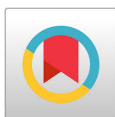RESEARCH ARTICLE

Direct Quantitation of D-serine in Human Plasma by Enantioselective Liquid Chromatography with Tandem Mass Spectrometry and its Application to a Clinical Study

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OBJECTIVES: Develop and validate a simple and rapid LC-MS/MS method for plasma D-serine measurement to support clinical development of a potential D-amino-acid oxidase (DAAO) inhibitor.

METHODS: Calibration standards in phosphate buffered saline (PBS), quality control samples (QCs) in both PBS and human plasma were extracted with methanol. Enantioselective separation of D- and L-serine was on a Regis® ChiroSil RCA(+) column with monitoring underivatized D-serine at m/z 106.1→ 60.1 and (D, L)-serine-d_3 internal standard at 109.0 → 63.0.

RESULTS: The validation showed all calibration curves with a correlation coefficient of $\geq$ 0.9997, and plasma QC samples' inter-run (n = 18) CV of $\leq$ 8.7% and REs from -7.0% to -6.1%. The plasma samples were stable for 6 freeze-thaw cycles, 36.5 h ambient storage, and 769 days at -20°C or lower.

CONCLUSIONS: The validated method was successfully applied to analyze plasma D-serine level in a Phase I clinical trial and the D-serine level was insignificantly impacted by the studied compound.

KEYWORDS: D-serine, human plasma, endogenous biomarker, enantioselective separation.

INTRODUCTION

D-serine, (2R)-2-amino-3-hydroxypropanoic acid, as shown in **Figure 1a**, is a well-known endogenous co-agonist of the N-methyl-D-aspartate-type glutamate receptor (NMDA receptor) that belongs to a family of neurotransmitter receptors in nerve cells and plays key roles in learning, memory and neuroplasticity functions [1-3]. D-serine is a neuromodulator that regulates the activities of neurons. Because of the importance of NMDA receptor in brain activities, D-serine levels in human plasma, cerebrospinal fluid (CSF), and urine are often considered as a biomarker in drug development for neuropathic pain, cognitive deficits by acute stress or impairment
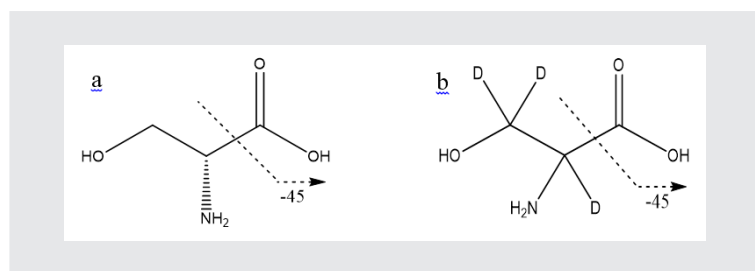
Figure 1. Structures of D-serine and D, L-serine-2,3,3-d₃ (internal standard); (a). D-serine (m.w. 105.09); (b). (D, L)-serine-2,3,3-d₃ (m.w. 108.11)

associated with schizophrenia (CIAS), and Alzheimer's disease (AD) *etc*. [4-7].
Several analytical methods were published for monitoring D-serine levels in various biological matrices [7-27]. In those methods, D-serine was quantitatively measured through either enzymatic assays using D-serine dehydratase [8], enzymatic-HPLC [9], or capillary electrophoresis (CE) [10-14], or chromatographic assays such as GC-MS, LC-UV, LC-fluorescence, and LC-MS/MS [15-27]. In order to improve method selectivity and sensitivity, chemical derivatization with various reagents such as 7-fluoro-4-nitrobenzoxadiazole [17], 9-fluorenylmethyl chloroformate and dansyl chloride [20], 1-fluoro-2-4-dinitrophenyl-5-L-alanine amide (Marfey's reagent) [21, 22, 27], o-phthalaldehyde (OPA), β-mercaptoethanol/OPA, N-tert-butyloxy-carbonyl-L-cysteine/OPA [23], naphthalene-2,3-dicarboxaldehyde (NDA), 4-(dimethylamino) azobenzene-4'-sulfonyl (dabsyl) chloride [24], and (R)-1-boc-2-piperidine carbonyl chloride [25] *etc*., were widely used in the reported D-serine measurement assays. Sample preparation procedures with chemical derivatization are capable of improving separation and ionization efficiency (for mass spectrometric detection) but often time-consuming and require proficient experimental skills. As a result, it often limits the throughput and robustness of an analytical method.
D-serine is a naturally endogenous compound in humans, and as such there are no analyte-free matrices available for the calibration standards and quality control samples preparation. Thus, a typical way to conduct the method validation is unrealistic. To overcome this challenge, different strategies have been employed, including using standard subtraction, or using surrogate matrices or surrogate analytes [26].
In this paper, we present a simple and rapid enantioselective LC-MS/MS method for direct determination of D-serine in human plasma without additional chemical derivatization step. Phosphate buffered saline (PBS) was used as the surrogate matrix for the preparation of calibration standards. The quality control samples (QCs) were prepared in both PBS and in real human plasma matrix. The method was fully validated and then has been applied for sample analysis in a phase I study for SEP-227900, a potential competitive inhibitor of D-amino-acid oxidase (DAAO) for treating chronic neuropathic pain, mentioned in [28].

MATERIALS AND METHODS
Chemicals, materials and apparatus
D-serine, as shown in **Figure 1a**, with a purity > 99% was purchased from Sigma-Aldrich (St. Louis, MO, USA). D, L-serine-2,3,3-d₃ (internal standard, IS), as shown in **Figure 1b**, with a purity > 99% was from CDN ISOTOPES, Inc. (Pointe-Claire, Quebec, Canada). HPLC grade 2-propanol was from J.T. Baker (Center Valley, PA, USA), AR grade ethylene glycol (100%) from Mallinckrodt (Center Valley, PA, USA), formic acid (98+%), HPLC grade methanol from EMD (Gibbstown, NJ, USA) and phosphate buffered saline (PBS, pH 7.4) in foil pouches (Part # P-5368) from Sigma-Aldrich (St. Louis, MO, USA).

Ultra-pure water with resistivity >18 MΩ cm^{-1} was generated by an in-house Milli-Q water purification system (Merck KGaA, Darmstadt, Germany). Blank human plasma with K$_2$-EDTA anticoagulant was purchased from Biological Specialties (Colmar, PA, USA).

A Sciex API 4000 triple quadrupole mass spectrometer (Concord, Canada) was interfaced to a Shimadzu LC10ADVP pump and a SIL HTC autosampler (Kyoto, Japan). ChiroSil RCA(+) 150 x 4.6 mm (5-µm) column from Regis (Morton Grove, IL, USA) was used for enantiomeric separation. Phenomenex C$_{18}$ Security Guard™ cartridge 4.0 x 2.0 mm (5-µm) (Torrance, CA, USA) was also used to protect the chiral separation column. The *Analyst* software version 1.4 from Sciex was used for data acquisition and calculation.

LC-MS/MS conditions

The optimized isocratic mobile phase used in this method was a mixture of 0.2% formic acid in water-0.2% formic acid in methanol at a ratio of 45:55 (v/v). The flow-rate was 0.80 mL/min. The column was operated at ambient temperature (~22 °C). The autosampler was with a cooler set at 5 °C. The injection volume was 10 µL. There was no carryover when the methanol containing 0.2% formic acid was used for injector wash. The per sample run time was about 7 min.

In this method, a turbo ionspray source operated in positive mode (ESI+) was employed to generate the molecular ions for mass spectrometric detection. The multiple-reaction mode (MRM) was used to acquire ion counts of the analyte and the IS at different time points. The optimized source parameters included a high voltage of 5.5 kV for the sprayer, the source temperature of 550 °C, the settings of Gas 1, Gas 2, curtain gas, and collision gas at 60, 60, 30, and 10 (arbitrary scale), respectively. Other optimized conditions included declustering potential (DP) of 34 V, entrance potential (EP) of 6 V, collision energy (CE) of 16 eV, and collision cell exit potential (CXP) of 11 V for both D-serine and the IS. MS/MS detection was by monitoring mass transitions at *m/z* 106.1 [M+H]$^+$→ 60.1 for D-serine and at *m/z* 109.0 [M+H]$^+$ → 63.0 for the IS, respectively. The fragmentations of D-serine and D-serine-d$_3$ were by breakdown of C-C bonds as indicated in **Figures 1a and 1b** and loss of a [H]-CO$_2$H group. The dwell times were set as 500 milliseconds for D-serine and 200 milliseconds for the IS. Both Q1 and Q3 quadrupoles were set at unit resolution.

Standard solutions and quality control (QC) samples

The D-serine stock solutions from two separate weighings were made at 250 µg/mL in methanol-water (1:1, v/v) by sonicating for approximately 15 min. The IS stock solution was made at 100 µg/mL in methanol-water (1:1, v/v) in the same way. The IS working solution was 5.00 µg/mL in methanol-water (1:1, v/v). All stock solutions and working standard solutions were stored in a freezer set at -20 °C. Under this condition, the stability of 293 days for both D-serine and the IS stock solution was confirmed.

D-serine is an endogenous entity in humans. For this plasma method validation, calibration standards were prepared at 100, 150, 200, 500, 1000, 2500, 4000 and 5000 ng/mL of D-serine in PBS. One set of QC samples were prepared at 200 (Low), 300 (mid-Low), 750 (Mid), 1500 (mid-High) and 3750 ng/mL (High) of D-serine in PBS and another set of QC samples were prepared at 394 (Low), 944 (Mid) and 3940 (High) ng/mL of D-serine in human plasma (endogenous + spiked). The endogenous D-serine level in the blank plasma pool used for these plasma QCs preparation was pre-determined to be 194 ng/mL based on the PBS calibration curve. The nominal plasma QC concentrations were then adjusted to account for this endogenous amount. A dilution-QC at 10,200 ng/mL in plasma and a lower limit of quantitation (LLOQ) QC at 100 ng/mL in PBS were also prepared.

Calibration standards and the QC pools were aliquoted into pre-labeled 1.5-mL conical polypropylene vials (approximately 0.3-mL sample per vial) and stored at –20 °C or lower.

These QC samples (both in PBS and plasma) were used for the entire method validation including short-term and long-term sample storage stability evaluation.

Sample preparation

For plasma samples, protein precipitation with methanol was used for sample preparation. Thawed samples were vortex-mixed well, and a 50.0 µL aliquot of each sample was transferred into small conical glass tubes. Both PBS blank and plasma blank were prepared. A 50.0-µL of the IS working solution (5.00 µg/mL) was added to the all samples except for double blanks and reagent blanks where the same volume of methanol-water (1:1, v/v) was added to make up the volume and composition. Then 500-µL of methanol was added to each tube, vortexed for 2 min, and centrifuged at approximately 3,000 rpm for 10 min. The supernatant was transferred into a clean round bottom glass tube containing 50-µL of keeper solution (ethylene glycol in 2-propanol), evaporated the extraction solvent under a stream of nitrogen at 45 °C using a water-bath for 25 mins. The residue was then reconstituted in 500-µL of methanol-water (1:1, v/v) by vortexing 2 min. A 10-µL aliquot of the resulted sample was injected for LC-MS/MS analysis.

Method validation

The validation protocol was established according to the food and drug administration (FDA) Bioanalytical Method Validation guidance for industry [29]. As D-serine is an endogenous biomarker in humans, there was no true negative (analyte-free) blank matrix available, so calibration standards were prepared in PBS buffer and QC samples were prepared in both PBS and human plasma, as described in previous section. During the validation, three analytical runs on three separate days were processed and analyzed for intra-run and inter-run precision and accuracy evaluation. Each run included duplicate sets of calibration standards, six replicates of each level non-dilution QC in PBS and in plasma including LLOQ QCs. One of the validation runs also included the dilution-QC samples for the examination of sample dilution integrity. In such analytical run, six replicates of dilution-QC samples (dilution factor of 5) and six replicates of mid-QC samples (dilution factor of 2.5) were also processed to demonstrate sample dilution linearity. The diluent used for dilution integrity evaluation was blank PBS solution as the blank plasma contained endogenous D-serine.

The short-term stability of the QC samples in both PBS and plasma were tested in one validation run, which included six freeze-thaw cycles from -20 °C to room temperature, and 36.5 h of room temperature (RT) sample stability. The extract stability and reinjection reproducibility were also evaluated. Extract stability of the QC samples was examined against a freshly prepared calibrator curve. Reinjection reproducibility was tested by re-injecting the entire run after a certain period of storage of extracts in the autosampler condition. Long-term frozen plasma QC sample storage stability was evaluated at multiple time points up to 769 days at both -20 °C and -70 °C. Whole blood processing stability was also assessed by fortifying fresh human whole blood with D-serine at low and high quality-control levels then storing for 0.5, 2 or 4 hours at room temperature or refrigerated condition (2 -8 °C) prior to isolating plasma.

Application

Human plasma samples were serially collected from a single ascending dose (SAD) first-in-human (healthy volunteers) study with the signed Inform Consent Form by each subject for an investigational compound SEP-227900. This clinical study consisted of 7 dose cohorts from 1 to 80 mg single oral dose. The study was placebo-controlled and

each dose cohort enrolled 12 healthy male subjects (9 received the active treatment and 3 received placebo). Each enrolled subject had blood sampling from predose through 312 h postdose for pharmacokinetics (PK) and D-serine biomarker measurements. The PK analysis was based on the SEP-227900 plasma concentrations measured by a fully validated LC-MS/MS method. The plasma D-serine measurement as a biomarker for the DAAO inhibition was by a validated enantioselective LC-MS/MS method described in this paper. The urine samples were also collected from this study for both PK and D-serine analyses, but will not be discussed in this report.

RESULTS AND DISCUSSION
Method Development
D-serine is an enantiomer of a chiral molecule serine (**Figure 1a**). To reliably quantify D-serine, an enantioselective baseline separation from L-serine is a must. To fulfill this goal, different chiral columns were screened and LC conditions were optimized during the method development. As a result, Regis® ChiroSil RCA (+) (150 × 4.6 mm, 5-μm) was selected as the analytical column and a mixture of 0.2% formic acid in water (Mobile Phase A)-0.2% formic acid in methanol (Mobile Phase B) at a ratio of 45:55 (v/v) was chosen as the mobile phase for the chromatographic separation. The method was validated under the above optimized conditions. As an often-seen phenomenon, the chiral stationary phase lot-to-lot variation may cause the retention time shifting even with the same manufacturer's products, thus a re-adjusting and fine-tuning chromatographic conditions might be needed when a new column is used. In this study, the analyte retention time shifting due to column variability was also observed later when the method was applied to analyze clinical samples. An acceptable range of mobile phase composition from 45% to 55% of mobile phase B was determined to be effective in compensating for the retention time shift. Depending on the degree of adjustment, an additional partial validation experiment might be needed before the method was used for clinical sample analysis. D-serine is often seen only a small portion (~1-2%) of "total serine" in human plasma samples even lower in the schizophrenia patients [4], therefore a good chromatographic separation between D- and L- serine is extremely important. Moreover, the use of an enantioselective column that could retain L-serine longer than D-serine, i.e. D-serine be eluted first, is quite critical. As L-serine is a dominant enantiomer and its peak tailing, as long as L-serine is eluted after D-serine, would not interfere with D-serine peak measurement and quantitation. Under the optimized chromatographic conditions, D-serine had a retention time of ~3.8 min, and L-serine had a retention time of ~4.8 min, D- and L- enantiomers were nearly baseline separated from each other. Typical chromatograms are presented in **Figure 2**. To minimize the effect of L-enantiomer on the peak integration for D-serine, peak height ratios of the analyte/ the IS were recommended for generating calibration curve and for unknown calculation. If D- and L-serine peaks were well separated on column, then the use of peak area ratio for calculation was equally satisfactory with no significant difference from using peak height ratio.

Method Validation
Specificity
Six lots of blank plasma were tested for the assay specificity. For each lot of plasma, plasma blank, plasma blank spiked with IS, plasma blank spiked with 200 ng/mL of D-serine were used to check interference and lot-to-lot matrix variation. For all six lots of blank plasma, no peaks were detected at the mass transition and retention time of the IS, indicating that (D,L)-serine-d$_3$ could be used as the internal standard for D-serine measurement.

The less abundant peak at the retention time of D-serine was attributable to the endogenous D-serine in human plasma. The drastically more abundant peak at the retention time of L-serine was confirming the observation of the typical serine's enantio-distribution in human plasma. Endogenous D-serine in these six blank plasma samples without IS ranged from 2451 to 4835 counts with a mean of 3652 (CV 23.8% across 6 individual lots) by peak height. The endogenous amounts of D-serine in these plasma samples after spiked with IS were measured against the calibration curve using PBS as the surrogate matrix, and the found D-serine concentrations ranged from 115 to 234 ng/mL. For each of these six lots, the endogenous D-serine concentration was determined as the average concentration measured in six replicates of blank plasma spiked with IS. Across these tested blank plasma samples from six individuals, endogenous D-serine variation showed a CV of 24.5% with a mean concentration of 181 ng/mL. The measured values of the above six lots of blank plasma post-spiked 200 ng/mL D-serine showed a 90.3% recovery of "total D-serine" and a CV of 5.0%, indicating an acceptable assay specificity and matrix lot-to-lot variation. After the specificity evaluation, a larger plasma pool was created by combining multiple individual lots of blank plasma together and then measured in six replicates to obtain a mean endogenous concentration of 194 ng/mL D-serine. This plasma pool and its endogenous level of 194 ng/mL D-serine were used for the preparation of plasma QC samples in this method validation study and the clinical sample analysis presented in this manuscript. Representative chromatograms of the PBS blank, LLOQ (STD1) in PBS (100 ng/mL), Low QC in PBS (200 ng/mL), mid-High QC in PBS (1500 ng/mL) are shown in **Figures 2a, 2b, 2c, and 2d**, respectively. Representative chromatograms of the pooled "blank" human plasma (unspiked, but with endogenous D-serine level of 194 ng/mL), the IS, Low QC in plasma (endogenous + spiked = 394 ng/mL), High QC in plasma (endogenous + spiked = 3940 ng/mL) are shown in **Figures 2e, 2f, 2g, and 2h**, respectively. These chromatograms show that an acceptable enantiomeric separation was achieved, and this was also true for the incurred plasma samples with a relatively small peak of D-enantiomer.

Sensitivity, linearity, recovery and matrix effect

The calibration curve range was 100 to 5,000 ng/mL of D-serine using a 50-μL sample volume. The lowest non-zero concentration that could be quantitated accurately and reproducibly, i.e. LLOQ of this method, was validated as 100 ng/mL. As shown in **Figure 2b**, the signal-to-noise (S/N) ratio was quite high (estimated to be ~75) at the LLOQ of 100 ng/mL, if necessary, this method could be modified to have a significantly lower LLOQ, even down to low ng/mL level if baseline level could be effectively reduced. The LLOQ of 100 ng/mL in this method was sufficient to support clinical trials. This was set for fit-for-purpose, not the limitation of the method and technology used. The linearity of the method was established by eight calibration standards in duplicate over the nominal concentration range. A linear, 1/concentration weighted, least-squares regression algorithm was used to describe the relationship between the peak height ratio and nominal concentration. The linear correlation coefficient was ≥ 0.9997 for the three precision and accuracy runs during the method validation. Back-calculated values and reproducibility from each level of the calibration standards are presented in **Table 1**. Back-calculated results for all calibration standards (n = 6) were with coefficient of variation (CV) of better than 2.7% and the accuracy of 98.5 to 102% (i.e. relative error -1.5 to 2.0%), including the lowest standard point.

The extraction recovery of D-serine and the IS from human plasma and PBS were evaluated by comparing the average analyte peak height of pre-extraction spiked samples
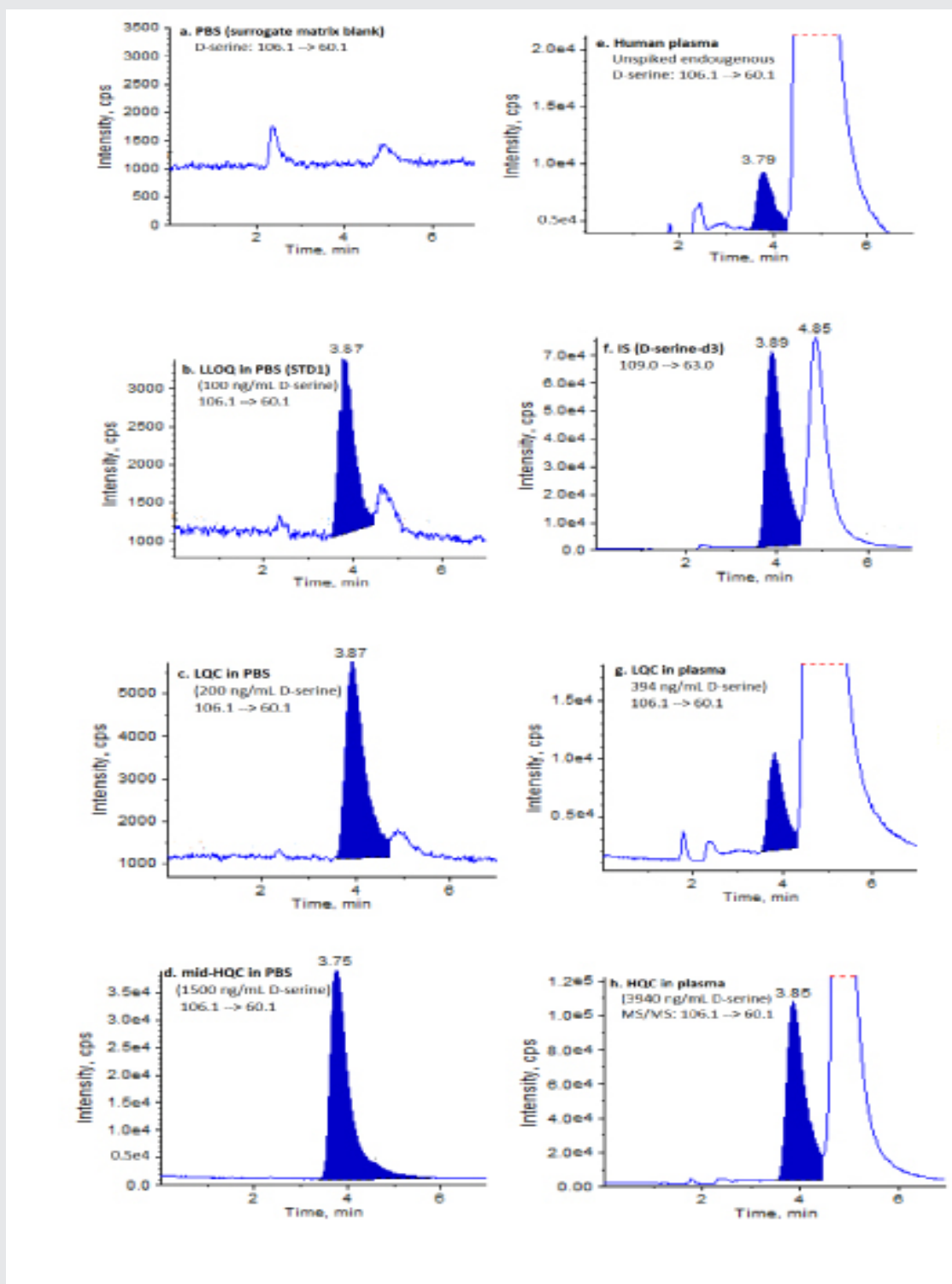
Figure 2. Representative chromatograms of PBS, LLOQ, human plasma, and QC samples

Table 1. Back-calculated calibration standards and the linearity of calibration curves for D-serine in PBS

Analytical run #	Calibration standard levels (ng/mL)								
	100	150	200	500	1000	2500	4000	5000	r^2
Run-1	101	155	195	489	956	2500	3970	5120	0.9998
	103	158	187	496	1010	2500	4000	4960	
Run-2	97.6	151	200	498	1010	2480	3900	5020	0.9998
	97.7	153	198	508	1010	2520	4110	4950	
Run-3	96.2	148	202	500	1000	2520	4030	4940	0.9997
	101	153	197	481	1040	2620	3890	4980	
Mean (n=6)	99.4	153	197	495	1000	2520	3980	5000	0.9998
CV (%)	2.6	2.2	2.7	1.9	2.7	2.0	2.1	1.4	
Accuracy (%)	99.4	102	98.5	99.0	100	101	99.5	100	

to the average analyte peak height of post-extraction spiked samples. The extraction recoveries from the PBS QC samples were found to be 88.8%, 87.5% and 99.0% for D-serine at low, medium and high concentrations, and 105% for the IS, respectively. The extraction recoveries from the plasma QC samples were found to be 84.1%, 78.4% and 76.4% for D-serine serine at low, medium and high concentrations, and 82.3% for the IS, respectively. These data showed that the method gave satisfactory extraction recovery from both plasma and PBS matrix.

Matrix-related suppression or enhancement was evaluated by comparing the average analyte peak height of post-extraction spiked samples to the average analyte peak height of pure solutions (non-matrix prepared samples). The data are shown in **Table 2** for PBS and human plasma. The matrix effect was 92.2 ± 6.5% for D-serine at low, medium and high QC concentrations in PBS, and 82.5% for IS, respectively. The matrix effect was 80.5 ± 14.0% for D-serine at low, medium and high QC concentrations in plasma, and 78.8% for IS, respectively. These data show that matrix suppressions in both PBS and plasma were between 10 – 20%, but this effect was compensated well by deuterated-label-internal standard. Therefore, the quantitation of D-serine with this method should be reliable.

Precision and accuracy of QC samples in PBS and in human plasma
As summarized in the previous section, for all validation curves, the back-calculation results for all calibration standards showed ≤ 2.7% CV and -1.5 to 2.0% RE, respectively. The precision and accuracy for the QC samples are given in **Table 3**. For all QC levels (LLOQ, Low, mid-Low, Mid, mid-High, and High) in PBS, precision and accuracy were ≤ 4.0% CV and -3.0 to 6.4% RE for intra-run assays (n = 6), and ≤ 4.7% CV and 1.0 to 3.3% RE for inter-run assays (n = 18), respectively. For all QC levels (Low, Mid, and High) in plasma, precision and accuracy were ≤ 6.7% CV and -15.0 to 0.0% RE for intra-run assays (n = 6), and ≤ 8.7% CV and -7.0 to -6.1% RE for inter-run assays (n = 18), respectively. These results demonstrated good precision and accuracy of the proposed method for the determination of D-serine in both PBS and plasma matrices, even at the lower end of

Table 2. Matrix effect

Matrix	QC samples in PBS			QC samples in Plasma		
	200 ng/mL	750 ng/mL	3750 ng/mL	394 ng/mL	944 ng/mL	3940 ng/mL
D-Serine	85.8%	92.0%	98.9%	66.4%	83.1%	94.5%
		Mean: 92.2 ± 6.5%			Mean: 80.5 ± 14.0%	
IS		5000 ng/mL			5000 ng/mL	
		82.5 %			78.8 %	

Note: Ion suppression (%) =100% - x%, where x% are the values listed in the table. The x% close to 100% means minimum matrix effect, less than 100% means a suppression, and greater than 100% means an enhancement.

Table 3. Intra-run and inter-run precision and accuracy of QC samples in PBS and in human plasma

P & A statistics		QC samples in PBS (ng/mL)						QC samples in Plasma (ng/mL)		
		100	200	300	750	1500	3750	394	944	3940
Run-1 (n = 6)	Mean	106	213	311	779	1570	3840	382	912	3810
	CV (%)	3.8	1.5	3.3	1.3	2.8	2.6	3.5	3.2	3.5
	RE (%)	5.8	6.4	3.7	3.9	4.7	2.5	-3.1	-3.4	-3.5
Run-2 (n = 6)	Mean	97.0	198	300	762	1540	3800	335	812	3410
	CV (%)	1.5	2.2	2.8	1.3	1.7	1.9	6.3	6.7	6.0
	RE (%)	-3.0	-1.0	0.0	1.6	2.4	1.3	-15.0	-14.0	-13.5
Run-3 (n = 6)	Mean	102	200	299	761	1540	3750	394	911	3870
	CV (%)	3.2	4.0	2.1	2.0	2.0	2.3	6.1	3.7	3.1
	RE (%)	2.1	0.0	-0.4	1.5	2.8	0.0	0.0	-3.5	-1.9
Inter-run (N = 18)	Mean	102	203	303	767	1550	3800	370	878	3700
	CV (%)	4.7	4.2	3.2	1.9	2.3	2.2	8.7	7.0	7.0
	RE (%)	2.0	1.5	1.0	2.3	3.3	1.3	-6.1	-7.0	-6.1

the quantitation dynamic range. This also indicated that PBS could be utilized as a surrogate matrix for the quantitation of D-serine in human plasma especially when a stable isotope-labeled analyte was used as the internal standard.

Dilution integrity, short-term and long-term sample storage stability

A 5-fold dilution for over-the-curve QC sample by PBS prior to extraction was used to evaluate sample dilution integrity. Six replicates of partial volume of Mid QC plasma samples with a 2.5-fold pre-dilution were also processed in a validation batch. Data (not shown) are acceptable with 3.3% CV and -6.3% RE for the 5-fold diluted over-the-curve QC in plasma, and 7.0% CV and -0.6% RE for the Mid QC with 2.5-fold dilution by PBS, respectively. The results indicated that taking partial volume and diluting plasma with PBS by up

to 5-fold did not produce significant deviation from their nominal values.

The short-term QC sample stability such as freeze-thaw and room temperature (RT) stability tests were performed as described, the results are summarized in **Table 4**. The QC samples undergoing six freeze-thaw cycles gave ≤ 2.1% CV and an accuracy of 97.3 – 98.9% in PBS, and ≤ 5.1% CV and an accuracy of 104 – 109% in plasma. The samples having been stored at room temperature (RT) for 36.5 h prior to extraction gave ≤ 2.4% CV and an accuracy of 95.0 – 99.1% in PBS, and ≤ 8.0% CV and an accuracy of 103 – 107% in plasma. It was also confirmed that the extracts from both PBS QC samples and plasma QC samples were stable for up to 149 h when they were kept at 5 °C (data not shown), thus extracted samples could be injected or re-injected within this established stability period.

The long-term frozen storage stability of the QC samples in plasma stored at -20 °C and -70 °C and the QC samples in PBS stored at -20 °C have also been tested and the data are included in **Table 4** as well. The results of the 8-day-old samples showed less than 7.0% CV (n = 6) and an accuracy of 92.2 – 102%, the results of the 322-day-old samples showed less than 5.2% CV (n = 6) and an accuracy of 88.4 – 99.1%, and the results of the 769-day-old samples showed less than 3.5% CV (n = 6) and an accuracy of 94.0 – 103%. These data illustrated that the plasma samples were stable for up to 769 days if they were stored frozen at -20 °C and -70 °C.

D-serine stability in K_2EDTA whole blood was also evaluated at Low and High quality control levels under both refrigerated and room temperature conditions. The whole blood samples being stored at room temperature for 4 hours showed a difference of 0.5 – 2.4% from the time zero control. The whole blood samples stored at 2 – 8 °C for 4 hours showed a difference of 0.0 – 1.2% from the time zero control. The above evaluation (data not shown) demonstrated that D-serine was stable in human whole blood for a period of 4 hours at either room temperature or refrigerated conditions. This should provide a grace period for clinical blood collection and processing to plasma for the reliable measurement of plasma D-serine levels.

Application to a Phase I Study

The above validated method has been successfully applied to analyze over 2200 plasma samples from a Phase I clinical study of SEP-227900. The study was a placebo-controlled single ascending dose (SAD) through oral administration. A total of seven cohorts (seven

Table 4. Short-term and long-term stability data of D-serine QC samples in PBS and in human plasma

Storage condition	In PBS		In Plasma	
QC Conc. (ng/mL)	200 (Low)	3750 (High)	394 (Low)	3940 (High)
6 Freeze-thaw cycles	97.3 (2.1)	98.9 (1.8)	109 (5.1)	104 (2.6)
36.5 h Ambient	95.0 (2.4)	99.1 (2.3)	103 (5.6)	107 (8.0)
8 days (-20 °C)	102 (4.5)	98.9 (1.6)	95.8 (6.2)	97.3 (2.5)
8 days (-70 °C)	NA	NA	92.2 (7.0)	95.4 (1.3)
322 days (-20 °C)	99.1 (2.3)	98.4 (1.1)	91.4 (3.3)	91.0 (5.2)
322 days (-70 °C)	NA	NA	88.4 (3.9)	91.8 (2.4)
769 days (-20 °C)	103 (3.5)	99.4 (0.9)	94.9 (2.8)	94.0 (1.7)
769 days (-70 °C)	NA	NA	100 (1.9)	101 (2.3)

Notes: "NA" – these were not done; the values in the parenthesis are CV (n = 6)

doses were 1, 2, 5, 10, 20, 40, and 80 mg) were clinically conducted in healthy male subjects. Predose plasma samples and the 24 h postdose plasma samples collected from all cohorts and all treatments were confirmed to be below the lower limit of quantitation for SEP-227900 (LLOQ was 0.050 ng/mL). The drug plasma concentrations were measured by a validated LC-MS/MS method. The mean C_{max} of SEP-227900 at each dose level was presented in **Table 5**. The tmax of SEP-227900 was typically 0.5 h across the dose range. The measured D-serine mean concentrations at each of predose, tmax and trough (24 h postdose) for each cohort, and normalized D-serine level (to predose) are also presented in **Table 5**, and D-serine results are graphically shown in **Figure 3**. The typical chromatogram of an incurred study sample was very similar to the blank plasma with endogenous D-serine, as shown in **Figure 2e**. To confirm the method reproducibility, 221 plasma sam-

Table 5. Human plasma D-serine biomarker results at different doses of SEP-227900

Cohort		1	2	3	4	5	6	7
Dose (mg)		1.0	2.0	5.0	10	20	40	80
$C_{max} \pm SD$ (ng/mL)		20.2±6.8	44.7±6.0	115±44	345±87	715±155	1650±310	3520±890
t_{max} (h)		0.5	0.5	0.5	0.5	0.5	0.5	0.5
D-serine (ng/mL)	Predose	150 (12.2)	141 (11.9)	128 (22.0)	184 (19.9)	191 (14.7)	161 (17.2)	156 (19.0)
	At t_{max}	152 (13.8)	142 (14.1)	143 (20.4)	190 (21.1)	198 (15.8)	158 (23.3)	163 (20.6)
	24 h	158 (9.1)	148 (10.9)	149 (20.2)	202 (22.0)	190 (13.6)	172 (16.6)	168 (19.0)
D-serine normalized to predose	Predose	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	At t_{max}	1.01	1.01	1.12	1.03	1.04	0.981	1.05
	24 h	1.05	1.05	1.16	1.10	0.995	1.07	1.08

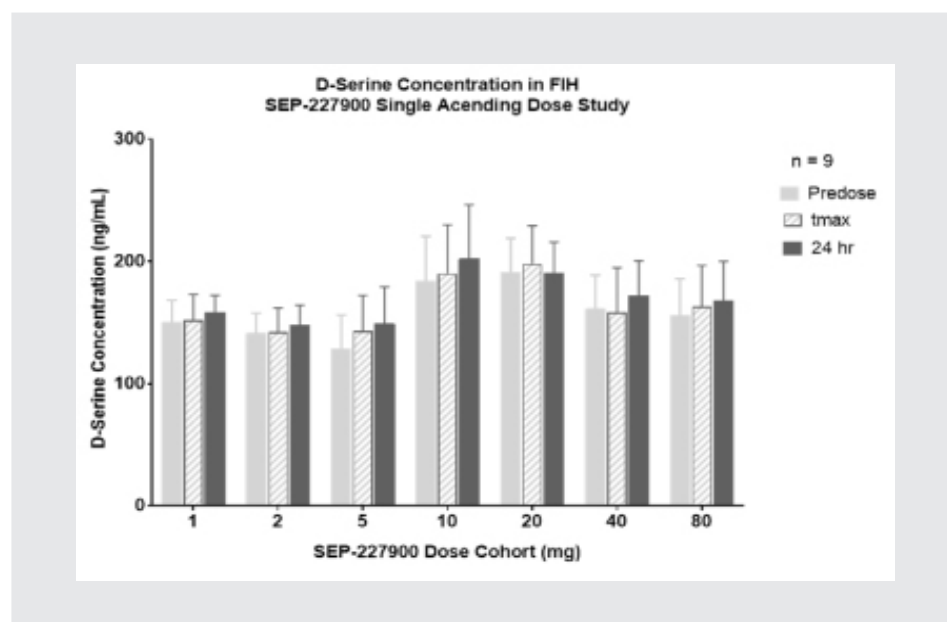Notes: The values in the parenthesis are CV (%) (n = 9).

Figure 3. D-serine levels in human plasma samples from healthy subjects treated with a single oral dose of 1, 2, 5, 10, 20, 40, and 80 mg SEP-227900 (n = 9)

ples, approximately ten percent (10%) of total number of plasma samples across 7 cohorts, were selected for incurred sample reassy (ISR) evaluation. An over 87% of the ISR plasma samples (193/221) had repeating and original results agreed within 20% of each other, demonstrating satisfactory assay performance for this newly validated method. As SEP-227900 was considered as a potential competitive inhibitor of DAAO, so D-serine level in humans was expected to be elevated with the treatment of this agent. However as summarized in **Table 5**, the plasma D-serine levels in the above SAD clinical study had no significant change from predose to tmax (at the peak concentration of the dosed compound) and 24 h post-dose, and from placebo to active treatments across the dose range used in this clinical trial. The normalized to predose D-serine level at all cohorts conducted had a value from 0.981 to 1.16. Although the C_{max} of SEP-227900 in plasma was increased from 20.2 ng/mL at 1 mg dose to 3520 ng/mL at 80 mg dose, normalized D-serine remained the same. The insignificant D-serine change as an outcome of the biomarker monitoring in this clinical study was very helpful to making "no-go" decision for the development of this investigational compound [30].

CONCLUSION

This paper presented the development and validation of a simple and robust enantioselective LC-MS/MS method for direct quantitative determination of D-serine in human plasma. To the best of our knowledge, this is the first direct and rapid quantitative analysis of D-serine in human plasma without a complex chemical derivatization step. The PBS buffer was proven to be a good choice as a surrogated matrix for the preparation of calibration standards. The quality control samples were prepared in both PBS and real matrix (plasma) for the method validation. The method validation was completed sometime before the new FDA guidance on Bioanalytical Method Validation was issued and part of the results was presented as a conference poster [31], but the validation design and entire procedure including the use of surrogate matrix for calibration standards and plasma matrix for quality control samples were well in line with the newly issued FDA guidance [29] where endogenous analytes and biomarker assays were specifically described. This method has been demonstrated to be reliable through validation and its successful application to a clinical Phase I study with acceptable incurred sample reanalysis reproducibility. This work demonstrated that the use of surrogate matrix standard curve and real matrix quality control (QC) samples could be considered as a feasible approach for endogenous biomarker monitoring in biomedical research and drug development.

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