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Capillary electrophoresis of carboxylated carbohydrates I. Selective precolumn derivatization of gangliosides with UV absorbing and fluorescent tags☆

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Abstract

We demonstrate that the precolumn derivatization reaction, recently introduced by our laboratory for the selective labeling of carboxylated monosaccharides, can be readily transposed to other glycoconjugates containing carboxylated sugar residues, namely sialogangliosides. The selective derivatization reaction described here involved the attachment of sulfanilic acid (a UV-absorbing tag) or 7-aminonaphthalene-1,3-disulfonic acid (a UV-absorbing and also fluorescing tag) to the sialic acid moiety of the gangliosides via the carboxylic group in the presence of water-soluble carbodiimide. This labeling of the sialic acid moiety of the gangliosides with a chromophore and/or fluorophore leads to the formation of an amide bond between the carboxylic group of the sugar residue and the amino group of the derivatizing agent, thus replacing the weak carboxylic acid group of the carbohydrate species by the stronger sulfonic acid group which is ionized over the entire pH range. Furthermore, novel electrolyte systems were introduced and evaluated for the separation of the derivatized and underivatized gangliosides. The addition of acetonitrile or α-cyclodextrin (α-CD) to the running electrolyte was necessary to break-up the aggregation of amphiphilic gangliosides and allowed for their efficient separation as monomers in aqueous media using capillary electrophoresis. Several operating parameters were investigated with these electrolyte systems including the additive concentration as well as the ionic strength, pH and nature of the running electrolyte. Acetonitrile at 50% (v/v) in 5 m*M* sodium phosphate at high and low pH or 15 m*M* α-CD in 100 m*M* sodium borate, pH 10.0, proved ideal, in terms of resolution and separation efficiency, for the group separation of mono-, di- and trisialogangliosides. On the other hand, the complete resolution of disialoganglioside isomers (e.g., G_{D1a} and G_{D1b}) necessitated the superimposition of a chromatographic component on the electrophoretic process. This was achieved by adding either a hydrophobic (e.g., decanoyl-N-methylglucamide–borate surfactant complex) or hydrophilic [e.g., poly(vinyl alcohol) or hydroxypropyl cellulose] selectors to the running electrolyte.

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1. Introduction

Gangliosides are sialic acid-containing glycosphingolipids found in nearly all vertebrate tissues [1,2]. They reside in the plasma membrane

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and can act as receptor binding sites on the cell surface. A ganglioside molecule has a hydrophilic sialooligosaccharide chain and a hydrophobic moiety, i.e. ceramide, that consists of a sphingosine and fatty acid [3] (see Fig. 1). The sialic acid residues, which determines the negative charge of the ganglioside molecule, seems to be involved in regulating the physiological capabilities of gangliosides such as reactivity with toxins, viruses, antibodies and growth factors [4–6].

Growing interest in the numerous and important biological functions of gangliosides has engendered the need for capable separation techniques for their isolation and determination. Chromatography in its various forms has been a widely used technique in the isolation of gangliosides [3,7–10]. As with most of other carbohydrate species, one of the major difficulties encountered in the analysis of gangliosides is the lack of chromophores in their molecules. This inherent property hinders their determination at low levels since their UV detection at wavelengths lower than 200 nm is met with relatively low sensitivity. The low detectability of underivatized gangliosides is also problematic because most gangliosides are often only available in minute amounts. Thus, besides the need for sensitive detection methodologies there is also a strong demand for microcolumn separation methods of high resolving power and small sample requirements. In this regard, high-performance capillary electrophoresis (HPCE) is the alternative technique for the analytical separation and determination of gangliosides. In fact, HPCE has already been briefly explored in the separation of underivatized gangliosides using low-wavelength UV detection [11,12].

To overcome the detectability problem, several precolumn derivatization reactions have been

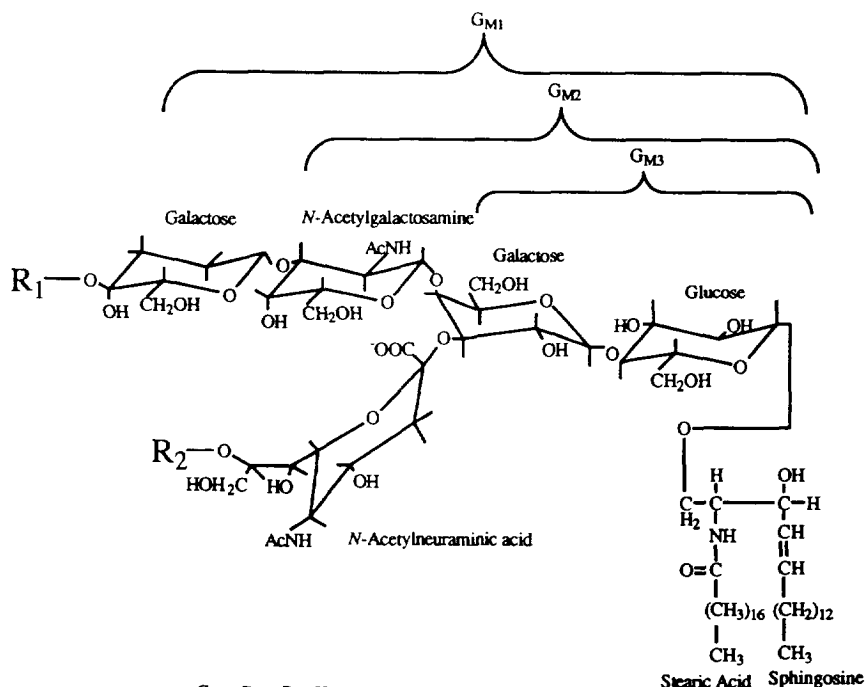


Fig. 1. Structures of the gangliosides.

already reported for the tagging of gangliosides, and in particular prior to separation by high-performance liquid chromatography (HPLC). UV-absorbing perbenzoyl [13,14] and *p*-nitrobenzyloxyamine derivatives [15] are typical examples. Although the sensitivity of these procedures was sufficiently high, the reactions were not specific for gangliosides and moreover, prewashing to remove reagents and by-products was necessary prior to separation. Nakalayashi et al. [10] introduced a specific precolumn derivatization of gangliosides based on the formation of an ester bond between *p*-bromophenancyl bromide reagent and the carboxylic group of the sialic acid moieties, and demonstrated its suitability for the analysis of the derivatives by HPLC. Although convenient to regenerate the original molecule, the susceptibility of the ester bond to hydrolysis makes this specific derivatization [10] rather unsuitable for the production of stable derivatives.

Similar to HPLC, with HPCE it is preferable that precolumn derivatization be selective. In addition, the tag should be charged or if it is neutral, its attachment to the carbohydrate molecule should not eliminate the already existing charge of the solute molecule so that separation in an electric field would be possible. Very recently, we have shown that these two requirements are readily met by exploiting the reactivity of the carboxylic group of acidic monosaccharides [16]. Since gangliosides are sialic acid-containing glycolipids, the carboxylic groups of the sialic acid residues can also be readily tagged as in the case of carboxylated monosaccharides. The tagging procedure [16] involves the formation of a stable amide bond between the carboxylic group of the sugar analyte and the amino group of the derivatizing agent, e.g. 7-aminonaphthalene-1,3-disulfonic acid or sulfanilic acid, in the presence of water-soluble carbodiimide. Besides providing the chromophore or fluorophore, the derivatization reaction utilized here replaces the weak carboxylic acid group of the gangliosides by the strong sulfonic acid group, thus ensuring permanent negative charges on the derivatives over the entire pH range.

This study is a logical continuation to our recent contribution to the area of separation of minute amounts of acidic carbohydrates by HPCE. Herein we report (i) the evaluation of the new derivatization procedure in the selective tagging of carboxylated glycoconjugates, e.g. gangliosides, and (ii) the introduction of novel electrolyte systems for the separation of gangliosides in capillary electrophoresis.

2. Experimental

2.1. Instruments and capillaries

The instrument for capillary electrophoresis was assembled in the laboratory from commercially available components [16–18]. It comprised two high-voltage power supplies of positive and negative polarity, Models MJ30P400 and MJ30N400, respectively, from Glassman High Voltage (Whitehouse Station, NJ, USA) and a UV–Vis variable-wavelength detector Model 200, equipped with a cell for on-column capillary detection from Linear Instruments (Reno, NV, USA). The detection wavelength was set at 247 nm for UV detection. Fluorescence detection of the derivatized gangliosides utilized a spectrofluorescence detector Model FL-750 BX from McPherson (Acton, MA, USA) with variable excitation wavelength. The excitation wavelength was set at 315 nm for fluorescence detection and 400 nm sharp cut-off long-wavelength absorption filter was used for collecting the emission wavelength. Electropherograms were recorded with a Shimadzu computing integrator Model CR5A (Columbia, MD, USA).

The absorption spectra of the derivatizing agents were performed on a UV–Vis spectrophotometer Model Lambda Array 3840 from Perkin-Elmer (Norwalk, CT, USA) by scanning from 190 to 350 nm.

Fused-silica capillary columns of 50 μm I.D. × 365 μm O.D. were obtained from Polymicro Technology (Phoenix, AZ, USA). All capillaries used in this study were uncoated since the analytes are negatively charged and would repulse from the like-charged capillary surface. The derivatized gangliosides were introduced

into the capillary as thin plugs by means of hydrodynamic injection for approximately 5 s at 15 cm differential height between inlet and outlet ends of the capillary.

2.2. Reagents and materials

Gangliosides G_{M1}, G_{D1a}, and G_{D3} were extracted from bovine brain as described below and elsewhere [19]. Ganglioside type III mixture (containing approx. 20% N-acetylneuraminic acid) and standard monosialoganglioside (G_{M1}) from bovine brain, as well as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) were purchased from Sigma (St. Louis, MO, USA). Standard disialogangliosides (G_{D1a}, G_{D1b}, and G_{D3}) and trisialoganglioside (G_{T1b}) were obtained from Sigma and from Matreya (Pleasant Gap, PA, USA). Neuraminidase from *Clostridium perfringens* and MEGA 10 (decanoyl-N-methylglucamide) surfactant were purchased from Calbiochem (LaJolla, CA, USA). The derivatizing agents 7-aminonaphthalene-1,3-disulfonic acid (ANDSA) and sulfanilic acid (SA) were purchased from TCI America (Portland, OR, USA). α-Cyclodextrin (α-CD) was donated by the American Maize-Products Co. (Hammond, IN, USA). Reagent-grade sodium phosphate monobasic, hydrochloric acid, sodium hydroxide, boric acid granular, and HPLC-grade acetonitrile (ACN) were obtained from Fisher Scientific (Pittsburgh, PA, USA). Poly(vinyl alcohol) (PVA), 87–89% hydrolyzed (M_r 124 000–186 000) and hydroxypropyl cellulose (HPC) of average M_r 100 000 were purchased from Aldrich (Milwaukee, WI, USA). A reversed-phase HPLC column, C_{18}-silica Microsorb-MW, with 5 μm mean particle diameter and 100 Å mean pore diameter was purchased from Rainin (Woburn, MA, USA). This column was used in purifying the side products of the derivatization reactions. Deionized water was used to prepare the running electrolytes, buffers and the sample solutions. The running electrolytes were filtered with 0.2-μm Whatman syringeless filters obtained from Baxter Diagnostics (McGaw Park, IL, USA) to avoid column plugging and minimize baseline noise.

2.3. Extraction of gangliosides

Approximately 500 g of bovine brain tissues were extracted. Tissues were homogenized and extracted twice with 1500 ml of chloroform–methanol (2:1, v/v) and once with isopropanol–hexanes–water (55:25:20, v/v/v). Following Folch's partition [20] the upper layer glycolipids were subjected to DEAE-Sephadex column chromatography to separate neutral and acidic glycolipids (sulfatide and gangliosides) [21]. The acidic glycolipids were eluted in three fractions with increasing salt concentration. The monosialogangliosides were eluted with 0.05 *M* NH_4OAc in MeOH, sulfatide and the disialogangliosides with 0.15 *M* NH_4OAc in MeOH and polysialogangliosides with 0.45 *M* NH_4OAc in MeOH. The lower-phase glycolipids were acetylated and subjected to column chromatography with Florisil [22]. Further purification of the gangliosides was performed by HPLC with a Varian Model 9012 HPLC system using a column (100 × 1 cm) of Iatrobeads (6RS-8010, Iatron, Tokyo, Japan) and eluted at 2.0 ml/min with a 200-min gradient of isopropanol–hexanes–water [19,23] from 55:44:1 (v/v/v) to 55:20:25 (v/v/v). Further purification of individual glycolipids was accomplished by preparative high-performance thin-layer chromatography (HPTLC) with the use of chloroform–methanol–water (55:40:10, v/v/v containing 0.02% $CaCl_2$). Separated glycolipids were visualized with UV light after being sprayed with 0.1% primuline in 80% aqueous acetone. Once visualized, individual glycolipids were recovered from HPTLC plates by scraping followed by sonication in isopropanol–hexanes–water (55:25:20, v/v/v).

2.4. Derivatization of gangliosides

Gangliosides were tagged with ANDSA or SA as previously described [16]. Briefly, an aliquot of 50 μl of 100 m*M* aqueous solution of EDAC, pH 5.0, was initially added to sub-microgram amounts of solid gangliosides and the mixture was stirred for 1 h. Then, 50.0 μl of 100 m*M* aqueous solution of the derivatizing agent were added and the mixture was stirred for an addi-

tional 1.5 h. Subsequently, the entire reaction mixture containing the derivatized gangliosides, excess derivatizing agent and other components of the reaction mixture was analyzed by capillary zone electrophoresis (CZE).

2.5. Neuraminidase treatment

An aliquot of 20.0 μl of the SA derivative of G_{T1b} aqueous solution was added to 7.0 μl of neuraminidase (i.e., 0.028 units), dissolved in 50.0 μM sodium acetate buffer, pH 5.0, and the mixture incubated at 37°C for 72 h [24–26]. Aliquots of the reaction mixture were taken at various time intervals and analyzed by CZE.

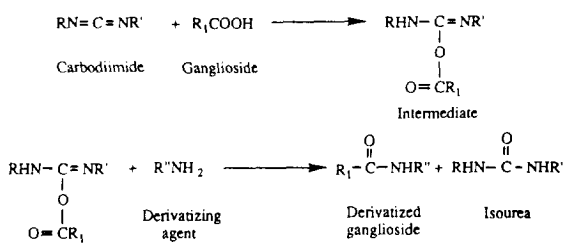
3. Results and discussion

Throughout the various studies concerning the electrophoretic behavior of derivatized gangliosides, the results were compared to those obtained with their underivatized counterparts in order to assess the various changes that might have been caused by the precolumn labeling of the different gangliosides.

In evaluating the suitability of the various electrophoretic systems under consideration, gangliosides type III mixture, which was purchased from Sigma, as well as individual gangliosides were utilized. The type III mixture may contain essentially G_{M1}, G_{D1a}, G_{D1b} and G_{T1b} since it is known that mammalian brains generally contain these four major gangliosides [3,27] in the concentration ratios of 2:3.5:1:1.5, respectively [27].

3.1. Precolumn derivatization of gangliosides

The use of low wavelengths (e.g., 195 or 185 nm) for the detection of underivatized gangliosides impairs the utility of many buffer systems because at these wavelengths the components of the electrolyte systems starts to absorb substantially in the UV, thus leading to high background signals. The lack of suitable chromophores in ganglioside molecules makes their detection impossible at high wavelengths where many useful running electrolytes are UV transparent. To overcome this difficulty, and permit the sensitive detection at higher wavelengths, the gangliosides were derivatized with SA and ANDSA according to established procedures for carboxylated carbohydrates which were recently introduced by our laboratory [16]. The precolumn derivatization reaction involves a condensation reaction between the free amino group of the derivatizing agent and the free carboxyl group of the ganglioside to form a peptide link by acid-catalyzed removal of water in the presence of EDAC. The carbodiimide EDAC, which is required to promote the formation of the peptide bond [28,29], was selected because of its high solubility in aqueous solutions.The derivatization proceeds according to the following scheme:



where $R''NH_2$ is either SA or ANDSA, and R_1COOH can be any ganglioside (e.g., Fig. 1).

Individual gangliosides as well as Sigma type III ganglioside mixture were derivatized according to the above scheme. Typical electropherograms of both SA and ANDSA derivatives of type III mixture are displayed in Fig. 2. The excess of derivatizing agent eluted at 23 min for SA (peak not shown) while it did not elute in reasonable time in the case of ANDSA (peak not shown) when 15 kV were used as the running voltage. The detection wavelength was set at 247 nm since the UV spectra of both derivatizing agents, i.e., SA and ANDSA, as well as the gangliosides derivatives showed maximum absorbance at 247 nm. Returning to Fig. 2, the signal of disialogangliosides (G_Ds) is much greater than that of G_{M1} and G_{T1b}. As stated above, there are more G_Ds in the mixture than mono- and trisialogangliosides. Since G_{T1b} and G_Ds have three and two derivatization sites, respectively, under exhaustive precolumn derivatiza-

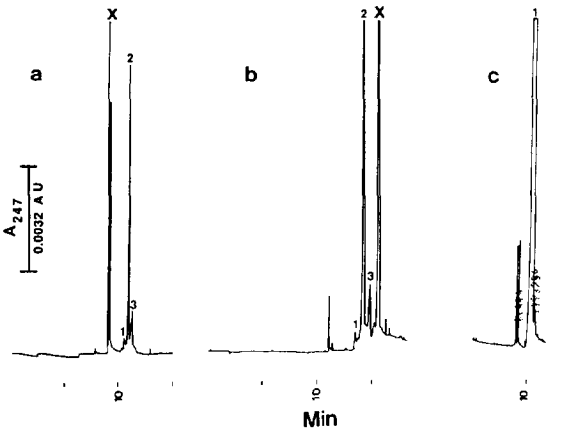


Fig. 2. Typical electropherograms of SA (a) and ANDSA (b) derivatives of the ganglioside mixture type III from Sigma and ANDSA derivative of standard G_{D1a} (c). Capillary, 80.0 cm (50.0 cm to detection) × 50 μm I.D.; running voltage, 15.0 kV in (a) and (b) and 20.0 kV in (c). Running electrolytes: (a) and (b): 150 m*M* borate containing 15.0 m*M* α-CD, pH 10.0; (c) 10 m*M* sodium phosphate, pH 7.0, containing 15.0 m*M* α-CD. Peaks in (a) and (b): 1 = G_{M1}; 2 = G_{D1a} and G_{D1b}; 3 = G_{T1b}, × = by-product; peak in (c): 1 = G_{D1a}.

tion conditions two and three chromophores could be attached to G_{T1b} and G_Ds, respectively. This would explain the difference between peak height ratios of derivatized and underivatized gangliosides (compare Figs. 2 and 6c).

As can be seen in Fig. 2a and b, in addition to the derivatized gangliosides, by-products are also produced in the precolumn derivatization reaction. The by-products were detected by UV at 247 nm (in the case of SA and ANDSA) and by fluorescence in the case of ANDSA. This suggests that both by-products possess the derivatizing agents as part of the molecule. This was further confirmed by the electrophoretic behaviors of the by-products when varying the pH of the running electrolyte. The SA by-product seems to be neutral at pH ≥ 5 and positively charged at pH < 5 as inferred from its migration with or ahead of the marker of the electroosmotic flow (EOF), respectively. The by-product of ANDSA has a net negative charge at pH ≥ 5 and neutral at pH < 5 as indicated by the migration time of its peak with respect to that of the EOF marker. The pK_a values of the amino groups of SA and ANDSA are in the range of 3–4 [30]. Together, these observations imply that the by-product of SA has involved reaction with the sulfonic acid group of the SA molecule while leaving its amino group unsubstituted. The same conclusion could be drawn regarding the ANDSA by-product whereby one sulfonic acid group of the molecule has been involved in the reaction while the other sulfonic acid group and the amino group were left unsubstituted. In fact, studies made by ^{1}H NMR and ^{13}C NMR on an HPLC purified fraction of the by-product of ANDSA agreed with these observations. Both modes of NMR suggested the presence of alkylated moieties attached to the derivatizing agent which may indicate that the by-product is the result of the reaction between the derivatizing agent and the carbodiimide.

The by-products were seen when the amount of ganglioside added to the reaction mixture was not well controlled. With almost all ganglioside samples we did not have sufficient quantities that would allow the accurate weighing of a known and desirable amount. In other words, the side product was observed only when the amount of carbodiimide far exceeded the amount of analyte in the reaction mixture. In fact, when the reaction was made such that for each mole of carboxylic group, one mole of EDAC and two moles of SA or ANDSA were added, the derivatization reaction did not lead to the formation of detectable amount of side product as was the case of G_{D1a} (Fig. 2c). The absence of by-products was also observed with acidic monosaccharides reported earlier [16] whenever the ratio of 1:1:2 of sugar:carbodiimide:derivatizing agent was utilized.

To confirm the occurrence of the derivatization at the sialic acid site, the derivatized trisialoganglioside SA-G_{T1b} was treated with neuraminidase (for structure, see Fig. 1). Neuraminidase is an exoglycosidase which catalyzes the hydrolysis of the linkage joining a terminal sialic acid residue to a D-galactose or a D-galactosamine residue [31]. Since G_{M1} is resistant to neuraminidase, and G_{T1b} is hydrolyzed more rapidly than G_Ds [32], the cleavage of G_{T1b} with neuraminidase should result in the production of G_{M1} and G_{D1b} [25,26]. This is shown in Fig. 3

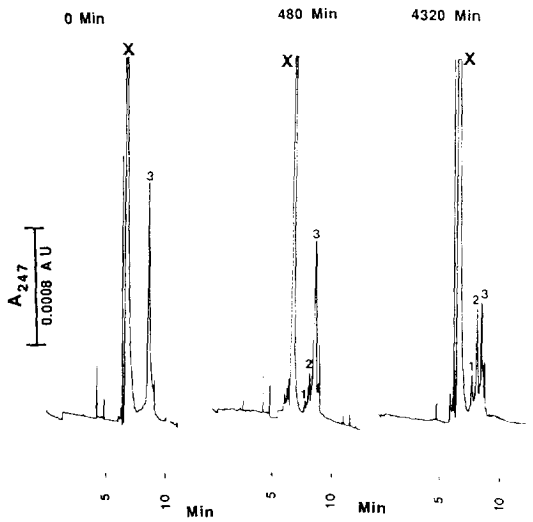


Fig. 3. Monitoring of the neuraminidase action on the SA derivative of GT1b by capillary electrophoresis. Conditions as in Fig. 2a and b. Peak: 1 = G_{M1}; 2 = G_{D1a}; 3 = G_{T1b}; × = by-product.

where SA-derivatized G_{T1b} gave one peak before treatment with neuraminidase and three peaks after exhaustive treatment with neuraminidase. Two additional peaks (not shown) have also appeared which were assumed to correspond to the cleaved mono- and disialic acid residues. The relatively intense peak that appeared at 19.2 min may be the SA derivative of sialic acid cleaved from G_{T1b} to yield G_{D1b} (i.e., the R_1 residue, see Fig. 1) while the less intense peak that eluted at 13.1 min may correspond to the SA derivative of disialic acid cleaved from G_{T1b} to yield G_{M1} (i.e., the R_2-sialic acid residues, see Fig. 1). The assumption that the less intense peak is that of the disialic acid and the more intense peak is that of the sialic acid monomer, corroborates well with the fact that the amount of G_{M1} produced is much less than that of the G_{D1b}. The excess SA was observed at 23.1 min.

The progress of the derivatization reaction with SA and ANDSA was monitored by CZE using the type III ganglioside mixture. Typical time course plots for SA-G_{T1b} and ANDSA-G_{T1b} are shown in Fig. 4 in terms of integrated peak heights versus time. In all cases, the precolumn derivatization reaction proceeded at a faster rate with SA than with ANDSA. The derivatization

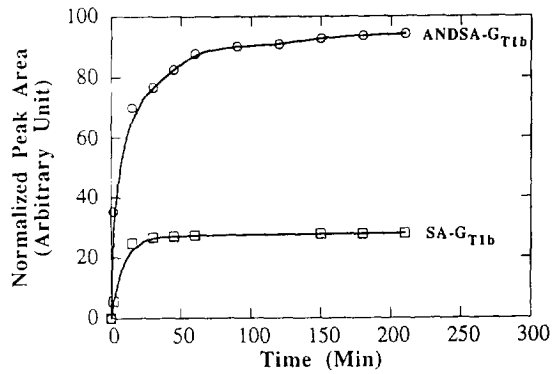


Fig. 4. Time course for the derivatization reactions of G_{T1b} with ANDSA and SA. The reaction mixture was subjected to CZE at different time intervals using conditions of Fig. 2a and b.

with SA reached a steady state in almost 1 h, while that with ANDSA required almost 2.5 h to reach the steady state. These results corroborate earlier findings with carboxylic monosaccharides [16], and may be attributed to differences in collision rates arising from differences in the molecular masses of the two derivatizing agents.

3.2. Capillary electrophoresis of gangliosides

It is well established that gangliosides exist as stable micelles in aqueous solutions with critical micellar concentration (CMC) in the range of 10^{-10}–10^{-8} M [10,33]. This phenomenon hinders the efficient separation of gangliosides as monomeric species in neat aqueous media. Consequently, an organic solvent or an additive capable of breaking the micelles is needed. In this study, acetonitrile and other additives were added individually to the running electrolytes used in separating the gangliosides. One virtue of the precolumn derivatization is that SA and ANDSA derivatives have maximum absorptivity at 247 nm far removed from low UV wavelengths (e.g., 195 nm) where only few solvents and light buffers can be used. In addition, ANDSA derivatives are readily detected by fluorescence.

HPLC-grade ACN was used as the organic modifier for the separation of gangliosides because its UV cut-off is 185 nm. Fig. 5 shows the separation of standard derivatized gan-

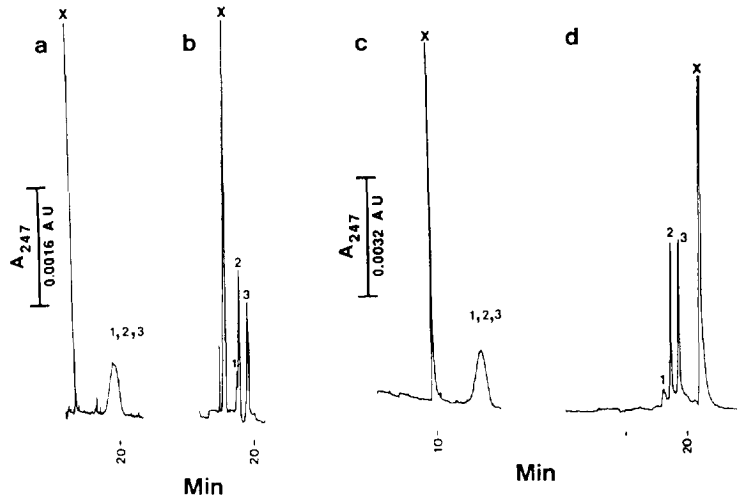


Fig. 5. Electropherograms of standard gangliosides derivatized with ANDSA at neutral (a and b) and high pH (c and d) in the presence (b and d) and absence (a and c) of acetonitrile in the running electrolyte. In (a) and (b): running electrolyte, 25 m*M* sodium phosphate, pH 7.0, at 0% (a) and 50% (v/v) (b) acetonitrile; running voltage, 25.0 kV. In (c) and (d): running electrolyte, 10 m*M* sodium phosphate, pH 10.0 at 0% (c) and 50% (v/v) (d) acetonitrile; running voltage, 20 kV. Samples: 1 = G_{M1}; 2 = G_{D1a}; 3 = G_{T1b}. Other conditions as in Fig. 2

gliosides at basic and neutral pH. At both pH values, and in the absence of ACN in the running electrolyte the ANDSA-gangliosides eluted as a single broad peak with no separation (see Fig. 5a and c). The inclusion of 50% ACN in the running electrolyte has apparently brought about the break up of aggregation and in turn the separation of the gangliosides. The same trend was also observed with the underivatized gangliosides (type III mixture) shown in Fig. 6, where 50% (v/v) ACN concentration seems to be the optimum amount which yielded the best separation in terms of resolution and sharpness of the migrating zones. The derivatization of the carbohydrate moiety of the gangliosides seems not to introduce undesirable effects as far as the aggregation of these species is concerned. In fact, both derivatized and underivatized gangliosides behaved similarly when electrophoresed with acetonitrile-rich electrolytes. In all cases, at ACN concentration below 30% (v/v), the gangliosides co-migrated indicating that the different analytes traveled through the capillary as a mixed micelle. As the ACN concentration increased above 30%, the different gangliosides migrated as monomers or oligomers and their migration mobility was greatly affected by their charge-to-mass ratio (Fig. 6). As can be seen in Fig. 6, only three different peaks were detected, which were identified by spiking the mixture with standard samples of the different gangliosides. The disialoganglioside isomers, G_{D1a} and G_{D1b},

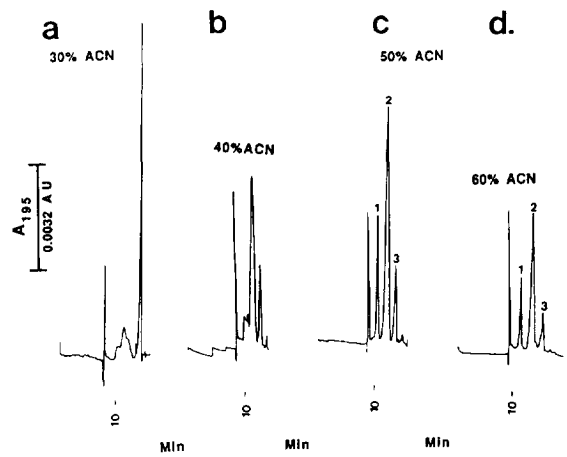


Fig. 6. Electropherograms of underivatized gangliosides (mixture type III). Running electrolytes: 5.0 m*M* sodium phosphate, pH 7.50, at various % (v/v) acetonitrile; running voltage, 15.0 kV. Peaks: 1 = G_{M1}; 2 = G_{D1a} and G_{D1b}; 3 = G_{T1b}. Other conditions as in Fig. 2.

co-migrated since both molecules have the same charge-to-mass ratio. Optimum separation conditions were achieved at 50% (v/v) ACN since at 60% ACN the gain in resolution was on the expense of broader peaks and at 70% ACN the efficiency dropped by a factor of almost 2.

To further optimize the hydro–organic electrolyte system just described, the effects of phosphate concentration and pH of the running electrolyte were examined. As expected, increasing the ionic strength of the running electrolyte decreased the electroosmotic flow which resulted in increasing the migration time of the gangliosides through the capillary (Fig. 7). More importantly, for amphiphilic gangliosides increasing the ionic strength of the running electrolyte reduces the CMC value. In fact, it has been shown that the CMC of G_{M1} decreased by a factor of three when going from pure water to 50 m*M* sodium acetate buffer [34]. As can be seen in Fig. 7, the effect of increasing buffer ionic strength seems to be more pronounced for the monosialoganglioside G_{M1} since electrostatic repulsion between the polar head groups of singly charged amphiphilic species can be shielded at much lower salt concentration, a phenomenon that favors aggregation and reduction of CMC in aqueous solution. This may explain the decrease in the sharpness of the G_{M1} peak as the concentration of phosphate buffer increased. Optimum separation efficiencies in terms of number of theoretical plates were achieved at 5.0 m*M* phosphate buffer concentration.

Increasing the pH of the running electrolyte from 5.0 to 8.0 did not greatly affect the electroosmotic flow (fluctuated between 8.5 and $9.0 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1}), nor the electrophoretic mobility of the different gangliosides (results not shown). For instance, the electrophoretic mobility of G_{M1} only increased from $2.0 \cdot 10^{-4}$ to $2.5 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1}. This might be due to the presence of the acetonitrile in the running electrolyte.

One of the shortcomings of the hydro–organic buffer systems evaluated above is that G_{D1a} and G_{D1b} were not separated. To provide other buffer systems that are useful for the separation of gangliosides in their monomeric forms, α-CD was used as a buffer additive. It has been shown that α-CD has the ability to break up the micellar form of the different gangliosides mixtures in a borate electrolyte [12]. In fact, and as can be seen in Fig. 8a, the ganglioside mixture type III could be separated into its mono-, di- and trisialogangliosides in the presence of α-CD. Although 7 m*M* α-CD was sufficient for keeping the different gangliosides as monomers, a 15 m*M* α-CD concentration was used because this

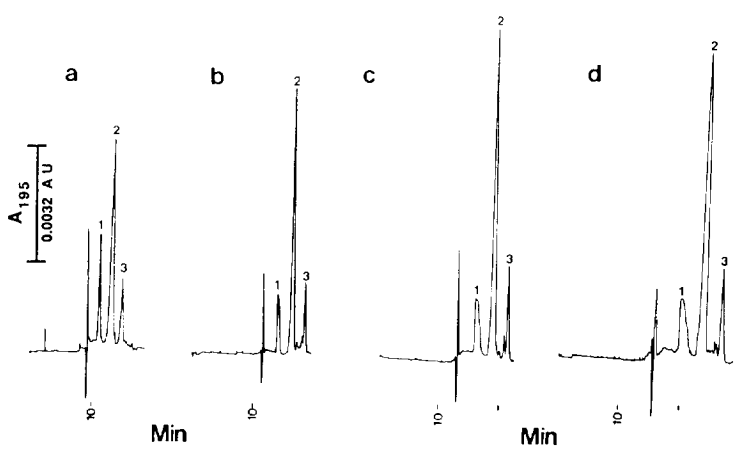


Fig. 7. Electropherograms of standard underivatized gangliosides. Running electrolytes: sodium phosphate buffers, pH 7.50, containing 50% (v/v) acetonitrile at 5.0, 15.0, 25.0 and 35.0 m*M* sodium phosphate in (a), (b), (c) and (d), respectively. Average number of theoretical plates per meter: 25 200, 14 400, 13 200 and 16 700 in (a), (b), (c) and (d), respectively. Peaks: 1 = G_{M1}; 2 = G_{D1a} and G_{D1b}; 3 = G_{T1b}. Other conditions as in Fig. 2.

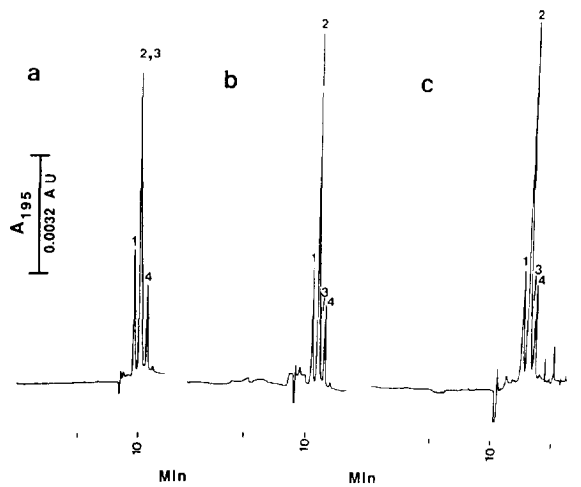


Fig. 8. Electropherograms of underivatized gangliosides (mixture type III). Running electrolytes, borate buffers containing 15.0 m*M* α-CD, pH 10.0, at 50, 100 and 150 m*M* borate in (a), (b) and (c), respectively. Peaks: 1 = G_{M1}; 2 = G_{D1a}; 3 = G_{D1b}; 4 = G_{T1b}. Other conditions as in Fig. 2.

amount yielded the highest plate count as shown in Fig. 9.

The effect of borate complexation with the oligosaccharide moiety of gangliosides was studied at three different borate concentrations to determine the best conditions for the separation of the two disialoganglioside isomers, G_{D1a} and G_{D1b}. As can be seen in Fig. 8, 100 m*M* borate provided partial separation between the two isomers and a further increase in borate concentration did not improve the resolution (Fig. 8c). This may indicate that the changes in the charge density between the isomers due to borate complexation may not be sufficiently high to allow their complete separation. As expected, increasing the borate concentration increased the migration time due to increasing the extent of complexation of the carbohydrate moieties of the gangliosides with borate and to decreasing the EOF. It should be noted that the electrophoretic velocity of the solutes is in the opposite direction to the EOF. The same trend was also observed with ANDSA-gangliosides when the borate concentration was varied in the presence of α-CD (Fig. 10). As with the underivatized gangliosides, increasing the borate concentration resulted in a partial separation between the isomers but at the expense of longer analysis time. As can be seen in Fig. 10, the complexation between borate and the individual gangliosides occurs to the same extent as indicated by the parallel increase of the migration time with the increase in borate concentration. This may indicate that the sialic acid does not contribute significantly to borate complexation since the only difference in the carbohydrate moieties of the various gangliosides is the number of sialic acids.

ANDSA is a fluorescent tagging agent. It has a maximum excitation signal at 315 nm and a maximum emission signal at 420 nm [35]. This

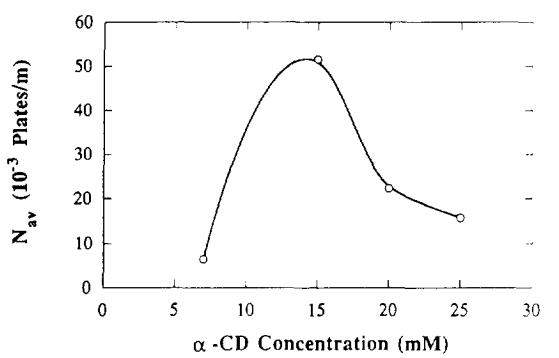


Fig. 9. Plot of average plate counts per meter at various α-CD concentrations in the running electrolyte. Running electrolytes, 150 m*M* borate, pH 10.0, at various concentration of α-CD. Other conditions as in Fig. 2.

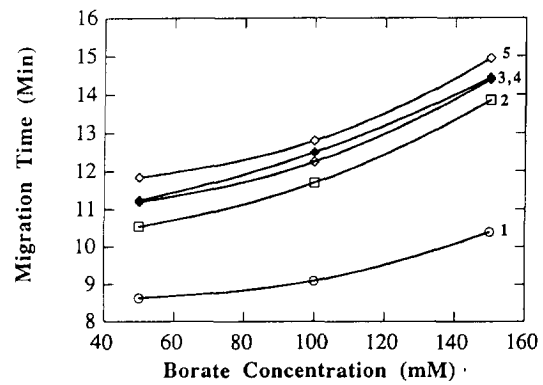


Fig. 10. Typical plots of migration time versus borate concentration in the running electrolyte obtained with ANDSA derivatives of standard gangliosides. 1 = Dimethyl sulfoxide; 2 = G_{M1}; 3 = G_{D1a}; 4 = G_{D1b}; 5 = G_{T1b}. Other conditions as in Fig. 8.

allowed the fluorescence detection of the ANDSA derivatives of gangliosides as shown in Fig. 11.

As shown above, the hydro–organic electrolyte system containing ACN and the α-CD buffer system did not provide adequate resolution for the disialoganglioside isomers, i.e., G_{D1a} and G_{D1b}, despite the fact that they were very efficient in breaking up micelle formation. Therefore, there is a need to add hydrophobic or hydrophilic selectors to the electrolyte to provide sufficient selectivity. To evaluate some potential additives, three model disialogangliosides were used including G_{D1a}, G_{D1b} and G_{D3}. G_{D3} has a smaller molecular mass than the two isomers G_{D1a} and G_{D1b} since its carbohydrate moiety is a tetrasaccharide with two sialic acid residues (Fig. 1). The charge-to-mass ratios of G_{D1a}, G_{D1b} and G_{D3} are $1.09 \cdot 10^{-3}$, $1.09 \cdot 10^{-3}$ and $1.35 \cdot 10^{-3}$, respectively.

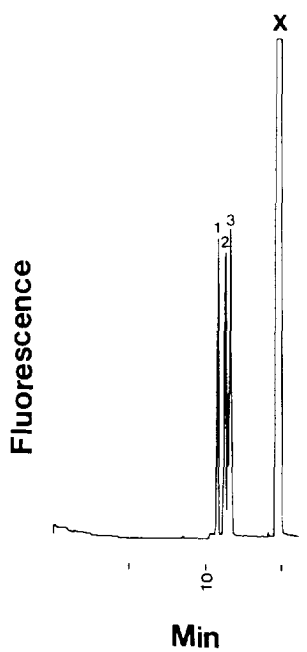


Fig. 11. Typical fluorescence electropherogram of mono-, di- and trisialogangliosides derivatized with ANDSA. Running electrolyte, 10 m*M* sodium phosphate, pH 12.0, containing 15.0 m*M* α-CD; running voltage, 18.0 kV. Peaks: 1 = G_{M1}; 2 = G_{D1a}; 3 = G_{T1b}; × = by-product. Other conditions as in Fig. 2.

In addition to the fact that the α-CD and ACN electrolyte systems provided marginal or no resolution between G_{D1a} and G_{D1b}, they did not allow the complete resolution of G_{D1a} and G_{D1b} from G_{D3} as shown in Fig. 12a and b. As can be seen in this figure, phosphate electrolyte containing 50% ACN yielded a better separation between G_{D3} and the other two disialoganglioside isomers. Similar results were obtained with the derivatized G_{D1a}, G_{D1b} and G_{D3}.

To enhance the selectivity of the electrophoretic system and to achieve separation of the G_Ds, hydrophilic interaction was superimposed on the electrophoretic process by including HPC or PVA in the running electrolytes in addition to α-CD. The amount of these additives were varied, and best results were attained at 0.5% (v/v) (see Fig. 13).

In another set of experiments, hydrophobic interaction was exploited to achieve the separation of the G_Ds. In this regard, the possibility of using micellar electrokinetic capillary chromatography was considered by investigating the usefulness of in situ charged micelles recently introduced by our laboratory [36,37]. In situ

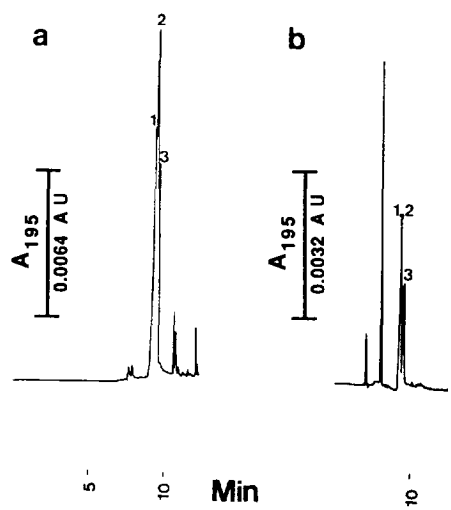


Fig. 12. Electropherograms of standard underivatized gangliosides. (a) Running electrolyte, 50 m*M* borate containing 15 m*M* α-CD, pH 10.0; running voltage 20.0 kV. (b) Running electrolyte, 10 m*M* sodium phosphate, pH 7.0 containing 50% (v/v) acetonitrile; running voltage, 25.0 kV. Samples: 1 = G_{D1a}; 2 = G_{D1b}; 3 = G_{D3}. Other conditions as in Fig. 2.

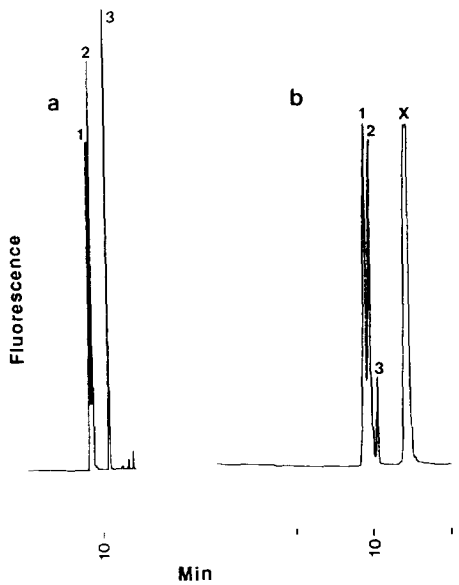


Fig. 13. Electropherograms of standard ANDSA derivatives of disialogangliosides. Running electrolytes, 10 m*M* sodium phosphate, pH 12.0, containing 15 m*M* α-CD and 0.5% HPC in (a) or 0.5% PVA in (b); running voltage, 18.0 kV. Peaks: 1 = G_{D1a}; 2 = G_{D1b}; 3 = G_{D3}; × = by-product. Other conditions as in Fig. 2.

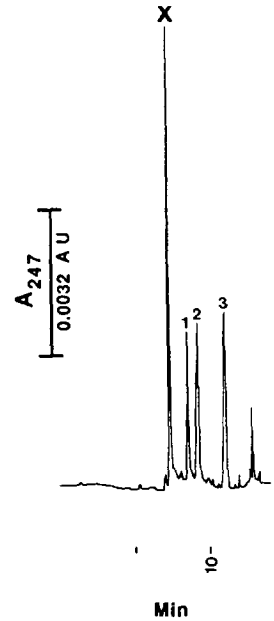


Fig. 14. Electropherogram of standard ANDSA-disialogangliosides. Running electrolyte, 50 m*M* borate, pH 6.0 containing 5.0 m*M* MEGA surfactant and 15.0 m*M* α-CD. Peaks and other conditions as in Fig. 13.

micellar systems are based on the complexation of alkylglycoside surfactants and borate, a phenomenon that provides micelles with adjustable surface charge density. As seen in Fig. 14, a micellar electrolyte system consisting of 50 m*M* borate, 5 m*M* MEGA 10 and 15 m*M* α-CD gave a baseline separation and a high plate count for the G_Ds. The pH of the electrolyte was adjusted to 6 to ensure a different migration time between the analytes and the by-product.

In conclusion, the selective precolumn derivatization of gangliosides involving the carboxylic acid groups of the sialic acid residues has proved suitable for the analysis of small amounts of these sialoglycolipids by HPCE. This attractive feature in conjunction with the unique selectivities offered by the various electrolyte systems developed in the present studies are expected to facilitate the determination of gangliosides in small tumors.

Acknowledgements

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References

[1] L. Svennerholm, *J. Neurochem.*, 10 (1963) 613.
[2] H. Wiegandt, in H. Weigandt (Editor), *Glycolipids*, Elsevier, Amsterdam, 1985, p. 199.
[3] R. Leeden and R. Yu, *Methods Enzymol.*, 83 (1982) 139.
[4] W. Van Heyningen, *J. Gen. Microbiol.*, 20 (1959) 291.

[5] S. Hakamori, *Proc. Natl. Acad. Sci. U.S.A.*, 67(1970) 1791.
[6] S. Spiegel and P. Fishman, *Proc. Natl. Acad. Sci. U.S.A.*, 84 (1987) 141.
[7] D.F. Smith and B.V. Torres, *Methods Enzymol.*, 179 (1989) 30.
[8] S. Ladisch and B. Gillard, *Methods Enzymol.*, 138 (1987) 300.
[9] R. Kannagi, K. Watanabe and S.-I. Hakomori, *Methods Enzymol.*, 138 (1987) 3.
[10] H. Nakalayashi, M. Iwamori and Y. Naga, *J. Biochem.*, 96 (1984) 977.
[11] Y. Liu and K.-F.J. Chan, *Electrophoresis*, 12 (1991) 402.
[12] Y. Yoo, Y. Kim, G.-J. John, and J. Park, *J. Chromatogr. A*, 652 (1993) 431.
[13] E. Bremer, S. Gross and R. McClure, *J. Lipid Res.*, 20 (1979) 1028.
[14] W. Lee, M. Westrick and B. Macher, *Biochim. Biophys. Acta*, 712 (1982) 489.
[15] T. Traylar, D. Koontz and E. Hogan, *J. Chromatogr.*, 272 (1983) 9.
[16] Y. Mechref and Z. El Rassi, *Electrophoresis*, 15 (1994) 627.
[17] W. Nashabeh and Z. El Rassi, *J. Chromatogr.*, 514 (1990) 57.
[18] W. Nashabeh and Z. El Rassi, *J. Chromatogr.*, 600 (1992) 279.
[19] G.K. Ostrander, S.B. Levery, S. Hakomori and E.H. Holmes, *J. Biol. Chem.*, 263 (1988) 3103.
[20] J. Folch, S. Arson and J.A. Meath, *J. Biol. Chem.*, 191 (1951) 819.
[21] R.K. Yu and R.W. Ledeen, *J. Lipid Res.*, 13 (1972) 680.
[22] T. Saito and S. Hakomori, *J. Lipid Res.*, 12 (1971) 257.
[23] K. Watanabe and Y. Arao, *J. Lipid Res.*, 22 (1981) 1020.
[24] F. Kilar and S. Hjertén, *J. Chromatogr.*, 480 (1989) 35.
[25] J. Montreuil, S. Bouquelet, H. Debray, B. Fournet, G. Spik and G. Strecrer, in M. Chaplin and J. Kennedy (Editors), *Carbohydrate Analysis —A Practical Approach*, IRL Press, Oxford, 1986, p. 143.
[26] R. Schauer, *Adv. Carbohydr. Chem. Biochem.*, 40 (1982) 132.
[27] S. Ando, N.-C. Chang and R. Yu, *Anal. Biochem.*, 89 (1978) 437.
[28] H. Khorana, *Chem. Rev.*, 53 (9153) 145.
[29] H. Khorana, *J. Chem. Soc.*, (1952) 2081.
[30] J.A. Dean, *Lange's Handbook of Chemistry*, McGraw Hill, New York, 1985.
[31] D. Voet and J. Voet, *Biochemistry*, Wiley, New York, 1990, p. 1018.
[32] R. Kuhn and H. Wiegandt, *Z. Naturforsch. B*, 18 (1963) 541.
[33] B. Ulrich-Bott and H. Wiegandt, *J. Lip. Res.*, 25 (1984) 1233.
[34] H. Rauvala, *Eur. J. Biochem.*, 97 (1979) 555.
[35] K.-B. Lee, Y.-S. Kim and R. Linhardt, *Electrophoresis*, 12 (1991) 636.
[36] J. Cai and Z. El Rassi, *J. Chromatogr.*, 608 (1992) 31.
[37] J.T. Smith, W. Nashabeh and Z. El Rassi, *Anal. Chem.*, 66 (1994) 1119.