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Journal of Chromatography B, 721 (1999) 239–247

JOURNAL OF CHROMATOGRAPHY B

Rapid quantification of indinavir in human plasma by high-performance liquid chromatography with ultraviolet detection

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Received 14 May 1998; received in revised form 28 September 1998; accepted 29 September 1998

Abstract

A rapid, sensitive and specific high-performance liquid chromatography (HPLC) procedure for the quantification of indinavir, a potent human immunodeficiency virus (HIV) protease inhibitor, in human plasma is described. Following C_{18} solid-phase extraction, indinavir was chromatographed on a reversed-phase C_8 column using a simple binary mobile phase of phosphate buffer–acetonitrile (60:40, v/v). UV detection at 210 nm led to an adequate sensitivity without interference from endogenous matrix components. The limit of quantification was 25 ng/ml with a 0.1 ml plasma sample. The standard curve was linear across the range from 25 to 2500 ng/ml with an average recovery of 91.4%. The mean relative standard deviations for concentrations within the standard curve ranged between 1.4 and 9.7%. Quality control standards gave satisfactory intra- and inter-assay precision (R.S.D. from 3.5 to 15.8%) and accuracy within 15% of the nominal concentration. Sample handling experiments, including HIV heat inactivation, demonstrated analyte stability under expected handling processes. The assay is suitable for the analysis of samples from adult and pediatric patients infected with HIV.

Keywords: Indinavir

1. Introduction

Indinavir (**I**, Fig. 1) is a potent and specific protease inhibitor, currently prescribed for the treatment of human immunodeficiency virus (HIV) infection. HIV protease is an enzyme that is required for cleavage of the *gag* and *gag-pol* polyprotein precursors of the proteins found in infectious HIV. Indinavir binds to this enzyme and inhibits its activity, and ultimately the absence of polyprotein cleavage results in immature, non-infectious virions [1–2]. Indinavir was granted accelerated approval by the US Food and Administration (FDA) in March 1996 [3], although safety and effectiveness for use in pediatrics were not determined [4].

A rapid, sensitive procedure to quantify indinavir in human plasma was desired in anticipation of sample analysis from large-scale clinical trials involving HIV-positive adult and pediatric patients, where 'real time' pharmacokinetic profiling may be required and where plasma volume is often limited.

Several methodologies have been previously reported in the literature. Early work, published in

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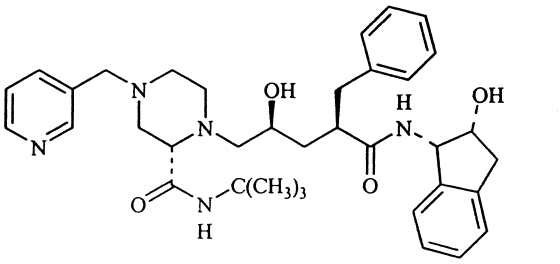
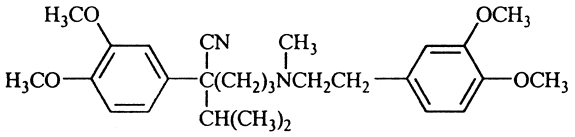
0378-4347/99/$ – see front matter
PII: S0378-4347(98)00451-4



I



II

Fig. 1. Chemical structures of indinavir (**I**) and internal standard (**II**).

1995, concentrated on the determination of indinavir in laboratory animals [5–6]. In that same year, Woolf et al., [7] published a high-performance liquid chromatography (HPLC) procedure with column switching for the quantification of indinavir from 1 ml of human plasma. This methodology was further utilized in 1996 for drug metabolism studies [8]. Woolf and Matuszewski [9] later released a modified HPLC procedure utilizing mass spectrometry to analyze the 1 ml plasma extract. All of these methodologies include liquid–liquid extraction techniques to isolate indinavir from matrix.

In this study, the development and validation of a simple HPLC method for the quantification of indinavir following solid-phase extraction of 0.1 ml of plasma is described.

2. Experimental

2.1. Reagents and materials

Solutions of compound **I** were prepared from a Crixivan 400 mg capsule (Merck, West Point, PA, USA). Compound **II** (internal standard, verapamil; Fig. 1) was purchased from Sigma (St. Louis, MO, USA) as hydrochloride salt. HPLC grade acetonitrile, potassium dihydrogen phosphate, *o*-phosphoric acid, 85% and Optima grade methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). Water was double distilled from a glass apparatus. Drug-free human plasma was purchased from UAB Hospital (Birmingham, AL, USA). Solid-phase extraction cartridges (C_{18} Sep-Pak Vac, 1 cc, 100 mg) were obtained from Waters (Franklin, MA, USA). Polypropylene micro-centrifuge tubes were purchased from USA/Scientific Plastics (Ocala, FL, USA). Helium was supplied by BOC Gases (Bessemer, AL, USA).

Following completion of the validation procedure, a source of pure indinavir was received from Merck Research Laboratories (Rahway, NJ, USA). A cross-validation was completed comparing authentic standards prepared with capsule-sourced **I** vs. pure **I**.

2.2. Preparation of indinavir solutions

Indinavir sulfate was obtained directly from the capsule formulation without chemical manipulation. The quantity of powder within a single capsule was determined (mass of intact capsule vs. mass of empty gelatin shell). This was compared to the known quantity of indinavir sulfate in the capsule [10] to obtain a mass factor. This mass factor and a factor to convert sulfate to free base mass were considered during the preparation of all solutions of **I** from capsule powder. Capsule excipients in solution were separated from **I** on the HPLC system.

2.3. Verification of identity of indinavir peak

A fraction of the indinavir chromatographic peak was obtained and analyzed by mass spectrometry. Direct injection electrospray ionization mass spectrometry on a PE-Sciex API III mass spectrometer was utilized. The sample in aqueous solution was injected into a 10 μg/ml flow of water–acetonitrile (50:50) containing 0.1% formic acid. A Harvard apparatus Model 22 syringe pump was used to deliver the flow to the electrospray interface.

2.4. Verification of indinavir peak homogeneity

Diode array (UV) spectral purity analyses were

performed by injection of the capsule powder solution onto the HPLC system. UV spectra were collected from 190 to 400 nm. HP Chemstation peak purity software was used to analyze the peak at the target retention time.

In addition, a simple thin-layer chromatography (TLC) procedure was developed to confirm target peak homogeneity. A sample taken across the target peak was spotted onto an Eastman Kodak Chromagram sheet (No. 13254 cellulose with fluorescent indicator). The sample was migrated up the chromagram using a methanol–acetone mixture (1:1, v/v).

2.5. Preparation of standards

Two stock solutions of **I** were prepared in 50% methanol (acidified to pH 3.1) at 1000 μg/ml. The acidified methanol solution was prepared in advance of stock solution preparation by mixing equal volumes of methanol and water and adjusting pH with *o*-phosphoric acid, 85%.

Serial dilutions of one of the stock solutions, for preparation of calibration standards, led to solutions of 100 μg/ml and 10 μg/ml. Calibration standards covering the concentration range between 25 and 2500 ng/ml were prepared by adding appropriate volumes of these diluted solutions to drug-free human plasma. Nine calibration concentrations were used to define the standard curve (25, 50, 100, 250, 500, 1000, 1500, 2000 and 2500 ng/ml). The second stock solution was used for the preparation of quality control standards (80, 1200 and 2200 ng/ml) which were prepared in drug-free plasma. All calibration and quality control standards were divided into polypropylene micro-centrifuge tubes as 150 μl aliquots and frozen at −20°C until assay.

A stock solution of internal standard (**II**) was prepared at 500 μg/ml in methanol and was diluted to 2 μg/ml in 50% methanol for use during sample preparation.

2.6. Sample preparation

Solid-phase extraction cartridges (C_{18}, 1cc, 100 mg) were conditioned sequentially with methanol (1 ml) and water (1 ml). An aliquot (100 μl) of the plasma sample was added to the cartridge and was allowed to pass through the bed with minimal suction. A water aliquot (1 ml) and internal standard solution were added to the cartridge (addition of internal standard to the water-filled cartridge, instead of the plasma, eliminates precipitation of plasma proteins by the methanolic internal standard solution). The cartridge bed was suctioned dry prior to washing. The bed was then washed with 50% methanol (1 ml) followed by 60% methanol (100 μl). After air-drying, **I** and the internal standard were eluted with methanol (500 μl). The eluent was evaporated to dryness under a nitrogen stream at ambient temperature, the residue was reconstituted in 50% methanol (100 μl) and an aliquot (80 μl) was injected onto the HPLC system.

2.7. Instrumentation/HPLC conditions

The HPLC system consisted of a Hewlett-Packard (Palo Alto, CA, USA) Model HP1050 quaternary pump and a Hewlett-Packard HP1050 Series 'C' variable wavelength UV detector, operating at 210 nm. Injections were facilitated via a manual, six-port Rheodyne (Cotati, CA, USA) injector valve system. Reversed-phase liquid chromatography was performed at ambient temperature using a Microsorb MV 5 μm (100 Å) C_8 analytical column, 250 mm× 4.6 mm I.D. (Rainin Instrument, Woburn, MA, USA). A Lichrosorb 100 RP-8 (5 μm) in-line guard column was obtained from Hewlett-Packard. The mobile phase consisted of acetonitrile–10 m*M* potassium dihydrogen phosphate (adjusted to pH 3.1 with *o*-phosphoric acid, 85%) at a ratio of 40:60, v/v. The mobile phase was filtered through a 0.2-μm membrane prior to use and was kept under continuous helium sparge to minimize baseline noise caused by solvent out-gassing. The flow-rate was 1.5 ml/min.

2.8. Calibration and calculation

Within each daily validation assay, a set of nine calibration standards was processed together with six quality control standards from each of the three concentration levels. Standard curve parameters were obtained from a weighted ($1/\text{concentration}^2$) least-squares linear regression analysis of the peak height ratio of drug to internal standard versus the prepared concentration of the calibration standards. The actual concentration of the standards (calibration and quality control) was then interpolated using these stan-

dard curve parameters and was compared to expected concentration.

2.9. Cross-validation

Following successful completion of the validation, assays to compare standards prepared with capsule formulation with standards prepared from pure standard were performed. Replicate extracts of quality control standards (at each concentration) prepared from capsule powder were analyzed with calibration standards prepared from pure **I**, and vice versa.

3. Results and discussion

3.1. Confirmation of identity and purity of indinavir from capsule formulation

Due to lack of pure standard availability, the initial validation of the assay for quantification of **I** in human plasma utilized indinavir sulfate obtained from the commercially available capsule formulation. The quantity of indinavir sulfate in the capsule powder was calculated as a percentage of the formulation. This percentage and a conversion factor (sulfate to free base) were used in the determination of solution concentrations for **I**.

The use of the commercially available capsule for solution preparation included excipients to the solution, necessitating verification of identity and purity of the peak assigned to **I** in the HPLC chromatogram. Mass spectral analysis of a fraction of this peak clearly showed a strong molecular ion peak at m/z 614, corresponding to the molecular mass of **I**.

In addition to peak identity, the verification of peak homogeneity for **I** was critical in the use of this procedure for quantification of **I** in unknown patient samples. Peak purity analysis of the target peak using HP Chemstation software had demonstrated conclusively that the peak obtained at the target retention time (previously identified via mass spectrometry as **I**) was greater than 99% pure. In addition, TLC analyses following HPLC separation of the target analyte produced a single spot (R_F = 0.55). Subsequent analysis of the recovered spot resulted in a single peak on the HPLC chromatogram, at the expected retention time.

3.2. Chromatographic system

Representative chromatograms of blank and spiked plasma samples are illustrated in Fig. 2. The chromatographic conditions as outlined above resulted in retention time windows of 5.2–5.8 min for **I** and 12.2–13.4 min for **II** (n=190). To eliminate potential interference from a late-eluting plasma peak in subsequent injections, the assay run time was extended to 19 min.

The aqueous solubility of **I** is known to be pH dependent [5]. It has been reported that **I** is soluble up to 60 mg/ml at pH 3.5, but only up to 0.3 mg/ml at pH 4.8 [6]. Therefore, the mobile phase composition used in this procedure was selected to facilitate a rapid elution of **I** with adequate resolution

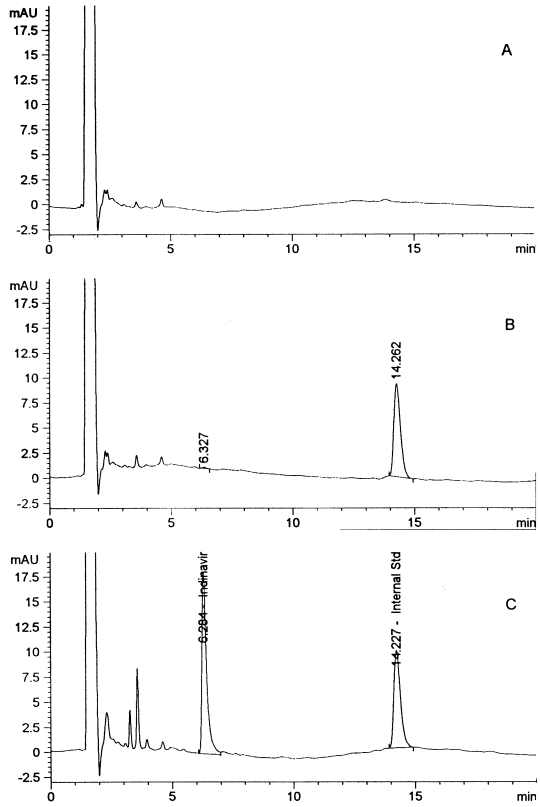


Fig. 2. Representative chromatograms of human plasma samples. (A) Blank plasma; (B) blank plasma spiked with 25 ng/ml of indinavir (lower limit of quantification) and internal standard; (C) blank plasma spiked with 2500 ng/ml of indinavir (upper limit of quantification) and internal standard.

from the solvent front, in addition to resolution from internal standard and potentially interfering endogenous plasma components.

The ultraviolet spectrum for **I** shows a λ_{max} at 260 nm but this wavelength proved to be unsuitable for this assay due to an inadequate sensitivity of the methodology.

3.3. Stability

HIV-infected patient samples are routinely heated at 58°C to inactivate the virus prior to handling. Heat deactivation stability studies were performed to verify the stability of **I** in plasma under these conditions. A set of quality control standards (80 and 2200 ng/ml, six replicates per concentration) was incubated at 58°C for 4 h. These samples were extracted simultaneously with an equivalent set of freshly thawed quality control standards. All standards were then assayed by the HPLC method previously described. The results show a minimal increase in concentration of **I** in the test samples (16.4% and 2.0% for low and high concentrations, respectively).

An additional stability test was performed to verify the stability of **I** (and the internal standard) in the injectate while awaiting HPLC analysis. Six aliquots of quality control standard at two concentrations (80 and 2200 ng/ml) were extracted, reconstituted with 50% methanol, and left on the laboratory bench top at room temperature for a minimum of 44 h. These samples were then analyzed along with a set of freshly extracted standards of the same concentrations. The results show minimal change in response for the test samples (4.9% and −1.5% for low and high concentrations, respectively).

The stability of **I** during sample handling was verified by subjecting samples to freeze–thaw conditions. A set of quality control standards (80 and 2200 ng/ml, six replicates per concentration) was subjected to three freeze–thaw cycles. These samples were then assayed along with an equivalent set of previously unfrozen standards. The results show minimal change in the concentration of **I** in the test samples (2.9% and 2.7% for low and high concentrations, respectively).

The data, presented in Table 1, demonstrates that **I** is very stable under routine sample handling conditions.

3.4. Selectivity

Plasma samples routinely pose a problem with assay selectivity, especially at low wavelength, due to the presence of UV-absorbing substances that survive the extraction procedure. However, analysis of six independent plasma sources demonstrated the lack of interference with the quantification of **I** extracted from plasma.

A review of recent data has demonstrated that long-term monotherapy using protease inhibitors is

Table 1
Stability of indinavir in human plasma[a]

	Expected (PHR) (n=6)	Found (PHR) (n=6)	R.S.D. (%)	Deviation (%)
Heat inactivation				
Low concentration	0.101	0.117	1.5	+16.4
High concentration	2.818	2.876	1.6	+2.0
Storage at room temperature				
Low concentration	0.101	0.106	5.7	+4.9
High concentration	2.818	2.775	1.3	−1.5
Three freeze–thaw cycles				
Low concentration	0.103	0.106	1.5	+2.9
High concentration	2.925	3.003	3.5	+2.7

[a] Abbreviations used: PHR=peak height ratio; R.S.D.=relative standard deviation.

inadequate for the treatment of AIDS, due to the emergence of viral resistance [11]. Potential antiretroviral agents used in combination therapy with indinavir including reverse transcriptase inhibitors (AZT, D4T, 3TC, ddI, ddC, nevirapine) and other protease inhibitors (saquinavir, nelfinavir, ritonavir, amprenavir) were also analyzed and were verified to be chromatographically resolved from **I** and internal standard.

3.5. Limit of detection and quantification

The limit of detection of **I**, defined by relative standard deviation (R.S.D.) less than 20%, was determined to be 0.4 ng on column (R.S.D.=19.0%, $n=6$). This corresponds to a concentration of 5 ng/ml for an 80 μl injection. The limit of quantification (LOQ) for this assay was set to 25 ng/ml and replicate injections gave an intra-assay R.S.D. of 8.4% ($n=6$). The inter-assay variability at this concentration was determined over the five day validation process to be 2.4%. A chromatogram of an LOQ sample is shown in Fig. 2.

3.6. Linearity

Weighted ($1/\text{concentration}^2$) least-squares linear regression calibration curves, obtained by plotting the peak height ratio of **I**/**II** versus calibration standard nominal concentration yielded coefficients of determination consistently greater than 0.99 over the concentration range 25 to 2500 ng/ml (Table 2).

3.7. Recovery

Extraction recovery of **I** was determined by comparing peak height ratios of extracts of plasma spiked with **I** (reconstituted with internal standard in 50% methanol) with non-extracted standards. The mean recovery of **I** after solid-phase extraction was 91.4% (R.S.D. 15.5%) over the entire concentration range (Table 3). However, the data shows that this calculation is skewed slightly due to improbably high values at the low end of the standard curve. Inspection of the data shows that extraction recovery can be more accurately estimated to be between 82 and 89%.

Table 2
Regression parameters calculated from calibration standard curves

Slope	Intercept	Coefficient of determination (r^2)
0.0013	0.0003	0.9927
0.0013	0.0050	0.9951
0.0013	−0.0030	0.9938
0.0012	0.0044	0.9976
0.0013	0.0083	0.9942

Table 3
Extraction recovery of indinavir from human plasma

Concentration (ng/ml)	Recovery (%)
25	127.3
50	96.5
100	82.2
250	81.8
500	85.5
1000	84.8
1500	87.6
2000	88.9
2500	88.4
Mean	91.4
R.S.D. (%)	15.5

3.8. Intra- and inter-day precision and accuracy

Five calibration standard curves were assayed during the course of this validation procedure. Quality control (QC) standards ($n=6$) at three concentrations of **I** were analyzed with each curve. Concentrations were calculated from the daily standard curve regression parameters.

The inter-assay accuracy and precision data for the calibration standards is shown in Table 4. The R.S.D. for the calibration standards ranged from 1.4% to 9.7% with accuracy within 7.0% across the curve range.

Over the five days, intra-assay R.S.D.s for the quality control standards ranged from 5.9 to 13.4%, from 2.7 to 12.1% and from 3.4 to 4.7% for the low, mid and high concentrations, respectively. Representative intra-assay precision data (from one of the five assay days) is shown in Table 5.

Inter-assay R.S.D.s for the quality control standards were 15.8%, 6.7% and 4.6% for low, mid and high concentrations, respectively. Quantification was

Table 4
Inter-assay reproducibility from calibration standards ($n=5$)

Nominal concentration (ng/ml)	Calculated concentration (ng/ml)	R.S.D. (%)	Deviation (%)
25	25.6	2.4	+2.5
50	46.5	4.8	−7.0
100	101.2	8.8	+1.2
250	251.4	4.7	+0.6
500	480.9	1.4	−3.8
1000	1034.1	9.7	+3.4
1500	1469.0	2.8	−2.1
2000	1974.0	3.0	−1.3
2500	2519.0	7.1	+0.8

Table 5
Intra-assay reproducibility from quality control standards ($n=6$)

Nominal concentration (ng/ml)	Measured concentration (ng/ml)	R.S.D. (%)	Deviation (%)
80	83.9	6.4	+4.9
1200	1259.3	4.2	+4.9
2200	2347.3	3.5	+6.7

accurate within 15% of the expected concentration (Table 6).

3.9. Cross-validation

The validation procedure described above for the quantification of **I** in human plasma utilized a mixed powder for compound **I**. The presence of undesired excipients was accounted for during the determination of solution concentration, to ensure accurate quantification of the analyte. HPLC isolation of **I** from these capsule excipients was utilized to produce a spectrally pure peak. At the time of validation, a source of pure **I** was not available. Subsequent to the validation, pure standard of **I** was made available by Merck Research Laboratories.

Comparative assay of capsule-source standards and pure-source standards with standard curve vs. QC standard crossover demonstrated a difference of less than 10% in the quality control standard concentrations for the two preparations (Table 7).

3.10. Application to biological samples

Using this novel HPLC methodology, indinavir plasma levels were determined following oral administration of 800 mg doses of Crixivan in AIDS patients enrolled in a large-scale population pharmacokinetic study within the AIDS Clinical Trials Group (ACTG) network. More than 250 patient samples were rapidly processed and representative chromatograms of patient plasma samples are shown

Table 6
Inter-assay reproducibility from quality control standards

Nominal concentration (ng/ml)	Replicates	Measured concentration (ng/ml)	R.S.D. (%)	Deviation (%)
80	28	91.8	15.8	+14.8
1200	30	1236.1	6.7	+3.0
2200	30	2303.0	4.6	+4.7

Table 7
Cross-validation of capsule-source standards: accuracy & precision of QC standards

	Measured concentration (ng/ml)	% Nominal	R.S.D. (%)
(i) Capsule-source calibration standards vs. pure-source QC standards			
Nominal concentration; pure source (ng/ml)			
79	72.8	92.3	4.9
1184	1078.6	91.1	4.2
2170	2143.0	98.8	1.8
(ii) Pure-source calibration standards vs. capsule-source QC standards			
Validated concentration; capsule source (ng/ml)			
92	96.7	105.4	1.9
1236	1268.2	102.6	2.5
2303	2327.6	101.1	4.5

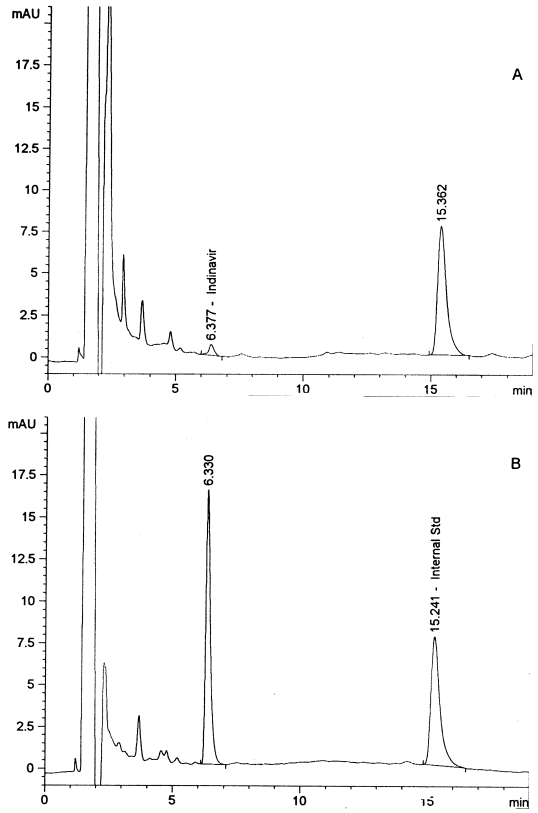


Fig. 3. Representative chromatograms of HIV-infected patient samples. (A) Eight hours post-dose (trough); measured concentration=80.2 ng/ml; (B) 1 h post-dose (peak); measured concentration (assayed as dilution)=7968.7 ng/ml.

in Fig. 3. It was determined during the routine assay of these patient samples that peak concentrations may exceed the upper limit of quantification for this assay. In these instances, patient sample dilution and re-assay was performed to provide chromatographic responses within the standard curve range.

4. Conclusions

Indinavir, a protease inhibitor, is currently prescribed for the treatment of HIV infection. We have developed a methodology which can measure the concentration of indinavir (**I**) in human plasma rapidly, specifically, precisely and accurately. The process requires a very small sample volume (100 μl) which allows the analysis of pediatric samples where blood volumes are limited. The simple extraction procedure and isocratic HPLC conditions combined with a modest run time (19 min) provide an assay well suited for real time analyses with as many as fifty samples easily processed within a single assay. The assay has been completely validated with respect to precision, accuracy, stability, selectivity, limit of detection and quantification, recovery and linearity. It has been successfully applied to clinical samples from patients infected with HIV.

Acknowledgements

This work was supported by a Pharmacology Core ATL grant provided by the AACTG. The authors wish to thank Dr. Xiao-Jian Zhou for his invaluable assistance during the course of this work. In addition, receipt of pure standard from Merck Research Laboratories enabled cross-validation of this work and is gratefully acknowledged.

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