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Simple and rapid quantification of the non-nucleoside reverse transcriptase inhibitors nevirapine, delavirdine, and efavirenz in human blood plasma using high-performance liquid chromatography with ultraviolet absorbance detection

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Abstract

A simple reversed-phase high-performance liquid chromatography assay for the simultaneous quantitative determination of three HIV non-nucleoside reverse transcriptase inhibitors (nevirapine, delavirdine, and efavirenz) in human blood plasma is described. The method was validated over the range of 10 ng/ml to 50 μg/ml for nevirapine, 25 ng/ml to 25 μg/ml for delavirdine, and 10 ng/ml to 10 μg/ml for efavirenz. The method is accurate (average accuracies over eight concentrations ranging from 87.3 to 113%), and precise (within-day and between-day precision measures ranging from 0.12 to 7.9% and 0.26 to 5.9%, respectively). All three non-nucleoside reverse transcriptase inhibitors proved to be stable under various conditions. Due to its simplicity, this assay can readily be used for investigational or clinical monitoring of plasma concentrations.

Keywords: Nevirapine; Delavirdine; Efavirenz

1. Introduction

Non-nucleoside reverse transcriptase inhibitors (NNRTIs) are one of three classes of drugs currently approved to treat human immunodeficiency virus (HIV) infection. This drug class, composed of structurally diverse compounds, inhibits the replication of HIV by noncompetatively binding directly to the reverse transcriptase enzyme and interfering with viral RNA to DNA-directed polymerase activities [1]. Currently, three NNRTIs are approved for use in the treatment of the treatment of HIV-1 infection: delavirdine, nevirapine, and efavirenz.

There is increasing evidence that virologic treatment failure is, at least in part, correlated with interindividual variations in the pharmacokinetic parameters of drugs [2,3]. As with protease inhibitors (PIs), plasma concentrations of non-nucleoside reverse transcriptase inhibitors may be of value in predicting efficacy and side effects [4].

Additionally, using therapy that combines NNRTIs and PIs is now being investigated, particularly in those patients who have experienced

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multiple prior courses of therapy, due to non-overlapping resistance profiles. However, in these combinations, the potential for pharmacokinetic drug interactions is significant, particularly between drugs metabolized by, and influencing the activity of, the cytochrome P450 enzyme system [5]. Understanding the pharmacokinetic consequences of combination therapy with NNRTIs is important.

To date, published analytical methods for measuring nevirapine, delavirdine and efavirenz use reversed-phase high-performance liquid chromatography (RP-HPLC) with UV detection [6,7,13–19], or for measuring delavirdine use RP with fluorescence detection [8]. However, no individual method for simultaneously assaying all three NNRTIs has been published.

We report here the development and validation of a high-performance liquid chromatographic assay with UV detection for the quantitative determination of NNRTIs in human blood plasma. The present assay is useful for pharmacokinetic research and therapeutic drug monitoring in HIV-1 infected patients treated with one or more NNRTIs.

2. Experimental

2.1. Chemicals

Nevirapine (Virammune, Roxane Labs., Columbus, OH, USA), delavirdine (Rescriptor, Pharmacia & Upjohn, Kalamazoo, MI, USA), and efavirenz (Sustiva, DuPont Pharmaceuticals, Wilmington, DE, USA) were prepared from pharmaceutical preparations. Hexobarbital (Sigma–Aldrich, Steinheim, Germany) was used as the internal standard for this assay. 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 HPLC system consisting of an Agilent Technologies (Wilmington, DE, USA) Model HP1100 binary pump, a HP1100 degasser, a HP1100 autosampler, a HP1100 UV-diode array detection (DAD) system, 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 all drugs were prepared in methanol at a concentration of 10 mg/ml. Each of these stock solutions was used for preparing a combination stock solution (1.0 ml from each drug stock solution was combined, and the mixture diluted to 10 ml with HPLC-grade water) containing nevirapine, delavirdine, and efavirenz at concentration of 1.0 mg/ml. An intermediate pooled human plasma stock solution was prepared at a concentration of 100 μg/ml (3% methanol content). Serial dilutions of this intermediate plasma stock solution were also performed with pooled human plasma. This plasma was devoid of any drugs, and obtained from whole blood anticoagulated with sodium EDTA (Biological Speciality, PA, USA). Eight to ten calibration standards defined the standard curve, spanning the concentration range from 0.01 to 50 μg/ml for each drug (each calibration standard contained $<$1.5% methanol). A second pooled human plasma intermediate stock solution of the three compounds was used for the preparation of quality control (QC) standards in plasma. The quality controls were prepared at three concentrations: 0.2, 2.0, and 20 μg/ml.

Solutions of potential drugs of interference (primarily protease inhibitors and nucleoside analogue reverse transcriptase inhibitors) were prepared from either intravenous or oral (after dissolution and filtering) pharmaceutical preparations. 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 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 100 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 was heated for 60 min at 58 °C to inactivate the HIV virus.

2.6. Solid-phase extraction (SPE) method

SPE AcuuBond columns (1.0 ml, 100 mg ODS; J&W Scientific, Folsom, 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 100 m*M* ammonium acetate (pH 7.4). With the vacuum off, 500 μl of 100 m*M* ammonium acetate buffer (pH 7.0) containing the internal standard (hexobarbital 2 μg/ml) was added, followed by the 500 μl plasma sample. Both I.S. and sample were allowed to pass through the column bed with minimal suction. The column was further washed with 1.0 ml of 100 m*M* ammonium acetate buffer (pH 7.4), and the bed was suctioned dry for 1 min. The drugs were then eluted with 600 μl of methanol. The eluent was evaporated to dryness under a nitrogen stream at 40 °C, and the residue was reconstituted with 100 μl mobile phase. The resulting solution was carefully vortexed for 30 s and centrifuged at 18 000 *g* for 3 min. The supernatant was transferred to 200-μl HPLC microvials (Agilent Technologies) and 75 μl 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 220 nm (0–14 min), 224 nm (14–20 min) and 248 nm (20–28 min).

An Eclipse XDB C_8 (150×4.6 mm, 5.0 μm particle size) analytical column, with an SB C_{18} guard column (5.0 μm particle size) was used.

The two mobile phase components were as follows: mobile phase A: 50 m*M* sodium phosphate buffer (pH 4.8). The 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 was mixed with 800 ml of acetonitrile. A linear gradient was programmed from 16 to 76% mobile phase B over 24 min followed by re-equilibration over the final 4 min. The analysis was performed at 35 °C, with a mobile phase flow-rate of 1.5 ml/min.

2.8. Specificity and selectivity

Interference from endogenous compounds was investigated by analysis of male and female blank plasma samples. Interference from 12 commonly used medications was also investigated, and included the protease inhibitors indinavir, amprenavir, saquinavir, nelfinavir, and ritonavir, the nucleoside analogue reverse transcriptase inhibitors stavudine, zalcitabine, zidovudine, lamivudine, abacavir, and didanosine, and the synthetic lipid-lowering agent atorvastatin (lipitor).

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) was defined as the concentration for which both the RSD and the percent deviation from the nominal concentration were less than 15% [9].

2.10. Stability

HIV-infected patient samples are routinely heated at 60 °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 handling was also verified, by subjecting samples to three freeze–thaw cycles, and storage for 7 days in the refrigerator at 4 °C prior to analysis.

QC samples at three concentrations (0.2, 2.0, and 20 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 linear least-squares regression for nevirapine, delavirdine and efavirenz, and showed coefficient of determination greater than

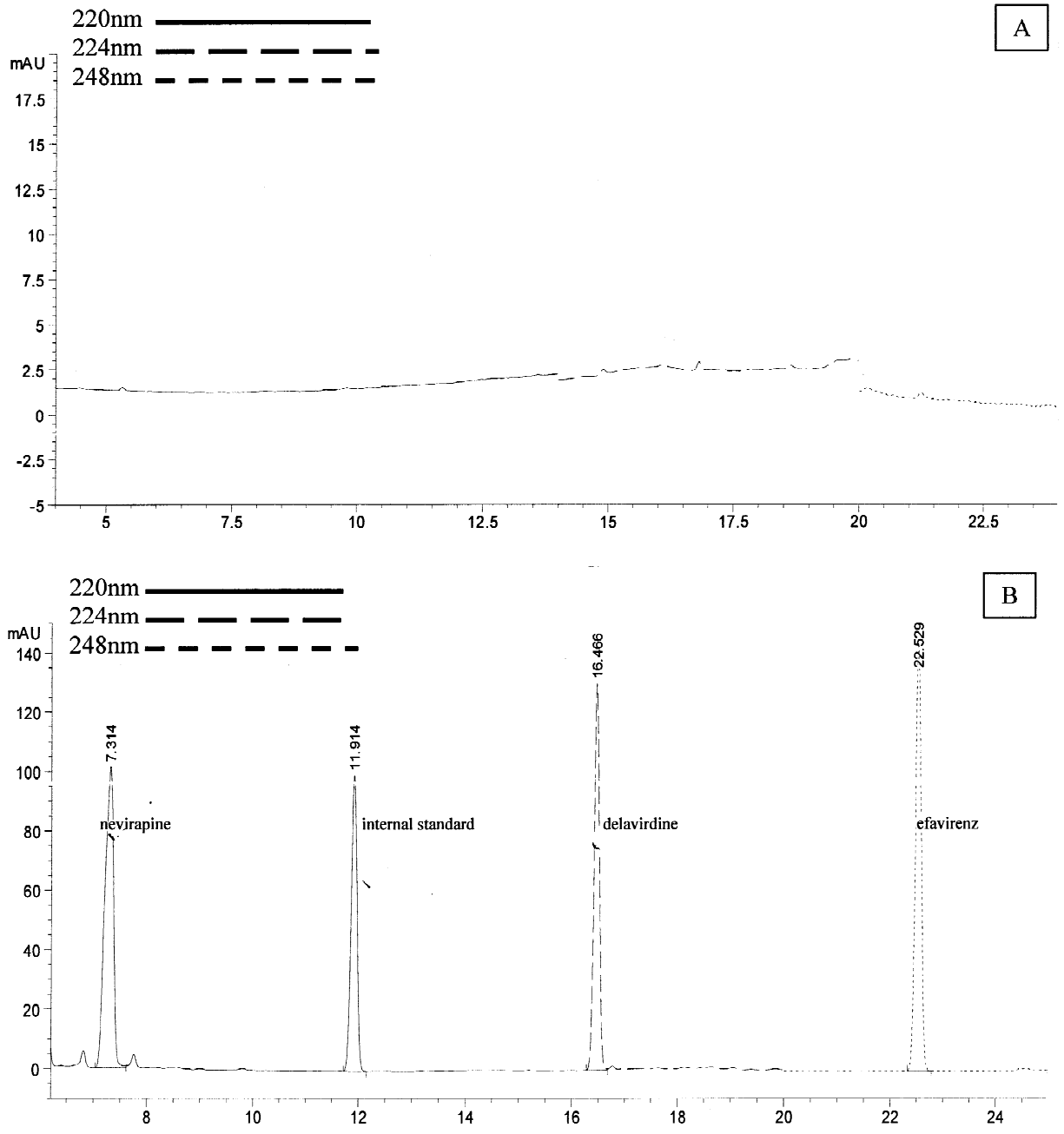

Fig. 1. (a) Sample chromatogram of blank pooled human plasma extract. (b) Sample chromatogram of an extracted plasma sample spiked with 1.0 μg/ml of nevirapine, 2.0 μg/ml of I.S., 1.0 μg/ml of delavirdine and 1.0 μg/ml of efavirenz.

Table 1
Retention times for drugs commonly prescribed to HIV-infected patients

Drug name	Retention time (min)
Abacavir	3.7
Amprenavir	16.6
Atorvastatin	18.9
Didanosine	1.4
Indinavir	13.7
Lamivudine	1.5
Nefinavir	20.3
Ritonavir	21.9
Saquinavir	16.8
Stavudine	1.8
Zalcitabine	1.2
Zidovudine	3.8

0.999 for all NNRTIs. The mean±SD of three standard curve slopes for nevirapine, delavirdine and efavirenz were 0.6857±0.0006, 0.5439±0.002, and 0.6845±0.0048, respectively.

3.2. Selectivity

Representative chromatograms of blank and spiked plasma samples are illustrated in Fig. 1. The approximate retention times for nevirapine, internal standard, delavirdine, and efavirenz were 7.31, 11.91, 16.47 and 22.53 min, respectively. No endogenous 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. The retention time of each individual compound is listed in Table 1.

3.3. The limit of quantification

Nevirapine was linear to 50 000 ng/ml, delavirdine to 25 000 ng/ml, and efavirenz to 10 000 ng/

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

Compound	Lower limit of quantitation (LLQ)			Upper limit of quantitation (ULQ)		
	Concentration (ng/ml)	Accuracy (%)	Inter-assay precision (%)	Concentration (ng/ml)	Accuracy (%)	Inter-assay precision (%)
Nevirapine	10	112	3.2	50 000	97.2	5.3
Delavirdine	25	113	1.2	25 000	95.4	5.3
Efavirenz	10	109	3.1	10 000	95.3	5.9

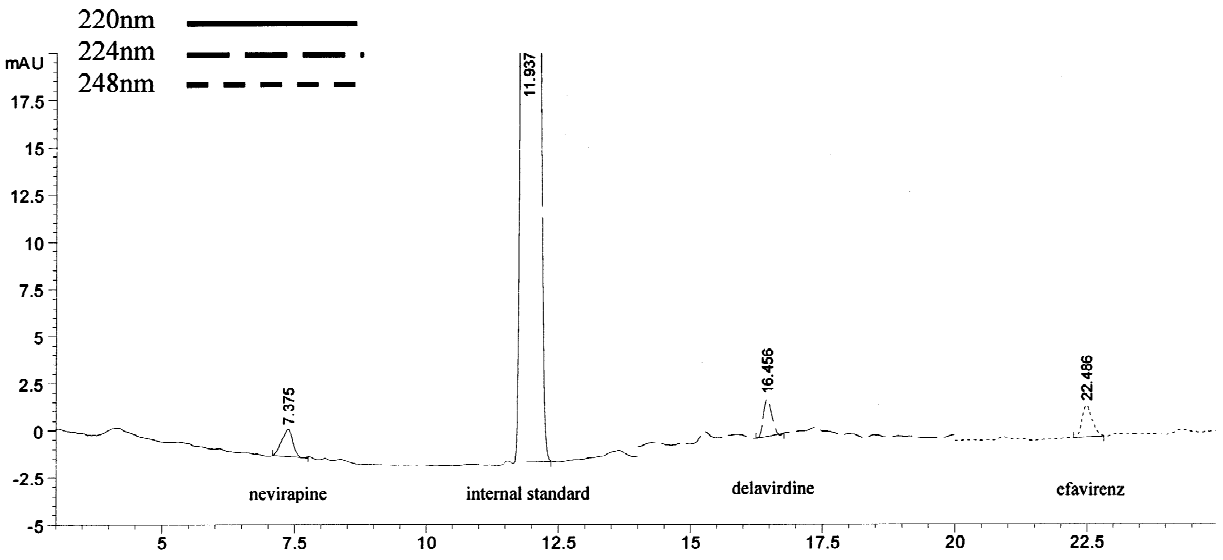

Fig. 2. Chromatogram of an extracted calibration standard at 25 ng/ml.

ml. The lowest limit of quantification was determined to be 10.0 ng/ml for nevirapine, 25 ng/ml for delavirdine and 10 ng/ml for efavirenz. Accuracy and precision at the lower and upper limits of quantitation are presented in Table 2. A chromatogram from one of the curve standards at a concentration of 25 ng/ml is shown in Fig. 2.

3.4. Accuracy, precision

The results from the validation of this method in human plasma are listed in Table 3. The accuracy of nevirapine ranged from 87.3 to 107% with a mean of 101%. The accuracy of delavirdine ranged from 95.1 to 113%, with a mean of 102%. The accuracy of efavirenz ranged from 95.4 to 109%, with a mean of 101%.

3.5. Recovery

The absolute recovery of NNRTIs from plasma extracted with SPE columns was calculated by comparing the peak areas. The ratio of three concentrations of NNRTIs in human blood plasma quality control samples that underwent solid phase extraction to that of identical concentrations of NNRTIs prepared in mobile phase without extraction. This method reliably eliminated interfering material from plasma, with good recovery for nevirapine (>98.0%), delavirdine (>88.2%), and efavirenz (>99.6%).

Table 3
Accuracy and precision for the analysis of nevirapine, delavirdine, and efavirenz in human blood plasma (n=6)

Compound	Concentration (ng/ml)	Accuracy		Precision (%)	
		%	RSD (%)	Between-day (n=3)	Within-day (n=2)
Nevirapine	10	112	1.71	3.2	7.4
	25	108	3.33	1.8	4.7
	50	110	6.16	2.8	0.12
	100	98.4	8.70	4.5	0.21
	500	98.5	8.31	0.38	2.7
	1000	103	6.89	0.46	2.5
	5000	97.7	8.41	0.49	1.4
	10 000	99.0	8.03	2.8	1.3
	25 000	87.3	0.74	1.5	1.9
	50 000	96.7	4.67	5.3	0.68
Delavirdine	25	113	2.35	1.2	7.9
	50	112	1.78	0.73	1.8
	100	101	2.19	3.6	2.5
	500	101	5.99	2.5	3.3
	1000	98.8	5.61	2.2	1.5
	5000	97.2	4.56	5.1	5.2
	10 000	95.6	7.77	2.7	7.5
	25 000	95.1	6.25	5.3	3.5
Efavirenz	10	109	8.03	3.1	2.5
	25	106	2.69	4.0	2.3
	50	102	4.19	2.4	4.0
	100	100	5.66	4.2	2.5
	500	98.5	3.99	0.26	0.33
	1000	97.7	5.04	0.48	5.7
	5000	96.7	7.59	3.7	5.4
	10 000	95.4	6.66	5.9	7.1

Table 4
Stability of nevirapine, delavirdine, and efavirenz in spiked human blood plasma ($n=3$)

Compound	Concentration (ng/ml)	1.0 h at 60 °C	24 h at at 25 °C	7 days at 4.0 °C	Three freeze–thaw cycles
Nevirapine	20 000	97.4±1.85	99.8±0.11	101±0.92	102±2.26
	2000	98.9±0.80	96.4±2.58	98.6±0.99	98.6±2.58
	200	101±1.10	99.5±0.34	102±2.26	99.7±0.34
Delavirdine	20 000	98.9±4.70	102±4.79	97.4±1.67	102±6.12
	2000	99.2±0.59	99.4±0.28	98.2±2.21	98.2±6.62
	200	101±4.70	97.5±3.81	102±7.73	95.6±5.00
Efavirenz	20 000	98.0±3.47	101±6.21	96.5±4.47	104±2.88
	2000	97.7±1.61	97.3±4.96	99.7±6.54	106±0.80
	200	95.2±1.40	98.0±2.11	101±6.31	95.2±2.24

All values are represented as the mean±SD of the percent of initial concentration. All samples were performed in triplicate.

3.6. Stability

The results of NNRTI stability under various conditions are shown in Table 4. Under all conditions tested, the three drugs proved to be stable. Concentrations measured at the end of the test period were at least 95.2% of the initial concentrations.

3.7. Analysis of patient samples

We examined the applicability of the described method by analyzing efavirenz in plasma samples collected from HIV-infected patients at different times after the administration of an oral 600 mg dose. Blood samples were taken at 0, 1, 2, 3, 4, 5, 6, 8, 12, 18, and 24 h after administration of the dose. Fig. 3a illustrates a chromatogram from one of these patients, also receiving lamivudine, stavudine and valacyclovir. Several sets of patient samples were also analyzed by an independent contract laboratory (Covance Bioanalytical Laboratory Service, Indianapolis, IN, USA) under Good Laboratory Practice (GLP) conditions (proprietary method). In Table 5, we report the results for one sample patient and compare them to the data obtained from the GLP laboratory. The difference between the two methods was <9% for all 11 samples.

Additionally, since we had no patients simultaneously receiving more than one NNRTI, Fig. 3b and c are chromatograms of nevirapine and delavirdine extracted from the plasma of two different patients.

Table 5
Cross-validation of the method with efavirenz

Sample No.	Time post-dose (h)	Current method (A)	Independent method (B)	A/B
1	0	1015.40	963.48	1.05
2	1	1096.43	1056.05	1.04
3	2	3298.17	3560.80	0.93
4	3	2738.89	2905.66	0.94
5	4	2295.87	2516.21	0.91
6	5	2058.17	2216.90	0.93
7	6	2008.02	2075.46	0.97
8	8	1624.60	1690.61	0.96
9	12	1279.76	1279.71	1.00
10	18	1003.02	1051.03	0.95
11	24	951.11	1005.76	0.95

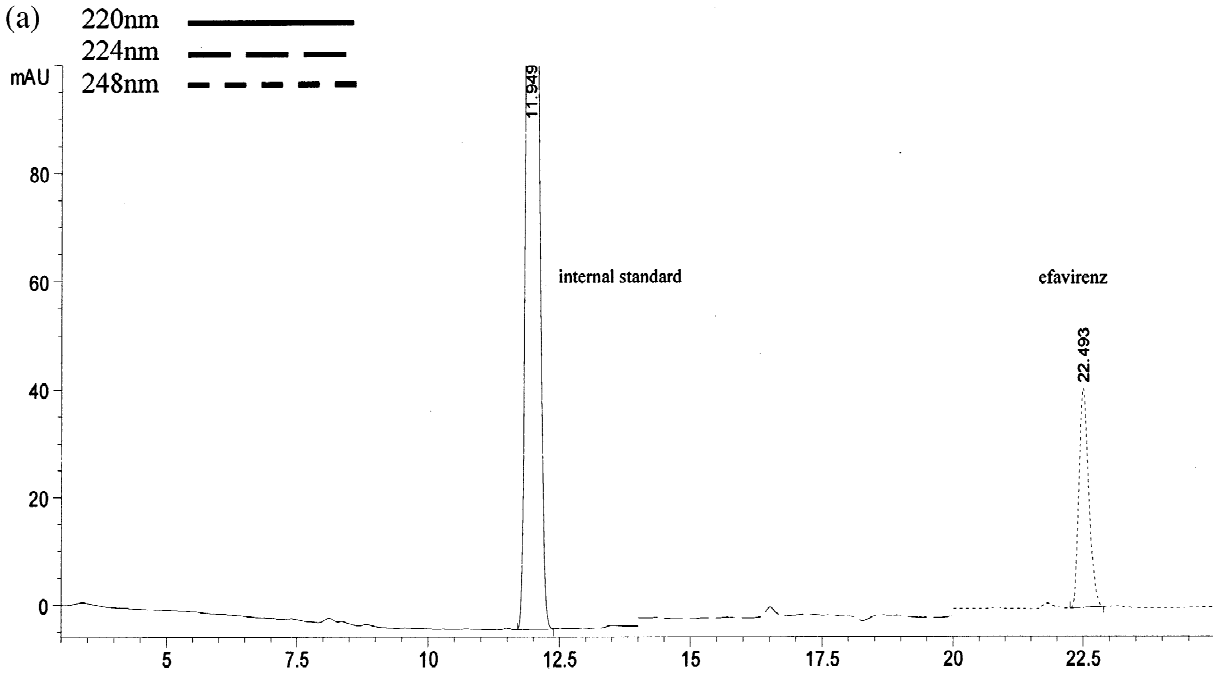

Fig. 3. Sample chromatograms of patient samples containing (a) efavirenz, (b) nevirapine, and (c) delavirdine.

4. Discussion

The published analytical methods to date are either for single NNRTIs, or single NNRTIs in combination with PIs [10–12]. No published method combines all three NNRTIs in one assay. This optimized HPLC method provides a simple procedure for simultaneously determining nevirapine, delavirdine, and efavirenz in the plasma of HIV-infected patients. This method is applicable to any simple binary HPLC system as it uses a standard mobile phase and a simple gradient elution. In addition, the preparation of standards, QCs, and patient samples is straightforward.

After testing four types of SPE cartridges (C-2 and Nexus from Varian, Oasis from Waters and AccuBond from Agilent), we selected AccuBond (ODS, 100 mg, 1.0 ml) to use with our method. The three other columns yielded extraction efficiencies less than 65% for nevirapine. However, adjusting the extraction conditions and using the AccuBond columns resulted in a clean sample baseline with extraction efficiencies greater than 88.0% for all analytes. Adding an I.S. standard to a large volume of acetate buffer, and subsequently adding the plasma sample inside the solid-phase extraction cartridge increased the accuracy and reproducibility of the method. In addition, the acetate buffer removed the plasma components from the column, and was found to be a better wash solution than water. The retained analytes of interest were successfully eluted clean, and improved the chromatogram baseline, peak shape, and specificity. Monitoring each analyte at a specific wavelength further increased the sensitivity and specificity of the method, and allowed us to quantitate NNRTIs at the lowest concentration possible. Furthermore, the simplicity of this method reduces the sample preparation and analysis time. With only 0.5 ml of plasma, we were able to achieve low quantifiable concentrations.

5. Conclusion

This report describes an accurate, precise, specific, and highly reproducible HPLC method for the direct measurement of nevirapine, delavirdine and

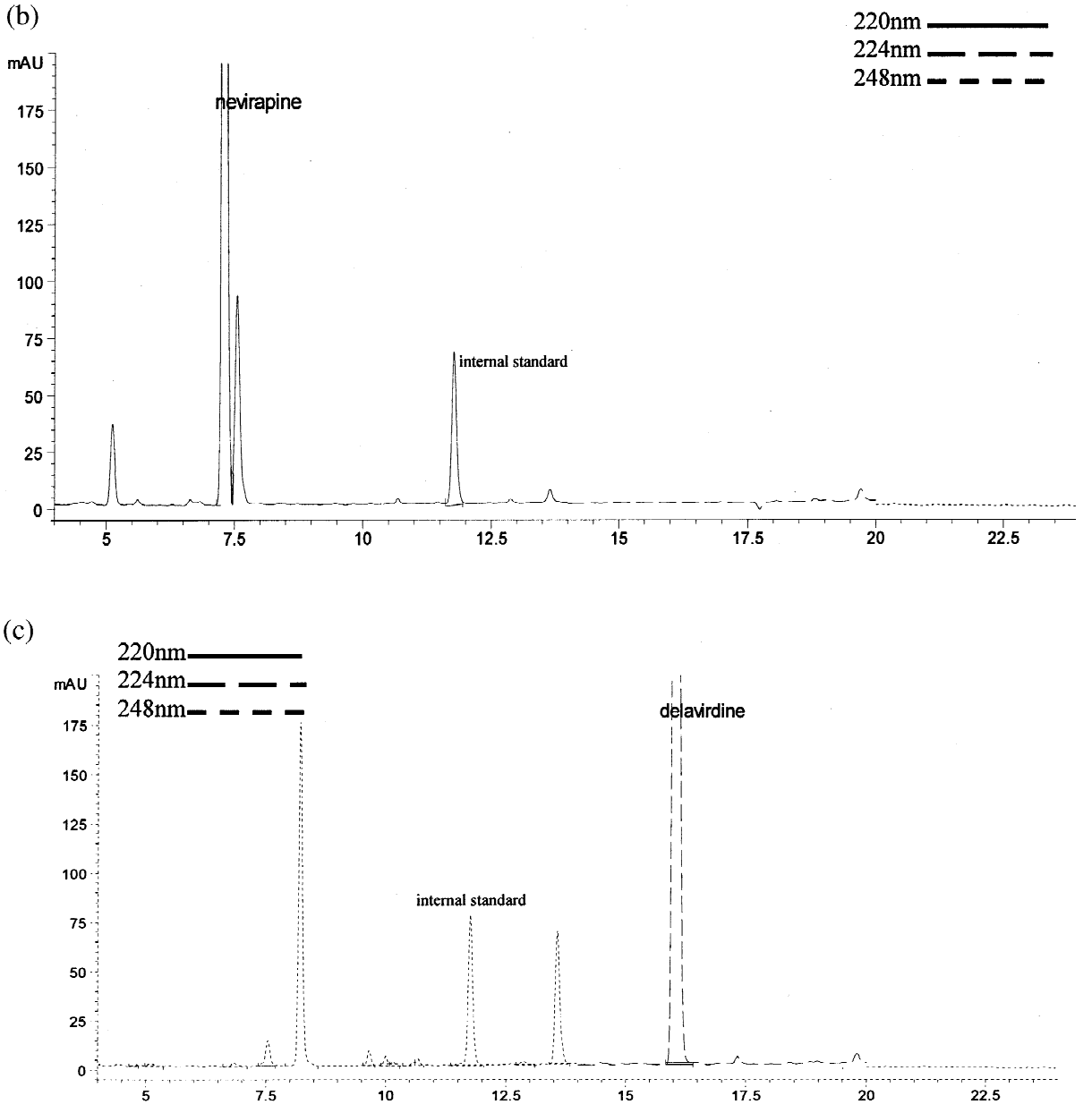

Fig. 3. (*continued*)

efavirenz in human plasma. A rapid, simple gradient system with an uncomplicated mobile phase is employed. The assay spans the concentration range of clinical interest, and is currently being used to analyze samples from HIV-1 infected patients.

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