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Short communication

Determination of free aspartic acid enantiomers in rat brain by capillary electrophoresis with laser-induced fluorescence detection

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Abstract

Quantification of aspartic acid enantiomers in rat brain by using a chiral capillary electrophoresis procedure is described. Amino acids were pre-column derivatized with naphthalene-2,3-dialdehyde. Enantiomeric separation was achieved by micellar electrokinetic chromatography in the presence of methanol and β-cyclodextrin as chiral selector. The chiral separation was coupled with laser-induced fluorescence detection. Contents of D- and L-aspartic acids in rats at different stages of growth (from 1 day before birth to 90 days after birth) were determined. D-Aspartic acid was detected in all the brain tissue samples tested, but at different levels. In the cerebrum of rats 1 day before birth, D-aspartic acid was found to be at the highest concentration of 81 nmol/g wet tissue. The level of D-aspartic acid in rat brain falls rapidly after birth, while the L-aspartic acid level increases with age. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Enantiomer separation; Aspartic acid

1. Introduction

Recent studies have shown that D-aspartic acid (D-Asp) is an endogenous chemical species in human and various animals [1–3]. It may play important roles in neurocrine and endocrine functions [4]. L-Asp racemization ratios in human femur have been used for age estimation [5]. An age-related accumulation of D-Asp in the human brain has been observed [6].

In the search for a better understanding of biological role of D-amino acids, enantiomeric determination of amino acids in biological samples has attracted much research interest. Several methods based on high-performance liquid chromatography (HPLC) [7–11], enzymatic assay [12], and immunohistochemistry [13] have been reported for the determination of D-Asp in body fluids, human brain, mammalian cells, and rat tissues. A comprehensive review on analysis of D-amino acids was given [14].

Capillary electrophoresis (CE) has advantages such as high separation efficiency, short run time, instrumentation simplicity, minimum operation cost, and compatibility with small sample volumes. It has been proven to be one of the most powerful techniques for the analysis of biological samples [15]. In

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this work, we describe for the first time the application of a chiral CE procedure to study the occurrence and postnatal changes of D-Asp in rat brain.

2. Experimental

2.1. Chemicals

Amino acids, β-cyclodextrin (β-CD), sodium dodecyl sulfate (SDS) and potassium cyanide (KCN) were purchased from Sigma (St. Louis, MO, USA). Naphthalene-2,3-dicarboxaldehyde (NDA) was from Molecular Probes (Eugene, OR, USA). All other chemicals and organic solvents used in this work were of analytical grade.

To prepare background electrolyte solution, 0.36 g SDS and 0.57 g β-CD were dissolved in 8.75 ml water, and then mixed with 3.75 ml methanol and 12.5 ml 100 m*M* borate buffer (pH 9.0). The solution contained 50 m*M* SDS, 20 m*M* β-CD, 50 m*M* borate, and 15% (v/v) methanol. It was filtered through a 0.22 μm syringe filter before use. KCN solution (20 m*M*) was prepared in water. NDA solution (2 m*M*) was prepared weekly in methanol and kept in the dark at 5°C. Milli-Q water was used throughout the work.

2.2. Apparatus

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. Fused-silica capillaries with an effective length of 50 cm (Supelco, Bellefonte, PA, USA) were used for separation. The buffer reservoir at the high-voltage end was enclosed in a plexiglass box.

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 on-column detection window was created by removing a 5-mm section of polyimide coating on the fused-silica 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 Griot, 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 laboratory-written software.

2.3. Tissue sample preparation and pre-column derivatization

Rats were anesthetized with CO_2. Cerebrum and cerebellum were dissected out on ice and stored at −80°C until analysis. A portion of the tissue sample (20–30 mg) was cut into pieces as small as possible by scissors, and then ground with 0.1 *M* HCl solution (150 μl) on ice using a tissue grinder. The homogenate was transferred to a 0.5-ml vial. The vial was sonicated for 5 min and centrifuged (2000 *g* for 10 min). The supernatant was transferred to another 0.5-ml vial and 30% (w/v) trichloroacetic acid solution (30 μl) was added. The solution was vortex-mixed and centrifuged (2000 *g* for 10 min). The pH value of the supernatant was brought to about 9 with 1 *M* NaOH solution. A portion (10 μl) of the sample solution was transferred to a 0.5-ml polyethylene centrifuge tube, then 0.1 *M* borate buffer solution (pH 9.5, 150 μl), 2 m*M* NDA solution (50 μl), and 20 m*M* KCN solution (50 μl) were added in sequence. The solution was vortex-mixed and kept at room temperature for 30 min before injection.

2.4. CE separation conditions

Separations were performed at 15 kV using a background electrolyte solution containing 50 m*M* sodium borate (pH 9.0), 50 m*M* SDS, 20 m*M* β-CD and 15% methanol. All samples were injected into the capillary by hydrodynamic flow at a height differential of 20 cm for 20 S. The capillary was rinsed sequentially with 0.1 *M* NaOH, water, and running buffer for approximately 3 min each between successive electrophoretic runs.

2.5. Quantification of D-/L-Asp

Calibration curve was prepared with authentic D- and L-Asp enantiomers. The concentration of each enantiomer in the derivative solution ranged from 0.1 to 1.5 μ*M* ($n=6$). Peak heights were used for the quantification. The following regression equation was obtained:

$y = 36.85x + 0.27$ ($r = 0.9992$)

where y is the peak height (mm), and x is the concentration of D- or L-Asp in the derivative solution (μ*M*). The amounts of D- and L-Asp in samples were calculated from the regression equation. Relative amounts of D-Asp (referred to as %D in the text) were calculated as %D = 100 D/(D + L).

3. Results and discussion

3.1. Separation of D-/L-aspartic acid enantiomers

The pre-column derivatization employed in this method enhances both detection sensitivity and separation efficiency. NDA reacts with amino acids in presence of cyanide, forming 1-cyano-2-benz-[*f*]isoindole (CBI) derivatives [16]. While NDA has little fluorescence, the CBI-amino acid derivatives are highly fluorescent with an excitation maximum at 445 nm, which is close to the 457.9 nm line from an argon ion laser. Many CBI-amino acid enantiomers can be well separated by β-CD-modified micellar electrokinetic chromatography (β-CD-MEKC) [17, 18]. However, the application of these enantioseparations to analyze some complex biological samples is limited due to the interference from co-existing amino acid enantiomers and other endogenous compounds. For example, the quantification of D-/L-Asp enantiomers by using the β-CD-MEKC separation proved to be unsuccessful due to serious peak overlapping. Fortunately, the separations can be dramatically improved by the addition of methanol to the β-CD-MEKC running buffer as an organic modifier. Fig. 1 shows two electropherograms obtained from separations of two rat brain samples. One rat was 1 day before birth (Fig. 1a), and another was 90 days after birth (Fig. 1b). As can be seen, the peaks of D- and L-Asp are well resolved with no interference from other amino acids or endogenous components in this biological sample matrix. To verify the peak identification, D-Asp or L-Asp was added to the rat brain sample solution at a concentration of 7.5 μ*M*, and the sample solution was again derivatized and separated. From the electropherogram obtained, only the D-Asp (or L-Asp) peak increased in size without other major changes in the electropherogram. From these chromatograms, the limit of detection for Asp enantiomer in rat brain tissue was estimated to be 2 nmol/g wet tissue (signal/noise = 3).

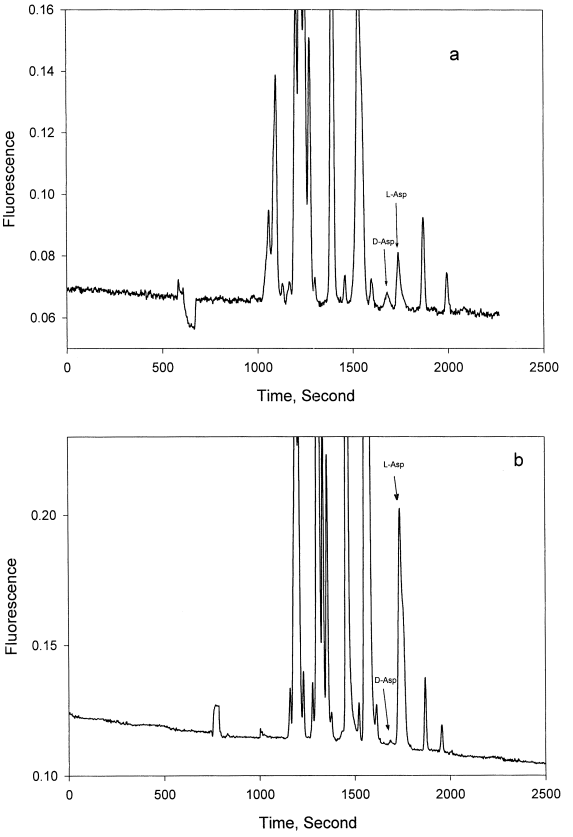

Fig. 1. Electropherograms obtained from separating rat brain samples by β-CD-MEKC in presence of methanol: (a) brain sample from a rat 1 day before birth, and (b) brain sample from a rat 90 days after birth. Electrolyte composition was 20 m*M* β-CD, 50 m*M* SDS, 15% (v/v) methanol, and 50 m*M* borate buffer (pH 9.0). A capillary of 75 μm I.D. was used. Applied voltage was 15 kV. LIF detection, $\lambda_{ex} = 457$ nm, $\lambda_{em} \geq 495$ nm.

3.2. Determination of D-/L-Asp in rat brain

Rat brain samples were taken from rats (groups of three) at different stages of growth. Two groups of animals were studied at different times for each stage of growth. Each brain tissue sample was analyzed in duplicate. The results are summarized in Fig. 2. The content of free D-Asp in rat brain diminishes dramatically to a low level within 1 week after birth, and then remains at this level during postnatal growth (Fig. 2a). In contrast, L-Asp content in rat brain increases steadily until 1 week after birth (Fig. 2b), and then remains nearly constant. The highest concentration of free D-Asp (81 nmol/g wet tissue) was detected in cerebrum (Fig. 2a). However, the highest ratio of D-Asp to total Asp (19.5%) was found in cerebellum (Fig. 2c). These results are in accordance with the previously reported results by other researchers. For example, using a HPLC method, Dunlop et al. [19] found the content of D-Asp was 100 nmol/g in the cerebrum of newborn rats and it decreased rapidly with increasing age. Using the same HPLC procedure, Hashimoto et al. studied in greater details the anatomical distribution and postnatal changes of D-Asp in rat brain and periphery with similar results [10].

4. Conclusions

An organic modifier-mediated β-CD-MEKC procedure is described for analysis of free aspartic acid enantiomers in rat brain. Tissue samples from rats at different stages of growth (from 1 day before birth to 90 days after birth) were analyzed. D-Aspartic acid was detected in all the brain tissue samples tested,

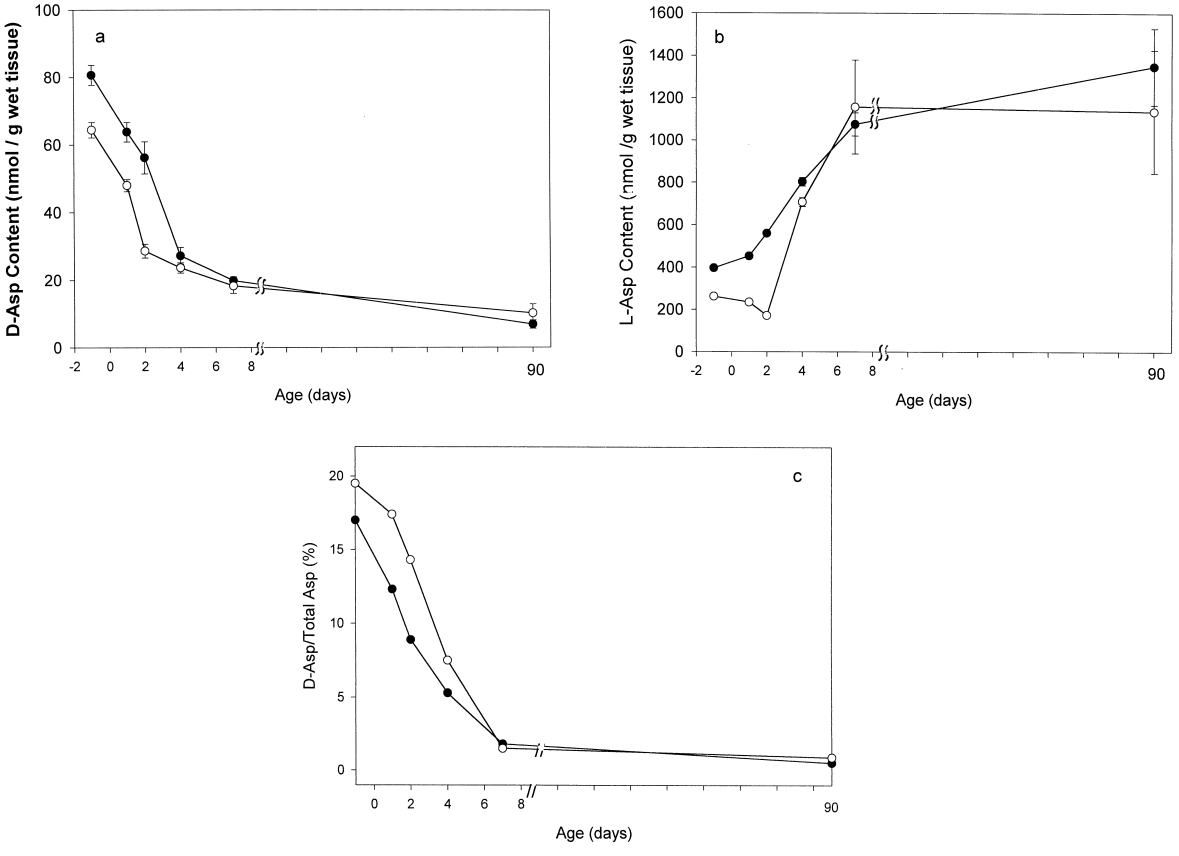

Fig. 2. Content changes of D-/L-aspartic acid with age in rat cerebrum (·) and cerebellum (○): (a) the content of free D-aspartic acid versus age, (b) the content of free L-aspartic acid versus age, and (c) the ratio of D-aspartic acid to total aspartic acid versus age.

which suggests that this amino acid enantiomer may be mainly of endogenous origin. The content of D-aspartic acid in rat brain diminishes rapidly after birth, and within 1 week it becomes nearly constant at a low level. In contrast, the level of L-aspartic acid increases rapidly after birth, and it becomes constant at a high level within 1 week. This is the first report on the quantitative measurements of free D-/L-aspartic acid enantiomers in rat brain by using a chiral CE–laser indcued fluorescence (LIF) detection procedure. As shown, this method can be a useful alternative to the HPLC methods. The most important advantage of this CE–LIF detection procedure is that it can be used to simultaneously quantify D- and L-aspartic acids in small mass/volume samples such as single cells. Research in this direction is underway.

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