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Short communication

# Separation and quantification of D- and L-phosphoserine in rat brain using Nα-(2,4-dinitro-5-fluorophenyl)-L-alaninamide (Marfey's reagent) by high-performance liquid chromatography with ultraviolet detection

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**Abstract**

D-Serine has recently been described to be present in the brain at high concentrations. However, while prior research has demonstrated that L-phosphoserine is the major precursor of L-serine in the brain, the possible role of D-phosphoserine as the direct precursor of D-serine is unknown. To address this problem, we developed an assay to separate and quantitate D- and L-phosphoserine. A very simple HPLC–UV procedure for the separation and quantification of D- and L-phosphoserine is presented using precolumn derivatization with a chiral reagent, Nα-(2,4-dinitro-5-fluorophenyl)-L-alaninamide (Marfey's reagent), and a conventional $C_{18}$ reversed-phase column. The procedure is sensitive to 11 pmol on-column and derivatives are stable for at least two weeks at room temperature. Rat brain regions (cortex, hippocampus, striatum, and cerebellum) were analyzed for the presence of D- and L-phosphoserine. It was determined that the brain regions studied contained exclusively L-phosphoserine.

## 1. Introduction

The discovery of high endogenous levels of D-serine in mammalian brain by Nagata et al. [1] and Hashimoto et al. [2] three years ago and later verified by other laboratories [3,4], created many questions as to the role of this amino acid in the mammalian central nervous system, not the least of which is its biosynthetic source. Nagata et al. [5] went on to show that it was indeed synthesized de novo; however, the actual pathways involved have yet to be determined.

L-Serine is an important intermediate involved in many biochemical pathways. Using elaborate radiolabelled precursor loading experiments in mouse brain extracts it was shown by Bridgers [6] that greater than 90% of the L-serine pool comes from the dephosphorylation of phosphoserine. In this pathway L-phosphoserine is produced by the transamination of 3-phosphohydroxypyruvate and L-glutamate. The L-phosphoserine formed from this reaction goes on to be dephosphorylated to L-serine by L-phosphoserine phosphatase (EC 3.1.3.3). Since phosphoserine phosphatase can utilize both D- and

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L-phosphoserine as substrates [7], an investigation into the presence of D-phosphoserine as the endogenous precursor of D-serine in rat brain was initiated.

Analytical methods for the quantification of phosphoamino acids have been reported [8,9]. However, these methods do not separate the optical isomers and are mostly used in the study of protein chemistry. N$\alpha$-(2,4-Dinitro-5-fluorophenyl)-L-alaninamide, FDAA, Marfey's reagent) has been used successfully to separate and quantitate D- and L-serine in mouse brain [1]; however, the presence of interfering peaks required extensive sample preparation prior to detection on a HPLC–UV system. The procedure reported here for D- and L-phosphoserine requires no elaborate clean-up steps and is sensitive to 11 pmol on-column.

## 2. Experimental

### 2.1. Reagents and materials

D-Phosphoserine, L-phosphoserine and sodium bicarbonate were obtained from Sigma (St. Louis, MO, USA). FDAA was purchased from Pierce (Rockford, IL, USA). D/L-2-Amino-5-phosphopentanoic acid (D/L-AP5) and ultrapure Tris were obtained from Tocris Cookson (Langford, Bristol, UK) and Bethesda Research Labs. (Gaithersburg, MD, USA), respectively. All solvents were of HPLC or better grade and were purchased from Baxter (Muskegon, MI, USA).

### 2.2. Preparation and derivatization of biological samples

Male Sprague–Dawley rats (200–250 g) were anesthetized with $CO_2$ and decapitated, after which the cortex, cerebellum, striatum and hippocampus were dissected out on ice and stored at −80°C. Cortex and cerebellum samples were homogenized in 1 ml and hippocampus and striatum samples in 0.5 ml of ice-cold 1.0 *M* HCl using a Kontes (Vineland, NJ, USA) microultrasonic cell disrupter. This homogenate was then centrifuged at 40 000 *g* for 45 min. Immediately following 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. [3]. Aliquots of the brain extracts (100 $\mu$l) or standards (100 $\mu$l made up in 1.0 *M* HCl) were added to 2.0-ml polypropylene microcentrifuge tubes (National Scientific, San Raphael, CA, USA) containing 20 $\mu$g of D/L-AP5 in 20 $\mu$l of water. Sodium bicarbonate (200 $\mu$l of a 1.0 *M* solution) was added to neutralize the acid (final pH was approximately 8), then 100 $\mu$l of FDAA (7.5 mg/ml) in acetone were added. The tubes were capped and heated at 50°C for 2 h. The samples were next dried to a residue under nitrogen in a water bath set at 60°C. To the final residue 250 $\mu$l of 50 m*M* Tris were added and thoroughly mixed with a vortex-mixer. The tubes were then centrifuged for 10 min in a Fisher Scientific microcentrifuge (Pittsburgh, PA, USA). A 200-$\mu$l aliquot of the clear supernatant was transferred to HPLC autosampler vials from which 20 $\mu$l were injected on the HPLC system.

### 2.3. Calibration

Known amounts of authentic D- and L-phosphoserine corresponding to 1–100 $\mu$g plus a fixed amount of the internal standard, D/L-AP5 (20 $\mu$g), were placed in reaction vials and carried through in parallel with each set of samples. The amount of D- and L-phosphoserine present was determined by comparing the peak-area ratio of analyte to internal standard obtained from the unknown samples to the standard curve generated using the ratios obtained from known concentrations of D- and L-phosphoserine. The first of the two AP5 peaks was used for all quantitations.

### 2.4. HPLC conditions

A Beckman (Fullerton, CA, USA) System Gold HPLC apparatus was used (Model 125 pump, 166 programmable detector and 507 autosampler) equipped with a 100-$\mu$l loop and a $C_{18}$ reversed-phase column (Beckman Ultrasphere ODS; 25 × 0.46 cm I.D.). The flow-rate was

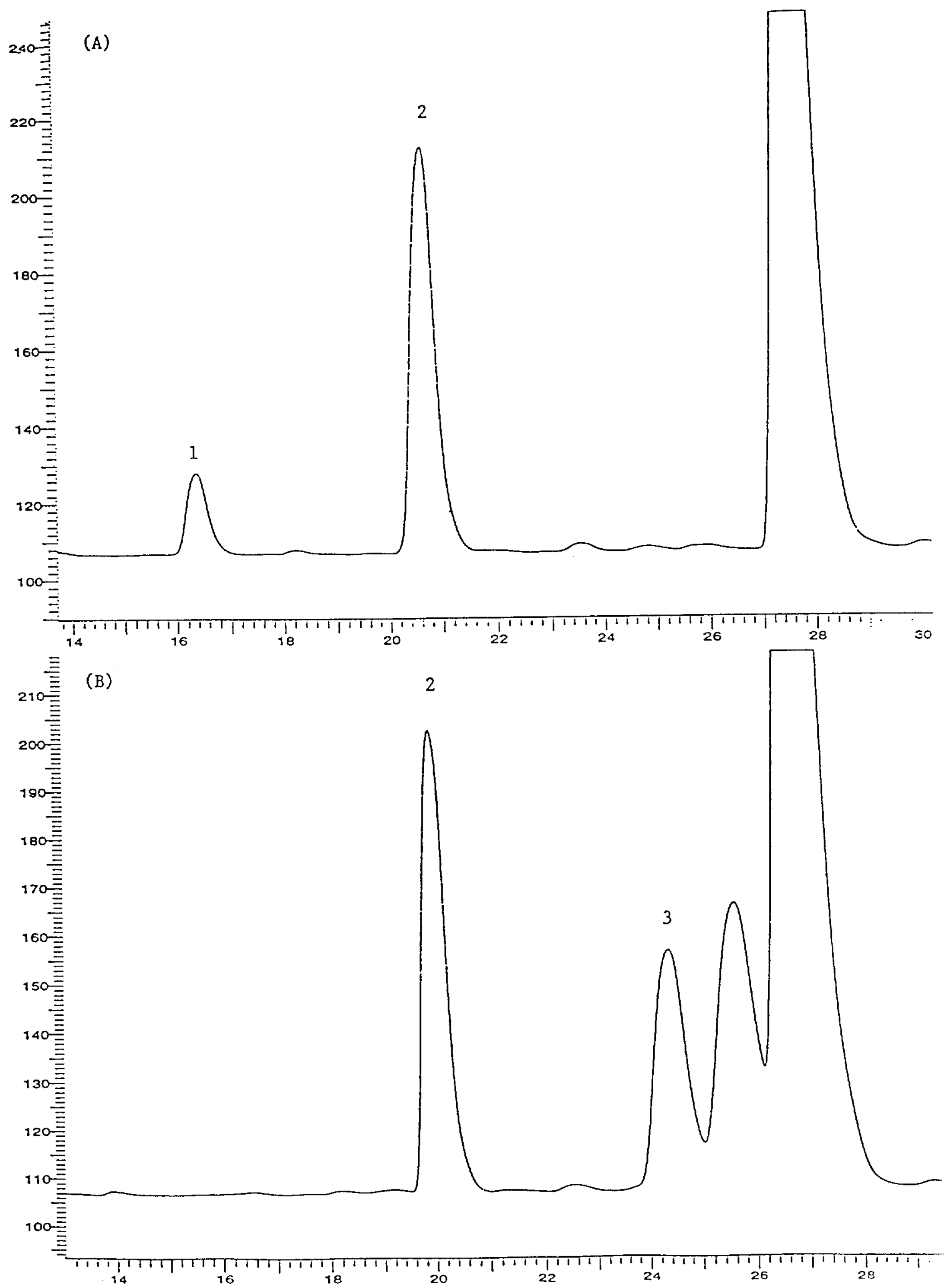


Fig. 1. Representative chromatograms of a rat cortex extract spiked with 10 μg D-phosphoserine (A) and a cortex extract spiked with 50 μg of D/L-AP5 (B). Peaks: 1 = D-phosphoserine; 2 = L-phosphoserine; 3 = D-AP5.

constant at 1.0 ml/min and the wavelength was set at 340 nm. The following solvent gradient system was used where solvent A was 50 m$M$ Tris pH 8.0 and solvent B acetonitrile: 100% A for 0 min; from 100 to 90% A over 30 min; from 90 to 30% A over 5 min; 30% A for 10 min; from 30 to 100% A over 5 min. Total run time was 55 min. The retention times of the analytes were 16.5 min for D-phosphoserine and 20.5 min for L-phosphoserine; AP5 eluted at 24.5 min.

## 3. Results and discussion

### 3.1. Assay validation

The reported procedure results in greater than 4 min baseline separation of the D and L isomers of phosphoserine (Fig. 1). Analytical standards of D- and L-phosphoserine were found to contain 2.5% of the opposite enantiomer. This impurity is believed to be due to the D- and L-phosphoserine and not FDAA as no detectable amount of D-phosphoserine was observed in the tissue samples. The standard curves generated using known concentrations of D- and L-phosphoserine versus AP5 were linear over the range indicated (1–100 $\mu$g) with correlation coefficients consistently $>0.99$. Controls at 1.5, 7 and 35 $\mu$g were run in parallel with each run and deviated from their expected values by less than 10%. A matrix effect was observed with the recovery of AP5 from tissue extracts being 183%. The total absence of D-phosphoserine in the tissue samples enabled us to evaluate the accuracy of using this internal standard by spiking tissue extracts with D-phosphoserine. Tissue extracts spiked with 1 $\mu$g of D-phosphoserine resulted in a recovery of 178%; however, the calculated value deviated from the expected one by less than 10%. From this data it was concluded that AP5 satisfactorily corrected for differences in recovery between the analytical standards and the tissue extracts. The absolute sensitivity of the detector to the phosphoserine derivatives was obtained by serial dilution of a 10-$\mu$g sample and was found to be linear to 2 ng (11 pmol) on column which is similar to the detection limit reported by Nagata et al. [1] for FDAA-derivatized D- and L-serine. Derivatized samples were stable, at room temperature, for at least two weeks.

### 3.2. Quantification of D- and L-phosphoserine in rat brain

No detectable amount of D-phosphoserine was observed in any of the brain regions studied. The regional distribution of L-phosphoserine is presented in Fig. 2. The levels of D- and L-serine in those brain regions are also shown for comparison. L-Phosphoserine levels are approximately two times higher than L-serine levels and like L-serine are consistent across the brain regions studied. No prior attempt, to our knowledge, has been made to quantitate the levels of D-phosphoserine in a mammalian species.

In summary, a simple, sensitive HPLC method is presented which allows the separation and quantification of D- and L-phosphoserine in biological samples. No detectable amount of D-phosphoserine was observed in any of the brain regions studied. It is therefore very unlikely that the observed D-serine in mammalian brain is the result of the dephosphorylation of D-phosphoserine.

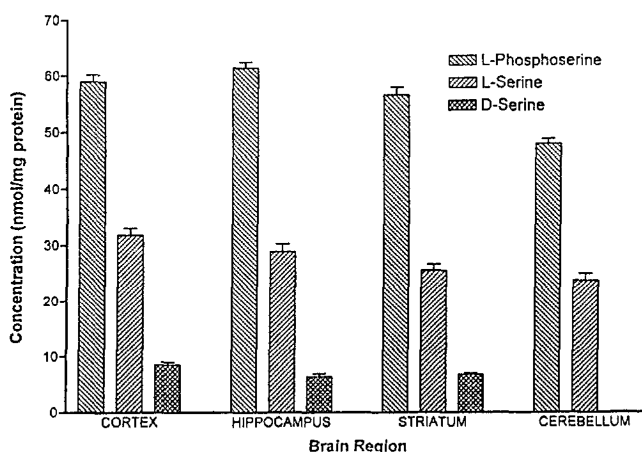


Fig. 2. Regional distribution of L-phosphoserine, L-serine and D-serine in rat brain regions. D-Serine and L-serine levels are taken from Goodnough et al. [10]. L-Phosphoserine levels are expressed as the mean ± S.E.M ($n = 14$).

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