ELSEVIER

Journal of Chromatography B, 757 (2001) 325–332

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

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

María Sarasa-Nacenta[a,*], Yolanda López-Púa[a], Josep Mallolas[b], José Luis Blanco[b], José M[a]. Gatell[b], Xavier Carné[a]

[a]*Clinical Pharmacology Unit, Hospital Clínic, Barcelona, Spain*

[b]*Infectious Diseases Service, Hospital Clinic, IDIBAPS (Institut d'Investigacions Biomèdiques August Pi i Sunyer), C/Villarroel 170, 08036 Barcelona, Spain*

Received 27 January 2000; received in revised form 13 February 2001; accepted 13 March 2001

Abstract

A rapid, simple and sensitive high-performance liquid chromatographic (HPLC) assay has been developed for the simultaneous quantification of the HIV-protease inhibitors indinavir, amprenavir, ritonavir, saquinavir and nelfinavir in human plasma. The method involved the solid-phase extraction of the five drugs and the internal standard (I.S., verapamil) from 400 μl of human plasma. The HPLC analysis used a reversed-phase C_{18} analytical column and a mobile phase consisting of a gradient with 15 m*M* phosphate buffer (pH 5.75)–acetonitrile and UV monitoring. The method was linear over the therapeutic concentration range for the five HIV-protease inhibitors. The accuracy of the method ranged from 98.2 to 106.7% and the precision values ranged from 1.4 to 8.1% for intra-day precision and from 3.1 to 6.4% for the inter-day values.

Keywords: Indinavir; Amprenavir; Ritonavir; Saquinavir; Nelfinavir

1. Introduction

The protease inhibitors (PIs) are new and potent antiretroviral drugs that have changed the treatment and evolution of infection with human immunodeficiency virus (HIV). Current treatment regimens of HIV infection consist of a combination of antiretroviral drugs. This multiple regimens include at least one protease inhibitor or a non-nucleoside HIV-reverse transcriptase inhibitor.

There is increasing evidence that virological treatment failure is, at least in part, correlated with variations in the pharmacokinetic parameters of drugs [1,2]. These variations may be due to drug–drug interactions, low bioavailability, inter-patient variability in drug disposition, specially variations in the activity of metabolic enzymes.

Therefore, the availability of a method able to quantitate plasma concentrations of various protease inhibitors in one analytical run could be very useful in the management on the infection. We wanted to

*Corresponding author. Tel.: +34-3-227-5766; fax: +34-3-451-5272.

E-mail address: msarasa@clinic.ub.es (M. Sarasa-Nacenta).

0378-4347/01/$ – see front matter
PII: S0378-4347(01)00172-4

explore the possibility of administering indinavir/ritonavir as a single daily dose combined with zidovudine plus lamivudine in a fixed-dose combination twice daily in HIV-infected antiretroviral naive patients. For all the protease inhibitors, separately, a method of analysis has been described elsewhere [3–8], but the objective of the present study was to develop a high-pressure liquid chromatographic assay able to determine in one analytical run, the five protease inhibitors indinavir (INV), amprenavir (AMV), ritonavir (RTV), saquinavir (SQV) and nelfinavir (NFV) in human plasma.

2. Experimental

2.1. Chemicals

Indinavir sulfate was a kind gift of Merck Sharp & Dohme (Rahway, NJ, USA), saquinavir mesylate was supplied by Roche Products (Welwyn Garden City, UK), ritonavir was provided by Abbott Laboratories (Abbott Park, IL, USA), nelfinavir mesylate was a gift from Agouron (La Jolla, CA, USA) and amprenavir was supplied by Glaxo Wellcome (County Durham, UK). Internal standard (I.S., verapamil) was purchased from Sigma (St. Louis, MO, USA) as the hydrochloride salt. Deionized water was prepared by a Milli-Q water purification system (Millipore, Bedford, MA, USA). Solid-phase extraction cartridges (OASIS®, 1 cc, 30 mg) were obtained from Waters (Franklin, MA, USA). HPLC-grade acetonitrile and methanol were obtained from Merck (Darmstadt, Germany). All other reagents were purchased from Merck.

2.2. Instrumentation

The HPLC system consisted of the following components: a Hewlett-Packard (Palo Alto, CA, USA) Model HP1100 quaternary pump, a HP1100 degasser, a HP1100 autosampler, a HP1100 UV detector and a HP Chemstation.

2.3. Chromatographic conditions

The chromatographic separation was performed with gradient elution at a variable wavelength. For the first 10 min, the wavelength was set at 210 nm, then changed to 240 nm, and in minute 19 the wavelength changed again to 220 nm.

The mobile phase components were acetonitrile–15 m*M* potassium phosphate adjusted to pH 5.75 with sodium hydroxide. The gradient elution conditions were increasing linearly the acetonitrile content from 37 to 57% over 23 min. The mobile phase flow-rate was set at 1 ml/min.

The analytical column was a Nova Pak C_{18} 5 μm particle size, 150×3.9 mm (Waters) with a Nova pak C_{18} guard column (Waters). The sample injection volume was 80 μl.

2.4. Preparation of standards

Individual stock solutions of all drugs and the I.S. were prepared in methanol. Each of these stock solutions was used for preparing a solution containing indinavir sulfate, amprenavir, ritonavir, saquinavir mesylate and nelfinavir mesylate. Serial dilutions of this stock solution with 50% methanol, led to solutions of different concentrations for all the PIs. Calibration standards in plasma covering the concentration range between 0.040 and 16 μg/ml for indinavir (as free base), 0.050 and 20 μg/ml for amprenavir, 0.1 and 20 μg/ml for ritonavir, 0.044 and 17.5 μg/ml for saquinavir (as free base) and 0.085 and 17 μg/ml for nelfinavir (as free base) were prepared by adding appropriate volumes of these solutions to drug-free plasma. Eight calibration concentrations were used to define the standard curves.

A second stock solution of the five drugs was used for the preparation of quality control (QC) standards in plasma. The quality controls were prepared at three concentration levels for each drug: 0.16, 0.603 and 2.41 μg/ml for indinavir (as free base), 0.2, 0.75 and 3.0 μg/ml for amprenavir, 0.2, 0.75 and 3.0 μg/ml for ritonavir, 0.175, 0.660 and 2.64 μg/ml for saquinavir (as free base) and 0.173, 0.648 and 2.59 μg/ml for nelfinavir (as free base). All calibration and QCs samples were divided into polypropylene micro-tubes as 1000-μl aliquots which were frozen at −80°C until assay.

A 1 mg/ml stock solution of I.S. was prepared in methanol and diluted to 25 μg/ml in 50% methanol for use during sample preparation.

2.5. Sample pre-treatment

Blood samples were collected in heparinized tubes, and centrifuged (10 min, 2000 *g*) as soon as possible. Plasma was decanted and heated for 60 min at 56°C to inactivate HIV virus before storing at −80°C.

2.6. Sample preparation

Solid-phase extraction cartridges (OASIS®, 1 cc, 30 mg, Waters) were conditioned sequentially with methanol (1 ml) and water (1 ml). A 400-μl aliquot of the plasma sample was added to the cartridge and allowed to pass through the bed with minimal suction. A 20-μl aliquot of the I.S. (25 μg/ml) was added to the cartridge, followed by a water aliquot (1 ml). The columns were further washed with 1 ml methanol–water (30:70, v/v). The bed was suctioned dry. The drugs were eluted with 0.5 ml of methanol. The eluent was evaporated to dryness under a nitrogen stream at 37°C. The residue was reconstituted with 100 μl of mobile phase and 80 μl were injected onto the HPLC system.

2.7. Calibration and calculation procedures

Daily standard curves were constructed for each drug using the ratio of the observed peak area for each PI to the I.S. Unknown concentrations were computed from the linear regression equation of the peak area ratio against the concentration of each PI. A weighting factor of [1/conc] was used for all the drugs.

2.8. Accuracy, precision and recovery

The accuracy and intra-day and inter-day precision of the method were estimated by assaying five replicate plasma QC samples at three different concentrations during three analytical runs. Precision was defined as the percent coefficient of variation (C.V) from five QC standards of three different concentrations analyzed on the same day. Inter-day variability was estimated from the analysis of the five QC standards on three separate days.

Recovery of the drugs after the solid-phase extraction was determined by comparing the observed peak areas in the extracted plasma, to those of non-processed standard solutions.

2.9. Specificity and selectivity

The interference from endogenous compounds was investigated by the analysis of six different blank matrices. Potential coadministered drugs used in combination therapy with INV, AMV, RTV, SQV, NFV including HIV-reverse transcriptase inhibitors (AZT, 3TC, d4T, ddI, ddC, nevirapine, efavirenz and abacavir) were also analysed and were verified to be chromatographically resolved from the five drugs and I.S.

2.10. Limit of quantification

The lower limit of quantification 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 the inter-day analytical runs.

2.11. Stability

HIV-infected patient samples are routinely heated at 56°C to inactivate the virus prior to handling. Heat deactivation studies were performed to verify the stability of all the drugs in plasma under these conditions. An additional stability test was performed to verify the stability of the drugs and I.S. in the injectate while waiting for HPLC analysis. The samples were left at room temperature for 24 h prior to analysis. The stability during sample handling was also verified, subjecting samples to three freeze–thaw cycles.

3. Results

3.1. Linearity

Peak area ratio values of calibration standards were proportional to the concentration of each drug in plasma over the range tested. The calibration curves were also fitted by linear least-squares regres-

sion for indinavir, amprenavir, ritonavir and saquinavir, and showed coefficients of determination greater than 0.999. For nelfinavir a cubic equation was found as the best relation. A weight factor of [1/conc] was used for the five drugs. The mean slopes±SD were 0.947±0.067, 0.890±0.080, 0.227±0.025, 1.323±0.049, and 0.47±0.056 for INV; AMV; RTV, SQV and NFV, respectively.

3.2. Selectivity

Representative chromatograms of blank and spiked plasma samples are illustrated in Fig. 1. Blank plasma showed no interfering endogenous substances neither with the five drugs nor with the I.S. Potentially coadministered drugs tested had retention times that were different from the drugs or the I.S. or were

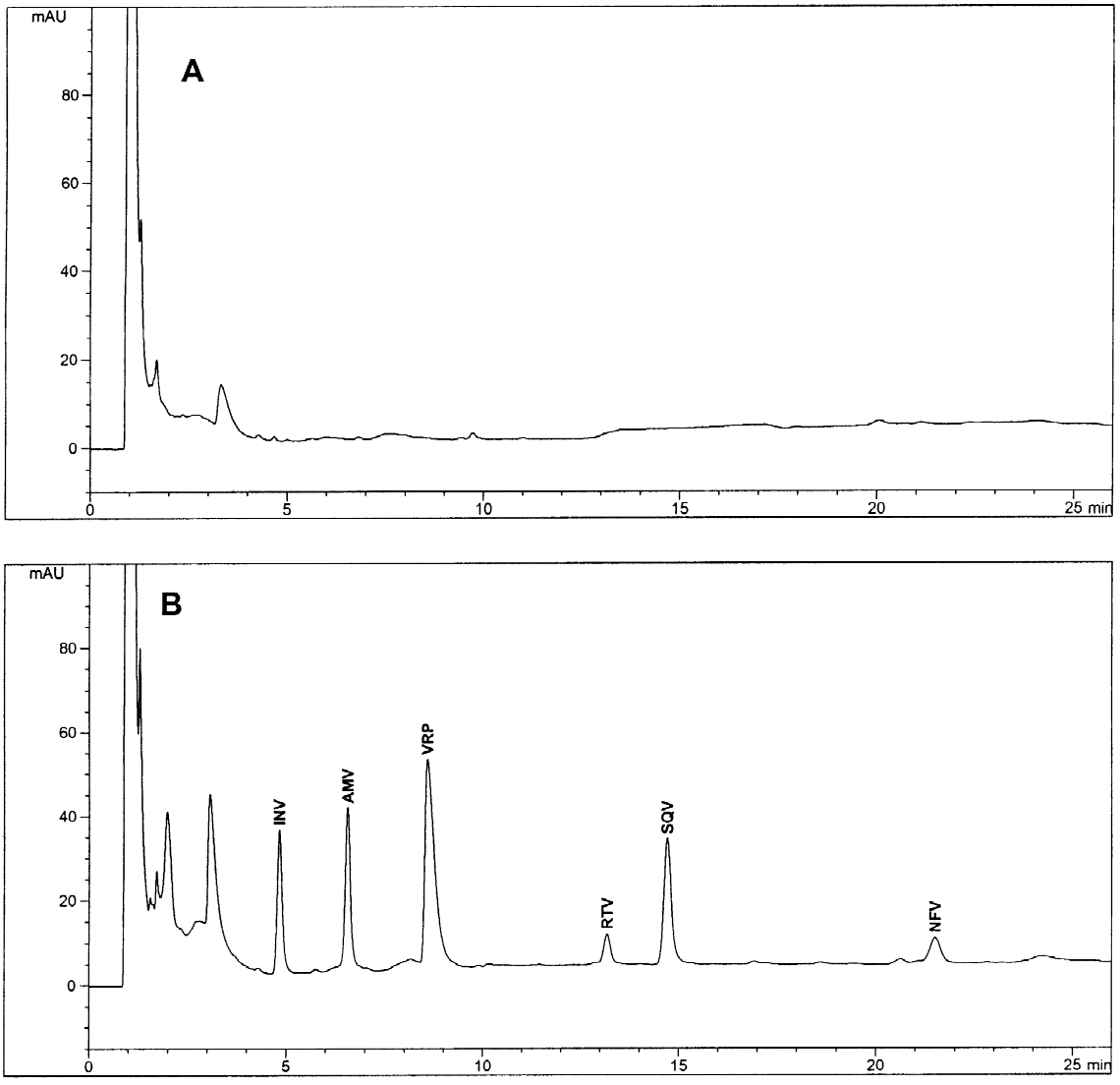

Fig. 1. Chromatograms of blank plasma (A) and plasma spiked with 0.4 μg/ml of indinavir (INV), 0.5 μg/ml of amprenavir (AMV), 0.5 μg/ml of ritonavir (RTV), 0.44 μg/ml of saquinavir (SQV) and 0.425 μg/ml of nelfinavir (NFV), and internal standard (VRP) (B).

Table 1
Determination of the low limit of quantification

Protease inhibitor	Concentration LOQ (μg/ml)	Accuracy (%)	Inter-assay precision (%)
Indinavir	0.040	103.4	8.9
Amprenavir	0.050	108.3	5.6
Ritonavir	0.100	96.5	12.3
Saquinavir	0.044	93.7	6.3
Nelfinavir	0.085	101.2	13.1

not detected with the described bioanalytical method. The approximate retention times for INV, AMV, I.S., RTV, SQV and NFV were 4.8, 6.6, 8.6, 12.9, 14.3 and 21.0 min, respectively.

3.3. Limit of quantification

The limit of quantification (LOQ) was defined as a concentration of 0.040 μg/ml for indinavir (free base), 0.050 μg/ml for amprenavir, 0.10 μg/ml for ritonavir, 0.044 μg/ml for saquinavir (free base) and 0.085 μg/ml for nelfinavir (free base). The results of accuracy and precision at the limit of quantification (LOQ) are presented in Table 1. A chromatogram at the LOQ for the five drugs is shown in Fig. 2.

3.4. Accuracy, precision and recovery

The results from the validation of the method in human plasma are listed in Table 2. These results indicate that the assay is accurate (average accuracy ranged from 98.2 to 106.7%), and precise (within-day precision ranged from 1.4 to 8.1% and between-day precision from 3.1 to 6.4%). The average recoveries are also shown in Table 2.

3.5. Stability

The results of stability under various conditions are shown in Table 3. Under all conditions tested the five drugs proved to be stable with concentrations of at least 92.1% of the initial concentration.

4. Discussion

If we look back over the development of anti-HIV therapies, a key event is undoubtedly the introduction of protease inhibitors (PIs) in 1995. As a component of antiretroviral therapy, PIs have produced a dramatic decrease in mortality and morbidity in HIV infection [9]. However, the reality of the present status is that combination therapy still lacks sufficient potency and durability. Treatment failure is produced for many causes, as poor adherence to therapy, development of viral resistance and pharmacokinetic reasons. Pharmacokinetic variability is particularly important in relation to PIs, because these drugs have considerable inter- and intra-patient variability in plasma levels and marked potential for drug interactions, as they act as inhibitors of the CYP3A4 to varying degrees. However, some of

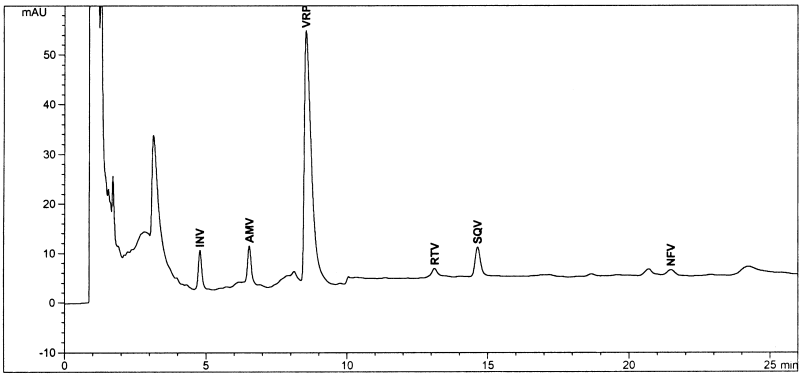

Fig. 2. Chromatogram of spiked plasma at the low limit of quantification (LOQ) for each drug.

Table 2
Accuracy and precision of the determination of indinavir, amprenavir, ritonavir, saquinavir and nelfinavir in plasma

Protease inhibitor	Concentration (μg/ml)	Accuracy (%)	Intra-assay precision (%) (n=5)	Inter-assay precision (%) (n=15)	Recovery Mean±SD (%)
Indinavir	0.160	104.4	2.7	3.6	89.4±6.5
	0.603	103.9	1.9	3.7	
	2.410	103.1	2.5	3.8	
Amprenavir	0.200	99.6	6.5	4.7	83.4±5.8
	0.750	102.4	2.2	4.2	
	3.000	101.3	3.2	3.1	
Ritonavir	0.200	105.5	8.1	6.4	70.1±5.5
	0.750	104.6	7.6	5.4	
	3.000	103.0	6.3	5.2	
Saquinavir	0.176	107.5	3.1	3.6	86.4±6.4
	0.660	98.3	1.4	5.1	
	2.640	98.3	2.2	3.7	
Nelfinavir	0.173	99.2	3.9	5.9	65.3±9.6
	0.648	99.1	2.9	2.8	
	2.590	96.2	2.2	6.3	

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

Protease inhibitor	N	Conc. (μg/ml)	30 min. at 56°C (Recovery % (SD))	24 h at 25°C (Recovery % (SD))	Three freeze–thaw cycles (Recovery % (SD))
Indinavir	5	0.160	103.3 (2.8)	95.9 (3.8)	100.2 (2.5)
	5	0.603	106.0 (3.1)	98.7 (2.6)	98.6 (3.1)
	5	2.410	100.7 (1.8)	96.4 (1.5)	101.1 (4.2)
Amprenavir	5	0.200	97.3 (1.2)	102.3 (2.5)	96.5 (2.8)
	5	0.750	105.1 (4.5)	101.5(6.2)	99.6 (4.8)
	5	3.000	93.2 (1.6)	101.2 (3.8)	105.9 (6.8)
Ritonavir	5	0.200	100.1 (3.5)	97.3 (2.8)	99.9 (5.8)
	5	0.750	106.1 (2.1)	99.5 (5.2)	102.3 (2.2)
	5	3.000	99.6 (1.9)	103.2 (5.5)	100.9 (5.2)
Saquinavir	5	0.176	101.8 (2.9)	102.8 (3.5)	103.3 (2.2)
	5	0.660	104.8 (3.5)	104.0 (7.8)	96.6 (3.7)
	5	2.640	97.8 (2.9)	99.7 (4.2)	95.9 (5.6)
Nelfinavir	5	0.173	100.2 (0.9)	96.4 (5.2)	98.7 (2.7)
	5	0.648	102.0 (3.3)	92.4 (1.2)	95.6 (2.5)
	5	2.590	96.8 (2.4)	99.9 (3.2)	99.9 (2.3)

these interactions could be very useful in the improvement of the treatment. The inhibitory effect of ritonavir on the CYP3A4 can be used to improve the pharmacokinetic profile of almost all the co-administered protease inhibitors (including indinavir) by increasing their exposure [10,11]. This pharmacokinetic interaction with the potent CYP3A4 inhibitor ritonavir, may lead to dose reductions and makes once or twice dosing of protease inhibitors combinations feasible.

These pharmacokinetic characteristics of the PIs suggest that the availability of an analytical method able to analyze protease inhibitors levels would be of clinical interest. A simultaneous assay for the protease inhibitors indinavir, amprenavir, ritonavir, saquinavir, and nelfinavir is both useful and convenient. Even more, when dual o triple protease therapy is being used.

Therefore, the objective of the present study was to develop a simple and rapid analytical method able to analyze the five PIs, INV, AMV, RTV, SQV and NFV in the same analytical run. There are other methods to determine protease inhibitors simultaneously [12–14], but only one of them is able to analyze also amprenavir [12], the others used liquid–liquid extraction process, and we preferred to use solid-phase extraction because it is rapid and reproducible. The starting point for the development of the method we describe was the validated assay for the quantification of indinavir in human plasma reported by Foisy and Sommadossy [3]. We optimized the method, for the simultaneous quantification of indinavir, amprenavir, ritonavir, saquinavir and nelfinavir. The modifications included determining the optimum mobile phase pH and the change from isocratic conditions to a gradient elution. We also needed to change the conditions of the solid-phase extraction by modifying the washing steps to obtain an acceptable recovery for all the drugs. The result is an assay rapid, accurate and reproducible, with a simple sample preparation.

We have analyzed more than 1000 patient samples with this method. One example, are the samples from the pilot study to find a INV/RTV regimen for once daily dosing. Patients started with Combivir® (ZDV plus 3TC) plus INV/RTV 800/100 bid with food. Plasma levels of INV and RTV (C_{min} and C_{max}) were measured at 4-week intervals, and according to these

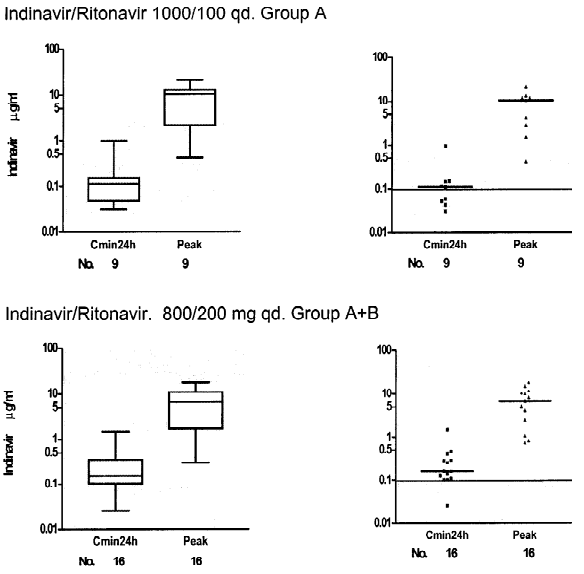

Fig. 3. Results of the pilot study to find a once a day dose of INV/RTV at week 8 (Group A) and week 12 (Groups A and B).

levels, dosage of INV/RTV were switched to 1000/100 qd and then 800/200 qd. If C_{min} of INV were too low (<0.1 μg/ml) with 1000/100 qd in first half of patients (Group A) it was planned to switch directly from 800/100 bid to 800/200 qd in the second half (Group B). The pharmacokinetic data at week 8 (Group A) and week 12 (Group A and B) are shown in Fig. 3. This study support the use of 800 mg INV in combination with 100 mg RTV in a twice daily regimen and emphasize that once daily regimen of INV/RTV (possibly 800/200 mg) is feasible and deserve further evaluation in larger randomized trials.

This study is an example of the possibility of combination therapies with PIs, but there are other combinations to explore. There are many situations where the possibility of determining PIs levels could be clinically useful.

5. Conclusion

In conclusion a validated assay is described. The assay is simple and can be readily used in any hospital laboratory for the quantitative determination of indinavir, amprenavir, ritonavir, saquinavir and

nelfinavir in human plasma. The assay covers the concentration range of interest and is currently being used to analyze samples of patients treated with one more protease inhibitors.

References

[1] D.M. Burger, R.M.W. Hoetelmans, J.W. Mulder, 12th World AIDS Conference, Geneva, Switzerland, July 1998, 42275.
[2] G.F. Vanhove, J.M. Gries, D. Verotta, L.B. Sheiner, R. Coombs, A.C. Collier, T.F. Blaschke, Antimicrob. Agents Chemother. 41 (1997) 2433.
[3] M.L. Foisy, J.P. Sommadossy, J. Chromatogr. B 721 (1999) 239.
[4] D.M. Burger, M. Graaff, E.W. Wuis, P.P. Koopmans, Y.A. Hekster, J. Chromatogr. B 703 (1997) 235.
[5] E.Y. Wu, J.M. Wilkinson, D.G. Naret, V.L. Daniels, L.J. Williams, D.A. Khalil, B.V. Shetty, J. Chromatogr. B 695 (1997) 373.
[6] S. Frappier, D. Breilh, E. Diarte, B. Ba, D. Ducint, J.L. Oellegrin, M.C. Saux, J. Chromatogr. B 714 (1998) 384.
[7] R.M.W. Hoetelmans, M. van Essenberg, M. Profijt, P.L. Meenhorst, J.W. Mulder, J.H. Beijnen, J. Chromatogr. B 705 (1998) 119.
[8] A.I. Veldkamp, R.P. van Heeswijk, R.M. Hoetelmans, P.L. Meenhorst, J.W. Mulder, J.M. Lange, J.H. Beijnen, J. Chromatogr. B 727 (1999) 151.
[9] F.J. Pallella, K.M. Delaney, A.C. Moorman, M.O. Loveless, J. Fuhrer, G.A.D.J. Aschman, S.D. Holmberg, New Engl. J. Med. 338 (1998) 853.
[10] L.L. Von Molte, D.J. Greenblatt, J.M. Grassi, B.W. Granda, S.X. Duan, S.M. Fogelman, J. Clin. Pharmacol. 38 (1988) 106.
[11] A. Hsu, G.R. Granneman, G. Cao, L. Carothers, A. Japour, T. El-Shourbagy, S. Dennis, J. Berg, K. Erdman, J.M. Leonard, E. Sun, Antimicrob. Agents. Chemother. 42 (11) (1998) 2784.
[12] P.W.H. Hugen, C.P.W.G.M. Verweij-van Wissen, D.M. Burger, E.W. Wuis, P.P. Koopmans, Y.A. Hekster, J. Chromatogr. B 727 (1999) 139.
[13] R.P.G. van Heeswijk, R.M.W. Hoetelmans, R. Harms, P.L. Meenhorst, J.W. Mulder, J.M.A. Lange, J.H. Beijnen, J. Chromatogr. B 719 (1998) 159.
[14] R.P. Remmel, S.P. Kawle, D. Weller, C.V. Fletcher, Clin. Chem. 46 (2000) 73.