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Short communication

Determination of atazanavir and other antiretroviral drugs (indinavir, amprenavir, nelfinavir and its active metabolite M8, saquinavir, ritonavir, lopinavir, nevirapine and efavirenz) plasma levels by high performance liquid chromatography with UV detection

E. Dailly[a,*], F. Raffi[b], P. Jolliet[a]

[a] *Clinical Pharmacology Department, Hôtel Dieu, Nantes, France*
[b] *Infectious Diseases Department, Hôtel Dieu, Nantes, France*

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Abstract

A global method is proposed for therapeutic drug monitoring of atazanavir, a novel protease inhibitor and of all other protease inhibitors (PI) and non nucleoside reverse transcriptase inhibitors (NNRTI) which are currently used to treat HIV patients. All drugs are extracted after a liquid–liquid extraction and separated on a C18 column with a binary gradient elution except lopinavir which is separated without this gradient. The absorbance is measured at 259 nm except for lopinavir (205 nm) and nevirapine (320 nm). This method is specific, accurate, precise (the intra-day and inter-day imprecision and inaccuracy are lower than 15%) and the limits of quantitation (0.40 mg/L for nevirapine, 0.10 mg/L for indinavir, 0.10 mg/l for M8, 0.05 mg/L for amprenavir, 0.10 mg/L for nelfinavir, 0.10 mg/L for saquinavir, 0.10 mg/L for ritonavir, 0.10 mg/L for efavirenz, 0.10 mg/L for atazanavir and 0.20 mg/L for lopinavir) are consistent with trough plasma concentrations allowing to use this method for therapeutic drug monitoring of PI and NNRTI.

Keywords: Indinavir; Atazanavir; Amprenavir; Nelfinavir; M8; Saquinavir; Ritonavir; Lopinavir; Nevirapine; Efavirenz

1. Introduction

Numerous methods were developed for therapeutic drug monitoring (TDM) of human immunodeficiency virus (HIV) drugs [1–13]. Among these assays, a few methods allow a monitoring of both non nucleoside reverse transcriptase inhibitors (NNRTI) (nevirapine and efavirenz) and the six protease inhibitors (PI) (indinavir, amprenavir, saquinavir, nelfinavir, ritonavir, lopinavir) currently used [7,9]. Presently, no published method allows a monitoring of nevirapine, efavirenz and all PI including M8, the active metabolite of nelfinavir and atazanavir, a novel protease inhibitor recently licensed by government agencies. The assays which were published to determine atazanavir plasma level are based on liquid chromatography coupled with tandem mass spectrometry which is not a currently available technology in the laboratories [14,15]. Moreover, these assays were not developed for a monitoring of others PI and NNRTI. The aim of this study is to present a high performance liquid chromatography assay (HPLC) with UV detection adapted for TDM of atazanavir and all other PI and NNRTI currently used to treat HIV patients. This assay is based on a previously published method which the extraction procedure was modified [16].

* Corresponding author. Present address: Laboratoire de Pharmacologie Clinique, Institut de Biologie, Centre Hospitalier Universitaire, 9 quai Moncousu, 44093 Nantes Cedex 1, France. Tel.: +33 2 40084087; fax: +33 2 40083996.
E-mail address: eric.dailly@chu-nantes.fr (E. Dailly).

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2. Experimental

2.1. Chemicals

Amprenavir mesylate (GLAXO WELLCOME, London, UK), nelfinavir mesylate and M8 (AGOURON PHARMACEUTICALS, La Jolla, CA, USA), indinavir sulfate (MERCK SHARP & DOHME-CHIBRET, West Point, PA, USA), the internal standard (A86093), lopinavir and ritonavir (ABBOTT, IL, USA), nevirapine (BOEHRINGER INGELHEIM, Ridgefield, CT, USA) saquinavir mesylate (F. HOFFMANN-LA ROCHE, Basel, Switzerland), efavirenz and atazanavir (BRISTOL-MYERS SQUIBB, New Brunswick, NJ, USA) were kindly provided by pharmaceutical companies. Tetramethylammonium perchlorate was purchased from ACROS ORGANICS (Geel, Belgium), 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). Sodium Carbonate decahydrate (for analysis) was from MERCK EUROLAB (Briare Le Canal, France).

2.2. Equipment

The high performance liquid chromatographic system consists of a model 9095 automatic sample injector, a model 9010 solvent delivery pump and a model Prostar 310 programmable wavelength UV detector. All these instruments were from VARIAN (Sunnyvale, CA, USA). The whole system is controlled by the LC Star Workstation Software (VARIAN). The separation is realized at room temperature on a Symmetry® 5 μm C18 column (250 × 4.6 mm i.d.) protected with a Symmetry® 5 μM C18 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)).

For all the drugs except lopinavir, the mobile phase was delivered at 0.9 mL/min with a gradient program: from time 0 (solvent A 30%–solvent B 70%) to 8 min (solvent A 80%–solvent B 20%), methanol is progressively replaced by acetonitrile; from time 8 to 30 min, the composition of the mobile phase is constant (solvent A 80%–solvent B 20%), from time 30 min to 40 min, the column is stabilized with the initial gradient (solvent A 30%–solvent B 70%) before the next injection. The compounds are detected at 320 nm for nevirapine and 259 nm for indinavir, M8, atazanavir, amprenavir, nelfinavir saquinavir, ritonavir, internal standard and efavirenz.

For lopinavir, the mobile phase (solvent A 80% and solvent B 20%) is delivered at 1 mL/min without a gradient elution program and the detection is performed at 205 nm.

2.4. Preparation of standards

Stock solution of M8 is prepared at a concentration of 500 mg/L (ethanol), stock solutions of indinavir, atazanavir, nelfinavir, ritonavir, internal standard and efavirenz at a concentration of 1000 mg/L (methanol for indinavir, atazanavir, efavirenz and ethanol for nelfinavir, internal standard, ritonavir) stock solutions of amprenavir and saquinavir at a concentration of 2000 mg/L (methanol), and stock solutions of nevirapine and lopinavir at a concentration of 2500 mg/L (methanol).

For the calibration samples, a working solution is realized by diluting the stock solutions in a mixture methanol/water (1:1 (v/v)) to a final concentration of 50 mg/L for M8; 100 mg/L for indinavir, atazanavir, amprenavir, nelfinavir, saquinavir, ritonavir; 200 mg/L for nevirapine, lopinavir and efavirenz. 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 microliters of these solutions were mixed with 900 μl of drug free human plasma to prepare calibration samples.

For the quality controls, a solution-high level is prepared by diluting the stock solutions in a mixture methanol/water (1:1 (v/v)) to a final concentration of 25 mg/L for M8 and 50 mg/L for other drugs. This solution-high level is used to prepare by dilution in methanol/water (1:1 (v/v)) a solution-medium level at 10 mg/L for M8 and 20 mg/L for other drugs; a solution-low level at 2.5 mg/L for M8 and 5 mg/L for other drugs. These solutions (solutions—high, medium and low levels) are diluted in free drug human plasma (1:9 (v/v)) to prepare quality controls high level (5 mg/L for all drugs except M8 2.5 mg/L), medium level (2 mg/L for all drugs except M8 1 mg/L)and low level (0.5 mg/L for all drugs except M8 0.25 mg/L). These diluted solutions are distributed in tubes and stored at −20 °C.

For the internal standard, a solution is prepared by diluting the stock solution of internal standard in a mixture methanol/water to a final concentration of 20 mg/L.

2.5. Specificity

The specificity of the method was investigated using plasma spiked with HIV drugs (zidovudine, stavudine, didanosine, lamivudine, tenofovir, abacavir) and possible co-administered drugs (itraconazole, sulfamethoxazole, trimethoprime, aciclovir, zolpidem, zopiclone, amitriptyline, bromazepam, alprazolam, tetrazepam, acetaminophen, aspirine, loperamide, phloroglucinol, omeprazole, naproxen, ibuprofen).

2.6. Sample treatment

The blood samples with lithium heparinate as anticoagulant 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 (20 mg/L), 1 ml of a sodium carbonate decahydrate solution (0.5 M, pH = 11.7) 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 upper organic phase are evaporated to dryness under a gentle stream of nitrogen at +40 °C. The lower phase is combined with 5 ml of a mixture of ethyl acetate/hexane (9:1 (v/v)). This combination is shaked for 10 min and the organic phase is separated by centrifugation at 3000 rpm (1800 × *g*) for 5 min at +4 °C. Three ml of the upper organic phase are added to the residue of the first extraction and evaporated to dryness under a gentle stream of nitrogen at +40 °C. The final residue is reconstituted by vortexing for 2 min by 0.3 mL of a solution containing acetonitrile, methanol and 0.025 M tetramethylammonium perchlorate in 0.2% aqueous trifluoroacetic acid (17:5:78 (v/v)). Fifty μl of the solution are injected.

2.7. Calibration curves

The calibration curves are calculated by unweighted least square linear regression. The range of standard concentrations tested are from 0.125 mg/L to 5 mg/L for M8, from 0.25 mg/L to 10 mg/L (for indinavir, atazanavir, nelfinavir, amprenavir, saquinavir, ritonavir) and from 0.5 mg/L to 20 mg/L for nevirapine, lopinavir and efavirenz.

3. Results

The quality controls (low, medium and high levels) were used for the imprecision and inaccuracy determination. For intra-day validation, five samples of each quality control were analyzed within the same day. For inter-day validation, concentrations of quality controls were determined on five separate days. The intra-day and inter-day imprecision and inaccuracy are less or equal than 15% for quality controls (Table 1). The quality controls were also used to calculate the recovery ratios (Table 2). With this extraction procedure, the limits of quantitation are 0.40 mg/L for nevirapine, 0.10 mg/L for indinavir, 0.10 mg/l for M8, 0.05 mg/L for amprenavir, 0.10 mg/L for nelfinavir, 0.10 mg/L for saquinavir, 0.10 mg/L for ritonavir, 0.10 mg/L for efavirenz, 0.10 mg/L for atazanavir and 0.20 mg/L for lopinavir. The calibration curves are linear (the correlation coefficients are higher than 0.99) at least up to 5 mg/L for M8; 10 mg/L for indinavir, atazanavir, nelfinavir, amprenavir, saquinavir, ritonavir and 20 mg/L for nevirapine, lopinavir and efavirenz. The stability of frozen plasma samples (−20 °C) is checked with

Table 1
Inter-day (ied; $n = 5$) and intra-day (iad; $n = 5$) imprecision and inaccuracy for antiretroviral drugs

	Low level			Medium level			High level		
	Nominal plasma level	Average measured plasma level (imprecision%–inaccuracy%)		Nominal plasma level	Average measured plasma level (imprecision%–inaccuracy%)		Nominal plasma level	Average measured plasma level (imprecision%–inaccuracy%)	
		iad	ied		iad	ied		iad	ied
Nevirapine	0.50	0.48 (2.05–4.00)	0.49 (4.79–2.00)	2.00	1.85 (5.30–7.50)	1.94 (13.30–3.00)	5.00	4.95 (2.10–1.00)	4.94 (9.07–1.20)
Indinavir	0.50	0.53 (3.01–6.00)	0.51 (7.06–2.00)	2.00	1.91 (3.50–4.50)	1.87 (4.35–6.50)	5.00	4.91 (3.01–1.80)	5.07 (11.19–1.40)
M8	0.25	0.22 (6.50–12.00)	0.24 (6.47–4.00)	1.00	0.89 (6.05–11.00)	0.90 (13.23–10.00)	2.5	2.70 (5.60–8.00)	2.43 (5.16–2.80)
Amprenavir	0.50	0.51 (8.00–2.00)	0.46 (4.01–8.00)	2.00	2.10 (4.51–5.00)	1.89 (2.15–5.50)	5.00	4.83 (1.50–3.40)	4.78 (4.55–4.40)
Nelfinavir	0.50	0.45 (1.50–10.00)	0.45 (7.84–10.00)	2.00	1.81 (10.50–9.50)	1.85 (14.57–7.50)	5.00	5.50 (5.66–10.00)	4.85 (6.15–3.00)
Saquinavir	0.50	0.44 (3.50–12.00)	0.43 (9.80–14.00)	2.00	1.75 (3.05–12.5)	1.97 (12.21–1.50)	5.00	4.41 (9.13–11.8)	4.39 (4.31–12.2)
Ritonavir	0.50	0.54 (2.50–8.00)	0.57 (1.73–14.00)	2.00	1.91 (5.01–4.50)	2.07 (3.79–3.50)	5.00	4.52 (3.05–9.60)	4.76 (11.53–4.80)
Efavirenz	0.50	0.55 (2.40–10.00)	0.51 (6.31–2.00)	2.00	2.10 (4.50–5.00)	1.89 (3.44–5.50)	5.00	4.35 (7.18–13.00)	5.14 (8.44–2.80)
Atazanavir	0.50	0.43 (5.04–14.00)	0.45 (4.68–10.00)	2.00	1.79 (1.90–10.50)	1.84 (4.39–8.00)	5.00	5.23 (10.41–4.60)	4.70 (5.94–6.00)
Lopinavir	0.50	0.55 (7.10–10.00)	0.54 (6.73–8.00)	2.00	2.15 (5.02–7.50)	2.12 (2.21–6.00)	5.00	5.06 (10.51–1.20)	5.01 (4.90–0.20)

Inaccuracy is defined as the percent of deviation from the nominal level [low, medium, high nominal levels are equal respectively to 0.50, 2.00, 5.00 mg/L for all drugs except for M8 (0.25 mg/L, 1.00 mg/L, 2.5 mg/L)] and imprecision as the coefficient of variation. The units of plasma concentration is mg/L.

Table 2
The recovery rates of antiretroviral drugs

	Recovery rate (%)
Nevirapine	88
Indinavir	81
M8	80
Amprenavir	88
Nelfinavir	90
Saquinavir	85
Ritonavir	95
Efavirenz	80
Atazanavir	82
Lopinavir	85

The recovery rate is calculated by comparing the peak areas of the quality controls samples after extraction with the peak areas of standards solution at the same concentration and not extracted. For each drug, this calculation is done for the three levels of quality controls and a mean value is calculated.

our storage conditions during at least 1 month. No interference was found with drugs listed in the paragraph "Specificity": with chromatographic conditions used for lopinavir, the retention time of sulfamethoxazole was 4.8 min and other tested drugs were not detected; with chromatographic conditions used for other PI and NNRTI, the retention time of abacavir was 4.5 min and the other tested drugs were not detected. The chromatograms of spiked plasma sample (8 mg/L for nevirapine, lopinavir, efavirenz; 4 mg/L for indinavir, atazanavir, amprenavir, nelfinavir, saquinavir, ritonavir; 2 mg/L for M8) are presented in Fig. 1 with chromatographic conditions used to determine plasma concentrations of nevirapine, efavirenz, indinavir, atazanavir, amprenavir, nelfinavir, saquinavir, ritonavir, M8 and in Fig. 4 with chromatographic conditions used to determine plasma concentrations of lopinavir. The corresponding chromatograms of blank plasma sample are presented in Figs. 2 and 5. Figs. 3 and 6 correspond to the chromatograms of patients treated respectively by atazanavir/ritonavir 300/100 mg once a day and lopinavir/ritonavir 533/133 mg twice a day.

4. Discussion

Increasing evidence suggest that the TDM for the PI and the NNRTI in the treatment of HIV infection is a promising therapeutic modality [17]. Consequently, a method to determine atazanavir plasma level adapted for TDM has to be developed. This method based on a HPLC assay coupled with UV detection allows a monitoring of all PI and NNTRI currently used with limits of quantitation which are consistent with therapeutic through plasma concentrations of PI and NNRTI [18–19]. In comparison with the previously published method [16], the modifications in the extraction procedure which allowed to have these limits of quantitation are the alkalinity adjustment of plasma (plasma is mixed with a sodium carbonate solution) which affects mainly efavirenz and lopinavir extraction, a double extraction in organic phase

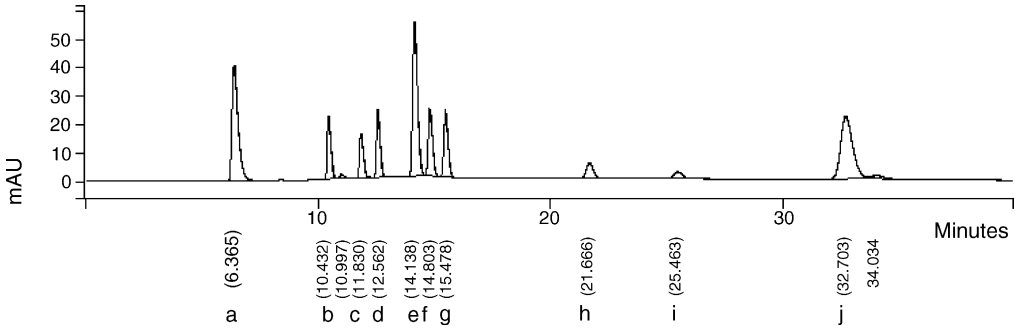

Fig. 1. Chromatogram of spiked plasma sample (8 mg/L for nevirapine, lopinavir, efavirenz; 4 mg/L for indinavir, atazanavir, amprenavir, nelfinavir, saquinavir, ritonavir; 2 mg/L for M8). Chromatography is performed at 320 nm before 8 min and 259 nm after 8 min with a gradient elution. The retention times are 6.365 min for nevirapine (a), 10.432 min for indinavir (b), 11.830 min for M8 (c), 12.562 min for atazanavir (d), 14.138 min for amprenavir (e), 14.803 min for nelfinavir (f), 15.478 min for saquinavir (g), 21.666 min for ritonavir (h), 25.463 min for A86093 (internal standard) (i) and 32.703 min for efavirenz (j). The unidentified peaks are blood interferences.

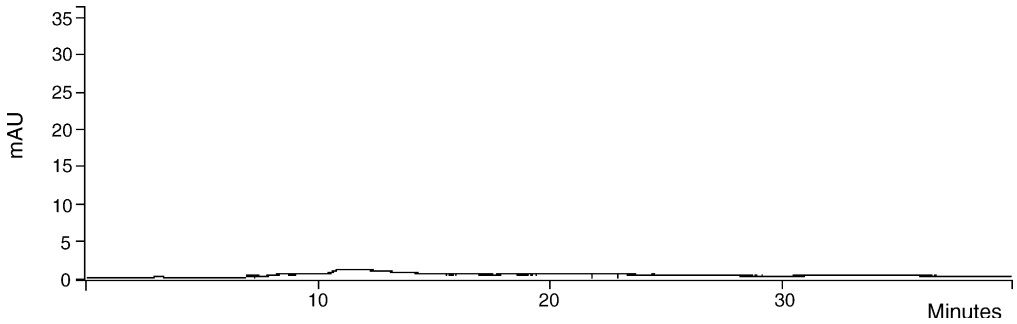

Fig. 2. Chromatogram of a blank plasma sample. Chromatography is performed at 320 nm before 8 min and 259 nm after 8 min with a gradient elution.

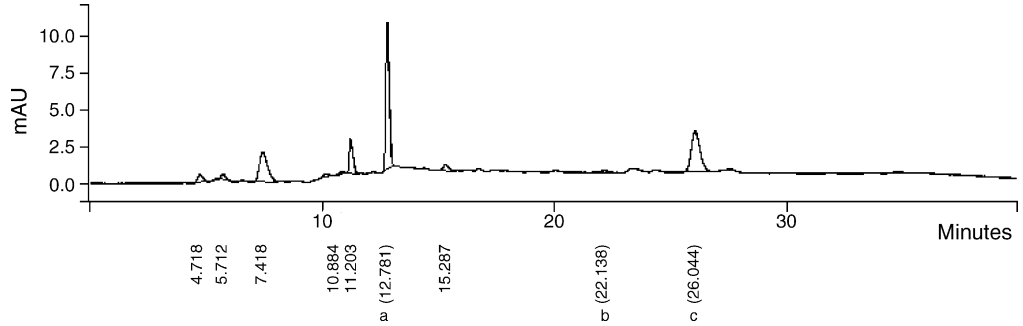

Fig. 3. Chromatogram obtained from a patient receiving atazanavir/ritonavir 300/100 mg once a day. The plasma concentration of atazanavir (a) is 1.40 mg/L. The plasma concentration of ritonavir (b) is lower than the limit of quantitation (<0.1 mg/L). A86093 (c) is the internal standard. The unidentified peaks are blood interferences.

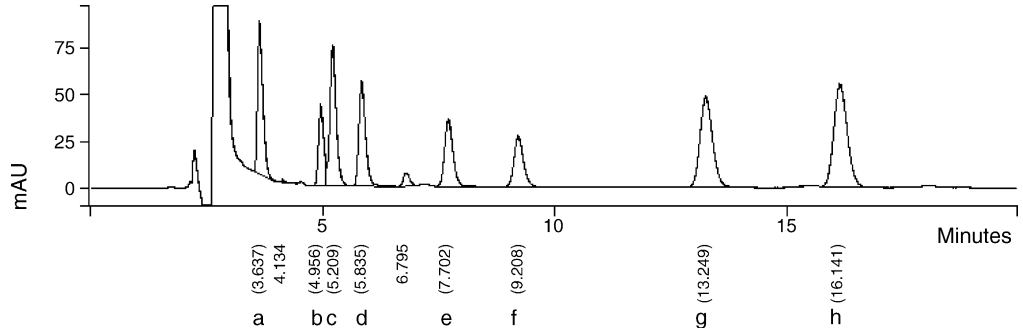

Fig. 4. Chromatogram of spiked plasma sample (8 mg/L for nevirapine, lopinavir, efavirenz; 4 mg/L for indinavir, atazanavir, amprenavir, nelfinavir, saquinavir, ritonavir; 2 mg/L for M8). Chromatography is performed at 205 nm without a gradient elution. The retention times of detected drugs with these chromatographic conditions are 3.637 min for atazanavir (a), 4.956 min for amprenavir (b), 5.209 min for nelfinavir (c), 5.835 min for saquinavir (d), 7.702 min for ritonavir (e), 9.208 min for A86093 (internal standard) (f), 13.249 min for lopinavir (g) and 16.141 min for efavirenz (h). The unidentified peaks are blood interferences.

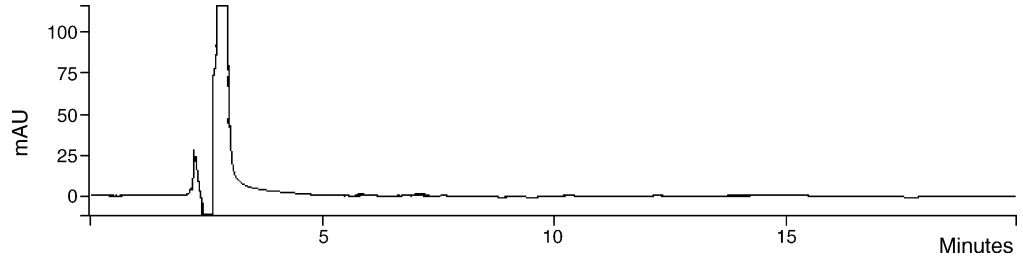

Fig. 5. Chromatogram of a blank plasma sample. Chromatography is performed at 205 nm without a gradient elution.

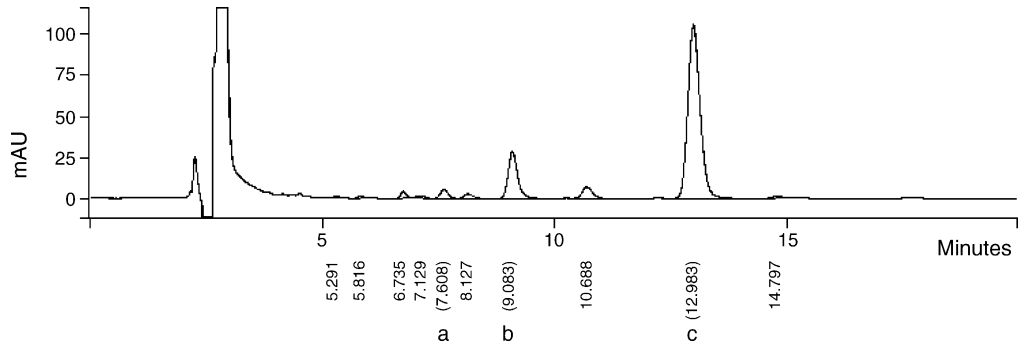

Fig. 6. Chromatogram obtained from a patient receiving lopinavir/ritonavir 533/133 mg twice a day. The plasma concentration of lopinavir (c) is 16.5 mg/L and 0.9 mg/L for ritonavir (a). A86093 (b) is the internal standard. The unidentified peaks are blood interferences.

rather than a simple extraction which improves mainly indinavir and M8 extraction and suppression of the final wash of the reconstituted residue by hexane which allows to detect atazanavir and efavirenz with this method. In conclusion, this method is enough specific, accurate and precise to be used in routine for TDM of all currently available NNRTI and PI including the novel protease inhibitor atazanavir.

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