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Separation of gangliosides using cyclodextrin in capillary zone electrophoresis

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ABSTRACT

Gangliosides are glycosphingolipids containing sialic acid. These glycolipids have been suggested to play important roles in biological processes such as cell growth, differentiation and malignant transformation. Based on these proposed biological functions, gangliosides can be used as diagnostic tools and therapeutics for various human diseases. In this study, capillary zone electrophoresis (CZE) was used to determine the major gangliosides G_{M1}, G_{D1a}, G_{D1b} and G_{T1b} in mammalian brains, in addition to G_{M3} and Lac. Enhancement of selectivity and efficiency of separation was obtained by using 50 m*M* borate–phosphate buffer containing 16.5 m*M* α-cyclodextrin (α-CD). Under this condition, several forms of gangliosides were successfully separated from extracts of deer antler, apricot seed and rat brain. The results demonstrate that the CD-modified CZE is a useful method for detecting glycolipids from various biological matrices.

INTRODUCTION

Gangliosides are carbohydrate-rich sphingolipids that contain acidic sugars as shown in Fig. 1 [1]. The acidic sugar is N-acetylneuraminate or N-glycolylneuraminate, and is also known as sialic acid. Gangliosides are found in high concentrations in nervous systems, plasma membranes of virtually all vertebrate tissues and some plant nuts. Although the biological functions of these glycolipids are not clearly known, gangliosides have been suggested to play important roles in the regulation of biological processes such as cell growth and differentiation [2,3]. Some investigators have shown that the concentrations of the ganglioside G_{M1} on the cell surface of the differentiated neuron cell was also increased by neuraminidase activation. A recent study showed that G_{M1} has some therapeutic effect on Alzheimer's disease and dementia [4]. As other gangliosides are overexpressed as antigens in several cancer cells, gangliosides could also be used in immunotherapy. Recently, gangliosides G_{M1}, G_{D1a}, G_{D1b}, G_{T1b} and their sulphate derivatives have also been reported to show antiviral activity towards the human immunodeficiency virus 1 (HIV-1) [5]. Based on these biological functions, gangliosides can be used as diagnostic tools and therapeutics for various diseases in humans.

Determination of gangliosides in biological

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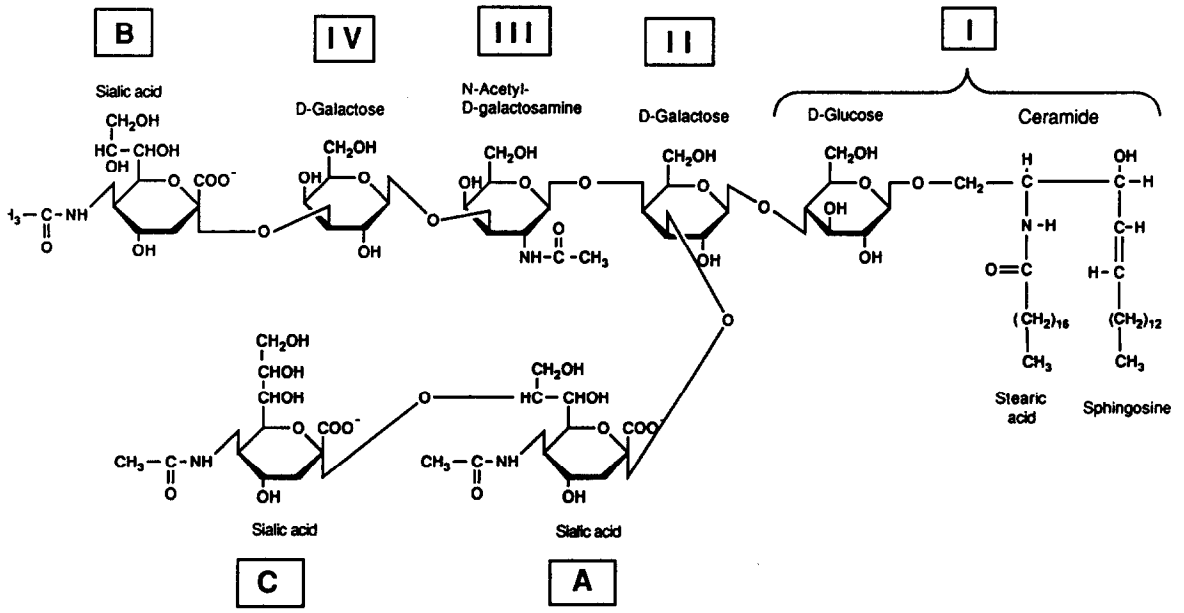

Fig. 1. Structures of some representative brain gangliosides. G_{M1}: I, II, III, IV, A. G_{D1a}: I, II, III, IV, A, B. G_{D1b}: I, II, III, IV, A, C. G_{T1b}: I, II, III, IV, A, B, C.

samples has frequently been carried out by high-performance liquid chromatography (HPLC) or thin-layer chromatography (TLC) [6]. The separation by HPLC usually employs aminosilica columns and a linear gradient of increasing salt concentration in the eluent. By using conventional UV techniques, the identification of gangliosides, which are characterized by a lack of chromophores in their structures, has been difficult owing to the low selectivity in addition to an unfavourable signal-to-noise ratio. In general, the identification of gangliosides after separation by HPLC is achieved using TLC with the UV peaks obtained and subsequent staining with resorcinol, which is specific for sialic acid [7]. Recently, the advent of photodiode-array UV detectors has improved the peak identification of gangliosides to some extent [8].

High-performance capillary electrophoresis is a recently developed and powerful technique with great potential for high-resolution separations and the determination of various molecules, from ions to biological macromolecules [9–18]. Separation by capillary electrophoresis is not limited to a single mode which is the case with liquid chromatography. Analytes can be determined by any of charge/mass ratio, hydrophobicity, size, affinity, absorption, etc. [19–22].

An elegant feature of capillary zone electrophoresis (CZE) is the ability to alter the electrophoretic performance by adding reagents such as surfactants, organic solvents, chelating agents and other additives to the mobile phase [23–26]. Cyclodextrins (CDs) are used as buffer additives to obtain better resolution. The most commonly occurring CDs contain six, seven and eight glucopyranose units, designated α-, β- and γ-CD, respectively [27]. These compounds are able to form inclusion complexes with many molecules. The formation of an inclusion complex is determined by the solute hydrophobicity and size [26,28–31].

In this study, we used CD-modified CZE to determine gangliosides. Parameters such as CD type and concentration and the pH of the mobile phase were varied to obtain the best resolution. The developed method was applied to the determination of gangliosides from various biological matrices including rat brain, deer antler and apricot seed. Gangliosides G_{M1}, G_{D1a} and G_{D1b} were found to exist in deer antler and G_{D1a} was detected in the electropherogram of apricot

seed. In rat brain, G_{M1} and G_{D1a} were the major components and G_{T1b} a minor component of the gangliosides.

EXPERIMENTAL

Instrumentation

The capillary electrophoresis instrument for this study was a Quanta 4000 capillary electrophoresis system (Millipore, Waters Chromatography Division, Milford, MA, USA), with detection using a fixed-wavelength UV detector equipped with a mercury lamp and a 185-nm filter. Some analyses were done with a zinc lamp and a 214-nm filter. The system was operated at a constant voltage of 20–30 kV. Fused-silica capillary tubes of 50 μm I.D. with a tube length ranging from 60 to 100 cm were used. All experiments were carried out at ambient temperature (*ca.* 25–28°C). Sample injections were made by raising the sample reservoir 10 cm higher than the collection reservoir for 10 s. The electropherograms were recorded on a Waters 746 data module.

Reagents

All chemicals were of analytical-reagent grade unless stated otherwise. Ganglioside standards, G_{M1}, G_{D1a}, G_{D1b}, G_{T1b}, G_{M3} and Lac, were purchased from Sigma (St. Louis, MO, USA). α-CD and β-CD were also obtained from Sigma and γ-CD from Merck (Darmstadt, Germany).

Deionized water was prepared with a Milli-Q system (Millipore, Bedford, MA, USA). Water of 18 MΩ was used for the preparation of all the solutions, electrolyte buffer and standards. All the solutions were passed through a 0.22-μm membrane filter unit (Green Cross Medical, Seoul, Korea) and carefully degassed before use.

Sample preparation

Standard ganglioside, *i.e.*, G_{M1}, G_{D1a}, G_{D1b}, G_{T1b}, G_{M3} and Lac, solutions were prepared by dissolution in deionized, 18-MΩ water. The final concentration of the standard samples was 0.5 mg/ml. For some experiments with the 214-nm wavelength filter unit 1 mg/ml sample solutions were used.

Extraction of gangliosides from biological matrices was performed by modified Folch–Suzuki methods [32,33]. The sample preparations from rat brain and apricot seed were homogenized with four volumes of water. Chloroform–methanol–water (4:8:3) was added to the homogenate and the mixture was centrifuged after shaking. Chloroform–methanol (2:1) was added to the supernatant. After partitioning the sample by the Folch method, the samples were vacuum distilled, dialysed, freeze-dried and dissolved in deionized, 18-MΩ water for CE analyses. Sample preparations from deer antler were subjected to further purification by Sephadex G-50 column chromatography and fractionation by the solvent method to collect the ganglioside fraction.

Procedure

To obtain good separation and reproducibility, the capillary tube was cleaned each time the buffer or sample solution was changed. All the cleaning procedures were done by vacuum purging at 12–15 p.s.i. (1 p.s.i. = 6894.76 Pa). The capillary tube was purged for 5 min with 0.5 *M* potassium hydroxide solution, flushed with water for 10 min and then purged with the new electrolyte solution for 10 min. The washed column was equilibrated with the working buffer for 30 min prior to use. After the analyses of biological matrices had been run 3–5 times, the capillary tube was washed as in the above procedure. In addition, the capillary tube was purged for 2 min automatically with the working electrolyte before each injection.

RESULTS AND DISCUSSION

CZE separation and inclusion pseudo-phase analyses using cyclodextrin

Initial studies were focused on investigating the separation of the standard gangliosides. Fig. 2 shows a comparison of electropherograms for plain CZE separation and inclusion pseudo-phase analyses using cyclodextrin of standard gangliosides G_{M1}, G_{D1a}, G_{D1b} and G_{T1b}. The electrolyte used for CZE was a 50 m*M* borate–phosphate buffer. In the CZE analysis for G_{M1}, G_{D1a}, G_{D1b} and G_{T1b}, only the monosialoganglioside G_{M1}, was separated at 8.3 min, with a broad peak. The di- and trisialogangliosides were

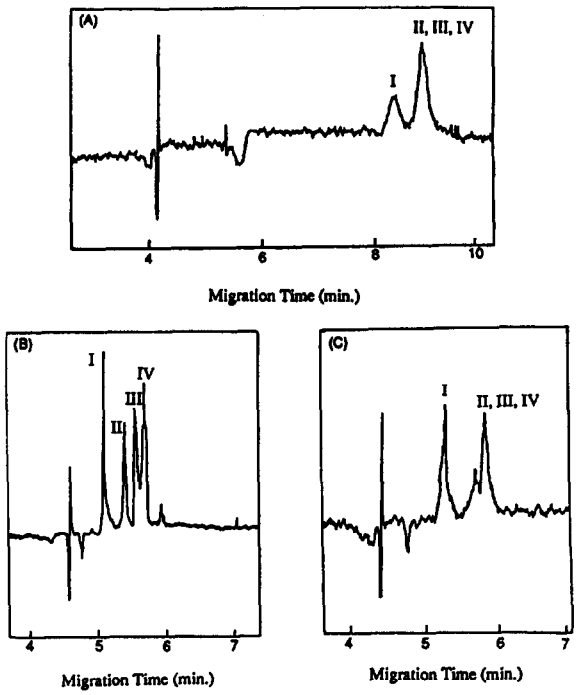

Fig. 2. Electropherograms of standard mixture of gangliosides with or without buffer additive. Conditions: 50 m*M* borate–phosphate buffer (pH 9.3); 20–30 kV; 10-s injection, hydrostatic; UV detection at 185 nm. Peaks: I = G_{M1}; II = G_{D1a}; III = G_{D1b}; IV = G_{T1b}. (A) No modifier; (B) with 11 m*M* α-CD; (C) with 11 m*M* β-CD.

not separated and co-migrated at 9.0 min (Fig. 2A).

As gangliosides contain not only hydrophilic sugar moieties but also hydrophobic lipid components in their structure, they have been known to form stable micelles. Sometimes, even mixed micelles were formed among mono-, di- and trisialogangliosides on prolonged incubation [34]. Although we did not attempt incubation to form mixed micelles in our CZE studies, Fig. 2A shows that mixed micelles were formed more easily between polysialogangliosides than poly- and monosialoganglioside at ambient temperature, *ca.* 25–28°C. However, when the gangliosides samples were pretreated by sonication or kept at room temperature for a long time, we noticed that the peak of G_{M1} merged with the peak of mixed micelles of polysialogangliosides (G_{D1a}, G_{D1b} and G_{T1b}). These observations are in agreement with the results of other investigators [34,35].

In order to separate di- and trisialogangliosides, micellar electrokinetic capillary chromatographic (MECC) methods using several surfactants such as sodium dodecyl sulphate (SDS), sodium cholate and sodium deoxycholate were attempted. Also, a number of buffer additives such as urea and some organic solvents were tried, but none of the above modifications improved the separations of the polysialogangliosides. The use of α-CD, however, improved the separation dramatically as shown in Fig. 2B. We also tried β- and γ-CD as buffer additives in CZE, but although some improvement in the resolution of the peaks was achieved, no separations of di- and trisialogangliosides were obtained, as shown for β-CD in Fig. 2C. When any of the CDs was added to the electrolyte buffer, the migration times of the gangliosides were slightly reduced.

CDs are neutral oligomers with different numbers of units of D-(+)-glucopyranose. In chromatographic analyses including separations of enantiomers, α-, β- and γ-CD with six, seven and eight glucose units, respectively, are most commonly used as modifiers. The structure of the CD forms a cavity that can contribute to partitioning of the solutes [27,36]. The inner diameters of the cavity for α-, β- and γ-CD are 0.47–0.52, 0.60–0.64 and 0.75–0.83 nm, respectively. Perhaps the size of the cavity of α-CD provides the best fit for the lipid moiety of gangliosides. Although we do not have any direct evidence through the measurement of light scattering, our results for the separations of gangliosides using CD in CZE suggest that the α-CD in the electrolyte buffer may also act as an inhibitor of ganglioside micelle formation.

Optimization of inclusion pseudo-phases with CD

Fig. 3 shows the separations of gangliosides with various concentrations of α-CD as a buffer additive. As the concentration of α-CD in the electrolyte increased, the migration speed of the peaks increased. As CDs are neutral, unlike SDS micelles, they are expected to have no electrophoretic velocity and to migrate faster than SDS.

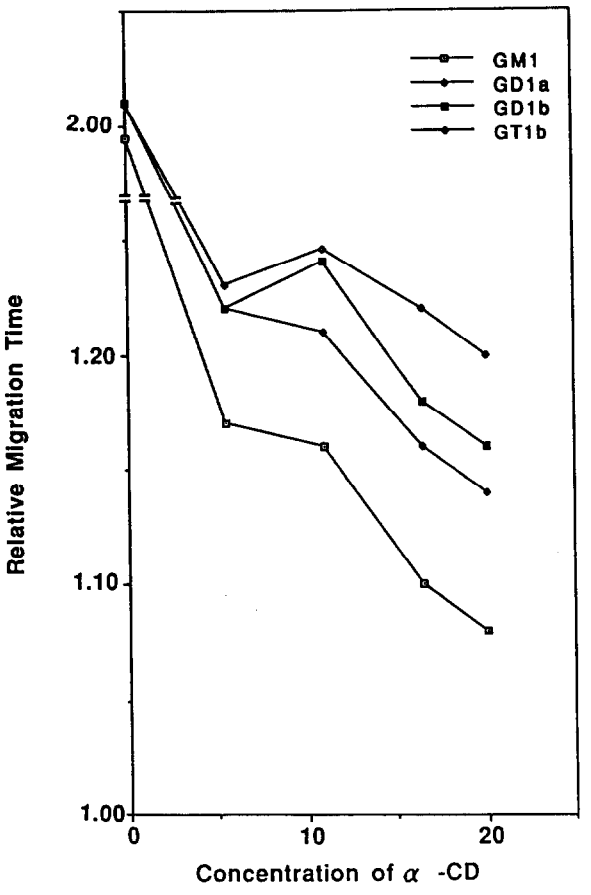

Fig. 3. Effect of concentration of α-CD in the electrolyte on relative migration times of gangliosides. Electrophoretic conditions as in Fig. 2A except for the concentration of α-CD. The relative migration time of the solutes were obtained with respect to the migration time of water, t_0.

Further, at higher concentrations of CD, the general tendency of solutes to be solubilized in the cavity would be increased. Therefore, as the concentration of α-CD increases, the inclusion complexation increases, and consequently the migration speeds of the solutes would be increased. Our results are in agreement with theoretical expectations and the findings of Yik *et al.* [36]. As the concentration of α-CD increases, the resolution of the peaks and the separations of the di- and trisialogangliosides were improved (Fig. 3). When the concentration of α-CD in the electrolyte was higher than 16.5 m*M*, the separation efficiency (number of theoretical plates, *N*) of the gangliosides appeared to be slightly impaired, as shown in Table I.

In order to optimize the α-CD concentration in the electrolyte for the determination of gangliosides, calculation of the separation efficiency was done in terms of *N*. As an indication of peak asymmetry due to tailing, the *B*/*A* ratio, *A* and *B* represent the distance from the centre of the peak to the left-hand side and right-hand side of the peak, respectively, at 10% of the peak maximum, was also measured. As a perfect Guassian peak has a *B*/*A* ratio of 1.0, any peak with a *B*/*A* ratio close to 1.0 would be preferable [37].

Table I shows the efficiency and *B*/*A* ratio for four individual peaks of gangliosides at three different concentrations of α-CD. The three higher concentrations of α-CD in Fig. 3 were

TABLE I

EFFICIENCY (THEORETICAL PLATES, *N*) AND *B*/*A* RATIO FOR GANGLIOSIDES WITH VARIOUS CONCENTRATIONS OF α-CD AS BUFFER ADDITIVE

The optimum experimental conditions (see text) were used, apart from the α-CD concentration. The number of theoretical plates was obtained based on the equation $N = 5.54\ (t_R/W_h)^2$, where t_R is the migration time of the peak and W_h the peak width at half-height. The asymmetry factor, *B*/*A*, was taken at 10% of the peak maximum [37] (a perfect Gaussian peak has *B*/*A* = 1.0).

Ganglioside	11 m*M* α-CD		16.5 m*M* α-CD		20 m*M* α-CD	
	N	*B*/*A*	*N*	*B*/*A*	*N*	*B*/*A*
G_{M1}	$6.2 \cdot 10^4$	1.4	$1.1 \cdot 10^5$	1.1	$8.9 \cdot 10^4$	1.2
G_{D1a}	$6.9 \cdot 10^4$	1.1	$1.1 \cdot 10^5$	1.0	$9.8 \cdot 10^4$	1.2
G_{D1b}	$4.6 \cdot 10^4$	0.6	$1.0 \cdot 10^5$	1.2	$1.0 \cdot 10^5$	0.7
G_{T1b}	$4.8 \cdot 10^4$	0.7	$1.1 \cdot 10^5$	1.1	$9.7 \cdot 10^4$	1.1

chosen to calculate N and the B/A ratio. When the concentration of α-CD was increased from 11 to 16.5 mM, N increased sharply by *ca.* 1.5–2.3 fold. A further increase in the α-CD concentration to 20 mM, however, caused N to decrease slightly. Although the B/A ratios were not affected significantly with variation in the α-CD concentration, the B/A ratios of the peaks at 16.5 mM showed the best result. Based on these observations, an α-CD concentration of 16.5 mM was chosen for subsequent work.

Attempts were made to perform separations in the pH range 4.5–9.3 (Fig. 4). In CZE analysis, ionic species are separated on the basis of the differential electrophoretic mobilities of the analytes. The resolution in CZE could, in principle, be improved by either increasing the difference in electrophoretic mobility of the separated zones or by reducing the electroosmotic flow of the running buffer [38]. At a higher pH, *i.e.*, above the pK_a of analytes, gangliosides exist as negative species and the interaction between the analytes and the capillary wall was minimized. Fig. 4 shows the changes in the relative migration time of each individual ganglioside on increasing the pH of the electrolyte. The migration time of water, t_0, in the electropherogram was used to obtain the relative migration times of the individual solutes. On increasing the pH from 4.5 to 5.5, the relative migration times of all the gangliosides were reduced significantly, although two of the disialogangliosides were not separated. The separation of gangliosides was improved at pH above 8.0. At pH 9.3, all four gangliosides were well separated, as shown in Fig. 4, and the peak shapes in the electropherogram showed the best result (data not shown).

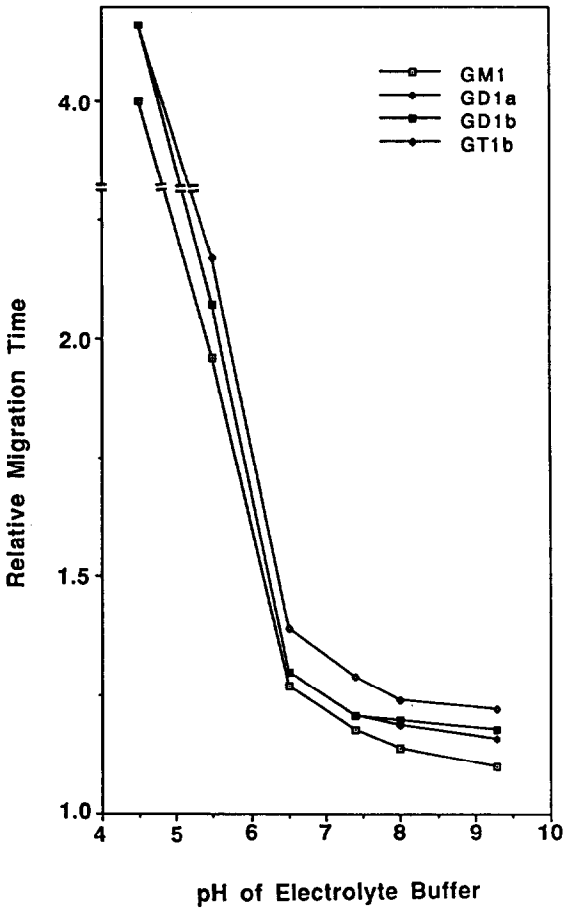

Fig. 4. Effect of pH of the electrolyte on relative migration times of gangliosides. Apart from the pH variation and the addition of 16.5 mM α-CD to the electrolyte buffer, the electrophoretic conditions were as in Fig. 2A.

Initial attempts to determine gangliosides were carried out by using a zinc lamp at 214 nm owing to the limited availability of commercial filter units at the time. When the 185-nm filter unit became available, the responses for gangliosides at 214 and 185 nm were compared. The molar absorptivity of many analytes increased with decreasing wavelength, sometimes significantly. Two drawbacks, *i.e.*, the low intensity of most of the light sources and the low transmission of most solvents in this range, have limited the use of sub-200-nm wavelengths in spectropho-

TABLE II

COMPARISON OF PEAK AREAS AT 185 AND 214 nm, AND SENSITIVITY RATIO

Experiments were performed under the optimum conditions with equal concentrations of sample.

Ganglioside	Peak area (arbitrary units)		Ratio
	214 nm	185 nm	
G_{M1}	1583	32 856	20.8
G_{D1a}	500	10 557	21.1
G_{D1b}	820	18 138	22.1
G_{T1b}	3960	82 242	20.8

tometry, HPLC and CZE. However, in CZE with a fixed-wavelength detection system, UV detection below 200 nm is feasible owing to the short path length of the light source and the separation medium being an isocratic aqueous solution. Table II gives the peak area (arbitrary units) of gangliosides obtained at 214 nm with a zinc lamp and at 185 nm with a mercury lamp. It can be seen that there is a dramatic increase in sensitivity of *ca.* twenty-fold for gangliosides when operating at 185 nm.

Separation of standard ganglioside mixture and reproducibility

Based on the above investigations, the optimum conditions for the determination of gangliosides were established. The electrolyte buffer was 50 m*M* borate–phosphate (pH 9.3) containing 16.5 m*M* of α-CD as buffer additive. The CE instrument was equipped with a 60 cm × 50 μm I.D fused-silica capillary column. The separation was performed at 30 kV. Fig. 5 shows the electropherogram for a standard mixture of gangliosides obtained using the optimum conditions.

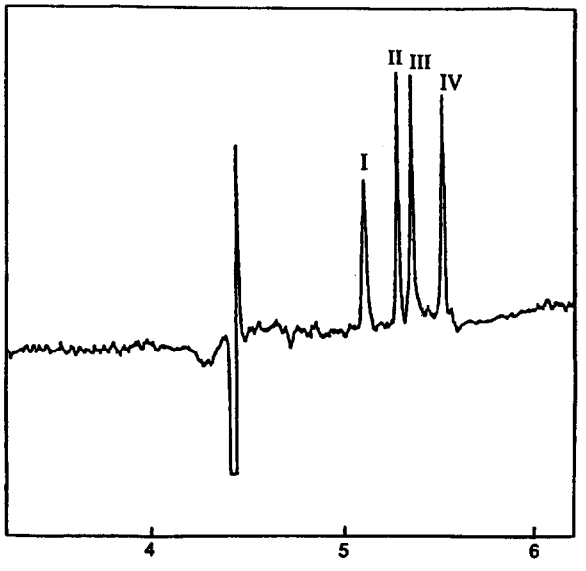

Fig. 5. Electropherogram of standard mixture of gangliosides under the optimum conditions (see text). Peaks: I = G_{M1}; II = G_{D1a}; III = G_{D1b}; IV = G_{T1b}.

To confirm the reproducibility of the migration time and peak area of the four standard gangliosides, G_{M1}, G_{D1a}, G_{D1b} and G_{T1b}, under the optimum conditions, the standard mixture was injected repeatedly, three times per day for three consecutive days. Almost no day-to-day variation observed, and the relative standard deviations ($n = 9$) for migration time and peak area were 0.12–0.24% and 2.0–2.7%, respectively.

Application to samples from biological matrices

The method was applied to the detection of gangliosides in the extracts of deer antler, apricot seed and rat brain. Other investigators have demonstrated that the sensitivity of CZE analysis for monosialoganglioside, *i.e.*, G_{M1}, was much greater (*ca.* 10^4–10^5 fold) than that of the resorcinol–hydrochloric acid method [34]. As the main purpose of this study was the improvement of the separation of gangliosides, especially from various biological matrices, we did not attempt to investigate quantitative aspects.

Fig. 6A depicts the detection of gangliosides in extracts of deer antler, which is commonly used in Chinese medicine as a tonic. To confirm the peaks of the individual components of gangliosides, the extracts were spiked with each standard ganglioside. In the extracts of deer antler, G_{D1a}, G_{D1b} and possibly G_{M1} were identified (Fig. 6A). Because the signal-to-noise ratio of peak I in Fig. 6A was low, not only was the standard G_{M1} added to the sample but also a comparison was made with the results of HPTLC. The standard G_{M1} co-migrated with peak I in the electropherogram and the HPTLC data also showed the possibility of the presence of G_{M1} in the extracts of deer antler. Although standard G_{M3} and Lac, which are thought to be the major ganglioside components in deer antler, were also added to the extracts, they appeared to co-migrate (*ca.* 4.7 min).

In the extracts of apricot seed, the migration time of standard G_{D1a} was matched with the peak at 5.4 min (Fig. 6B). As shown in Fig. 6C, G_{M1} and G_{D1a} were detected in rat brain, although a small peak at 5.5 min co-migrated with the added G_{T1b}. This observation is in good agreement with the results of other investigators [2]. Fig. 6 shows a distinct peak of an unknown

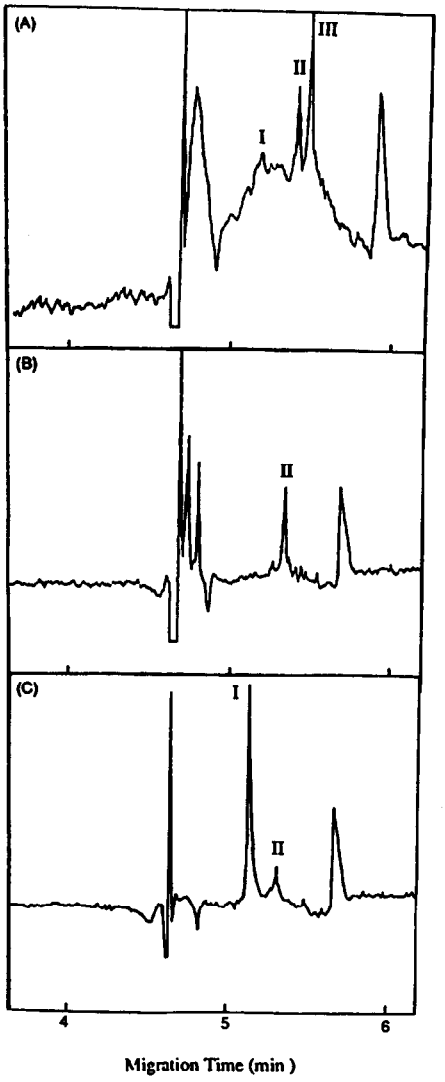

Fig. 6. Electropherogram of gangliosides in biological matrices: (A) deer antler; (B) apricot seed; (C) rat brain. Conditions as in Fig. 5. Peaks: I = G_{M1}; II = G_{D1a}; III = G_{D1b}.

compound around 5.7 min (Fig. 6B and C) or 5.9 min (Fig. 6A). Although this peak has not been identified, presumably the properties of the compound might be similar to those of glycosphingolipids.

CONCLUSIONS

We have investigated the separation conditions for the gangliosides G_{M1}, G_{D1a}, G_{D1b} and G_{T1b}, by using CD-modified CZE. The optimum conditions established were 50 m*M* borate–phosphate buffer (pH 9.3) electrolyte containing 16.5 m*M* α-CD, 60 cm × 50 μm I.D. column and applied voltage 30 kV.

The developed method was applied to the determination of gangliosides from various types of biological matrices, *i.e.*, extracts of deer antler, apricot seed and rat brain. G_{D1a}, G_{D1b} and possibly G_{M1} were shown to exist in deer antler and G_{D1a} was detected in the electropherogram of apricot seed. In rat brain, G_{M1} and G_{D1a} were major components and G_{T1b} was a minor component.

The results demonstrate that CZE using CD is a useful method for detecting glycosphingolipids, which have previously been difficult to detect especially in various biological matrices owing to the lack of chromophores and mixed micelle formation.

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