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Simultaneous quantitative determination of the HIV protease inhibitors indinavir, amprenavir, ritonavir, lopinavir, saquinavir, nelfinavir and the nelfinavir active metabolite M8 in plasma by liquid chromatography

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Abstract

A simple HPLC method that quantitates all six currently available protease inhibitors and the nelfinavir active metabolite M8 in one assay is presented. A 500-μl plasma sample was treated by liquid–liquid extraction with a mixture of heptane and ethyl acetate. After evaporation, the residue was redissolved in sodium dihydrogenphosphate and acetonitrile and washed twice with heptane. Chromatography was performed with an analytical C_{18} column. Ultraviolet detection at 210 and 239 nm was used. The present method is associated with high accuracy and low imprecision in the concentration range of 25–5000 ng/ml of all six protease inhibitors and M8. This makes it suitable for monitoring purposes.
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Keywords: Indinavir; Amprenavir; Ritonavir; Lopinavir; Saquinavir; Nelfinavir

1. Introduction

The HIV protease inhibitors have contributed to the improvement of life of many HIV-infected patients the last few years. Inhibition of the HIV protease leads to the production of non-infectious viruses. The result is declining levels of HIV RNA in plasma, which correlates to reduced morbidity and mortality [1]. The protease inhibitors are usually administered as one or two in combination with two nucleoside reverse transcriptase inhibitors. The treatment though is not without problems. Patients experience adverse reactions and because of this they sometimes have to stop treatment. Other patients do not respond to treatment and never achieve low plasma HIV RNA levels. More and more data suggest that there is a relationship between the plasma concentrations of the protease inhibitors and adverse reactions and levels of plasma HIV RNA [2–6]. There is a wide inter-individual variation in steady-state plasma protease inhibitor concentrations. This might be attributable to the metabolism by the

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cytochrome P450 enzymes, primarily CYP3A4, which is expressed with some variation between individuals. Furthermore, the multi-drug transporter P-glycoprotein, located primarily in the upper intestine and liver might add to the variability in the pharmacokinetic behaviour of the protease inhibitors. These factors are also responsible for the extensive drug–drug interaction potential with the protease inhibitors, which can alter the pharmacokinetics of both the protease inhibitors and the co-administered drug substantially. At the present time, there are six protease inhibitors, indinavir, amprenavir, ritonavir, lopinavir, saquinavir and nelfinavir, which have been approved by the FDA by the accelerated approval regulations and by the EMEA, The European Agency for the Evaluation of Medicinal Products (July 2001), for the treatment of HIV-infected patients (Fig. 1). It is important further to explore the possible relationship between plasma protease inhibitor concentrations and effects. If such a relationship exits, therapeutic drug monitoring would lead to an individualised treatment of HIV-infected patients with protease inhibitors. Consequently there will be a need for methods that allow quantitation of the protease inhibitors in plasma from HIV-infected patients. Several HPLC methods for the simultaneous quantitative determination of up to five protease inhibitors and a single one including all six have been described. None of these methods included the nelfinavir active metabolite M8 [7–9]. The metabolite M8 is important because it contributes considerably to the antiretroviral effect of nelfinavir treatment. It has almost the same in vitro activity against HIV as nelfinavir and can be found in concentrations higher than the nelfinavir concentration [10]. The concentration of M8 changes and gets higher if nelfinavir is combined with a CYP3A4 inhibitor, such as ritonavir, since M8 is metabolised by CYP3A4. A simple HPLC method that quantitates all six currently available protease inhibitors and the metabolite M8 in one assay is presented in this paper.

2. Experimental

2.1. Chemicals

All substances used were of analytical grade and all solvents were of HPLC grade. Indinavir was a kind gift from Merck & Co (Rahway, NJ, USA). Amprenavir was a kind gift from Glaxo-Welcome Operations (Middlesex, UK). Ritonavir and lopinavir (ABT-378) were kind gifts from Abbott Laboratories (Abbott Park, IL, USA). Saquinavir was a kind gift from Roche Discovery Welwyn (Welwyn Garden City, UK) and nelfinavir and M8 (AG 1402) were kind gifts from Agouron (La Jolla, CA, USA). Hydrochloric acid, sulphuric acid, sodium hydroxide, sodium dihydrogenphosphate, potassium dichromate and ammonium acetate were purchased from Merck (Darmstadt, Germany). Acetonitrile and methanol were from BDH Laboratory Supplies (Poole, UK). Triethylamine was from Bie & Berntsen (Rodovre, Denmark). Heptane and ethyl acetate were from Rathburn Chemicals Ltd. (Walkerburn, UK). Blank drug-free plasma was obtained from the Transfusion Service at the Department of Clinical Immunology, Odense University Hospital (Odense, Denmark). Distilled water was used throughout the analysis.

2.2. Equipment

The HPLC system consisted of Merck-Hitachi (Tokyo, Japan) L-6200 Intelligent Pump, AS-2000 A Autosampler, La Chrom UV Detector L-7400 and D-2500 Chromato-Integrator. The analytical column was a Nova-Pak C_{18} column, 3.9×150 mm from Waters (Wexford, Ireland). The flatbed mixer was a HS 501 digital from IKA-Labortechnik (Staufen, Germany). Duran centrifuge conical glass tubes were from Schott (Mainz, Germany) and ordinary centrifuge glass tubes was from Bjorn Nielsen Hospitalartikler A/S (Solbjerg, Denmark).

2.3. Standard preparation

Stock solutions of indinavir, amprenavir, ritonavir, lopinavir, saquinavir nelfinavir and M8 were prepared by dissolving the appropriate amount of reference substance, accurately weighed, in methanol to a concentration (as free base) of 500 000 ng/ml. Stock solutions were kept at 4 °C. Working solution was prepared by diluting stock solution with water, to a concentration of 50 000 ng/ml. The working solution was used for the preparation of calibration standards and controls. Appropriate amounts of working solution were diluted with drug-free plasma

to span a calibration standard range of 100–5000 ng/ml (100, 500, 1000, 3000 and 5000 ng/ml). Controls, low (150 ng/ml) and high (2500 ng/ml), were made in a similar way using a separate stock solution. The calibration standards and controls were kept at −20 °C.

2.4. Sample preparation

On the day of analysis, plasma from patients, calibration standards and controls were thawed at room temperature protected from light. A 500-μl sample of plasma from patients, calibration standards

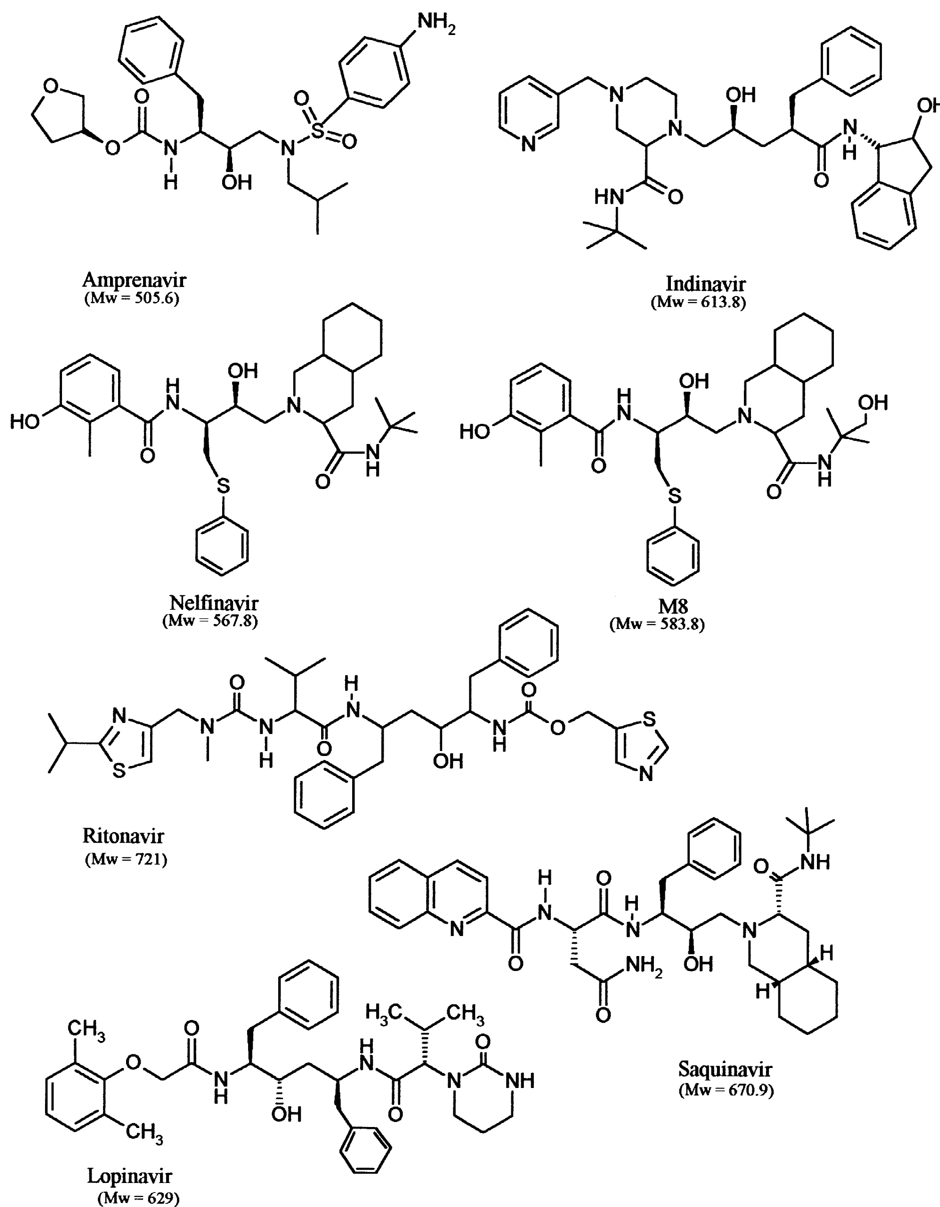

Fig. 1. Molecular structures of the available protease inhibitors. M_w, molecular weight.

or controls were mixed with 50 μl of 1 *M* ammonium acetate, pH 6.8, and 5 ml of a mixture of heptane and ethyl acetate, 1:1, in a centrifuge glass tube and shaken horizontally for 10 min on a flatbed mixer, 300 strokes/min. The mixture was then centrifuged for 8 min at 3000 *g*. Of the organic phase, 4.2 ml was transferred to a conical chromosulphuric acid rinsed glass tube and evaporated to dryness under a gentle stream of nitrogen at 37 °C. The residue was redissolved in 300 μl of a solution prepared from 6 ml of 5 m*M* sodium dihydrogenphosphate, pH 3.5, and 4 ml of acetonitrile. The redissolved residue was washed twice with 3 ml of heptane and 100 μl of the aqueous solution was transferred to autosampler vials and 50 μl was injected.

2.5. Chromatography

Chromatographic separation was obtained isocratic on an analytical C_{18} column at room temperature. The mobile phase was prepared from 140 ml of acetonitrile, 75 μl triethylamine and 160 ml of 5 m*M* sodium dihydrogenphosphate, pH 6. The run-time of the assay was 30 min. The spectrophotometer was operated at 210 nm from 0 to 9.8 min for the detection of indinavir, amprenavir, M8, ritonavir and lopinavir and from 9.8 to 15 min the absorbance was measured at 239 nm for the detection of saquinavir, and then again at 210 nm for the detection of nelfinavir. The detector was programmed to switch automatically between wavelengths. The flow-rate was 1 ml/min. Two sets of controls and blank drug-free plasma were included in every analytical run.

2.6. Specificity and selectivity

Sixteen different samples of drug-free plasma was analysed to see if any endogenous substances were causing interference.

Frequently co-administered drugs like the nucleoside reverse transcriptase inhibitors zidovudine, lamivudine, didanosine, stavudine, abacavir and zalcitabine and the non-nucleoside reverse transcriptase inhibitors delavirdine, nevirapine and efavirenz in patient samples were investigated to see if any interference with the protease inhibitors occurred.

Table 1
Drugs, other than antiretrovirals, used in the treatment of HIV infected patients, which was tested for interference with the analysis

Acyclovir	Fluconazole	Pantoprazole
Aspirin	Furosemide	Phenoxymethylpenicillin
Azithromycin	Ganciclovir	Pivampicillin
Bendroflumethiazide	Granisetron	Pyrazinamide
Benzylpenicillin	Hydrocortisone	Pyridoxine
Candesartancilexetil	Isoniazid	Rifabutin
Chlorprothixene	Lamotrigine	Simvastatin
Citalopram	Levothyroxine	Spironolactone
Clarithromycin	Loperamide	Sulfamethoxazole
Clomipramine	Lovastatin	Sulfasalazine
Codeine	Methadone	Tramadol
Diazepam	Nitrazepam	Trimethoprim
Estazolam	Olanzapine	Warfarin
Ethambutol	Oxazepam	Zopiclone

Samples from HIV-infected patients treated with therapeutic doses of the drugs listed in Table 1 were analysed with regards to interference. This would also show a possible interference from metabolites.

2.7. Accuracy, precision, linearity and recovery

Samples made from working solution and drug-free plasma with a concentration of 150 and 2500 ng/ml of each of the protease inhibitors and M8 were analysed four times in each of four different runs on 4 separate days. Accuracy and precision were then calculated by applying one-way ANOVA on data obtained. As a test parameter for linearity, the ratio lack of fit/pure error was used. The ratio was calculated by applying one-way ANOVA and regression analysis on the peak heights of indinavir, amprenavir, ritonavir, lopinavir, saquinavir, nelfinavir and M8, respectively, from four randomly selected calibration curves. Recovery was calculated as the extraction yield. By relating the response of the calibration standards to the response of a solution containing 2000 ng/ml of the protease inhibitors and M8, an extraction yield was obtained.

2.8. Limit of detection and limit of quantitation

The detection limit was determined according to IFCC's Recommendation on Quality Control in Clinical Chemistry. Assessment of Analytical Methods for Routine Use [11]. This means that the

standard deviation (SD) is calculated at a value in the lower part of the measuring range as defined by the calibration standards. The limit of detection is then defined as 3×SD. The limit of quantitation was defined as the concentration where the obtained results had a coefficient of variation of more than 20%. Ten samples of the 100-ng/ml calibration standard were diluted 1:2 and 1:4 to a concentration of 50 and 25 ng/ml and analysed to determine the limit of quantitation.

2.9. Stability ofstock solutions, calibration standards, controls and patient samples

Stock solutions and patient samples went through three freeze–thaw cycles. All samples were collected in EDTA or lithium–heparin tubes and centrifuged for 20 min at 800 *g* and plasma obtained within 2 h after collection. Patient samples were stored at −80 °C until analysis. Blood from patients treated with one or two protease inhibitors as part of a combination was left to stand at 4 or 20 °C for 6 h, before it was centrifuged and plasma obtained to evaluate the stability of protease inhibitor concentrations in blood. It is recommended that blood is processed within 2 h of collection [12].

2.10. Analysis of samples from patients and healthy volunteers

Plasma samples from three HIV-infected patients treated with combinations of protease inhibitors and two healthy volunteers given single doses of protease inhibitors were analysed with the described method. This was done to demonstrate the applicability of the assay and to investigate the possible interference with metabolites derived from the protease inhibitors. One patient received 1000 mg of saquinavir in combination with 100 mg of ritonavir twice a day together with zidovudine and lamivudine. The second patient received 800 mg of indinavir in combination with 100 mg of ritonavir twice a day together with zidovudine and lamivudine. The third patient received 400 mg of lopinavir in combination with 100 mg of ritonavir (Kaletra) twice a day together with lamivudine, stavudine and nevirapine. Nine blood samples were drawn during 12 h after ingestion of the drugs in the morning. One healthy volunteer was given a single dose of nelfinavir, 750 mg, in combination with 100 mg of ritonavir and 17 blood samples were drawn during 12 h after ingestion of the drugs in the morning. The other healthy volunteer was given 600 mg of amprenavir twice a day for 9 days to achieve steady-state and on day 10 after ingestion of the drug in the morning, 15 blood samples were drawn during 24 h. The samples were from different pharmacokinetic studies approved by the local Ethics Committee and the Danish Medicines Agency (Laegemiddelstyrelsen).

3. Results

3.1. Development phase

Different mobile phases with changing concentrations of acetonitrile, methanol and tetrahydrofurane were tried, but the one selected gave the best separation between the protease inhibitors. In connection with the choice of mobile phase, different wavelengths were also tested. Ultraviolet detection of ritonavir at 239 nm had previously been described, but 210 nm was chosen because of a better result with the mobile phase chosen [7].

During sample preparation, the washing procedure with heptane twice showed to be crucial to eliminate interference from endogenous compounds. This was especially important in case of low concentrations of indinavir or amprenavir.

The conical glass tubes used were rinsed with chromosulphuric acid. All attempts to use a more environmentally friendly rinsing procedure were unsuccessfully. The washing with chromosulphuric acid was especially important in relation to the retrieval of indinavir.

In the development phase, A-86093.0 supplied by Abbott was tried as internal standard, but unfortunately it co-eluted with saquinavir. It was not possible to use one of the protease inhibitors as an internal standard, because the samples received for monitoring purposes often represent all six protease inhibitors when analysed together in one run. Furthermore the information on the requisition form is not always correct which could make interpretation of the result difficult. Because of the seven compounds already included and the satisfactory per-

formance characteristics with the described method, no further efforts were made to find a suitable internal standard.

3.2. Specificity and selectivity

As seen from the typical chromatograms, no endogenous compounds interfere with the drugs or M8 (Figs. 2–4). The retention times of the drugs and M8 were for indinavir 2.4 min (2), amprenavir 3.4 min (3.3), M8 6.7 min (7.4), ritonavir 7.5 min (8.5), lopinavir 8.9 min (10.1), saquinavir 10.6 min (12.3) and nelfinavir 21.6 min (26) (k' in parenthesis, $t_0 =$ 0.8 min). The t_0 was determined as the first signal that deviated from the baseline and was read directly from the computer-generated chromatogram. The nucleoside reverse transcriptase inhibitors (zidovudine, lamivudine, didanosine, stavudine, abacavir, zalcitabine) and the non-nucleoside reverse transcriptase inhibitors (delavirdine, nevirapine, efavirenz) in samples from patients were shown not to interfere with the protease inhibitors. None of the listed drugs in Table 1 interfered with the analysis.

3.3. Accuracy, precision, linearity and recovery

Performance characteristics, accuracy and precision data for the analysis of protease inhibitors and M8 are shown in Table 2. Accuracy varied from 91.9 to 100.3%. Within-day precision varied from 1.6 to 5.5% and between-day from 0.0 to 4.6%. The correlation coefficients (r^2) of the calibration curves for each of the protease inhibitors and M8 were >0.99 as determined by least-squares analysis over a concentration range of 100–5000 ng/ml. Within this range, the calibration curves of the protease in-

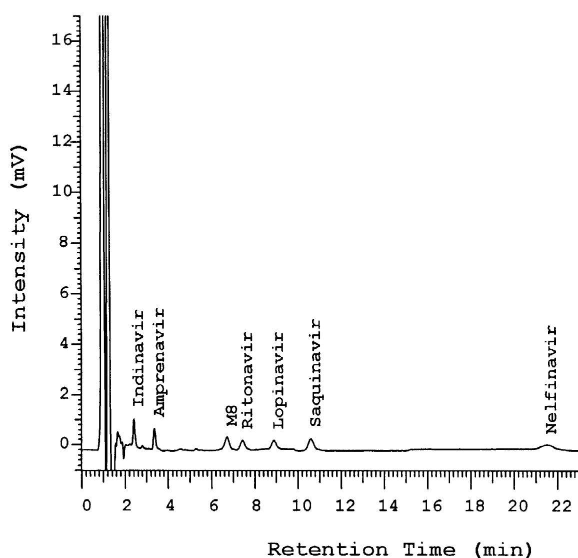

Fig. 3. Chromatogram of the low control.

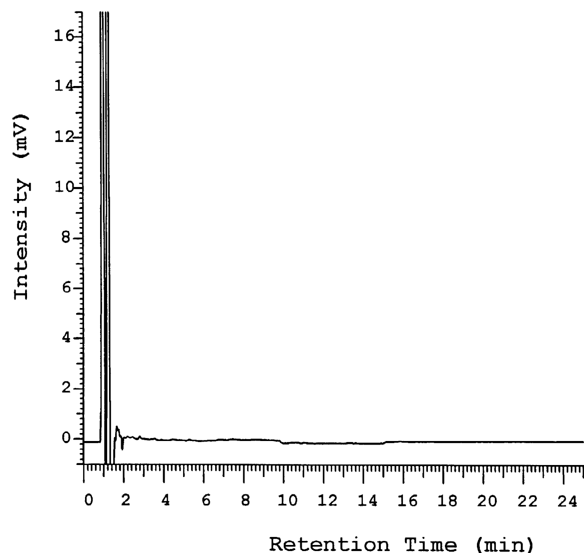

Fig. 2. Chromatogram of drug-free plasma.

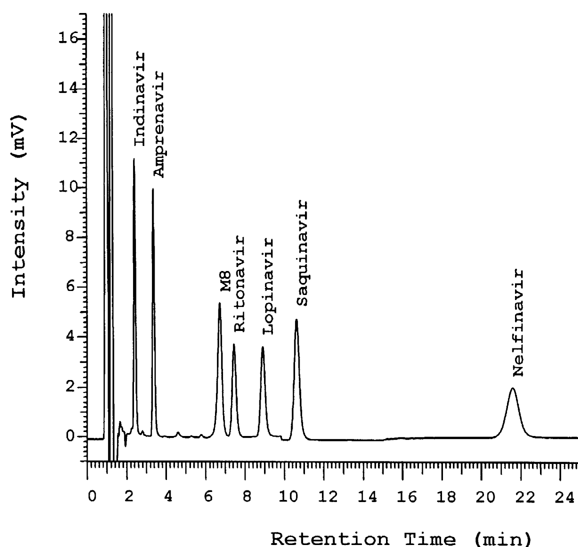

Fig. 4. Chromatogram of the high control.

Table 2
Accuracy and precision data

Protease inhibitor	Concentration (ng/ml)	Accuracy	Precision (%)			n
			C.V. (%) total	C.V. (%) between-day	C.V. (%) within-day	
Indinavir	150	95.1	2.5	0.4	2.5	16
	2500	96.6	1.8	0.5	1.7	16
Amprenavir	150	100.3	5.1	4.6	3.0	16
	2500	97.4	2.3	1.5	1.8	16
M8	150	95.1	1.6	0.0	1.6	16
	2500	95.9	2.3	0.0	2.4	16
Ritonavir	150	91.9	3.1	1.5	2.8	16
	2500	94.0	3.2	0.0	3.3	16
Lopinavir	150	93.4	4.3	1.1	4.2	16
	2500	95.0	4.9	0.0	5.1	16
Saquinavir	150	92.5	3.0	1.1	2.9	16
	2500	98.0	3.3	0.0	3.6	16
Nelfinavir	150	94.8	3.7	1.2	3.5	16
	2500	94.7	5.2	0.0	5.5	16

hibitors and M8 were shown to be linear using the test parameter lack of fit/pure error ratio as an indicator of linearity of each regression model. A weight factor of [1/conc.] was used for all the protease inhibitors in question. By relating the response of the calibrators to the response of a solution containing 2000 ng/ml of protease inhibitor, an extraction yield of each of the drugs and M8 of nearly 100% was found.

3.4. Limit of detection and limit of quantitation

Using the coefficient of variations listed in Table 2, standard deviations of 3.6, 7.7, 4.3, 6.0, 4.2, 5.3 and 2.3 ng/ml were obtained for indinavir, amprenavir, ritonavir, lopinavir, saquinavir, nelfinavir and M8, respectively, at 150 ng/ml. Defining the detection limit as 3×SD, the following detection limits were obtained: Indinavir: 10.8 ng/ml, amprenavir: 23.1 ng/ml, ritonavir: 12.9 ng/ml, lopinavir: 18.0 ng/ml, saquinavir: 12.6 ng/ml, nelfinavir: 15.9 ng/ml and M8: 6.9 ng/ml. The coefficient of variation when analysing at a nominal concentration of 25 ng/ml was for indinavir 6.7%, amprenavir 3.9%, M8 10.6%, ritonavir 4.3%, lopinavir 12.6%, saquinavir 4.2% and nelfinavir 11.7%, and the limit of quantitation was therefore defined as 25 ng/ml.

3.5. Stability of stock solutions, calibration standards, controls and patient samples

Stock solutions are stable for at least 3 months at 4 °C. Calibration standards and controls are stable for at least 6 months at −20 °C. The concentrations of the protease inhibitors in plasma have been thoroughly investigated under different circumstances and have been shown to be very stable [7,13,14]. The stability of the protease inhibitors in blood from patients treated with one or two protease inhibitors that were left to stand at 4 or 20 °C for 6 h before centrifugation, are shown in Figs. 5 and 6. Of the samples analysed, 14% (12/85) at 4 °C and 8% (7/85) at 20 °C were outside a concentration range of 90–110% when compared to the concentration in samples that were immediately processed. The ranges of the analysed samples were for indinavir: 230–11 301 ng/ml, amprenavir: 76–1127 ng/ml, ritonavir: 51–2025 ng/ml, lopinavir: 1743–3667 ng/

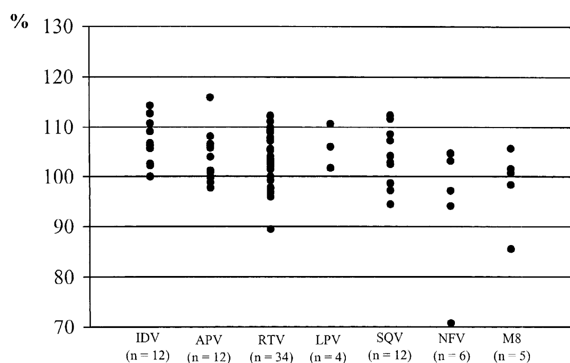

Fig. 5. Stability of the protease inhibitors and M8 in blood after 6 h at 4 °C before centrifugation, expressed as percentage of the concentration found in blood, which was immediately processed. Amprenavir (APV), indinavir (IDV), lopinavir (LPV), nelfinavir (NFV), ritonavir (RTV) and saquinavir (SQV).

ml, saquinavir: 75–2449 ng/ml, nelfinavir: 390–2598 ng/ml and M8: 195–864 ng/ml.

3.6. Analysis of samples from patients and healthy volunteers

In Fig. 7, plasma concentration–time curves of indinavir/ritonavir, amprenavir, lopinavir/ritonavir, saquinavir/ritonavir and nelfinavir/M8/ritonavir are shown. The C_{max}, T_{max} and C_{min} of the different regimens are comparable to results obtained from clinical studies. No metabolites of the protease inhibitors, apart from the nelfinavir active metabolite M8, were shown to interfere significantly with the analysis.

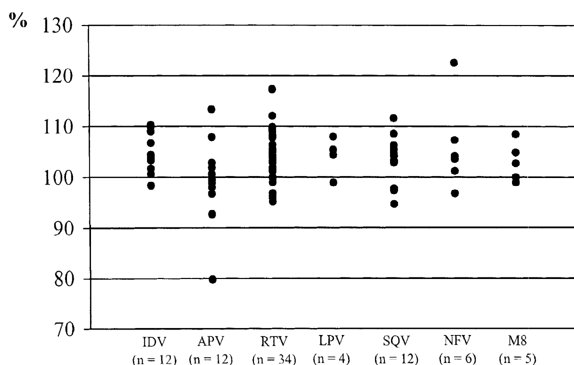

Fig. 6. Stability of the protease inhibitors and M8 in blood after 6 h at 20 °C before centrifugation, expressed as percentage of the concentration found in blood, which was immediately processed. Amprenavir (APV), indinavir (IDV), lopinavir (LPV), nelfinavir (NFV), ritonavir (RTV) and saquinavir (SQV).

4. Discussion

This paper describes the development, validation and routine use a HPLC method for the simultaneous quantitative determination of all the currently approved HIV protease inhibitors (and one active metabolite) for the treatment of HIV-infected patients. In a clinical setting with combinations of two or more protease inhibitors, it is necessary to have a method capable of doing simultaneous analysis, thereby being cost effective.

The quantitative determinations of protease inhibitor concentrations below 100 ng/ml for monitoring purposes are usually not required, which is why the lowest calibration standard was chosen at this level. If required or in case of e.g. pharmacokinetic studies, a calibration standard of 25 ng/ml is included in every analytical run.

In this context, it is also important that methods used for the quantitative determination of protease inhibitors are subjected to external quality assurance programmes if the results from different studies are to be compared [15]. This is in part because of the many different methods used. Furthermore, it is imperative to secure good quality, due to the important decisions made on the basis of these concentrations. An international interlaboratory quality control program for the therapeutic drug monitoring in HIV infection is currently being directed from the Department of Clinical Pharmacy, University Medical Center Nijmegen, Nijmegen, The Netherlands, with the participation of more than 13 laboratories from Europe, Canada, USA and Australia [16]. The participation in such programmes should be part of the quality control of every hospital laboratory doing quantitative determinations of the HIV protease inhibitors for clinical use.

In our work, we included a small study to evaluate the stability of protease inhibitor concentrations in blood kept at 4 or 20 °C for 6 h before processing. The results showed that the majority of concentrations did not change to a greater extent, and accordingly the 2-h limit may not be critical.

In conclusion, the present method is associated

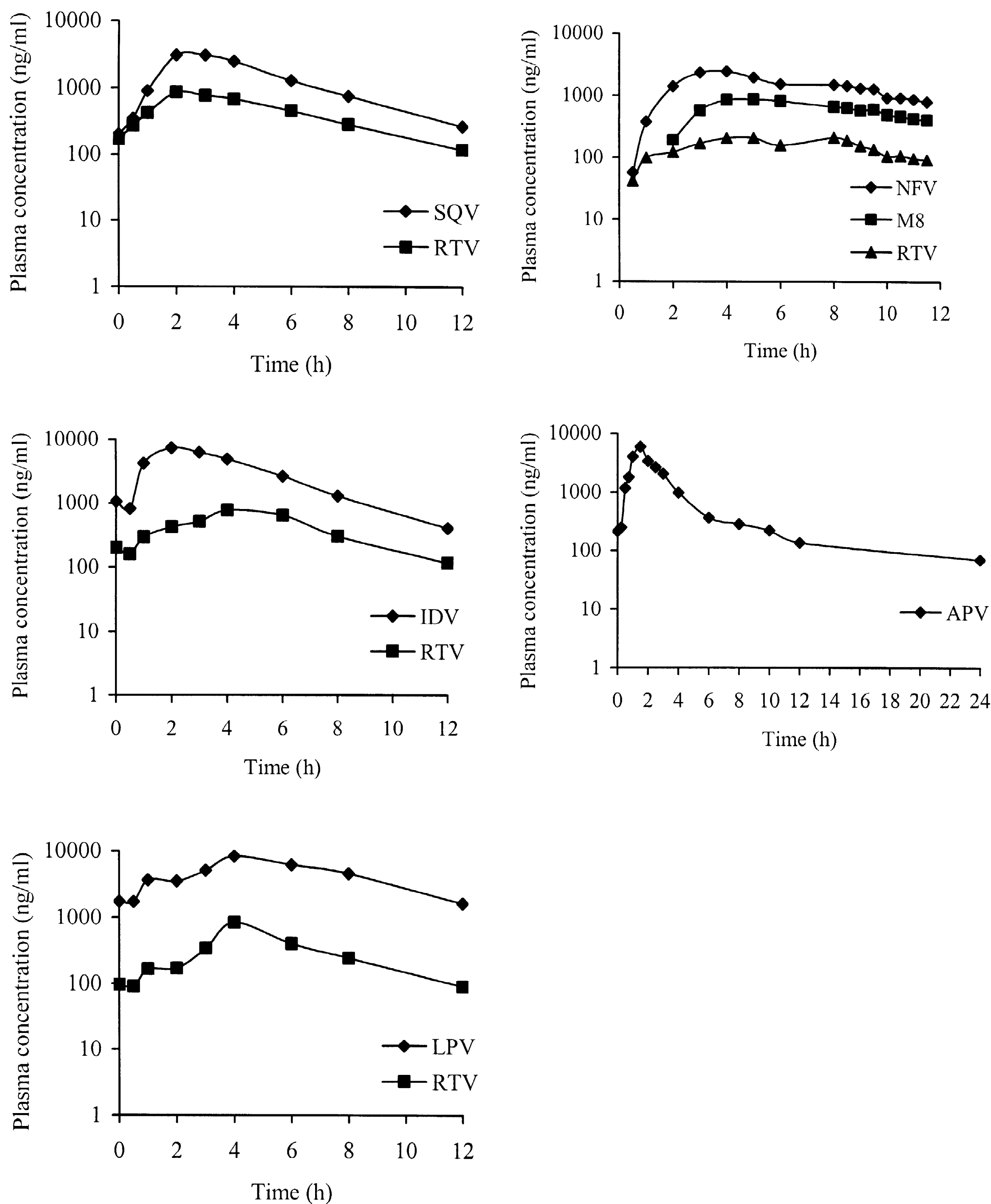

Fig. 7. Concentration–time curves of patients and healthy volunteers receiving protease inhibitors. Amprenavir (APV), indinavir (IDV), lopinavir (LPV), nelfinavir (NFV), ritonavir (RTV) and saquinavir (SQV).

with high specificity and accuracy together with low imprecision. This makes it suitable for monitoring purposes.

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