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Short communication

Quantification of serine enantiomers in rat brain microdialysate using Marfey's reagent and LC/MS/MS

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Abstract

The ability to selectively measure serine enantiomer concentrations in rat brain microdialysate is essential during drug discovery to study the interaction of D-serine with the *N*-methyl-D-aspartate (NMDA) subtype of the glutamate receptor. NMDA receptor-stimulating agents, such as D-serine, have been shown to reduce the negative symptoms and cognitive dysfunction in individuals with schizophrenia when added to conventional or atypical antipsychotic drug regimens. In the work presented here, an LC/MS/MS assay was developed and validated to simultaneously measure D-serine and L-serine concentrations in rat brain microdialysate. Reverse phase chromatographic resolution of the enantiomers was obtained through derivatization with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent). The assay was validated to determine concentrations over the range of 10–7500 ng/mL using electrospray ionization and multiple reaction monitoring (MRM). Both intra- and inter-day precision and accuracy were less than 16.5% (RE) and 7% (CV) for both analytes, respectively, and assay throughput was increased significantly relative to existing methodologies.

Keywords: D-Serine; L-Serine; Marfey's reagent; LC/MS/MS; Schizophrenia

1. Introduction

The amino acid D-serine is found in brain tissues at high concentrations, challenging the paradigm that D-amino acids do not play an important role in mammalian biochemistry. D-Serine is converted from L-serine by serine racemase, which results in D-serine levels in the brain that exceed those of many L-amino acids such as asparagine, valine, isoleucine, and tryptophan [1]. Current research suggests that decreased serum levels of D-serine may play an important role in schizophrenia [2]. D-Serine is an agonist at the glycine binding site of the *N*-methyl-D-aspartate glutamate receptor (NMDA), and hypofunction of the NMDA receptor has been implicated in the pathophysiology of schizophrenia [3]. This hypothesis is supported by findings that antagonists of the NMDA subtype of the glutamate receptor, such as ketamine, can elicit the full range of symptoms associated with schizophrenia [4], and by fMRI studies in rat brain [5]. It has also been suggested that D-serine administration, in combination with conventional or atypical antipsychotics, may result in significant reduction in negative and cognitive symptoms [6]. Therefore, the ability to selectively and sensitively measure serine enantiomers in brain tissues can serve as an important tool in the search for treatments for schizophrenia and associated cognitive disorders.

A number of papers on the analysis of D-amino acids in general, and D-serine in particular, exist in the literature. General strategies to D-amino acid analysis were reviewed by Hamase et al. and include chromatographic, electrophoretic, and enzymatic approaches [7]. The use of Marfey's reagent for chiral amino acid analysis was reviewed by Bhushan and Bruckner in which the reagent 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide was used to chemically convert amino acid enantiomers to diasteromers [8]. Specific approaches to D-serine measurements include reversed-phase chromatography with pre-column derivatization to form diastereomers [9,10], capillary electrophoresis [11,12], and gas chromatography-mass spectrometry [13]. In addition, McLellan et al. recently presented a reversed-phase chiral assay to determine D/L-serine and glycine which utilized LC/MS/MS [14]. This assay used an Astec Chirobiotic T column to resolve the serine enantiomers in ~12 min; the injection cycle time was

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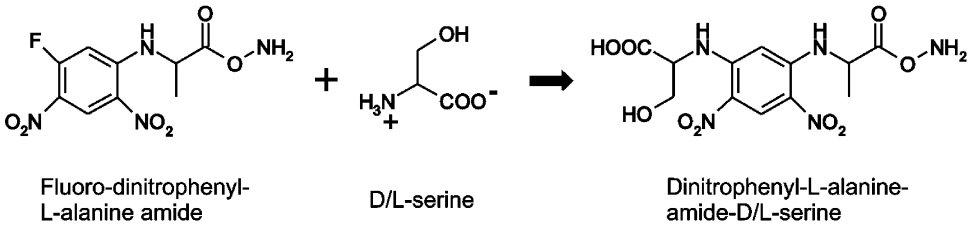

Fig. 1. Reaction of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent) with D-serine and L-serine to form dinitrophenyl-L-alanine-amide-D/L-serine diastereomers that are resolved by reverse phase chromatography.

21 min. Shortcomings of some of these approaches include difficult and time-consuming derivatization steps, poor selectivity and robustness, and low sample throughput.

In the work presented here, we developed an LC/MS/MS assay to quantitate D-serine and L-serine in rat brain microdialysate. Resolution of the serine enantiomers was accomplished with chemical derivatization using Marfey's reagent followed by reverse-phase chromatography. The reaction of D/L-serine with Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine) was complete within 1 h (60 °C) and produced dinitrophenyl-5-L-alanine-D/L-serine diastereomers (DNPA-D/L-serine, Fig. 1). Detection was by negative electrospray ionization (ESI) mass spectrometry in the multiple reaction monitoring mode. The assay was validated to quantify both D-serine and L-serine over the range of 10.3–7500 ng/mL and used D/L-(d_3)-serine as a stable isotope labeled internal standard. DNPA-D-serine and DNPA-L-serine were chromatically resolved in less than 3 min, and the injection cycle time was 3.5 min.

2. Experimental

2.1. Materials and reagents

D-Serine, L-serine, and Marfey's reagent (Nα-(2,4-dinitro-5-fluorophenyl)-L-alaninamide) were purchased from Sigma–Aldrich (St. Louis, MO), and D/L-(d_3)-serine was from Cambridge Isotope Laboratories (Andover, MA). HPLC grade water, acetonitrile, and methanol; and ammonium acetate, sodium bicarbonate, and phosphate buffered saline (PBS) were from Fisher Scientific (Pittsburg, PA).

Stock solutions of D-serine, L-serine, and D/L-(d_3)-serine were prepared at 100 μg/mL by placing 1.0 mg in 10 mL of HPLC grade water Q.S. An internal standard working solution of 500 ng/mL D/L-(d_3)-serine was prepared by diluting 50 μL of the 100 μg/mL stock solution with 10 mL of PBS Q.S. All solutions were stored in polypropylene containers at −20 °C when not in use.

2.2. Mass spectrometric conditions

Mass spectrometric detection was accomplished using a MDS Sciex API 4000 Q Trap (Ontario, Canada) operated in negative turbo ionspray mode. The following mass spectrometer settings were used: spray voltage −4500 V, curtain gas 15 AU, gas-1 50 AU, gas-2 25 AU, collision gas pressure medium, EP −10 V, Q1/Q3 resolution unit, and TEM 550 °C. The multiple reaction monitoring (MRM) transition for DNPA-D/L-serine was 356.1 → 192.0 (dwell 150 ms, DP −65 V, CE −24 V, CXP −11 V) and for DNPA-D/L-(d_3)-serine was 359.1 → 192.0 (dwell 150 ms, DP −65 V, CE −24 V, CXP −11 V). The mass spectrometer acquisition time was 3.5 min.

2.3. Chromatographic conditions

Chromatographic resolution of DNPA-D-serine and DNPA-L-serine was accomplished using a Shimadzu HPLC system (Wood Dale, IL) that consisted of two LC10AD vp pumps, an SCL-10Avp system controller, and an HTS PAL Leap autosampler (Carrboro, NC). A Sprite Armor C18 HPLC column (5 μm, 40 mm × 2.1 mm), obtained from Analytical Sales and Services (Pompton Plains, NJ), was used for the HPLC separation. A linear gradient of mobile phase A (1000:3 water/5 M ammonium acetate, v/v) and mobile phase B (800:200:3 methanol/acetonitrile/5 M ammonium acetate, v/v/v) was delivered using the following profile (min/% MP B): 0.0/5, 3.0/40, 3.0/5, 3.5/5. The flow rate was 0.5 mL/min and the injection volume was 50 μL of 1000:3 water/5 M ammonium acetate (v/v).

2.4. Preparation of standard and quality control samples

Standard samples used for calibration were prepared fresh daily in PBS at 7500, 2500, 833, 278, 92.6, 30.9, and 10.3 ng/mL by serial dilution of the 100 μg/mL stock solutions of D/L-serine. Each standard was prepared in duplicate on each day of analysis, and a standard curve was run before and after each batch of samples. Quality control samples used in the validation were prepared fresh daily in the same fashion as the standard samples at 7500, 278, and 10.3 ng/mL.

2.5. Sample preparation procedure

The rat brain microdialysis samples were stored at −70 °C prior to analysis. On the day of analysis, the study samples were thawed at room temperature. After thawing, a 25 μL aliquot of each sample was transferred to a 96-well plate. A 25 μL aliquot of the internal standard working solution was added to each sample except the double blank (no std or IS), which received 25 μL of PBS. A 10 μL aliquot of 200 mM (pH 7.5) sodium bicarbonate solution was added to each sample followed by 10 μL of 1% Marfey's reagent (w/v in acetone). The samples were

vortex mixed and incubated at 60 °C for 1 h. Next, the samples were concentrated to dryness at 60 °C under a stream of nitrogen, and the dried residues were reconstituted with 200 μL of 12.5% methanol in 15 mM ammonium acetate. The samples were centrifuged at ~600 × *g* for 5 min, and 50 μL aliquots were diluted by the addition of 200 μL of 1000:3 water/5 M ammonium acetate (v/v). Injections of 50 μL were used for LC/MS/MS analysis.

2.6. Data analysis

Calibration curves were obtained by plotting the peak area ratio of D/L-serine to their internal standard against concentration. A weighted (1/concentration^2) least squares regression analysis was used to obtain a linear equation over the range of the calibration. The origin was not used in the standard curve calculations.

2.7. Validation procedures

Assay accuracy and precision were evaluated on each of three days by analyzing five replicates of quality control samples at three concentrations: 10.3, 278, and 7500 ng/mL. The quality control samples were bracketed by duplicate standard curves that spanned the dynamic range of 10.3–7500 ng/mL. The assay intra- and inter-day accuracy (%RE) and precision (%CV) were calculated for D-serine and L-serine, and are summarized in Table 1.

Analyte carryover and selectivity were evaluated during each batch analysis during the validation and analysis of study samples. Carryover was evaluated by analyzing duplicate carryover blanks (no analytes, IS present) following the highest standard sample. The percent absolute carryover and relative carryover were calculated and are presented in Section 3. Assay selectivity was evaluated by analyzing blank (with IS) and double blank (no analytes or IS) samples and evaluating for the presence of interfering peaks from the matrix or internal standard that could potentially interfere with the quantitation of the analytes.

Due to the stage of the work presented here (i.e. early discovery), formal stability experiments to evaluate room temperature matrix, long-term freezer, and freeze/thaw stability were not performed. However, because of the unknown potential for the DNPA-D/L-serine derivatives to degrade at room temperature, it was decided to bracket the samples with duplicate standard curves. Since the standard curves were co-regressed, any issues with analyte stability during a batch analysis would be obvious in the back-calculated residual errors of the standard samples. In addition, stock solution stability was evaluated by analyzing system suitability samples prepared from stored stock solutions (−20 °C) on each day of analysis and looking for a negative trend in suitability response over time.

3. Results

The upper (ULOQ) and lower (LLOQ) limits of quantitation were defined as the highest and lowest concentrations in the standard curve that had acceptable accuracy (+/−25% RE) and precision (≤25% CV). The LLOQ for D-serine and L-serine was 10.3 ng/mL, and the ULOQ was 7500 ng/mL. The accuracy and precision results are shown in Table 1. Extracted ion chromatograms showing the LLOQ for DNPA-D-serine and DNPA-L-serine are presented in Fig. 2, and Fig. 3 shows representative extracted ion chromatograms obtained from a blank sample.

Analyte carryover and selectivity were evaluated during the validation. Absolute carryover present in a blank sample that followed the ULOQ standard was found to be 0.03% for DNPA-D-serine and 0.06% for DNPA-L-serine. This represented ~17% of the response of the LLOQ standard for both analytes. Selectivity was investigated through the analysis of blank (w/IS) and double blank samples (no IS or analytes). There were no peaks present in the blank samples resulting from addition of the internal standard that interfered with DNPA-D-serine or DNPA-

Table 1a
Intra-day and inter-day accuracy and precision statistics for D-serine

Nominal (ng/mL)	*N*	Mean (ng/mL)	%RE	%CV
Intra-day 1				
10.3	5	9.9	−3.7	10.2
278	5	274.0	−1.5	2.6
7500	5	7556	0.7	1.8
Intra-day 2				
10.3	5	10.0	−3.0	15.5
278	5	272.6	−2.0	3.6
7500	5	7332	−2.2	4.1
Intra-day 3				
10.3	5	10.4	0.6	6.1
278	5	273.0	−1.8	4.3
7500	5	7252	−3.3	5.0
Inter-day				
10.3	15	10.1	−2.0	10.6
278	15	273.1	−1.8	3.3
7500	15	7381	−1.6	4.0

Table 1b
Intra-day and inter-day accuracy and precision statistics for L-serine

Nominal (ng/mL)	*N*	Mean (ng/mL)	%RE	%CV
Intra-day 1				
10.3	5	10.0	−3.0	12.3
278	5	281.7	1.3	7.2
7500	5	7934	5.8	6.4
Intra-day 2				
10.3	5	10.8	5.1	17.8
278	5	276.5	−0.5	4.7
7500	5	8047	7.3	5.9
Intra-day 3				
10.3	5	9.8	−4.3	20.8
278	5	283.1	1.8	6.2
7500	5	8089	7.8	6.4
Inter-day				
10.3	15	10.2	−0.8	16.5
278	15	280.4	0.9	5.8
7500	15	8023	7.0	5.9

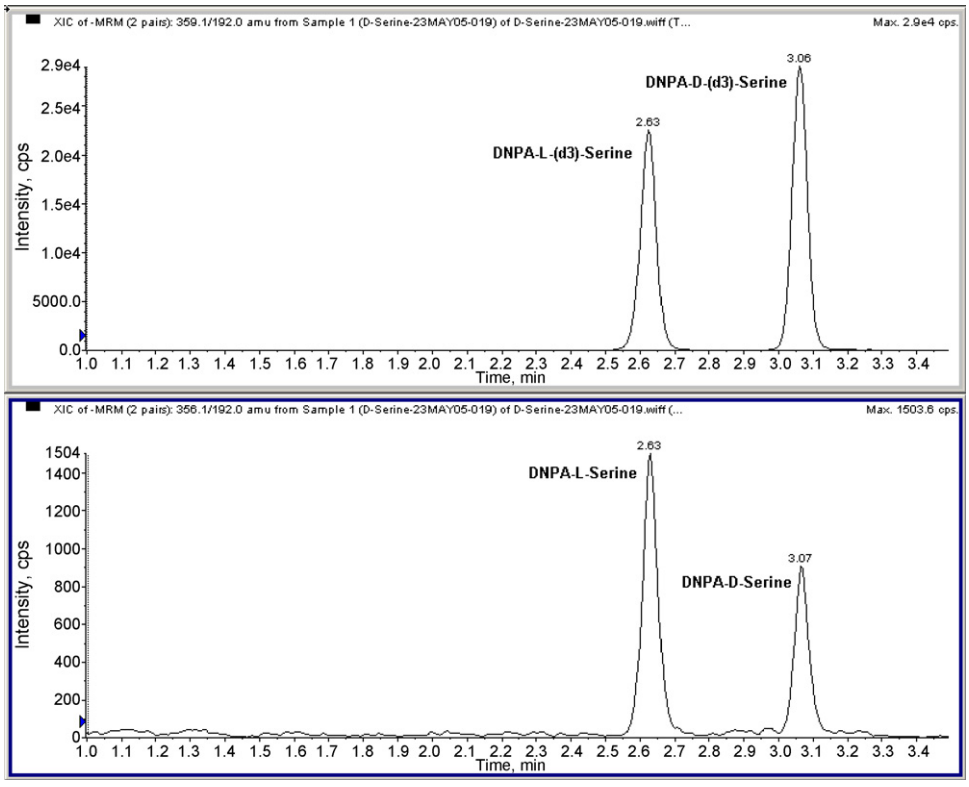

Fig. 2. Extracted ion chromatogram of DNPA-L-serine and DNPA-D-serine at the LLOQ (10.3 ng/mL), and DNPA-D/L-(d_3)-serine stable isotope labeled internal standard.

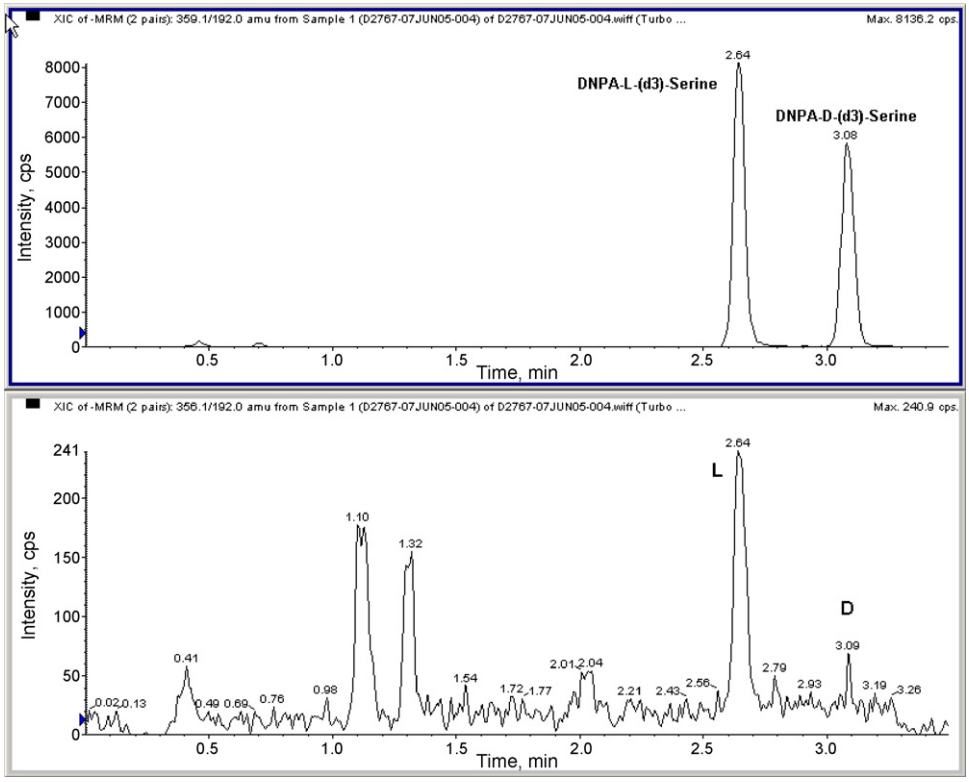

Fig. 3. Extracted ion chromatogram of a blank sample: the retention times of DNPA-L-serine [L] and DNPA-D-serine [D] are indicated. The interfering peak at [L] from the Marfey's reagent co-elutes with DNPA-L-serine and is ~15% of the LLOQ.

L-serine measurement. However, an interfering peak was present in the reagent blank at the retention time of DNPA-L-serine; the reagent blank contained all of the sample preparation reagents, including Marfey's reagent, but no analytes or internal standard. By selectively isolating each of the reagents in the derivatization scheme, it was found that the interfering peak originated in the Marfey's reagent. We believe that a side reaction in the derivatization step produced a peak that was isobaric to DNPA-L-serine and chromatographically unresolved. As the interference was significantly less than the DNPA-L-serine LLOQ, it did not affect L-serine measurement over the range of the assay. Furthermore, L-serine concentrations are in the μg/mL range in rat hippocampus microdialysate, while the L-serine interference was ∼1.5 ng/mL. The interfering peak also impacted the measurement of DNPA-L-serine carryover, because it was difficult to differentiate the carryover from the interference. This provides a plausible explanation for why DNPA-L-serine had twice the absolute carryover as DNPA-D-serine, which was not impacted by an interfering peak.

4. Discussion

An enantioselective LC/MS/MS assay for the determination of D-serine and L-serine was described. Derivatization of D/L-serine enantiomers was accomplished using Marfey's reagent, which created DNPA-D/L-serine diastereomers that were easily resolved under reverse-phase chromatographic conditions. A distinct advantage of the present assay is the simplicity and efficiency of the chemical derivatization, which precludes the use of difficult and oftentimes unreliable normal phase chiral chromatography. In addition, LC/MS/MS provided highly sensitive and selective detection and relatively high throughput (1 sample every 3.5 min). Sample throughput using this approach is significantly higher than an existing chiral LC/MS/MS approach that has a run time of 21 min. In addition, by modifying the sample extraction procedure, this approach would be amenable to CSF and plasma analyses.

D-serine and L-serine were isolated from rat brain tissues using microdialysis. A shortcoming of microdialysis procedures is the difficulty in determining analyte recovery across the dialysis probe. In the work presented here, this issue was addressed by relying on relative comparisons of D-serine and L-serine concentrations in individual rat brain tissues rather than on absolute D-serine levels. Microdialysate obtained from the hippocampus of four male Sprague Dawley rats had basal L-serine levels of 1.86 (±0.27) μg/mL and D-serine levels of 214 (±0.18) ng/mL. Furthermore, D-serine levels were found to increase significantly in rat hippocampus following IP administration of D-serine while L-serine levels remained constant (unpublished data).

During method development, and interfering peak was found at the retention time of L-serine. By isolating each of the reagents used during the derivatization procedure, the interfering peak was found to result from the Marfey's reagent. We believe that a side reaction during the derivatization produced a peak that was isobaric to DNPA-L-serine; however, it should be mentioned that the interfering peak was only ∼15% of the DNPA-L-serine LLOQ (10.3 ng/mL). Furthermore, because normal L-serine levels in rat hippocampus microdialysate are in the μg/mL range, the impact of this interference was insignificant. However, the presence of this interference provides a plausible explanation for the relatively large %CV at the LLOQ for L-serine compared to D-serine, which was still well within the range of the acceptance criteria.

This assay will provide an essential tool for studying D-serine and L-serine concentrations in rat brain tissues in the search for effective treatments for cognitive related disorders.

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