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Determination of twelve antiretroviral agents in human plasma sample using reversed-phase high-performance liquid chromatography

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Abstract

A new high-performance liquid chromatography (HPLC) with UV detection assay was developed for the simultaneous determination of protease inhibitors (PIs), nucleoside and non-nucleoside reverse transcriptase inhibitors (NRTIs, NNRTIs) using a single 1-ml plasma samples. A solid–liquid extraction procedure without internal standard was coupled with two separate reversed-phase HPLC systems; one for the determination of amprenavir, efavirenz, indinavir, nelfinavir, ritonavir, saquinavir (run time=32 min) and one for the determination of abacavir, didanosine, lamivudine, stavudine, nevirapine, zidovudine (run time=40 min). The first requires a mobile phase containing sodium phosphate buffer+ion pair–acetonitrile (50:50, v/v) through a C_{18} Symmetry column (250×4.6 mm I.D., 5 μm particle size), using variable wavelengths (241, 254 and 261 nm). The second system requires three mobile phases (potassium phosphate buffer+ion pair–acetonitrile) for different elution through a C_{18} Symmetry Shield column (250×4.6 mm I.D., 5 μm), using a single wavelength (260 nm). Peak-areas are linear; correlation coefficients are better than 0.998 for all compounds, with both inter- and intra-day relative standard deviations lower than 12%. Extraction recoveries are higher than 93% for PIs and NNRTIs and higher than 70% for NRTIs. The method is specific and sensitive and was used to determine trough and peak levels of antiretroviral drugs in HIV infected patients under various combinations of RTIs and PIs.

Keywords: Protease inhibitors; Nucleoside reverse transcriptase inhibitors; Non-nucleoside reverse transcriptase inhibitors

1. Introduction

Human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS) encodes at least three enzymes: protease, reverse transcriptase, endonuclease. To inhibit the viral replication, three therapeutic classes have been developed: (i) nucleoside reverse transcriptase inhibitors (NRTIs): abacavir, didanosine, dideoxycytidine, lamivudine, stavudine, zidovudine, (ii) non-nucleoside reverse transcriptase inhibitors (NNRTIs): delavirdine, efavirenz, nevirapine, and (iii) protease inhibitors (PIs): amprenavir, indinavir, nelfinavir, ritonavir, saquinavir.

Therapeutic strategy regimens require the combination of these antiretroviral (ARV) drugs. The increasing number of drugs available rapidly increases the number of different combinations. Some very promising combination regimens contain two PIs and/or one NNRTI and at least two NRTIs [1].

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PIs and NNRTIs are both potent CYP450 enzymes inhibitors [2–5] and inductors [6,7], so drug–drug interactions have to be monitored [8,9]. Furthermore, substantial inter-individual variations in exposure to these drugs occur. There is no consensus that therapeutic drug monitoring should be performed for NRTIs. However, relationships between plasma concentrations and virologic efficacy or toxicity are described for didanosine [10] and zidovudine [11]. These relations are also described for saquinavir [12] or ARV drug combinations [13]. Furthermore, for research purposes, as combination therapy is the gold standard, it is very helpful to quantify the respective activity of each drug [14]. Therefore therapeutic drug monitoring of all drugs may warrant an adjustment of doses and combinations to ensure an optimal therapy for HIV infected patients.

In the literature, quantification methods for ARV use reversed-phase high-performance liquid chromatography (RP-HPLC) with UV detection or mass spectrometry (MS). MS is an accurate and selective detection system used to quantify dideoxycytidine [15], indinavir [16] and saquinavir [17]. UV detection is widely used for relatively simple bioanalytical assays. Numerous individual methods have been developed to quantify ARV drugs in human biological fluids: indinavir [18–22], nelfinavir [23], ritonavir [24,25], saquinavir [26,27], lamivudine [28–31], stavudine [32,33], didanosine [34,35], zidovudine [36–39], nevirapine [40], delavirdine [41,42] and efavirenz [43].

No individual method for assaying amprenavir or abacavir has yet been published. Few methods for the simultaneous determination of drugs of the same therapeutic class have been published for PIs: ritonavir+saquinavir [44], indinavir+ritonavir+saquinavir+nelfinavir [45], indinavir+amprenavir+ritonavir+saquinavir+nelfinavir [46] and for three NRTIs: dideoxycytidine+didanosine+zidovudine [47]. But no simultaneous method for the determination of ARVs from different therapeutic classes is available. Furthermore, each method (individual or simultaneous) involves a sample preparation procedure: liquid–liquid or solid–liquid extraction, or protein precipitation. Such methods increase the difficulty (time and cost) of quantifying all ARVs received by one single HIV-infected patient undergoing multiple therapy with drugs from different therapeutic classes. Consequently, quantification of drug plasma levels from several patients is a quite complicated operation. At the patient individual level, these assays required a very large volume of plasma sample. Therefore, there is a need for relatively simple bioanalytical assays for use with standard laboratory equipment, to quantify plasma concentrations of various ARVs in a single blood sample. We have developed and validated such a simple method using one extraction procedure fitted to all ARV drugs and two analytical runs for the simultaneous determination of 12 ARVs in a single plasma sample. The method is available for drug monitoring, determination of pharmacokinetic profiles, and evaluation of drug–drug interactions. Individualized design of effective antiviral regimens may benefit from monitoring of all ARV drugs.

2. Experimental

2.1. Chemicals

The compounds were kindly obtained from the respective pharmaceutical companies: amprenavir (A) mesylate, abacavir (Ab) hemisulfate, lamivudine (3TC), zidovudine (AZT) from Glaxo-Wellcome (London, UK); didanosine (DDI) from Bristol-Myers Squibb (Princeton, NJ, USA); efavirenz (E) from DuPont-Pharma (Wilmington, DE, USA); indinavir (I) sulfate from Merck Sharp & Dohme-Chibret Labs. (West Point, PA, USA); nelfinavir (N) mesylate from Agouron (La Jolla, CA, USA); nevirapine (Nvp) from Boehringer Ingelheim (Ridgefield, CO, USA); ritonavir (R) from Abbott (Illinois, IL, USA); saquinavir (S) mesylate from Roche Products (Basel, Switzerland); delavirdine (D) mesylate from Pharmacia and Upjohn (Michigan, MI, USA); stavudine (D4T) and 2′,3′-*o*-isopropylidene uridine were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Acetonitrile (LiChrosolv, Merck, Darmstadt, Germany) and methanol (Uvasol, Merck) were HPLC grade. Disodium hydrogenphosphate dihydrate (Na_2HPO_4) (Merck), potassium dihydrogenphosphate (KH_2PO_4) (Merck), octane sulfonic acid (OSA) 0.25 *M* low UV (Waters, Milford, MA, USA) and water (distilled: Fresenius France Pharma,

Louviers, France) were analytical-reagent grade. Blank drug-free plasma was obtained from the hospital blood bank.

Stock solutions of each compound (1 mg/ml, except Nvp: 5 mg/ml) were prepared by dissolving 10 mg of equivalent free and pure base (except for Nvp: 50 mg) in 10 ml of methanol or distilled water. The NRTIs except AZT were dissolved in water and stored at +4°C. The other compounds (IP, NNRTIs and AZT) were dissolved in methanol and kept at −20°C. Stock solutions are stable for at least 1 year. Stock solutions of DDI, D4T and AZT were diluted with water to achieve working solutions of 100 μg/ml. Stock solutions of S and E were diluted in methanol to achieve working solutions of 500 μg/ml. All working solutions stored at +4°C were stable for at least 1 month.

2.2. Instrumentation

The first HPLC system used to assay PIs and E consisted of an isocratic P1000 pump (TQ: Thermo Quest, Fremont, CA, USA), an AS3000 autosample injector (TQ), a PC-1000 integrator (TQ), a UV-1000 variable-wavelength ultraviolet detector (TQ) connected on-line (for D detection) to a RF-551 fluorescence HPLC monitor (Shimadzu; Dyson Instrument, Hetton, UK). Separation was performed at 37°C using a column heater (Temperature Control System, Waters) on a Symmetry 5 μm C_{18} column (250×4.6 mm I.D.) protected by a Guard-Pak, μBondapack C_{18} pre-column (Waters).

The second HPLC system used to assay NRTIs and Nvp consisted of three 114M pumps (Beckman, Berkeley, CA, USA), a Wisp 717 plus autosample injector (Waters), two switch valves (Lea Switch I & T, Labmetrics Technology, Roissy, France), a 481 variable-wavelength ultraviolet detector model (Waters), a System Gold 2 integrator (Beckman). Separation was performed at 30°C with a column heater Croco-Cil (Cil Cluzeau, Paris la Defense, France) on a Symmetry Shield 5 μm C_{18} column (250×4.6 mm I.D.) (Waters) protected by a 2 μm Upchurch filter (USA). Molecular spectra of all compounds and peak purity were checked under the chromatographic conditions using a UV–Vis diode-array detector coupled to the PC-1000 software (TQ) (Fig. 1).

2.3. Chromatographic conditions

For PI and E assay: the mobile phase is composed of 0.04 *M* Na_2HPO_4 buffer with 4% (v/v) OSA filtered through a 0.22-μm filter (GVWP 047 Durapore, Millipore, Bedford, MA, USA) and acetonitrile (50:50, v/v), then degassed ultrasonically for 5 min. The salt mixture (Na_2HPO_4 and OSA) stored at +4°C is stable for 1 month. Before mixing with acetonitrile, the salt mixture was stabilized at room temperature to prevent salt precipitation. The mobile phase was delivered at a flow-rate of 1.3 ml/min with an average operating pressure of 1.6 kp.s.i. (1 p.s.i.=6894.76 Pa) The spectrophotometer was set at 261 nm between time 0 and 9 min, at 241 between time 9 and 20 min, at 254 nm between time 20 and the end of the run (32 min). The fluorescence monitor used for D detection was set at 305 and 425 nm for excitation and emission, respectively. The sample injection volume was 100 μl.

For NRTI and Nvp assay: three mobile phases (MPs) were prepared using M/15 KH_2PO_4 buffer with 1% OSA and different acetonitrile proportions (v/v): 5% for MP1 delivered at a flow-rate of 1 ml/min, 20% for MP2 delivered at the same flow-rate, 70% for MP3 delivered at a flow-rate of 1.2 ml/min. The average operating pressure of the system was below 1.9 kp.s.i. The mobile phases were degassed ultrasonically for 5 min. The sample injection volume was 150 μl. The spectrophotometer was set at 260 nm.

The NRTI–Nev system consists of three pumps with three mobile phases depending on the switch valve positions. Switch 1 was connected to the AI 406 module interface programmed by the Beckman System Gold 2 software. Switch 2 was connected (event times) to the Waters autosample injector and programmed in step function. The first pump was connected through a six-way switching valve to the analytical column. During the injection of the sample, switch 1 was in position 1 and the eluent from MP1 was directed to the column; switch 2 was in position 2 and MP2 and 3 were directed to waste. Switching valve 1 was activated to position 2 at time 12 to 35 min and MP1 was directed to the waste, valve 2 was in position 1 then MP2 was directed to the column through valve 1. At time 30 min, switching valve 2 was set back to position 2 and

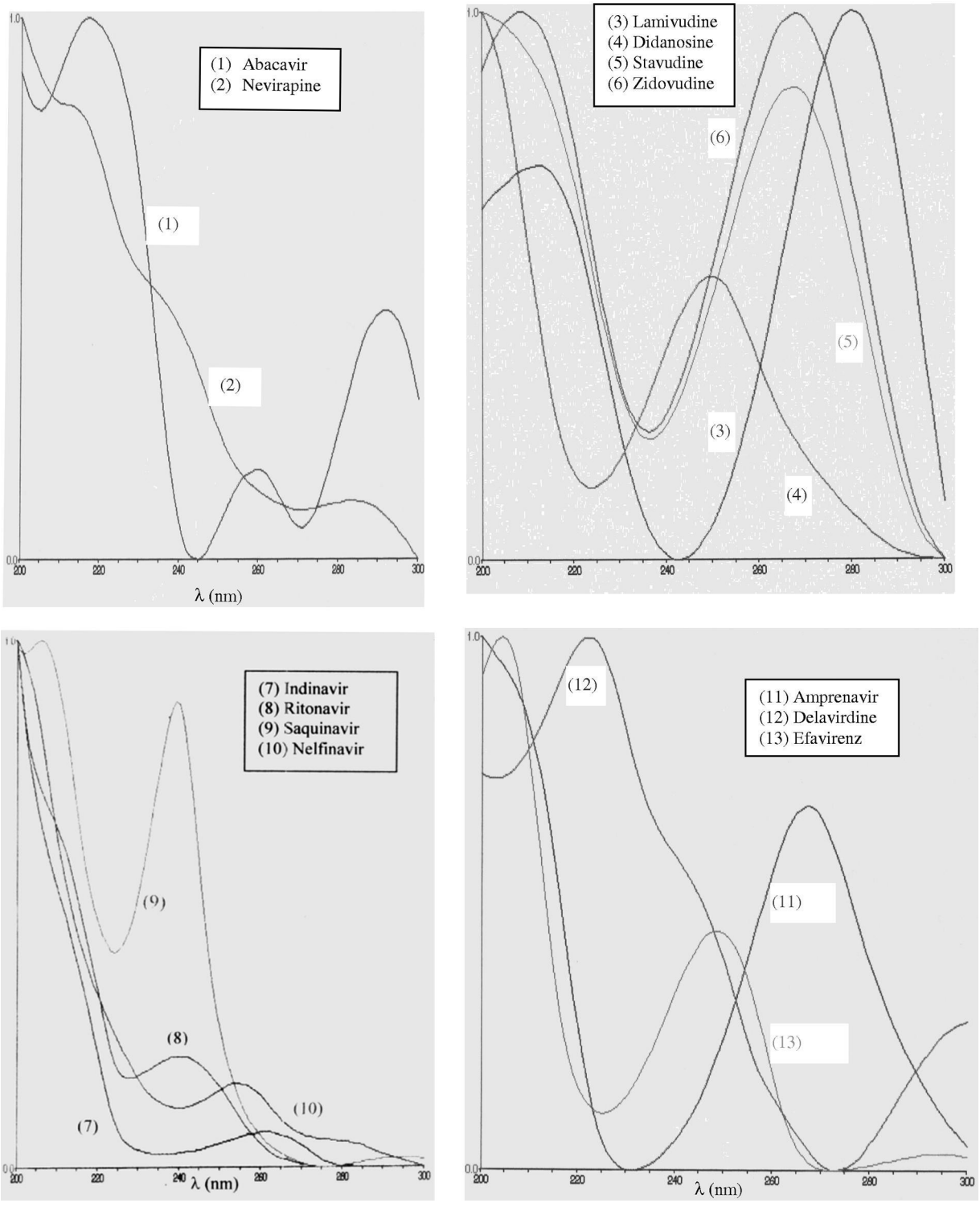

Fig. 1. Molecular spectra of the molecules obtained under the chromatographic conditions using a UV–Vis diode-array detector.

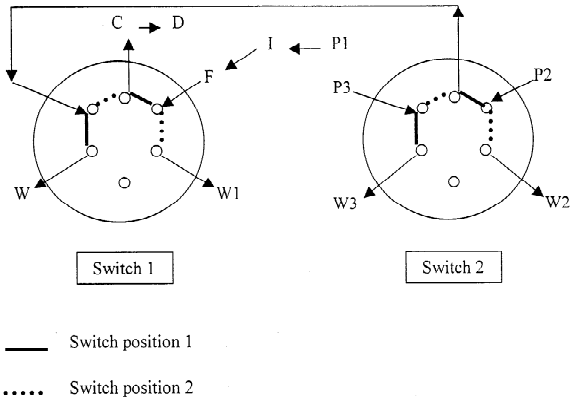

Fig. 2. Block diagram of HPLC column-switching system. I= Injector; F=filter; P=pump; C=column; D=detector; W=waste.

MP3 was directed to the column to rinse it. From time 35 min to the end of run (40 min), the column was reequilibrated with MP1 before the next injection (Fig. 2).

At the end of each chromatographic session, the Symmetry column was washed with methanol–water (50:50, v/v) and acetonitrile–water (80:20, v/v); the Symmetry Shield column was washed with water and methanol.

2.4. Preparation of the calibration curves

A spiked plasma sample with NRTIs and Nvp corresponding to the highest concentration level was prepared by adding to a 100-ml flask: 500 μl of each DDI, AZT and D4T working solutions, 100 μl of 3TC stock solution, 200 μl of A and Nvp stock solutions and drug-free human plasma. This spiked plasma was mixed, aliquoted in volumes of 5.5-ml and stored at −20°C before use. The spiked plasma sample with all compounds corresponding to the highest concentration level was prepared extemporaneously from the one containing NRTIs and Nvp: 50 μl of A, I, N stock solutions, 100 μl of R stock solution and 50 μl of S and E working solution were added to a 15-ml silicone tube (Venoject, Terumo, Belgium). The mixture was evaporated to dryness under a gentle stream of nitrogen in a water bath at 40°C. The residue was then reconstituted with 50 μl of methanol. The tube was vortex-mixed before adding a 5-ml volume of the spiked plasma with NRTIs and Nvp, then mixed.

A calibration curve based on peak-area was constructed for each assay by diluting in a 5-ml silicone tube known volumes (10 to 1000 μl) of global spiked plasma with drug-free human plasma to obtain a 1 ml final volume for each calibration sample. Nine different spiked plasma samples (see the range in Table 1) were assayed. Correlation coefficients of calibration curves were assessed by least-squares regression analysis.

2.5. Sample extraction procedure

C_{18} extraction columns (J.T. Baker, Deventer, The Netherlands) were placed on a vacuum elution manifold and rinsed with 3 ml of methanol, followed

Table 1
Range of calibration curves, detection wavelength (λ), retention times (t_R), capacity factors (K') and average recoveries (ρ) of antiretroviral (ARV) drugs from spiked human plasma sample

ARV	Range calibration curve (ng/ml)	λ (nm)	t_R (min)	K'	$\rho \pm$RSD (%)
Indinavir	100–10 000	261	4.8	2.0	99.3±3.3
Amprenavir	100–10 000	261	5.6	2.5	99.8±3.5
Ritonavir	200–20 000	241	12.9	7.1	96.4±3.1
Efavirenz	50–5000	241	15.2	8.5	97.2±2.7
Saquinavir	50–5000	241	16.8	9.5	98.0±3.5
Nelfinavir	100–10 000	254	29.2	17.3	99.4±2.5
Lamivudine	20–1000	260	8.5	2.7	80.0±4.2
Didanosine	10–500	260	9.6	3.2	73.0±3.1
Stavudine	10–500	260	11.1	3.8	78.0±4.1
Zidovudine	10–500	260	17.4	6.6	77.0±4.1
Abacavir	20–2000	260	20.9	8.1	70.0±6.1
Nevirapine	100–10 000	260	27.9	11.1	93.0±5.1

by 3 ml of distilled water. Care was taken that the columns did not run dry. A 1-ml volume of the plasma samples was then transferred to the columns and drawn into them by applying pressure. The columns were then washed with 2 ml of distilled water followed by vacuum suction for 1 min. Elution of the absorbed analyte was performed with 2.6 ml of methanol into silicone tubes. The eluted methanolic solution was mixed well and separated into two aliquots of 1 and 1.6 ml, respectively used for PI–E and NRTI–Nvp assay. These aliquots were evaporated to dryness under a gentle stream of nitrogen in a water bath at 40°C. The residues were kept at −20°C until reconstitution with 200 μl of water for NRTI–Nvp assay or with 150 μl of mobile phase for IP–E assay.

2.6. Specificity and selectivity

The concomitant therapeutic agents most likely to be encountered in the plasma of HIV positive patients were screened under the HPLC assay conditions. Drugs are checked as pure solution in mobile phase or distilled water to reach 500 to 1000 μg/ml, then injected in both systems, and compared to the peak retention time of the 12 molecules (Table 2). ''Interfering drug'' is defined as a molecule which exhibits a retention time close to 0.3 min from any ARV drug.

2.7. Limit of detection and limit of quantification

The limit of detection (LOD) in plasma was defined by the concentration that yields a signal-to-noise ratio of 3. At this concentration a significant difference between the spiked and blank samples is required in plasma. 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%.

Table 2
Co-administered drugs for specificity, on both systems (NRTI–Nvp and PI–E)

Acebutolol	Clozapine	Imipramine	Phenytoin
Acetaminophen	Cocaine	Indomethacin	Piracetam
Acetylcysteine	Codeine	Interferon alfa	Prazosin
Acetylsalycilic acid	Cortisol	Isoniazid	Prednisolone
Aciclovir	Cyamemazine	Itraconazol	Prednisone
Albendazole	Dantrolene	Josamycin	Primidone
Alimemazine	Dexamethasone	Ketoconazole	Propranolol
Alizapride	Dextropropoxyphene	Levomepromazine	Quinidine
Amikacin	Diazepam	Lidocaine	Quinine
Amiodarone	Diclofenac	Loperamide	Ranitidine
Amphotericin B	Digoxin	Loratadine	Ribavirin
Ampicillin	Dihydroergotamine	Losartan	Rifabutine
Bepridil	Diltiazem	Mefloquine	Rifampicin
Buprenorphine	Doxycycline	Meprobamate	Roxithromycin
Butobarbital	Ethambutol	Methadone	Salicylic acid
Caffeine	Flecainide	Methylprednisolone	Simvastatin
Calcium folinate	Fluconazole	Metoclopramide	Sulfadiazine
Captopril	Flunitrazepam	Metronidazole	Sulfamethoxazole
Carbamazepine	Fluoxetine	Mianserin	Sulpiride
Carbutamide	Fluvoxamine	Moclobemide	Thalidomide
Chloroquine	Foscarvir	Morphine	Theophylline
Ciprofloxacin	Furosemide	Nifedipine	Trimethoprim
Clindamycin	Ganciclovir	Niflumic acid	Valproic acid
Clofazimine	Gentamicin	Nitrofurantoin	Venlafaxine
Clofibrate	Glibenclamide	Omeprazole	Vigabatrin
Clonazepam	Granisetron	Paroxetine	Viloxazine
Clonidine	Halofantrine	Pentamidine	Zolpidem
Cloxacillin	Haloperidol	Phenobarbital	Zopiclone

2.8. Accuracy, precision, linearity and recovery

Accuracy, between-day and within-day precision of the method were determined by assaying 10 replicate plasma samples at three different concentrations (low, medium, high) included in the calibration curves, in 10 analytical runs. Spiked plasmas used for within-day and between-day precision can be considered as quality controls (they were prepared from different stock and working solutions than those of spiked plasmas used for calibration curves). Accuracy was calculated as the percent deviation from the nominal concentration. Within-day and between-day precision were expressed as the standard deviation at each concentration. The maximum linearity range (up per past of the calibration curves) was tested by analysis of individuals spiked plasmas containing each drug at 60 000 ng/ml for R; 40 000 ng/ml for A, I, N and Nvp; 20 000 ng/ml for E and S; and 5000 ng/ml for each NRTI, except Ab 10 000 ng/ml. A spiked plasma containing all the drugs at the same concentration as the individual spiked plasmas, was also analyzed. All spiked plasmas were analyzed in triplicate.

Average recovery of each drug ($n=30$) was determined by comparing the peak-area ratios of the extracts with those obtained by direct injection of the same amount of drug in mobile phase or distilled water at different concentrations (low, medium, high).

2.9. Analysis of patient samples

In our hospital, antiretroviral drug monitoring is undertaken when the efficacy decreases, when non-compliance or malabsorption is suspected, when drug interactions occurred, and in patients with renal failure. Blood samples are usually obtained from HIV-1 infected subjects before dosing (trough level) and/or at the time when the concentration is maximum: 1 h for I, Ab, DDI, E, 3TC, Nvp, D4T, AZT, and 3 h for A, N, R, S. The blood was drawn in heparinized tubes, plasma was separated by centrifugation at 3000 *g* for 10 min and immediately stored at −20°C until analysis. A patient who had been treated with multiple therapy called ''GIGAHAART'': 3TC (150 mg×2/j), DDI (400 mg×1/j), D4T (40 mg×2/j), Ab (300 mg×2/j), E (600 mg×1/j), R (400 mg×2/j), I (400 mg×2/j), A (600 mg×2/j), was sampled both at through (before ingestion) and maximal concentrations (2 h after ingestion). Pharmacokinetic profiles of I (800 mg×3/j) and E (600 mg×1/j) were determined for patients with renal failure. Blood samples were drawn from time 0 to 7 h for I, and from time 0 to 24 h for E. Samples were half diluted when found concentrations were above the highest calibration level.

2.10. Stability of spiked plasmas and extracts

Stability at −20°C of NRTIs and Nvp in human plasma at the highest concentrations (high calibration level) was tested for 6 months. The spiked plasma samples assayed in triplicate at 1-, 2-, 4- and 6-month intervals and compared to the samples prepared extemporaneously. Stability at −20°C for all compounds in human plasma at three concentration levels was also tested: each level was assayed in triplicate at 1 and 2 months and compared to the respective sample prepared extemporaneously. Stability at −20°C of all compounds as dry residue extracts at three level concentrations was checked in triplicate at 1 week. Stability at room temperature of all compounds in reconstituted extracts (with distilled water or PI–E mobile phase) at three concentration levels was checked in triplicate at 48 h.

3. Results

3.1. Chromatography and detection

Chromatograms obtained from drug-free human plasma and spiked plasma sample are shown in Figs. 3 and 4. As shown, PIs and E are well resolved. In addition the six drugs are well separated without any interference from endogenous compounds. NRTI and Nvp chromatograms show good separation from endogenous interferences, drug peaks are well resolved at 260 nm. The different drug retention times and capacity factors (K') are listed in Table 1.

Over 500 samples can be analyzed on each column without significant loss of resolution.

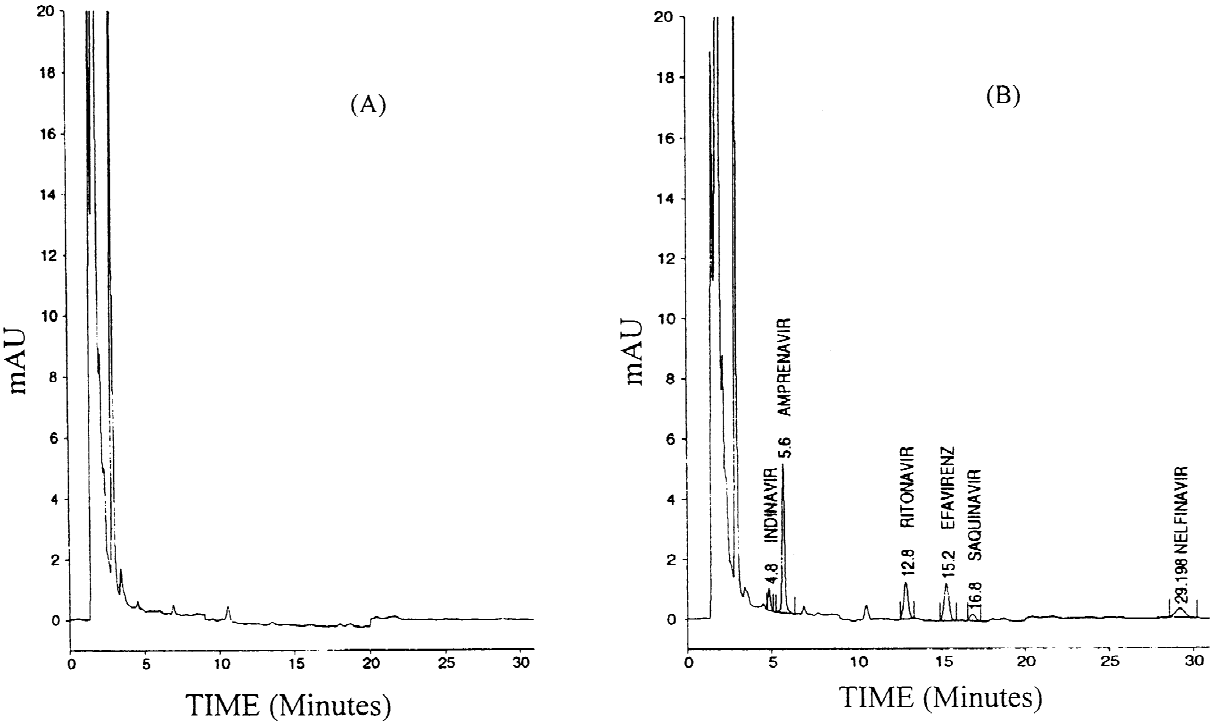

Fig. 3. Chromatograms obtained from: drug-free plasma sample (A) and spiked plasma sample at low level concentration (B) (100 ng/ml of A, I, N, 50 ng/ml of E, 200 ng/ml of R, 10 ng/ml of S) under the chromatographic conditions of the PI–E system with UV detection.

3.2. Validation of accuracy, precision, linearity and recovery

The results from the validation of the method in human plasma are listed in Table 3.

The method proved to be accurate (relative error at three concentrations 0.01 to 11% of the nominal concentration for all drugs) and precise (within-day precision ranged from 1.5 to 9.5% and between-day precision ranged from 0.5 to 12%). Correlation coefficients (r) of calibration curves of each ARV were higher than 0.998 as determined by least-squares analysis. The recoveries are listed in Table 1. The percent deviation between expected and found concentrations of compounds in individual spiked plasmas and spiked plasma containing all compounds at very high concentrations (3–10-fold higher than the highest calibration level) were below 10%. Thus, the calibration curves of A, I, N and Nvp were linear up to 40 000 ng/ml; of R up to 60 000 ng/ml; of E and S up to 20 000 ng/ml; of NRTIs up to 5000 ng/ml except for Ab up to 10 000 ng/ml. There was no saturation at this level and no analytical interaction between the 12 drugs occurred.

3.3. Specificity

Table 2 shows the 111 molecules checked for interference. A small number of potential interferences were noted and were of concern: dihydroergotamine–flecainide–quinine–rifampicin–zolpidem with I, delavirdine–flunitrazepam with A, salicylic acid with AZT, sulpiride with Ab, simvastatine with Nvp.

3.4. Limits of quantification

The LOQs as defined were 5 ng/ml for S; 10 ng/ml for DDI, D4T and AZT; 20 ng/ml for 3TC and Ab; 25 ng/ml for A; 50 ng/ml for E, I, N and R; and 100 ng/ml for Nvp.

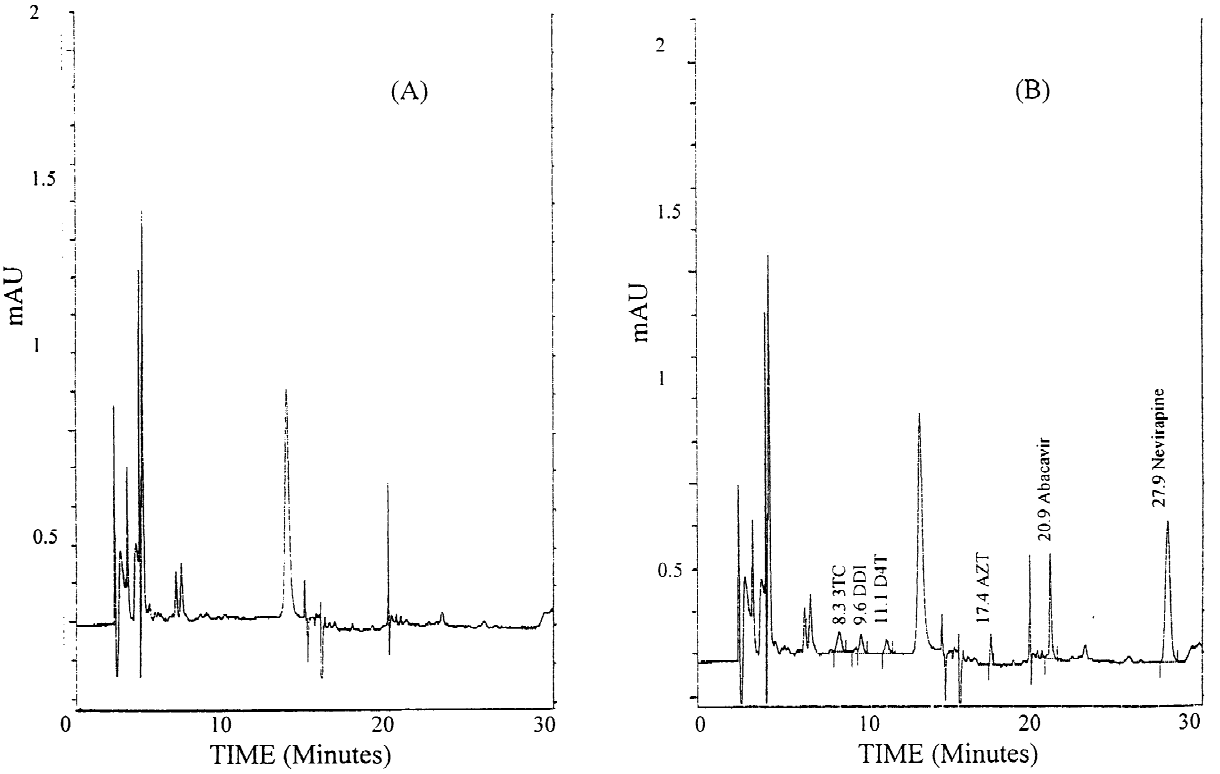

Fig. 4. Chromatograms obtained from: drug-free plasma sample (A) and spiked plasma sample at low level concentration (B) (25 ng/ml of DDI, D4T and AZT, 50 ng/ml of 3TC, 100 ng/ml of Ab, 500 ng/ml of Nvp) under the chromatographic conditions of the NRTI–Nvp system.

Table 3
Accuracy, within-day (WD) and between-day (BD) [mean (RSD %)] precision for the analysis of drugs in human plasma sample ($n=10$)[a]

	Low level concentrations			Medium level concentrations			High level concentrations		
	Theory	Found		Theory	Found		Theory	Found	
		WD (RSD %)	BD (RSD %)		WD (RSD %)	BD (RSD %)		WD (RSD %)	BD (RSD %)
Amprenavir	200	202 (3.9)	204 (5.8)	4000	4025 (2.2)	4008 (4.3)	10 000	10 017 (1.0)	10 038 (4.4)
Efavirenz	100	110 (2.9)	108 (4.0)	2000	2014 (1.6)	2007 (5.0)	5000	4939 (1.5)	5017 (3.8)
Indinavir	200	192 (4.4)	193 (11.0)	4000	3912 (2.2)	3943 (4.7)	10 000	10 024 (2.0)	10 065 (3.4)
Nelfinavir	200	174 (2.6)	185 (9.0)	4000	3864 (1.5)	3892 (1.4)	10 000	10 224 (1.2)	10 778 (3.5)
Ritonavir	400	433 (3.1)	392 (5.4)	8000	8038 (1.5)	7902 (2.5)	20 000	20 247 (1.8)	22 215 (3.8)
Saquinavir	20	20 (5.0)	21 (12.0)	400	412 (1.5)	408 (1.2)	5000	5061 (3.2)	5095 (4.1)
Abacavir	80	82 (9.5)	78 (9.0)	800	782 (5.2)	773 (9.0)	2000	1992 (0.8)	1964 (4.0)
Didanosine	20	19 (4.3)	20 (5.7)	200	197 (3.9)	196 (5.0)	500	501 (0.5)	470 (3.2)
Lamivudine	40	38 (6.8)	39 (8.0)	400	401 (5.0)	397 (5.0)	1000	1002 (1.6)	963 (4.2)
Nevirapine	400	374 (4.6)	413 (8.5)	4000	4022 (3.5)	3964 (6.0)	10 000	10 109 (3)	10 482 (5.9)
Stavudine	20	20 (6.7)	20 (5.2)	200	199 (3.8)	195 (5.0)	500	500 (1.7)	480 (4.2)
Zidovudine	20	23 (5.2)	19 (7.4)	200	201 (5.2)	191 (9.0)	500	502 (0.5)	485 (4.7)

[a] The units are in ng/ml.

3.5. Analysis of patient samples

Fig. 5 shows the chromatograms of a plasma sample obtained from a patient included in the GIGAHAART trial. In this patient, R concentration is lower 2 h after ingestion than before (1929 vs. 2640 ng/ml) suggesting a problem of compliance.

Fig. 6 shows the pharmacokinetic profiles of I and E in patients with renal failure.

3.6. Stability of spiked plasma and extracts

No degradation (<10%) of NRTIs and Nvp in human plasma at the highest concentrations (last calibration level) occurred for 6 months. No degradation of any other compound in human plasma at three concentration levels occurred. Stability when kept at −20°C was good for the compounds in dry residue extracts at three concentration levels. Stability at room temperature of all compounds in reconstituted extracts at three concentration levels was validated as well.

4. Discussion

Most of the published methods for PI and Nvp determination used an ion-pair for the mobile phase [19,20,24–27,40,46]. An ion-pair (OSA) is also used in our method for both chromatographic systems, PI–E and NRTI–Nvp. It improves specificity, thanks to the formation of complexes with exogenous components (co-administered drugs) selectively delaying their chromatographic peaks. Amounts of salt and ion-pair have determined the pH of the mobile phase for PI–E and NRTI–Nvp (7.1 and 4.2, respectively). Villani et al. [43] used a non-specific mobile phase (water–acetonitrile) to assay E, and they showed no analytical interactions with nine co-administered drugs and antiretroviral drugs, unfortunately A and R were not tested. A volume of 50% of acetonitrile is required to separate S from E, slowing down selectively S. D is the only NNRTI quantified with fluorescence instead of UV detection. Indeed, peak retention time is very close to A retention time as reported by van Heeswijk et al. [46]. More than 100 samples from patients who received ARV except D were analyzed under these conditions, and neither endogenous interferences nor analytical interaction with A were observed.

The chromatographic system for NRTI–Nvp is sophisticated: three pumps and two switch valves are needed to mimic a pseudo gradient of elution. When available, the system can be simplified by using a binary gradient pump, which dramatically improves resolution. MP-1 used to quantify 3TC, DDI and D4T allows the separation of DDI and D4T from an endogenous interference. MP-2 used to quantify AZT, Ab and Nvp allows the separation of Nvp from an exogenous interference, identified as the sulfamethoxazole. MP-3 used to wash the column, thus avoiding appearance of late peaks (PI, E, co-administered drugs). Furthermore, MP-3 is mandatory to separate 3TC from D4T. Because of the very large difference in acetonitrile proportion between MP-3 and MP-1, the retention time of 3TC is strongly dependent on the equilibration time. We take advantage of this property to selectively speed up 3TC relatively to D4T and DDI. Retention times of NRTIs and Nvp remain stable for a whole set of analytical runs (Δt_R<0.3 min in a 50-sample run).

Numerous 250×4.6 columns were tested for the separation of NRTI–Nvp. C_{18} columns (Symmetry, Supelcosil) and phenyl columns (Inerstil, Hypersil, Spherisorb, μBondapak) allowed a good resolution of the six compounds. Unfortunately 3TC, DDI and endogenous interfering peaks collapsed irreversibly after 100–200 injections. The Symmetry Shield column allows over 500 injections without any loss of resolution, possibly related to the hydrophilic element between silica and C_{18}.

To increase sensitivity, some methods for I [18–22,45,46], N [23], R [24]or A [46] are performed at 205–220 nm. As a consequence of the increased sensitivity, small plasma volumes can be used: 100 μl [18,20], 300 μl [19] or 250 μl [23]; under these conditions, Jayewardene et al. [19] and Burger et al. [20] obtained a LOQ of 10 and 50 ng/ml, respectively for I. A problematic non-specificity is, however, the rule at 210 nm added to the fact that, the mobile phase has a very high cut-off, and interfering peaks are detected near I or A. The detection at 261 nm correspond to the second maximal wavelength (λ_{max}) for I and close to λ_{max} for A, with a molar extinction coefficient lower than at 210 nm (Fig. 1). The LOQs for I (50 ng/ml) and A (25 ng/ml) are satisfactory

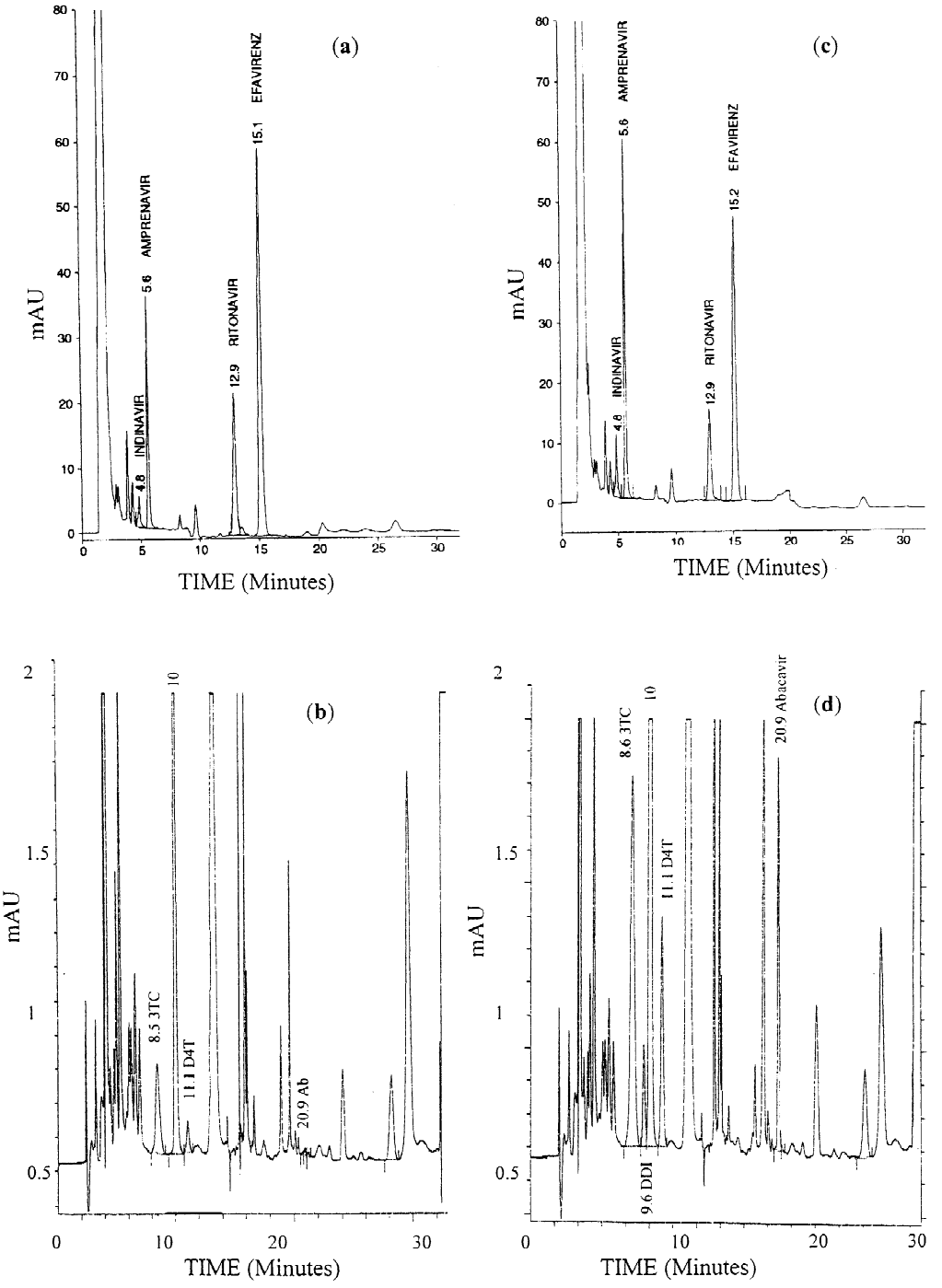

Fig. 5. Chromatograms obtained from a patient administered multiple therapy. Before ingestion (except for efavirenz: 12 h after ingestion): (a) containing 920 ng/ml of indinavir (I), 715 ng/ml of amprenavir (A), 2640 ng/ml of ritonavir (R), and 1825 ng/ml of efavirenz (E); (b) containing 345 ng/ml of lamivudine (3TC), 73 ng/ml of stavudine (D4T), below the LOQ for didanosine (DDI) and abacavir (Ab). Two hours after ingestion (except for E: 14 h after ingestion): (c) containing 1712 ng/ml of I, 1235 ng/ml of A, 1929 ng/ml of R, 1448 ng/ml of E; (d) 1430 ng/ml of 3TC, 511 ng/ml of D4T, 176 ng/ml of DDI and 556 ng/ml of Ab.

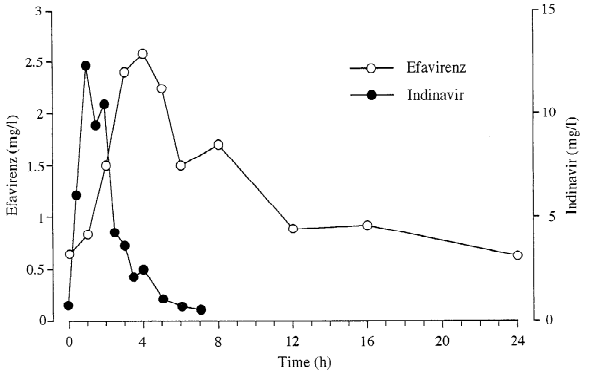

Fig. 6. Efavirenz and indinavir plasma concentrations as a function of time in patients with renal failure, receiving 800 mg three times daily of indinavir and 600 mg once day of efavirenz.

regarding the expected trough concentrations, additionally a very low LOQ for S is mandatory when S is administered alone [5]. These LOQs were obtained from an equivalent 400 μl volume of plasma sample. Van Heeswijk et al. [46] validated a comparable LOQ for A starting from 600 μl plasma sample with a detection set at 210 nm. For N, R, S and E, the wavelengths used in our method are around the λ_{max} (Fig. 1). Hugen et al. [45] were able to reach the same LOQ for I, R and N, and higher for S (45 ng/ml), setting wavelength at 215 nm for detection, and using a 500-μl plasma sample volume extracted by liquid–liquid procedure.

NRTI–Nvp detection mode has been chosen to be performed with relatively standard instrumentation. The wavelength adopted was 260 nm, knowing that the maximum absorption wavelength of NRTIs reach 250 to 280 nm, except for Ab (Fig. 1). Van Heeswijk et al. [40] obtained a better LOQ than ours for Nvp (52 versus 100 ng/ml) using 282 nm detection starting from a 250-μl plasma sample volume only. As far as DDI, D4T, AZT are concerned, the LOQs are equivalent or better than those obtained in separate methods. Hoetelmans et al. [28] obtained a LOQ twice lower than ours for 3TC (20 versus 10 ng/ml) with the same plasma sample volume. LOQs for NRTIs and NNRTIs (except DDI) are, respectively lower and much lower than trough concentrations observed from treated patients. It should be pointed out, that as DDI administration is once daily, and its half-life is short, trough concentrations are often below the LOQ. Our method does not quantify DDC (t_R=5.4 min).

Various methods involving precipitation of proteins have been published: acetonitrile to quantify I [20], R [25], Nvp [40] and D [41,42], perchloric [36] or trichloroacetic acid [37,38] to assay AZT and its glucuronide metabolite. Ammonium sulfate is also used to quantify AZT, DDC and DDI simultaneously [47]. In our hands precipitation with acetonitrile was successful (comparable reproducibility) for DDI, D4T, AZT, 3TC and Nvp, but the recovery was below 60% for D4T and DDI. Furthermore, peaks corresponding to Ab and N were broad, column degradation occurred very quickly (200 injections), and supernatant evaporation time was long (40 min). The acetonitrile precipitation procedure was therefore rejected. Liquid–liquid extraction method was rejected as well, because NRTIs are hydrophilic compounds which are poorly, if at all, extractable in organic solvents, while PI, E and Nvp are lipophilic molecules, easily extractable in solvents like methyl *tert*.-butyl ether [43,45]. A solid–liquid extraction procedure remained therefore the only method available for the simultaneous extraction of all drugs from one single plasma sample. Best results were obtained using C_{18} stationary phase. Others authors have shown better specificity and recovery for D4T [32] and 3TC [28] assay using silica column over C_{18}, but as mentioned this is not suitable for lipophilic molecules like PIs and E. Two types of column were tested: polymeric (Oasis, Waters) and C_{18} (J.T. Baker), loaded with 60 and 500 mg, respectively. Recoveries at low and high concentration levels obtained with an Oasis column were 10–25% lower than with the J.T. Baker column. Van Heeswijk et al. [46] reported recoveries of 70–94% for the five PIs (versus 96.4–99.8% in our method) using a C_2 extraction column, loaded with 100 mg. Hence, a heavily loaded column is probably necessary to extract highly concentrated drugs. In our method, PIs and E exhibit very good extraction recoveries in a wide range of concentrations, with both low variability (RSD≤3.5%) and good linearity (r>0.998). Methods using solid–liquid extraction have obtained similar recoveries [18,20,27,44]. On an Oasis column, Poirier et al. [22] obtained a rather low but constant extraction ratio (66%) for I extraction. Altogether, these recoveries were definitely higher than those obtained with liquid–liquid extraction

procedures: 83% for R [24]; 87% [23] and 73% [45] for N; 70–103% [19] and 80% [21] for I; and 88.6–92.4% for E [43].

Using solid–liquid extraction procedures, individual methods to assay NRTIs showed recoveries higher than 90% for 3TC [28] and D4T [32] on silica column, and DDI [34,35] and AZT [39] on the C_{18} column, as low as 50% for 3TC by ionic exchange [29] or not defined for D4T on C_{18} [33]. Our recoveries for NRTIs reached 70–80%, which are slightly lower than in dedicated methods, and we obtained a very low variability (RSD: 3.1–6.1%) and excellent linearity. Furthermore, for a given compound, the recovery remained constant whether the molecule was extracted alone or simultaneously with other drugs, thus it did not appear necessary to use an internal standard (I.S.). The method was first developed with 2′,3′-*o*-isopropylidene uridine as an I.S. The I.S. was well extracted (recovery=80±2%), and eluated before AZT in the chromatographic system (t_R=16.4 min), with good resolution and without interference. Occasionally however, an interfering peak was detected on chromatogram from patients. This interference was not further identified.

The water washes we used enable elution of proteins and other plasma hydrophilic molecules. A small proportion of NRTIs might be eluted under these conditions, which would explain the relatively low recoveries. In addition, water rinsing, without modification of pH or ionic strength makes the extraction procedure less specific, as co-administered drugs can be extracted as well. For the elution to be optimal the best way is to use methanol. Indeed, a large volume of methanol ensures elution of all antiretroviral drugs. This elution procedure although less specific than other, allows much better recoveries. Van Heeswijk et al. [46] used 1 ml of 0.1 *M* ammonium acetate–acetonitrile (3:7, v/v) for PIs and Poirier et al. [22] acetonitrile 23% for I extraction. Finally, the rather large solvent front on chromatograms suggests the intermediate specificity of the extraction procedure as compared to liquid–liquid [45] or other solid–liquid extractions [46], and confirms, the need for highly specific chromatographic conditions.

The preparation of calibration standards consists in a simple and rapid dilution of spiked plasma with drug-free human plasma. Care should be taken that as little volume of methanol as possible is contained in spiked plasma samples, in order to avoid elution of NRTIs during the extraction procedure, hence diminution of recoveries. When the residue is directly dissolved in spiked NRTI–Nvp plasma, recovery and linearity for PIs and E decline (<50% versus 97.2%). This has been shown by Ha et al. [26] for the S quantification. Many authors checked ARV drugs and/or some others drugs for interference; and, as already mentioned, a very careful investigation for assay specificity is mandatory when detection is performed at 210 nm. Actually, a few co-administered drugs showed some potential for interference. Therefore, when antiretroviral plasma concentrations in patients appear much higher than expected, samples are injected again in the system fitted to a UV–Vis diode-array detector. It then becomes possible to compare the molecular absorption spectra and rule out any analytical interference.

AZT is metabolized into two metabolites: 3′-amino-3′-deoxythymidine and the glucuronide G-AZT (t_R: 5.4 and 13.3 min, respectively). The two metabolites are well separated from NRTIs and Nvp under the described chromatographic conditions; occasionally some interferences with unknown endogenous compounds might occur. The 3TC's sulfoxide metabolite is eluated in the solvent front under our conditions. For DDI [48], D4T [49], Ab [50] no relevant metabolite has been described. Among 300 patients plasma samples analyzed with our method no interfering peak from PI or E metabolites was observed.

5. Conclusion

In conclusion, a sensitive, specific and validated assay for the simultaneous quantitative determination of 12 ARV drugs from different therapeutic classes is described. The HPLC method can be used in a hospital laboratory for the monitoring of ARV concentrations in plasma, and for pharmacokinetic studies in HIV-infected patients. Thanks to the absence of interference with other medications, the method is usable in this heavily co-medicated patient group. This is the first method validated for the simultaneous quantification of 12 commonly administered ARV drugs, requiring only 1 ml of human plasma sample. The method appears to be applicable to determine plasma concentrations of all ARV drugs

and is currently being used to analyze samples of patient treated with approved combination therapy regimens.

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