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Rapid and sensitive procedure for the separation and quantitation of D- and L-serine in rat brain using gas chromatography–mass spectrometry

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Abstract

A very simple and rapid GC–MS procedure for the separation and quantitation of D- and L-serine has been developed utilizing a conventional bonded-phase capillary column. The procedure involves initial esterification with isobutanol followed by acylation with the chiral derivatizing reagent *S*-(−)-N-(heptafluorobutyryl)prolyl chloride (HPC). This procedure requires neither extraction nor clean-up steps and is sensitive to 50 pg on-column. Total time of the procedure is under 3 h and derivatives are stable at room temperature for at least 5 days, making this procedure ideal for automated injections. A simple, one-day synthesis of HPC is described which yields >99.9% optical purity.

1. Introduction

D-Serine is a stereospecific agonist for the N-methyl-D-aspartate (NMDA)-coupled glycine receptor [1]. In addition, this amino acid is inactive at the strychnine-sensitive glycine receptor [2] and has low affinity for glycine uptake systems [3]. These properties make D-serine an ideal pharmacological tool for studying the NMDA-coupled glycine receptor [4].

High concentrations of D-serine in rodent brain were first reported by Nagata et al. [5] and Hashimoto et al. [6]. These results were later verified by other laboratories [7,8]. D-Serine was reported to be from de novo synthesis in mice [9], but the actual pathways involved have not been determined. Furthermore, the regional distribution of D-serine, but not glycine, has been shown to be positively correlated with NMDA receptor density in rat brain [10] and negatively correlated with regional levels of D-amino acid oxidase (E.C. 1.4.3.3 [11]) in mouse brain, however, regional dynamics of D-serine pools have yet to be determined. More recent studies have also quantitated D-serine in human brain [7,12] and plasma [8,13] supporting the importance of this D-amino acid in the CNS. An assay which allows rapid and sensitive quantitation of D-serine will be of great assistance in elucidating the synthesis, degradation, and biological significance of this interesting amino acid.

The separation and quantitation of D- and L-serine has been previously described for high-performance liquid chromatography (HPLC)

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[5,14], GC–MS [7] and GC [6,8]. Unfortunately, these procedures involve laborious sample preparations and/or involve the use of non-bonded, labile, chiral columns. The procedure reported here is rapid, sensitive, the resultant derivatives are stable at room temperature and separation is achieved on commonly used bonded-phase columns.

2. Experimental

2.1. Reagents and materials

D- and L-Serine were obtained from Sigma (St. Louis, MO, USA). [$^2H_3$]-D/L-Serine was purchased from Cambridge Isotope Laboratories (Woburn, MA, USA). Isobutanol was purchased from J.T. Baker (Phillipsburg, NJ, USA). Thionyl chloride and heptafluorobutyric anhydride were purchased from Aldrich (Milwaukee, WI, USA). All solvents were of HPLC or better grade and were purchased from Baxter (Muskegon, MI, USA). *S*-(−)-N-(Heptafluorobutyryl)prolyl chloride (HPC) was synthesized as described below.

2.2. Synthesis of S-(−)-N-(heptafluorobutyryl)prolyl chloride (HPC)

HPC was synthesized using a modification of the procedure reported by Lim et al. [15]. Heptafluorobutyric anhydride (10 mmol) was added to a mixture of L-proline (11 mmol) and ethylacetate (30 ml) at room temperature. The reaction was allowed to proceed for 2 h, after which the ethylacetate layer was washed with 5 ml of 0.1 *M* HCl, dried with anhydrous Na_2SO_4, gravity-filtered through a Whatman G/F-B filter disc, and then evaporated under vacuum at room temperature. The resultant clear, colorless liquid was approximately a 1:1 ratio of N-heptafluorobutyrylproline and heptafluorobutyric acid. To 5 mmol of diacid (10 total mmol), 9 mmol of triethylamine (TEA) was added and thoroughly mixed. Any residual TEA was removed by applying vacuum for 5 min. This residue was then cooled to 0°C in an ice-bath and 250 ml of ice-cold 2.0 *M* thionyl chloride in dichloromethane was added. The reaction was allowed to proceed for 2 h at room temperature after which the excess thionyl chloride (b.p. 79°C) and the C_3F_7COCl (b.p. 39°C) formed were removed under vacuum at room temperature. To the resultant residue (a mixture of the hydrochloride salt of TEA and HPC) 100 ml of hexane was added, thoroughly mixed, and allowed to settle. The hexane layer was collected, leaving the hydrochloride salt of TEA behind. The hexane was then removed under vacuum at room temperature and the final residue (a clear, colorless liquid) was reconstituted in hexane to a concentration of 0.2 *M* and stored at −20°C.

NMR of the final product indicated that only one compound with the proline backbone was present and that only one C_3F_7 species was present. Optical purity of the synthetized HPC was evaluated by the derivatization of D- and L-serine and was observed to be >99.9%.

2.3. Preparation and derivatization of biological samples

Male Sprague–Dawley rats (200–250 g) were anesthetetized with CO_2, decapitated and the cortex, cerebellum, striatum and hippocampus were dissected out on ice and stored at −80°C. These regions were then homogenized in 1 ml of ice-cold 0.1 *M* HCl using a Kontes (Vineland, NJ, USA) micro ultrasonic cell disrupter. This homogenate was then centrifuged at 40 000 *g* for 45 min. Immediately following the centrifugation the supernatant was decanted and stored at −80°C until the time of analysis. The pellet obtained was analyzed for protein content by the method of Chouinard et al. [7]. Aliquots of the brain extract (80 μl for hippocampus and striatum and 40 μl for cortex and cerebellum) or standard (10 μl, made up in 0.1 *M* HCl) were added to small reaction vials (containing 2000 ng of [$^2H_3$]-D/L-serine in 20 μl of 0.1 *M* HCl) and dried under nitrogen at room temperature. The dry samples were then redissolved in 200 μl of an isobutanol-HCl solution (one drop of concentrated HCl per ml of isobutanol), capped and heated at 100°C to derivatize the carboxylic acid

moiety [16]. After 15 min, the vials were allowed to cool to room temperature, and then evaporated at room temperature under a stream of nitrogen. To this residue HPC (20 μl in 200 μl of hexane) and TEA (2 μl in 100 μl of hexane) were added. The vials were vortex-mixed briefly and then allowed to stand at room temperature for 30 min. After 30 min the vials were dried under nitrogen at room temperature. A 100-μl volume of hexane was then added to the residue and vortex-mixed. An aliquot of the hexane (1 μl) was then injected onto the GC–MS system.

2.4. Calibration

Known amounts of authentic D- and L-serine corresponding to 5–2500 ng plus a fixed amount of the internal standard, [$^2H_3$]-D/L-serine (2000 ng) were placed in reaction vials and carried through in parallel with each set of samples. The amount of D- and L-serine present was determined by comparing the ratio of the nondeuterated to the deuterated fragment ions in the tissue samples to the standard curve generated using the ratios obtained with known concentrations of D- and L-serine. The ion pairs used for quantitation were *m/z* 550/553, 455/458, 353/356, and 335/338. The standard curves generated by each of these ion pairs were identical so the mean ion ratio of the four ion pairs was used. Correlation coefficients were routinely >0.99. Internal standard was added to the brain supernatant prior to drying.

2.5. Gas chromatographic and mass spectrometric conditions

A Finnigan MAT Ion Trap (electron-impact ionization mode) equipped with a DB-17 (50% phenylmethylpolysiloxane stationary phase, 0.1-μm film thickness) capillary column (30 m × 0.32 mm I.D., J and W, Folsom, CA, USA) was used for routine mass spectral analysis. The oven temperature program for the GC was as follows: initial temperature of 180°C, held for 0 min; increasing to 260°C at a rate of 8.5°C/min, held for 2.09 min and then increasing to 300°C at 25°C/min where it was maintained until the end of the run. The total run time was 17 min. The SPI (septum equipped programmable injector) was programmed from 180°C to 300°C at 25°C/min then maintained at 300°C until the end of the run. The mass range was set for 300 to 560 amu with the background mass set at 299 amu.

Verification of derivatives was carried out using the above GC conditions on a Fisons (Manchester, UK) Autospec in full-scan mode with an ionization voltage of −70 eV.

3. Results and discussion

3.1. Synthesis of HPC

S-(−)-Trifluoroacetylprolyl chloride (TPC) is a chiral derivatizing agent which has been used successfully in the separation and quantitation of the optical isomers of fluoxetine and norfluoxetine [17], tranylcypromine [18], and amphetamine derivatives [19] and is commercially available (Aldrich, Milwaukee, WI, USA). The optical purity of several different lots of TPC, however, was very inconsistent ranging from 85 to 90%, which was unacceptable for our purposes. The use of HPC as a chiral derivatizing reagent was reported by Lim et al. [15] for the separation and quantitation of (+) and (−)-methylphenidate and by Roy and Lim [20] for the quantitation of the optical isomers of methoxyphenamine and its metabolites. HPC is not available commercially and the synthetic procedure reported by Lim et al. [15] did not result in pure HPC in our hands. A new synthetic procedure was investigated. The synthesis reported in this report follows the same concept as the Lim et al. [15] process except that L-proline is used in excess instead of heptafluorobutyric anhydride. The excess L-proline is then removed by extraction with dilute acid. The heptafluorobutyric acid is removed by first converting it to the acid chloride along with the heptafluorobutyryl proline. The heptafluorobutyryl chloride is then evaporated off with the excess thionyl chloride and solvent. However, we found that heptafluorobutyric acid was not completely converted to the acid chloride unless

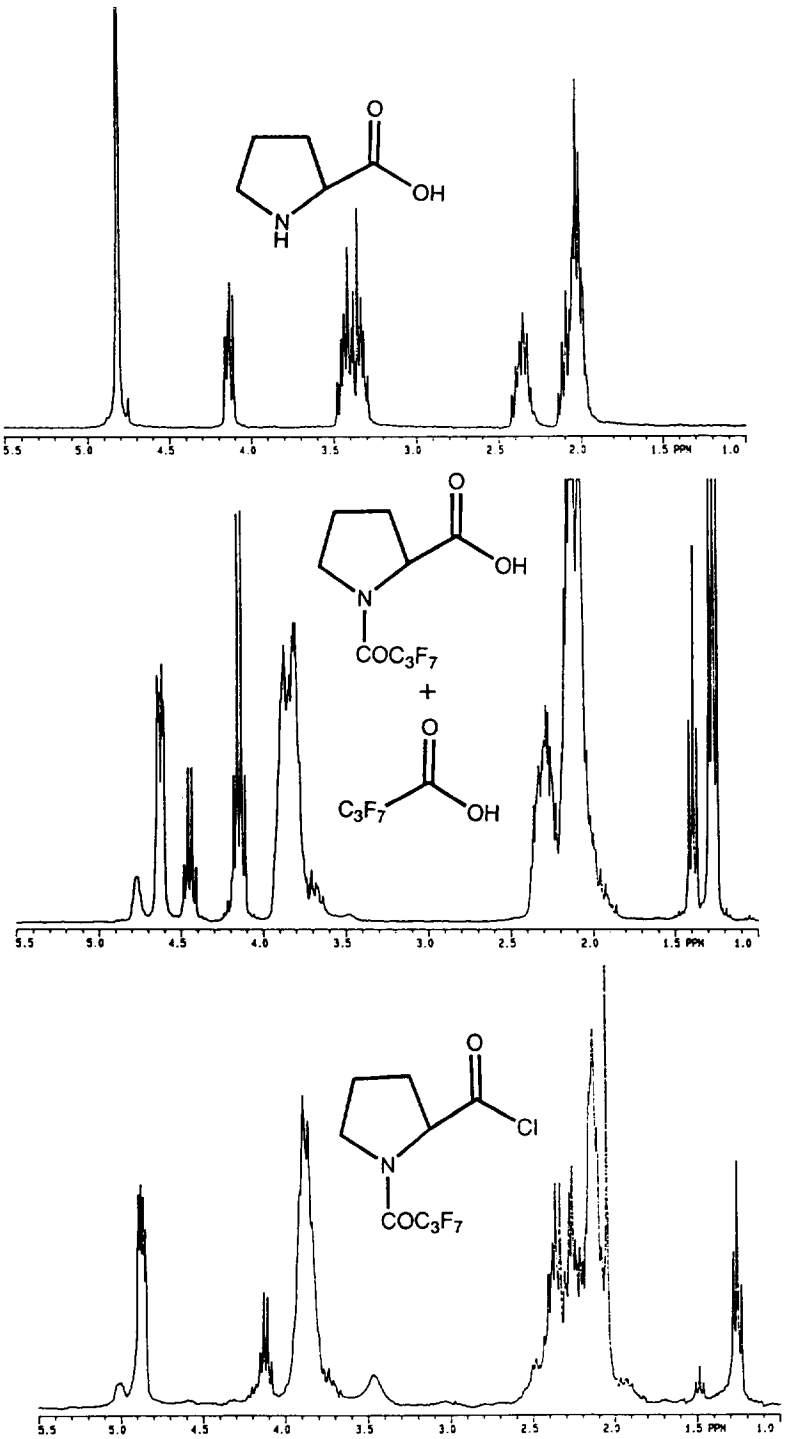

Fig. 1. Representative ^{1}H NMR traces of the synthetic pathway to HPC.

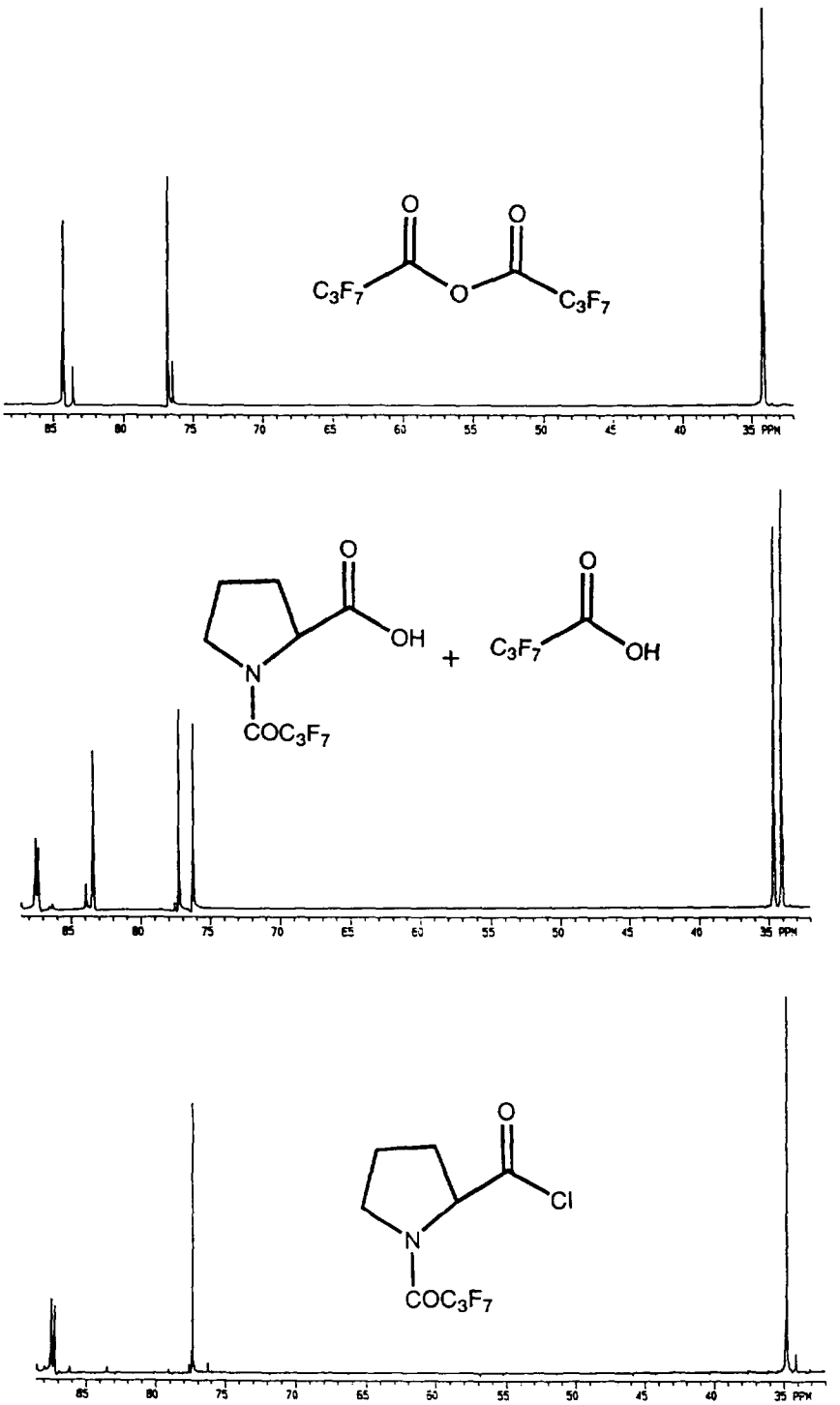

Fig. 2. Representative ^{19}F NMR traces of the synthetic pathway to HPC.

the thionyl chloride reaction was performed in refluxing ethyl acetate or a proton acceptor (TEA) was added. Refluxing in ethyl acetate resulted in significant epimerization (3–10%), but adding a proton acceptor allowed the reaction to proceed rapidly to completion at room temperature with no observable epimerization.

The course of the reaction can easily be

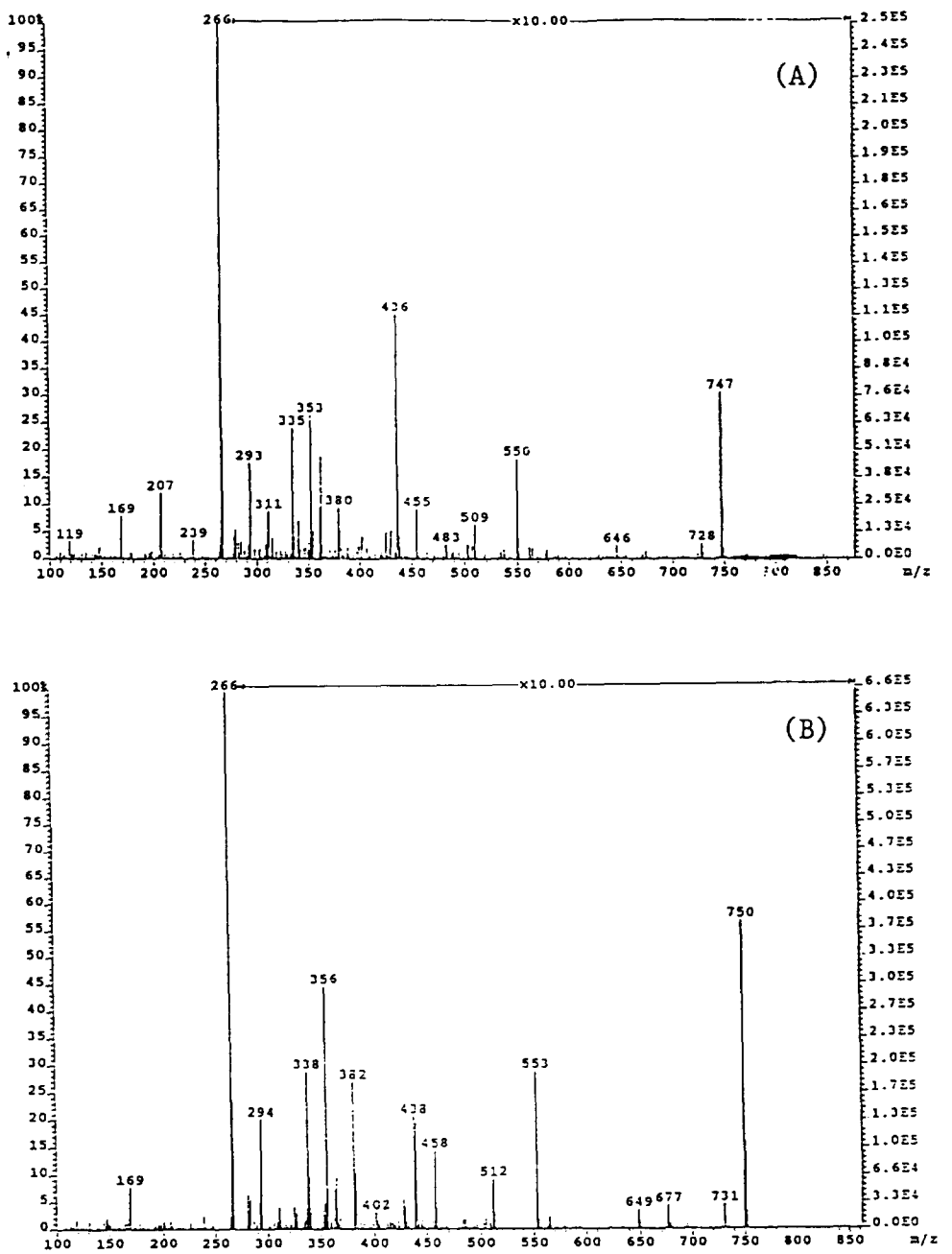

Fig. 3. Mass spectra of the isobutyl/HPC derivatives of D-serine (A) and the trideuterated analog of D-serine (B).

monitored by both fluorine and hydrogen nuclear magnetic resonance (^{1}H NMR and ^{19}F NMR, respectively, Figs. 1 and 2). The proton on the asymmetric carbon of proline gives a clear doublet–doublet peak at 4.12 ppm (D- and L-proline give identical spectra). This proton in N-heptafluorobutyryl-L-proline gives two doublet–doublets which are shifted downfield to 4.64 and 4.78 ppm, respectively. The two peaks have a ratio of approximately 85:15 with the 4.64 peak being the major one. These two peaks are believed to be the result of the two geometric isomers of the amide bond. The spectrum of this proton is further shifted downfield in HPC to 4.88 and 5.02 ppm. Again the ratio of the two peaks is approximately 85:15 with 4.88 being the dominant peak. Thus by monitoring the spectra of the proton on the asymmetric carbon the course and the completeness of the reaction can be followed. Similarly, the ^{19}F spectra are useful in that the major contaminants (heptafluorobutyric acid and/or heptafluorobutyryl chloride) give different spectra than N-heptafluorobutyrylproline and HPC and are not detectable by ^{1}H NMR. Near-complete removal of these components was observed under the specified reaction conditions (Fig. 2).

The optical purity of HPC was assessed by reacting it with the isobutyl ester of pure D- and L-serine and then analyzing the derivatives by GC–MS. Optical purity, as determined by this method, was found to be >99.9% (see Fig. 5)

3.2. Separation and quantitation of D- and L-serine

The separation and quantitation of D- and L-serine has been reported by a number of groups, however these processes involved laborious sample preparations [5,8,14] or the use of a labile, non-bonded chiral capillary column [6–8]. The procedure reported here involves an initial esterification with an isobutanol-HCl solution followed by acylation with HPC in the presence of an organic base (TEA) in hexane. The carboxylic acid, amine and hydroxy functional groups are derivatized in this procedure.

Analysis of the HPC-derivatives of D- and L-serine was carried out by GC–MS with trideuterated D/L-serine as the internal standard. This method showed excellent calibration linearity with correlation coefficients consistently >0.99. The accuracy of the method was determined at 50, 100, 250, 500, and 1000 ng and at each concentration the deviation of the calculated from the actual values was <10%. Intra-day variability was less than 5% and inter-day variability was less than 10%. Reproducibility was determined by running ten 500-ng samples (5 ng on-column) through the procedure and resulted in a coefficient of variation of <5%. The sensitivity of the procedure is approximately 50 pg on column, which was more than adequate for the analysis of the prepared brain extracts which generally resulted in levels of D-serine from 1–3

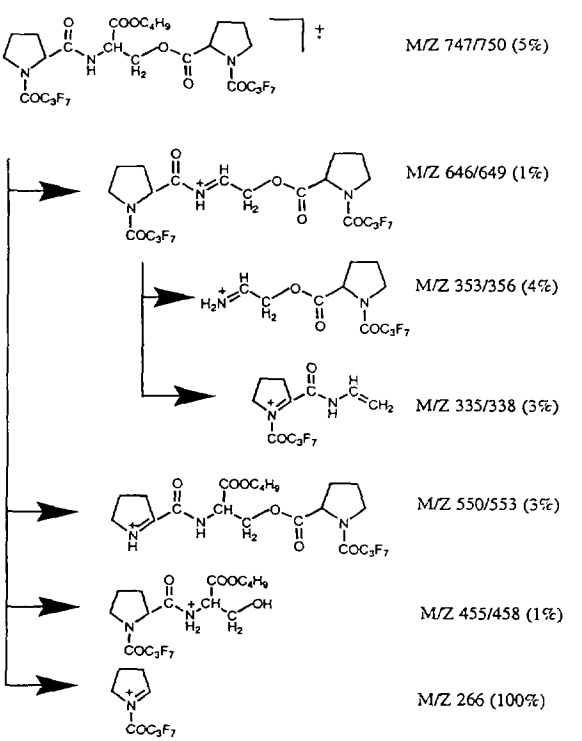

Fig. 4. The proposed fragmentation pathway of the isobutyl/HPC derivatives of D/L-serine and its trideuterated analog. Values in parentheses indicate the percent abundance relative to the base ion (*m/z* 266).

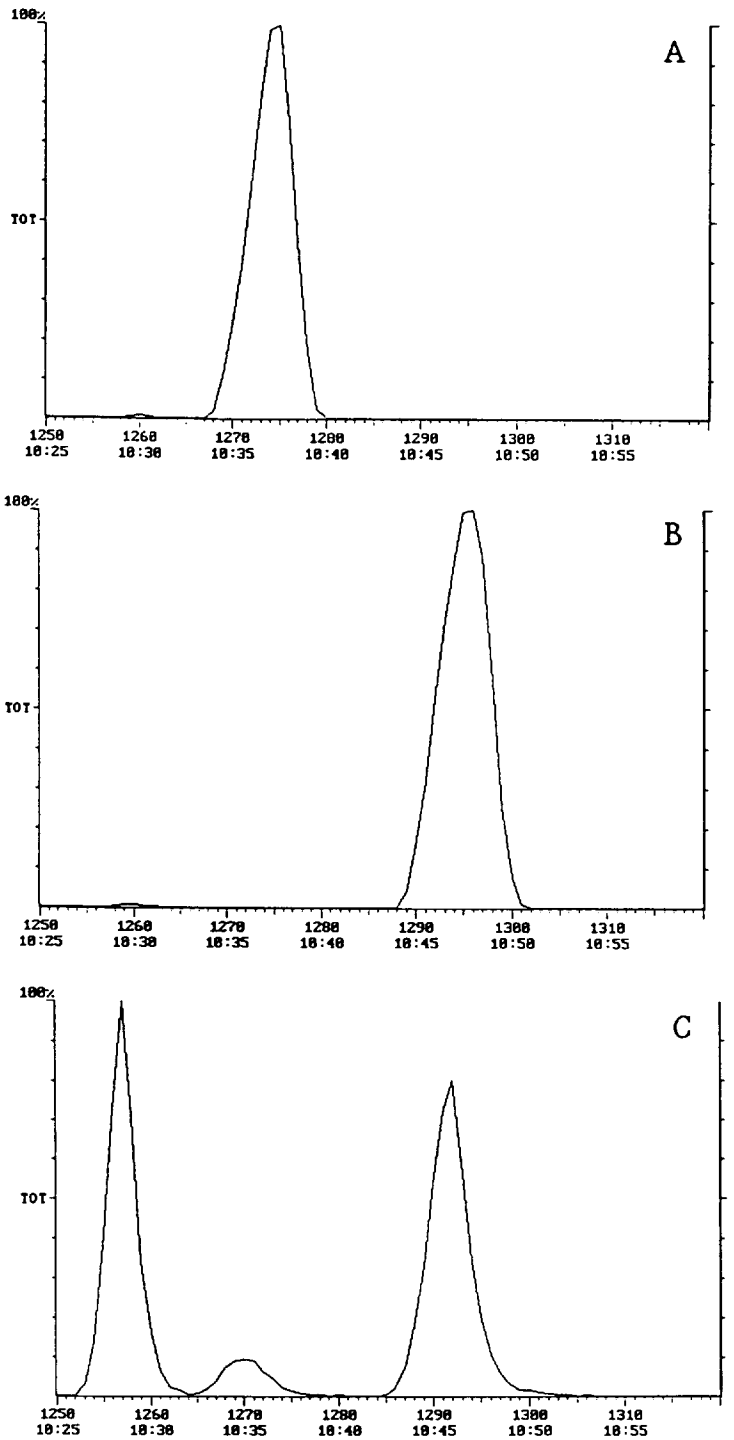

Fig. 5. Representative total-ion GC–MS traces of the isobutyl/HPC derivatives of authentic D-serine (A), L-serine (B), and a cortex extract (C).

Table 1
Levels of D- and L-serine in rat brain regions

Brain region	D-Serine (nmol/mg protein)	L-Serine (nmol/mg protein)	Percent D-serine
Cortex	8.52 ± 0.50	31.9 ± 1.2	21.0 ± 0.8
Hippocampus	6.41 ± 0.50	28.9 ± 1.4	18.0 ± 0.6
Striatum	6.73 ± 0.27	25.5 ± 1.1	20.9 ± 0.2
Cerebellum	0.230 ± 0.016	23.6 ± 1.3	1.0 ± 0.1

Values are expressed as the mean ± S.E.M. ($n = 9$).

ng on-column and L-serine from 4–9 ng on-column.

The mass spectra of the isobutyl/HPC derivatives of D-serine and its trideuterated analog are shown in Fig. 3. The proposed fragmentation pathways of these compounds are shown in Fig. 4. D- and L-serine were observed to exhibit identical mass spectra. Representative GC–MS traces of the isobutyl/HPC derivatives of authentic D-, L-serine, and a cortical extract are presented in Fig. 5. The two optical isomers of serine were successfully separated from each other in less than 11 min using the method described (Fig. 5).

The levels of D- and L-serine were measured in rat brain regions and the results are presented in Table 1. In general, these levels and the ratio of D-serine to L-serine are in agreement with the levels reported in the literature [6,7,9,14].

In summary, a simple, one-day synthesis is described for HPC which results in a chiral derivatizing reagent of greater than 99.9% optical purity. A rapid and sensitive procedure for the separation and quantitation of D- and L-serine in biological tissue extracts using a conventional bonded-phase capillary column and no laborious clean-up or extraction steps is presented. Furthermore, the HPC derivatives formed are stable at room temperature for at least one week making the procedure ideal for automated injection systems.

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