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Simultaneous determination of the HIV nucleoside analogue reverse transcriptase inhibitors lamivudine, didanosine, stavudine, zidovudine and abacavir in human plasma by reversed phase high performance liquid chromatography

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Abstract

A reversed phase high performance liquid chromatography method was developed for the simultaneous quantitative determination of the nucleoside reverse transcriptase inhibitors (NRTIs) lamivudine, didanosine, stavudine, zidovudine and abacavir in plasma. The method involved solid-phase extraction with Oasis MAX cartridges from plasma, followed by high performance liquid chromatography with a SymmetryShield RP 18 column and ultraviolet detection set at a wavelength of 260 nm. The assay was validated over the concentration range of 0.015–5 mg/l for all five NRTIs. The average accuracies for the assay were 92–102%, inter- and intra-day coefficients of variation (CV) were <2.5% and extraction recoveries were higher than 97%. This method proved to be simple, accurate and precise, and is currently in use in our laboratory for the quantitative analysis of NRTIs in plasma.

Keywords: Nucleoside reverse transcriptase inhibitors; Abacavir; Didanosine; Lamivudine; Stavudine; Zidovudine; HPLC; HIV

1. Introduction

Nucleoside reverse transcriptase inhibitors (NRTIs) were the first class of drugs that were introduced as antiretroviral agents for the treatment of infection with human immunodeficiency virus (HIV). Four additional drug classes have since been developed: protease inhibitors (PIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), nucleotide reverse transcriptase inhibitors (NtRTIs), and fusion inhibitors. The current standard of care, referred to as "highly active antiretroviral therapy" (HAART), is to combine at least three antiretroviral drugs. Two NRTIs should be combined with either one or two PIs, an NNRTI or a third NRTI [1]. The six NRTIs, approved for use in HIV infection are: abacavir (ABC), didanosine (ddI), lamivudine (3TC), stavudine (d4T), zalcitabine (ddC) and zidovudine (AZT). Zalcitabine is not recommended by HIV treatment guidelines and is therefore rarely used. NRTIs are prodrugs that require intracellular phosphorylation to their corresponding triphosphate derivates, which are the active inhibitors of HIV reverse transcriptase. There is no clear correlation between NRTI plasma concentrations and virological response or intracellular concentrations of the corresponding triphosphate derivates. Nevertheless, plasma concentration measurements may be important for research purposes and in patient care to check adherence to NRTIs, to guide dosing in patients with renal failure and to evaluate drug-drug interactions. Separation methods for NRTIs used for treatment of HIV-1 infection

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were reviewed in 2001 by Pereira et al. [2] Most of these reviewed methods allowed the quantification of only one drug. Only one method was capable of measuring abacavir, didanosine, lamivudine, stavudine and zidovudine simultaneously [3]. Since that time two other high performance liquid chromatography (HPLC) methods with UV detection for the simultaneous determination of NRTIs have been published [4,5]. Aymard et al. [3] showed chromatograms with a low signal-to-noise ratio possibly due to the use of an ion-pair reagent in their mobile phase. Ion-pair HPLC in a gradient mode is usually not recommended [6,7]. Simon et al. [4] had high standard deviations for their recovery and high quantification limits. Rezk et al. [5] used four different ultraviolet wavelengths for detection which can make their method less robust. They all used conventional silica-based solid-phase extraction sorbents and with these columns, care should be taken that the columns did not run dry during conditioning, in order to obtain reproducible results. This could complicate manual SPE extraction of large amounts of samples and compromise the accuracy of the assay. We considered none of these methods suitable for application in our laboratory.

Therefore, the aim of this study was to develop a simple and robust HPLC-UV method that could be used in our hospital for the simultaneous assay of abacavir, didanosine, lamivudine, stavudine and zidovudine in patient plasma samples.

2. Experimental

2.1. Chemicals

Abacavir sulphate was kindly supplied by Glaxo Smith Kline (London, UK), didanosine and stavudine were kindly provided by Bristol-Myers Squibb (Princeton, NJ, USA), lamivudine was purchased from Moravek Biochemicals (Brea, CA, USA) and zidovudine was obtained from Sigma (St. Louis, MO, USA). Chemical purity for all compounds was >99%. Super gradient acetonitrile and HPLC quality methanol were purchased from Labscan Analytical Sciences (Dublin, Ireland), and HPLC quality water from Baker (Deventer, The Netherlands). All other reagents were obtained from Merck (Darmstadt, Germany). The drugs that were investigated for possible interference with the assay were obtained from Sigma (St. Louis, MO, USA) or were extracted from commercial products.

2.2. Preparation of standards and quality control samples

Two series of stock solutions of each NRTI were prepared in methanol–HPLC-grade water (1:9) at a concentration of 0.5 mg/ml and kept at −20 °C. For the preparation of standard and quality control (QC) samples, both series of stock solutions were diluted with blank plasma to obtain a concentration that equalled the highest standard concentration (5 mg/l). To achieve 6 standard concentrations of 0.015, 0.050, 0.150, 0.50, 1.500 and 5 mg/l, appropriate amounts of one of the 5 mg/ml stocks in plasma were added to blank plasma. For the QC samples, concentrations of 0.10, 0.4 and 2.0 mg/l were prepared from the other 5 mg/ml plasma stock. The standard and QC samples were stored at −20 °C.

2.3. Equipment

The HPLC system consisted of a model P4000 solvent delivery pump, a model AS3000 autosampler, a model UV2000 programmable wavelength UV detector and a ChromJet integrator. All these instruments were from Thermo Electron (Breda, The Netherlands). The analytical column was a SymmetryShield RP 18 column (150 mm × 4.6 mm i.d./particle size 3.5 μm) protected by a SymmetryShield RP 18 guard column (20 mm × 3.9 mm i.d./particle size 3.5 μm), both from Waters (Etten-Leur, The Netherlands).

2.4. Solid-phase extraction method

Solid-phase extraction (SPE) columns (1 cc, 30 mg Oasis MAX Waters) were placed on a vacuum elution manifold (Baker spe 24G Column Processor) and rinsed with 0.5 ml of methanol, followed by 0.25 ml of distilled water. Next, 0.5 ml of distilled water and 0.5 ml of plasma sample were transferred onto the columns and were drawn into them by applying reduced pressure (flow rate <1 ml/min). The columns were then washed with two aliquots of 0.15 ml of distilled water, followed by vacuum suction for 5 min. Elution of the absorbed analytes was performed by using two volumes of 0.25 ml of methanol–HPLC quality water (80:20, v/v) and reduced pressure (flow rate <1 ml/min). The eluent was evaporated to dryness under a nitrogen stream at 40 °C. The residues were redissolved in 0.2 ml of acetonitrile–HPLC quality water (5:95, v/v), mixed on a vortex mixer for 20 s, and centrifuged for 5 min. The clear supernatants were placed in autosampler vials with inserts and aliquots of 25 μl were injected onto the column.

2.5. Chromatography and detection

The chromatographic separation was performed at 30 °C with a gradient elution. The mobile phase flow rate was set at 1.0 ml/min. The mobile phase components were an acetate buffer (20 mM potassium acetate adjusted to pH 4.60 with 20 mM acetic acid) and acetonitrile. Mobile phase (A) consisted of acetate buffer:acetonitrile (95:5, v/v) and mobile phase (B) consisted of acetate buffer:acetonitrile (76:24, v/v). After 10 min 100% mobile phase (A), the mobile phase (B) concentration was increased in 14 min linearly from 0 to 100%. After that, mobile phase (A) concentration returned to 100% in 2 min. The column was then re-equilibrated for 9 min. UV detection was performed at 260 nm. Peak height was used for integration.

2.6. Specificity and selectivity

The interference from endogenous compounds was investigated by analyzing blank plasma of six different individuals, who did not use an NRTI. No peaks interfering with the peaks of the NRTIs were allowed. Possible interference by all antiretroviral drugs and regularly co-administered drugs in HIV patients was tested by analyzing samples that contained concentrations of 50 mg/l of each drug. Metabolites of NRTIs were tested for interference by analyzing patient plasma samples containing each of the NRTIs.

2.7. Accuracy, precision, recovery and lower limit of quantification

Five replicates of three different concentrations of QC samples were analyzed on three different days in order to determine the accuracy and precision. One-way analysis of variance (ANOVA) was used to calculate the intra- and inter-day variation in these parameters.

Average recovery of abacavir, didanosine, lamivudine, stavudine and zidovudine was determined by comparing responses with those obtained by direct injection of the same amount of drug in mobile phase at six different concentrations (0.015, 0.050, 0.150, 0.500, 1.500, 5.00 mg/l) in three separate runs.

For the lower limit of quantification the percent deviation from the nominal concentration and the relative standard deviation had to be less than 20%. Samples for determining the lower limit of quantification were assayed as five replicates.

2.8. Stability

The stability during sample handling of abacavir, didanosine, lamivudine, stavudine and zidovudine was verified by subjecting samples to three freeze-thaw cycles, testing the stability in plasma at room temperature and at −20 °C, all at different concentration levels. As a reference for comparison, fresh, unfrozen samples at the same concentration levels were used. The stability of processed extracts in the autosampler was assessed by re-injection of standards and three QC samples in duplicate. The QC concentrations were compared with the original QC concentrations. Stability of the standard solutions in methanol–water (1:9, v/v) at −20 °C was determined by reanalyzing aliquots of the stored stock solution with aliquots of a fresh stock solution.

2.9. Participation in interlaboratory quality control

We participated four times in the interlaboratory proficiency testing program of the AIDS Clinical Trials Group [8]. Each time three plasma samples containing unknown amounts of both abacavir, lamivudine and zidovudine had to be analyzed. The proficiency testing program did not provide samples containing didanosine and stavudine.

3. Results

3.1. Development phase

Lamivudine, didanosine and stavudine are very polar compounds and tend to stay in the water phase. Therefore, we chose solid-phase instead of liquid–liquid extraction.

In search for a robust and simple manual solid-phase extraction, several polymeric reversed-phase sorbents were evaluated. These sorbents are water-wettable and unlike silica based cartridges, reproducible results are obtained even when the cartridges run dry. Five polymeric sorbents were tested: Nexus (Varian), Speedos H_2O Phallic DVB (J.T. Baker), Oasis HLB, Oasis MCX and Oasis Max. The cleanest baselines and best recoveries (>97%) were achieved with the Oasis Max sorbent. To improve flow during loading and to prevent blockages, plasma was diluted with the same amount of water on top of the column. We used a water wash step and a methanol-water (80:20, v/v) elution of the absorbed analytes. Elution with 100% methanol yielded a lower recovery for didanosine (65%) and elution with lower percentages of methanol gave less elution of zidovudine and abacavir. Since didanosine is an acid-labile compound, we chose for reconstitution of the evaporated extracts in water–acetonitrile (95:5, v/v) instead of using the acid eluent. With extraction recoveries higher than 97% there was no need for an internal standard.

To test the batch-to-batch reproducibility of the Oasis Max cartridges for our assay, five replicates of three different batches were used for extraction of an 800 ng/ml plasma sample. Coefficients of variation of the extraction recovery were less then 3.5% for all five NRTIs.

As regards to the chromatographic separation of the NRTIs, we originally validated our method on an Inertsil 5 ODS2 column. Symmetrical peaks and good resolutions were achieved, but column lifetime appeared to be short (sometimes less then 100 injections). The search for a more suitable column was complicated by two endogenous peaks that interfered with didanosine and stavudine and were very difficult to separate. We tested several phenyl columns (Inertsil Phenyl, X Terra phenyl) and C18 columns (Inertsil 5 ODS3, Hypersil ODS 5, OmniSpher 5 C18, X Terra RP18, Aquasil C18, Prevail C18). A SymmetryShield RP 18 column (150 mm × 4.6 mm i.d./particle size 3.5 μm) finally yielded best separation and column lifetime. So far, we have been able to analyse more than 1500 plasma samples on this column. To evaluate the reproducibility of our separation method on this column, we compared the chromatographic separations of the NRTIs on the SymmetryShield column in use with those obtained on three different batches. Chromatographic separations and resolutions were identical on all columns.

Acetonitrile and a 20 mM acetate buffer pH 4.60 were chosen as mobile phase. At pH 4.60 all NRTIs were separated from interfering plasma peaks. Because of its pK_a value (4.8), acetate buffer has the best buffer capacity in this area. A column temperature of 30 °C provided the best resolution of

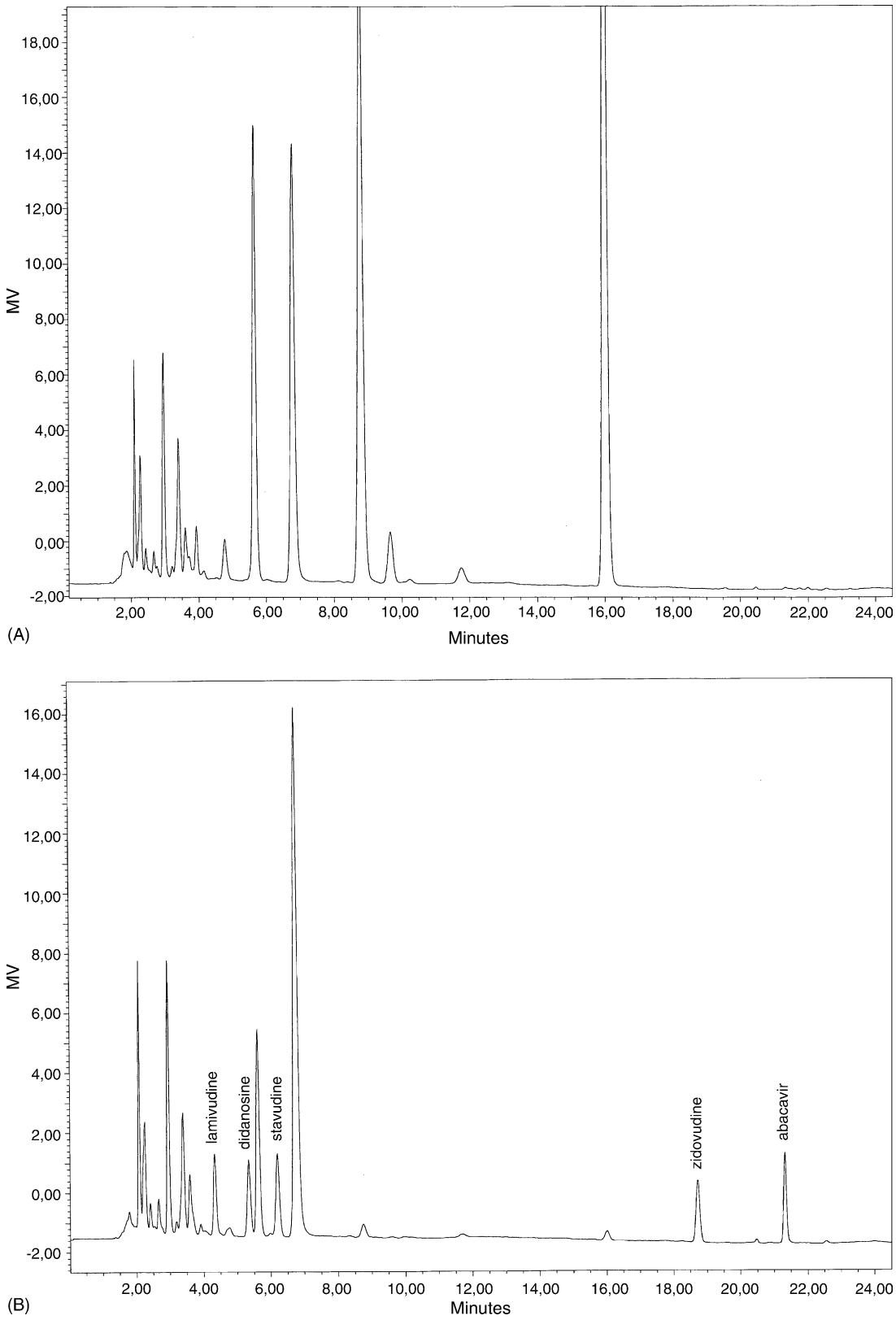
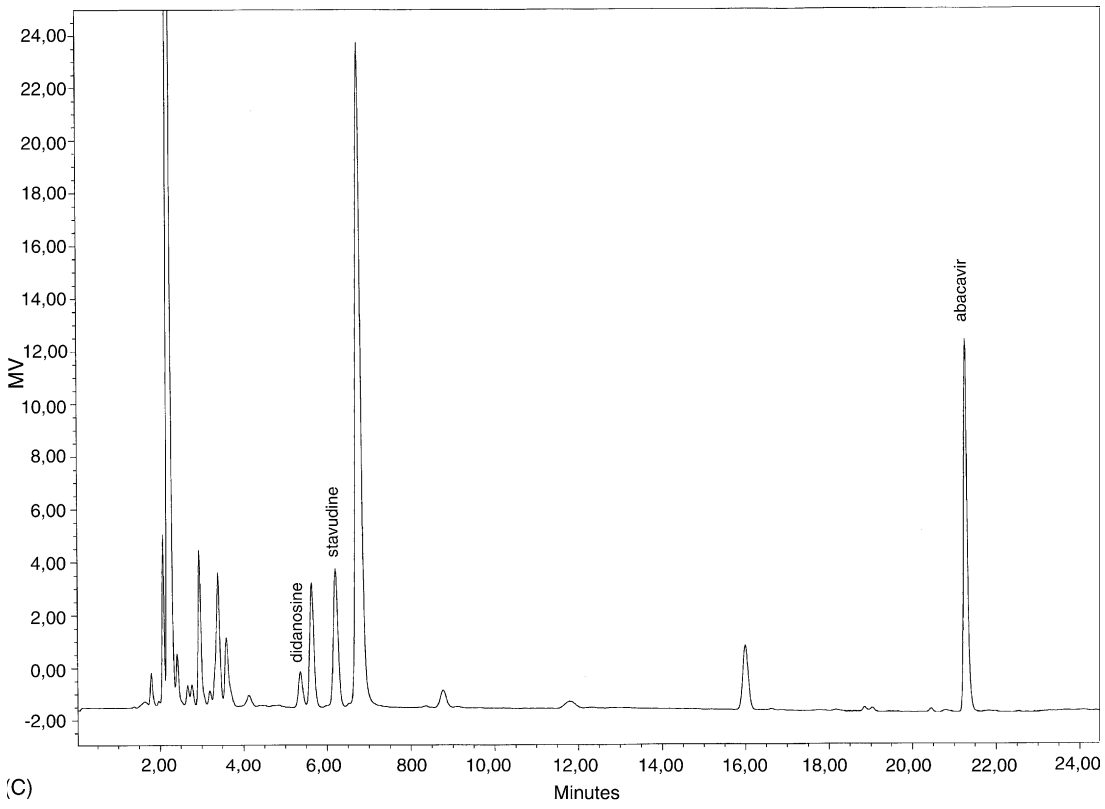
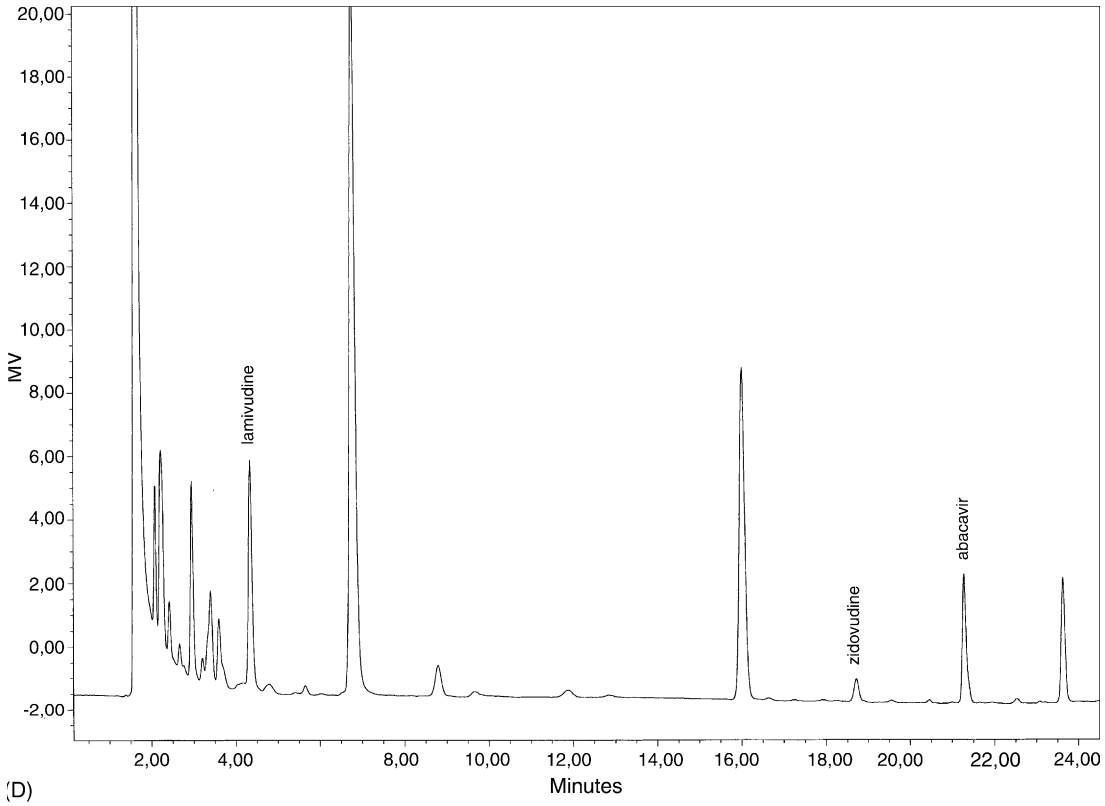


Fig. 1. (A) Chromatogram of a blank plasma sample; (B) chromatogram of spiked plasma sample containing 150 ng/ml lamivudine, didanosine, stavudine, zidovudine and abacavir; (C) chromatogram of a patient plasma sample (87 ng/ml didanosine, 297 ng/ml stavudine and 688 ng/ml abacavir); (D) chromatogram of a patient plasma sample (387 ng/ml lamivudine, 51 ng/ml zidovudine and 225 ng/ml abacavir).



(C)



(D)

Fig. 1. (*Continued*).

stavudine. The detection wavelength was set at 260 nm. This wavelength is not the wavelength of maximal absorption for all components but it provided a good signal-to-noise ratio and a good selectivity.

Fig. 1A and B shows chromatograms of an extracted blank plasma and a standard plasma, respectively, containing all five NRTIs. The approximate retention times were 4.3 min for lamivudine, 5.3 min for didanosine, 6.2 min for stavudine, 18.7 min for zidovudine, and 21.3 min for abacavir.

3.2. Specificity and selectivity

The six blank plasma samples showed no peaks that co-eluted with the NRTIs. Potentially co-administered drugs tested (Table 1) had retention times that were different from the NRTIs or were not detected with the described bioanalytical method. There was no interference from the metabolites of the NRTIs.

3.3. Accuracy, precision, recovery and lower limit of quantification

The results of the determination of accuracy and precision of the assay are presented in Table 2. These results show that this method is accurate (average accuracy from 92 to 102%) and precise (inter-day coefficient of variation (CV) ranged from 0 to 2.4% and intra-day CV from 1.1 to 2.3%).

The average recoveries (determined in three separate runs) were 100.4% (S.D. 2.7%) for lamivudine, 96.8% (S.D. 2.2%) for didanosine, 99.7% (S.D. 3.0%) for stavudine, 100.1% (S.D. 3.3) for zidovudine and 101.0% (S.D. 2.8) for abacavir. Recovery was consistent across the concentration range, less than 2% difference was found between the average recovery of the lowest and highest concentration.

The lower limit of quantification was found to be 0.015 mg/l for all five NRTIs. The calibration curves were linear over the concentration range of 0.015–5 mg/l.

3.4. Stability

The results of stability tests under various conditions are listed in Table 3. Under all conditions tested, abacavir, didanosine, lamivudine, stavudine and zidovudine proved to be stable with recoveries of at least 94% of the initial concentration.

Table 1
Co-administered drugs for specificity

Acetaminophen	Dapsone	Lidocaine	Rifabutin
Acyclovir	Domperidon	Lopinavir	Rifampicine
Amphotericin B	Efavirenz	Methadone	Ritonavir
Amoxycillin	Erythromycin	Nelfinavir	Saquinavir
Amprenavir	Ethambutol	Nevirapine	Sulfamethoxazole
Atovaquone	Famotidine	Ofloxacine	Sulfametrol
Caffeine	Fluconazole	Oxazepam	Tenofovir
Calcium folinate	Ganciclovir	Pentamidine	Trimethoprim
Carbamazepine	Indinavir	Phenobarbital	Valproic acid
Clarithromycin	Isoniazid	Phenytoin	Zalcitabine
Clindamycin	Itraconazole	Pyrazinamid	
Clofazimine	Ketoconazole	Pyrimethamine	

3.5. Participation in interlaboratory quality control

Participation in an interlaboratory quality control program allowed us to evaluate the accuracy of our method. In this program 20% limits around the nominal concentration of the drugs are considered to be appropriate thresholds for a satisfactory measurement. Results obtained from all measurements of all four rounds of the interlaboratory quality control program were within these limits. For lamivudine all 12 results deviated less then 7% from the nominal concentration. For zidovudine 11 results deviated less then 4% and one deviated 14% from the nominal concentration. For abacavir 10 results deviated less then 7% and the other two deviated 11% and 19% from the nominal concentration.

3.6. Application of the method

The applicability and robustness of the assay was proven by analyzing more than 1500 plasma samples without any problems. The method has been used for pharmacokinetic studies and plasma concentration monitoring in patient care (therapeutic drug monitoring). Representative chromatograms of patient samples are shown in Fig. 1C and D. The plasma concentration-time profile in patients after ingestion of standard doses of lamivudine (150 mg twice daily), zidovudine (300 mg twice daily) and abacavir (300 mg twice daily) is shown in Fig. 2A and of didanosine (400 mg once daily) and stavudine (40 mg twice daily) is shown in Fig. 2B.

4. Discussion

This paper describes the development and extensive validation of a bioanalytical method for the determination of five approved NRTIs. Use of an HPLC method for the simultaneous measurement of several NRTIs saves considerable time and costs.

When we started method development, only the assay of Aymard et al. [3] was available. Since that time Simon et al. [4] and Rezk et al. [5] published two other HPLC-UV methods for the simultaneous determination of NRTIs in plasma.

Contrary to the methods developed by Aymard et al., Simon et al. and Rezk et al., who all used conventional silica-based SPE sorbents, we used a polymeric reversed phase sorbent for extraction. This sorbent is water-wettable and reproducible results can be obtained even when the cartridges run dry. Therefore, manual solid-phase extractions of large amounts of samples are supposed to be more rugged and less complicated to perform. Indeed, high recoveries were achieved (>97%) and low coefficients of variation (2.2–3.3%), using less plasma sample than other existing

Table 2
Accuracy and precision of the determination of lamivudine, didanosine, stavudine, zidovudine and abacavir in plasma

Analyte	Concentration (mg/l)	Mean-measured concentration	Accuracy (%)	Intra-day precision (%)	Inter-day precision (%)
Lamivudine	0.098	0.090	92	1.8	1.9
	0.397	0.381	96	2.3	0.7
	1.984	1.950	98	1.4	2.2
Didanosine	0.110	0.110	100	1.8	1.8
	0.438	0.447	102	2.1	1.5
	2.192	2.194	100	1.8	2.4
Stavudine	0.130	0.129	99	1.7	1.6
	0.520	0.524	101	2.2	1.6
	2.600	2.556	98	1.7	1.3
Zidovudine	0.108	0.107	99	1.7	0.5
	0.432	0.433	100	2.0	0.0[a]
	2.160	2.192	101	1.5	0.0[a]
Abacavir	0.110	0.107	97	1.7	2.3
	0.438	0.435	99	1.9	1.4
	2.192	2.182	100	1.1	0.2

[a] In cases where the inter-day precision is 0.0%, no additional variation is observed as a result of performing the assay on different days.

methods. 1500 plasma samples could be analyzed without problems.

For chromatographic separation, we chose to avoid the use of ion-pair reagent and used only potassium acetate buffer and acetonitrile for the mobile phase. The slow equilibration of the column with ion-pair reagents can create problems if a gradient elution is used. Retention may be less reproducible, baselines can be more erratic and other problems, like artifactual peaks, may arise. For this reason, ion-pair HPLC in a gradient mode is usually not recommended [6,7]. Aymard et al. used an ion-pair (OSA) for the mobile phase and this could be responsible for their low signal-to-noise ratio. In addition they required a complicated system that used three different mobile phases to mimic a gradient. This system could be simplified by using a binary gradient pump. However, the problems mentioned above would still remain.

We used one wavelength set at 260 nm for UV detection. This is not the maximum absorption wavelength for all

Table 3
Stability experiments

Analyte	Concentration range (mg/l)	Condition	Matrix	Time interval	Recovery mean percent (S.D.)
Lamivudine	0.1–2	20 °C	Plasma	7 days	103 (1.0)
	0.1–2	−20 °C	Plasma	9 months	100.7 (0.6)
	0.1–2	Freeze-thaw	Plasma	3 cycles	99.9 (2.2)
	0.1–2	Autosampler	Processed extracts	7 days	102 (2.8)
	495	−20 °C	Water–methanol 90:10	26 months	101.9 (0.1)
Didanosine	0.1–2.2	20 °C	Plasma	7 days	98.7 (2.6)
	0.1–2.2	−20 °C	Plasma	9 months	93.7 (4.8)
	0.1–2.2	Freeze-thaw	Plasma	3 cycles	95.0 (1.1)
	0.1–2.2	Autosampler	Processed extracts	7 days	105.5 (2.0)
	548	−20 °C	Water–methanol 90:10	26 months	95.4 (0.0)
Stavudine	0.1–2.6	20 °C	Plasma	7 days	96.0 (1.0)
	0.1–2.6	−20 °C	Plasma	9 months	101.0 (1.0)
	0.1–2.6	Freeze-thaw	Plasma	3 cycles	94.7 (1.2)
	0.1–2.6	Autosampler	Processed extracts	7 days	94.0 (1.5)
	567	−20 °C	Water–methanol 90:10	26 months	98.9 (0.8)
Zidovudine	0.1–2.2	20 °C	Plasma	7 days	102.7 (4.4)
	0.1–2.2	−20 °C	Plasma	9 months	104.0 (1.0)
	0.1–2.2	Freeze-thaw	Plasma	3 cycles	96.0 (2.1)
	0.1–2.2	Autosampler	Processed extracts	7 days	97.5 (0.7)
	559	−20 °C	Water–methanol 90:10	26 months	99.5 (0.7)
Abacavir	0.1–2.2	20 °C	Plasma	7 days	102.3 (1.1)
	0.1–2.2	−20 °C	Plasma	9 months	101.0 (1.7)
	0.1–2.2	Freeze-thaw	Plasma	3 cycles	95.7 (1.2)
	0.1–2.2	Autosampler	Processed extracts	7 days	95.5 (0.7)
	529	−20 °C	Water–methanol 90:10	26 months	100 (2.8)

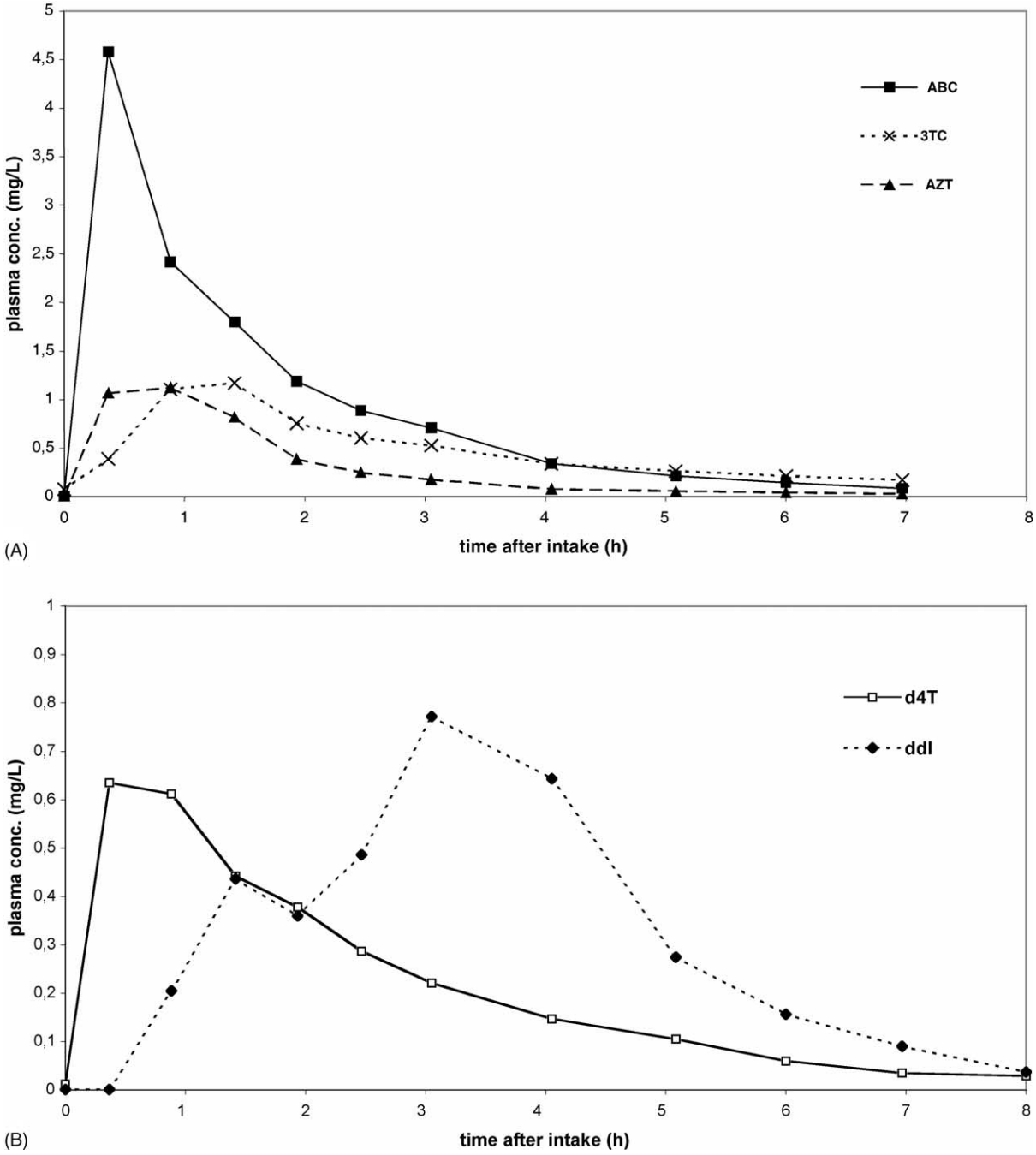


Fig. 2. (A) Plasma concentration vs. time curves for HIV-infected patients after ingestion of standard doses of abacavir (ABC), lamivudine (3TC) and zidovudine (AZT); (B) plasma concentration vs. time curves for HIV-infected patients after ingestion of standard doses of stavudine (d4T) and didanosine (ddI).

components but it provides a good signal-to-noise ratio and good selectivity. Rezk et al. used four different ultraviolet wavelengths for detection, to make their method more specific and sensitive and to achieve 10 ng/ml detection limit for all analytes. The absorption wavelength was switched at 14 min just after the didanosine peak (retention time: 13.6) and at 24 min just after the zidovudine peak (retention time: 23.8). In our opinion this could be problematic because a small shift in retention time will compromise the signal by the UV detector.

Validation of our assay included testing of all antiretroviral and other possible co-administered drugs in HIV patients for interference. Simon et al. tested no drugs, Rezk et al. tested 10 antiretroviral drugs and Aymard et al. tested 111 drugs for interference. Stability of the NRTIs was tested under various conditions. Recovery, specificity, accuracy, intra- and inter-assay precision and the lower limit of quantification have all been validated. Recovery rates of our method were higher than with Aymard et al., Simon et al. and Rezk et al. and smaller inter and intra day variability were

achieved than with other previously published methods. Finally, batch-to-batch reproducibility of the extraction cartridges and the analytical column for this method was tested. We participated four times in an interlaboratory quality control program to confirm and maintain the accuracy of the method.

5. Conclusion

We developed a HPLC-UV method for the simultaneous quantitative determination of NRTIs in plasma, which is more robust, offers higher recoveries and has less inter- and intra-day coefficients of variation than previously published methods. In our hospital the assay has been in use for pharmacokinetic studies and patient care for more than 18 months and for more than 1500 patient samples.

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