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Short communication

An isocratic liquid chromatography method for determining HIV non-nucleoside reverse transcriptase inhibitor and protease inhibitor concentrations in human plasma

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Abstract

An efficient, isocratic high performance liquid chromatography (HPLC) method for determining human immunodeficiency virus (HIV) non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs) in plasma is advantageous for laboratories participating in clinical trials and therapeutic drug monitoring (TDM) programs, or conducting small animal research. The combination of isocratic reversed phase chromatography using an S-3, 3.0 mm × 150 mm column along with low plasma volume (200 μl), rapid liquid–liquid extraction, and detection at a single wavelength (212 nm) over a short run time makes this method valuable. Within and between assay variability ranges from 0.8 to 3.5% and 1.2–6.2%, respectively. Accuracy ranges from 91.0 to 112.8% for four quality controls (50, 100, 1000, and 10,000 ng/ml) for all drugs measured (efavirenz, nevirapine, amprenavir, atazanavir, indinavir, lopinavir, nelfinavir, ritonavir, and saquinavir).

Keywords: HIV; Non-nucleoside reverse transcriptase inhibitors; Protease inhibitors; HPLC-UV; Isocratic; Liquid–liquid extraction; Therapeutic drug monitoring

1. Introduction

Antiretroviral therapy for human immunodeficiency virus (HIV) infections typically consists of combinations of nucleoside/tide reverse transcriptase inhibitors (NRTIs/NtRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), or in later stages of disease, fusion inhibitors. Relationships between plasma drug concentrations and clinical response (efficacy and toxicity) are widely documented for agents within the NNRTI and PI drug classes [1–4]. As a result, plasma concentrations of NNRTIs and PIs are frequently evaluated as part of HIV clinical trials and clinical therapeutic drug monitoring (TDM) programs, requiring that high quality, reproducible analytical methods are available.

There are currently three NNRTIs and nine PIs approved for the treatment of HIV infections. The NNRTIs include delavirdine, efavirenz, and nevirapine, however only efavirenz and nevirapine are used routinely in clinical practice. The PIs include amprenavir, atazanavir, darunavir, indinavir, lopinavir co-formulated with low-dose ritonavir, nelfinavir, ritonavir, saquinavir, and tipranavir. Several multi-drug high performance liquid chromatography (HPLC) assays that measure a number of these NNRTIs and PIs have been described in the literature in recent years [5–10]. Most published methods are gradient-based and often involve complicated chromatography techniques. In this paper we describe a novel, purely isocratic HPLC assay with ultraviolet (UV) detection for quantifying the following antiretrovirals in human plasma: efavirenz, nevirapine, amprenavir, atazanavir, indinavir, lopinavir, nelfinavir, ritonavir, and saquinavir. A combination of several characteristics makes this assay a unique and useful analytical tool for quantifying these drugs. The plasma preparation involves a rapid liquid–liquid ether extraction, and the chromatography conditions are simple with detection at a single wavelength over a short run time. This assay also accommodates small volume plasma samples making it widely applicable for HIV clinical trials or clinical TDM programs in both adult and

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pediatric populations, or for HIV research involving small animals.

2. Experimental

2.1. Chemicals and reagents

Amprenavir, atazanavir sulfate, efavirenz, indinavir sulfate, nevirapine, ritonavir, and saquinavir were obtained through the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH. Lopinavir was a gift from Abbott Laboratories (Abbott Park, Illinois). Nelfinavir mesylate, the M8 metabolite of nelfinavir, and delavirdine mesylate were provided by Pfizer Incorporated (Groton, Connecticut). Sodium hydroxide and potassium phosphate monobasic were purchased from Sigma (St. Louis, Missouri). Methyl alcohol, acetonitrile and methyl-*tert*-butyl ether were all HPLC grade and along with 2-propanol, were obtained from Fisher Scientific (Fair Lawn, New Jersey). EDTA derived human plasma was purchased from Biological Specialty Corporation (Colmar, Pennsylvania).

2.2. Chromatographic and extraction apparatus and supplies

The HPLC system was a series 1100 HP consisting of a G1322A quaternary pump, G1322A degasser, G1330A thermostatic control, G1316A column heater, G1329A autosampler, and a G1314A ultraviolet detector (Agilent Technologies, Palo Alto, California). The HPLC column was an S-3, 3.0 mm × 150 mm YMC-Pack Octyl C8 column (Waters Corporation, Milford, Massachusetts). Sample preparation and extraction procedures included the use of a Beckman model J-6B centrifuge (Beckman Coulter Incorporated, Fullerton, California), a Jouan GR 4.22 centrifuge (Jouan Incorporated, Winchester, Virginia), a Zymark model Turbo Vap LV nitrogen evaporator (Zymark Corporation, Hopkinton, Massachusetts), a Fisher Genie 2 vortex (Fisher Scientific, Hanover Park, Illinois), and both air-displacement and positive displacement pipettes (Gilson, Woburn, Massachusetts). Data acquisition and analysis were accomplished using Chrom Perfect® Spirit™, version 5.1.0 (Justice Innovations, Denville, New Jersey).

2.3. Standard, quality control, and internal standard preparation

Individual primary methanolic stock solutions of amprenavir, atazanavir sulfate, efavirenz, indinavir sulfate, lopinavir, nelfinavir mesylate, nevirapine, ritonavir, and saquinavir were prepared at concentrations high enough, so that when combined, a secondary dilution of each in a 10 ml volume of methanol resulted in free base concentrations of 200 μg/ml for each drug. This became the high working standard in methanol. This standard was non-serially diluted to concentrations of 40, 10, 2, and 0.5 μg/ml. A 20 μl addition of each of these solutions to 200 μl of blank EDTA derived human plasma, resulted in a standard curve with concentrations of 50, 200, 1000, 4000, and 20,000 ng/ml. Primary quality control methanolic stocks of each drug were also prepared as described above with separate weighings, and combined to achieve the same concentration in methanol (200 μg/ml). This solution when diluted directly in human plasma to a concentration of 10 μg/ml resulted in the high quality control (QCH) at 10,000 ng/ml. This QCH was non-serially diluted in plasma to make concentrations equal to the lowest standard (QCLLOQ), the low quality control (QCL), and the medium quality control (QCM), at final concentrations of 50, 100, and 1000 ng/ml, respectively. These four quality controls were assayed in triplicate on five assays to determine within and between assay performance for accuracy and variability. Following validation, the three higher quality controls were assayed in duplicate for continuous assay monitoring. Finally, a working solution of internal standard (delavirdine mesylate) was made from a higher primary methanolic concentration so that the delavirdine concentration was 40 μg/ml. An addition of 25 μl of this solution to each extraction resulted in a final plasma concentration of delavirdine of 5000 ng/ml.

2.4. Sample extraction and chromatography conditions

All standards, quality controls, plasma, and reagents were allowed to warm to room temperature prior to extraction. Duplicate 20 μl pipettings of each working standard stock solution were added to 13 mm × 100 mm borosilicate glass culture tubes. A 200 μl volume of EDTA human plasma was added to each standard tube as well as a duplicate set of blank tubes. A 200 μl volume of plasma was used for analysis. A 25 μl volume of the internal standard working solution was added to all tubes except the plasma blanks. A 200 μl volume of 0.01 M sodium hydroxide was added to all tubes and vortexed briefly followed by an addition of 2 ml of methyl-*tert*-butyl ether. The samples were vortexed for 10 s and centrifuged at 1000 × *g* for 5 min. The aqueous layer was frozen in a dry-ice isopropyl alcohol bath for 10 min. The methyl-*tert*-butyl ether layer was decanted into clean 12 mm × 75 mm borosilicate glass tubes, warmed to room temperature, and vortexed briefly. The methyl-*tert*-butyl ether was dried under nitrogen at 40 °C on a Turbo Vap LV evaporator. The residue was reconstituted in 200 μl of fresh mobile phase and 25 μl was injected for analysis.

The mobile phase consisted of 52% 25 mM monobasic potassium phosphate (pH 4.90), 48% acetonitrile, vol:vol. The stationary phase was an S-3, 3.0 mm × 150 mm YMC-Pack Octyl C8 column protected by the addition of a 0.5 μm filter apparatus. Column temperature was maintained at 35 °C. The mobile phase flow rate was 0.4 ml/min. Detection wavelength was at 212 nm for all drugs.

All standard curves were calculated using peak height ratios with the internal standard, and were linear fit with $1/X$ weighting for all drugs except nevirapine for which $1/X^2$ weighting was used. Extraction recoveries were measured in triplicate at the 1000 and 20,000 ng/ml calibrator concentrations by calculating mean peak area ratios of extracted calibrators to mobile phase spiked at these same levels. Variability and accuracy for each drug were measured using calculated concentrations from the four quality controls (50, 100, 1000, and 10,000 ng/ml). These

values were analyzed using a single factor analysis of variance (ANOVA) to determine within and between assay variability. In addition to these within laboratory quality control validations, the analytical laboratory also participated in a blinded external proficiency testing program for the measurement of antiretroviral drug concentrations, which is sponsored by the AIDS Clinical Trials Group [11].

Several drugs commonly prescribed for HIV/AIDS patients along with PIs and NNRTIs were tested for interference with the assay. These included the nucleoside reverse transcriptase inhibitors abacavir, didanosine, lamivudine, stavudine and zidovudine, the nucleotide reverse transcriptase inhibitor tenofovir, as well as atorvastatin, atovaquone, dapsone, fenofibric acid, fluconazole, sulfamethoxazole, and the M8 metabolite of nelfinavir. We also evaluated potential metabolite interferences in samples from an HIV infected patient taking two second-generation PIs, atazanavir sulfate and lopinavir co-formulated with ritonavir, as part of a clinical study.

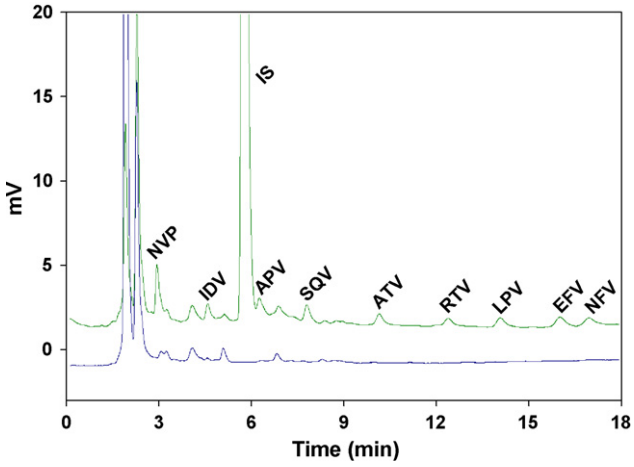

Fig. 1. Representative chromatograms of blank human plasma (lower) and a low quality control (100 ng/ml) (upper). NVP: nevirapine; IDV: indinavir; IS: internal standard (delavirdine); APV: amprenavir; SQV: saquinavir; ATV: atazanavir; RTV: ritonavir; LPV: lopinavir; EFV: efavirenz; NFV: nelfinavir. mV: microvolts; min: minutes.

3. Results

The assay was linear for all nine drugs with correlation coefficients equal to or greater than 0.99. Chromatograms from extractions of blank human plasma and a low quality control (100 ng/ml) are shown in Fig. 1. Retention times for nevirapine, indinavir, delavirdine, amprenavir, saquinavir, atazanavir, ritonavir, lopinavir, efavirenz, and nelfinavir were 2.9, 4.5, 5.7, 6.2, 7.7, 10.1, 12.3, 14.0, 15.0, and 17.1 min, respectively. No significant interfering endogenous plasma peaks were found in the blank extraction over this time period. An injection timing sequence of 19–20 min avoided interference from late eluting peaks. The recoveries and coefficients of variation (CV %) resulting from triplicate unextracted and extracted determinations at the 1000 and 20,000 ng/ml concentrations are shown in Table 1.

The ANOVA performed on the four quality controls resulted in 10 and 4 degrees of freedom for within and between assay comparisons, respectively. Results for accuracy and variability are shown in Table 2. All variability is expressed as CV %. Within assay variability ranged from 0.8 to 3.5% and between assay variability ranged from 1.2 to 6.2%. Overall accuracy was determined by using the grand mean of each calculated concentration, and ranged from 91.0 to 112.8% for the four target concentrations for all nine drugs.

Of the drugs tested for assay interference, the M8 metabolite of nelfinavir eluted slightly later than saquinavir preventing quantification of saquinavir in patients on combination therapy consisting of these two PIs. Dapsone and nevirapine coeluted preventing quantification of this NNRTI. The nucleotide reverse transcriptase inhibitor, tenofovir, and fluconazole eluted very early, less than 2.14 min, and did not interfere. Atorvastatin, atovaquone, fenofibric acid, and sulfamethoxazole had retention times of 7.10, 3.26, 9.54, and 3.23 min, respectively, but did not interfere. An 8-h post-dose chromatogram from a patient on combination therapy with atazanavir 300 mg and lopinavir 400 mg co-formulated with ritonavir 100 mg is shown in Fig. 2. No interference from metabolites was observed at this time point or at any time point over a 12-h sampling period.

4. Discussion and conclusion

A high quality analytical method for simultaneously measuring plasma concentrations of NNRTIs and PIs is fundamental for

Table 1
Extraction recoveries for nine antiretroviral drugs

1000 ng/ml		NVP	IDV	APV	SQV	ATV	RTV	LPV	EFV	NFV
Unextracted	CV %	0.8	0.6	0.7	0.5	0.6	0.4	0.3	0.3	0.2
Extracted	CV %	2.3	3.8	2.7	5.5	2.4	2.3	3.3	7.3	7.6
Recovery	%	93.5	86.0	101.0	86.0	97.0	94.7	91.9	83.6	77.7
20,000 ng/ml		NVP	IDV	APV	SQV	ATV	RTV	LPV	EFV	NFV
Unextracted	CV %	1.2	1.1	1.0	0.7	0.9	0.8	0.7	0.5	0.4
Extracted	CV %	5.8	7.3	6.0	7.6	5.7	5.8	6.9	8.5	9.8
Recovery	%	94.1	93.4	107.3	93.5	102.6	100.6	96.6	84.4	82.8

Recovery: (mean extracted/mean unextracted) × 100; CV %: coefficient of variation; NVP: nevirapine; IDV: indinavir; APV: amprenavir; SQV: saquinavir; ATV: atazanavir; RTV: ritonavir; LPV: lopinavir; EFV: efavirenz; NFV: nelfinavir.

Table 2
Assay accuracy and variability

Accuracy (%)

	ng/ml	NVP	IDV	APV	SQV	ATV	RTV	LPV	EFV	NFV
QCLLOQ	(50)	93.2	101.6	112.8	107.6	104.8	106.0	105.6	97.0	100.0
QCL	(100)	96.6	105.4	96.8	99.9	99.7	101.8	101.0	100.5	100.9
QCM	(1000)	98.5	97.5	91.0	93.2	96.5	96.5	95.8	101.5	102.0
QCH	(10000)	97.2	100.6	100.4	101.2	100.3	100.5	100.8	104.0	104.3

Within assay variability (CV %)

	ng/ml	NVP	IDV	APV	SQV	ATV	RTV	LPV	EFV	NFV
QCLLOQ	(50)	1.9	1.7	1.8	2.2	1.1	3.3	2.3	2.5	3.4
QCL	(100)	1.9	1.4	3.5	3.1	1.3	2.5	2.2	2.9	2.7
QCM	(1000)	2.2	1.4	4.5	2.5	1.5	1.5	2.0	2.9	3.4
QCH	(10000)	1.2	0.8	1.6	1.8	1.0	1.3	2.1	2.8	3.2

Between assay variability (CV %)

	ng/ml	NVP	IDV	APV	SQV	ATV	RTV	LPV	EFV	NFV
QCLLOQ	(50)	3.0	3.3	4.0	3.0	1.2	2.0	2.5	5.6	5.3
QCL	(100)	2.5	6.2	2.2	4.4	2.2	2.1	2.2	4.3	4.1
QCM	(1000)	4.0	3.6	3.3	2.5	3.4	2.6	2.7	3.2	2.6
QCH	(10000)	2.8	2.6	1.9	2.5	1.8	1.7	2.0	4.2	4.4

QCLLOQ: quality control at the lowest standard; QCL: low quality control; QCM: medium quality control; QCH: high quality control; NVP: nevirapine; IDV: indinavir; APV: amprenavir; SQV: saquinavir; ATV: atazanavir; RTV: ritonavir; LPV: lopinavir; EFV: efavirenz; NFV: nelfinavir; CV: coefficient of variation.

HIV clinical trials with pharmacology endpoints and for clinical antiretroviral TDM programs. Our HPLC-UV assay measures the most frequently prescribed NNRTIs and PIs and has certain advantages over similar methods [5–10]. Sample preparation is simple, utilizing an efficient, rapid liquid–liquid extraction. The assay is entirely isocratic involving only two stable solvent components with low volatility, a pH-adjusted phosphate solution and acetonitrile. Isocratic elution should improve assay reproducibility and reduce inter- and intra-laboratory variability associated with reconstructing conditions on different HPLC systems during gradient elution [5–9]. The use of a pH-adjusted phosphate mobile phase, while not buffering at pH 4.90, works well in this assay. The background blank baseline is clean with little interference from endogenous peaks. Also, equilibration is rapid, absorbance at 212 nm is transparent, and the run time is less than 18 min.

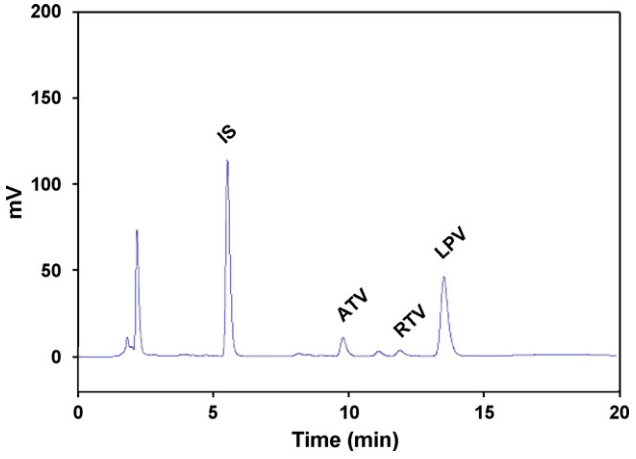

Fig. 2. An 8-h post-dose chromatogram from a patient taking lopinavir 400 mg co-formulated with ritonavir 100 mg twice daily in combination with atazanavir 300 mg once daily. Atazanavir, ritonavir, and lopinavir concentrations were 1589, 683, and 7309 ng/ml, respectively. IS: internal standard (delavirdine); ATV: atazanavir; RTV: ritonavir; LPV: lopinavir. mV: micro-volts; min: minutes.

Another important characteristic is the use of only 200 μl of plasma for analysis combined with an injection volume of only 25 μl. The small volume of plasma extracted to achieve a sensitivity of 50 ng/ml is especially useful for pediatric samples, small animal studies, or other situations where sample volume would be limited. Absolute responses (peak heights in microvolts) ranged from approximately 210 for ritonavir to over 1800 for nevirapine. Previous experience with this type of response indicates further reduction in the required plasma volume could be accommodated if necessary. The small volume of reconstituted extract used for injection is also important for maintaining column and assay integrity with large assays over a long period of time. It is important to note that all preliminary assay development, assay validation, proficiency testing, and patient or subject sample analysis runs for this assay were carried out over a period of more than one year, on a single column, with no observable changes in assay performance as it relates to variability, accuracy, or column pressure.

Our assay maintained a constant pressure of approximately 100 bar over 24 h of run time. If new drugs need to be added, a higher flow rate and/or the addition of a gradient elution could be beneficial and easily adaptable with this procedure. Tipranavir, for example, is less polar than the other drugs in its class with a partition coefficient ($X\log P$) value of 8.579 [12]. As a result, tipranavir would likely have a retention

time much longer than any of the drugs now included on the assay.

In conclusion, we believe that this HPLC-UV method is unique and can be advantageous for laboratories that measure NNRTI and PI plasma concentrations for adult or pediatric HIV clinical trials and TDM programs, or for HIV research involving small animals.

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