Simultaneous Analysis of D- and L-Serine in Cerebrospinal Fluid by Use of HPLC

Rama Sethuraman,¹ Malathi G. Krishnamoorthy,¹ Tat-Leang Lee,¹ Eugene Hern C. Liu,¹ Siau Chiang,¹ Wataru Nishimura,² Masato Sakai,² Toshiaki Minami,² and Shinro Tachibana¹*

Background: D-Serine is a coagonist for the glycine-binding site of the N-methyl-D-aspartate receptors and has been implicated in various neuropsychiatric functions such as learning, memory, and nociception, as well as schizophrenia and Alzheimer disease. We developed an HPLC method for D- and L-serine in cerebrospinal fluid (CSF).

Methods: The dabsylated racemic serine peak, automatically collected using a previously reported HPLC separation process for CSF amino acids, was desalted and subjected to a chiral resolution HPLC step with a Sumichiral column using an ultraviolet-visible detector.

Results: The limits of quantification (signal-to-noise ratio = 10) for D- and L-serine were 0.8 and 1.3 μmol/L, respectively. The mean imprecision values (CVs) for within-day measurements of D- and L-serine were 2.1% and 1.8%, respectively, and for between-day were 6.2% and 6.6%. Mean recovery of CSF serine (sum of D-serine + L-serine) applied to the Sumichiral column was 87%. The mean (SD) D-serine concentrations in 45 CSF samples obtained from 16 patients with chronic pain due to degenerative osteoarthritis of the knees, 16 with postherpetic neuralgia, and 13 with no pain were, respectively, 3.97 (0.44), 1.85 (0.21), and 2.72 (0.32) μmol/L.

Conclusion: D- and L-Serine can be quantified with ultraviolet-visible detection of dabsyl derivatives. The dabsyl derivatives are stable and allow duplicate analysis of CSF samples in multisample runs.

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¹ Department of Anaesthesia, Yong Loo Lin School of Medicine, National University of Singapore, Singapore.
² Department of Anesthesiology, Osaka Medical College, Osaka, Japan.
* Address correspondence to this author at: Department of Anaesthesia, National University of Singapore, 5 Lower Kent Ridge Rd., Singapore 119074.

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phase, and nonchiral derivatization with fluorescent reagents followed by HPLC separation with chiral columns (3, 14). The latter method was improved on by a column-switching system (15) and applied to detect D- and L-serine in CSF (8). The fluorescence derivatives are light sensitive and relatively unstable (13), however, and many of these methods require special instrumentation. Hence, there is a need for a more practical method for analysis of D-serine in human tissues and fluids (14, 16).

We report here a method for measuring D-serine in CSF using 4-(dimethylamino) azobenzene-4′-sulfonyl (dabsyl) chloride labeling and HPLC separation techniques with a simple UV-visible detector.

Materials and Methods

CHEMICALS

We purchased dabsyl chloride from Pierce. Oxalic acid, citric acid, tartaric acid, lithium hydroxide, potassium perchlorate, guanidine thiocyanate, D-tert-leucine, norleucine, γ-aminobutyric acid (GABA), taurine, citrulline, glutamine, asparagine, and amino acid standard mixture (AA-S-18) were from Sigma. Triton X-100 was from Bio-Rad; dimethyl formamide (DMF), acetone, methanol, and acetoni trile (HPLC grade) were from Merck. Ethanol (HPLC grade) and triethylamine (TEA) were from Fluka.

HPLC APPARATUS

We used a Gynkotek HPLC system with a P 580A HPG pump, autosampler, and UV-visible photodiode array detector (UVD 340S) and a Shimadzu Prominance HPLC system with a LC20AD pump, SIL20A autosampler, and SPD M20A UV-visible photodiode array detector.

CSF SAMPLES

The protocol for collecting CSF samples was approved by our Institutional Review Board, and written informed consent was obtained from the patients. We obtained 1 mL of CSF from each patient during subarachnoid puncture. Patients with moderate to severe degenerative osteoarthritis (OA) affecting the knees had earlier consented for joint replacement surgery under spinal anesthetic technique, whereas the patients with postherpetic neuralgia (PHN) had given their consent to receive intrathecal steroid therapy for pain management. Pain-free patients were undergoing prostatic surgery and inguinal hernia repair surgery under spinal anesthesia. We age-matched the participants in the 3 groups to reduce variation in certain amino acid concentrations due to age differences (17).

We froze all CSF samples at −80 °C immediately after collection. The samples were thawed and aliquots were removed just before derivatization and analysis.

DERIVATIZATION AND HPLC ANALYSIS

Dabsyl chloride derivatization and HPLC separation of 23 compounds including D-serine. The 1st step involved dabsyl chloride derivatization of the sample followed by separation on a LiChrosphere 100 RP-18 (Merck, 5 μm, 2 by 250 mm) column. We followed the previously reported dabsyl chloride derivatization procedure for standard amino acid mixture and CSF samples and gradient HPLC analysis (18), with the exception that 1 nmol norleucine was added as an internal calibrator before dabsylation to confirm complete derivatization. We collected the dabsyl serine peak from 2 runs (20-μL injection volume for each run) using an automatic Foxy Jr fraction collector as 30-s (0.1-mL) fractions. The concentrations of total DL-serine collected from the LiChrosphere column HPLC were calculated against known concentrations of DL-serine run on the same day.

We pooled the collected dabsyl serine peak fractions from the 2 runs, diluted them 10 times with distilled water, and acidified them to pH = 3 using 0.01 mol/L HCl. We added 200 pmol/2 μL dabsylated D-tert-leucine to this sample as an internal standard. The sample was loaded onto 2 Waters Sep-Pak C 18 cartridges preactivated with aqueous 10 mL/L acetonitrile containing 1 mL/L trifluoroactic acid and connected in series to desalt the sample. We performed elution using 2 mL of a 1:1 mixture of acetonitrile and methanol and then used nitrogen gas to evaporate the mixture to dryness.

Resolution of D- and L-serine peaks. We reconstituted the above desalted dabsyl racemic serine sample in 10 μL DMF, from which we injected 3 μL onto a Sumichiral OA 3200 (5 μm, 2 by 250 mm) column (Sumika Chemical Analysis Service). Mobile phase A consisted of 0.1 mL/L guanidine thiocyanate in methanol, and mobile phase B consisted of 0.005 mol/L oxalic acid and 20 mL/L TEA in a 3:2 mixture of methanol and ethanol. A 0.18 mL/min isocratic flow of 70% B in mobile phase A was used for elution, and detection was set at 438 nm. We performed duplicate runs to minimize variance. We calculated the concentrations of D- and L-serine using the peak areas in the standard DL-serine run on the same day and corrected the amounts based on the internal standard dabsyl D-tert-leucine recovery in the Sumichiral run.

METHOD VALIDATION

We added known concentrations (0, 4.2, 8.4, 16.8, and 33.6 μmol/L) of DL-serine and the internal standard D-tert-leucine to a CSF sample containing 22.66 μmol/L endogenous DL-serine and performed microbore Sumichiral separation (n = 5 for each concentration). We subjected the data to linear regression analysis and calculated the slope, intercepts, SEs of the slope and intercept, and SD about the regression line. ANOVA was used to compare the differences between-day and within-day and estimate the within-day and between-day imprecision for our method. We analyzed 6 different concentrations (2.1, 4.2, 8.4, 16.8, 33.6, and 67.2 μmol/L) of D-serine and L-serine 5 times on the same day and calculated within-day reproducibility. On 21 separate days, 33.6 μmol/L each of D- and L-serine were run, and on 7 separate days, 16.8 and
8.4 μmol/L of these enantiomers were run. The peak areas on each day were used to calculate between-day reproducibility. We defined the limit of quantification (LOQ) as signal-to-noise ratio = 10 in the HPLC run during linear regression analysis using CSF samples described above. Recovery tests for d- and l-serine peaks from the desalting and the Sumichiral separation step were calculated as shown below. The concentration of DL-serine collected from the first RP-18 separation step was calculated (18), and the concentrations of d- and l-serine in the final Sumichiral separation step were calculated based on the corresponding peak areas for duplicate runs of 45 CSF samples:

Recovery % = (concentration of d-serine + concentration of l-serine calculated after Sumichiral separation)/(concentration of DL-serine applied to the Sumichiral column from the first RP-18 HPLC run) × 100.

ANALYSIS OF D-SERINE AND L-SERINE IN CSF SAMPLES

Forty-five CSF samples obtained from 3 groups of patients—chronic pain due to OA of the knees (n = 16), chronic pain resulting from PHN (n = 16), and pain-free participants (n = 13)—were analyzed for d- and l-serine. These CSF samples were dabsylated and the amino acids separated as reported (18). In this 1st step, we calculated the concentrations of 23 compounds, including the physiologic amino acids and citrulline, taurine, and GABA, for the 45 CSF samples based on the peak areas in this HPLC run. The dabsyl DL-serine peak from this run was automatically collected, desalted, and subjected to chiral separation on HPLC using a Sumichiral column as described above.

All the amino acid concentrations are reported as mean (SE). We evaluated differences in amino acid concentrations among 3 groups using ANOVA and Bonferroni post hoc tests. We used SPSS (version 14.0, SPSS) statistical software and considered a P value <0.05 as statistically significant.

**Results**

The Sumichiral column separation of dabsyl D-serine, L-serine, and the D-tert-leucine in the standard and a CSF sample are shown in Fig. 1.

The technical brochure on Sumichiral OA 3200 column recommends a methanol-ammonium acetate solvent system for the resolution of racemic compounds and advises that water or water-containing solvents could damage the column matrix. We investigated the effects of various organic solvents (methanol, ethanol, acetonitrile, and DMF), carbonic acids (acetic acid, oxalic acid, tartaric acid, and citric acid), and chaotropic agents on the resolution of racemic DL-serine mixture. We found that oxalic acid (0.005 mol/L) produced the best resolution for the D- and L-serine peaks and the dabsyl D-tert-leucine internal standard. Although there was merging of the dabsyl D- and L-serine peaks when citric or oxalic acid in methanol was used in the mobile phase B, they gradually separated when the acid was neutralized by addition of ammonia. Furthermore, the salt form of the carbonic acids was also studied by the addition of TEA instead of ammonia. The best separation was achieved by adding 20 mL/L TEA in the mobile phase B with 0.005 mol/L oxalic acid. Among the various organic solvents studied, the ethanol:methanol mixture in the ratio of 2:3 was standardized as the ideal solvent for the mobile phase B. A chaotropic agent guanidine thiocyanate (0, 10, or 20 mL/L) was tested, and 10 mL/L guanidine thiocyanate in methanol in mobile phase A improved the resolution of the dabsyl D- and L-serine peaks on the Sumichiral column.

**METHOD VALIDATION RESULTS**

**Linearity.** A linear relationship between peak areas and concentrations was observed in the interval of 1.7 to 333.3 μmol/L when evaluated on dabsyl D-serine and L-serine calibrators with r > 0.99 (data not shown). The linearity was also determined by adding known amounts of dabsyl D-serine, L-serine, and D-tert-leucine to a CSF sample as described in Materials and Methods. Regression analysis showed r > 0.99 for all 3 compounds (Table 1).

<table>
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<th>Amino acid</th>
<th>Slope, m</th>
<th>Intercept y</th>
<th>Slope SE</th>
<th>Intercept SE</th>
<th>SD</th>
<th>Correlation coefficient</th>
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</thead>
<tbody>
<tr>
<td>D-Serine</td>
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<td>0.0004</td>
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<td>0.03</td>
<td>0.999</td>
</tr>
<tr>
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<td>0.0007</td>
<td>0.036</td>
<td>0.06</td>
<td>0.997</td>
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<tr>
<td>D-Tert-leucine</td>
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<td>-0.016</td>
<td>0.0029</td>
<td>0.148</td>
<td>0.23</td>
<td>0.992</td>
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</table>

Fig. 1. Separation of dabsyl D-serine, L-serine, and D-tert-leucine on a Sumichiral column.

Chromatograms of dabsyl derivatives of the 3 compounds (100 pmol each) in standard amino acid mixture (A) and a CSF sample from a patient suffering from OA of the knees (B) separated using an isocratic flow of 70% buffer B in A. Peaks: 1, D-tert-leucine; 2, D-serine; 3, L-serine; 4, L-glutamine.
LOQ. The LOQ values for dabsyl d-serine, l-serine, and d-tert-leucine were 0.8, 1.3, and 0.3 μmol/L, respectively.

Recovery. Recovery calculations for the 45 CSF samples showed a mean 87% recovery for d-serine and l-serine from the Sumichiral column (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol53/issue8).

Reproducibility. The mean within-day reproducibility (CV) was 2.1% and 1.8% for d-serine and l-serine peaks, respectively, and the mean between-day reproducibility was 6.2% and 6.6% for d- and l-serine on the Sumichiral column (see Table 2 in the online Data Supplement).

CSF ANALYSIS RESULTS

Multiple comparisons by ANOVA showed significant differences among the 3 groups: d-serine (F 10.477, P <0.001), l-serine (F 7.522, P = 0.002). Bonferroni post hoc test showed that the d-serine concentrations in the OA patient group were significantly higher than those in the no-pain controls (P = 0.043), whereas the d-serine concentrations in the PHN group were lower, although not statistically significant, vs the no-pain controls (Fig. 2).

In addition, the concentrations of the other amino acids in these 3 groups of CSF samples were calculated as described in Materials and Methods. ANOVA also showed significant differences in citrulline (F 3.570, P = .037) and glycine (F 4.297, P = 0.02) concentrations among the 3 groups. Post hoc tests showed no significant difference in the concentrations of l-serine, glycine, citrulline, aspartate, or glutamate between the OA and the no-pain groups. On the other hand, only the citrulline concentrations were significantly increased in the PHN group compared with the controls (P = 0.039; Fig. 2).

Discussion

There have been some reports on quantitative analysis of d-serine in the human brain, serum, and CSF. Except for 2 early reports that used gas chromatography, the common derivatization procedures reported include tert-butyloxycarbonyl-l-cysteine and OPA/chiral thiols such as N-acetyl-l-cysteine (15, 19, 20). All of these methods require fluorescence detection. However, the OPA/chiral thiol system has the additional disadvantage that the resulting isoindole derivatives are relatively unstable (13, 20). In comparison, the dabsyl derivatives used as described in this report are more stable and can be stored for up to 48 h at room temperature when protected from light and up to 1 week when stored at 4 °C (18), making this method more suitable for automated analysis of multiple CSF samples. In addition, the CSF samples, once dabsylated, can be repeatedly analyzed (such as in duplicate) to minimize variance.

This method involves isocratic elution from the Sumichiral column, and separation of these mixtures takes ≤20 min. The microbore size column (2 by 250 mm) allows the detection of d-serine with higher sensitivity (LOQ 0.8 μmol/L for d-serine) than the frequently used fluorescence detection (15). This method offers reasonable recovery and also reproducibility (within-day and between-day) for both the d- and l-serine peaks.

In using this method as described, during the 1st HPLC step in which the RP-18 column is used for collection of the dabsyl d-serine peak, the amounts of 22 other amino acids, including citrulline, GABA, and taurine, can be calculated. Thus this procedure has an additional advantage in that it can quantitatively analyze d- and l-serine in CSF along with other amino acid neurotransmitters and neuromodulators, explicating any possible interplay between d-serine and these neuromodulatory substances.

The concentration of glutamine in CSF is normally >10 times higher than other amino acids. On the LiChrosphere column the dabsyl serine peak was found to elute close to the huge glutamine peak during the 1st step of this procedure (18). Hence, some glutamine was inevitably included in the d-serine peak collected by automatic fraction collection from the 1st run, accounting for the presence of the dabsyl glutamine peak in addition to the dabsyl d- and l-serine peaks and dabsyl d-tert-leucine internal standard peaks when the desalted sample was injected onto the Sumichiral column (Fig. 1). Another possible source of contamination of the dabsyl serine peak was dabsyl citrulline, which emerged just before the serine peak during the 1st step. Under the Sumichiral separation conditions described in this report, however, the dabsyl citrulline emerged after the glutamine peak and hence did not interfere with the quantitative analysis of d- and l-serine.
There have been reports on CSF \( \alpha \)-serine analysis in pathophysiology of diseases such as Alzheimer disease and schizophrenia (7, 8, 21, 22). We chose to evaluate the possible roles of \( \alpha \)-serine in pain, a multidimensional sensory experience. We analyzed the CSF obtained from 2 chronic pain conditions with different pathology (OA and PHN) and compared the results with those of another group of no-pain individuals. The significantly higher concentrations of \( \alpha \)-serine in the OA group \[[3.97 (0.44) \mu\text{mol/L}] \] compared with the no-pain group \[[2.72 (0.32) \mu\text{mol/L}] \] suggest that NMDA receptors that require \( \alpha \)-serine for full activation are involved in pain arising from OA. The lower concentration of \( \alpha \)-serine in the PHN group \[[1.85 (0.21) \mu\text{mol/L}] \] was unexpected, however. Pain in OA patients can be classified as chronic inflammatory pain, whereas PHN is a chronic neuropathic pain after viral infection. These results suggest that the pain transmission between inflammatory and neuropathic pain could be different with respect to NMDA activation mechanisms. This role of \( \alpha \)-serine in inflammatory pain is supported by a recent report that \( \alpha \)-serine enhanced C-fiber responses in spinal dorsal horn neurons of normal rats at single-unit recording experiments, whereas it failed to increase these nociceptive responses in a carrageenan-injection inflammation model, probably owing to the saturation of the glycine binding sites of the NMDA receptors with endogenous glycine and/or \( \alpha \)-serine for full activation are involved in pain arising from OA. The lower concentration of \( \alpha \)-serine in the PHN group \[[1.85 (0.21) \mu\text{mol/L}] \] was unexpected, however.

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Citrulline, a byproduct of nitric oxide (NO) synthesis, was significantly increased in the PHN group compared with the no-pain controls. This finding suggests that NO and not \( \alpha \)-serine may be involved in the pain mechanisms of PHN. To the best of our knowledge, there have been no reports on NO concentrations in the CSF of herpetic patients, although inducible-type NO synthase (NOS) was highly expressed in the human skin lesions caused by acute herpes zoster infection (23). It is possible that S-nitrosylation of some constituent proteins of NMDA receptors influences the neurotransmission of pain by changing inward ion current (24). The apparent inverse relationship between \( \alpha \)-serine and NO is supported by 2 recent reports using the human glioblastoma cell line U87. The activity of \( \alpha \)-serine racemase was inversely regulated by \( \alpha \)-serine and NO (25). \( \alpha \)-Serine indirectly caused dose- and time-dependent inhibition of neuronal NOS without affecting endothelial NOS. The activity of \( \alpha \)-amino acid oxidase catalyzing the oxidative deamination of \( \alpha \)-amino acid was enhanced by NO in a dose-dependent manner (26).

Furthermore, not only \( \alpha \)-serine \((P < 0.01) \) but also L-serine \((P < 0.01) \) and glycine \((P < 0.05) \) concentrations in OA were significantly higher than those in PHN. Pearson correlation analysis showed significant correlations between \( \alpha \)-serine and L-serine or glycine (both \( P < 0.01) \). This finding also supports the involvement of \( \alpha \)-serine in NMDA receptor activation, because \( \alpha \)-serine is synthesized from L-serine by serine racemase and glycine has been known to be the precursor of L-serine (2, 3). This is the 1st report that associates \( \alpha \)-serine in CSF to human chronic inflammatory pain. These results also suggest that NO might play a role in the pain mechanism in PHN patients.

The \( \alpha \)-serine concentration of the no-pain group \[[2.72 (0.32) \mu\text{mol/L}, 69.0 (7.8) years old, \( n = 13 \)] \] was slightly higher than that of the published normal control data \[[1.8 (1.2) \mu\text{mol/L}, 66.0 (7.4) years old, \( n = 5 \)] \], and 1.39 (0.29) \mu\text{mol/L}, 27.3 (range, 22–44) years old, \( n = 17 \) (8)]. However, the \( \alpha \)-serine of the pain-free group \[[20.53 (1.31) \mu\text{mol/L}] \] was almost the same as that of the published normal control data \[[18.2 (4.78) \mu\text{mol/L}, 27.3 (range, 22–44) years old, \( n = 17 \) (8)]. The slight discrepancy between the current and the published \( \alpha \)-serine concentrations in the control groups might result from the differences in the method for analysis: age [younger (8)], sex [only male (7)], and method of CSF collection [ventricular (7)].

\( \alpha \)-serine has been recently identified as a gliotransmitter influencing numerous key processes in the CNS, including glutamatergic neurotransmission, regenerative firing, and synaptic plasticity (1, 12). In light of such increasing evidence for multifarious roles of \( \alpha \)-serine in the CNS, this new method with enhanced sensitivity will be a valuable tool for evaluating the involvement of \( \alpha \)-serine in the pathophysiology of various CNS diseases and in normal neuropsychiatric functions, including learning, memory, and nociception.

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References


