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Simultaneous determination of 16 anti-HIV drugs in human plasma by high-performance liquid chromatography

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Abstract

Therapeutic drug monitoring (TDM) is pivotal to improve the management of HIV infection. Here, a HPLC–UV method has been developed to quantify simultaneously seven HIV protease inhibitors (amprenavir, atazanavir, indinavir, lopinavir, nelfinavir, ritonavir, and saquinavir; PIs), seven nucleoside reverse transcriptase inhibitors (abacavir, didanosine, emtricitabine, lamivudine, stavudine, zalcitabine, and zidovudine; NRTIs), and two non-nucleoside reverse transcriptase inhibitors (efavirenz and nevirapine; NNRTIs) in human plasma. The volume of the plasma sample was 600 μL. This method involved automated solid-phase extraction with Oasis HLB Cartridge 1 cc (divinylbenzene and *N*-vinylpyrrolidone) and evaporation in a water bath under nitrogen stream. The extracted samples were reconstituted with 100 μL methanol. Twenty microliters of these samples were injected into a HPLC–UV system, the analytes were eluted on an analytical C_{18} Symmetry™ column (250 mm × 4.6 mm I.D.) with a particle size of 5 μm. The mobile phase (0.01 M KH_2PO_4 and acetonitrile) was delivered at 1.0 mL/min with linear gradient elution. The total run time for a single analysis was 35 min, the anti-HIV drugs were detected by UV at 240 and 260 nm. The calibration curves were linear up to 10 μg/mL. The absolute recovery ranged between 88 and 120%. The in vitro stability of anti-HIV drugs (0.005–10 μg/mL) in plasma has been studied at 24.0 °C. On these bases, a two to four analyte method has been tailored to the individual needs of the HIV-infected patient. The HPLC–UV method here reported has been validated and is currently applied to monitor PIs, NRTIs, and NNRTIs in plasma of HIV-infected patients. It allows to monitor the largest number of anti-HIV drugs simultaneously, appearing useful in a routine laboratory, and represents an essential step to elucidate the utility of a formal therapeutic drug monitoring for the optimal follow-up of HIV-infected patients.

Keywords: HIV protease inhibitors; HIV nucleoside reverse transcriptase inhibitors; HIV non-nucleoside reverse transcriptase inhibitors; HPLC–UV; Therapeutic drug monitoring

1. Introduction

HIV nucleoside reverse transcriptase inhibitors (NRTIs) in combination with protease inhibitors (PIs) and/or with non-nucleoside reverse transcriptase inhibitors (NNRTIs) have transformed the short-term and prognosis of HIV-infected patients (see [1,2]). The aim of therapeutic drug monitoring (TDM) consists in individualizing dosages for maximizing the efficacy of treatment while minimizing its toxicity. The combination of pharmacokinetic–pharmacodynamic relationships for antiretroviral therapy and the presence of a wide interpatient variability in drug exposure supports the application of TDM in HIV-infected patients. Prospective clinical trials assessing the clinical usefulness of this strategy have shown contradictory results, pointing out the need to consider different issues when performing TDM. It may be useful in patient management because it contributes to ensure adequate and efficacy drug levels, avoiding or reducing, in many scenarios, the drug associated adverse effects. Then, TDM may warrant an adjustment of doses and combinations

Abbreviations: HPLC, high-performance liquid chromatography; LOD, limit of detection; LOQ, limit of quantification; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; PI, protease inhibitor; SPE, solid-phase extraction; TDM, therapeutic drug monitoring

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to ensure an optimal therapy for HIV infected patients (see [3–8]).

Anti-HIV therapeutic strategy regimens require the administration of several antiretroviral drugs. The increasing number of anti-HIV drugs available rapidly increases the number of different combinations. Some very promising combination regimens contain PIs and NRTIs (see [9–13]). Therefore, an analytical method for anti-HIV drug determination in blood on a routine basis may represent a useful clinical tool, enabling the study of the relationship between plasma levels, metabolic disorders and virological response failure, and treatment fine-tuning. Moreover, it may contribute to ameliorate patient management, in particular in evaluating drug–drug interactions and indicating relationships between drug concentration and associated toxicity (see [14–16]).

Several HPLC–UV methods have been reported to quantify anti-HIV drugs in human biological fluids, e.g. abacavir (see [17]), amprenavir (see [18–20]), atazanavir (see [18,19]), didanosine (see [17]), efavirenz (see [18–21]), emtricitabine (see [22]), indinavir (see [23]), lamivudine (see [17]), lopinavir (see [19,20,24,25]), nelfinavir (see [18–20,24]), nevirapine (see [18–20,24]), ritonavir (see [23]), saquinavir (see [19,20]), stavudine (see [3]), zalcitabine (see [17]), and zidovudine (see [17]). Furthermore, each method (individual or simultaneous) involves a sample preparation procedure: liquid–liquid or solid–liquid extraction or protein precipitation. The application of solid-phase extraction (SPE) of analytes from biological matrix allows either higher recoveries or the elimination of some possible interferences from co-administrated drugs (see [3,17–25]).

Here, we report the setting up and validation of a HPLC–UV method for the simultaneous separation and quantitation of 16 anti-HIV drugs, i.e., abacavir, amprenavir, atazanavir, didanosine, efavirenz, emtricitabine, indinavir, lamivudine, lopinavir, nelfinavir, nevirapine, ritonavir, saquinavir, stavudine, zalcitabine, and zidovudine, in plasma from HIV-infected patients. The determination of 16 anti-HIV drugs by a single method appears useful in a routine laboratory since different drug cocktails are administered to HIV-infected patients.

2. Materials and methods

2.1. Chemicals

Amprenavir (from Vertex/Kissei/Glaxo Wellcome), atazanavir (from Bristol-Meyers Squibb), abacavir (from Glaxo Wellcome), efavirenz (from Dupont Merck), didanosine (from Bristol-Myers Squibb), emtricitabine (from Triangle Pharmaceuticals), indinavir (from Merck), lamivudine (from Iaf Biochem. Int./Glaxo Wellcome), lopinavir (from Abbott), nelfinavir (from Agouron/Japan Tobacco), nevirapine (from Boehringer Ingelheim), ritonavir (from Abbott), saquinavir (from Roche), stavudine (from Bristol-Myers Squibb), zalcitabine (from Hoffman-La Roche), and zidovudine (from Glaxo Wellcome) were obtained through the NIH AIDS Research Reagent Program, Division of AIDS, NIAID, National Institute of Health (Bethesda, MD, USA). All anti-HIV drugs were of analytical grade and used without further purification. Acetonitrile, methanol, and KH_2PO_4 (from Carlo Erba reagenti, Rodano, Milano, Italy) were of HPLC grade. Deionized water (18.2 mΩ, total organic carbon <100 ppb) was produced on-site.

2.2. Chromatographic system

The chromatographic system consisted of a Waters 600 pump and a Waters autosample 717 PLUS equipped with a spectrophotometric UV–vis dual-wavelength system Waters 2487 set at 240 and 260 nm (Milford, MA, USA). Anti-HIV drug separation was performed at 24.0 °C on an analytical C_{18} Symmetry^{TM} column (250 mm × 4.6 mm I.D.) with a particle size of 5.0 μm (Waters) equipped with a Waters Sentry guard column (20 × 3.9 mm I.D.) filled with the same packing material (Waters). The 'Millenium' software (Waters) was used to pilot the HPLC–UV instrument and to process the data (i.e., area integration, calculation, and plotting of chromatograms) throughout the method validation and sample analysis.

2.3. Mobile phase solutions

The mobile phase is composed of solution A (0.01 M KH_2PO_4) and B (acetonitrile). Both solutions were degassed by sparging with helium. The injection volume was 20 μL. The mobile phase was delivered at 1.0 mL/min. The gradient program conditions are reported in Table 1.

2.4. Stock, working, and plasma solutions

Stock solutions of abacavir, amprenavir, atazanavir, didanosine, efavirenz, emtricitabine, indinavir, lamividine, lopinavir, nelfinavir, nevirapine, ritonavir, saquinavir, stavudine, zalcitabine, and zidovudine (1.0 mg/mL) were prepared by dissolving 5.0 mg of each anti-HIV drug in 5.0 mL of methanol. Stock solutions were appropriately diluted with methanol for the preparation of working solutions (final concentration ranging between 0.005 and 10 μg/mL). The anti-HIV drug concentration in plasma calibration samples ranged between 0.005 and 10 μg/mL. All working solutions were stored at +4.0 °C and were stable for at least 6 months.

2.5. Sample preparation

According to the protocol approved by the Ethics Committee of the Istituto Nazionale per le Malattie Infettive I.R.C.C.S.

Table 1
Gradient elution program

Time (min)	Flow (mL/min)	Solution A (%)[a]	Solution B (%)[b]	Gradient curve profile[c]	pH
0	1	94	6	–	5.0
10	1	40	60	7	4.5
20	1	40	60	1	4.5
25	1	0	100	1	–
35	1	94	6	1	5.0
40	1	94	6	1	5.0

[a] 0.01 M KH_2PO_4.
[b] Acetonitrile.
[c] For details see [28].

'Lazzaro Spallanzani' (Roma, Italy) and with the written informed consent of the patients, blood samples were drawn from HIV-infected patients. Patients were instructed not to take their morning pills prior to the consultation. The patient selection criteria were pharmacological steady state and efficient response to the therapy.

Blood samples (6.0 mL) were collected in monovetters Li heparinate and centrifuged at 3000 rpm for 20 min at 24.0 °C. Then, human plasma was separated from blood cells and stored at −20.0 °C.

Human plasma samples were cleaned-up by off-line solid-phase extraction using Oasis HLB Cartridge 1 cc (30 mg) (Waters). The SPE cartridges were conditioned with 1.0 mL methanol followed by 1.0 mL water Milli-Q. One hundred microliters of methanol were added to 600 μL of human plasma, the solution was vortexed for 1.0 min and centrifuged at 13,000 rpm for 6.0 min, at 24.0 °C. The supernatant (ca. 650 μL) was diluted by adding water Milli-Q (1.0 mL) and loaded in the cartridge. Then, cartridges were washed with 1.0 mL of 5% (v/v) methanol in water Milli-Q. Analytes were eluted by washing cartridges with 550 μL 0.01 M KH_2PO_4 followed by 2.0 mL absolute methanol. The eluate was evaporated in a water bath at 36.0 °C under a stream of nitrogen. The extracted sample was reconstituted with 100 μL absolute methanol and transferred to an injection vial.

2.6. Calibration curves

The calibration curves were established over the 0.005–10 μg/mL range for amprenavir, atazanavir, lamivudine, lopinavir, nevirapine, saquinavir, and ritonavir, the 0.025–10 μg/mL range for abacavir, didanosine, indinavir, and zidovudine, and the 0.10–10 μg/mL range for efavirenz, emtricitabine, nelfinavir, stavudine, and zalcitabine. Under all the experimental conditions, the response/amount ratio was linear.

2.7. Recovery

The efficiency of SPE was determined with control samples at 0.625, 5.0, and 10 μg/mL. The absolute recovery of abacavir, amprenavir, atazanavir, didanosine, efavirenz, emtricitabine, indinavir, lamividine, lopinavir, nelfinavir, nevirapine, ritonavir, saquinavir, stavudine, zalcitabine, and zidovudine from plasma was obtained as the peak-area response of the processed samples, expressed as the percentage of the response of the anti-HIV drugs contained in the 20-μL injection volume and not subjected to SPE.

2.8. Development of a two to four analyte method

The liquid cromatography resources 'Gradient Scouting Run Evaluation' tool [26] was used to develop a two to four analyte method. The initial and final concentration (%) of the B mobile phase were calculated for each anti-HIV drug cocktail using the following parameters: length and diameter of the analytical C_{18} Symmetry™ column (250 mm × 4.6 mm I.D.), dwell volume (=0.10 mL), flow rate (=1.0 mL/min), initial and final concentration of the B mobile phase (=6 and 100%, respectively), and single run time (=35 min) for the simultaneous determination of 16 anti-HIV drugs, as well as the retention times given in Table 1. Chemicals, chromatographic system, mobile phases, sample preparation, calibration curves, and recovery of the two to four analyte method were identical to those used for the simultaneous determination of 16 anti-HIV drugs.

3. Results

3.1. Chromatograms

The HPLC method here reported provides a simple procedure to determine simultaneously the concentration of abacavir, amprenavir, atazanavir, didanosine, efavirenz, emtricitabine, indinavir, lamividine, lopinavir, nelfinavir, nevirapine, ritonavir, saquinavir, stavudine, zalcitabine, and zidovudine in plasma by UV detection at 240 and 260 nm. The gradient program used for anti-HIV drug separation and the retention times of anti-HIV drugs are repoted in Tables 1 and 2, respectively. Data obtained at 240 nm (see Tables 1–7) are superimposable to those obtained at 260 nm (data not shown) within the experimental error.

Fig. 1 shows the chromatogram of a standard mixture of 16 anti-HIV PIs, NNRTIs, and NRTIs (10 μg/mL) (panel A), of a drug-free human plasma sample from a healthy donor (panel B), of a healthy donor plasma sample spiked with 100 μL of 16 anti-HIV drugs (10 μg/mL) (panel C), and of plasma samples from HIV-infected patients (panels D and E).

Table 3 shows the antiretroviral regimens and anti-HIV drugs plasma concentration of HIV-infected patients. Values here reported agree with literature (http://aidsinfo.nih.gov). This method was validated with regard to specificity, selectivity, linearity, limits of detection and quantification, recovery, precision, and accuracy.

3.2. Specificity and selectivity

Blank samples showed no interfering endogenous substances eluting at the retention time of anti-HIV drugs. The selectivity

Table 2
Retention time of anti-HIV drugs

Anti-HIV drug	Retention time (min)
Lamivudine	4.1
Zalcitabine	6.2
Emtricitabine	7.8
Didanosine	8.6
Stavudine	9.7
Abacavir	15.1
Zidovudine	16.2
Nevirapine	16.6
Indinavir	18.1
Saquinavir	19.2
Amprenavir	19.9
Nelfinavir	21.1
Ritonavir	23.1
Lopinavir	24.5
Efavirenz	28.4
Atazanavir	32.0

Table 3
Anti-HIV regimens and anti-HIV drug plasma concentration of HIV-infected patients[a]

Patient	Anti-HIV drug	Dose (mg)	Plasma concentration (μg/mL ± S.D.)
1	Atazanavir	400.0^{qid}	1.8173 ± 0.741
	Lamivudine	300.0^{qid}	0.3817 ± 0.114
	Ritonavir	100.0^{qid}	0.2633 ± 0.244
2	Lamivudine	150.0^{bid}	0.1736 ± 0.006
	Lopinavir	133.3^{bid}	0.5800 ± 0.221
	Ritonavir	33.30^{bid}	1.8543 ± 0.214
	Zidovudine	300.0^{bid}	0.3182 ± 0.013
3	Abacavir	300.0^{bid}	0.2533 ± 0.025
	Lopinavir	133.3^{bid}	1.0533 ± 0.125
	Ritonavir	33.30^{bid}	0.3138 ± 0.202
4	Lamivudine	150.0^{bid}	0.4351 ± 0.025
	Lopinavir	133.3^{bid}	0.1104 ± 0.082
	Ritonavir	33.30^{bid}	0.3138 ± 0.202
	Zidovudine	300.0^{bid}	0.2803 ± 0.010
5	Lamivudine	300.0^{bid}	0.0376 ± 0.019
	Lopinavir	133.3^{bid}	0.0284 ± 0.006
	Ritonavir	33.30^{bid}	0.3249 ± 0.494
6	Atazanavir	400.0^{qid}	1.0775 ± 1.016
	Lamivudine	300.0^{qid}	0.8841 ± 0.111
7	Efavirenz	600.0^{qid}	0.6561 ± 0.372
	Lamivudine	300.0^{qid}	0.6421 ± 0.008
8	Lamivudine	300.0^{qid}	0.0962 ± 0.016
	Lopinavir	133.3^{bid}	0.0383 ± 0.053
	Ritonavir	33.30^{bid}	0.3808 ± 0.375
9	Lamivudine	300.0^{bid}	0.0371 ± 0.040
	Lopinavir	133.3^{bid}	0.0618 ± 0.026
	Ritonavir	100.0^{bid}	0.2835 ± 0.243
10	Atazanavir	400.0^{qid}	0.4090 ± 0.345
	Lamivudine	300.0^{qid}	0.0884 ± 0.026
11	Lamivudine	300.0^{qid}	0.0363 ± 0.016
	Nevirapine	200.0^{bid}	0.0188 ± 0.005
12	Lamivudine	300.0^{qid}	0.1932 ± 0.126
	Ritonavir	100.0^{bid}	0.3132 ± 0.232
13	Abacavir	300.0^{bid}	0.1072 ± 0.076
	Lamivudine	300.0^{qid}	0.3300 ± 0.035
	Lopinavir	133.3^{bid}	0.0339 ± 0.011
	Ritonavir	33.30^{bid}	0.0320 ± 0.005
14	Atazanavir	400.0^{qid}	0.6858 ± 0.208
	Lamivudine	300.0^{qid}	0.5070 ± 0.392
	Ritonavir	100.0^{qid}	0.6971 ± 0.080
15	Atazanavir	400.0^{qid}	0.1583 ± 0.040
	Lamivudine	300.0^{qid}	0.0416 ± 0.031
16	Lopinavir	133.3^{bid}	0.0914 ± 0.125
	Ritonavir	33.30^{bid}	0.1108 ± 0.076
	Stavudine	40.00^{bid}	0.5245 ± 0.193
17	Didanosine	400.0^{bid}	0.6835 ± 0.392
	Lamivudine	300.0^{qid}	0.0692 ± 0.049
	Nelfinavir	250.0^{bid}	0.1021 ± 0.028
18	Lamivudine	150.0^{bid}	0.0165 ± 0.003
	Lopinavir	133.3^{bid}	0.6400 ± 1.031
	Ritonavir	33.30^{bid}	0.0949 ± 0.072
	Zidovudine	300.0^{bid}	0.1912 ± 0.138
19	Efavirenz	600.0^{qid}	1.0646 ± 0.006
	Lamivudine	150.0^{bid}	0.0793 ± 0.103
	Zidovudine	300.0^{bid}	0.3661 ± 0.097
20	Lamivudine	300.0^{qid}	0.0209 ± 0.002
	Lopinavir	133.3^{bid}	0.0424 ± 0.049
	Ritonavir	33.30^{bid}	0.0901 ± 0.062
21	Lamivudine	150.0^{bid}	0.0152 ± 0.003
	Lopinavir	133.3^{bid}	0.0271 ± 0.006
	Ritonavir	33.30^{bid}	0.1912 ± 0.044
	Zidovudine	300.0^{bid}	0.4396 ± 0.014
22	Abacavir	300.0^{bid}	0.0255 ± 0.005
	Lamivudine	300.0^{qid}	0.0131 ± 0.010
	Lopinavir	133.3^{bid}	0.0296 ± 0.001
	Ritonavir	33.30^{bid}	0.2540 ± 0.076
23	Abacavir	300.0^{bid}	0.1628 ± 0.028
	Amprenavir	1400^{bid}	0.7282 ± 0.185
24	Indinavir	800.0^{bid}	1.1987 ± 0.012
	Lamivudine	300.0^{qid}	1.7248 ± 0.603
	Ritonavir	100.0^{bid}	0.3308 ± 0.047
	Stavudine	30.00^{bid}	<LOD
25	Abacavir	300.0^{bid}	0.6104 ± 0.022
	Lamivudine	300.0^{qid}	7.6649 ± 0.087
	Ritonavir	100.0^{bid}	0.1144 ± 0.078
	Saquinavir	400.0^{bid}	1.6421 ± 0.058
26	Emtricitabine	200.0^{qid}	0.4729 ± 0.112
	Ritonavir	100.0^{bid}	0.2880 ± 0.258

^{qid}: once a day. ^{bid}: twice a day.
[a] Data referring to HIV-infected patients 1 and 4 correspond to those reported in panels D and E, respectively, of Fig. 1.

Table 4
Anti-HIV drug calibration curve parameters

Anti-HIV drug	Calibration curve	r^2
Lamivudine[a]	$y = 0.1314x + 0.0117$	0.9814
Zalcitabine[c]	$y = 0.2008x - 0.1034$	0.9738
Emtricitabine[c]	$y = 0.2563x - 0.0688$	0.9971
Didanosine[b]	$y = 0.2963x - 0.0599$	0.9903
Stavudine[c]	$y = 0.2653x - 0.0621$	0.9977
Abacavir[b]	$y = 0.6369x + 0.1339$	0.9919
Zidovudine[b]	$y = 0.0391x + 0.0028$	0.9862
Nevirapine[a]	$y = 0.0231x + 0.0174$	0.9928
Indinavir[b]	$y = 0.5681x + 0.0109$	0.9879
Saquinavir[a]	$y = 0.8278x - 0.0794$	0.9955
Amprenavir[a]	$y = 0.1011x - 0.0162$	0.9808
Nelfinavir[c]	$y = 0.1713x + 0.0195$	0.9994
Ritonavir[a]	$y = 1.2623x - 0.1744$	0.9855
Lopinavir[a]	$y = 1.1766x - 0.033$	0.9977
Efavirenz[c]	$y = 0.4849x - 0.0850$	0.9969
Atazanavir[a]	$y = 0.1242x - 0.0105$	0.9859

[a] The response range was 0.005–10 μg/mL.
[b] The response range was 0.025–10 μg/mL.
[c] The response range was 0.10–10 μg/mL.

Table 5
Recovery for each anti-HIV drug after extraction from human plasma

Anti-HIV drug	Recovery (% ± S.D.)[a]		
	0.625 μg/mL	5.0 μg/mL	10 μg/mL
Lamivudine	100.1 ± 6.4	107.8 ± 6.2	96.1 ± 0.6
Zalcitabine	99.1 ± 4.5	115.1 ± 5.8	104.5 ± 5.3
Emtricitabine	97.8 ± 2.3	113.0 ± 9.3	116.4 ± 5.1
Didanosine	99.8 ± 5.9	115.6 ± 4.8	98.4 ± 3.8
Stavudine	98.2 ± 2.9	105.2 ± 7.7	88.7 ± 3.9
Abacavir	98.1 ± 2.8	101.1 ± 10.0	93.6 ± 6.1
Zidovudine	100.9 ± 7.8	106.2 ± 7.2	93.8 ± 5.0
Nevirapine	98.1 ± 2.8	107.7 ± 6.3	105.4 ± 1.2
Indinavir	99.7 ± 5.6	114.8 ± 6.3	100.8 ± 6.2
Saquinavir	98.0 ± 2.7	107.4 ± 6.4	102.5 ± 6.1
Amprenavir	110.5 ± 8.1	116.3 ± 3.7	114.2 ± 8.4
Nelfinavir	99.8 ± 5.8	118.1 ± 1.7	99.3 ± 6.3
Ritonavir	120.4 ± 8.9	114.8 ± 6.3	104.7 ± 6.1
Lopinavir	117.8 ± 1.9	117.9 ± 1.8	90.5 ± 9.8
Efavirenz	113.9 ± 7.6	113.2 ± 3.4	96.9 ± 7.2
Atazanavir	119.5 ± 2.7	120.0 ± 6.8	91.4 ± 9.4

[a] Results are the mean of six experiments.

was determined by injecting onto the HPLC column all currently prescribed anti-HIV drugs and/or employed in the treatment/prophylaxis of opportunistic infections.

3.3. Linearity

The standard curves for abacavir, amprenavir, atazanavir, didanosine, efavirenz, emtricitabine, indinavir, lamividine, lopinavir, nelfinavir, nevirapine, ritonavir, saquinavir, stavudine, zalcitabine, and zidovudine are satisfactorily described by unweighted least-squares linear regression. The response/amount ratio was linear between 0.005 and 10 μg/mL for amprenavir, atazanavir, lamivudine, lopinavir, nevirapine, saquinavir, and ritonavir, between 0.025 and 10 μg/mL for abacavir, didanosine, indinavir, and zidovudine, and between 0.10 and 10 μg/mL for efavirenz, emtricitabine, nelfinavir, stavudine, and zalcitabine (Table 4). Data obtained dissolving drugs in methanol and plasma are superimposable. The calibration curves for the determination of amprevavir, lopinavir, nelfinavir, and saquinavir concentration are shown in Fig. 2.

3.4. Limits of detection and quantification

The limit of detection (LOD) in plasma of anti-HIV drugs was defined as the concentration that yields a signal-to-noise ratio of 3:1. For the concentration to be accepted as the lowest limit of quantification (LOQ), the percent deviation from the nominal concentration (measure of accuracy) and the relative standard deviation (measure of precision) has to be less than 20% [27]. LOQ values were 0.005 μg/mL for amprenavir, atazanavir, lamivudine, lopinavir, nevirapine, saquinavir, and ritonavir, 0.025 μg/mL for abacavir, didanosine, indinavir, and zidovudine, and 0.10 μg/mL for efavirenz, emtricitabine, nelfinavir, stavudine, and zalcitabine.

3.5. Recovery

The absolute recovery was calculated by comparing the peak areas obtained from standard working solutions with the peak areas from standard extracts. Recovery experiments were carried out at 0.625, 5.0, and 10 μg/mL anti-HIV drug concentration in plasma samples. Unspiked samples were used as a control. Results are shown in Table 5.

3.6. Precision and accuracy

Intra- and inter-day precision and accuracy were studied at six different concentrations. The precision was calculated as the relative standard deviation (R.S.D.) within a single run (intra-day) and between different assays (inter-day):

$$\text{R.S.D. (\%)} = \left(\frac{\text{S.D.}}{\text{mean}}\right) \times 100$$

where S.D. is the standard deviation. The accuracy was calculated as the percentage of the deviation between the nominal and the found concentration:

$$\text{Accuracy (\%)} = \left(\frac{\text{found} - \text{nominal}}{\text{nominal}}\right) \times 100$$

results are shown in Table 6. For all anti-HIV drugs both precision and accuracy were <20%, according to literature [27].

3.7. The two to four analyte method

The two to four analyte method was tailored to the individual needs of the patient based on that developed for the simultaneous determination of 16 anti-HIV drugs. The gradient program parameters used for the separation of two to four anti-HIV drugs and the retention times of the anti-HIV drugs for infected patients are reported in Table 7. The calibration curves for abacavir, amprenavir, atazanavir, didanosine, efavirenz, emtricitabine, indinavir, lamividine, lopinavir, nelfinavir, nevirapine, ritonavir, saquinavir, stavudine, zalcitabine, and zidovudine are satisfactorily described by unweighted least-squares linear regression, overlapping those reported in Fig. 2 and Table 4. The anti-HIV drug plasma concentration of HIV-infected patients obtained by the two to four analyte method corresponds to that obtained by the simultaneous determination of 16 anti-HIV drugs.

4. Discussion

Here, we report a new fairly simple HPLC–UV method that provides the simultaneous determination of 16 anti-HIV drugs (i.e., PIs, NRTIs, and NNRTIs) in human plasma from HIV-infected patients. Note that no HPLC–UV methods are available for the simultaneous determination of PI, NRTI, and NNRTI plasma levels.

The volume of the plasma sample used here is 600 μL. Based on the different sensitivity of HPLC–UV methods for abacavir [17], amprenavir [18–20], atazanavir [18,19], didanosine [17], efavirenz [18–21], emtricitabine [22], indinavir [23], lamivudine

Table 6
Intra- and inter-day anti-HIV drug determination

Anti-HIV drug	Intra-day[a]				Inter-day[a]			
	Nominal concentration (μg/mL)	Found concentration (μg/mL)	Precision (%)	Accuracy (%)	Nominal concentration (μg/mL)	Found concentration (μg/mL)	Precision (%)	Accuracy (%)
Lamivudine	0.625	0.59 ± 0.04	7.6	5.2	0.625	0.62 ± 0.01	1.7	0.5
	5.0	4.81 ± 0.23	4.8	2.3	5.0	5.01 ± 0.10	2.1	−0.2
	10	8.41 ± 0.51	6.1	15.0	10	9.53 ± 0.39	4.1	4.6
Zalcitabine	0.625	0.58 ± 0.05	8.1	5.6	0.625	0.58 ± 0.02	0.3	7.1
	5.0	4.88 ± 0.22	4.5	2.2	5.0	4.71 ± 0.55	11.8	6.0
	10	9.09 ± 0.93	10.0	9.0	10	9.12 ± 1.02	11.1	8.7
Emtricitabine	0.625	0.57 ± 0.05	9.5	7.7	0.625	0.59 ± 0.02	4.3	4.8
	5.0	4.84 ± 0.31	6.4	3.1	5.0	4.52 ± 0.45	10.2	10.0
	10	9.02 ± 0.82	9.3	9.7	10	9.46 ± 0.49	5.2	5.4
Didanosine	0.625	0.58 ± 0.05	8.5	6.8	0.625	0.58 ± 0.03	6.2	6.4
	5.0	4.90 ± 0.19	4.1	1.9	5.0	5.11 ± 0.10	1.9	−2.1
	10	9.12 ± 0.72	8.1	9.1	10	9.66 ± 0.57	5.9	3.3
Stavudine	0.625	0.57 ± 0.05	9.2	7.5	0.625	0.57 ± 0.06	10.5	8.5
	5.0	4.86 ± 0.27	5.5	2.7	5.0	4.82 ± 0.32	6.6	3.0
	10	8.95 ± 0.55	6.5	10.5	10	9.45 ± 0.95	10.1	5.5
Abacavir	0.625	0.54 ± 0.02	4.6	12.0	0.625	0.56 ± 0.05	9.9	8.8
	5.0	4.83 ± 0.33	6.8	3.2	5.0	4.66 ± 0.30	6.5	6.7
	10	9.88 ± 0.81	8.9	1.2	10	9.4 ± 0.33	3.5	5.8
Zidovudine	0.625	0.55 ± 0.02	3.6	11.4	0.625	0.60 ± 0.02	3.0	3.2
	5.0	4.87 ± 0.25	5.3	2.5	5.0	4.80 ± 0.17	3.6	3.7
	10	8.81 ± 0.61	7.0	12.0	10	9.52 ± 0.45	4.7	4.8
Nevirapine	0.625	0.54 ± 0.02	3.7	12.5	0.625	0.63 ± 0.06	8.8	−2.1
	5.0	4.88 ± 0.23	4.8	2.3	5.0	4.93 ± 0.06	1.3	1.2
	10	9.91 ± 0.66	6.3	0.4	10	8.93 ± 0.12	1.4	10.6
Indinavir	0.625	0.55 ± 0.02	3.3	11.9	0.625	0.58 ± 0.04	7.3	6.4
	5.0	4.83 ± 0.25	5.3	3.3	5.0	4.90 ± 0.19	4.1	1.9
	10	9.89 ± 0.77	7.8	1.0	10	9.53 ± 0.39	4.1	4.6
Saquinavir	0.625	0.57 ± 0.06	10.0	7.7	0.625	0.58 ± 0.03	5.1	7.2
	5.0	4.66 ± 0.29	6.3	6.7	5.0	4.70 ± 0.28	6.0	5.9
	10	9.90 ± 0.71	7.2	0.8	10	8.78 ± 0.21	2.4	12.1
Amprenavir	0.625	0.58 ± 0.05	9.3	6.5	0.625	0.62 ± 0.01	1.7	0.5
	5.0	4.69 ± 0.28	6.0	6.0	5.0	4.90 ± 0.19	4.1	1.9
	10	10.1 ± 0.33	3.7	−0.8	10	9.32 ± 0.74	7.9	6.8
Nelfinavir	0.625	0.58 ± 0.05	9.7	6.7	0.625	0.56 ± 0.01	2.7	9.3
	5.0	4.73 ± 0.29	6.2	5.2	5.0	4.92 ± 0.12	2.4	1.5
	10	10.0 ± 0.52	5.4	−0.2	10	9.60 ± 0.70	7.4	3.9
Ritonavir	0.625	0.62 ± 0.05	9.0	0.2	0.625	0.58 ± 0.02	2.6	7.7
	5.0	4.66 ± 0.29	6.3	6.6	5.0	4.92 ± 0.12	2.4	1.5
	10	9.96 ± 0.62	6.0	0.3	10	9.41 ± 0.60	6.4	5.8
Lopinavir	0.625	0.61 ± 0.04	7.2	2.6	0.625	0.57 ± 0.03	5.2	8.8
	5.0	4.73 ± 0.29	6.2	5.4	5.0	4.99 ± 0.007	0.2	0.1
	10	9.68 ± 0.84	8.8	3.1	10	9.85 ± 0.07	0.7	1.4
Efavirenz	0.625	0.59 ± 0.04	8.3	4.1	0.625	0.57 ± 0.05	9.5	7.7
	5.0	4.70 ± 0.28	6.0	5.9	5.0	4.80 ± 0.10	2.1	4.0
	10	9.83 ± 0.82	9.1	1.6	10	9.91 ± 0.66	6.3	0.5
Atazanavir	0.625	0.61 ± 0.04	7.3	1.8	0.625	0.58 ± 0.05	8.5	6.8
	5.0	4.81 ± 0.40	8.4	3.6	5.0	5.13 ± 0.15	2.9	−2.6
	10	9.42 ± 0.87	9.1	5.7	10	9.88 ± 0.81	8.9	1.2

[a] Results are the mean of six experiments.

Table 7
Elution parameters for the two to four analyte method

Patient[a]	Solution B (%)		Anti-HIV drug	Retention time (min)
	Initial	Final		
1, 14	5	85	Lamivudine	5.1
			Ritonavir	22.7
			Atazanavir	28.2
2, 4, 18, 21	5	64	Lamivudine	2.3
			Zidovudine	4.6
			Ritonavir	5.8
			Lopinavir	7.9
3	34	64	Abacavir	2.8
			Ritonavir	6.9
			Lopinavir	7.3
5, 8, 9, 20	5	64	Lamivudine	2.4
			Ritonavir	5.7
			Lopinavir	7.5
6, 10, 15	5	85	Lamivudine	5.1
			Atazanavir	28.5
7	5	75	Lamivudine	2.3
			Efavirenz	23.0
11	5	43	Lamivudine	4.2
			Nevirapine	8.3
12	5	61	Lamivudine	2.4
			Ritonavir	6.0
13, 22	5	64	Lamivudine	2.4
			Abacavir	3.1
			Ritonavir	5.8
			Lopinavir	7.9
17	5	55	Lamivudine	3.2
			Didanosine	7.2
			Nelfinavir	8.2
23	34	52	Abacavir	2.7
			Amprenavir	3.5
16	20	64	Stavudine	3.0
			Ritonavir	5.9
			Lopinavir	7.7
19	5	75	Lamivudine	2.3
			Zidovudine	9.1
			Efavirenz	23.2
24	5	61	Lamivudine	2.4
			Stavudine	3.1
			Indinavir	4.4
			Ritonavir	5.8
25	5	61	Lamivudine	2.4
			Abacavir	3.2
			Saquinavir	4.5
			Ritonavir	5.8
26	15	61	Emtricitabine	2.9
			Ritonavir	8.6

[a] For details, see Table 3.

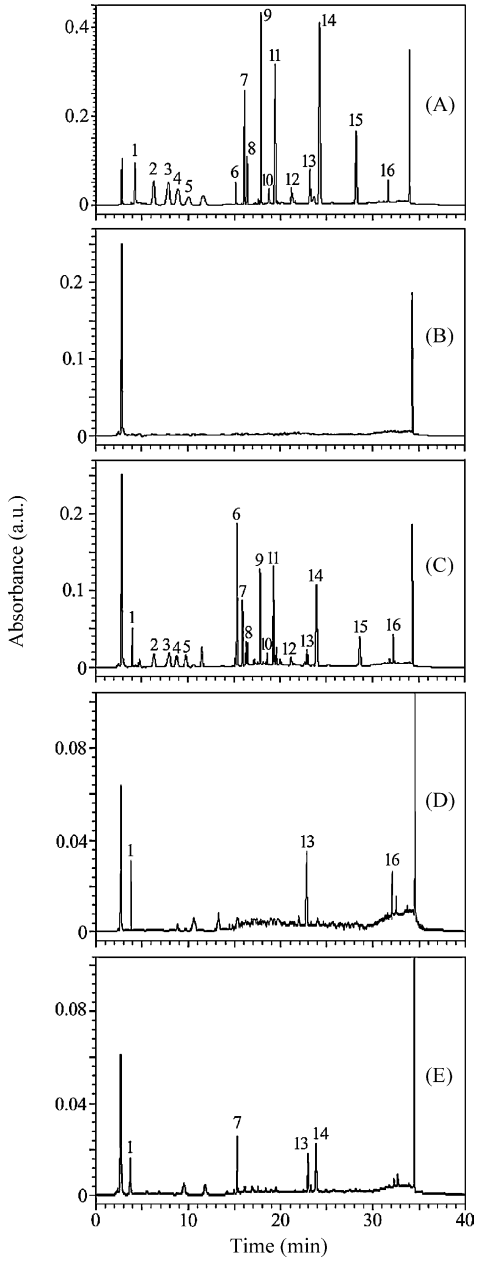

Fig. 1. Simultaneous detection of 16 anti-HIV drugs by HPLC–UV. Chromatogram of a standard mixture of 16 PIs, NNRTIs, and NRTIs (10 μg/mL) (panel A). Chromatogram of a drug-free human plasma sample from a healthy donor (panel B). Chromatogram of a healthy donor plasma sample spiked with 100 μL of 16 anti-HIV drugs (10 μg/mL) (panel C). Chromatogram of plasma samples from HIV-infected patients (panels D and E). Data shown in panels D and E correspond to those of HIV-infected patients 1 and 4, respectively, reported in Table 3. Lamivudine, 1; zalcitabine, 2; emtricitabine, 3; didanosine, 4; stavudine, 5; abacavir, 6; zidovudine, 7; nevirapine, 8; indinavir, 9; saquinavir, 10; amprenavir, 11; nelfinavir, 12; ritonavir, 13; lopinavir, 14; efavirenz, 15; and atazanavir, 16. For details, see text.

[17], lopinavir [19,20,24,25], nelfinavir [18–20,24], nevirapine [18–20,24], ritonavir [23], saquinavir [19,20], stavudine [3], zalcitabine [17], and zidovudine [17] determination, plasma volumes ranged between 500 and 1000 μL. Anti-HIV drug extraction was achieved by divinylbenzene and *N*-vinylpyrrolidone and evaporation in a water bath under nitrogen stream. The extracted samples were reconstituted with methanol and injected into a HPLC–UV system, the analytes were eluted on an analytical C_{18} Symmetry^{TM} column with a particle size of 5 μm. The C_{18} Symmetry^{TM} column gives good separation results (see Fig. 1, panels A and B) and the retention times (see Table 2) of

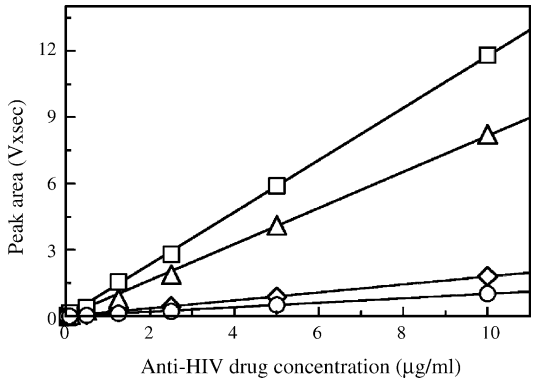

Fig. 2. Calibration curves for the determination of amprevavir (circles), lopinavir (squares), nelfinavir (diamonds), and saquinavir (triangles). The anti-HIV drugs were dissolved in plasma. Calibration curves obtained by dissolving the anti-HIV drugs in methanol are superimposable. The linearity of standard curves was excellent ($r^2 > 0.98$). For details, see text.

anti-HIV drugs are stable for a whole set of analytical runs (Δt_R <0.2 min in a 40-sample run). During the gradient chromatography, pH changes from 4.5 to 5.0 (see Table 1).

LOQ values achieved with this method were 0.005 μg/mL for amprenavir, atazanavir, lamivudine, lopinavir, nevirapine, saquinavir, and ritonavir, 0.025 μg/mL for abacavir, didanosine, indinavir, and zidovudine, and 0.10 μg/mL for efavirenz, emtricitabine, nelfinavir, stavudine, and zalcitabine. LOQ values here reported are somewhat lower than those given in the literature for amprenavir (0.025–0.2 μg/mL) [18–20], atazanavir (0.10–0.2 μg/mL) [18,19], lamivudine (0.015 μg/mL) [17], lopinavir (0.025–0.20 μg/mL) [19,20], nevirapine (0.010–0.40 μg/mL) [18–20], ritonavir (0.025–0.10 μg/mL) [19,20], and saquinavir (0.010–0.10 μg/mL) [19,20]. Present LOQ values are similar to those previously reported for abacavir (0.015 μg/mL) [17], didanosine (0.015 μg/mL) [17], and zidovudine (0.015 μg/mL) [17] determination. However, LOQ values obtained from literature for efavirenz (0.010–0.2 μg/mL) [18–20], indinavir (0.010–0.10 μg/mL) [19,20], nelfinavir (0.025–0.2 μg/mL) [18–20], stavudine (0.005 μg/mL) [3], and zalcitabine (0.015 μg/mL) [17] are lower than those here reported. Therefore, this method appears to be more sensitive than those previously reported for amprenavir, atazanavir, lamivudine, lopinavir, nevirapine, ritonavir, and saquinavir quantification.

According to recommendations [27], the linearity of standard curves was excellent ($r^2 > 0.97$) (Fig. 2 and Table 4), the absolute recovery of anti-HIV drugs ranged between 88 and 120% (Table 5), the standard deviation ranged between ±0.6 and ±10% (Table 5), and both precision and accuracy were always <20% (Table 6).

Based on the simultaneous determination of 16 anti-HIV drugs, a two to four analyte method was developed. By the algorithm 'Gradient Scouting Run Evaluation' the initial and final concentration (%) of the B mobile phase was rapidly identified being known the drug cocktail of the HIV-infected patient. Although the time of the single run and the anti-HIV drug retention time of the two to four analyte method are considerably shorter than those reported for the simultaneous determination of 16 anti-HIV drugs, the latter method appears useful in a routine laboratory since different drug cocktails are administered to HIV-infected patients. Moreover, the two to four analyte method needs specific column equilibration for each anti-HIV drug cocktail.

As a whole, the HPLC–UV method here reported is sensitive and specific, allowing the simultaneous determination of the largest number of anti-HIV drugs (i.e., 16 PIs, NRTIs, and NNRTIs). Therefore, it appears very promising to examine several anti-HIV drug combination regimens. This method is used routinely at the Istituto Nazionale per le Malattie Infettive I.R.C.C.S. 'Lazzaro Spallanzani' (Roma, Italy) for TDM in HIV-infected patients.

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