampler vials with inserts.

2.5. Chromatography

The chromatographic analysis was performed at ambient temperature using a C_{18} analytical column as previously described and a mobile phase composed of acetonitrile plus distilled water containing 25 mM sodium acetate and 25 mM hexane-1-sulfonic acid and adjusted to pH 6.0 with hydrochloric acid 37% (40.5:59.5, v/v). Prior to use, air was removed by leading helium through the solution. The run-time of the assay was 20 min. During the first 6 min of the run, absorbance was measured at 210 nm for detection of amprenavir and indinavir. Six minutes after injection the detection wavelength was automatically switched to 239 nm for detection of ritonavir and saquinavir, and 12 min after injection the detection wavelength was automatically switched back to 210 nm for detection of nelfinavir. The

flow-rate was maintained at 1.5 ml/min. Aliquots of 75 µl were injected.

2.6. Specificity and selectivity

The separation from endogenous compounds was investigated by the analysis of six different blank plasma samples. The following compounds were investigated for interference with the analytical method (including the sample pretreatment): delavirdine, didanosine, fluconazole, folinic acid, ganciclovir, lamivudine, methadone, methotrexate, nevirapine, oxazepam, pyrazinamide, ranitidine, rifampin, stavudine, sulphamethoxazole, trimethoprim, zalcitabine, zidovudine, and zidovudine glucuronide in a final concentration of 20 µg/ml in plasma.

2.7. Limit of detection and limit of quantitation

For the concentration to be accepted as the lower limit of quantitation (LLQ), the percent deviation from the nominal concentration (measure of accuracy) and the relative standard deviation (measure of precision) were to be less than 20%. The upper limit of quantitation (ULQ) of all compounds was arbitrarily defined as 25 µg/ml.

2.8. Accuracy, precision, linearity and recovery

Accuracy, between-day and within-day precision of the method were determined by assaying six replicate plasma samples at four different concentrations (101, 505, 1,012 and 10 120 ng/ml) in three analytical runs. Accuracy was calculated as the percentage of the nominal concentration. The within-day and between-day precision were obtained by analysis of variance (ANOVA) for each concentration, using the analytical run as the grouping variable.

Linearity of three calibration curves was tested with the F-test for lack of fit, using a weight factor of [1/conc.] for amprenavir, indinavir, saquinavir and ritonavir and a weight factor of [1/conc.^{2}] for nelfinavir [9,10]. For the construction of each calibration curve for amprenavir, indinavir, nelfinavir, ritonavir and saquinavir, nine spiked plasma samples were analyzed in duplicate.

The average recovery of each of the protease inhibitors was determined in three analytical runs by calculating the ratio of the slope of a calibration curve in extracted plasma and the slope of a calibration curve of non-processed solutions in mobile phase.

2.9. Stability

Blank plasma samples were spiked with an aliquot of diluted protease inhibitor solution to yield initial concentrations of approximately 870 and 7200 ng/ml. These samples were stored for 1 h at 60°C, 24 h at 25°C, 7 days at 4°C, 30 days at −30°C and 30 days at −30°C plus three freeze–thaw cycles. For each concentration and each storage condition four replicates were analyzed. The concentration of each protease inhibitor was related to the initial concentration as determined for the samples that were freshly prepared and processed immediately.

2.10. Analysis of patient samples

Plasma samples from three HIV-1 infected patients who used a combination of two protease inhibitors as part of a combination regimen were analyzed with the currently described method. One patient used 600 mg saquinavir tid plus 750 mg nelfinavir tid, one used 400 mg saquinavir bid plus 400 mg ritonavir bid and the third used 800 mg indinavir bid plus 100 mg ritonavir bid. Twelve heparinized blood samples were drawn during 8 h after ingestion of the morning dose of saquinavir and ritonavir, and of indinavir and ritonavir, after an overnight fast. For the patient who used nelfinavir in combination with saquinavir only ten blood samples were drawn during 6 h after ingestion of the antiretroviral drugs. Plasma was separated by centrifugation at $3000 \times g$ for 10 min and was immediately stored at −30°C until analysis.

2.11. Statistics

All statistical calculations were performed with the Statistical Product and Service Solutions (SPSS) for Windows, version 6.1 (SPSS, Chicago, IL, USA). Correlations were considered statistically significant if calculated *P*-values were 0.05 or less.

3. Results and discussion

3.1. Chromatography and detection

Basis of the current methodology was our validated assay for the determination of saquinavir in human plasma, saliva and cerebrospinal fluid [11]. This method was initially used for the simultaneous analysis of amprenavir, indinavir, nelfinavir, ritonavir, and saquinavir, but resulted in poor resolution of saquinavir and nelfinavir. This problem was resolved by adjusting the pH of the mobile phase from 4.0 to 6.0. (Fig. 2).

Subsequently, UV spectra of amprenavir, indinavir, saquinavir, ritonavir, and nelfinavir in mobile phase were recorded. All protease inhibitors demonstrated significant absorbance at 210 nm. Saquinavir and ritonavir, however, also possessed significant absorption at 239 nm, while amprenavir displayed an absorption maximum at 266 nm. Because UV detection at higher wavelengths is more specific, we preferred saquinavir and ritonavir detection at 239 nm. Amprenavir could not be detected at 266 nm in our chromatographic system because the small difference in retention time of indinavir and amprenavir did not allow for programmed wavelength changes. However, no endogenous compounds were detected at 210 nm with the same retention time as amprenavir. Thus, during the first 6 min after injection of a sample amprenavir and indinavir were detected at 210 nm. After 6 min the wavelength was automatically changed to 239 nm for
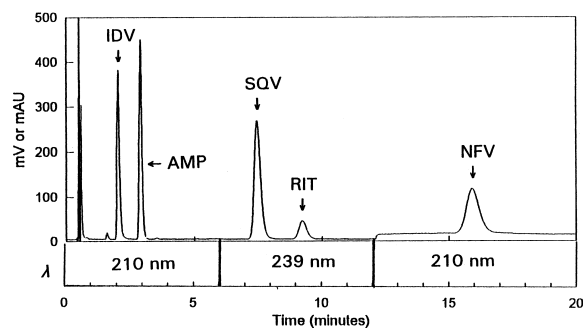

Fig. 2. Chromatogram of a solution of 1000 ng/ml of amprenavir (AMP), indinavir (IDV), nelfinavir (NFV), ritonavir (RIT), and saquinavir (SQV) in mobile phase.

Table 1
Range of calibration curves, detection wavelength, retention times, specific extinctions and average recoveries of protease inhibitors from spiked human plasma

Protease inhibitor	Range of calibration curve	Detection wavelength (nm)	Retention time (min)	Specific extinction	Recovery (mean±S.D.) (%)
Indinavir	25 ng/ml–25 μg/ml	210	2.0	399	93.4±1.1
Amprenavir	25 ng/ml–25 μg/ml	210	2.9	486	94.4±1.4
Saquinavir	25 ng/ml–25 μg/ml	239	7.4	659	89.3±1.7
Ritonavir	50 ng/ml–25 μg/ml	239	9.3	123	73.7±2.9
Nelfinavir	50 ng/ml–25 μg/ml	210	16.1	689	70.1±2.7

Abbreviations: S.D.=standard deviation.

detection of ritonavir and saquinavir, and after 12 min the wavelength was changed back to 210 nm for detection of nelfinavir (Fig. 2). The specific extinctions of amprenavir, indinavir, saquinavir, ritonavir, and nelfinavir at the reported wavelengths were 486, 399, 659, 123, and 689 nm, respectively. The specific extinction of ritonavir (being the lowest) is not expected to hamper detection of ritonavir concentrations in plasma because these are in the μg/ml range.

A suitable internal standard was not available at

the time of development of the assay. However, the assay as described gives satisfactory validation results without the use of an internal standard.

3.2. Sample pretreatment and recovery

Knebel et al. reported solid-phase extraction of 250 μl of human plasma with high recovery (>95%) of saquinavir with use of C_2 extraction columns [12]. In order to increase the LLQ of our RP-HPLC assay for quantification of saquinavir we used 600 μl of

Table 2
Accuracy and precision for the analysis of amprenavir, indinavir, nelfinavir, ritonavir and saquinavir in human plasma

Compound	Concentration (ng/ml)	Accuracy (%)	C.V. (%)	Precision (%)		*n*
				Between-day	Within-day	
Amprenavir	101	90.6	5.8	3.2	5.0	17
	506	99.0	2.8	2.4	2.0	18
	1011	101.7	4.6	2.1	4.3	18
	10 109	96.0	5.5	5.6	5.6	18
Indinavir	101	96.4	6.5	2.1	6.2	17
	507	98.6	3.7	3.6	2.1	18
	1013	100.7	4.9	1.7	4.7	18
	10 130	98.9	5.4	5.5	5.5	18
Nelfinavir	508	100.2	7.5	6.3	5.4	18
	1015	91.8	5.8	4.0	4.7	18
	10152	103.9	6.0	6.4	6.4	18
Ritonavir	101	98.9	9.4	7.6	6.8	17
	506	105.4	4.1	3.8	2.6	18
	1011	109.2	5.7	1.0	5.6	18
	10 113	102.3	8.0	6.7	6.7	18
Saquinavir	101	102.4	4.8	2.8	4.2	17
	506	105.0	3.6	3.7	1.8	18
	1012	106.1	4.9	0.7	4.9	18
	10 123	100.7	5.3	5.5	5.5	18

Abbreviations: C.V.=coefficient of variation; *n*=number of replicates.

human plasma [13]. The same methodology yielded high recoveries for indinavir and amprenavir and somewhat lower recovery for ritonavir and nelfinavir. Average recoveries over the concentration range of the standard curve for amprenavir, indinavir, saquinavir (all range from 25 ng/ml to 25 μg/ml), nelfinavir and ritonavir (range from 50 ng/ml to 25 μg/ml) as determined in three analytical runs are shown in Table 1. The relatively lower recoveries of ritonavir and nelfinavir (i.e. 73.7 and 70.1%, respectively) had no negative effects on the assay performance (Table 2).

3.3. Specificity and selectivity

Blank plasma from six different individuals showed no interfering endogenous substances in the analysis of amprenavir, indinavir, nelfinavir,

saquinavir and ritonavir (Fig. 3A). Potentially co-administered drugs or metabolites tested had retention times (t_r) that were different from the protease inhibitors (methadone, $t_r = 4.6$ min, rifampin, $t_r = 7.0$ min, oxazepam, $t_r = 1.3$ min, and delavirdine, $t_r = 2.6$ min) or were not detected with the described bioanalytical method.

3.4. Limit of quantitation

The lower limit of quantitation (LLQ) was defined as the concentration for which the percent deviation from the nominal concentration and the relative standard deviation were both less than 20%, as determined in three separate analytical runs.

For amprenavir, indinavir and saquinavir a concentration of 25 ng/ml was defined to be the LLQ. For nelfinavir and ritonavir the concentration defined
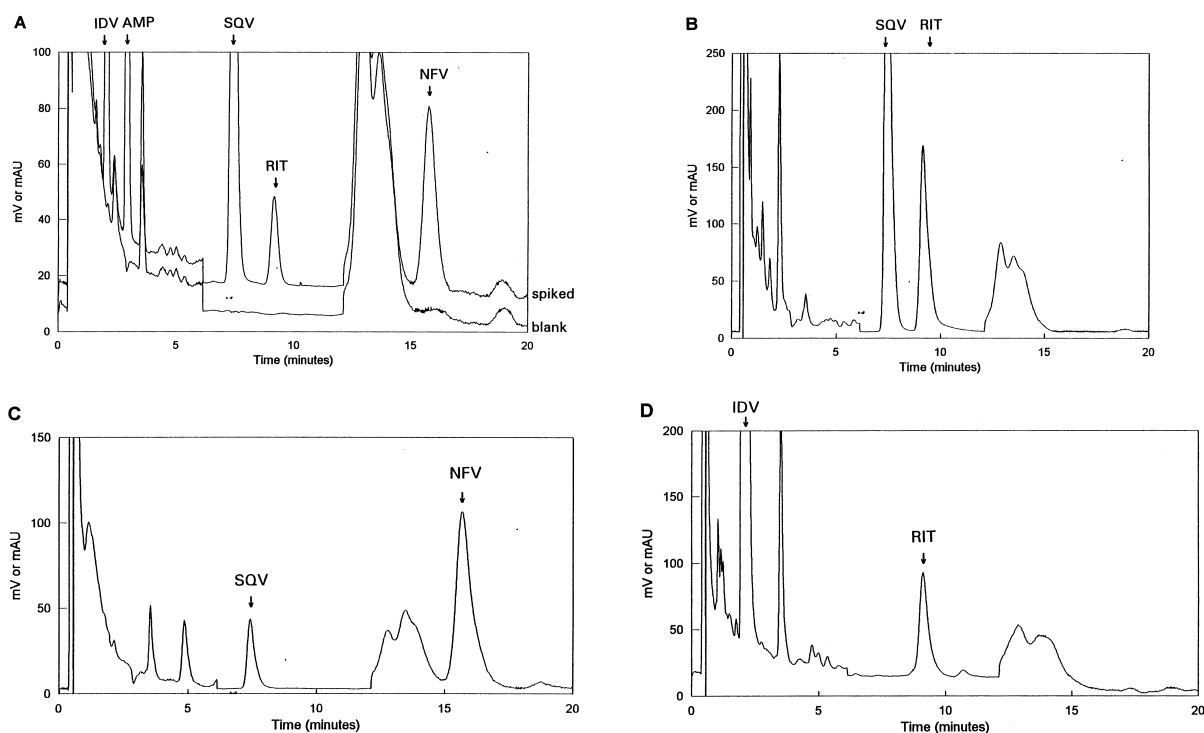

Fig. 3. (A) Chromatograms of blank human plasma and a spiked plasma sample of 1010 ng/ml of amprenavir (AMP), indinavir (IDV), nelfinavir (NFV), ritonavir (RIT), and saquinavir (SQV). (B) Chromatogram of a patient sample containing 2515 ng/ml of saquinavir (SQV) and 7796 ng/ml of ritonavir (RIT). (C) Chromatogram of a patient sample containing 237 ng/ml of saquinavir (SQV) and 1901 ng/ml of nelfinavir (NFV). (D) Chromatogram of a patient sample containing 19 191 ng/ml of indinavir (IDV) and 3450 ng/ml of ritonavir (RIT).

to be the LLQ was 50 ng/ml. At all other concentrations up to the ULQ (25 μg/ml for all protease inhibitors) the percent deviation from the nominal concentration and the relative standard deviation were less than 15%.

3.5. Validation: accuracy, precision, linearity and stability

The results from the validation of the method in human plasma are listed in Table 2. The use of the peak area in combination with a weight factor of [1/conc.] for amprenavir, indinavir, ritonavir and saquinavir and of [1/conc.^{2}] for nelfinavir resulted in a minimal deviation from nominal concentrations. The method proved to be accurate (average accuracy at four concentrations 90.6 to 109.2% of the nominal concentrations for all five protease inhibitors) and precise (within-day precision ranged from 1.8 to 6.7%, and between-day precision ranged from 0.7 to 7.6%). Correlation coefficients (r^2) of calibration curves of each of the protease inhibitors were >0.99 as determined by least-squares analysis. The calibration curves for amprenavir, indinavir and saquinavir proved to be linear in the range of 25 ng/ml to 25 μg/ml with use of the F-test for lack of fit as an indicator of linearity of the regression model. The same test proved linearity of the calibration curves of nelfinavir and ritonavir in the range from 50 ng/ml to 25 μg/ml.

The stability of amprenavir, indinavir and nelfinavir under various conditions, at two concentrations, is shown in Table 3. The stability of ritonavir and saquinavir under identical conditions has already been investigated in our laboratory [11,13]. Under all conditions amprenavir, indinavir and nelfinavir are stable with concentrations of at least 89.0% of the initial concentration. Similar results have been reported for saquinavir and ritonavir [11,13].

3.6. Analysis of patient samples

The applicability of the assay for pharmacokinetic research in HIV infected patients was demonstrated by analyzing plasma samples from three HIV-1 infected patients receiving different combinations of two protease inhibitors as part of their antiretroviral therapy. Typical examples of chromatograms of

Table 3
Stability of amprenavir, indinavir, and nelfinavir in spiked human plasma samples

Storage condition	Concentration (ng/ml)	Recovery (%)	C.V. (%)	n
Amprenavir				
1 h at 60°C	877	90.3	6.5	4
	7144	93.9	3.4	4
24 h at 25°C	877	91.8	5.7	4
	7144	96.7	4.4	4
7 days at 4°C	877	93.9	2.8	4
	7144	94.4	10.8	4
30 days at −30°C	877	101.8	3.5	4
	7144	104.1	1.0	4
30 days at −30°C,	877	95.4	8.8	4
3 freeze–thaw cycles	7144	105.3	2.5	4
Indinavir				
1 h at 60°C	861	89.0	5.9	4
	7142	93.7	3.5	4
24 h at 25°C	861	90.8	6.2	4
	7142	97.4	4.3	4
7 days at 4°C	861	93.9	4.0	4
	7142	94.4	12.4	4
30 days at −30°C	861	99.6	4.2	4
	7142	104.3	1.1	4
30 days at −30°C,	861	99.5	5.9	4
3 freeze–thaw cycles	7142	106.9	2.1	4
Nelfinavir				
1 h at 60°C	777	105.4	2.7	4
	8193	99.8	4.7	4
24 h at 25°C	777	96.8	1.3	4
	8193	98.1	4.3	4
7 days at 4°C	777	101.0	1.6	4
	8193	95.6	8.5	4
30 days at −30°C	777	99.3	1.5	4
	8193	102.0	3.4	4
30 days at −30°C,	777	92.2	6.3	4
3 freeze–thaw cycles	8193	98.5	6.1	4

Abbreviations: C.V.=coefficient of variation; n=number of replicates.

patient samples are shown in Fig. 3B–D. The plasma concentration–time profiles of the protease inhibitors in these patients as determined by the currently described bioanalytical method are shown in Fig. 4A–C.

4. Conclusion

In conclusion, a validated assay for the simultaneous quantitative determination of amprenavir, in-
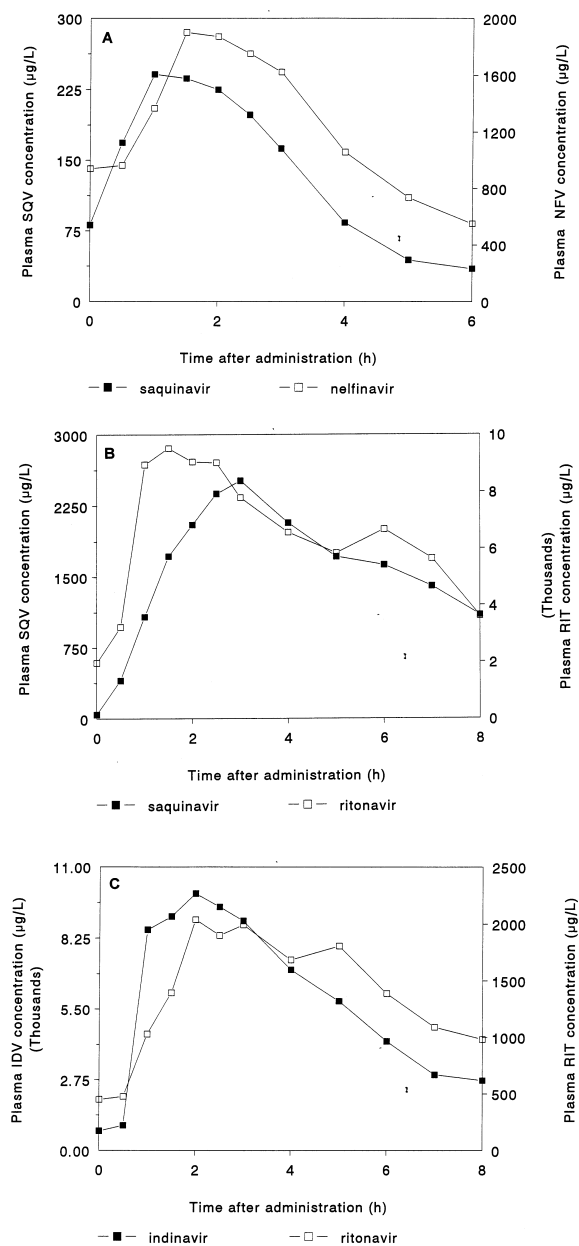
Fig. 4. Plasma concentration versus time curves of saquinavir 600 mg (SQV)+nelfinavir 750 mg (NFV) (A); saquinavir 400 mg (SQV)+ritonavir 400 mg (RIT) (B); and of indinavir 800 mg (IDV)+ritonavir 100 mg (RIT) (C), after oral administration with breakfast to an HIV-1 infected patient (chronic use).

dinavir, nelfinavir, ritonavir, and saquinavir in human plasma is described. The assay meets all current requirements as to the validation of a bioanalytical methodology and covers the concentration ranges of interest for use in pharmacokinetic studies with amprenavir, indinavir, nelfinavir, ritonavir and saquinavir, alone or in combination, in HIV-infected patients. The described HPLC assay can readily be used in a hospital laboratory for the monitoring of protease inhibitor concentrations in plasma.

Acknowledgements

References

[1] R.M.W. Hoetelmans, P.L. Meenhorst, J.W. Mulder, D.M. Burger, C.H.W. Koks, J.H. Beijnen, Pharm. World Sci. 19 (1997) 159.

[2] P. Lorenzi, S. Yerly, K. Abderrakim, M. Fathi, O.T. Rutschmann, J. von Overbeck, D. Leduc, L. Perrin, B. Hirschel, the Swiss HIV Cohort Study, AIDS 11 (1997) F95.

[3] G. Moyle, Exp. Opin. Invest. Drugs 7 (1998) 413.

[4] C. Merry, M.G. Barry, F. Mulcahy, K.L. Halifax, D.J. Back, AIDS 11 (1997) F117.

[5] C. Merry, M.G. Barry, F. Mulcahy, M. Ryan, J. Heavey, J.F. Tija, S.E. Gibbons, A.M. Breckenridge, D.J. Back, AIDS 11 (1997) F29.

[6] N. Roberts, J.C. Criag, J. Sheldon, AIDS 12 (1998) 453.

[7] R.M.W. Hoetelmans, R.P.G. van Heeswijk, D.M. Burger, J.W. Mulder, P.L. Meenhorst, M. Keuter, P.P. Koopmans, K. Brinkman, J.H. Beijnen, submitted.

[8] D.M. Burger, P.P. Koopmans, K. Brinkman, M. Keuter, W. Dolmans, R.M.W. Hoetelmans, P.L. Meenhorst, J.W. Mulder, E.W. Wuis, Y.A. Hekster, ICAAC 37th (1997) A-19 [abstract].

[9] H.T. Karnes, C. March, J. Pharm. Biomed. Anal. 9 (1991) 911.

[10] D.L. Massart, B.G.M. Vandeginste, S.N. Deming, Y. Michotte, L. Kaufman, Chemometrics: a Textbook, Elsevier, New York, 1988, p. 84.

[11] R.M.W. Hoetelmans, M. van Essenberg, P.L. Meenhorst, J.W. Mulder, J.H. Beijnen, J. Chromtogr. B. 698 (1997) 235.

[12] N.G. Knebel, S.R. Sharp, M.J. Madigan, J. Mass Spectrom. 30 (1995) 1149.

[13] R.M.W. Hoetelmans, M. van Essenberg, M. Profijt, P.L. Meenhorst, J.W. Mulder, J.H. Beijnen, J. Chromatogr. B. 705 (1998) 119.