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Short communication

Detection of D-Serine in rat brain by capillary electrophoresis with laser induced fluorescence detection

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Abstract

A capillary zone electrophoresis method with laser induced fluorescence detection for the chiral separation of highly fluorescent enantiomeric derivatives of D/L-Serine from 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-D/L-Serine) was developed and optimized. Enantiomeric separation of NBD-D/L-Serine was accomplished by using 40 mM hydroxypropyl-β-cyclodextrin (HP-β-CD) contained in 100 mM borate buffer, pH 10.0. A 70 cm (effective length of 50 cm) uncoated fused-silica capillary at a voltage of 15 kV was used for the separation. The optimized electrophoretic conditions were subsequently applied to the analysis of D-Serine in rat brain, and satisfactory analytical results with respect to accuracy were obtained. This assay showed acceptable precision, with linearity in the D-Serine concentration range of 0.2–20.0 μM. The limit of detection for D-Serine was 3.0×10^{-7} M.

Keywords: Capillary zone electrophoresis; Enantiomeric separation; D-Serine determination

1. Introduction

High levels of D-Serine occur in mammalian brain, where it appears to be an endogenous ligand of the glycine site of *N*-methyl-D-aspartate (NMDA) receptor [1]. A series of studies have been done with the results that suggest D-Serine acts in concert with glutamate to stimulate nerve cells to fire [2–5]. D-Serine has been found to modify behavioral changes associated with higher brain functions such as memory, convulsion, anxiety, psychotomimetic-induced abnormal behavior and cerebellar ataxia, and has been considered that D-Serine might be an intrinsic positive modulator of the brain NMDA receptor containing the R2B subunit and plays a pivotal role in controlling behavioral expression in mammals [6]. On the other hand, a recent study showed that serum levels of D-Serine in the patients with schizophrenia involving hyperfunction of NMDA receptors were significantly lower as compared with those in normal controls [7]. Thus, D-Serine supplement was tested for the treatment of schizophrenia. Patients who received D-Serine treatment revealed significant improvements in their positive, negative, and cognitive symptoms as well as some performance in Wisconsion Card Sorting Test (WCST) [8,9]. Therefore, researches in these areas have promoted the development of novel analytical procedures for the determination of D-Serine present in biological samples.

Several methods based on high performance liquid chromatography (HPLC) [10–13] have been reported for the determination of D-Serine in rat brain, human brain, and rat tissues. However, this conventional method suffers from high consumption of samples and expensive reagents. Capillary electrophoresis (CE) has many advantages such as high separation efficiency, instrumentation simplicity, minimum operation cost, and compatibility with small sample volumes. It has been proven to be one of the most pow-

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erful techniques for analysis of biological samples [14]. CE-based separations of serine enantiomers have been developed [15–17], and have been applied to the analysis of D-Serine in rat vitreous perfusates [18]. Recently, several microdialysis-chiral CE procedures were reported for the determination of D-Serine in dialysate colleted from the rat striatum [19], and larval tiger salamander retinal homogenates [20]. The methods employed a 5 μm i.d. separation capillary and UV laser induced fluorescence detection for sensitive and high throughput analysis. In these methods, amino acid enantiomers including D/L-Serine tagged with fluoresceine-5-isothiocyanate (FITC), dansyl chloride, *o*-phthaldialdehyde (OPA), and 3-(4-carboxybenzoyl)-2-quinoline-carboxaldehyde (CBQCA) were resolved by chiral CE. However, derivatization of amino acids with FITC or dansyl chloride tends to produce various by-products, which complicates the subsequent separation [21–23]. And it is well known that the stability of OPA derivatives of amino acids depend on the thiol used in the reaction [24–26], and the derivatization reaction of CBQCA with amino acids takes 1 h.

4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) is a fluorescence tagging reagent for amino acids [27] and peptides [28]. Compared to other fluorescent reagents, NBD-F has several important advantages. First, it can react with almost all common amino acids, including both primary and secondary amino acids. Second, its reaction with amino acids is very fast, and there are only two fluorescent side products. In addition, NBD-F has high derivatization efficiency for amino acids [29]. On the other hand, NBD-F tagged amino acids exhibit a fluorescence excitation maximum at ∼470 nm, which is very close to the wavelength of the 457.9 and 488 nm laser line from a popular argon ion laser. Therefore, CE separations coupled with highly sensitive laser induced fluorescence detection (CE–LIF) have been developed for the separation and determination of D/L-Phe [30] and D/L-Asp [31] after derivatization with NBD-F. In this work, a simple CE method for the enantiomeric separation of NBD-D/L-Serine has been developed using hydroxypropyl-β-cyclodextrin (HP-β-CD) as the chiral selector in a borate pH 10.0 buffer. And the developed method has been then applied to detect the D-Serine in rat brain.

2. Experimental

2.1. Chemicals and reagents

HP-CDs (α, β, and γ) were purchased from Aldrich Chemical Company (Milwaukee, WI, USA). Amino acids, β-CD, sodium borate and NBD-F were obtained from Sigma Chemicals (St. Louis, MO, USA). All the other chemicals and organic solvents used in this work were of analytical grade. Analytes were dissolved in 0.1 M HCl solutions. NBD-F solution (2 mM) was prepared in acetonitrile and kept in dark at 5 °C. Milli-Q water was used throughout.

2.2. Apparatus

Capillary electrophoresis (CE) was performed using a laboratory-built system. A high-voltage supply (0–30 kV, Glassman High Voltage, Whitehouse Station, NJ, USA) was used to drive the electrophoresis. Uncoated fused-silica capillaries with 50 μm i.d./360 μm o.d. (Polymicro Technologies, Phoenix, AZ, USA) were used for the separation. Capillary lengths were 75 cm, and the detection window was located 50 cm from the injection end of the capillary. The buffer reservoir at the high-voltage end was enclosed in a plexiglass box. Samples were injected into the capillary by hydrodynamic flow at a height differential of 20 cm for 10 s. The 457.9 nm line from an argon ion laser (INNOVA 90C FreD, Coherent, Santa Clara, CA, USA) was focused with a 25 mm focal length fused-silica lens onto the detection window of the separation capillary. The detection window was made by removing a 5 mm section of polyimide coating on the capillary. Fluorescence emission was collected from the detection window at an angle of 90° relative to the laser beam via a 40× microscope objective (Melles Griod, Irvine, CA, USA). The image of the collected fluorescence was focused on a spatial filter and passed through a GG495 cut-off filter (Melles Griot) before reaching the photomultiplier tube (R374 equipped with a C1556-50 DA-type socket assembly, Hamamatsu, Shizuoka, Japan). The photomultiplier tube was operated at −950 V provided by a Hamamatsu C1309 regulated high voltage power supply. The output signal was recorded and processed with an IBM compatible computer using home made written software.

2.3. Rat brain sample preparation

Male rats (250–300 g) were anesthetized with CO_2. Brain was dissected out on ice and stored at −80 °C until be analysed. Whole cerebrum tissue was cut into pieces as small as possible by scissors, and then a portion of (30–60 mg) tissue sample was ground with 0.1 M HCl solution (150 μL) on ice using a tissue grinder. The homogenate was transferred into a 0.5 ml vial. The vial was sonicated for 5 min and centrifuged (2000 × *g* for 10 min). The supernatant (100 μL) was transferred into another 0.5 ml vial and 30% (w/v) trichloroacetic acid solution (30 μL) was added. The solution was vortexed and centrifuged (2000 × *g* for 10 min). The pH value of the supernatant was brought to about 9 with 1 M NaOH solution (20 μL).

2.4. Precolumn derivatization

Sample solution (10 μL) was transferred to a 0.3 mL microcentrifuge vial, then 0.1 M borate buffer solution (pH 9.0, 40 μL), 2 mM NBD-F solution (50 μL) were added in sequence. The solution was vortexed and heated at 60 °C for 5 min. The derivative solution was injected for CE separation without further purification.

3. Results and discussion

3.1. Separation of NBD-F tagged serine enantiomers

Experimental conditions for the chiral separation of NBD-D/L-Serine enantiomers were optimized. Some experimental factors, such as type of CD, CD concentration, pH of running buffer and running buffer concentration have been examined

3.1.1. Choice of CD type

Enantioseparation of NBD-D/L-Serine in CE is obtained through formation of an inclusion complex with CD. The size of the nonpolar cavity of CD is a critical factor for formation of the inclusion complex. In this study, four chiral selectors, i.e. β-CD, HP-α-CD, HP-β-CD and HP-γ-CD were choosed to determine which CD forms inclusion complex most effectively with NBD-D/L-Serine. It was found that no enantioseparation was observed when HP-α-CD or HP-γ-CD was added to running buffer, which indicate that HP-α-CD or HP-γ-CD does not interact significantly with NBD-Serine. When β-CD or HP-β-CD was as chiral selector, the migration time of NBD-Serine was increased, and chiral separation of NBD-D/L-Serine enantiomers was observed which suggest that NBD-Serine forms an inclusion complex with β-CD or HP-β-CD, but the baseline enantioseparation for NBD-D/L-Serine was observed only by using HP-β-CD (Fig. 1). It indicated that the size of the cavity of β-CD and HP-β-CD should be more suitable for NBD-Serine to form effective inclusion complex than other kinds of CD. Furthermore, the hydrogen bonding ability of HP-β-CD plays an important role.

3.1.2. Effect of HP-β-CD concentration

The effect of HP-β-CD concentration on the chiral separation of NBD-D/L-Serine was investigated over the concentration range 10–50 mM. The resolution increases with the increase of HP-β-CD concentration. A complete resolution can be obtained at HP-β-CD concentrations greater than 30 mM. Then, a 40 mM HP-β-CD concentrations is considered optimal as it provides a best resolution.

3.1.3. Effect of running buffer pH

The effect of running buffer pH was examined using the same electrolyte composition at different pH values in the range of 8.0–10.5. The enantiomeric resolution increases slightly with increasing pH up to 10.0 where maximum separation is reached. Further increases the running buffer pH result a decrease in resolution. According to the results, a running buffer of pH 10.0 was chosen for further experiments.

3.1.4. Effect of running buffer concentration

After optimizing the pH of the running buffer, another series of experiments were performed at the optimal pH of 10.0 with 40 mM HP-β-CD to determine the optimum running buffer concentration. The enantiomeric resolution increases with the increase of running buffer concentration from 25 to 120 mM. Higher resolution could be achieved for NBD-D/L-Serine at higher borate concentration; however, an increase of borate concentration led to a reduction of the electroosmotic flow, and as a consequence, to very long separation times. Furthermore, the excessive Joule heating would lead to the peak tail at a higher borate concentration. Therefore, it would be ideal to use a concentration of 100 mM borate buffer.

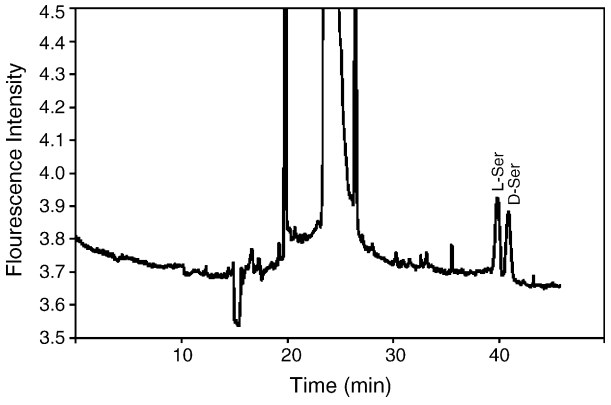

Fig. 1. Separation of NBD-Ser enantiomers. Running buffer: 100 mM borate (pH 10.0), 40 mM HP-β-CD. Capillary was 50 μm i.d. × 50 cm effective length. Voltage applied was 15 kV. The concentratraion of D- and L-Ser was 4.0 μM.

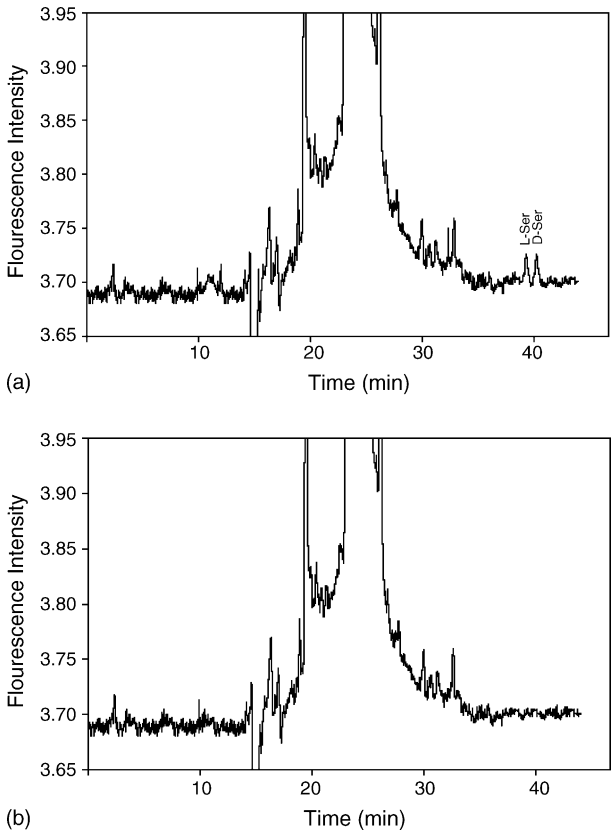

Fig. 2. Electropherograms obtained from an analysis of a sample near the detection limit containing 4.0×10^{-7} M serine racemate (a) and an analysis of a blank sample (b). Separation conditions were as in Fig. 1.

3.2. Linearity and the limit of detection

A series of D-Serine standard solutions were tested to determine the linearity between the D-Serine concentration and fluorescence intensity. Linear regression analysis of the results yielded the following equation:

$$F = 0.0521X - 0.0051 \quad (r = 0.9993)$$

where F is the fluorescence intensity, and X is the concentration of D-Serine in the derivative solution (μM). The calibration curves exhibited an excellent linear behavior over the concentration range of from 1.0 to 20.0 μM. Fig. 2 shows the electropherogram obtained from an analysis of a sample near the detection limit containing 4.0×10^{-7} M serine racemate and an analysis of a blank sample. From this electropherogram, the limit of detection (signal/noise = 3) was estimated to be 3.0×10^{-7} M D-Serine.

3.3. Analysis of rat brain samples

Cerebrum tissue samples from four adult rats were analyzed. A typical electropherogram obtained from separations of rat cerebrum samples is shown in Fig. 3a. The two peaks corresponding to D- and L-Serine can be well identified. To verify the peak identification, D-Serine at 2.0 μM and L-Serine at 20 μM was added to the sample and the sample was again derivatized and separated. The electropherogram obtained is shown in Fig. 3b. As can be seen by comparing Fig. 3a with Fig. 3b, only the D- and L-Serine peaks increases in size without other major changes in the electropherograms.

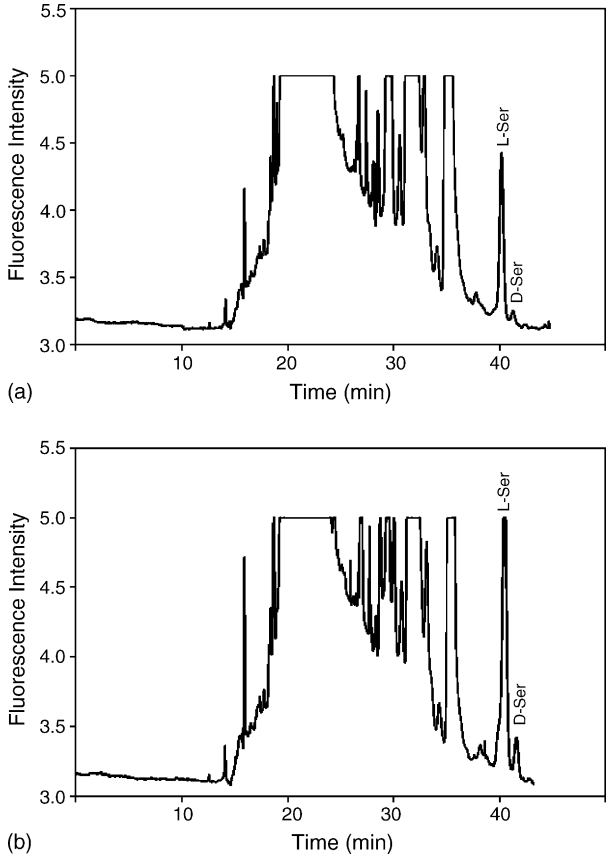

Fig. 3. Electropherogram obtained from the separation of a rat cerebrum sample (a) and the separation of a rat brain sample that was spiked with D-Ser at 2 μM and L-Ser at 20 μM (b). Separation conditions were as in Fig. 1.

Table 1
Results of D-Serine determination in rat cerebrum samples

Sample number	Content of D-Serine (nmol/g)	R.S.D. (%, $n = 4$)
1	132.5	4.2
2	139.3	3.4
3	95.4	5.1
4	101.2	4.7

The determination results are summarized in Table 1. The average of the D-Serine results was 117.1 nmol/g. The precision of the method was evaluated by repeatedly analyzing each cerebrum tissue sample for four times within one working day. The relative standard deviations (R.S.D.) were between 3.4 and 5.1%. Recovery of D-Serine from a rat cerebrum sample was studied. D-Serine was spiked to three portions of a rat brain sample at 2 μM. The recovery was found to be 95.7 ± 2% (mean of the three measurements).

4. Conclusions

A chiral CE method for the separation of D/L-Serine enantiomers fluorescently labeled with 4-fluoro-7-nitro-2,1,3-benzoxadiazole was developed. The chiral separation was achieved by employing HP-β-CD as chiral selector. Coupled with laser induced fluorescence detection, the separation method was well suited for sensitive determination of D-Serine in rat brain tissues. Cerebrum tissue samples from four adult rats were analyzed by the present CE–LIF method. The results are in accordance with the results reported in ref. [32], which was obtained by an HPLC procedure. Compared to other CE–LIF method for the determination of D-Serine, although the analysis time of the present method was relatively long, but none of other endogenous compounds such as biogenic amines and amino acids would interfere with the determination of D-Serine in this separation system, which was well suited for determination of D-Serine in the matrix of complex samples. If a shorter separation capillary and a higher voltage applied can use in this method, it will increase the throughput of the analysis.

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