Published in final edited form as:

J Pharm Biomed Anal. 2014 February 15; 89: . doi:10.1016/j.jpba.2013.10.028.

Development and Validation of a Sensitive LC-MS/MS Method for the Determination of D-Serine in Human Plasma

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Abstract

A validated LC-MS/MS method was developed for the determination of D-Serine in human plasma. The method was fully validated for use with human plasma samples and was linear from 0.19 nmol/ml to 25 nmol/ml. The coefficient of variation was 5% for the high QC standards and 8% for the low QC standards in plasma. D-Serine and L-Serine were resolved by pre-column derivatization using (R)-1-Boc-2-piperidine carbonyl chloride as the derivatizing agent. The method was used to determine the concentration of D-Serine in plasma samples obtained in patients receiving a continuous 5-day intravenous infusion of (R,S)-ketamine. The changes in D-Ser levels varied in the 6 patients, with circulating D-Ser levels increasing as much as 35% in a patient, while decreasing 20% in a patient. While only preliminary data, the results suggests the potential importance in determining the D-Ser levels in plasma and their potential role in physiological response.

Keywords

Enantiomeric separation; Chiral derivatization; Ketamine; Complex regional pain syndrome (CRPS)

1. Introduction

D-Serine (D-Ser) is a major endogenous *N*-methyl-D-aspartate (NMDA) receptor co-agonist that specifically activates pre-synaptic NMDA receptors [1]. In vitro studies have suggested that D-Ser activation is associated with NMDA-related neurotoxicity [2] and clinical and postmortem studies have indicated that D-Ser concentration are associated with a number of central nervous system (CNS) diseases and pathological states [3], including schizophrenia,

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aging, Alzheimer's disease, convulsion, anxiety, cerebellar ataxia, Parkinson's disease, neuropathic pain and depression. Specifically, changes in D-Ser levels in the central nervous system were observed in patients with schizophrenia and amyotrophic lateral sclerosis (ALS) [4, 5]. In addition, a reduction of endogenous D-Ser concentration in the rostral anterior cortex of the rat attenuated pain-related negative emotions, and led to the suggestion that reducing D-Ser concentrations and, thereby, NMDAR activity, may be a new strategy for reducing chronic pain-induced emotional distress [6]. Recently, we have demonstrated that treatment of PC-12 cell with gabapentin and (S)-pregabalin decreased intracellular D-Ser concentrations, which may be the source of the efficacy of these compounds in the treatment of neuropathic pain [7]. D-Ser is produced by enantio-conversion of L-serine (L-Ser) by serine racemase (SR), and the changes in the endogenous D-Ser levels due to inhibition and augmentation in the activity and expression of SR are currently areas of pharmacological and clinical interest [2, 8].

Complex Regional Pain Syndrome (CRPS) is a chronic debilitating disease characterized by significant pain often resulting in severe disability and lost productivity. While there are very limited clinical treatments that produce relief of the symptoms, there has been recent success with the treatment of CRPS with a 5-day continuous infusion of a sub-anesthetic dose of (R,S)-ketamine ((R,S)-Ket). It has been shown that the treatment response does not correlate with the circulating plasma levels of (R,S)-Ket or norketamine, in fact, we have previously demonstrated that the major circulating metabolite after three days of continuous infusion was (2S,6S;2R,6R) hydroxynorketamine, followed by (R,S)-dehydronorketamine (DHNK). In addition, it was demonstrated that while (R,S)-Ket did not inhibit the $\alpha 7$ nicotinic receptor, its downstream metabolites were negative allosteric modifiers of this receptor [9], with (R,S)-DHNK having the most pronounced effect with an IC_{50} of 50 nM. Subsequently, it was demonstrated that nicotinic receptor antagonists, including (R,S)-DHNK, resulted in a decrease in intracellular D-Ser levels and that inhibition of $\alpha 7$ subtype induced novo SR protein expression via multiple signaling cascades that converge at mTOR [10]. Thus, the accurate quantification of D-Ser in complex biological matrices is highly desirable.

Currently, there are several methods for the quantification of D-Ser in rat brain microdialysate [11], mouse brain [12], cell matrix [13], and human cerebrospinal fluid and plasma [14]. More recently, Visser et al. [14] described the determination of several amino acids, including D-Ser using a chiral derivatizing reagent and UPLC-MS/MS. However, the method was not validated for use in the analysis of plasma or tissue samples as the calibration curves were developed using aqueous solutions and the curves were then applied to the analysis of biological matrices. Therefore, we have developed and fully validated LC-MS/MS method for the determination of D-Ser levels in human plasma. The method utilizes pre-column derivatization using (R)-1-Boc-2-piperidine carbonyl chloride. The lower limit of quantification (LLOQ) of the method is 0.19 nmol/ml, which is ten times lower than the endogenous levels found in human plasma and CSF. The method was applied to the sequential changes in plasma D-Ser and L-Ser levels in 6 CRPS patients receiving a continuous 5-day intravenous infusion of (R,S)-Ket.

2. Materials and Methods

2.1. Materials

(D-serine (purity 98%), L-serine (purity 99%), D-arginine (the internal standard, IS, purity 98%), (R)-1-Boc-2-piperidineacetic acid (purity 98%), cyanuric chloride (purity 99%), triethylamine (TEA) (purity 99%) and trifluoroacetic acid (TFA, purity 99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, of HPLC grade, was also obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetone was analytical grade and

obtained from Avantor Performance Materials (Phillipsburg, NJ, USA). Water was distilled and purified using a Milli-Q Water Purification System (Millipore, Bedford, MA, USA).

2.2 Chromatographic conditions

2.2.1 Instrumentation—The chromatographic experiments were carried out on a Shimadzu Prominence HPLC system (Shimadzu, Columbia, MD, USA). The samples were introduced to the analytical column using Shimadzu SIL-20A autosampler and maintained at 4°C in the autosampler tray, and injections of 20 µl were made. The samples were run on a 5500 QTRAP triple quadrupole mass spectrometer equipped with a Turbo V electrospray ionization source (AB Sciex, Concord, ON, Canada).

2.2.2 Enantioselective separations—The chromatographic separation was achieved on a Zorbax Eclipse XDB-C18 column (4.6 mm × 150 mm, 5 µm; Agilent Technologies, Santa Clara, CA, USA) protected with an Agilent C18 guard column at room temperature. The mobile phase consisted of water with 0.3% TFA (elute A) and methanol with 0.3% TFA (elute B). The gradient eluent at a flow rate of 0.4 ml/min was programmed as follows: 0–15 min, 5–9% B; 15–22 min, 15% B; 22–25 min, 5% B. The total run time was 25 min and the injection volume per sample was 20 µl.

2.2.3 Mass spectrometry conditions—MS/MS analysis was performed using a triple quadrupole mass spectrometer model API 5500Q system from Applied Biosystems/MDS Sciex equipped with Turbo V electrospray ionization source (TIS)® (Applied Biosystems, Foster City, CA, USA). The data was acquired and analyzed using Analyst version 1.5.1 (Applied Biosystems). Positive electrospray ionization data were acquired using multiple reaction monitoring (MRM). The TIS instrumental source settings for temperature, curtain gas, ion source gas 1 (nebulizer), ion source gas 2 (turbo ion spray), collision energy and ion spray voltage were 550°C, 20 psi, 45 psi, 80 psi, 15V and 4500 V, respectively. The TIS compound parameter settings for declustering potential, entrance potential, and collision cell exit potential were 80 V, 10 V, and 10 V, respectively. The standards were characterized using the following MRM ion transitions: D-Ser derivatization product (m/z 231.5 to 106.1) and D-Arg derivatization product (m/z 300.4 to 175.0).

2.3 Sample preparation

2.3.1. Synthesis of the (R)-1-Boc-2-piperidinecarbonyl chloride—28 µl of a 72 µM TEA solution in acetone was added to a 2 mM (R)-1-Boc-2-piperidineacetic acid in 500 µl of acetone and 500 µl of a 1 mM cyanuric chloride in acetone. This mixture was incubated by stirring at 1000 rpm for 3 h at 28 °C. The reaction was terminated on ice and the product (R)-1-Boc-2-piperidinecarbonyl chloride was stored at –70 °C before use (Fig. 1A).

2.3.2. Plasma samples—Plasma samples (100 µl) were combined with 20 µl aliquot of IS (10 nmol/ml in acetone) and 400 µl acetone and then centrifuged at 13,000 × g for 10 min at 4 °C. A 400 µl aliquot of the supernatant was subsequently derivatized with 300 µl (R)-1-Boc-2-piperidinecarbonyl chloride. After derivatization by stirring at 1000 rpm for 2 h at room temperature, 200 µl of TFA was added to each sample and then incubated for 1 h. The terminal product was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 100 µl of a mixed solution (methanol/water, 10:90, v/v) and transferred to the autosampler for analysis.

2.4 Validation

2.4.1 Stock solution—A concentrated D-Ser stock solution was prepared containing 1 $\mu\text{mol/ml}$. The solution was prepared in ultra-pure water and stored in polypropylene tubes at -4°C . Serial dilutions of the stock solution were used to prepare the samples for the calibration curves and quality control standards (QC).

2.4.2 General procedures—The quantification of D-Ser was accomplished using area ratios calculated using the derivatized D-Ser and the derivatized D-Arg as the IS. QC standards were prepared daily by adding 10 μl D-Ser to the tubes and immediately evaporating to dryness under nitrogen gas. Then 100 μl of pooled human plasma from Bioreclamation (East Meadow, NY, USA) was added into each tube followed by vortex-mixing for 2 min. Then the blank plasma area was subtracted from all the spiked standards to obtain the calibration curve.

2.4.3 Calibration curves and QC standards (plasma)—An eight point calibration curve was constructed in the range of 0.19–25.0 nmol/ml for human plasma by plotting peak area ratio (y) of spiked D-Ser to IS versus nominal concentration (x), and the linearity was assessed. One standard curve along with at least three sets of quality control samples was prepared each day of analysis. The quality control concentrations were as follows: 0.39, 3.13 and 20.0 nmol/ml for low quality control (QC_1), middle quality control (QC_2) and high quality control (QC_3), respectively.

2.4.5. Matrix effects—Blank plasma was spiked with D-Ser and derivatized D-Arg post-extraction and the peak areas of the post-spiked sample was compared to the peak areas of the derivatized D-Ser and D-Arg spiked in standard solution. The matrix effect percentage was calculated as the mean peak area of the reconstituted solution over the mean peak area of the reference solution for each QC.

2.4.5. Intra and Inter-day Validation—Intra-day accuracy and precision (relative standard deviation, R.S.D.) was evaluated by analyzing QC samples in five replicates over 1 day, while inter-day accuracy and precision was evaluated from the analysis of each control once on each of 5 days. The accuracy was estimated for each QC sample by comparing the measured concentration to the actual concentration. The LLOQ was defined as the lowest concentration in the calibration curve, where the accuracy and precision were below 20%.

2.4.6. Stability—Analyte stability was evaluated under a variety of conditions. Prepared sample stability was determined by quintuplicate measurements of the QC's stored at 4°C for 24 h after preparation and 1 h, 2h and 4 h at room temperature (bench top stability). Additionally, stability was demonstrated for spiked plasma with three freeze–thaw cycles. Acceptable stability was achieved if the sample could be quantified within $\pm 10\%$ of the expected value.

2.5 Application

The patients received a five day inpatient (R,S)-Ket infusion using a modification of the protocol of Harbut and Correll [15]. In addition, Midazolam (Versed) 2 mg was administered intravenously (IV) prior to the infusion and every 4 hours during the (R,S)-Ket infusion. Clonidine 0.1 mg was administered every 12 hours during the 5 day procedure. (R,S)-Ket in 0.9% saline solution (1 gram (R,S)-Ket per liter of saline) was administered IV using the following schedule. On the first day, the infusion was started at 10 mg of (R,S)-Ket per hour (mg/hr) and increased in 10 mg/hr increments every 2 hours until a maximum dose of 40 mg/h was achieved. After completion of five days the (R,S)-Ket infusion was tapered in the same manner as it was initiated. The patient was observed for 6 hours after the

(R,S)-Ket had been discontinued, and pending status, was discharged. Plasma samples were drawn pre-infusion, at day 3 or day 4 (mid-infusion) and after (R,S)-Ket treatment was stopped (post-infusion). Plasma samples were obtained from all subjects after giving informed consent as approved by the Drexel University School of Medicine Institutional Review Boards (IRB).

3. Results and Discussion

3.1 Quantitation of D-Serine

The method development for an endogenous compound in its authentic biological matrix, is, at times, difficult as the matrix contains an unknown concentration of the analyte. For D-Ser, the circulating plasma levels in humans ranges between 2.3 ± 0.6 nmol/ml [15]. In order to circumvent this problem, a modified standard addition method was used, where the addition of D-Ser to a pooled plasma sample was carried out and the endogenous levels were subtracted from the spiked standards. Quality controls were carried out in a similar fashion. In order to improve the sensitivity of D-Ser for detection by MS/MS and to allow the resolution of the enantiomers of serine, (R)-1-Boc-2-piperidine carbonyl chloride was prepared and used as the derivatizing agent (Fig. 1). The determination of D- and L-Ser in human plasma was carried out using a Zorbax Eclipse XDB-C18 column and D-Arg was used as the IS. The retention factors for the derivatized D-Ser and L-Ser in the analysis of plasma samples was 2.42 and 2.92 respectively, and the retention factor for the internal standard was 5.08. The derivatized D and L- Serine were resolved with $\alpha = 1.21$ and $R_s = 1.5$, improved from previously reported methods, where $R_s=1.3$ was obtained, calculated using the published chromatograms [14].

The developed method exhibited good specificity and selectivity as demonstrated in Fig. 2. Chromatograms of the blank pooled plasma (Fig 2A), the blank pooled plasma spiked with 3.13 nmol/ml of D-Ser and the internal standard (Fig 2B) and plasma from a CRPS patient receiving a continuous infusion of Ket, spiked with the internal standard (Fig 2C) are shown.

3.2 Validation

In plasma, an eight point calibration curves (0.19–25 nmol/ml) and three quality controls (0.39 nmol/ml (QC1); 3.13 (nmol/ml) (QC 2); 20 nmol/ml (QC 3)) were prepared using (R)-1-Boc-2-piperidine carbonyl chloride as the derivatizing agent and D-Arg as the IS. The calibration curve was linear with $y=0.0167x+0.0022$ ($r^2=0.9983$) in human plasma. The method was then validated for intra-day and inter-day accuracy and precision. Table 1 summarizes the intra- and inter-day precision and accuracy evaluated by assaying the QCs. The intra- and inter- day precision was less than 8.38% and the accuracy was from 92.93% to 102.29% for each QC level. The matrix effects were less than 25% for all levels of QC samples, indicating limited ion suppression. From our assay, the endogenous D-Ser levels in the pooled plasma was determined to be around 1.48 nmol/ml, which is similar to those reported previously in other studies [16, 17]. An LLOQ of 0.19 nmol/ml was obtained for D-Ser, which was a significant improvement over existing methods [11]. While Visser et al., obtained a lower calculated LOD of 0.22 nM, their calibration curve was in water [14] and not in the biological matrix.

After three freeze-thaw cycles, the concentrations of analytes in human plasma deviated less than $\pm 15\%$ from their nominal concentrations (from -9.28% to 5.26%). In the short-term stability and autosampler stability test, the relative errors for two levels of QC samples were within $\pm 10.99\%$ (Table 2). Stock solutions of D-Ser and IS were also stable for at least 4 weeks at 4°C (data not shown). These results indicate that the method is reliable and robust within the analytical range.

The results of these studies indicate that the achiral analytical method is accurate and reproducible within accepted limits, and can be used to analyze plasma samples obtained from human patients.

3.3 Application

The optimized chromatographic conditions were applied to the analysis of plasma samples obtained from 6 CRPS patients receiving a 5-day continuous infusion of (R,S)-Ket. The D-Ser levels of the 6 patients ranged from 1.61 nmol/ml to 3.61 nmol/ml with the average pre-infusion concentration at 2.61 ± 0.74 nmol/ml, which is similar to the range observed in healthy samples 2.3 ± 0.6 nmol/ml [16]. Of interest, was the difference in the effect of (R,S)-Ket on D-Ser plasma levels, Fig. 3. In 3 of the patients (pt 27, pt 310 and pt 312) (R,S)-Ket administration produced a continuous drop in D-Ser levels with a maximum decrease of 20% (pt 27). Treatment with (R,S)-Ket produced increased D-Ser levels in pt 258 and pt 307, with a 35% and 21% increase, respectively, while pt 308 had an initial 17% increase in circulating D-Ser levels on Day 3 followed by 8% reduction at the end of the treatment. The correlation of these changes to clinical response will be discussed elsewhere, but these initial results indicate that it is necessary in determining the D-Ser levels in plasma and their potential role in physiological response.

Conclusion

A highly sensitive LC-MS/MS method for the determination of D-Ser in plasma was developed and validated. The analysis of the plasma data from 6 CRPS patients suggests that D-Ser may be playing a role in response/non-response, which is the subject of ongoing studies.

Acknowledgments

This research was supported by the Intramural Research Program of the National Institute on Aging/NIH.

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Highlights

Chiral derivatization with (R)-1-Boc-2-piperidine acyl chloride

Quantitation of D-Ser in human plasma using standard addition method

Full-Validation of the LC-MS/MS method in human plasma

Determination of circulating plasma D-Ser levels in CRPS patients

Figure 1.

A. Synthesis of the derivatizing agent (R)-1-Boc-2-piperidinecarbonyl chloride B. Synthesis of the derivatized D-Ser and D-Arg.

Figure 2.

The chromatographic trace from the analysis of D-Ser in a blank plasma sample (A); a plasma sample spiked with 3.13 nmol/ml of D-Ser and the IS (B); and a plasma sample obtained after administration of (R,S)-Ket in a CRPS patient (C).

Figure 3. Relative changes in circulating plasma levels of D-Ser in 6 CRPS patients receiving a continuous infusion of (R,S)-Ket for 5 days determined using a Zorbax Eclipse XDB-C18 column (4.6 mm × 150 mm, 5 μm) from pre-infusion samples. Changes are seen in relative % increase or decrease of circulating D-Ser levels from mid infusion and post infusion over pre-infusion levels.

Table 1

Intra- and inter-day precision and accuracy of D-Ser assay in human plasma.

	Nominal concentration (nmol/ml)	Measured concentration (mean $\pm$ S.D., nmol/ml)	Precision (R.S.D., %)	Accuracy (%)
Intra-day ($n = 5$)	0.39	0.37 ± 0.03	8.38	95.41
	3.13	2.90 ± 0.02	0.71	92.93
	20.00	20.46 ± 0.82	4.03	102.29
Inter-day ($n = 15$)	0.39	0.38 ± 0.02	4.99	96.90
	3.13	2.93 ± 0.12	4.16	93.81
	20.00	19.32 ± 1.02	5.29	96.61

Table 2Autosampler stability and freeze-thaw stability and short-term stability, of D-Ser in human plasma ($n = 5$).

Sample condition	Nominal concentration (nmol/ml)	Measured concentration (mean, nmol/ml)	Accuracy (%)	R.S.D. (%)
Autosampler (24 h, 4 °C)	0.39	0.36	91.62	11.11
	3.13	2.89	92.21	5.13
	20.00	17.80	89.01	6.48
Three freeze-thaw cycles	0.39	0.41	105.26	9.66
	3.13	2.84	90.87	8.91
	20.00	18.14	90.72	4.82
Room Temp 1h	0.39	0.38	97.52	13.35
	3.13	3.10	99.08	9.69
	20.00	19.48	97.41	8.05
Room Temp 2h	0.39	0.38	96.06	13.41
	3.13	2.94	94.21	7.27
	20.00	19.14	95.68	8.52
Room Temp 4h	0.39	0.37	94.25	12.15
	3.13	2.88	92.01	6.37
	20.00	18.57	92.84	3.27