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Determination of lopinavir cerebral spinal fluid and plasma ultrafiltrate concentrations by liquid chromatography coupled to tandem mass spectrometry

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Abstract

A method for the determination of lopinavir (LPV) concentrations in cerebral spinal fluid (CSF) and plasma ultrafiltrate (UF) was developed and validated to analyze clinical specimens from patients receiving antiretroviral treatment with lopinavir/ritonavir. The CSF (400 μL sample volume) final calibration range for LPV was 0.313–25.0 ng/mL. The final calibration range for UF (50 μL sample volume) was 1.25–100 ng/mL. The samples were prepared using liquid–liquid extraction, concentrated, and analyzed using a reversed phase isocratic separation. Detection was achieved in positive mixed reaction monitoring mode on a triple quadrupole mass spectrometer. Isolation of LPV through chromatographic separation and proper selection of calibration matrix were important factors in achieving accurate results. Plasma UF was found to be an equivalent calibration matrix to CSF whereas plasma matrix produced a positive bias in samples with unknown concentrations. Artificial CSF media prepared chemically were biased and less superior than UF. Sources of plasma for the UF did not affect accuracy. Several CSF sources were tested for specificity of the method and LPV concentrations were accurately produced with atmospheric pressure chemical ionization source producing more accurate results than the electrospray source. The method successfully measured LPV concentrations in CSF that were previously undetectable by HPLC as well as UF from protein binding studies.

Keywords: Cerebral spinal fluid; Ultrafiltrate; Protein binding; Lopinavir; Chromatography coupled to mass spectrometry

1. Introduction

Lopinavir (LPV)-based antiretroviral therapy (ART) is one of multiple HIV-1 protease inhibitors that is in use as an effective therapy to reduce the plasma HIV-1 viral load below the limit of detection [1,2]. However, limited research has been done describing the pharmacokinetics of lopinavir in the central nervous system (CNS). The CNS is described as a protected compartment due to factors that limit the distribution of protease inhibitors across the blood–brain barrier such as high plasma protein binding and membrane efflux transporters [3]. Cerebral spinal fluid (CSF) drug concentrations and viral load do not always correlate with the plasma compartment allowing the CNS to act as a sanctuary for viral replication resulting in evolving patterns of resistance that are different compared to the plasma compartment. These observed low CSF protease inhibitor (PI) concentrations may also contribute to HIV-associated cognitive impairment [4]. Therefore, there is a need to develop accurate analytical methods that can be used to further our understanding of plasma/CSF pharmacokinetics for HIV-1 PIs that highly protein bound such as lopinavir [3].

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The measurement of PIs in sanctuary sites provides a challenge for analytical researchers. In addition to sample matrix limitations, the low PI concentration approaches the edge of our current technical capabilities [5,6]. Previously, the lowest measurable lopinavir concentration in CSF was 50 ng/mL. Several additional publications have cited lower CSF LPV limits of 10 and 3.7 ng/mL, respectively [7,8]. These methods utilize prior plasma PIs assays. None of these publications provide data on CSF matrix equivalency, suitability or accuracy.

The free concentration of LPV has been measured and reported by several researchers [3,7]. Boffito et al. reported LPV protein binding in 23 patients receiving 400 mg LPV/100 mg ritonavir twice daily [3]. The method used measured both ultrafiltrate and plasma concentrations from these patients, but little data was presented on the details of the methodology, such as choice of calibration matrix or standard curve preparation and range. Therefore, our objective was to develop and validate a sensitive and specific method for the measurement of lopinavir in CSF and UF by liquid chromatography coupled to tandem mass spectrometry.

2. Materials and methods

2.1. Chemicals and reagents

Lopinavir was obtained from the NIH Reagent Program (Germantown, MD). (5S,8S,10S,11S)-9-hydroxy-2-cyclopropyl-5-(1-methylethyl)-1-[2-(1,methylethyl)-4-thiazoly]-3,6-dioxo-8,11-bis(phenylmethyl)-2,4,712-tetraazatrid acan-13-oic acid, 6-thiazolylmethylester (A86093) was kindly provided by Abbott (Chicago, IL). Atazanavir (ATV) and nelfinavir (NFV) were obtained from the NIH reagents program (Germantown, MD). A correction for purity was applied during the weighing of material for the preparation of stock solution if necessary. Acetonitrile, ammonium acetate, HPLC grade water and HPLC grade methanol were obtained from Fisher Scientific (Fair Lawn, NJ). Acetic acid, ethyl acetate, hexane and sodium hydroxide were obtained from VWR (South Plainfield, NJ).

All chemicals used for this method were of reagent grade or better.

The mobile phases consisted of 5 mM ammonium acetate buffer, pH 3.5, adjusted pH with glacial acetic acid, mixed with acetonitrile. Mobile Phase A was 95:5 buffer:acetonitrile; mobile phase B was 5:95 buffer:acetonitrile. All lots of heparinized human plasma were purchased from Valley Biomedical (Knoxville, TN). Sources of cerebral spinal fluid were obtained through an institutional review board approved protocol allowing residual clinical CSF samples to be donated for research purposes. None of these patients were receiving LPV.

2.2. Apparatus

Validation was conducted using an Agilent high pressure liquid chromatography (HPLC) system (Agilent Technologies, Palo Alto, CA): Agilent Series 1100 Autosampler, Agilent Series 1100 Degasser and Agilent Series 1100 LC Pumps. The HPLC system was coupled to an Applied Biosystems PE/Sciex API 3000 Mass spectrometer (MS) (Applied Biosystems, Foster City, CA) and controlled by Analyst Software, Version 1.4 (Applied Biosystems). The output data was collected with the same program.

The chromatographic separation was conducted at ambient temperature using a Waters Symmetry® C18, 2.1 by 30 mm (Milford, MA) shielded by a guard column, 2.1 by 10 mm of the same material and manufacturer. The flow rate of the mobile phase was held at 350 μL per minute. A six and one half minute isocratic separation at 59% mobile phase A and 41% mobile phase B was utilized (composition described previously). After analyte and internal standard (IS) elution the % B was changed to 90% for 1 min to flush the column and the column was then re-equilibrated for three and one half minutes prior to the next injection. The mass spectrometer was operated in the mixed-reaction-monitoring (MRM) positive ion mode using an Atmospheric Pressure Chemical Ionization (APCI) or Turbo Ionspray® (ESI) interface. When the APCI source was used, the desolvation temperature of the interface was 400 °C and the needle current was 3 μA. When the ESI source was used, the desolvation temperature was set at 400 °C and the sprayer voltage was set at 5000 V. Nitrogen was used as the desolvation, nebulizer and collision gas. The detection of LPV and its internal standard, IS was monitored in MRM as 629.5/447.6 and 747.5/322.3, respectively [9]. Fig. 1 displays the fragmentation pattern of LPV and the IS (5S,8S,10S,11S)-9-hydroxy-2-cyclopropyl-5-(1-methylethyl)-1-[2-(1,methylethyl)-4-thiazoly]-3,6-dioxo-8,11-bis(phenylmethyl)-2,4,712-tetraazatrid acan-13-oic acid, 6-thiazolylmethylester.

2.3. Preparation of calibrators and quality control solutions

Calibrators were prepared by dissolving 5 mg of reference grade LPV powder in 5 mL of methanol to create 1 mg/mL stock. Subsequently, the stock was diluted with methanol to prepare working standard solutions at 100, 50, 25, 12.5, 5.0, 2.50 and 1.25 ng/mL. Calibrators for CSF experiments were prepared by adding 100 μL of working standard solution to 400 μL matrix:100 μL plasma (v/v) resulting in a calibration range of 0.25–20 ng/mL for the matrix mix or 0.313–25 ng/mL for CSF alone. Calibrators for UF experiments were prepared by combining 50 μL blank UF with 50 μL working standard solution resulting in a calibration range of 1.25–100 ng/mL.

Matrices for artificial CSF matrix equivalents 1 (ART CSF A) and 2 (ART CSF B) were prepared as indicated by Stuart [10]. UF medium was prepared by centrifuging 1 mL aliquots of plasma with Millipore Centrifree™ devices as directed by the manufacturer. All UF prepared was pooled for use except when lot specific plasma was tested.

Separate 1 mg/mL LPV stocks were prepared for quality controls (QC). Quality Controls were prepared by dissolving 5 mg of reference grade LPV powder in 5 mL of methanol to create 1 mg/mL quality control stock and subsequently diluting the stock with methanol to prepare QC spiking solutions at 5000,

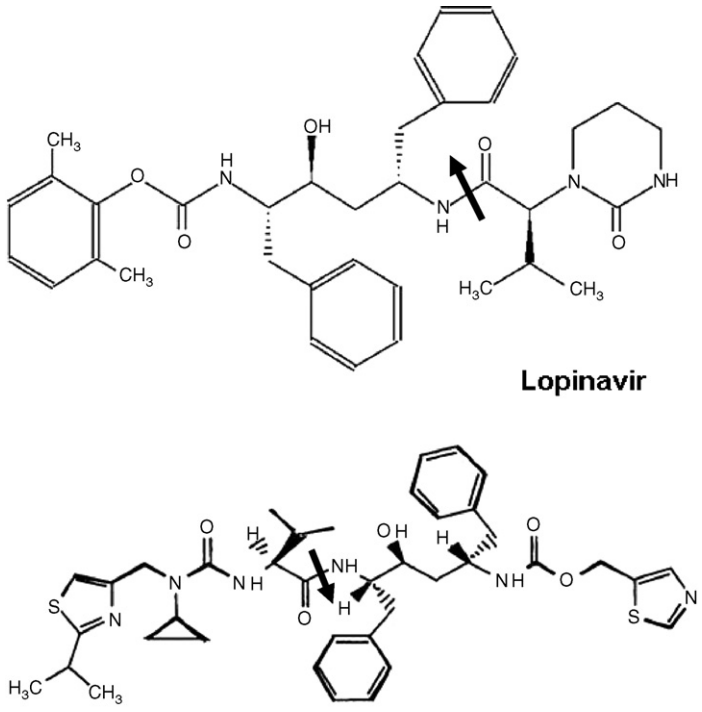

Fig. 1. Structures of lopinavir and internal standard (5S,8S,10S,11S)-9-hydroxy-2-cyclopropyl-5-(1-methylethyl)-1-[2-(1,methylethyl)-4-thiazoly]-3,6-dioxo-8,11-bis(phenylmethyl)-2,4,712-tetraazatrid_acan-13-oic acid, 6-thiazolylmethylester. Arrows depict fragmentation.

500 and 50 ng/mL. High, middle and low QC were prepared in 10.0 mL volumetric flasks with the blank matrix; the total volume of spiking solution to matrix was ≤1.2%. The final concentration of LPV in the QC was 0.6, 1.6 and 16 ng/mL. For UF quality controls, no plasma was added. UF quality controls were prepared at five-fold greater concentrations than CSF quality controls, 3, 8 and 80 ng/mL.

To prepare an IS A86093 was prepared in methanol stock, and diluted to 5 ng/mL in methanol for a working solution. All stocks, calibrators and controls were stored at −70 °C.

2.4. Liquid–liquid extraction

Calibrators, standards, QC and blanks were prepared for liquid–liquid extraction by first adding blank heparinized plasma to the matrix (previously described in calibrator and QC preparations above) and vortexing well. For patient CSF samples, 0.1 mL of methanol and 0.1 mL of blank plasma [11] was added to 0.4 mL CSF and vortexed. IS (200 μL) was added to samples, calibrators and QC, followed by 0.5 mL 0.1 M NaOH, and vortexed well after each addition. Five milliliters of 25:75 hexane to ethyl acetate was added to the sample mix, shaken for 25 min, centrifuged at 2800 × *g* for 15 min, and the top organic layer (extract) was retained. The extract was then evaporated to dryness under air in a water bath at 50 °C for 30 min, reconstituted with 100 μL, vortexed for 15 s, transferred to a microcentrifuge, and spun at 6660 × *g* for 5 min to remove any particulates. Reconstituting solution consisted of 59:41 mobile phase A:mobile phase B (v/v). Twenty microliters injections were required for analysis.

2.5. Development studies

2.5.1. Calibration matrix

To determine if a suitable matrix alternative to CSF was obtainable, calibrators were spiked into two laboratory-prepared artificial CSF mediums prepared as referenced above (ART CSF A, ART CSF B) and UF medium. Six separate CSF lots were also spiked with one calibrator concentration to determine which matrix based calibration curve would produce the most accurate results for the CSF samples. To verify equivalence of the best matrix for CSF concentration determination, plasma and other matrix alternatives were spiked with calibrators and assayed. Results were compared to one another using each separately as the basis of the calibration curve and calculating the percent accuracy of the others.

2.5.2. Recovery and matrix effects

To determine recovery and matrix effects several lots of CSF ($n = 6$) were tested to determine whether endogenous interferences or matrix suppression or enhancement would occur and if matrices independent of the calibrators and validation samples would provide accurate results. To accomplish this, seven extracts of each matrix were prepared: three spiked with LPV and IS before extraction (pre-spike); three spiked with LPV and IS after extraction (post-spike); and one was left as blank. Actual analyte concentrations in the different sources of matrix were calculated as unknowns using the UF calibration curve. Percent recoveries for were calculated by averaging the peak areas of post- and pre-spike replicates for each matrix separately and dividing the mean pre-spike result

by the mean post-spike result for each matrix. To determine the effect of matrix alone, mean responses from triplicate injection of analyte in mobile phase were compared to the mean response of the three plasma samples spiked after extraction. During development three chemicals were added as internal standards to determine which would be best for LPV detection and quantitation: A86093, ATV and NFV. To determine a suitable internal standard the response ratios (LPV response to IS response) and the final accuracy in matrices were compared.

2.6. Validation studies

One day of calibration with triplicates of each level of QC were tested to determine accuracy of the QC preparations. Three days of calibration curves, with six replicates of each of three QC levels were performed to determine intraassay variation and accuracy. An additional day of triplicate QCs was performed with unknowns. The criteria acceptability of ±15% was used for accuracy and precision for all results.

System suitability was always checked prior to sample analyses by evaluating four injections of a test solution for chromatography performance. Quality controls were dispersed throughout the analyses to assess and address drift.

Test CSF samples were prepared at high and low concentrations, then frozen and stored at −70 °C. Stability experiments included three freeze–thaw cycles [from −70 °C to room temperature] and room temperature stability was accessed under normal laboratory conditions for 18 h in polypropylene tubes. The control group (untreated) was thawed only once and immediately assayed. Results for the treated groups were compared to the untreated group using an unpaired *t*-test. Processed sample stability was examined by allowing the freshly analyzed samples to be stored at 4 °C for 96 h and then analyzing samples. After validation this method was applied to CSF samples from patients receiving Kaletra®.

2.7. Analysis of patient CSF samples

After this method was successfully validated, a standard operating procedure was constructed based on the validation parameters and sample limitations derived from the validation process. One sample was drawn from each of 10 patients after receiving lopinavir/ritonavir (400/100 mg). Samples were collected at approximately 10 h after therapy was administered. CSF was aliquoted to cryovials and immediately frozen at −70 °C. All samples were subsequently analyzed for LPV concentrations. Another eight samples from a previously published study where LPV was determined as undetectable were also analyzed [12]. The initial analyses were performed in 2001 and the samples were stored at −70 °C in the interim time. It should be noted that the stability of these specimen over the 5 years is not known nor is there any data in the literature to support their stability. The intent, however, was determine if samples that were previously undetectable could be detected using this more sensitive method.

2.8. Analysis of UF samples

Plasma was spiked to contain 10 μg/mL of LPV. Three replicates of the spiked plasma were ultra-filtered using CentriFree® devices at 37 °C as instructed by the manufacturer after a water wash of the membrane. The plasma samples were assayed in triplicate using a previously reported HPLC method to measure accurately the LPV concentration of the spike [11]. The free LPV concentrations in the three ultrafiltrates were assayed in triplicate using only 0.1 mL of the volume per extract. The UF concentration values determined in triplicate were averaged and a standard deviation determined.

2.9. Calculations

Statistical tests for significance were performed using Minitab ™ software (Version 14, State College PA). Percent target was calculated as a percent of observed concentration divided by target concentration. Variability or coefficient of variation (CV), was calculated as a percent of the relative standard deviation from the mean. Calibration curves and calculations of unknowns or controls were calculated using Analyst™, Version 1.4 (Applied Biosystems). Calibration curves used a $1/x^2$ weighting with a linear fit. The LOD was calculated each day by dividing five times the concentration of the calibrator used by the ratio of the height of the lopinavir peak to baseline signal-to-noise height.

3. Results

3.1. Optimization of method

The assays optimized characteristics are shown in Table 1. A86093 was chosen as the best IS because it produced the most

Table 1
Optimized assay characteristics

Parameter	Variables
Chromatography Reversed phase isocratic	*Column* Waters Symmetry® C18, 2.1 by 30 mm (Milford, MA) *Mobile phase* Acetonitrile: 5 mM acetate buffer, pH 3.5 41:59
Tandem mass spectrometry detection	MRM mode APCI source (conditions) Desolvation temperature: 400 °C Needle current: 3 Ua Source and collision gas: nitrogen LPV: 629.5/447.6 Internal standard: 747.5/322.3
Limit of detection (ng/mL)	<0.1
Limit of quantitation (ng/mL)[a]	0.3125 Cerebral spinal fluid[a] (CSF) 1.25 Plasma ultrafiltrate (UF)
Range of quantitation (ng/mL)	0.3125–25.0[a] CSF 1.25–100 UF
Calibration matrix Control matrix	UF CSF

[a] Adjusted for sample volume.

accurate results in all tested matrices (95 ± 8%) as compared to others tested (data not shown). Although the LOD was fairly low at <0.1 ng/mL each day, the LOQ of 0.25 ng/mL was chosen due to unacceptable variability at the 0.1 ng/mL calibration standard tested during the first day of validation (data not shown). The chosen calibration matrix was plasma ultrafiltrate for reasons explained below.

3.2. Suitability of alternative matrices

The best alternative matrix was determined to be plasma UF for the calibrators. Using UF as calibrator matrix, the mean of 6 CSF lots' accuracy was 101 ± 8% versus 81 ± 6% for ART A and 94 ± 7% for ART B. While ART B provided a mean accuracy within ±15%, the results were negatively biased. Using plasma as a calibration matrix, UF tested 113 ± 7% accurate and CSF tested 116 ± 4% accurate, indicating a positive bias or that plasma matrix created a suppression of the LPV response. When two separate sources of plasma were used to prepare UF and tested against the calibrator UF source, results remained accurate. Lot 1 and Lot 2 averaged 95 and 93% accurate across all calibrator concentrations (range 84–103%) indicating that the source of plasma for UF would not alter the accuracy of the method.

3.3. Matrix testing of CSF lots with two source interfaces for mass spectrometer

Across the six lots of CSF, recovery of LPV was 99% with less variation measured by the APCI source (2% versus 4%). Recovery of A86093 was 84% using the APCI source and the changes in recovery mirrored the changes in LPV recovery more closely as the CSF lots' accuracy results were all within ±15% of target and less variable (8% versus 10%). Electrospray results were positively biased and 3/6 inaccuracies were greater than 15% of the target value.

3.4. Accuracy and variability of calibration standards and quality controls. LOQ and LOD

Calibration curve performances over 5 days were well within acceptable parameters with mean % deviation ranging −10% to +13% and CV of <9%. Coefficients of determination (R2) were greater than 0.993 over the course of validation. The final validated range of quantitation was 0.250–20.0 ng/mL for LPV calibrators for CSF analyses and 1.25–100 ng/mL for UF calibrators. Fig. 2a illustrates the typical chromatographic responses of LPV and the IS at the lowest calibrator level (0.25 ng/mL).

Table 2 summarizes the accuracy and variation accomplished by the method during validation at the LOQ (lowest calibrator) and all quality controls levels. Variation at the lowest calibrator concentration (LOQ) ranged 2–11% over each of the 4 days with a median of 9.5%. Across all days the interassay variation at the LOQ was 10%. Accuracy was within ±5%. The calculated LOD across all 5 days was always less than 0.010 ng/mL, indicating that the LOQ was operated at >25× the LOD.

QC performances within each day (or "intraassay") were acceptable with all mean values within 85–115% of target and CV as low as 1–3% and as high as 9–16%. Across 5 days, which included sample assay days, the interassay variation and accuracy achieved was successful. Variation was 8, 7 and 12% for low, medium and high concentrations, respectively. Accuracy was within ±10%, trending lower than the target concentrations. Fig. 2b provides an example chromatogram for the highest quality control.

3.5. Stability

Experiments during method validation provided no evidence of LPV stability issues for high or low concentration CSF samples after three-times freeze–thaw processes or room temperature exposure for 18 h ($P > 0.05$). The freshly prepared and analyzed QC results were compared to the results for prepared samples that were held at 4 °C for 96 h using a paired *t*-test.

3.6. Analysis of patient CSF samples

This method was successfully used to measure LPV in CSF from patients. Fig. 3 provides an example chromatogram for a patient CSF sample. The CSF sample was reported previously undetectable for LPV [12]. CSF samples concentrations measured from two clinical studies. Table 3 displays the results measured for each patient.

3.7. Analysis of UF samples

The total LPV concentration of the spiked plasma measured 10.4 μg/mL. Fig. 4 shows the chromatogram for this sample. Free concentration of the plasma measured 108 ng/mL or 1.04 (± 0.31)% of the total plasma concentration. These results were equivalent to previously reported patient ultrafiltrate ranges of 0.94 (±0.33)% to 1.05 (±0.32)% at 9.5 and 9.3 μg/mL total LPV plasma concentration, respectively [3].

4. Discussion

Our data indicate that mass spectrometry provides a more sensitive assay for determining LPV in CSF than UV detection. All CSF samples were measurable with mass spectrometry however, since the study 1 samples were frozen for a long duration, additional experiments will be needed to confirm LPV stability in CSF. In addition, matrix effects are a concern when developing a method to accurately measure compounds in biological samples. In fact, the FDA encourages that whenever possible the matrix of the sample be used for the bioanalytical method: "A calibration curve should be prepared in the same biological matrix as the samples in the intended study by spiking the matrix with known concentrations of the analyte" [13]. The data in this report clearly demonstrate a positive bias when CSF concentrations are measured using a plasma calibration standard curve. The use of plasma UF provided the best alternative to using CSF as the calibrator matrix. Use of the plasma UF also allows for

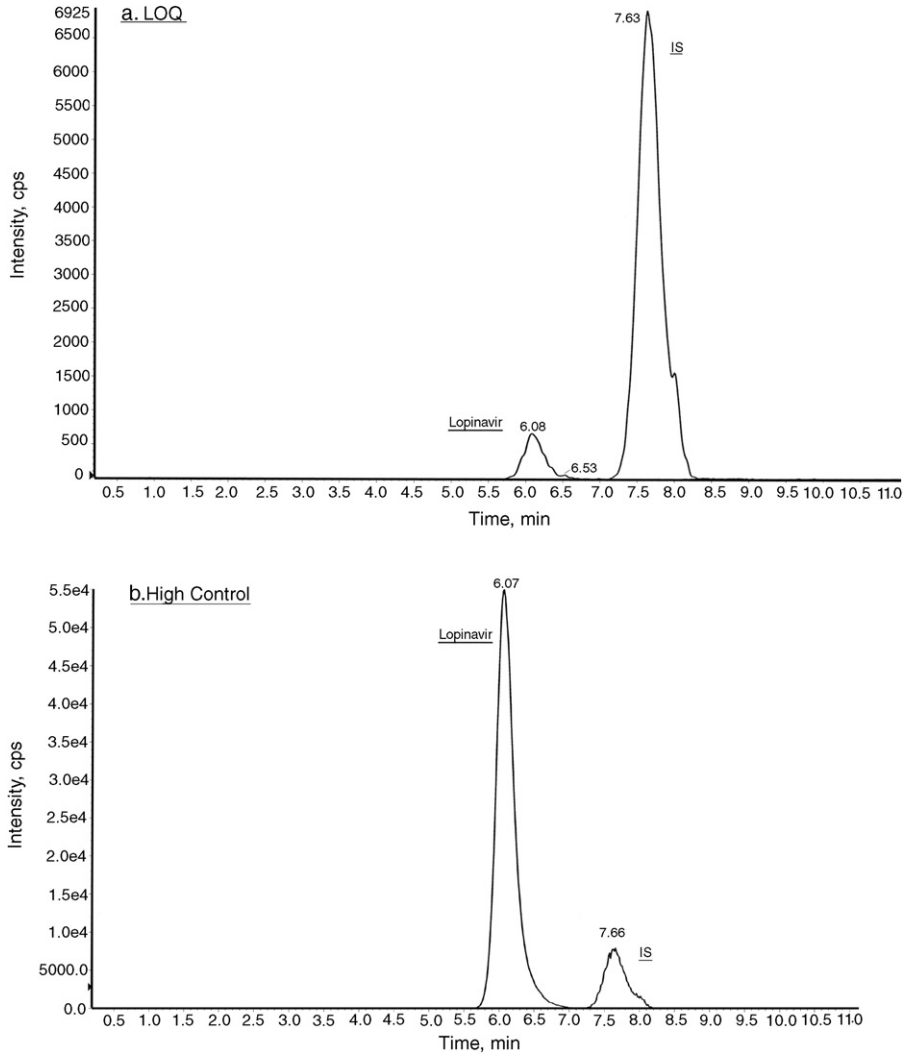

Fig. 2. (a) Chromatogram at lowest calibration standard, 0.25 ng/mL. (b) Chromatogram at high quality control, 16 ng/mL.

the accurate measure of free LPV concentrations by this same method. Data from different lots of plasma UF were equivalent.

The use of a larger sample volume, often available from a lumbar puncture, allows for pre-concentration of the sample and a gain in sensitivity from previously reported limits of quantitation, 3.7 ng/mL [5], 10 ng/mL [6] using tandem mass spectrometry assays. Since lopinavir trough concentrations in plasma are usually >1000 ng/mL and protein binding can be higher than 99%, the concentration of free drug will be low consequently this method also provides an accurate method for measuring plasma UF from clinical specimens. As shown in our analyses, smaller UF volume is sufficient. Therefore, smaller clinical samples for measuring pediatric samples would be allowable with this sensitive method.

The lower results for these measured patient CSF concentrations (as compared to previous literature) may be attributed to the use of a compatible matrix for calibration. The results of this method development and validation show that when plasma is

Table 2
Validation statistics

	LOQ	LQC	MQC	HQC
Target concentration (ng/mL)	0.313	0.600	1.60	16.0
Median of intraassay variation	9.47	2.36	3.72	5.95
Median of intraassay accuracy	+4	−9%	−11%	−5%
Days for intraassay (*n* per day)	4 (5–6)	3 (6)	3 (6)	3 (5–6)
Interassay variation	10.1	7.5	7.4	11.6
Interassay accuracy	+5%	−8%	−10%	−8%
Days for interassay (*n*)	4 (23)	5 (24)	5 (24)	5 (23)

Table 3
Cerebral spinal fluid (CSF) lopinavir concentrations (ng/mL) measured in HIV+ patients

Study 1—Samples drawn between 2 and 7 h after dose (400/100)		Study 2—Samples drawn between 10 and 12 h after dose (400/100)	
Subject no.	LPV (ng/mL)	Subject no.	LPV (ng/mL)
1	<0.10	1	9.49
2	10.5	2	16.4
3	2.88	3	20.9
4	8.06	4	19.3
5	22.2	5	3.00
6	14.8	6	6.76
7	14.0	7	11.2
8	29.5	8	11.2
		9	4.06
		10	16.1

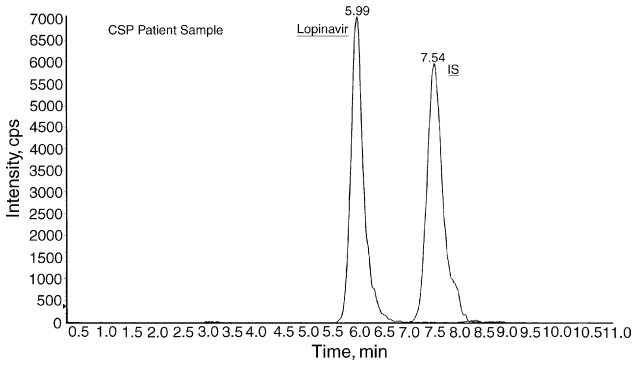

Fig. 3. Chromatogram of CSF patient sample.

used for a calibration matrix, multiple CSF matrices measure 11–22% higher than when a more appropriate matrix is used. This could be due in part to recovery, matrix ion suppression and/or chromatographic separation from co-eluting endogenous and medication compounds [14,15]. The UF results compare well to literature values.

The internal standard structure and behavior adequately provided for the method, however an alternative approach would be the use of a deuterated form of lopinavir. Use of the deuterated form, in theory might also allow for the accurate quantitation of LPV concentration in CSF and UF using a plasma-based calibration matrix. Although the matrix effect of the many components in plasma might lower the sensitivity of the method.

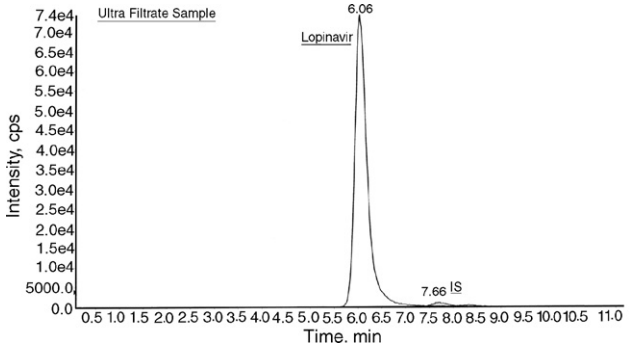

Fig. 4. Chromatogram of plasma ultra filtrate.

5. Conclusion

The method developed accurately measures LPV in CSF and UF samples. This method measured lower CSF values than reported in the literature and UF values in agreement with published results. The use of this method will allow for better modeling of lopinavir into sanctuary compartments.

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