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Simultaneous determination of nine antiretroviral compounds in human plasma using liquid chromatography

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Abstract

A rapid, sensitive and specific high-performance liquid chromatographic (HPLC) method using UV detection was developed for the determination of nine antiretroviral compounds commonly found in plasma from patients receiving antiretroviral therapy. Analytes include indinavir, saquinavir, ritonavir, amprenavir, lopinavir, delavirdine, efavirenz, nelfinavir and its M8 metabolite. Analytes were isolated from plasma using *tert*.-butyl methyl ether and separation achieved via reversed-phase liquid chromatography on a C_8 column with a gradient mobile phase. Detection at 210 nm provided adequate sensitivity. Limit of quantification is 50 ng/ml and all analytes demonstrated linearity across 50–10 000 ng/ml from a single 200-μl plasma sample. Recovery from plasma was consistently high (>80%). This novel HPLC methodology allows us to simultaneously determine plasma concentrations of nine antiretrovirals, including lopinavir, in HIV-infected patients on a single HPLC system.

Keywords: Antiretroviral compounds

1. Introduction

Since approval of zidovudine (ZDV) in 1987, the number of drugs available for treatment of HIV has increased dramatically, and new mechanisms of antiretroviral action have been discovered [1,2]. Nucleoside reverse transcriptase inhibitors (NRTIs) prevent ongoing viral DNA synthesis by being incorporated into the DNA nucleoside chain being produced by reverse transcriptase and stopping attachment of further nucleosides [3]. Non-nucleoside reverse transcriptase inhibitors (NNRTI) bind to reverse transcriptase making it unable to function by restricting its mobility. Protease inhibitors (PI) prevent proteolytic cleavage of large precursor polyproteins rendering the new virion non-infectious [4]. Among these three antiretroviral classes, clinicians and patients now have 16 different chemical agents available for HIV pharmacotherapy.

Standard treatment for HIV infection, commonly termed highly active antiretroviral therapy (HAART), includes at least three drugs from two different classes. Maintenance of an undetectable viral load is the goal of antiretroviral therapy and is necessary to prevent emergence of a resistant virus. In spite of increased therapeutic options, only 40–

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50% of patients in a clinical setting achieve this goal [5,6]. Possible explanations for treatment failure include poor regimen adherence and inadequate drug exposure. Recently, attention has focused on the importance of plasma concentrations of antiretrovirals and the role that inadequate drug concentrations may play in treatment failure. Concentration–response relationships have been noted with various antiretrovirals [7]. Drug concentrations below the expected target range have been associated with decreased virologic response and increased probability for emergence of resistance isolates. Monitoring plasma concentrations, or therapeutic drug monitoring (TDM), has been shown in a limited number of studies to improve efficacy and decrease toxicity [8]. The possibility of incorporating TDM into the clinical care of patients receiving antiretrovirals results in the need for rapid, sensitive, and precise assays to determine drug concentrations. This study describes an HPLC methodology with ultraviolet (UV) detection for simultaneous quantification of all currently available PIs plus the NNRTIs efavirenz (EFV) and delavirdine (DLV).

2. Experimental

2.1. Reagents and materials

Drug standards (Fig. 1) were gratefully obtained from pharmaceutical companies as follows: indinavir (IDV), as sulfate salt, from Merck (Albany, GA, USA); amprenavir (APV) from Vertex Pharmaceuticals Inc. (Cambridge, MA, USA); saquinavir (SQV) mesylate from Roche Discovery (Hertfordshire, UK); efavirenz from DuPont Pharmaceuticals (Wilmington, DE, USA); ritonavir (RTV), lopinavir (LPV) and internal standard (IntStd, A-86093) from Abbott Laboratories (Abbott Park, IL, USA) and nelfinavir (NFV) and M8 metabolite from Agouron Pharmaceuticals (San Diego, CA, USA). Delavirdine mesylate was purchased from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA, USA). HPLC grade methanol, acetonitrile, *tert.*-butyl methyl ether, *o*-phosphoric acid (85%), and potassium dihydrogen phosphate were purchased from Fisher (Pittsburgh, PA, USA). Water was double distilled in glass on-site. Human K-EDTA plasma

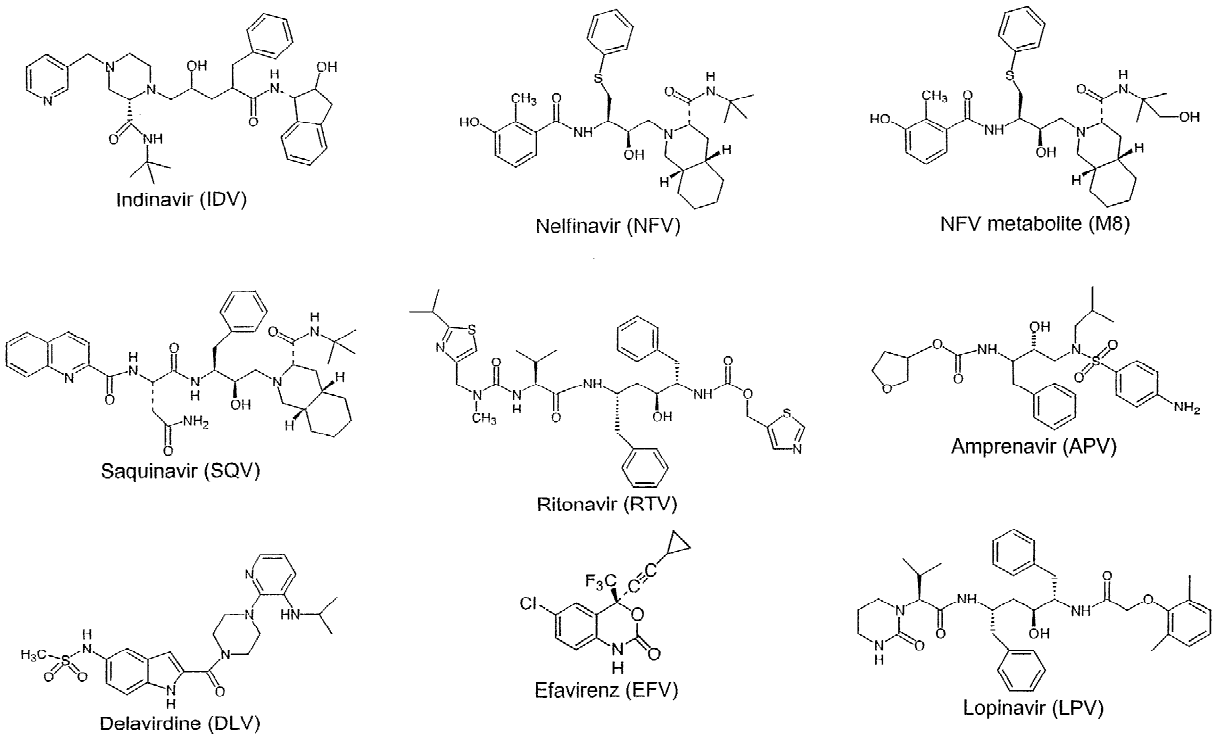

Fig. 1. Chemical structures of protease inhibitor and non-nucleoside reverse transcriptase inhibitors.

was purchased from Biological Specialty Corporation (Colmar, PA, USA). Polypropylene micro-centrifuge tubes were purchased from Fisher.

2.2. Preparation of solutions and standards

Individual master stock solutions of IDV, APV, SQV, DLV, RTV, LPV, EFV, NFV and M8 were prepared at 500 μg/ml concentrations by weighing drug powder into separate volumetric flasks and dissolving each to volume with methanol. Master stock solutions were prepared to reflect free-base concentration of each drug analyte in solution, regardless of the raw material used. Duplicate master stocks were prepared for each analyte. A 50 000 ng/ml solution was prepared for preparation of calibration curve standards by mixing a 1-ml aliquot from one master stock solution per analyte in a 10-ml volumetric flask, followed by dilution to volume with H_2O. This 50 000 ng/ml solution was further diluted to 5000 ng/ml. Calibration standards covering the concentration range from 50 to 10 000 ng/ml were prepared by adding appropriate volumes of these diluted solutions to drug-free, K-EDTA human plasma. Six calibration concentrations were used to define the standard curve (50, 100, 500, 1000, 5000 and 10 000 ng/ml). The duplicate set of master stock solutions was used for the preparation of a second 50 000 ng/ml solution for preparation of quality control (QC) standards (200, 2000 and 8000 ng/ml) in drug-free K-EDTA human plasma. Typically, 10 ml of each calibration standard and 25 ml of each QC standard were prepared and divided into multiple tubes for storage at −30 °C until use. A second set of QC standards was prepared in Na-heparin plasma for evaluation of matrix additive equivalence. A master stock solution of internal standard was prepared at 100 μg/ml in methanol and was diluted to 10 μg/ml in 50% methanol for use during sample preparation.

2.3. Assay procedure

Individual 200 μl plasma samples (e.g. blank plasma, calibration standard, QC standard) were transferred into separate 1.5-ml polypropylene micro-centrifuge tubes. Internal standard was added to each tube and the samples were briefly vortex mixed. One milliliter of the extraction solvent, *tert*.-butyl methyl ether, was added to each tube and the samples were capped. The tubes were vortex mixed for 20–30 min and then centrifuged to separate the aqueous and organic layers. The samples were placed in an ultra-low temperature freezer (−80 °C). When the aqueous layer was frozen, the organic layer was decanted into clean polypropylene micro-centrifuge tubes and left overnight on the laboratory bench to evaporate. Dried residues were re-suspended in mobile phase, transferred to injector vials and injected onto the HPLC system as described below.

2.4. Instrumentation and HPLC conditions

The HPLC consisted of a Waters (Milford, MA, USA) Alliance liquid chromatography system, including a Model 2695 Separations Module and a Model 2487 Dual Wavelength UV detector. Reversed-phase liquid chromatography was carried out at 27 °C using a Supelguard™ Discovery® C_8 analytical column, 5 μm, 25 cm×4.6 mm I.D. (Supelco Inc., Bellefonte, PA, USA). A Discovery C_8 (2 cm×4 mm I.D., 5 μm) in-line guard column was used to extend the life of the analytical column. The ultraviolet detector was set to monitor the 210 nm wavelength. Separation was facilitated via gradient elution at 1.5 ml/min flow-rate. The mobile phase consisted of (A) 25 m*M* potassium phosphate buffer, pH 3.1; (B) acetonitrile and (C) methanol according to the following program, with linear adjustments in a 40-min run time:

Time		% A	% B	% C
Initial		55	25	20
At min	15	55	40	5
At min	16	55	45	0
At min	29	55	45	0
At min	30	55	25	20

2.5. Calibration and calculation

Within each daily validation assay, a set of six calibration standards containing all drug analytes was processed together with six replicates from each of three QC concentration levels. Standard curve parameters were obtained from a weighted ($1/\text{concentration}^2$) least-squares linear regression analysis of the calibration standard concentrations (50–10 000 ng/ml) versus the drug-to-internal standard peak height ratio. The actual concentration of the standards (calibration and QC) was then interpolated using these standard curve parameters and was compared to expected concentrations. The assay procedure was subjected to a complete validation, which included six complete and separate assays. Each daily assay was subjected to pre-defined specifications for acceptance. A full stability investigation was also completed to assess the stability of each individual analyte following heat deactivation of HIV, exposure to room temperature conditions for 8.75 days and multiple freeze/thaw cycles. Specificity studies included demonstration of selectivity from endogenous matrix components and other potential concomitant antiviral medications. Assay recovery, limit of detection and quantification were also assessed. Currently, this assay is routinely used to measure antiretroviral plasma concentrations of any combination of IDV, APV, SQV, DLV, RTV, LPV, EFV, NFV and M8 in patients after oral administration.

3. Results

3.1. Chromatographic system

Representative chromatograms of blank and spiked plasma samples are illustrated in Fig. 2. Under the specified chromatographic conditions, typical retention times were approximately: IDV, 6.2 min; M8, 7.0 min; DLV, 8.0 min; APV, 9.9 min; NFV, 11.6 min; SQV, 13.1 min; RTV, 22.9 min; EFV, 24.2 min; LPV, 25.0 min and IntStd, 27.8 min. The exact retention time of each drug analyte was seen to vary slightly from assay to assay and was attributed to column wear and daily mobile phase buffer preparation. This shift was most evident in the retention times of the later-eluting quartet of peaks and never exceeded ±1 min. The order of analyte elution remained constant, regardless of shifts in retention time.

3.2. Stability

Stability testing was carried out to verify the accurate response for all analytes under several test conditions. Quality control standards at low and high concentration for all drugs were left at room temperature for 210 h (>8 days). A second set of quality control standards of equivalent concentration was subjected to six freeze–thaw cycles. In addition, a third set of quality control standards was subjected to heating in a water bath for 30 min at 58 °C. This application of heat to the samples is typical for laboratory deactivation of active HIV. All test standards were analyzed simultaneously with freshly thawed standards of equivalent concentrations and data are presented in Table 1. Results show that specimens under stability test conditions assay within 16% of the mean of non-test samples, demonstrating that all nine analytes are stable in plasma for up to six freeze–thaw cycles, 8.75 days at room temperature and heat deactivation of HIV for 30 min at 58 °C.

3.3. Matrix considerations

The matrix chosen for validation of this assay was EDTA plasma. In a clinical setting, however, it is possible that specimens may be collected in heparinized blood collection tubes. It was determined that verification of equivalence of these matrices was required to prepare for future possible need. Quality control standards containing IDV, APV, SQV, DLV, RTV, LPV, EFV, NFV and M8 were prepared in heparinized plasma at concentrations equivalent to low and high concentration EDTA quality control standards. Analysis and comparison of the EDTA and heparin standards demonstrates equivalence (mean concentrations within 20%) of the two matrices for quantification of these antiviral drugs. The slightly higher accuracy margin was allowed for this

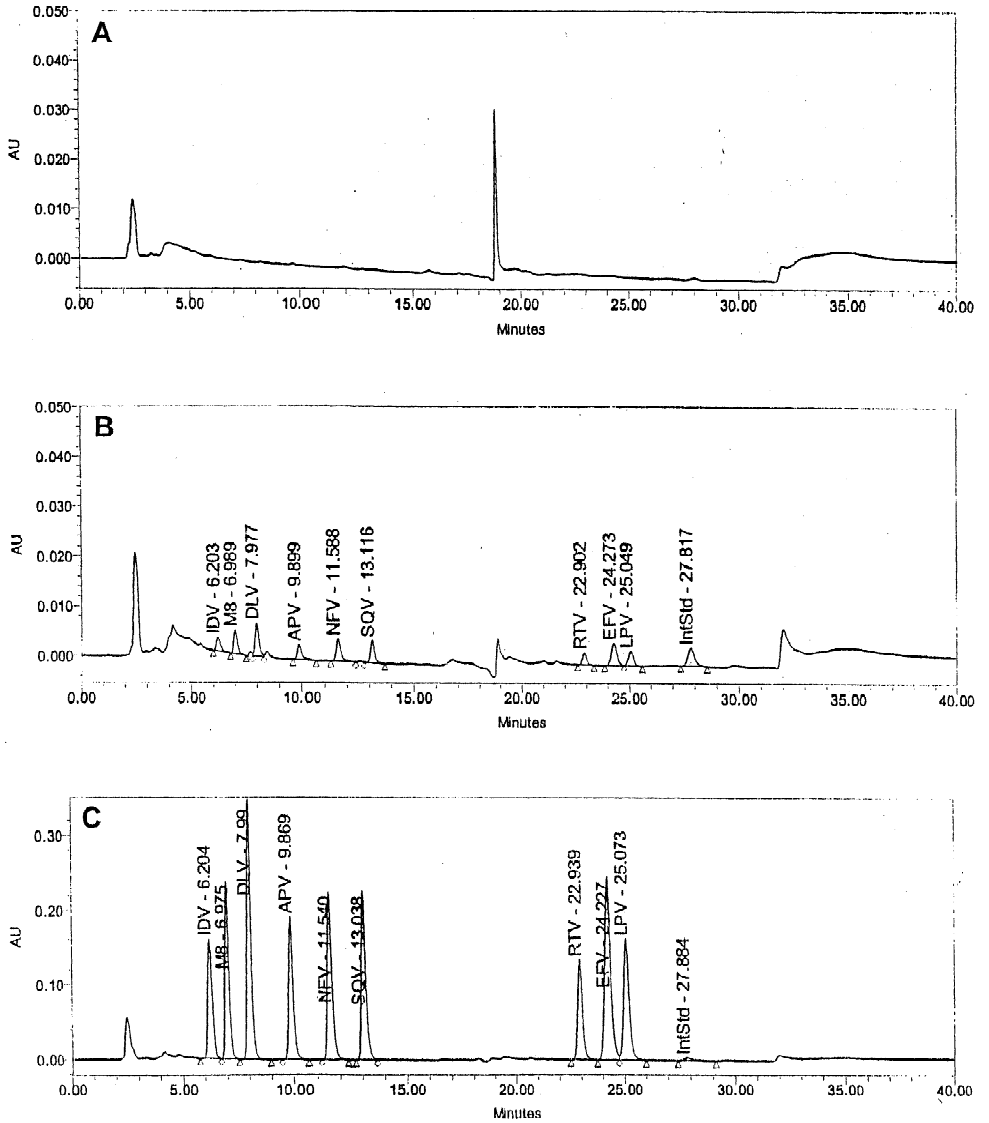

Fig. 2. Chromatograms of: (A) blank plasma; (B) low concentration QC standard; and (C) upper limit of quantification calibration standard, STD10000.

testing because the reference and test specimens did not originate from the same aliquot, and a 10% accuracy limit might have resulted in false-negative results (i.e. allowance for pipette accuracies). Results are summarized in Table 2. Long-term storage stability of these analytes in heparin plasma is currently being examined.

3.4. Specificity

Plasma extracts pose a potential interference hazard at target analyte retention times due to the presence of UV-absorbing components that survive the extraction procedure. In order to demonstrate non-interference from endogenous matrix compo-

Table 1
Room temperature, freeze–thaw and heat deactivation stability

Analyte	LOW Reference Stds Avg. conc. (CV%)	210 h at RT Avg. conc. (CV%)	Deviation (%)	HIGH Reference Stds Avg. conc. (CV%)	210 h at RT Avg. conc. (CV%)	Deviation (%)
Room temperature (RT) stability (n=5)						
IDV	213.5 (17.1)	211.8 (17.8)	99.2	8304.9 (6.0)	8722.9 (10.3)	105.0
NFV	176.6 (10.3)	148.2 (5.8)	83.9	6579.7 (1.3)	6867.4 (6.3)	104.4
M8	217.5 (16.5)	187.8 (6.5)	86.2	8201.9 (2.0)	8901.4 (10.1)	108.5
SQV	203.7 (12.7)	187.0 (4.1)	91.8	7347.7 (1.3)	7625.3 (6.8)	103.8
RTV	198.8 (2.4)	193.4 (2.4)	97.2	8820.3 (2.4)	8226.7 (0.7)	93.3
APV	191.7 (12.1)	171.3 (5.3)	89.4	8787.1 (1.2)	9318.6 (6.8)	106.0
DLV	201.3 (13.5)	200.9 (4.8)	99.8	7582.1 (1.6)	8176.5 (8.8)	107.8
EFV	199.1 (6.0)	193.8 (4.1)	97.3	7917.3 (2.3)	7746.1(3.5)	97.8
LPV	192.9 (4.6)	198.6 (5.3)	103.0	7646.7 (1.8)	7789.6 (2.0)	101.9
		Six FT cycles Avg. conc. (CV%)			Six FT cycles Avg conc. (CV%)	
Freeze–thaw (FT) stability (n=5)						
IDV	178.5 (3.6)	199.3 (4.6)	111.7	8932.9 (7.4)	9001.7 (1.8)	100.8
NFV	167.2 (2.0)	167.5 (2.7)	100.2	6556.2 (3.9)	6607.7 (1.5)	100.8
M8	206.3 (4.6)	210.3 (3.1)	101.9	8427.0 (7.4)	8605.4 (1.9)	102.1
SQV	193.3 (3.7)	187.6 (1.7)	97.1	7282.7 (3.9)	7457.4 (1.8)	102.4
RTV	214.2 (6.9)	218.4 (5.7)	101.9	8529.6 (3.2)	8669.3 (0.9)	101.6
APV	187.7 (1.8)	189.2 (1.7)	100.8	8523.0 (4.0)	8539.5 (1.8)	100.2
DLV	191.1 (2.9)	193.1 (3.9)	101.0	8096.7 (5.4)	8163.5 (1.4)	100.8
EFV	204.5 (2.3)	202.0 (1.9)	98.8	7818.7 (6.7)	7975.8 (1.8)	102.0
LPV	188.8 (2.5)	184.1 (2.7)	97.5	7275.5 (3.2)	7412.2 (1.1)	101.9
		30 min HD Avg conc. (CV%)			30 min HD Avg. conc. (CV%)	
Heat deactivation (HD) stability (n=5)						
IDV	197.6 (3.6)	198.1 (6.6)	100.3	8596.4 (14.9)	8256.9 (8.9)	96.1
NFV	209.0 (2.9)	209.5 (5.0)	100.2	6333.6 (2.7)	6197.0 (5.7)	97.8
M8	209.0 (6.4)	211.2 (7.0)	101.0	8226.4 (12.8)	8159.2 (7.3)	99.2
SQV	202.3 (1.8)	195.4 (3.6)	96.6	6919.0 (7.7)	6957.2 (4.5)	100.6
RTV	220.6 (2.9)	205.0 (2.0)	92.9	8424.7 (2.3)	7995.3 (4.5)	94.9
APV	184.0 (3.5)	199.5 (4.9)	108.4	8169.5 (2.5)	7946.4 (5.6)	97.3
DLV	202.5 (3.5)	198.9 (5.2)	98.2	7974.3 (6.3)	7712.3 (7.4)	96.7
EFV	209.9 (6.9)	188.8 (4.3)	89.9	7821.2 (4.9)	7436.3 (4.0)	95.1
LPV	196.0 (1.5)	190.1 (2.1)	97.0	7375.4 (5.6)	7528.8 (12.5)	102.1

Table 2
Heparin versus EDTA plasma ($n=5$)

Analyte	LOW EDTA Stds Avg. conc. (CV%)	LOW Heparin Stds Avg. conc. (CV%)	Deviation (%)	HIGH EDTA Stds Avg. conc. (CV%)	HIGH Heparin Stds Avg. conc. (CV%)	Deviation (%)
IDV	168.4 (9.3)	184.7 (7.8)	109.7	8596.4 (14.9)	6964.0 (10.2)	81.0
NFV	138.2 (6.8)	159.0 (3.1)	115.0	6333.6 (2.7)	5799.3 (4.0)	91.6
M8	180.1 (4.4)	204.5 (5.8)	113.6	8226.4 (12.8)	6949.4 (8.7)	84.5
SQV	163.3 (1.4)	181.7 (3.3)	111.3	6919.0 (7.7)	6227.9 (8.0)	90.0
RTV	158.0 (9.9)	181.2 (10.5)	114.7	8424.7 (2.3)	7745.3 (4.0)	91.9
APV	157.7 (5.0)	176.4 (5.2)	111.9	8169.5 (2.5)	7294.0 (3.6)	89.3
DLV	168.6 (3.4)	185.1 (5.2)	109.7	7974.3 (6.3)	6858.2 (5.3)	86.0
EFV	170.1 (9.1)	196.5 (9.6)	115.5	7821.2 (4.9)	7314.4 (4.0)	93.5
LPV	148.5 (4.9)	175.8 (15.3)	118.4	7375.4 (5.6)	6721.0 (7.6)	91.1

nents, a minimum of 15 aliquots of blank plasma were assayed. No significant matrix interferences were found in the chromatograms at the retention times for IDV, APV, SQV, DLV, RTV, LPV, EFV, NFV and M8. In addition, standards of NRTIs commonly prescribed in combination HIV therapy were assayed and verified no interference with the target analytes. Specimens from several patients on ZDV-containing regimens were also analyzed and verified no interference from ZDV-glucuronide metabolite.

3.5. Limit of quantification

The limit of quantification (LOQ) was determined to be 50 ng/ml for a 200-μl plasma aliquot. Blank (drug-free) plasma was spiked at a concentration of approximately 50 ng/ml for each of the nine compounds under investigation. Six aliquots of this standard were assayed. Intra-assay accuracy and precision for these six LOQ aliquots were as follows:

	% Nom	CV%
IDV	113.6	7.5
APV	100.2	14.7
SQV	102.6	2.1
DLV	98.4	6.4
RTV	103.1	6.9
LPV	93.0	11.1
EFV	109.4	6.2
NFV	107.1	5.4
M8	102.6	9.7

Table 3
Intra- and inter-assay accuracy (%nom) and precision (CV%) of nine antiretrovirals in plasma

	Nominal concentration (ng/ml)	Intra-assay ($n=6$)			Inter-assay ($n=36$)		
		Mean (ng/ml)	% nom	CV%	Mean (ng/ml)	% nom	CV%
IDV	207	212	102.4	8.6	204	98.6	10.5
	2069	2050	99.1	7.6	1990	96.2	9.3
	8277	8053	97.3	5.5	8285	100.1	8.3
NFV	201	214	106.7	4.9	212	105.5	6.1
	2006	1970	98.2	3.4	2017	100.6	5.9
	8024	7447	92.8	3.0	7708	96.1	5.2
M8	228	219	96.0	7.3	216	94.9	6.9
	2280	2093	91.8	6.3	2088	91.6	7.5
	9120	7836	85.9	4.6	8098	88.8	7.0
SQV	199	205	102.7	3.8	204	102.0	6.2
	1994	1898	95.1	2.8	1909	95.7	5.8
	7977	7198	90.2	2.6	7375	92.5	4.2
RTV	208	223	107.4	3.8	222	106.9	5.4
	2080	1954	94.0	1.6	1992	95.8	5.3
	8320	7565	90.9	2.1	7720	92.8	2.7
APV	204	202	98.9	8.3	197	96.5	7.7
	2040	1869	91.6	4.1	1833	89.8	5.4
	8160	7393	90.6	4.5	7640	93.6	4.5
DLV	202	202	100.0	7.3	201	99.9	7.3
	2016	1854	92.0	5.1	1913	94.9	6.1
	8063	7142	88.6	4.0	7355	91.2	5.0
EFV	204	214	104.8	6.3	216	105.6	12.8
	2040	1847	90.5	2.1	1931	94.7	6.9
	8160	7007	85.9	2.0	7121	87.3	2.5
LPV	212	201	95.0	6.0	195	91.8	7.1
	2120	1821	85.9	1.6	1846	87.1	4.3
	8480	7028	82.9	2.0	7077	83.5	3.0

3.6. Linearity

Weighted (1/concentration^2) least-squares linear regression calibration curves, obtained by plotting the peak height ratio of drug/internal standard versus calibration standard concentration yielded coefficients of determination consistently greater than 0.99 (CV%=0.5) across six validation assays for all drug analytes. Linear slope and intercept parameters were also determined, with good inter-assay slope precision (CV%<10). Concentrations ranging from 50 to 10 000 ng/ml can be accurately measured from a 200-μl patient plasma sample.

3.7. Recovery

Extraction recovery of each analyte was determined by comparing detector response of plasma extracts with non-extracted standards of equivalent concentration, across the range of calibration standard concentrations. Multiple aliquots (n=6) at each of four different concentrations, including LOQ, were assayed. Mean extraction recoveries were 97.9% for IDV, 90.4% for DLV, 80.4% for APV, 85.9% for SQV, 87.8% for RTV, 90.7% for EFV, 90.3% for LPV, 85.3% for NFV and 85.7% for M8. Precision (CV%) was within 15% for IDV and APV and less than 8% for all other analytes.

3.8. Precision and accuracy

Six calibration standard curves were assayed during the course of this validation procedure. Quality control standards (n=6) containing all drugs were assayed at three concentration levels with each calibration standard curve. Measured concentrations of the quality control standards were calculated daily from each associated calibration standard curve. The intra- and inter-assay accuracy and precision for the quality control standards is presented in Table 3.

Table 4 describes the accuracy range across the calibration standard curve for each analyte. Each calibration standard concentration was assayed once over six separate days. Table 4 summarizes the accuracy and precision results for each analyte across the entire calibration standard curve.

Table 4
Inter-assay reproducibility for calibration standards

Analyte	Accuracy range (% nom)	Precision range (CV%)
IDV	92.5–105.6	2.5–7.3
NFV	99.2–101.3	2.9–6.8
M8	98.0–102.7	1.5–9.6
SQV	96.5–103.8	3.0–7.3
RTV	98.0–102.4	2.8–5.8
APV	92.8–107.0	2.9–6.5
DLV	98.3–102.2	2.0–4.9
EFV	98.1–103.1	2.2–8.0
LPV	97.3–102.0	2.0–5.2

3.9. Partial aliquot

Occasionally, especially for pediatric specimens where blood volumes are limited, it may not be possible to obtain 200 μl of plasma for sampling. For this reason, partial aliquot sampling was investigated. Multiple 50- and 100-μl aliquots of known concentration quality control standards were measured and were brought to 200 μl with pre-screened drug-free (blank) plasma. Three aliquots of each partial volume were assayed and were compared to non-diluted (200 μl) quality control standards. Mean accuracy ranged from 99.9 to 106.2% (1.4–8.6 CV%) for the 100-μl aliquot and 95.6 to 103.1% (1.1–5.3 CV%) for the 50-μl aliquot, demonstrating excellent accuracy and precision for reduced volume sampling.

4. Discussion

Multi-drug antiretroviral regimens have rapidly become the standard of care for treatment of HIV infection, especially in the setting of salvage therapy. As new antiretroviral agents emerge, analytical procedures to isolate and assay each drug are needed. Early analytical procedures focused on assays of a single antiretroviral, which generally followed the standard practice of monotherapy. Increased use of multi-drug regimens over the past several years has presented a unique challenge for the drug testing laboratory with respect to analytical method capabilities. The laboratory must be able to analyze any combination of the available antiretrovirals in patient

plasma. Sometimes these analyses must be completed rapidly, as with TDM. It has therefore become desirable for laboratories to develop methodologies to assay several antiretrovirals simultaneously in order to maximize laboratory resources. Fig. 3 depicts a concentration–time profile from a commonly used twice-daily dual PI regimen derived from patient plasma samples.

Methodologies have been published for the simultaneous assay of two to four [9–12], five to six [13–16], eight [17], 12 [18] and 13 [19] antiretroviral drugs, although the latter two methods cannot assay all drugs simultaneously on one HPLC system. For one of these methods [19], seven PI and NNRTI drugs can be analyzed from a 1-ml serum aliquot. However, assay of the six NRTI drugs requires a separate aliquot for extraction and must be chromatographed separately. We found the required 1-ml sample aliquot impractical for assay of our pediatric patient samples. Similarly, we found another methodology for analysis of 12 antiretrovirals from a single plasma extract unsuitable for use in our laboratory [18]. Solid-phase extraction (SPE) facilitates isolation of all 12 drugs from a 1-ml aliquot of plasma, but the methodology requires splitting the SPE eluent into two aliquots for assay on two chromatographic systems. One HPLC system requires two detectors for assay of PIs, EFV and DLV. The second requires a complicated set-up of three pumps with valve switching for assay of NRTIs and NVP.

Using a simple liquid extraction process, we were able to isolate and quantify nine PI and NNRTI drugs, including lopinavir, in the 50–10 000 ng/ml concentration range from a single 200-μl patient plasma extract on a single HPLC system with UV detection. A recent literature search resulted in no other published methodologies that include lopinavir in the simultaneous assay of approved antiretrovirals. Despite a lower specimen volume, the LOQ reported here is similar to previously published data [10,12–14,20]. In some cases, we were able to detect drug plasma levels up to five or more times lower than previously available methods [16]. For example, an LOQ of 400 ng/ml has recently been reported for RTV [21]. This is approximately eight times higher than the LOQ for our assay.

A primary challenge while developing this assay was the successful chromatographic separation of 10 compounds in the presence of potential plasma interferences within a 30-min run time. Initial binary mobile phase conditions (buffer/acetonitrile) resulted in insufficient retention and resolution of the early-eluting, polar drugs (IDV, M8, DLV, APV) and

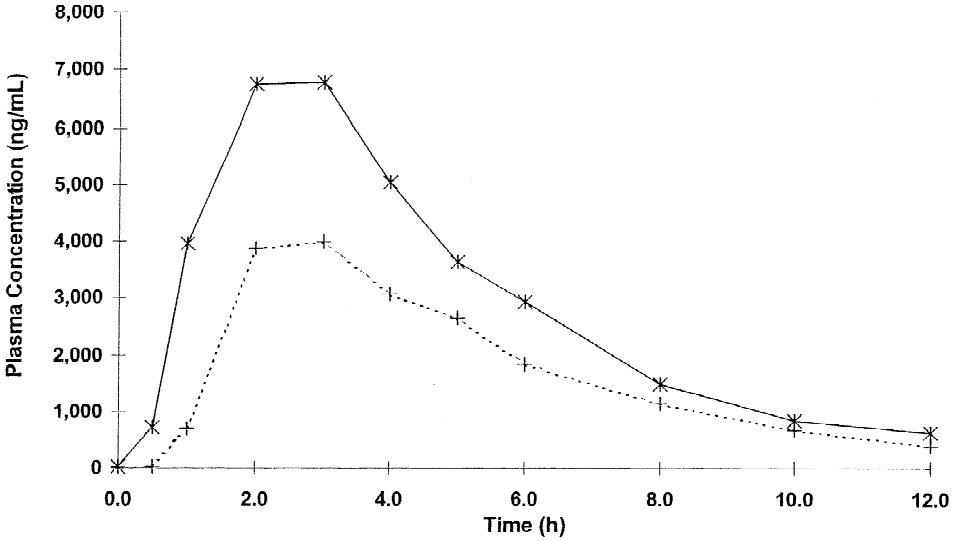

Fig. 3. Representative concentration–time profile from a patient receiving 800 mg of IDV BID (solid line) and 200 mg of RTV BID (dashed line) concomitantly.

complete co-elution of NFV/SQV and EFV/LPV. Replacing acetonitrile with methanol in the mobile phase decreases solvent strength (k') and increases retention of all analytes. This resulted in adequate retention with baseline resolution of the polar compounds but had an adverse effect on the retention times of the more retained non-polar drugs (RTV, EFV, LPV and IntStd), increasing the run time to over 60 min. Selectivity (relative retention) was also affected by the change in the organic component, resulting in successful separation of the NFV and SQV peaks. To optimize resolution, selectivity, and run time, a tertiary mobile phase was selected. The selected composition of buffer/acetonitrile/methanol at sample injection is adequate to provide resolution for the polar drugs. However, in order to optimize the retention of the non-polar drugs, the percentage of methanol in the mobile phase must be slowly decreased. In addition, we found that maintaining constant column temperature was crucial to ensure baseline separation between EFV and LPV. Seasonal changes in room temperature and the proximity of the HPLC system to heating/cooling vents had a marked effect on the resolution between the EFV and LPV peaks. Adding a column heater helped to maintain baseline resolution among all peaks in the chromatogram. While RTV and LPV only move slightly, we found that increasing the column temperature will move the EFV peak closer to the RTV peak, improving baseline resolution from LPV where necessary. We found a delicate balance in solvent strength and temperature that maximizes peak resolution and minimizes total run time resulting in baseline resolution of all drugs, sufficient for proper software integration and identification of all compounds.

5. Conclusion

Using this novel HPLC methodology, we were able to simultaneously determine the plasma con-

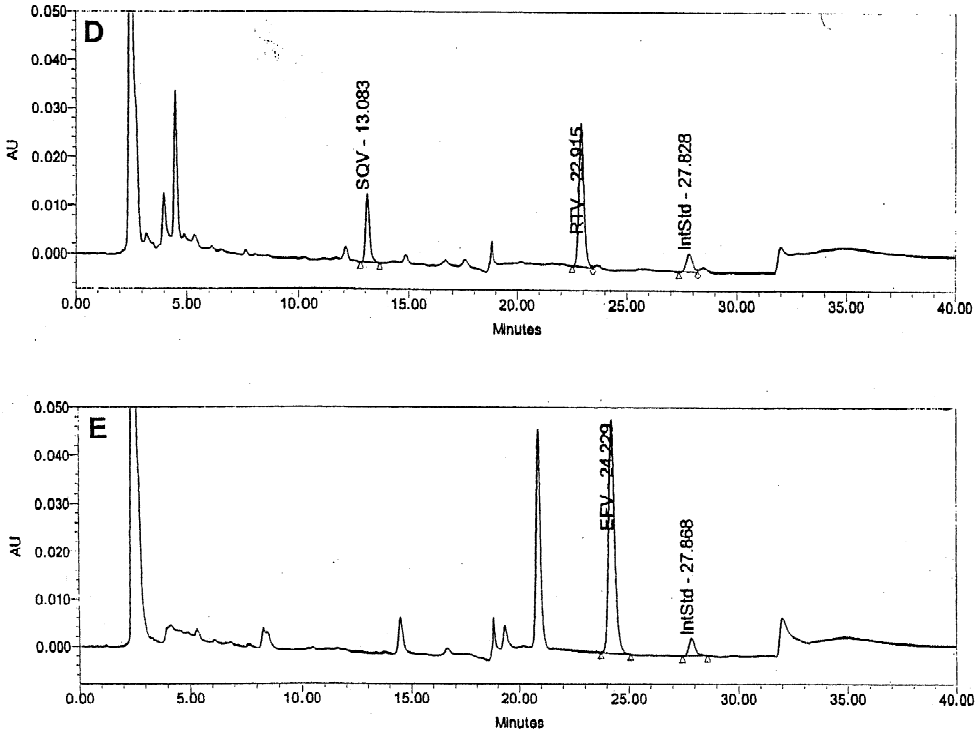

Fig. 4. Chromatograms of: (D) adult patient, SQV/RTV-containing regimen (calculated 959 ng/ml SQV and 1034 ng/ml RTV); and (E) adult patient, EFV-containing regimen (calculated 2176 ng/ml EFV).

centrations of PIs and NNRTIs following oral administration in HIV-infected patients. Analyses of samples from patients on many different PI- and NNRTI-containing regimens were completed in support of multi-center protocols for the Adult and Pediatric AIDS Clinical Trials Groups (ACTG). More than 300 patient plasma concentrations of various antiretrovirals have been determined using this methodology and representative chromatography of patient plasma samples is shown in Fig. 4. The low-volume plasma aliquot (200 μl) has proven critical for assay of pediatric patient samples, where blood volumes are typically limited.

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