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Simultaneous determination of six HIV nucleoside analogue reverse transcriptase inhibitors and nevirapine by liquid chromatography with ultraviolet absorbance detection

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Abstract

An accurate, sensitive and specific reversed-phase high-performance liquid chromatography assay for the simultaneous quantitative determination of the nucleoside reverse transcriptase inhibitors zalcitabine, lamivudine, didanosine, stavudine, zidovudine, and abacavir with the non-nucleoside reverse transcriptase inhibitor nevirapine in human blood plasma is described. The new Polarity dC C_{18} silica column used in this method provides better resolution and peak shape than all other columns tested. Also, four different ultraviolet wavelengths were used for accurate and specific quantitation of the analytes. The method was validated over the range of 10–10 000 ng/ml for all analytes except zalcitabine (10–5000 ng/ml). This method is accurate (average accuracies of three different concentrations ranged from 97.2 to 105%), and precise (within- and between-day precision measures ranged from 0.5 to 5.1% and 0.5 to 5.6%, respectively), and is currently being used for determination of plasma drug concentrations in our laboratory.

Keywords: Zalcitabine; Lamivudine; Didanosine; Stavudine; Zidovudine; Abacavir; Nevirapine

1. Introduction

Combination antiretroviral therapy is the most effective approach to managing HIV infection [1–3]. Current treatment guidelines state that antiretroviral regimens should contain at least two nucleoside reverse transcriptase inhibitors (NRTIs), and either a non-nucleoside reverse transcriptase inhibitor (NNRTI) such as nevirapine, or a protease inhibitor [4]. To date, six nucleoside reverse transcriptase inhibitors (NRTIs) have been approved for the treatment of HIV infection: zidovudine (ZDV), didanosine (ddI), zalcitabine (ddC), stavudine (d4T), lamivudine (3TC) and abacavir (ABC). Since multiple drugs are used in a single patient, sensitive and specific analytical methods are needed for simultaneously determining plasma concentrations for as many HIV medications as possible [5]. A clinician may use such methods to provide valuable information about patient treatment in several regards: malabsorption, drug interactions, adherence, and individual drug pharmacokinetics [6].

To date, most published analytical methods for the NRTIs and nevirapine use reversed-phase high-per-

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formance liquid chromatography (RP-HPLC) with ultraviolet (UV) detection or mass spectrometry (MS). UV detection is widely used for relatively simple bioanalytical assays. The majority of these methods are for single drugs: didanosine [7,8], zidovudine [9–12], lamivudine [13–16], stavudine [17–19], abacavir and its two major metabolites [20] and nevirapine [21,28]. Several other methods have been published for two or more NRTIs [6,22–25].

This paper describes the development and validation of the first RP-HPLC method with UV detection for the simultaneous determination of all six NRTIs and nevirapine in human blood plasma after an optimized solid-phase extraction procedure.

2. Experimental

2.1. Chemicals

Zidovudine, didanosine, stavudine, and hexobarbital were purchased from Sigma (St. Louis, MO, USA). Nevirapine, lamivudine, abacavir, and zalcitabine were obtained from the NIH AIDS Research & Reference Reagent Program (McKesson HBOC BioServices, Rockville, MD, USA). HPLC grade chemicals were purchased from Fisher Scientific (Norcross, GA, USA). Purified compressed nitrogen gas used was obtained from National Welders Supply (Charlotte, NC, USA).

2.2. Equipment

A high-performance liquid chromatography (HPLC) system consisting of an Agilent Technologies (Wilmington, DE, USA) Model HP1100 binary pump, an HP1100 degasser, an HP1100 autosampler, an HP1100 UV-DAD-detector, and HP ChemStation software (Version A.08.03) run on an HP-Kayak XU-800 computer, was used for this method.

2.3. Preparation of standards

Individual stock solutions of ddC, ddI and d4T were prepared in HPLC-grade water at a concentration of 1.0 mg/ml. Stock solutions of 3TC, ABC, AZT and Nvp were prepared in a solution of 60% methanol and 40% HPLC-grade water. The working solution was prepared as a composite of all seven analytes (1.0 ml each), adjusted to a final concentration of 100 μg/ml by HPLC-grade water. The methanol content of this solution was 24%. This 100-μg/ml standard was used to prepare working solutions in methanol–HPLC-grade water (1:3) at concentrations of 100–100 000 ng/ml. Plasma calibration samples at 10, 50, 100, 500, 1000, 5000, and 10 000 ng/ml of all seven drugs combined were prepared by using a 1:10 dilution of the respective working solutions to blank plasma. From another 100 μg/ml working stock solution, concentrations of 1600, 8000 and 40 000 ng/ml were prepared in methanol–HPLC-grade water (1:3). Plasma quality control samples at 160, 800, 4000 ng/ml were prepared using a 1:10 dilution of their respective working solutions to blank plasma. This plasma was devoid of any drugs, and obtained from whole blood anticoagulated with sodium EDTA (Biological Specialty Corporation, Colmar, PA, USA). The final methanol content of all calibrators and quality controls was $<2.5\%$.

Solutions of potential drugs of interference (primarily protease inhibitors and other non-nucleoside reverse transcriptase inhibitors) were prepared from clear filtered extracts of the pharmaceutical formulations. These solutions were prepared in 100% methanol to a final concentration of 1.0 mg/ml, and diluted with mobile phase before injection onto the HPLC system at concentrations of 10 μg/ml. Metabolites were not considered.

2.4. Internal standard (I.S.) preparation

Hexobarbital (10 mg) was dissolved in acetonitrile to achieve a final concentration of 1.0 mg/ml (stock solution). From this solution, an aliquot was diluted in 25 m*M* ammonium acetate buffer (pH 7.0) to a final concentration of 2.0 μg/ml (working solution).

2.5. Sample pre-treatment

Blood samples were collected in sodium EDTA tubes and centrifuged (2800 rpm for 15 min at 4 °C) within 15 min after collection. Plasma was transferred to clean cryovials and stored at −70 °C. Prior

to extraction, all plasma samples were heated for 60 min at 58 °C to inactivate the HIV virus.

On the day of analysis, 800 μl from patient samples, QCs and calibrators were transferred to centrifuge tubes containing 300 μl of the internal standard working solution. The solutions were mixed by vortexing for 30 s and centrifuged at 18 000 *g* for 5 min. One milliliter of the supernatant was transferred to the SPE extraction cartridge.

2.6. SPE extraction method

Solid-phase extraction columns (1.0 ml, 100 mg BOND ELUT-C_{18} Varian, Harbor City, CA, USA) were placed in a vacuum elution manifold (20-SPE system, Waters, Milford, MA, USA). The cartridges were conditioned with 1.0 ml of methanol and equilibrated with 1.0 ml of 100 m*M* ammonium acetate (pH 7.0). An amount of 1.0 ml of the plasma/internal standard mixture supernatant was allowed to pass through the column bed with minimal suction (2–5 mmHg). The column was further washed with 1.0 ml of 100 m*M* ammonium acetate buffer (pH 7.0) then the bed was suctioned dry for 1 min. The drugs were then eluted with 800 μl of methanol. The eluent was evaporated to dryness under a nitrogen stream at 40 °C, and the residue was reconstituted with 100 μl of mobile phase. The resulting solutions were carefully vortexed for 30 s and centrifuged at 18 000 *g* for 3 min. The supernatants were transferred to 200-μl HPLC microvials (Agilent Technologies) and 80 μl were injected onto the column.

2.7. High-performance liquid chromatographic conditions

The chromatographic separation was performed with gradient elution. During the gradient the absorbance wavelength was set at 269 nm (0–11 min), 250 nm (11– 14 min), 271 nm (14–24 min) and 230 nm (24–33 min). A Polarity dC_{18} (150×3.9 mm, 5.0-μm particle size, Waters, Milford, MA, USA) analytical column, with a Polarity dC_{18} (20×3.9 mm, 5.0-μm particle size, Waters) guard column were used for separation.

The two mobile phase components were as follows. *Mobile Phase (A):* 10 m*M* ammonium acetate buffer (pH 6.5), pH adjusted with diluted acetic acid. This buffer solution was filtered through a 0.45-μm membrane filter (Millipore, Milford, MA, USA) before use; and *Mobile Phase (B):* 200 ml of mobile phase A (pH 6.5) were mixed with 500 ml of acetonitrile and 300 ml of methanol. A linear gradient was programmed as 4% mobile phase B over the first 15 min, then from 4 to 64% mobile phase B over next 15 min, followed by 3 min at 64% of mobile phase B, with re-equilibration over the final 7 min. The analysis was performed at 40 °C, with a mobile phase flow-rate of 1.1 ml/min.

2.8. Specificity and selectivity

Interference from endogenous compounds was investigated by analysis of six male and female blank plasma samples. Interference from 10 commonly used medications was also investigated. These included the protease inhibitors indinavir, amprenavir, saquinavir, nelfinavir, ritonavir and lopinavir, the non-nucleoside reverse transcriptase inhibitors delavirdine and efavirenz, the nucleotide analogue reverse transcriptase inhibitor tenofovir, and the synthetic lipid-lowering agent atorvastatin.

2.9. Limits of quantitation

The lower limit of quantitation (LLQ) was defined as the concentration for which both the relative standard deviation (RSD) and the percent deviation from the nominal concentration were less than 20%. The upper limit of quantitation (ULQ) defined as the concentration for which both the relative standard deviation and the percent deviation from the nominal concentration were less than 15% [26].

2.10. Stability

HIV-infected patient samples are routinely heated at 58 °C to inactivate the virus prior to handling. Heat deactivation studies were performed to verify the stability of all the drugs in plasma under these conditions. An additional stability test was performed to verify the stability of the drugs in the autosampler tubes while waiting for HPLC analysis. The samples were left at room temperature for 24 h prior to analysis. The stability during sample hand-

ling was also verified by subjecting samples to three freeze–thaw cycles, and storage for 7 days in the refrigerator at 4 °C prior to analyses. Quality controls (QC) samples at three concentrations (0.16, 0.8, and 4 μg/ml) were utilized in the stability test.

3. Results

3.1. Linearity

The peak area ratio values of the calibration standards were proportional to the concentration of each drug in plasma over the range tested. The calibration curves were fitted by unweighted least-squares linear regression. The mean±SD of three standard curve slopes for zalcitabine, lamivudine, didanosine, stavudine, zidovudine, abacavir and nevirapine were 0.7817±0.078, 1.0879±0.044, 1.6533±0.022, 1.3960±0.025, 1.2281±0.033, 1.8719±0.035and 2.1452±0.011, respectively. The concentration range was 10–10 000 ng/ml for all analytes except ddC (range of 10–5000 ng/ml). The regression coefficient (r^2) of all calibration curves was greater than 0.998.

Table 1
Retention time for seven analytes and internal standard

Drug name	Retention time (min)
Zalcitabine	5.9
Lamivudine	8.6
Didanosine	13.6
Stavudine	15.7
Zidovudine	23.8
Abacavir	25.1
Nevirapine	27.3
I.S.	30.6

3.2. Selectivity

Representative chromatograms of the blank plasma samples are illustrated in Fig. 1. The approximate retention times for all seven analytes and internal standard are listed in Table 1. No endogenous

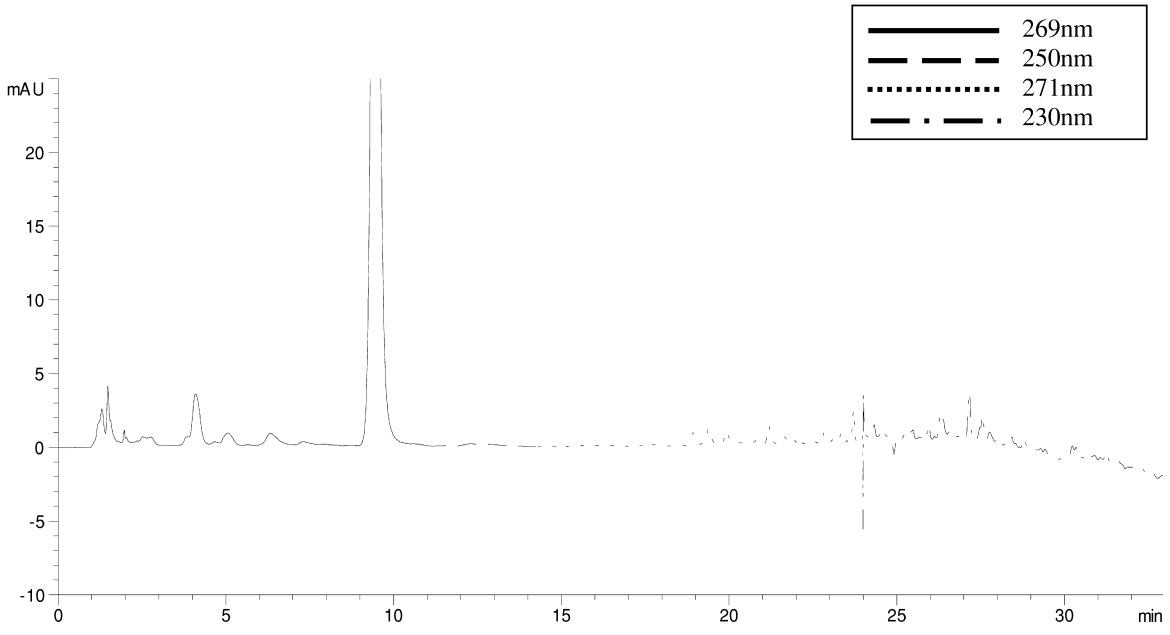


Fig. 1. Sample chromatogram of blank pooled human plasma extract.

substances interfered with any of the analytes in blank plasma extracts. Potentially coadministered drugs tested had retention times that were either very different from the compounds of interest, or were not detected with the described bioanalytical method.

3.3. The limit of quantification

All analytes were linear in the range of 10.0–10 000 ng/ml except for ddC, which was linear in the range of 10–5000 ng/ml. The lowest limit of quantification was determined to be 10.0 ng/ml for all analytes. Accuracy and precision at the lower and upper limits of quantitation are shown in Table 2. Chromatograms from the standard curves at concentrations of 10 and 5000 ng/ml are shown in Fig. 2.

3.4. Accuracy, precision

Results from the validation of this method in human plasma were acceptable. The accuracy of all analytes ranged from 94.8 to 109% with a mean of 103%. Precision and accuracy of the control samples at the lower and upper limit of quantitation are given in Table 2. Throughout the concentration range of the control samples, the mean intra-assay precision was always lower than 7%. Overall, the mean inter-day precision for each the seven analytes was similar, with mean RSDs ranging from 2.9 to 5.2%. The inter-assay deviation from the nominal concentration was always <10.4% and the range of inter-day deviation of the seven analytes was between −5.7 and +5.5%.

3.5. Recovery

The absolute recovery of NRTIs and nevirapine from plasma extracted with solid-phase SPE columns was calculated by comparing peak areas. This was calculated from the quality control samples as the ratio of the analyte concentration in human blood plasma undergoing solid-phase extraction to that of the identical concentrations of NRTIs and nevirapine prepared in mobile phase without extraction. This extraction method reliably eliminated interfering material from plasma, with good recovery for zalcitabine (>75.2%) lamivudine (>90.0%), didanosine (>98.1%), stavudine (>97.4%), zidovudine (>97.1%), abacavir (>96.8%) and nevirapine (>96.1%).

3.6. Stability

The NRTIs, NVP and internal standard were stable under all conditions tested, with all results falling within the acceptance criteria of ±15% deviation from the nominal concentration.

3.7. Analysis of patient samples

We examined the applicability of the described method by analyzing plasma samples collected from HIV-infected patients. Fig. 4 shows chromatograms from three of these patients. Fig. 4a is a patient

Table 2
Precision and accuracy at the upper and lower limits of quantitation

Compound	Lower limit of quantitation (LLQ)			Upper limit of quantitation (ULQ)		
	Conc. (ng/ml)	Accuracy (%)	Inter-assay precision (%)	Conc. (ng/ml)	Accuracy (%)	Inter-assay precision (%)
Zalcitabine	10	95.5	8.7	5000	92.9	5.6
Lamivudine	10	95.0	9.1	10 000	94.4	8.7
Didanosine	10	100	6.9	10 000	96.1	9.8
Stavudine	10	94.8	8.9	10 000	104	3.6
Zidovudine	10	102	3.5	10 000	102	9.2
Abacavir	10	101	7.6	10 000	90.5	7.0
Nevirapine	10	99.8	6.7	10 000	96.0	9.0

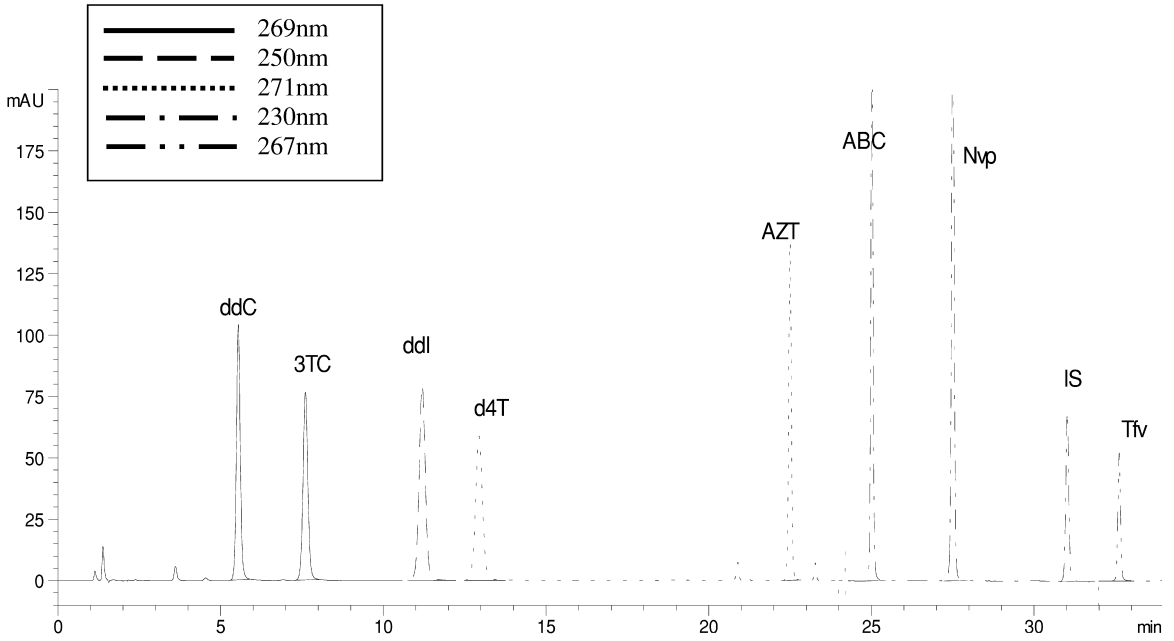


Fig. 2. Chromatogram of the assay analytes, tenofovir, and internal standard at 10 μg/ml in mobile phase (direct injection). ZDV, zidovudine; ddI, didanosine; ddC, zalcitabine; d4T, stavudine; 3TC, lamivudine; ABC, abacavir; NVP, nevirapine; I.S., internal standard; TFV, tenofovir.

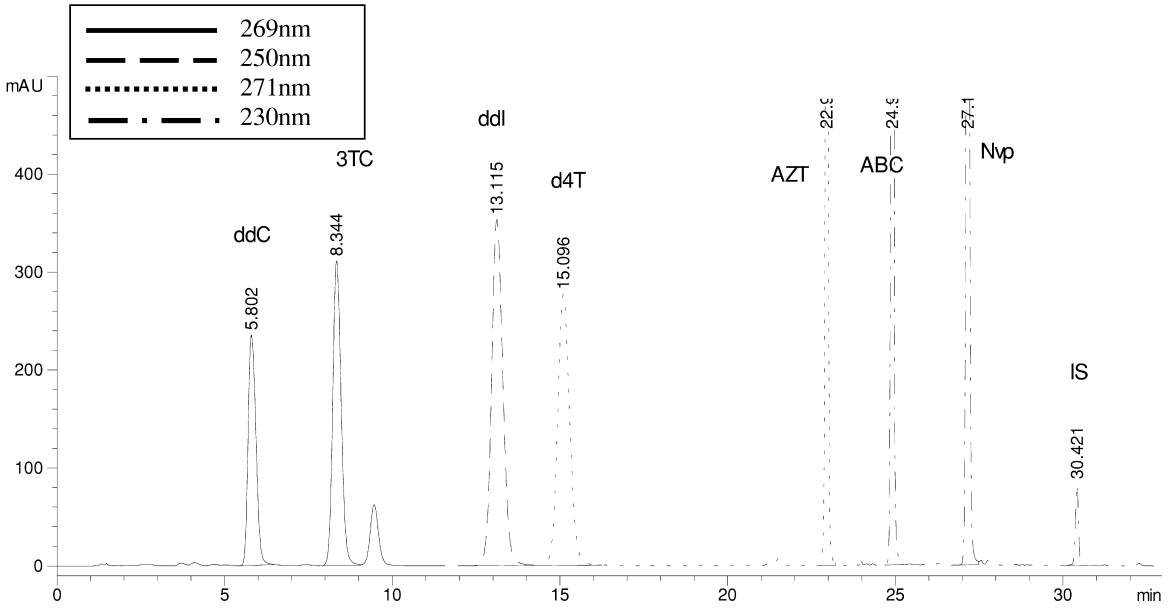
plasma sample containing 3TC and d4T, Fig. 4b contains ZDV, ABC, and NVP and Fig. 4c contains 3TC and ddI. Reproducibility of the analyzed samples was tested on different days (Table 3). We were unable to find any patients currently receiving ddC.

4. Discussion

At present, two HPLC–UV methods have been published for the simultaneous determination of multiple NRTIs [6,24]. However, the method of Aymard et al. [24] does not include zalcitabine or internal standard, and the method recently published by Simon et al. [6] does not include nevirapine or internal standard. A capillary electrochromatographic method for NRTIs was published by Mesplet et al. [27], but did not include abacavir or nevirapine.

Our optimized HPLC method provides a sensitive and accurate procedure for simultaneously determining the six NRTIs and nevirapine in the plasma of HIV-infected patients. Since many drug combinations in HIV therapy include two or more NRTIs with nevirapine, an analytical method is needed for these medications. This method relied on the optimization of the solid-phase extraction column, the analytical column, and the monitored UV wavelengths.

We tested 10 types of solid-phase extraction cartridges from Varian (C_2, C_8, C_{18}, and Nexus), Waters (Oasis-Max, Oasis-HLB, and Sep-Pak Vac C_{18}), Fisher (PrepSep C_{18}), and Agilent (Zorbax C_{18} EC and AccuBond C_{18}). We found that only two types of cartridges (Oasis-HLB from Waters and C_{18} from Varian) retained the seven analytes and yielded extraction efficiencies for ddC of greater than 60% (most other cartridges yielded <20% for ddC). For Oasis-HLB the extraction efficiency for ddC was> 80%, and for the other analytes was >95%. For the Varian C_{18} 50 mg column, the extraction efficiency for ddC was >60%, and for the other analytes was >88%. Although the Oasis column had better extraction efficiencies, the Varian column yielded a cleaner baseline. Once we switched from a Varian C_{18} 50-mg column to a 100-mg column, the extraction efficiency of ddC increased to >75%, 3TC to >90%, and the other analytes to >96%. The 200-mg Varian C_{18} cartridge was also tested, but was



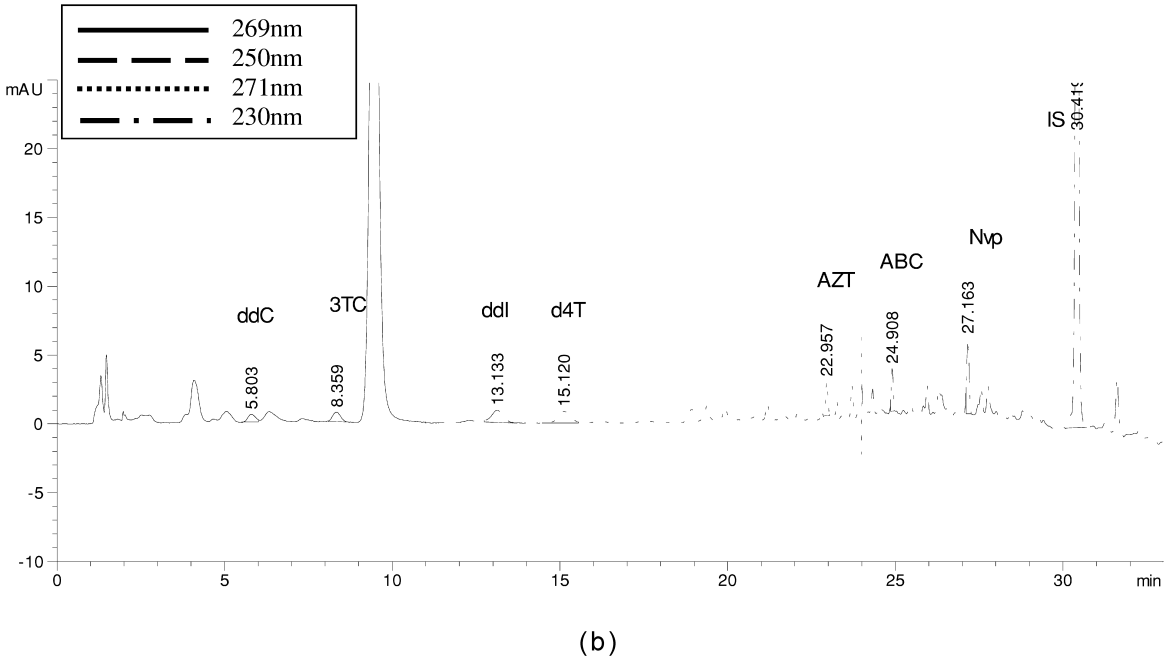


Fig. 3. (a) Chromatogram of calibrators at 10 ng/ml. (b) Chromatogram of calibrators at 5000 ng/ml. ZDV, zidovudine; ddI, didanosine; ddC, zalcitabine; d4T, stavudine; 3TC, lamivudine; ABC, abacavir; NVP, nevirapine; I.S., internal standard.

Table 3
Reproducibility of analyzing patient samples in different runs

Compound	First run (ng/ml)	Second run (ng/ml)	Variation (%)	RSD (%)
Lamivudine	664.3	644.6	97.0	2.1
Didanosine	74.0	76.0	103	1.9
Stavudine	362.8	341.6	106	5.3
Zidovudine	124.8	137.9	110	7.1
Abacavir	1208.5	1067.7	88.3	8.7
Nevirapine	5429.0	4898.0	90.2	7.3

found unsuitable due to some difficulty in drawing the sample through the solid-phase.

We also chose the Polarity column for this method, which is suitable for both polar and non-polar compounds. The silica-based C_{18} derivatized material was able to retain the cationic nucleosides ddC (pK_a=4.4) and 3TC (pK_a=4.4). The sulfur atom in 3TC could be involved in an additional hydrogen-bond interaction with the Polarity stationary phase leading to a greater retention for 3TC (t_R=8.6 min) than ddC (t_R=5.9 min). Resolution between ZDV (t_R=23.2 min) and ABC (t_R=25.1 min) improved by using 30% methanol in mobile phase B. The resolution of ddI (t_R=13.6 min) and d4T (t_R=15.7 min) is highly aqueous phase-dependent.

We originally developed this method using a Zorbax SB-C_{18} (150×4.6 mm, 3.5 μm) column and achieved peak resolution at 60 °C. However, the first eluting peaks had poor symmetry and plasma components interfered with the latter eluting peaks. Using the Polarity column with the same mobile phase composition and a pH of 6.5 improved the peak shape. Also, with Polarity we obtained better resolution at a lower temperature (40 °C), even though the number of theoretical plates of the Polarity column is less than that of the Zorbax SB-C_{18}. We also tested the Polarity column resolution at pH 8.0, but an unknown plasma peak eluting between ddC and 3TC interfered with the 3TC peak.

Recently, Stewart and Fan [22] combined zidovudine, lamivudine and nevirapine in one assay. In this method, all three analytes were measured at 265 nm. Simon et al. [6] measured all six NRTIs at 250 nm. Although Aymard et al. [24] listed each analyte's absorbance curve and λ_{max}, these investigators measured five NRTIs and nevirapine at the single 260-nm wavelength. Our method utilizes optimal absorption for each drug: ddC and 3TC are measured at 271 nm, ddI is measured at 250 nm, d4T and ZDV are measured at 269 nm, and ABC, nevirapine and I.S. (hexobarbital) are measured at 230 nm. These four different wavelengths make our method more specific and highly sensitive, and allow us to achieve the low 10 ng/ml detection limit for all analytes.

We also evaluated the potential of including tenofovir in this assay. Tenofovir is an acyclic nucleotide reverse transcriptase inhibitor, approved for use by the FDA in 2001. Due to its potency, once-daily dosing, and favorable resistance profile, it has become a popular choice in combination with other NRTIs. Although no optimal wavelength for tenofovir has previously been published, we monitored this compound at wavelength of 267 nm, and by direct injection, found a good peak at approximately 33 min (shown in the chromatogram in Fig. 2). Unfortunately, due to its water solubility, tenofovir was not retained on our SPE cartridge of choice, and could not be included in the validation of this assay.

5. Conclusion

The HPLC assay here described represents an accurate, precise, specific, and highly reproducible HPLC method for the direct measurement of seven ARV drugs in plasma. The solid-phase extraction method was optimized to concentrate the samples and provided excellent clean up. This method yields high recoveries, shows good linearity, precision and accuracy within a wide concentration range for each drug. The method is currently being used to analyze

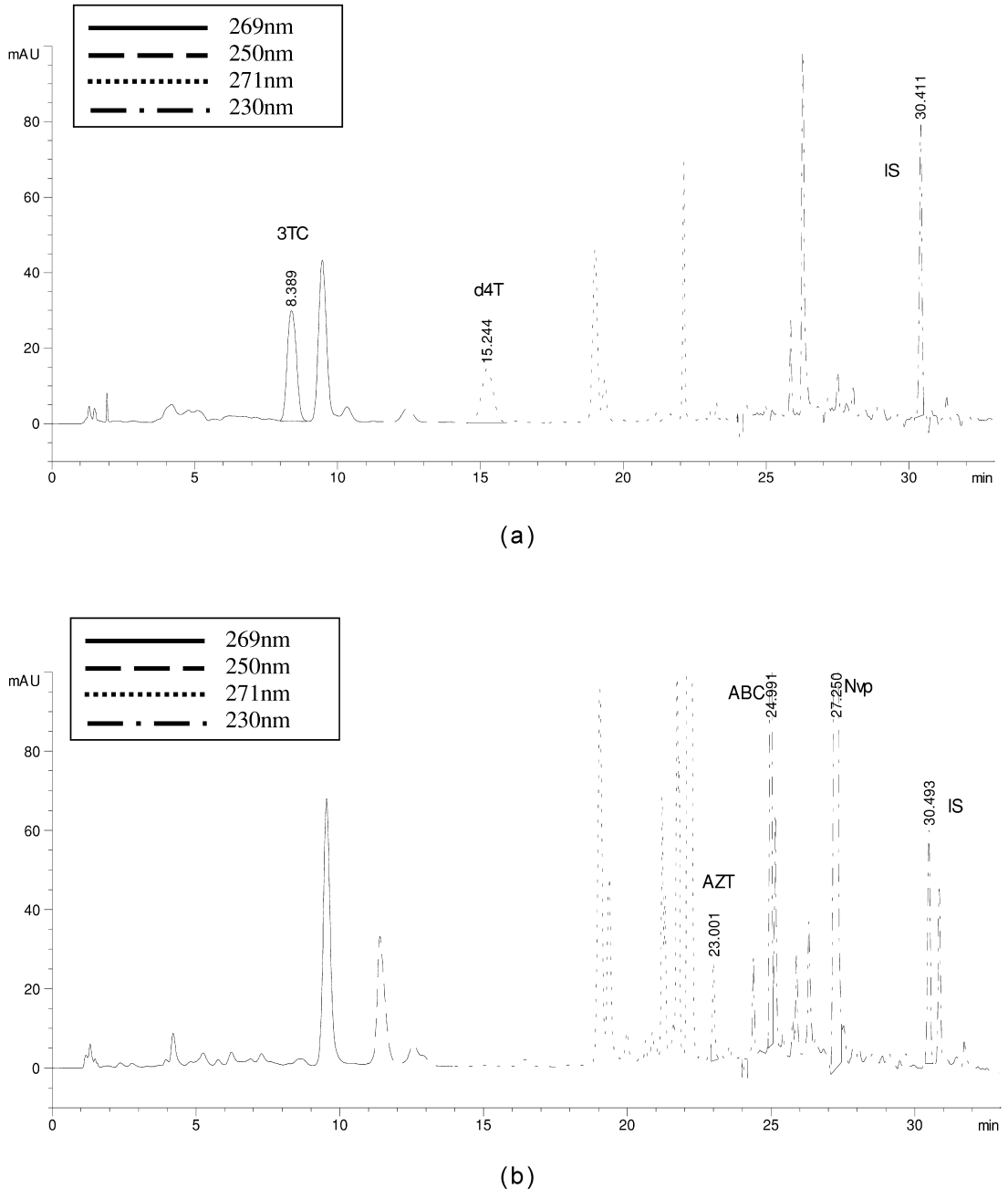
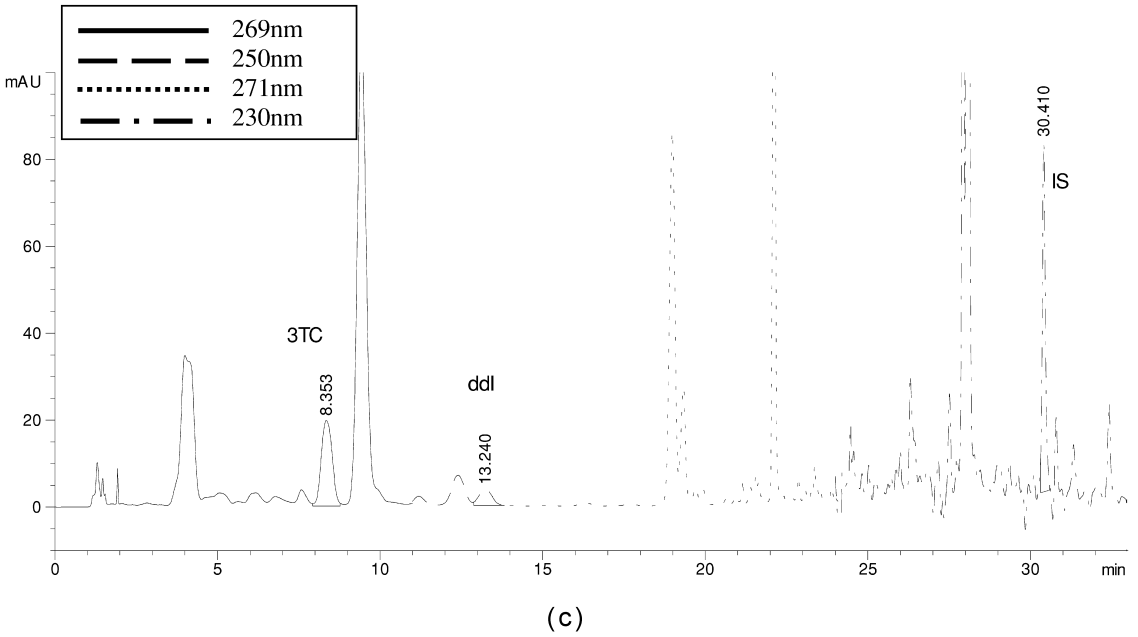


Fig. 4. (a) Chromatogram of a patient sample containing 3TC and d4T. The concentration of 3TC was 851 ng/ml, and of d4T was 315.3 ng/ml. (b) Chromatogram of a patient sample containing ZDV, ABC, and NVP. The concentration of ZDV was 124.8 ng/ml, of ABC was 1208.5 ng/ml, and of NVP was 5429.0 ng/ml. (c) Chromatogram of a patient sample containing 3TC and ddI. The concentration of 3TC was 423.1 ng/ml, and of ddI was 76.0 ng/ml.



(c)

Fig. 4. (*continued*)

samples of patients treated with combination therapy regimens.

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