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High-performance liquid chromatographic assay to determine the plasma levels of HIV-protease inhibitors (amprenavir, indinavir, nelfinavir, ritonavir and saquinavir) and the non-nucleoside reverse transcriptase inhibitor (nevirapine) after liquid–liquid extraction

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Abstract

A single HPLC assay was developed for therapeutic drug monitoring of 5 HIV protease inhibitors (indinavir, amprenavir, saquinavir, ritonavir, nelfinavir) and a non-nucleoside reverse transcriptase inhibitor (nevirapine) in human plasma. After liquid–liquid extraction in a mixture ethyl acetate–hexane, compounds are separated on a C_{18} column with a gradient elution of solvent A [acetonitrile and 0.025 *M* tetramethylammonium perchlorate in 0.2% aqueous trifluoroacetic acid (55:45 (v/v))] and solvent B [methanol and 0.025 *M* tetramethylammonium perchlorate in 0.2% aqueous trifluoroacetic acid (55:45 (v/v))]. The compounds are detected at various wavelengths: 320 nm (nevirapine), 259 nm (indinavir), 254 nm (amprenavir, nelfinavir, saquinavir) and 239 nm (ritonavir). The intra-day and inter-day precision and accuracy are lower than 15%. The limits of quantitation are 0.05 mg/l (amprenavir), 0.2 mg/l (indinavir, saquinavir, nelfinavir) and 0.4 mg/l (ritonavir, nevirapine). This method which allows to estimate simultaneously plasma levels of protease inhibitors and nevirapine can be used for therapeutic drug monitoring.

Keywords: Amprenavir; Indinavir; Nelfinavir; Ritonavir; Saquinavir; Nevirapine

1. Introduction

The concentrations of the HIV protease inhibitors (PI) and the non-nucleoside reverse transcriptase inhibitor (NNRTI) in human plasma became a useful parameter in the clinical management of HIV disease. The plasma levels of PI and NNRTI seem to be connected with virologic efficacy [1–3]. Different methods were developed for simultaneous determination of the plasma levels of antiretroviral agents [4–8]. The methods described for determination of 5 PI integrate a solid-phase extraction [4–7]. Our method was developed to evaluate the plasma concentrations of 6 antiretroviral agents (5 PI and 1 NNRTI, nevirapine) in a single run after liquid–liquid extraction. This assay is based on a method

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developed for determination of ritonavir level and modified to evaluate concentrations of others PI [9].

2. Experimental

2.1. Chemicals

Amprenavir mesylate (Glaxo Wellcome, London, UK), nelfinavir mesylate (Aguron pharmaceuticals, La Jolla, CA, USA), indinavir sulfate (Merck Sharp & Dohme-Chibret, West Point, PA, USA), the internal standard (A 86093) and ritonavir (Abbott, IL, USA), nevirapine (Boehringer Ingelheim, Ridgefield, CT, USA) and saquinavir mesylate (Roche products, Basel, Switzerland) were kindly provided by pharmaceutical laboratories. Tetramethylammonium perchlorate was purchased from Sigma–Aldrich (Saint-Louis, MO, USA), and trifluoroacetic acid was purchased from Fluka Chemie AG (Buchs, Germany). Acetonitrile and ethyl acetate were from SDS (Peypin, France). Hexane and methanol (for analysis) were from Merck (Darmstadt, Germany).

2.2. Equipment

The high-performance liquid chromatographic system consists of a model 9095 automatic sample injector, a model 9010 solvent delivery pump, a model Prostar 310 programmable wavelength UV detector and a model 9065 diode array detector. All these instruments were from Varian (Sunnyvale, CA, USA). The UV detector is used to quantify signals (peak areas) and the diode array detector to check purity and identity of peaks. The whole system is controlled by the LC Star Workstation Software (Varian). The separation is realized at room temperature on a Symmetry® 5 μm C_{18} column (250×4.6 mm I.D.) protected with a Symmetry® 5 μ*M* C_{18} pre-column (20×3.9 mm I.D.). The column and the pre-column were from Waters (Milford, USA).

2.3. Chromatographic conditions

The solvent A was composed of acetonitrile and 0.025 *M* tetramethylammonium perchlorate in 0.2% aqueous trifluoroacetic acid (55:45 (v/v))and the solvent B was composed of methanol and 0.025 *M* tetramethylammonium perchlorate in 0.2% aqueous trifluoroacetic acid (55:45 (v/v)). The solvents are ultrasonicated. The mobile phase was delivered at 0.9 ml/min and the gradient program conditions are given in Table 1. From time 0 to 8 min, methanol is progressively replaced by acetonitrile. From time 8 to 25 min, the composition of the mobile phase is constant. From time 25 min to the end of the run (35 min), the column is stabilized with the initial gradient before the next injection. The compounds are detected at different wavelengths: nevirapine (320 nm), indinavir (259 nm), amprenavir nelfinavir saquinavir (254 nm), ritonavir and internal standard (239 nm). The UV detector program conditions are presented in Table 2.

Table 1
Gradient elution program

Time (min)	Solvent A (%)	Solvent B (%)
0	30	70
8	80	20
25	80	20
35	30	70

2.4. Preparation of standards

Stock solutions of nevirapine, indinavir, amprenavir, saquinavir, nelfinavir, ritonavir, internal standard are prepared at concentrations of 1000 mg/l except for nevirapine at a concentration of 2500 mg/l. These solutions are stable for at least 6 months at −20°C. The working solution of PI and nevirapine is realized by diluting the stock solutions in a mixture methanol–water (1:1 (v/v)) to a final concentration of 100 mg/l for all compounds, except for ritonavir and nevirapine (200 mg/l). A working solution of the internal standard is similarly prepared to a final concentration of 40 mg/l. The working

Table 2
UV detector program

Time (min)	Wavelength (nm)
from 0 to 6	320
from 6 to 10	259
from 10 to 20	254
from 20 to 35	239

solution of PI and nevirapine is used to prepare calibration samples and quality controls. Known volumes of this working solution (25–1000 μl) are diluted in methanol–water (1:1 (v/v)) to obtain a 1000 μl final volume. One hundred μl of these solutions were mixed with 900 μl of drug free human plasma to prepare calibration samples and quality controls.

2.5. Sample treatment

Blood samples were collected in Venoject® from Terumo (Leuven, Belgium)with lithium heparinate as anticoagulant. No viro-inactivation procedure is realized. The Venoject® are centrifuged at 3000 rpm (1800 *g*) for 10 min at +4°C. A 1 ml aliquot of plasma (patient samples, calibration samples, quality controls) is combined with 100 μl of the internal standard solution (40 mg/l) and 5 ml of a mixture of ethyl acetate–hexane (9:1 (v/v)). The tubes are horizontally shaken for 10 min followed by centrifugation at 3000 rpm (1800 *g*) for 5 min at +4°C. Three ml of the organic phase are evaporated to dryness under a gentle stream of nitrogen at +40°C. The residue is reconstituted by vortexing for 2 min by 0.4 ml of a solution containing acetonitrile, methanol and 0.025 *M* tetramethylammonium perchlorate in 0.2% aqueous trifluoroacetic acid (17:5:78 (v/v)). Each reconstituted sample is washed twice with 3 ml hexane by vortexing for 2 min prior to being centrifugated at 3000 rpm (1800 *g*) at +4°C. The hexane layer was removed by aspiration. Fifty μl of the washed aqueous layer are injected.

2.6. Specificity

Currently prescribed anti HIV drugs (zidovudine, stavudine, didanosine, lamivudine, delavirdine, abacavir, efavirenz) and possible co-administered drugs (aspirine, paracetamol, amoxicilline, amitriptyline, sulfamethoxazole, trimethoprime, ofloxacine, alprazolam, bromazepam) were tested.

2.7. Calibration curves

The calibration curves are calculated by unweighted least square linear regression. The range of standard concentrations tested are from 0.25 to 10 mg/l (for indinavir, nelfinavir, amprenavir, saquinavir and from 0.5 to 20 mg/l for ritonavir and nevirapine.

2.8. Accuracy, precision, limit of quantitation

The quality controls are spiked plasma samples (0.3 mg/l – 2.5 mg/l – 6 mg/l for saquinavir, indinavir, amprenavir, nelfinavir and 0.6 mg/l – 5 mg/l – 12 mg/l for nevirapine, ritonavir). These samples were used for the precision and accuracy determination. Accuracy is defined as the percentage of deviation from the nominal concentration and precision is defined as the coefficient of variation. For intra-day validation, triplicate of quality controls were analyzed within the same day. For inter-day validation, concentrations of quality controls were determined on five separate days. The limit of quantitation is defined as the lowest concentration in plasma sample such as the deviation between measured and nominal concentration is less than 20% [10].

2.9. Stability

A batch of spiked plasma samples were analyzed to investigate stability. These samples were stored at −20°C for 6 months. Another batch of spiked plasma sample submitted three freeze–thaw cycles and were analyzed.

2.10. Recovery

The efficiency of the method was determined with quality controls samples. The recovery was calculated by comparing the peak areas of the quality controls samples after extraction with the peak areas of standard solutions at the same concentration.

3. Results and discussion

The sample extraction procedure allows to extract simultaneously 5 PI and nevirapine. The cost of this liquid–liquid extraction was compared with the cost of the liquid–solid extraction procedure developed by Aymard et al. which allows to extract 5 PI and nevirapine [7]. For each sample, liquid–solid ex-

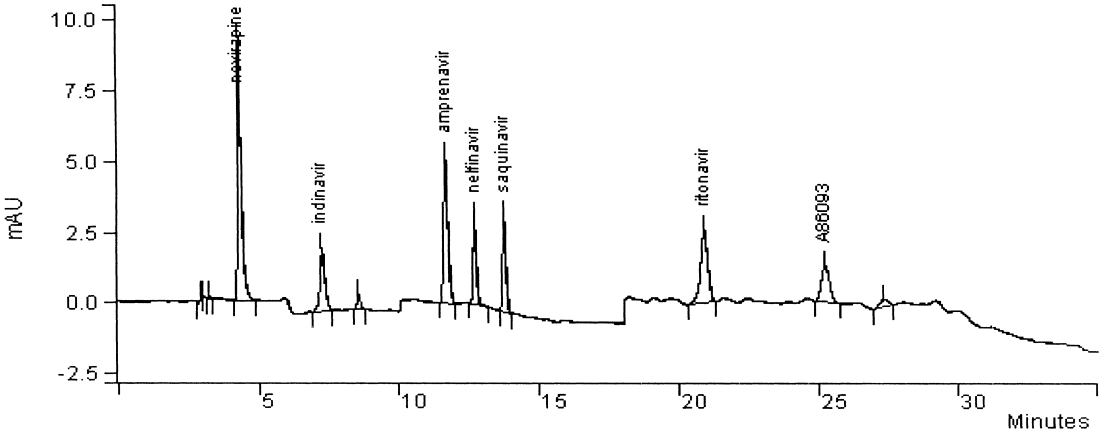

Fig. 1. Chromatogram of spiked plasma sample (4 mg/l for indinavir, amprenavir, nelfinavir, saquinavir, ritonavir and 8 mg/l for ritonavir and nevirapine). The retention times are 4.3 min (nevirapine), 7 min (indinavir), 12 min (amprenavir), 13 min (nelfinavir), 14 min (saquinavir), 21 min (ritonavir) and 25 min (internal standard, A 86093).

traction procedure is seven time as expensive than liquid–liquid extraction procedure since the single used C_{18} extraction columns used in liquid–solid extraction are more expensive than solvents (ethyl acetate, hexane).

The gradient of elution allows to separate the compounds in a single run. In the beginning of the run, the ratio of methanol is superior to acetonitrile in the mobile phase. Methanol which is a more polar solvent than acetonitrile increases the retention times of compounds in our method. Thus, the retention of compounds such as nevirapine and indinavir which are little delayed by column is increased. In the second part of the run (from time 8 to 25 min), the ratio of acetonitrile in the mobile phase is superior to methanol to accelerate the elution of the other compounds. Thus, the runtime is limited to 35 min. With this gradient elution, the retention times are respectively 4.3 min, 7 min, 12 min, 13 min, 14 min, 21 min and 25 min for nevirapine, indinavir, amprenavir, nelfinavir, saquinavir, ritonavir, internal standard as described in Fig. 1. A blank plasma is presented in Fig. 2. Fig. 3 shows the plasma chromatogram obtained from a HIV patient 13 h after indinavir (800 mg twice daily), ritonavir (100 mg twice daily), stavudine (40 mg twice daily),

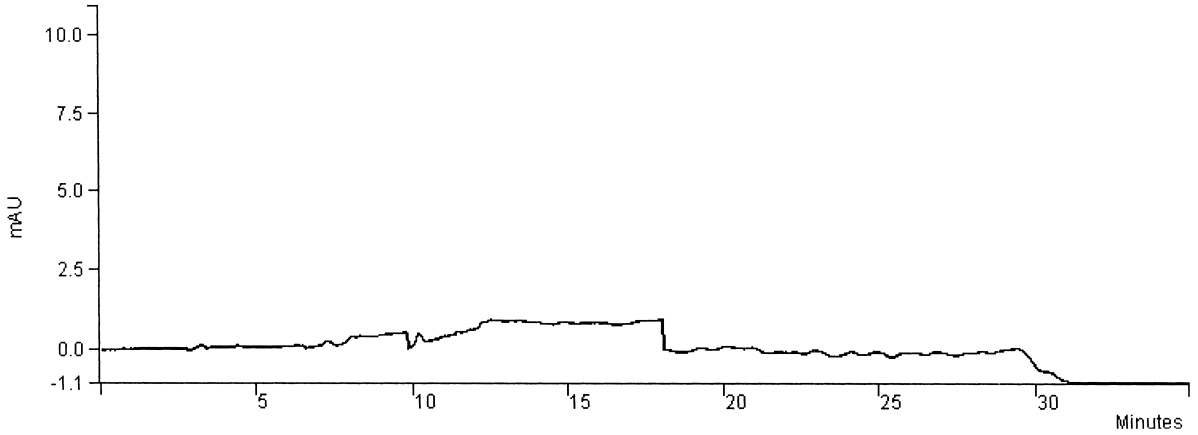

Fig. 2. Chromatogram obtained from drug free plasma sample.

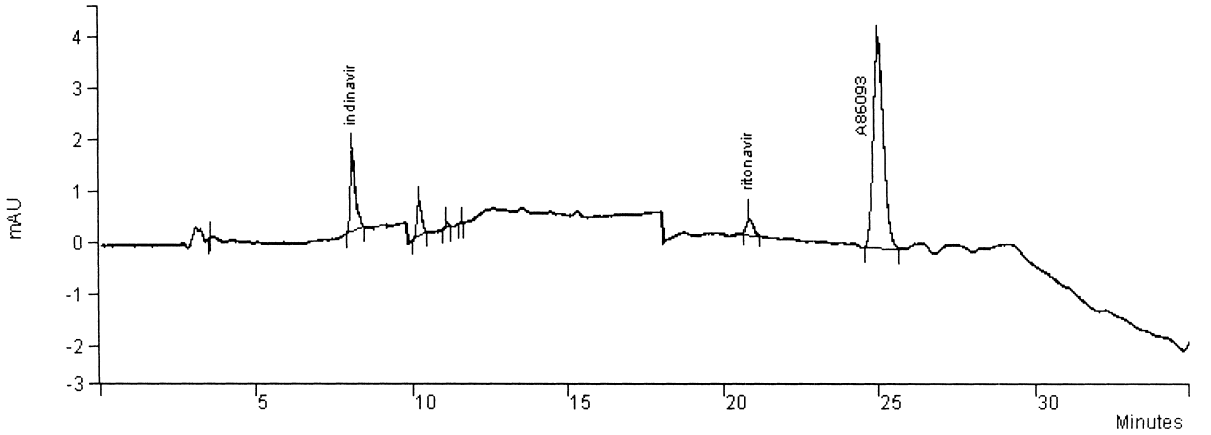

Fig. 3. Chromatogram obtained from a patient receiving indinavir, ritonavir, stavudine, lamivudine. The trough levels of indinavir and ritonavir are respectively 0.55 mg/l and 0.45 mg/l. A86093 is the internal standard.

lamivudine (150 mg twice daily) intakes. Fig. 4 shows the plasma chromatogram obtained from a HIV patient 12 h after saquinavir (600 mg twice daily), ritonavir (100 mg twice daily), efavirenz (600 mg once daily). No interference was detected with drugs listed in 2–6. The retention times of co-administered drugs established from spiked human plasma samples are respectively 3.1, 3.2, 3.8 and 4 min for abacavir, ofloxacine, delavirdine and sulfamethoxazole. The others compounds are not detected. Moreover, purity and identity of peaks are controlled by diode array detector for each analysis. The method is currently used to analyze sample of patients treated and no interfering drugs were found. The retention time of sulfamethoxazole which is frequently a co-administered drug with antiretroviral is 4 min versus 4.3 min for nevirapine. Sulfamethoxazole could interfere with nevirapine. To prevent this possible interference, the wavelength detection for nevirapine was established at 320 nm which is not optimum for its detection but avoids interference with sulfamethoxazole. At 320 nm, the limit of quantitation is 0.4 mg/l for nevirapine. The value is clinically relevant since the average plasma trough level of nevirapine (400 mg once daily) is 4.0 mg/l in a previous study [3].

For the other compounds, the limits of quantitation are 0.4 mg/l (ritonavir), 0.2 mg/l (indinavir,

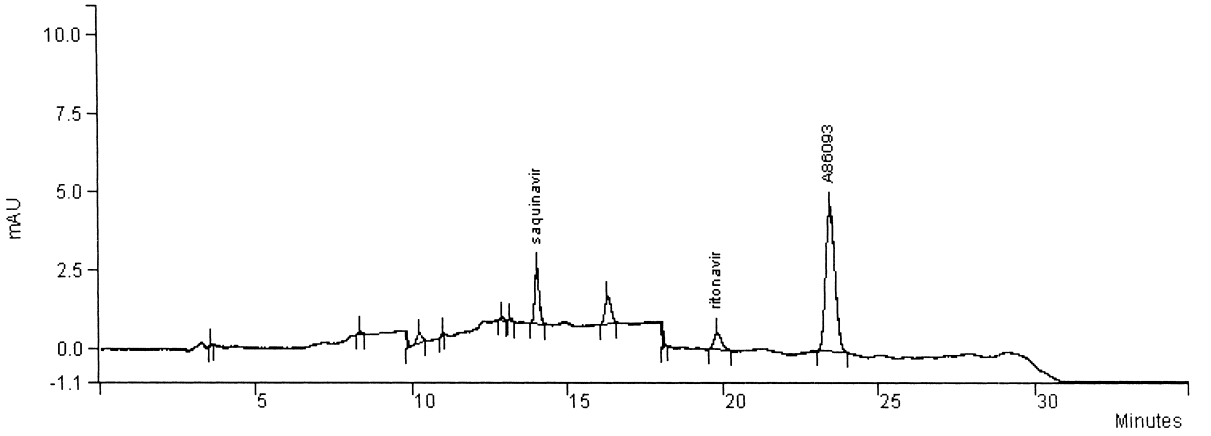

Fig. 4. Chromatogram obtained from a patient receiving saquinavir, ritonavir, efavirenz. The trough levels of saquinavir and ritonavir are respectively 0.37 mg/l and 0.42 mg/l. A86093 is the internal standard.

Table 3
Inter-day (ied) and intra-day (iad)accuracy for antiretroviral agents Accuracy is defined as the percent of deviation from the nominal level[a]

	Low level			Medium level			High level		
	Nominal plasma level	Average measured plasma level (accuracy%)		Nominal plasma level	Average measured plasma level (accuracy%)		Nominal plasma level	Average measured plasma level (accuracy%)	
		iad $n=3$	ied $n=5$		iad $n=3$	ied $n=5$		iad $n=3$	ied $n=5$
nevirapine	0.60	0.58 (3.33)	0.57 (5.00)	5.00	5.52 (10.4)	5.18 (13.67)	12.00	11.14 (7.17)	11.01 (8.25)
indinavir	0.30	0.33 (10.00)	0.29 (3.33)	2.50	2.53 (1.2)	2.40 (4.00)	6.00	5.61 (12.2)	5.55 (7.50)
amprenavir	0.30	0.31 (3.33)	0.34 (13.33)	2.50	2.65 (6.00)	2.54 (1.60)	6.00	5.61 (12.2)	5.57 (7.17)
nelfinavir	0.30	0.28 (6.66)	0.27 (10.00)	2.50	2.79 (11.60)	2.66 (6.40)	6.00	5.87 (2.17)	5.83 (2.83)
saquinavir	0.30	0.31 (6.66)	0.29 (6.66)	2.50	2.73 (9.20)	2.63 (5.20)	6.00	5.83 (2.83)	5.80 (3.33)
ritonavir	0.60	0.63 (5.00)	0.58 (3.33)	5.00	4.95 (1.00)	5.03 (0.60)	12.00	11.28 (6.00)	11.05 (7.92)

[a] The units of plasma level are mg/l.

saquinavir, nelfinavir) and 0.05 mg/l (amprenavir). The optimum wavelengths are respectively 254 nm, 250 nm and 238 nm to detect amprenavir, nelfinavir and saquinavir. The similar retention times of amprenavir, nelfinavir, saquinavir do not allow to change the detection wavelength for each compound. The wavelength is established at 254 nm for these compounds. This wavelength is optimum for detection of amprenavir which explains the low limit of quantitation for amprenavir. The limit of quantitation of saquinavir is clinically relevant with plasma trough level when saquinavir is combined with another PI such as ritonavir [11]. Ritonavir which acts as a potent inhibitor of CYP3A4 may therefore result in elevation of saquinavir level. These combinations are interesting since the trough levels obtained with saquinavir alone are near the lower limit of antiretroviral efficacy.

The intra-day and inter-day precision and accuracy are less or equal than 15% for quality controls (Tables 3 and 4). The correlation coefficients are higher than 0.99 for each compound. The calibration curves are linear at least up to 10 mg/l for indinavir, amprenavir, nelfinavir, saquinavir and 20 mg/l for ritonavir and nevirapine. The average values for recovery calculated with quality controls are from 76 to 91%. The stability of frozen samples is checked with our storage conditions and treatment. These

Table 4
Inter-day (ied) and intra-day (iad) precision for antiretroviral agents[a]

	Low level			Medium level			High level		
	Nominal plasma level	Average measured plasma level (precision%)		Nominal plasma level	Average measured plasma level (precision%)		Nominal plasma level	Average measured plasma level (precision%)	
		iad $n=3$	ied $n=5$		iad $n=3$	ied $n=5$		iad $n=3$	ied $n=5$
nevirapine	0.60	0.58 (2.99)	0.57 (5.00)	5.00	5.52 (11.13)	5.18 (3.58)	12.00	11.14 (1.04)	11.01 (8.28)
indinavir	0.30	0.33 (12.37)	0.29 (14.63)	2.50	2.53 (10.48)	2.40 (7.57)	6.00	5.61 (1.87)	5.55 (1.17)
amprenavir	0.30	0.31 (7.07)	0.34 (14.78)	2.50	2.65 (14.96)	2.54 (3.88)	6.00	5.61 (0.76)	5.57 (0.58)
nelfinavir	0.30	0.28 (5.39)	0.27 (5.99)	2.50	2.79 (8.53)	2.66 (4.38)	6.00	5.87 (1.67)	5.83 (0.59)
saquinavir	0.30	0.31 (3.33)	0.29 (2.40)	2.50	2.73 (11.01)	2.63 (3.53)	6.00	5.83 (2.58)	5.80 (0.50)
ritonavir	0.60	0.63 (5.00)	0.58 (10.32)	5.00	4.95 (1.00)	5.03 (2.44)	12.00	11.28 (6.00)	11.05 (3.07)

[a] Precision is defined as the coefficient of variation. The units of plasma level are mg/l.

results allow to validate this method which can be used for the therapeutic drug monitoring of PI and nevirapine in HIV disease.

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