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A liquid chromatography–tandem mass spectrometry assay for quantification of nevirapine, indinavir, atazanavir, amprenavir, saquinavir, ritonavir, lopinavir, efavirenz, tipranavir, darunavir and maraviroc in the plasma of patients infected with HIV

J. Martin[a], G. Deslandes[a], E. Dailly[a,b,*], C. Renaud[a], V. Reliquet[c], F. Raffi[c], P. Jolliet[a,b]

[a] *Clinical Pharmacology Department, CHU de Nantes, Nantes, France*
[b] *EA 4275 Biostatistique, Recherche Clinique et Mesures Subjectives en Santé, Faculté de Médecine–Pharmacie, Université de Nantes, Nantes, France*
[c] *Infectious Diseases Department, CHU de Nantes, Nantes, France*

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ABSTRACT

A liquid chromatography–tandem mass spectrometry assay for simultaneous determination of the plasma concentration of 11 antiretroviral agents (nevirapine, indinavir, atazanavir, amprenavir, saquinavir, ritonavir, lopinavir, efavirenz, tipranavir, darunavir and maraviroc) has been developed. Sample pre-treatment is limited to protein precipitation with a mixture of methanol and zinc sulfate. After centrifugation the supernatant is injected in the chromatographic system, which consists of on-line solid phase extraction followed by separation on a phenyl–hexyl column. This method, with its simple sample preparation provides sensitive, accurate and precise quantification of the plasma concentration of antiretroviral drugs and can be used for therapeutic drug monitoring in patients infected with HIV.

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1. Introduction

Several liquid chromatography assays coupled with ultra violet [1–10] or tandem mass spectrometry detection [11–14] have been developed for simultaneous determination of antiretroviral agents in the plasma of patients infected with HIV. However, a few assays allowing simultaneous measurement of the plasma concentration of darunavir, the last protease inhibitor to be licensed, and other antiretroviral drugs [10–13] and a single assay measuring the plasma concentration of the CCR5 co-receptor antagonist maraviroc plasma concentration have been previously published [14]. However, no chromatographic method has been developed to determine simultaneously the plasma concentrations of maraviroc and other antiretroviral drugs which are the most commonly prescribed (the protease inhibitors amprenavir, atazanavir, darunavir, indinavir, lopinavir, ritonavir, saquinavir, tipranavir and the non-nucleoside reverse transcriptase inhibitors nevirapine and efavirenz). This work presents a liquid chromatography–tandem mass spectrometry method with a simple sample pre-treatment which can be used for the monitoring of these antiretroviral drugs.

2. Experimental

2.1. Chemicals

Methanol, zinc sulfate, acetic acid and ammonium acetate were purchased from VWR International (Fontenay sous Bois, France). The antiretroviral agents are listed in Table 1. Amprenavir mesylate (GlaxoSmithKline, London, UK), indinavir sulfate salt (Merck, Rahway, NJ, USA), the internal standard (A 86093), lopinavir and ritonavir (Abbott, IL, USA), nevirapine and tipranavir (Boehringer Ingelheim, Ridgefield, CT, USA) saquinavir mesylate (Roche Products, Basel, Switzerland), efavirenz and atazanavir (Bristol-Myers Squibb, New Brunswick, NJ, USA), darunavir (Tibotec, Mechelen, Belgium) and maraviroc (Pfizer, Groton, USA) were kindly provided by the pharmaceutical companies. The chemical structures of these compounds are presented in Fig. 1.

2.2. Preparation of standards

Stock solutions were prepared as follows: indinavir, atazanavir, ritonavir and internal standard A86093 at a concentration of 1000 mg/L (methanol for indinavir, atazanavir and ethanol for

* Corresponding author at: Laboratoire de Pharmacologie clinique, Hôtel Dieu, 9 Quai Moncousu, 44093 Nantes Cedex, France. Tel.: +33 2 40 08 40 95; fax: +33 2 40 08 39 96.
E-mail address: eric.dailly@chu-nantes.fr (E. Dailly).

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Table 1
Antiretroviral drugs and MRM transitions used for detection, declustering potential (DP), entrance potential (EP), cell entrance potential (CEP), collision energy (CE) and cell exit potential (CXP) for API 3200, and retention times for the phenyl–hexyl HPLC column.

	MRM transition (*m*/*z*)	Retention time (min)	DP	EP	CEP	CE	CXP
Amprenavir	506.2–156.2	1.91	51	4	28	39	4
Atazanavir	705.4–168.2	1.97	66	11	30	79	6
Darunavir	548.2–392.1	1.91	51	7	26	23	32
Efavirenz	316.0–243.9	1.94	36	11	24	19	8
Indinavir	614.2–421.0	1.95	71	4	34	37	26
Lopinavir	629.2–183.1	2.01	31	9.5	52	25	14
Nevirapine	267.1–225.8	1.92	46	5	14	39	8
Ritonavir	721.3–296.0	1.99	56	4.5	22	31	10
Saquinavir	671.3–225.2	1.98	81	4.5	26	77	8
Tipranavir	603.1–172.2	1.95	61	5.5	54	67	7
Maraviroc	514.2–280.1	1.92	51	9.5	28	37	8
A86093	747.2–322.0	2.00	51	10	33.4	33	10

ritonavir and internal standard); nevirapine, lopinavir, efavirenz and darunavir at a concentration of 2500 mg/L (methanol); amprenavir and saquinavir at a concentration of 2000 mg/L (methanol); maraviroc at a concentration of 500 mg/L (methanol), and tipranavir at a concentration of 5000 mg/L (methanol).

For the calibration samples, a working solution was prepared by diluting the stock solution in a mixture of methanol/water [1:1 (v/v)] to a final concentration of 200 mg/L for nevirapine, lopinavir, efavirenz and darunavir; 100 mg/L for indinavir, amprenavir, atazanavir and saquinavir; 20 mg/L for ritonavir and maraviroc, 800 mg/L for tipranavir. Six-point calibration curves and blanks were prepared for the calibration of each analyte by diluting known volumes of the working solution in methanol/water [1:1 (v/v)] to obtain a final volume of 1000 μL. Fifty microliters of these solutions were mixed with 450 μL of drug-free human plasma to prepare the calibration samples. The calibrators were further treated as described in Section 2.3.

For the quality controls, a high-level solution was prepared by diluting the stock solutions in a mixture of methanol/water [1:1 (v/v)] to a final concentration of 300 mg/L for tipranavir, 50 mg/L for nevirapine, indinavir, amprenavir, atazanavir, saquinavir, darunavir, lopinavir and efavirenz, and 8 mg/L for ritonavir and maraviroc. This high-level solution was used to prepare a medium-level solution by dilution in methanol/water [1:1 (v/v)] at 120 mg/L for tipranavir, 20 mg/L for nevirapine, indinavir, amprenavir, atazanavir, saquinavir, darunavir, lopinavir and efavirenz, and 3.2 mg/L for maraviroc and ritonavir, and a low-level solution at 30 mg/L for tipranavir, 5 mg/L for nevirapine, indinavir, amprenavir, atazanavir, saquinavir, darunavir, lopinavir and efavirenz, and 0.8 mg/L for maraviroc and ritonavir. Fifty microliters of these solutions were mixed with 450 μL of drug-free human plasma to prepare quality controls for high, medium and low levels. The quality controls were further treated as described in Section 2.3. The final plasma concentrations of the low, medium and high controls were 0.5, 2 and 5 mg/L for nevirapine, indinavir, amprenavir, atazanavir, saquinavir, lopinavir, efavirenz, darunavir; 0.08, 0.32 and 0.8 mg/L for ritonavir, maraviroc and 3, 12, and 30 mg/L for tipranavir.

2.3. Sample preparation

The blood samples with lithium heparinate as anticoagulant were centrifuged at 3000 rpm (1800 × g) for 10 min at 4 °C. One hundred microliters of plasma were treated with 200 μL of precipitation reagent [methanol/0.2 M zinc sulfate (80/20 v/v)] including 0.5 mg/L A86093, used as internal standard, in a 1.5 mL conical plastic Eppendorf test tube. Samples were immediately vortexed and then centrifuged at 13,000 g for 15 min at 4 °C. Then, 150 μL of supernatant were transferred into a sample vial with a 200 μL volume micro-insert and 2 μL were injected into the chromatographic system.

2.4. Calibration curves

Six-point calibration curves (0.4, 2, 4, 8, 16, 40 mg/L for tipranavir; 0.1, 0.5, 1, 2, 4, 10 mg/L for lopinavir, efavirenz, darunavir and nevirapine; 0.05, 0.25, 0.5, 1, 2, 5 mg/L for amprenavir, atazanavir, saquinavir and indinavir; 0.01, 0.05, 0.1, 0.2, 0.4, 1 for ritonavir and maraviroc) were calculated (area ratio using the internal standard versus nominal concentration) and fitted either by a linear regression ($1/x$ and $1/x^2$ weighting) or a quadratic regression ($1/x$ and $1/x^2$ weighting). The concentrations were back calculated and the model with the lowest deviation between the calculated and nominal concentrations was retained.

2.5. Instruments

The instrument setup is shown in Fig. 2. The chromatographic system consisted of Agilent (Palo Alto, CA, USA) 1200 Series components including a binary pump, isocratic pump, column oven, 2 ten-port switching valves and an autosampler. The hardware configuration included an Applied Biosystems (Foster City, CA, USA) API 3200 equipped with a TurboIonspray source.

On-line extraction was performed using a perfusion column (POROS R1/20, 2.1 mm × 30 mm, Applied Biosystems, Foster City, CA, USA). The HPLC column was a short phenyl–hexyl column (Phenomenex Luna 5 μm Phenyl–Hexyl, 2 mm × 50 mm, Aschaffenburg, Germany).

Data analysis was performed using the Analyst 1.4.2 software package (Applied Biosystems, Foster City, CA, USA).

2.6. On-line solid phase extraction (SPE) and chromatographic separation

The chromatographic conditions are presented in Fig. 2 and the pumps and valves configurations are summarized in Table 2. During the first-dimension chromatography the binary pump supplied eluent A (100% water, 10 mM ammonium acetate, 0.1% acetic acid) for binding of whole molecules on a Poros column at a flow rate of 2700 μL/min for 1 min (0.1–1.1 min). At zero time, a 2 μL aliquot of supernatant from each prepared sample was injected. Simultaneously eluent C (methanol/water 97/3 (v/v), 10 mM ammonium acetate and 0.1% acetic acid) was introduced to the HPLC–tandem mass spectrometry detection at a flow rate of 500 μL/min, supplied by the isocratic pump. The SPE elution and analytes transfer to the HPLC column were performed by switching valve 1 after 1.1 min. The configuration developed offered complete SPE elution in flush mode and analyte transfer to the HPLC column and tandem

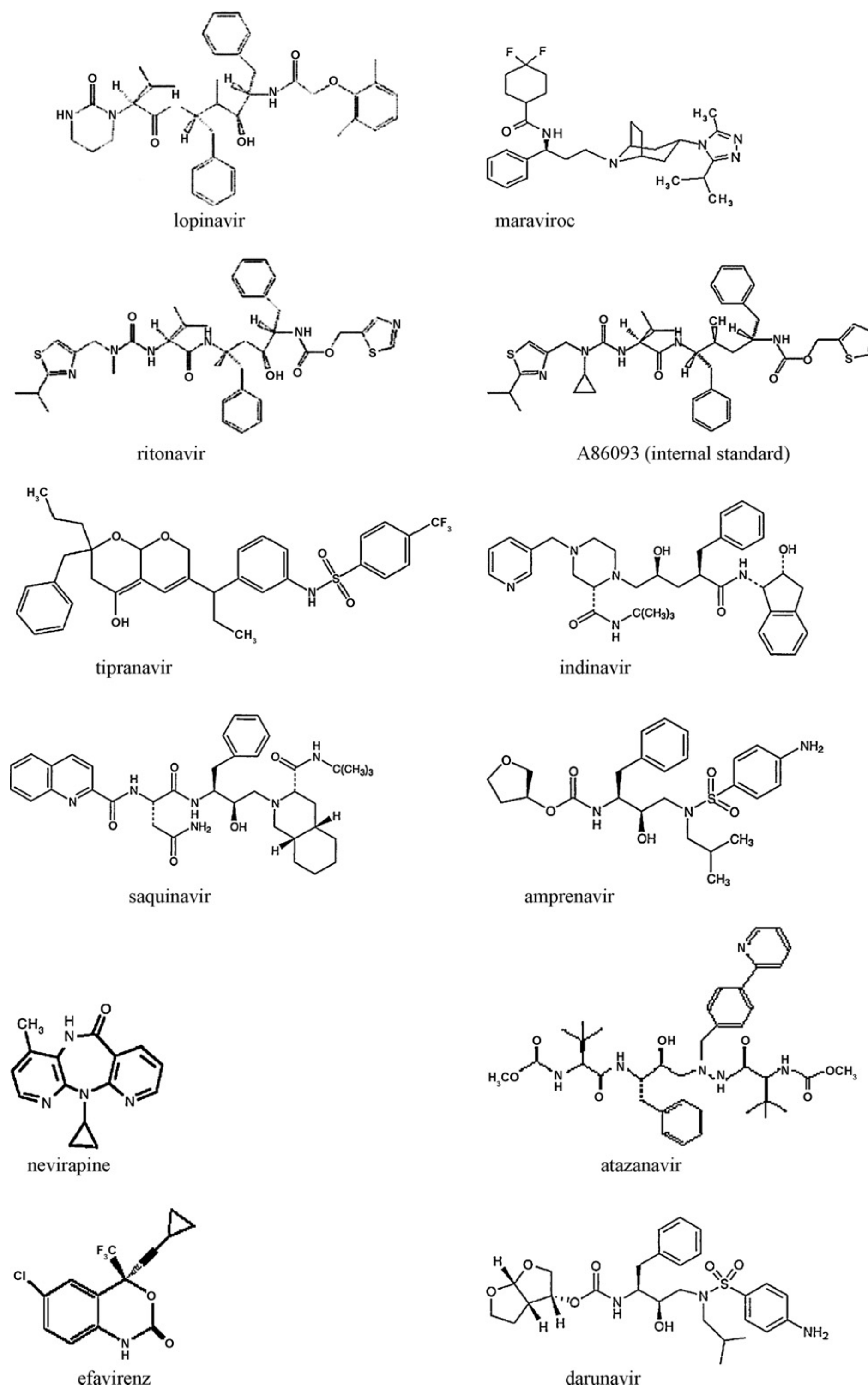


Fig. 1. Chemical structures of antiretroviral drugs and internal standard.

mass spectrometer by means of eluent C at 500 μL/min, which was well compatible with the TurboIonspray source. Eluent C was not able to separate the analytes completely using the phenyl–hexyl HPLC column but fortunately the highly selective tandem mass spectrometry detector in multiple reaction monitoring (MRM, precursor/production) detection mode was suitable for simultaneous detection of more than one analyte without retention time differences. The retention times are shown in Table 1. During this analyzing step the flow rate of eluent A was reduced to 500 μL/min. After 4.0 min, valve 1 was switched back to the start position and the binary pump supplied eluent B (100% methanol, 10 mM ammonium acetate, 0.1% acetic acid) to wash the Poros column at a flow rate of 2700 μL/min. After 5 min, the binary pump stopped supplying eluent B for eluent A for 1 min at a flow rate of 500 μL/min to re-equilibrate the Poros column. A total of analysis time of 6 min was obtained for all molecules including the internal standard.

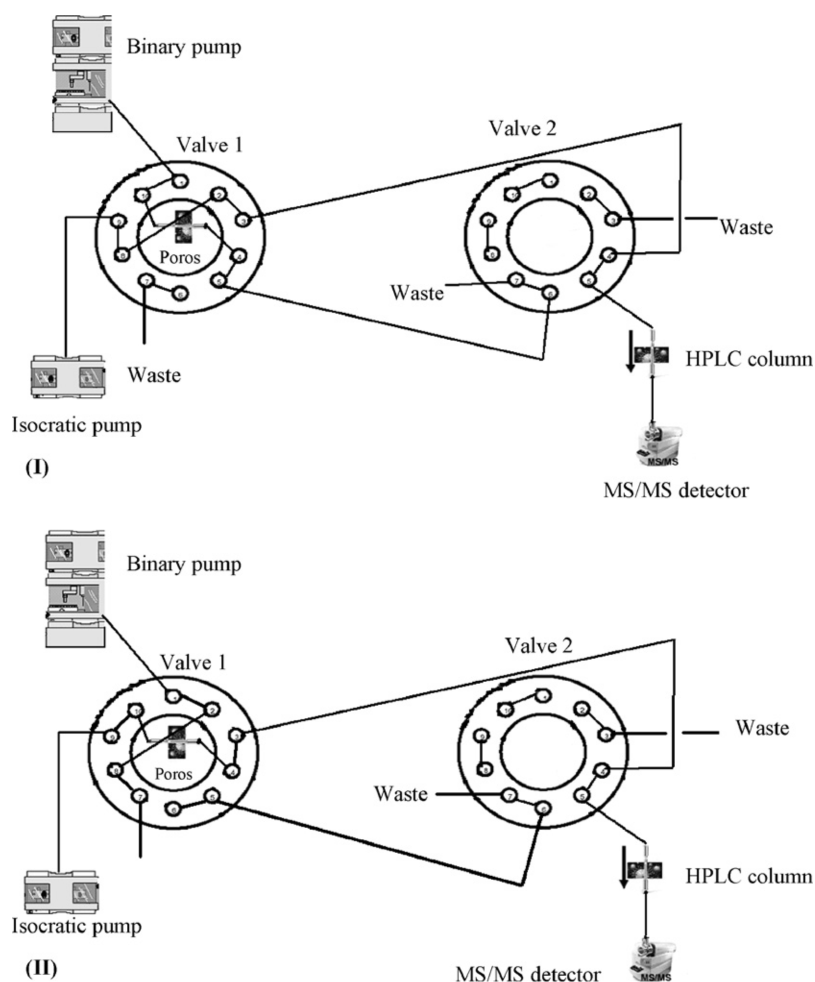


Fig. 2. Column switching system. (I), system configuration for loading, analysis, rinsing and re-equilibration. (II), system configuration for transfer.

2.7. Tandem mass spectrometry analysis

One positive ion mode MRM transition was used for each antiretroviral drug and internal standard. All transitions are listed in Table 1 including declustering potential (DP), entrance potential (EP), cell entrance potential (CEP), collision energy (CE) and cell exit potential (CXP). The dwell time was set to 50 ms for each MRM transition. The TurboIonspray interface settings and collision gas pressure were manually optimized (Ion spray voltage: 5500 V, temperature: 400 °C, collision gas: 5.0 psi, curtain gas: 25.0 psi). Tandem mass spectrometry was performed with nitrogen as collision gas. The procedure was completely automatic and was performed using the Analyst 1.4.2 software package.

2.8. Analytical method validation

2.8.1. Inaccuracy, imprecision and limit of quantitation

Inaccuracy and imprecision were evaluated by analyzing quality control samples at low, medium and high concentrations on 5 different days. For intra-day validation, 5 samples of each quality control were analyzed on the same day. For inter-day validation,

Table 2
Configuration for the pumps and valve.

Time (min)	Binary pump (eluents A and B)		Isocratic pump (eluent C)		10-port switching valve 1
	Flow rate eluent (μL/min)	A (%)	B (%)	Flow rate eluent (μL/min)	Position
0.0	500	100	0	500	A
0.1	2700	100	0	500	A
1.1	2700	100	0	500	A
1.2	500	100	0	500	B
3.4	500	100	0	500	B
3.5	2700	0	100	500	B
4.0	2700	0	100	500	A
4.9	2700	0	100	500	A
5.0	2700	100	0	500	A
5.5	2700	100	0	500	A
5.6	500	100	0	500	A
6.0	500	100	0	500	A

Table 3
Inter-day (ied; $n = 5$) and intra-day (iad; $n = 5$) precision and accuracy for antiretroviral drugs: Inaccuracy is defined as the percentage deviation from the nominal level and the imprecision as the coefficient of variation. The units for plasma concentration are mg/L.

	Low level (mg/L) average measured plasma level (imprecision%–inaccuracy%)		Medium level (mg/L) average measured plasma level (imprecision%–inaccuracy%)		High level (mg/L) average measured plasma level (imprecision%–inaccuracy%)	
	iad	ied	iad	ied	iad	ied
Amprenavir	0.55 (1.91–10.00)	0.53 (6.29–6.00)	2.05 (4.63–2.50)	2.03 (11.91–1.50)	5.65 (2.73–13.00)	5.41 (6.61–8.20)
Atazanavir	0.54 (4.13–8.00)	0.54 (8.57–8.00)	2.03 (4.38–1.50)	2.00 (5.54–0.00)	5.59 (2.60–11.80)	5.52 (10.73–10.40)
Darunavir	0.57 (2.89–14.00)	0.56 (2.80–12.00)	1.99 (5.09–0.50)	1.99 (5.14–0.50)	5.32 (1.30–6.4)	5.23 (4.65–4.6)
Indinavir	0.55 (4.10–10.00)	0.54 (3.52–8.00)	2.15 (6.16–7.5)	1.95 (6.96–2.5)	5.70 (1.77–14.0)	5.21 (11.92–4.20)
Lopinavir	0.53 (3.81–6.00)	0.51 (4.93–2.00)	2.02 (3.50–1)	1.86 (7.75–7.00)	5.23 (2.01–4.60)	4.95 (3.68–1.00)
Ritonavir	0.086 (1.91–7.5)	0.080 (9.43–0.00)	0.310 (4.07–3.13)	0.300 (3.15–6.25)	0.850 (2.40–6.25)	0.810 (9.70–1.25)
Saquinavir	0.55 (4.41–−10.00)	0.52 (4.68–4.00)	2.09 (3.57–4.50)	1.90 (6.70–5.00)	5.4 (1.73–8.00)	5.10 (6.85–2.00)
Tipranavir	3.30 (8.92–10.00)	3.03 (12.60–1.00)	12.50 (4.53–4.17)	11.52 (10.52–4.00)	29.76 (4.02–0.80)	27.95 (11.74–7.00)
Efavirenz	0.57 (3.98–14.00)	0.52 (9.53–4.00)	2.19 (4.49–9.5)	2.08 (4.71–4.00)	5.70 (1.92–14.00)	5.40 (6.65–8.00)
Nevirapine	0.57 (6.12–14.00)	0.53 (5.90–6.00)	2.08 (3.52–4.00)	1.91 (8.16–4.50)	5.55 (2.72–11.00)	5.01 (7.60–0.20)
Maraviroc	0.088 (3.73–10.00)	0.090 (3.10–12.50)	0.316 (3.16–1.25)	0.310 (4.39–3.13)	0.883 (3.92–10.38)	0.860 (9.16–7.50)

concentrations of the quality control samples were determined on 5 separate days. Inaccuracy is defined as the percentage of deviation from the nominal level and imprecision as the coefficient of variation within a single run (intra-assay) and between different assays (inter-assay). The imprecision and the inaccuracy should not exceed 15% except for the lower limit of quantitation for which 20% deviation was allowed.

2.8.2. Matrix effect and specificity

The matrix effect was investigated by the post-column infusion method defined by Bonfiglio et al. [15] and by a method proposed by Matuszewski [16] which is based on the precision of standard line slopes. According to Bonfiglio et al. [15], blank extracts (five different lots of plasma) were injected into the chromatographic system during post column infusion of the antiretroviral drugs and internal standard at a concentration of 200 ng/ml. The ion suppression or enhancement in the response of the infused analyte due to interferences with endogenous compounds was assessed using this post column infusion experiment. According to Matuszewski [16], the precision of the slope of the calibration lines in five different lots of plasma was used as an indicator of relative matrix effects. The relative standard deviation should not exceed 3–4% for the method to be considered practically free from relative matrix effects. To investigate this matrix effect, 5 points (the upper level of the calibration curves was deleted) calibration curve were calculated and fitted by linear regression ($1/x$ weighting) since non-linearity was observed for all drugs except darunavir and efavirenz. Under these conditions, deviations of the back calculated concentrations were within 85% and 115% of the nominal concentrations.

2.8.3. Recovery

The recovery ratios were determined by comparing the peak areas of the quality controls samples after extraction with the peak areas of the standard solutions at the same concentration and not extracted for the three levels of quality controls.

2.8.4. Stability

The stability of the analytes (aliquots of each level of quality control samples) was investigated in plasma stored at −20 °C, after 3 freeze and thaw cycles, and thawed at room temperature and kept at this temperature for 4 h. Stability was assured when 85–115% of the nominal concentration was found in the stored stability samples compared with a freshly prepared calibration curve. The stability of the stock solutions of drugs and internal standard stored at −20 °C was also evaluated.

2.8.5. Incurred sample reproducibility

Duplicate analysis of incurred samples has been performed. The bias of repeat [(result 1 − result 2)/result 1) × 100] should not exceed 15%.

3. Results

The calibration curves were satisfactorily fitted by quadratic regression ($1/x$ weighting) for all drugs except darunavir and efavirenz for which linear regression ($1/x$ weighting) was retained. Deviations of the back calculated concentrations were within 85% and 115% of the nominal concentrations (80% and 120% for the lower level which is the limit of quantitation) and the correlation coefficients for all calibration curves were above 0.990. The intra-day and inter-day imprecision and inaccuracy were less than 15% for quality control samples. These results are presented in Table 3. The limits of quantitation were 0.01 mg/L for maraviroc and ritonavir, 0.05 mg/L for amprenavir, atazanavir, saquinavir and indinavir, 0.1 mg/L for lopinavir, darunavir, efavirenz and nevirapine, and 0.4 mg/L for tipranavir. Fig. 3 shows one significant ion suppression area between 1.5 and 1.8 min for all transitions just before the eluting peaks of the analytes. The relative standard deviations of the slopes of the calibration lines in five different lots of plasma did not exceed 3–4% (Table 4). The average values for recovery calculated with the quality control samples were from 75% to 98%. The stability of the frozen plasma samples and stock solutions for a period of at least 2 months under our storage conditions and the stability of the analytes in plasma after 3 freeze and thaw cycles and thawed at room temperature and kept at this temperature 4 h were checked as the variation for each drug was within the ±15% of the nominal concentrations. Fig. 4a and b show the chromatograms for the lowest calibration

Table 4
Precision values (coefficient of variation, %CV) for the slopes of standard lines constructed for five different lots of plasma.

	slope (mean ±standard deviation)	%CV
Amprenavir	0.364 ± 0.014	3.84
Atazanavir	2.215 ± 0.079	3.57
Darunavir	0.126 ± 0.004	3.17
Indinavir	0.307 ± 0.012	3.91
Lopinavir	0.339 ± 0.013	3.83
Ritonavir	0.909 ± 0.030	3.30
Saquinavir	0.250 ± 0.002	0.80
Tipranavir	0.052 ± 0.001	1.92
Efavirenz	0.101 ± 0.001	0.99
Nevirapine	0.246 ± 0.009	3.66
Maraviroc	1.653 ± 0.030	1.81

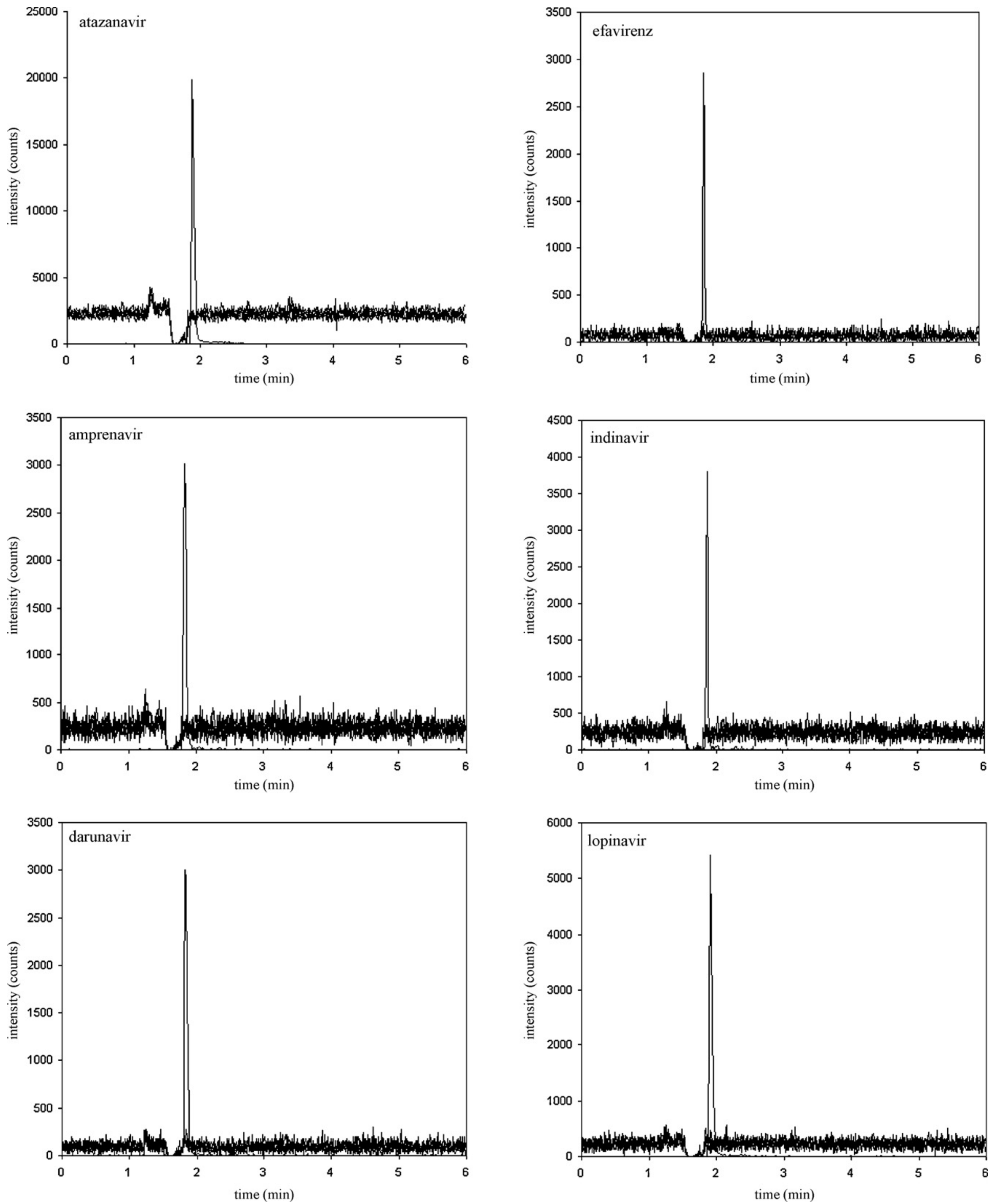


Fig. 3. Chromatograms of 5 blank plasma extracts with a post column infusion of solutions containing tipranavir, lopinavir, efavirenz, darunavir amprenavir, atazanavir, saquinavir, indinavir, maraviroc,ritonavir and A86093 at 200 ng/ml. Chromatogram of a calibration sample is also shown.

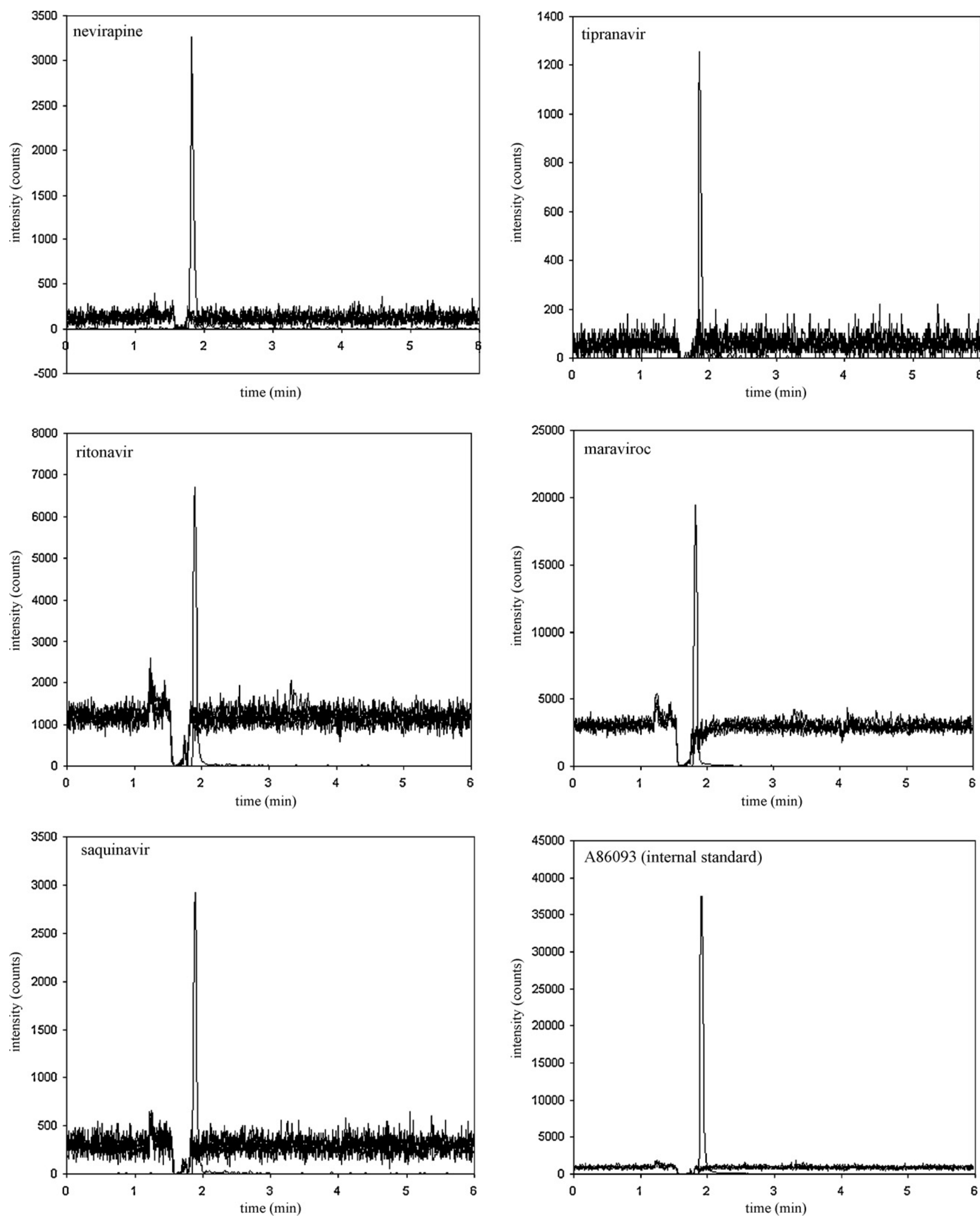


Fig. 3. (*Continued*).

sample and the blank methanol sample analyzed just after the high-level standard curves. No carry-over effects were observed. Fig. 5 shows the chromatograms of a woman infected with HIV who was treated with darunavir/ritonavir at a dosage of 600 mg/100 mg twice-daily in combination with efavirenz 600 mg once-daily and abacavir/lamivudine 600 mg/300 mg once-daily. The incurred sample reproducibility was checked for each drug (Table 5).

4. Discussion

This method allows accurate and precise determination of plasma concentrations of 11 antiretroviral agents including the novel antiretroviral agent maraviroc. Various methods based on chromatography coupled with mass spectrometry [12,13] or ultraviolet detection [10] have been developed recently to measure

plasma concentrations of the new protease inhibitor darunavir and other commonly used protease inhibitors and non-nucleoside reverse transcriptase inhibitors. The advantages of our method include the simultaneous quantification of these drugs with a limited sample (100 μl versus 500 μl [10]), a minimal and cost-effective sample preparation (a protein precipitation versus a manual solid phase extraction procedure [10]) and a short analytical run time (6 min versus 28 min [10] versus 25 min [12] versus 10 min [13]). Compared with a direct liquid chromatography–tandem mass spectrometry assay [13], the one-line solid phase extraction before liquid chromatography–tandem mass spectrometry prevents the introduction of endogenous compounds into the mass spectrometer which limits interference from endogenous compounds and the matrix effects as previously demonstrated [17]. According to these previous results, the post infusion column method showed that eluting peaks of analytes are separated from the ion suppression area. This result is confirmed by the method based on the precision of slopes of the calibration lines as the relative standard deviations of these slopes are low. No carry-over effect was highlighted by direct analysis of blank methanol sample after measuring the high-level standard curves. The limits of quantification are consistent with trough plasma concentrations of antiretroviral agents [18,19].

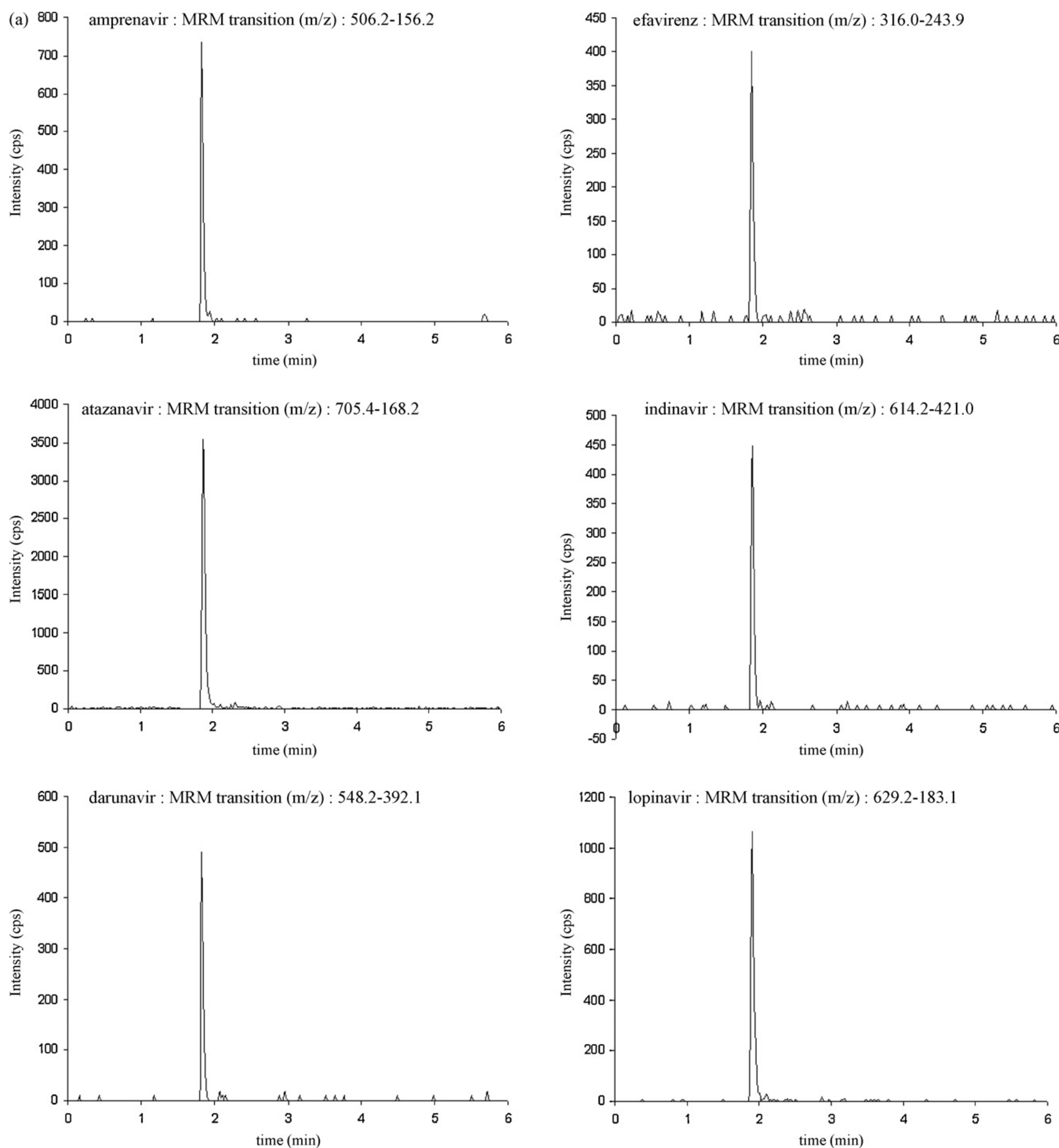
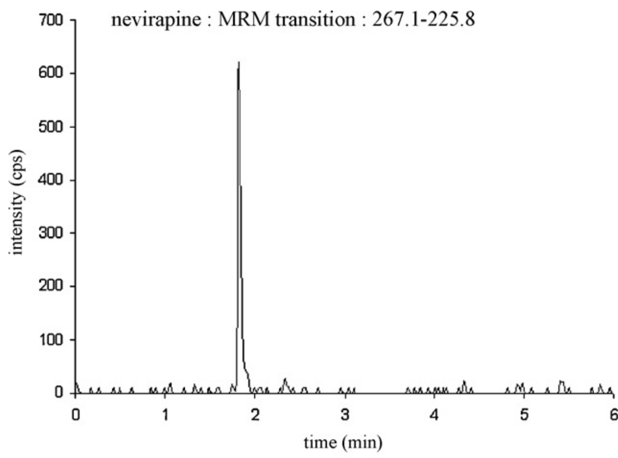
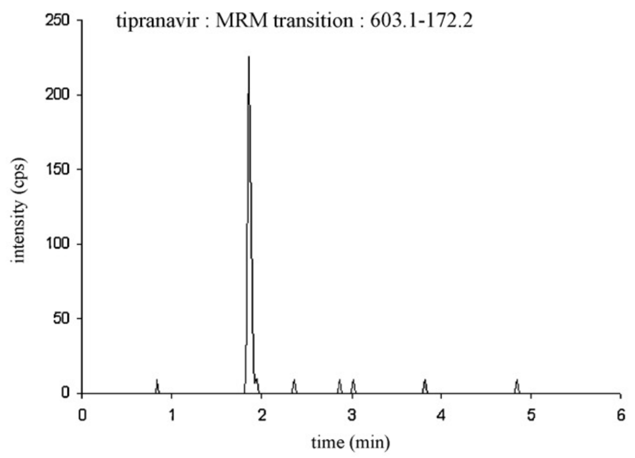
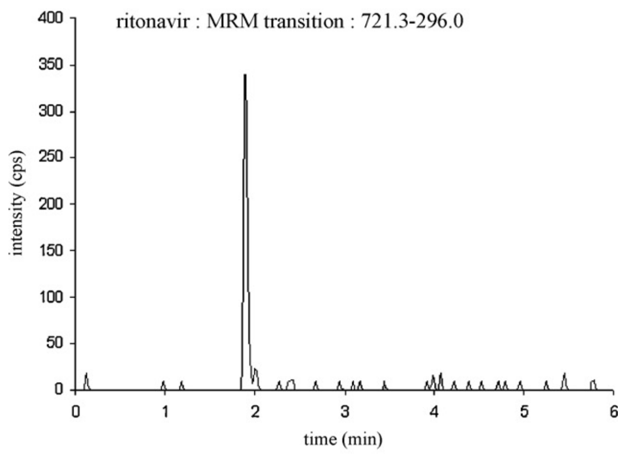
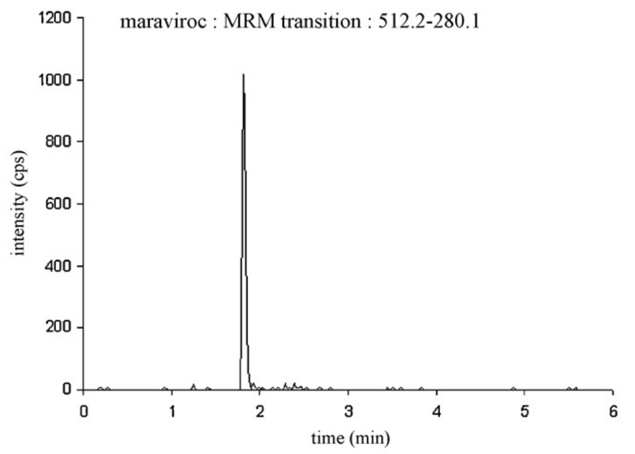


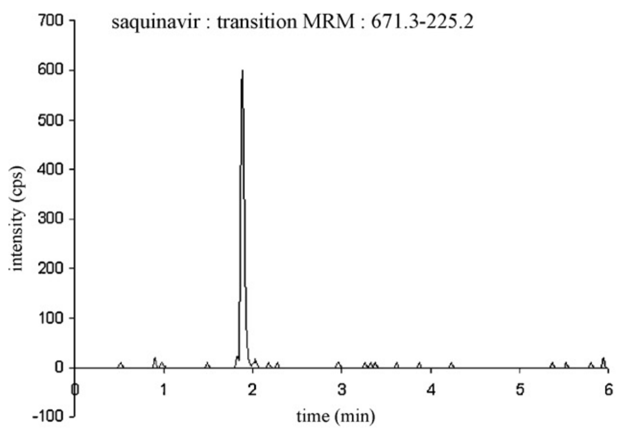
Fig. 4. (a) Chromatograms of the lowest calibration sample (0.01 mg/L for maraviroc and ritonavir, 0.05 mg/L for amprenavir, atazanavir, saquinavir and indinavir, 0.1 mg/L for lopinavir, darunavir, efavirenz and nevirapine, and 0.4 mg/L for tipranavir). (b) Chromatogram of a blank methanol sample analyzed just after the top level of standard curves.











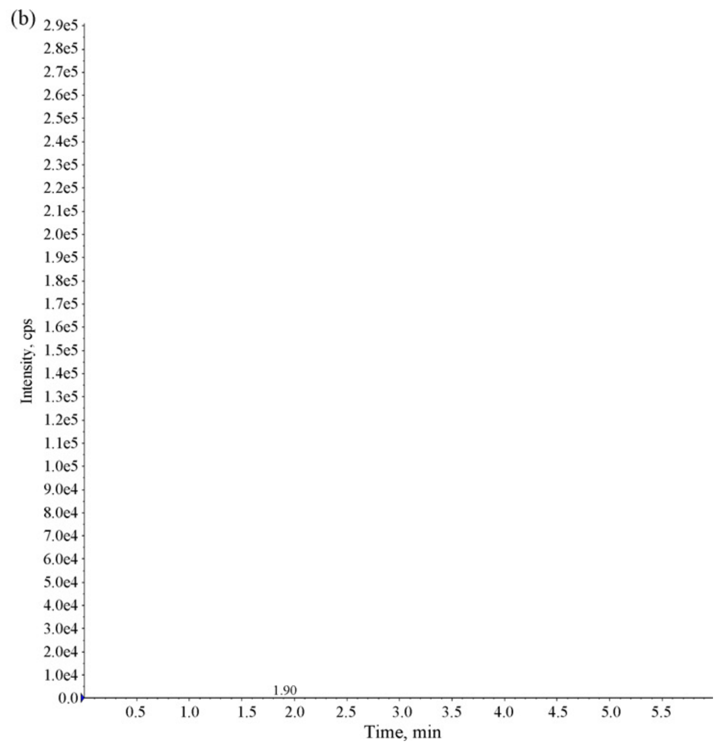


Fig. 4. (*Continued*).

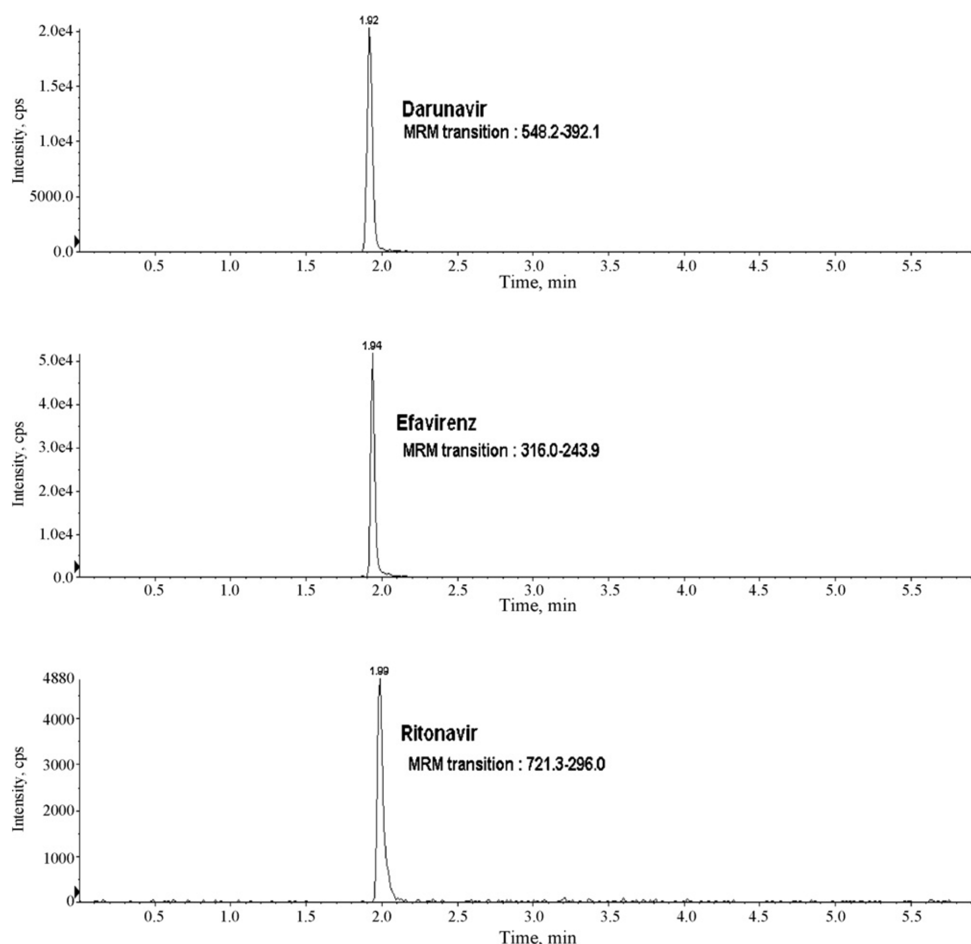


Fig. 5. Chromatograms of darunavir, efavirenz and ritonavir obtained from a woman infected with HIV who was receiving efavirenz 600 mg once a day and darunavir/ritonavir 600/100 mg twice a day. The plasma concentration of darunavir is 2.68 mg/L, 10.4 mg/L for efavirenz and 0.108 mg/L for ritonavir.

Table 5
Incurred sample reproducibility based on a duplicate analysis of trough plasma concentrations of antiretroviral drugs measured 12–24 h after administration in patients infected with HIV.

	Result 1: plasma concentration (mg/L)	Result 2: plasma concentration (mg/L)	bias of repeat [(result 1 − result 2)/result 1] × 100
Amprenavir	0.348	0.331	+4.9%
Atazanavir	1.70	1.61	+5.3%
Darunavir	1.03	1.02	+1%
Indinavir	0.473	0.516	−9.1%
Lopinavir	2.16	2.22	−2.8%
Ritonavir	0.089	0.093	−4.5%
Saquinavir	0.44	0.42	+4.5%
Tipranavir	9.05	9.20	−1.7%
Efavirenz	1.06	0.987	+6.9%
Nevirapine	5.00	5.16	−3.2%
Maraviroc	0.287	0.300	−4.5%

5. Conclusion

Plasma concentrations of the novel antiretroviral drug maraviroc and the most frequently used protease inhibitors and non-nucleoside reverse transcriptase inhibitors were determined using the proposed method. The results indicate that this method is sensitive, accurate, precise and can be used for therapeutic drug monitoring of these antiretroviral agents in patients infected with HIV.

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