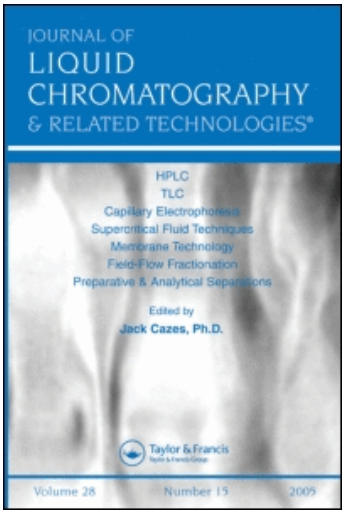

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Separation and Determination of Gangliosides Using High Performance Capillary Electrophoresis

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SEPARATION AND DETERMINATION OF GANGLIOSIDES USING HIGH PERFORMANCE CAPILLARY ELECTROPHORESIS

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ABSTRACT

The application of high performance capillary electrophoresis to the separation and determination of gangliosides is described. With a capillary of 60 cm x 25 μm I.D., filled with 100 mM borax containing 16.5 mM α-cyclodextrin as running buffer, a complex mixture of gangliosides can be separated and determined quantitatively by using a UV detection method. Several experimental parameters were investigated with the electrolyte system, including the composition of the running buffer, as well as the additive concentration and the electric field applied. Phenyl-β-D-glycopyranoside was used to calculate relative migration times for identification and as internal standard for quantitative analysis. Linearity of calibration graphs is observed for about two orders of magnitude with 5-15 μg/mL detection limits. The applicability of the method to the component analysis of brain tissues was demonstrated.

INTRODUCTION

Gangliosides, glycosphingolipids containing at least one sialic acid moiety, are an important group of functional membrane components, such as surface antigens and receptor molecules.[1-3] The distribution of gangliosides varies among animal species and tissues, suggesting that a specific molecule or its metabolite is closely associated in an unknown manner with various biological phenomena.[4-7] An earlier observation showed that gangliosides have antiviral activity towards the human immunodeficiency virus,[8] and a therapeutic effect on Alzheimer's disease and dementia.[9] Based on these biological functions, gangliosides have been suggested as diagnostic tools or as therapeutics for various human diseases. In order to elucidate the biological role of gangliosides, it will be important to obtain the information on ganglioside composition and contents in biological samples.

Many methods are available for the analysis of gangliosides in biological materials, including high performance liquid chromatography [HPLC],[10,11] thin-layer chromatography (TLC),[12] or high performance thin-layer chromatography (HPTLC)[13] followed by destructive densitometry, colorimetry or gas chromatography (GC).[14-16] The TLC and HPLC methods have been widely employed for the quantitation of gangliosides. The TLC method usually involves separation of individual gangliosides on a silica gel thin-layer plate and detection of the separated bands with a TLC scanner at 580 nm. The method generally requires relatively large amounts of gangliosides for a single determination and its use for small sized samples is, therefore, limited. Quantitative application of HPLC has frequently been carried out by converting gangliosides to UV-absorbing perbenzoyl[10] or p-nitrobenzyloxyamine derivatives.[11] Although these methods are sensitive, the reactions were not specific for gangliosides. In addition, they require a rather tedious sample clean-up procedure to achieve the separation of gangliosides.

Capillary electrophoresis (CE) is a powerful separation technique that has become an important analytical tool in many areas.[17] It provides fast separation, special selectivity, small sample requirement, and virtually no waste production. Previous investigations in this laboratory and by other researchers have shown the practicality of the use of CE for the separation of some gangliosides.[18-20] However, simultaneous separation of the mono- and polysialogangliosides has not been investigated, and the methods reported are not quantitative.

In this work, the separation of a complex ganglioside mixture and the quantitative determination of brain ganglioside compositions with CE is explored.

EXPERIMENTAL

Electrophoresis

CE was performed on a Beckman P/ACE system 2050 equipped with an autosampler and a temperature control system. An IBM-compatible computer with the System Gold Software was used for instrument control and data handling. A 25 μm I.D. fused-silica capillary (60 cm effective separation length) was obtained from Yong Nian Photoconductive Fibber Factory, Hebei, China. The column was etched with 0.5 M NaOH for 30 min before running each day, followed by rinse with water for 10 min. It was then filled with the running buffer and allowed to equilibrate for 30 min. The capillary was washed with running buffer for 3 min between runs. Samples were injected by pressure for 2 s each. The separated bands were detected at 200 nm and 1 Hz. Electropherograms were printed on an Epson 1600 printer.

Chemicals

Ganglioside standards G_{M1}, G_{D1a}, G_{D1b}, and G_{T1b} were purchased from Sigma Chem. Co. (St. Louis, MO, USA), G_{M2} and G_{M3} from BioCard Chemicals (S-223 Lund, Sweden). α-Cyclodextrin was obtained from Tokyo Chemical Industry Co. (Japan) and phenyl-β-D-glycopyranoside from Packard Becker B.V. (the Netherlands). All other chemicals were of analytical reagent grade.

Buffer and Sample Preparation

Running buffers were prepared by directly dissolving the appropriate amounts of sodium tetraborate and α-cyclodextrin in doubly distilled water. They were filtered through a 0.2 μm filter before use. Mixed standard sample was prepared with buffer to give 0.30-0.65 mg/mL of each gangliosides, respectively. For quantitative analysis, phenyl-D-glycopyranoside was used as internal standard, at a concentration of 0.020 mg/mL.

Gangliosides from brain tissue was extracted according to reference [21]. Briefly, the brain tissue (10-20g) was homogenized with 19 volumes of chloroform-methanol (2:1, v/v). The homogenate was chilled in an ice bath and filtered. The residue was extracted a second time with 10 volumes of chloroform-methanol (1:2, v/v) containing 5% water. After partitioning the sample by Folch's method,[22] the sample was concentrated, dialyzed,

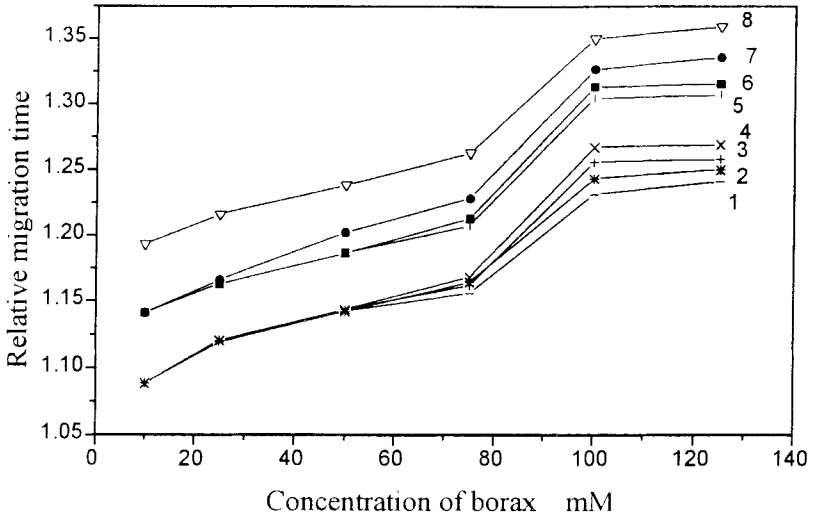

Figure 1. Effect of concentration of borax in the electrolyte on relative migration times of gangliosides. Buffer additive: 16.5 mM α-cyclodextrin; pH: 9.4; electric field strength: 250 v/cm; column temperature: 22°C; 1-G_{M3}; 2-G_{M2}; 3,4-G_{M1}; 5,6-G_{D1a}; 7-G_{D1b}; 8-G_{T1b}. The relative migration times of the solutes were obtained with respect to the migration time of methanol, t_0.

lyophilized, and subjected to further purification by chromatography on Iatrobeads (Iatron Industries, Inc., Tokyo, Japan).[20,23] The ganglioside fractions were collected and dissolved in buffer for CE analyses.

RESULTS AND DISCUSSION

Capillary Electrophoresis

Since the ganglioside compositions of most tissues are rather complex, it is very important to achieve high resolution of individual ganglioside species for quantitative analysis. Nevertheless, gangliosides cannot be separated by CE with normal buffers.[24] At the beginning of this project, we found that, by using β-cyclodextrin as an additive, gangliosides containing different numbers of sialic acid moieties can be well separated, but the isomers with same number of sialic acid, such as G_{D1a} and G_{D1b}, co-migrated.[18] At the same time, Yoo et al. investigated the separation conditions for a mixture of four gangliosides.[19] The

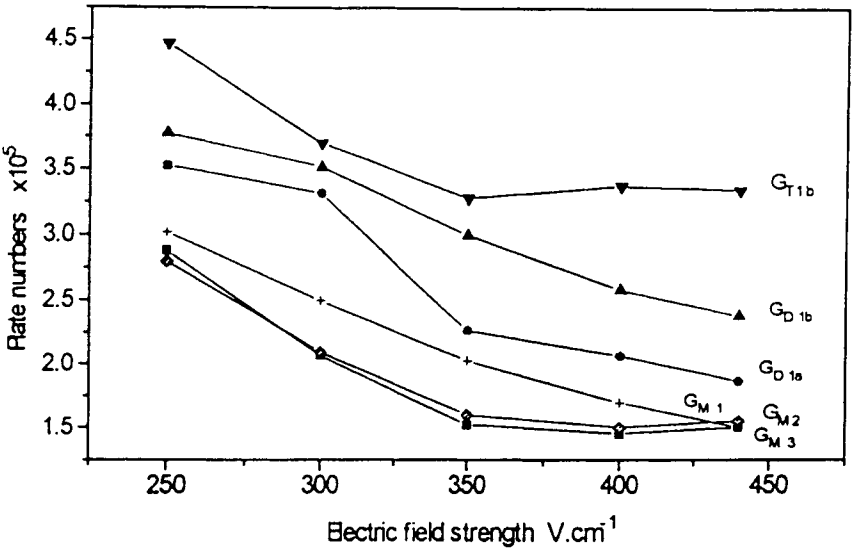

Figure 2. Effect of electric field strength on the separation efficiency of gangliosides. Running buffer: 100 mM borax (pH 9.4) containing 16.5 mM α-cyclodextrin. The number of theoretical plates obtained was based on the equation $N = 5.54\ (t_R/W_h)^2$, where t_R is the migration time of the peak and W_h the peak width at half-height.

separation of gangliosides G_{M1}, G_{D1a}, G_{D1b}, and G_{T1b} was achieved by using α-cyclodextrin as an additive. However, monosialogangliosides G_{M1}, G_{M2}, and G_{M3} were found to be co-eluted using the same running conditions. Thus, insufficient selectivity for a complex mixture appeared to be the key problem in these separation procedures.

We have tried unsuccessfully to improve the resolution by optimizing concentration of cyclodextrin and pH in the buffer. Further experiments, however, found that successful separation of a complex mixture depends not only upon the above mentioned factors, but on the electrolyte composition and applied electric field. Figure 1 shows the effect of borax concentration on the relative migration times of gangliosides. When the concentration of borax was lower than 50 mM, the separation of isomers for G_{D1a} and G_{D1b} as well as monosialogangliosides was not possible. In contrast, all the isomers were well separated when the concentration of borax was higher than 100 mM. The applied electric field was also found to have an important influence on the separation of gangliosides, especially of monosialogangliosides. Under the given conditions, increasing in the electric field strength caused the separation

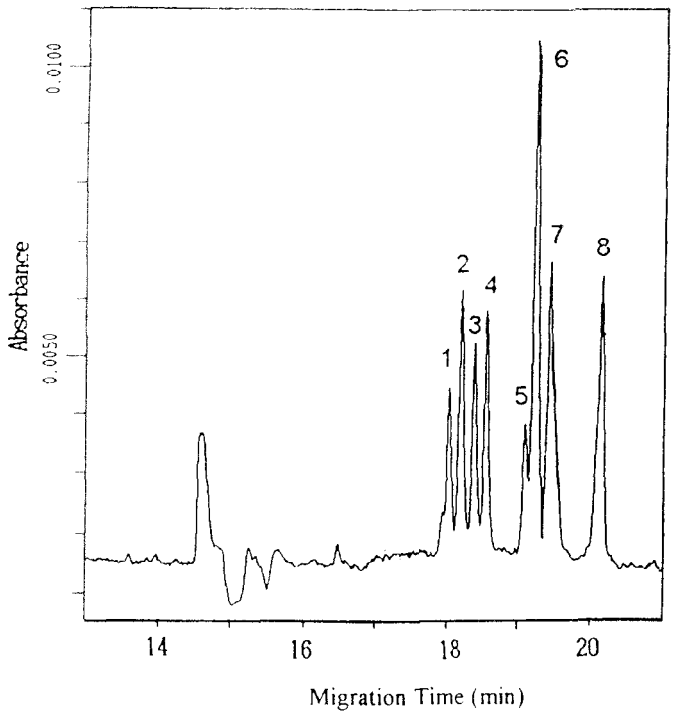

Figure 3. Electropherogram of standard mixture of gangliosides. Running buffer: 100 mM borax (pH 9.4) containing 16.5 mM α-cyclodextrin; electri field strength: 250 v/cm; current: 47.8 μA; column temperature: 22°C. Further details, see text. Peak numbers as in Table 1.

efficiency (number of theoretical plates) of all gangliosides to decrease, as shown in Figure 2. The pH of the buffer solution was optimized at 9.40. A general trend of a decrease on resolution with decrease in pH was observed, which is agreement with the observations reported in earlier studies.[18,19]

Figure 3 shows a typical electropherogram for a standard mixture of gangliosides obtained using 100 mM borax buffer (pH 9.40) electrolyte containing 16.5 mM α-cyclodextrin and applied electric field 250 v/cm. It can be seen that all the ganglioside species is satisfactorily separated. Peaks 1, 2, 7 and 8 were assignable to G_{M3}, G_{M2}, G_{D1b} and G_{T1b}, respectively. It is interesting that under the given separation conditions, the commercial standards G_{M1} [Lot 73H4024] and G_{D1a} [Lot 84H4120] gave two Peaks in an electropherogram,

respectively, showing that each of these commercial standards may contain two types of components or isomers. The presence of isorners in these standards has not been mentioned before.

Qualitative and Quantitative Analysis

For the analysis of a biological sample, an internal standard, phenyl-β-D-glycopyranoside, was added to the sample solution. Relative migration time was then calculated by measuring t_R/t_S, where t_R is the elution time of the sample and t_S of the internal standard. To select the internal standard, several substances were tested, and phenyl-β-D-glycopynanoside was selected because it does not exist in any biological sample. Besides, it has high solubility in the running buffer and is eluted from the column with a shorter migration time than the gangliosides in the sample.

Table 1 shows the relative migration times of individual ganglioside species tested and their within-day and between-day coefficients of variation. These relative migration times were used for the peak identification. The migration reproducilbility depends on several operational factors such as age of capillary, ionic strengtih of the buffer, previous capillary treatment, applied voltage, and external capillary temperature.[25] Results showed that control over the capillary temperature was important and the migration time decreased as the room temperature increased. The within-day relative standard deviations (R.S.D.) of the migration times were in the range of 2.5-4.6% and the between-day relative standard deviation values were 3.5-6.7% when the room temperature varied in the range of 20-25°C. The reproducibility is not as good as is required for qualitative analysis. An alternative way to eliminate this problem is to use the relative migration time. In this case, the reproducibility of relative migration times was satisfactory with a within-day R.S.D. in the range of 0.20-0.30% and a between-day R.S.D. ranging from 0.27 to 0.42%, as shown in Table 1.

The usefulness of the method for quantitative monitoring of the ganglioside tested was evaluated by means of calibration and reproducibility experiments. To study the relative peak area reproducibility and the variation of the relative peak areas with concentration, several consecutive runs using different standard mixtures with variable concentrations of the gangliosides and a fixed concentration(0.020 mg/ml) of phenyl-β-D-glycopyranoside were made. The standard curves were based on peak area measurement relative to internal standard. As shown in Table 2, valid calibration plots could be obtained for the gangliosides tested. The precision of five consecutive determinations was evaluated at concentrations of 0.30 (G_{M3}), 0.40 (G_{M2}, G_{D1b} and G_{T1b}), and 0.65

Table 1

Relative Migration Times ands Within-Day Realtive Standard Deviations and Between-Day Relative Standard Deviations

Gangliosides	Peak Numbers	Relative Migr. Time	Within-Day R.S.D. (%)*	Between-Day R.S.D. (%)**
I.S.[a]		1.000	---	---
G_{M3}	1	1.020	0.20	0.33
G_{M2}	2	1.030	0.25	0.38
G_{M1}	3	1.045	0.29	0.37
	4	1.050	0.23	0.27
G_{D1a}	5	1.082	0.29	0.39
	6	1.091	0.30	0.42
G_{D1b}	7	1.100	0.25	0.33
G_{T1b}	8	1.142	0.20	0.34

* n=8; ** n=5. [a] Internal standard (phenyl-β-D-glycopyranoside).

Table 2

Calibration Data Between 0.03 to 3.0 mg/mL for the Gangliosides Tested

Component	G_{M1}	G_{M2}	G_{M3}	G_{D1a}	G_{D1b}	G_{T1b}
Slope	0.80	0.80	0.79	1.03	1.01	1.33
y Intercept	0.022	0.020	0.023	0.10	0.05	0.13
Regression Coefficient	0.9993	0.9994	0.9991	0.9992	0.9991	0.9993

mg/mL (G_{M1} and G_{D1a}). The R.S.D. of relative peak area were in the range of 2.0-4.3%, which is quite good. The detection limits for gangliosides tested achieved at two times the signal-to-noise were 5-15 μg/mL, depending on sample components.

It is known that small changes in electroendosmosis can lead to changes in peak area. To overcome this problem, peak areas should be normalized to one of the migration times in each experiment.[26] The relative standard deviations obtained in this study were considered satisfactory without using normalized peak areas.

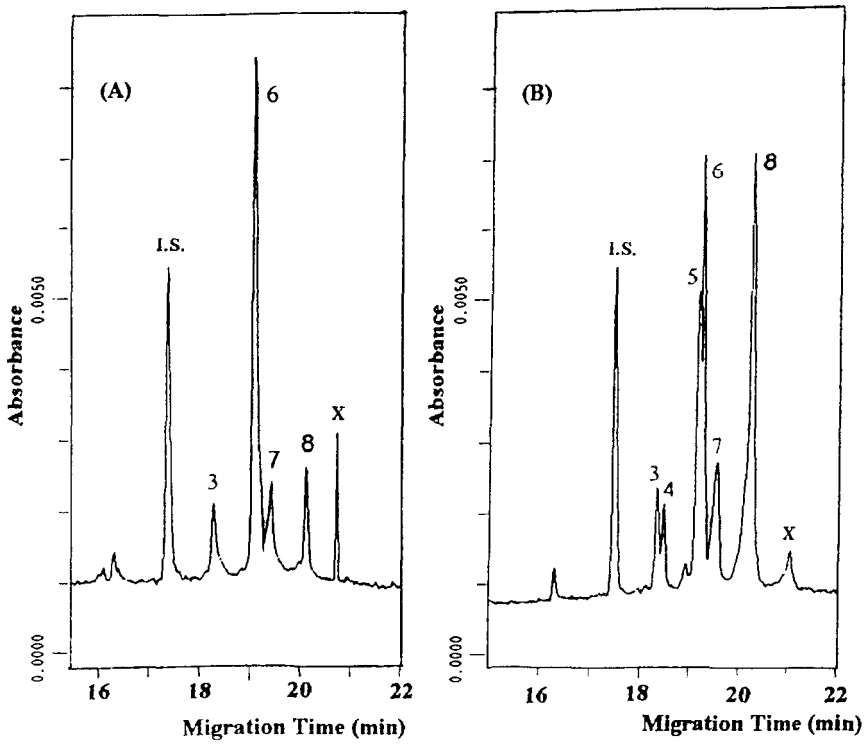

Figure 4. Electropherograms of gangliosides from extracts of brain tissue. (A) Human brain of premature delivery; (B) Bovine brain. The running conditions are the same as in Figure 3. Peak: I.S.- internal standard (phenyl-β-D-glycopyranoside); x-unknown peak; all other peak identities as in Table 1.

Application to the Component Analysis From Brain Tissues

The ganglioside extracts from bovine brain and human brain of premature delivery were directly analyzed by the method described above. The electropherograms are shown in Figure 4. The peak identifications were made by comparing the relative migration times with those of standard. The major gangliosides for both brain tissues were found to be G_{M1}, G_{D1a} G_{D1b} and G_{T1b}.

The determined values of these components are in good agreement with our earlier observation.[27] Note that both G_{M1} and G_{D1a} from extract of bovine brain split into two peaks on electropherogram, respectively. The exact cause of peak splitting from these gangliosides is not known. A likely

explanation is that the peaks reflect the molecular heterogeneity of these gangliosides. Further work is needed to identify the components responsible for these peaks.

CONCLUSIONS

The CE conditions for the simultaneous separation of the mono- and polysialogangliosides have been developed. The optimum conditions established were 100 mM borax buffer (pH 9.40) electrolyte containing 16.5 mM α-cyclodextrin, 60 cm x 25 μm I.D. capillary, and applied electric field 250 v/cm. By using phenyl-β-D-glycopyranoside as internal standard, reproducible qualitative and quantitative analysis of various ganglioside species was achieved. The results demonstrated that CE is a convenient and efficient method for separating and determining the components of gangliosides extracted from small biological samples.

ACKNOWLEDGMENTS

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