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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF GANGLIOSIDES, COMBINED WITH IMMUNOLOGICAL DETECTION AND FAST ATOM BOMBARDMENT MASS SPECTROMETRIC CHARACTERIZATION

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SUMMARY

A complete strategy for the isolation of individual mono- and disialogangliosides has been elaborated. We have used straight-phase silica gel chromatography or partitioning to obtain a crude ganglioside fraction. This fraction was then peracetylated and run through a second silica gel column. After anion-exchange chromatography the gangliosides were separated by straight-phase high-performance liquid chromatography with chloroform–methanol–water mixtures as eluting solvents. The method is suitable for preparative isolation of gangliosides and subsequent structural characterization by thin-layer chromatography–enzyme-linked immunosorbent assay, fast atom bombardment mass spectrometry and/or gas chromatography–mass spectrometry, which is demonstrated by several examples, including the separation of GalNAc-II^{3}NeuAc-GgOse$_4$Cer from GalNAc-isoII^{3}NeuAc-GgOse$_4$Cer.

INTRODUCTION

Gangliosides are complex acidic glycosphingolipids that are normal constituents of all mammalian cell membranes and most abundant in neuronal cells [1,2]. The complexity of gangliosides is related to variations in their ceramide and/or oligosaccharide moieties. The acidic function of the gangliosides is provided by a varying number of sialic acids, and anion-exchange chromatography is often used as an initial step in ganglioside separation [3–5]. Anion-exchange chromatography has also been utilized to separate all major gangliosides by three-dimensional gradients [6]. Another common approach to the separation

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of individual gangliosides has been straight-phase high-performance liquid chromatography (HPLC) [7–14]. In the present report we combine the use of anion-exchange chromatography with straight-phase HPLC to obtain optimal separation and purity of individual gangliosides.

Most of the elution solvents for the separation of gangliosides by HPLC have been based on 2-propanol–hexane–water mixtures [7–9,13,14], but in this study we have used chloroform–methanol–water. These latter elution mixtures have two major advantages: the gangliosides are readily soluble in them, and they give a good resolution of closely allied gangliosides. The present system utilizes a Hypersil silica gel column and a gradient of increasing water content. It can be used for injections of up to 1 μmol ganglioside sialic acid on an analytical column (200 mm×4 mm I.D.), and is therefore also suitable for preparative purposes, which is demonstrated with several examples.

EXPERIMENTAL[a]

Chemicals

Precoated high-performance thin-layer chromatographic (HPTLC) plates with silica gel 60 were obtained from Merck (Darmstadt, F.R.G.). HPLC columns (200 mm×4.0 mm I.D. and 150 mm×8 mm I.D.) packed with 5-μm Hypersil silica gel were purchased from HPLC-Teknik (Robertsfors, Sweden). The monoclonal antibodies (MAb) used against 3′-LM1 (TR:3), 3′-isoLM1 (C-50) and cholera toxin for detection of GM1, were all produced in our laboratory. Gangliosides used as standards and references were also isolated in our laboratory. Ganglioside GM1 was labelled in the sphingosine double bond with the $Pd(OAc)_2/NaB^3H_4$ procedure [15] and diluted with unlabelled GM1 ganglioside to a specific activity of 190 000 dpm/nmol.

Tissues

Human adult and infant brains were obtained from the Department of Forensic Medicine (Gothenburg, Sweden). Gangliosides were also extracted from the nude mice xenografts of the human medulloblastoma cell line, TE671 [16].

HPLC instrumentation

Two LKB 2150 HPLC pumps were used with an LKB-2152 gradient controller. A 2.0-ml sample loop, on a Rheodyne injector, was used to load the column.

[a] Abbreviations of gangliosides according to IUPAC-IUP [25]. GM3 = II^3NeuAc-LacCer; GM2 = II^3NeuAc-$GgOse_3$Cer; GM1 = II^3NeuAc-$GgOse_4$Cer; 3′-isoLM1 = IV_3NeuAc-nLc-Ose_4Cer; GD3 = $II^3(NeuAc)_2$-LacCer; GD2 = $II^3(NeuAc)_2$-$GgOse_3$Cer; GD1a = IV^3NeuAc,II^3NeuAc-$GgOse_4$Cer; GD1b = $II^3(NeuAc)_2$-$GgOse_4$Cer; 3′,8′-LD1 = $IV^3(NeuAc)_2$-n$LcOse_4$Cer; GT1b = IV^3NeuAc,$II^3(NeuAc)_2$-$GgOse_4$Cer; GQ1b = $IV^3(NeuAc)_2II^3$-$(NeuAc)_2$-$GgOse_4$Cer.

Isolation of a crude ganglioside fraction

The tissue was homogenized in water, and the lipids were extracted in chloroform–methanol–water (4:8:3, v/v) [17]. A crude ganglioside extract was obtained by solvent partition [17], or chromatography on a silica gel column.

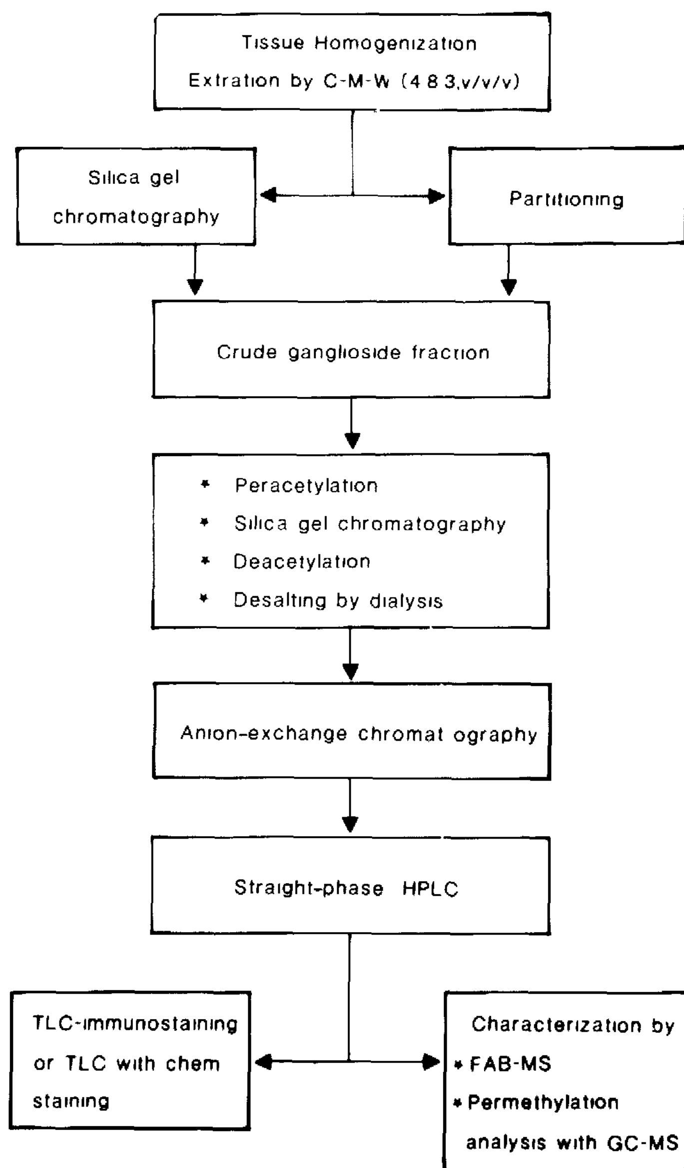


Fig. 1. Flow-chart of the ganglioside isolation and separation procedure. C–M–W = chloroform–methanol–water.

The crude ganglioside extract, corresponding to 10–25 g of tissue (wet weight) was dissolved in chloroform and applied to a 10–25 g silica gel column. Lipids were eluted batchwise according to increasing polarity. Non-polar lipids were eluted with 15 vol. (w/v) of chloroform and most phospholipids, sulphatides and cerebrosides were eluted with 8 vol. (w/v) of chloroform–methanol–water (65:25:4, v/v). A crude ganglioside fraction was eluted with 15 vol. (w/v) of chloroform–methanol–water (5:4:1, v/v) and a last fraction was eluted with 5 vol. (w/v) of chloroform–methanol–water (3:6:2, v/v). The final two fractions were evaporated and dialysed against tap water for three days. They were then evaporated and dissolved in chloroform–methanol–water (60:30:4.5, v/v), and insoluble residues were removed by centrifugation. The two fractions were then combined to form the crude ganglioside fraction (Fig. 1).

Peracetylation of the crude ganglioside fraction

Ganglioside sialic acid (1 μmol) was peracetylated [18] in 0.5 ml of pyridine–acetic anhydride (3:2, v/v) for 16 h at 60°C and dried under a gentle stream of nitrogen. The dry residue was dissolved in chloroform and applied to a column containing 1.0 g of silica gel. After washing with 10 ml of chloroform, the acetylated gangliosides were eluted with 15 ml of chloroform–methanol (85:15, v/v) and evaporated to dryness. They were then dissolved in 0.3 ml of chloroform and deacetylated by the addition of 0.6 ml of 0.2 *M* sodium hydroxide in methanol at 21°C for 16 h. The extracts were then neutralized by acetic acid and desalted by dialysis against a 20 μM potassium carbonate buffer (pH 7.5).

Anion-exchange chromatography

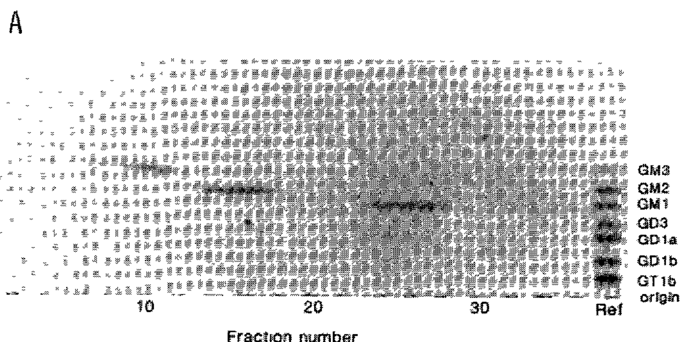
The total ganglioside extract was separated into fractions according to their number of sialic acids by anion-exchange chromatography on Spherosil DEAE-dextran [4] or DEAE-Sepharose (fast flow). When DEAE-Sepharose was used the gangliosides were dissolved in chloroform–methanol–water (60:30:4.5, v/v) to a final concentration of 1.0 μmol ganglioside sialic acid per ml. The column was loaded with 1.0 μmol of ganglioside sialic acid per ml gel, and neutral glycolipids were eluted with 10 vol. of chloroform–methanol–water (60:30:4.5, v/v). Mono-, di-, tri- and polysialogangliosides were eluted with 10 vol. of 0.01, 0.02, 0.03 and 0.05 *M* potassium acetate in methanol, respectively.

HPLC separation of gangliosides

The straight-phase Hypersil silica gel column (200 mm × 4.0 mm I.D.) was regenerated with chloroform–methanol–water (65:25:3 or 60:30:4.5, v/v) for mono- and disialoganglioside separation, respectively, at a flow-rate of 0.25 ml/min for 4 h before every injection. Samples containing 0.05–1.0 μmol of ganglioside sialic acid were dissolved in 1.0 ml of chloroform–methanol–water (65:25:3 or 60:30:4.5, v/v), filtered through a 0.45-μm filter and injected on

the column at a flow-rate of 1.0 ml/min. The monosialogangliosides were eluted with a gradient of chloroform–methanol–water from 65·25:3 to 60:35:8 (v/v) (Fig. 2B) and disialogangliosides were eluted with a separate gradient of chloroform–methanol–water from 60:30:4.5 to 60:35:8 (v/v) (Fig. 3B). Fractions of 1.0 ml were collected and the elution was monitored by assaying 10 μl of each fraction by thin-layer chromatography (TLC) on HPTLC silica gel plates with chloroform–methanol–0.25% potassium chloride in water (5.4:1, v/v) or 1-propanol–0.25% potassium chloride in water (3:1, v/v) as developing solvent. Gangliosides were detected by the resorcinol reagent [19] or by immunostaining with monoclonal antibodies and alkaline phosphate-linked second antibodies [20]. All chromatographic procedures were performed at constant temperature (21°C).

A semipreparative column (150 mm×8 mm I.D.) was used for injections of



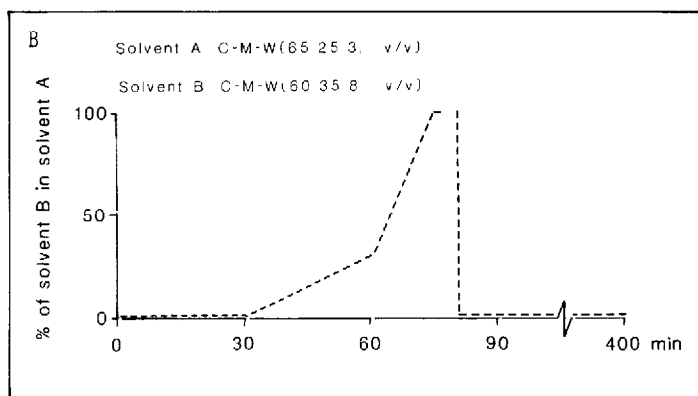


Fig. 2. (A) TLC of known monosialoganglioside standards (GM3, GM2 and GM1) separated by HPLC. The gangliosides were visualized by the resorcinol reagent after separation of 10 μl of each fraction (1–35) by TLC, run in chloroform–methanol–0.25% potassium chloride in water. (B) Gradient profile used for the monosialoganglioside separation shown in (A) C–M–W = chloroform–methanol–water.

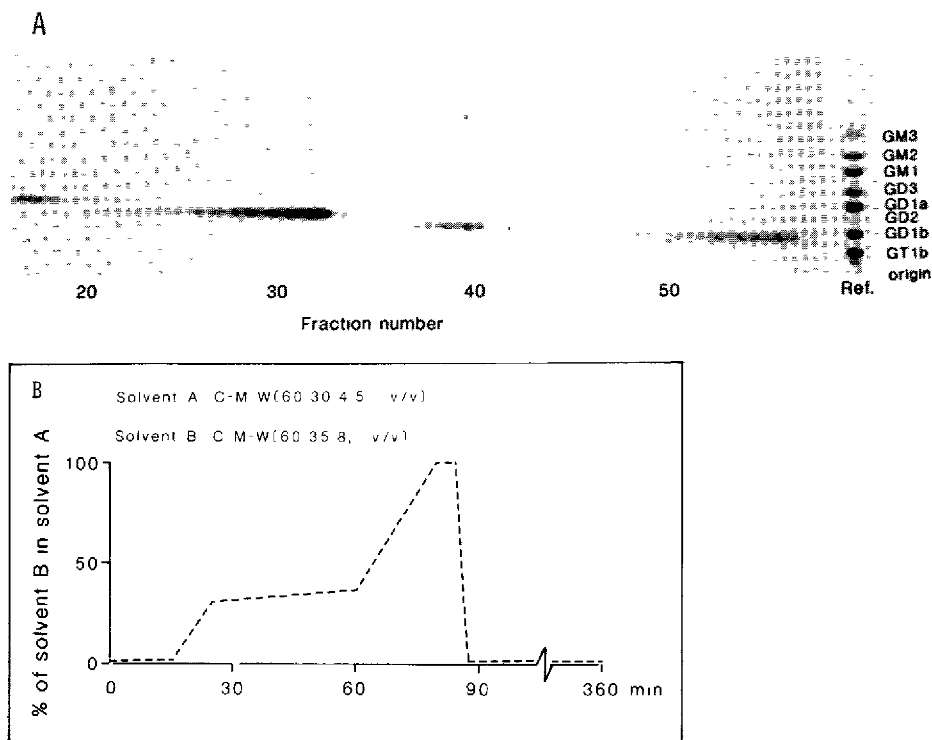


Fig. 3. (A) TLC of known disialoganglioside standards (GD3, GD1a, GD2 and GD1b) separated by HPLC. The gangliosides were visualized by the resorcinol reagent after separation of 10 μl of each fraction (15–58) by TLC, run in chloroform–methanol–0.25% potassium chloride in water. (B) Gradient profile used for the disialoganglioside separation shown in (A). C–M–W = chloroform–methanol–water.

1–5 μmol of ganglioside sialic acid. The elution was performed as described for the analytical column with the following modifications: the flow-rate was increased from 1.0 to 2.0 ml/min; the same gradient profile was used but the total elution time was increased to 180 min.

Preparation of GalNAc-GM1 and GalNAc-isoGM1 from GalNAc-GD1a

Ganglioside GalNAc-GD1a (Fig. 4) was hydrolysed in 0.1 *M* formic acid for 60 min at 80°C in a water-bath. The reaction mixture was neutralized with sodium hydroxide and desalted on Sephadex G-25 [21]. The resulting monosialogangliosides were separated from the starting material by anion-exchange chromatography on DEAE-Sepharose (fast flow).

Analytical methods

The patterns of ganglioside mixtures were determined on HPTLC plates developed with the solvents 1-propanol–0.25% aqueous potassium chloride

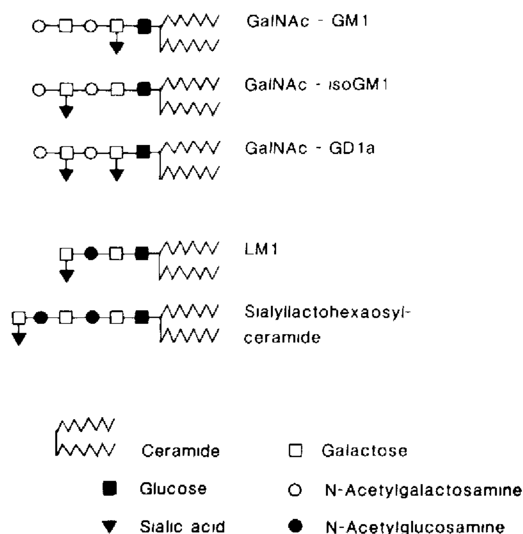


Fig. 4. Structures of some gangliosides included in the present study.

(3:1, v/v) or chloroform–methanol–0.25% aqueous potassium chloride (5:4:1, v/v). The gangliosides were visualized by the resorcinol reagent [19] and quantified by densitometry at 620 nm. Fast atom bombardment mass spectrometry (FAB-MS) was carried out as previously described [22,23].

RESULTS

Sample purification procedure

Ganglioside fractions isolated with partitioning or on open silica gel columns contained protein contaminants that interfered with the subsequent chromatographic steps. Therefore a further purification step was developed, including the acetylation of the gangliosides. The ganglioside recovery of this step was tested on human brain and lung tissues. The recovery of total lipid-bound sialic acid was 95.4% (S.D. = 2.1%, $n = 8$). Densitometric scanning before acetylation and after deacetylation revealed that the recovery was quantitative for gangliosides GM1, GD3, GD1a, GD1b, and GT1b, but for GQ1b it was 80.0% (S.D. = 3.6%).

Optimization of the separation procedure

Several different mixtures of chloroform–methanol–water or 2-propanol–hexane–water were tested for the separation of gangliosides on a straight-phase HPLC system. Chloroform–methanol–water was chosen as the best solvent system since it more readily dissolved the gangliosides and thus required smaller elution volumes than the 2-propanol–hexane–water solvent system.

Optimal elution of gangliosides was found using different gradients for mono- and disialogangliosides (Figs. 2B and 3B). The elution of known standards of mono- and disialogangliosides are shown in Figs. 2A and 3A, respectively. HPLC separation of tri- and polysialogangliosides was not required, since they were well separated by the anion-exchange chromatography. The elution characteristics of several Hypersil silica gel columns were tested with a ganglioside mixture from human brain with added GM3, GM2 or GD3. The difference between individual columns was so small that no alteration to the mobile phase was required. More than 100 sample injections have been made on the same column without any loss of efficiency.

Recovery

The recovery of gangliosides injected into the HPLC system was assessed by measurement of the sialic acid content, and of tritium-labelled GM1 added to a monosialoganglioside fraction, before and after separation by HPLC. The recoveries for 50–1000 nmol of gangliosides, containing both mono- and oligosialogangliosides, were 95–100%; the recovery for monosialogangliosides was 96–105%. The mean recovery was 98% (S.D. = 4.3%, n = 14, Table I). No significant change in the ganglioside pattern, determined by densitometry on five different samples, was observed.

TABLE I

RECOVERY OF GANGLIOSIDES AFTER HPLC SEPARATION

Gangliosides (50–1000 nmol), isolated as described in Experimental, were dissolved in 1 ml of chloroform–methanol–water (65:25 3, v/v) and injected into the HPLC system. The recoveries of the gangliosides were determined by assay of the sialic acid content, except for [^{3}H]Cer-GM1, which was assayed by liquid scintillation counting. The tritiated substance was diluted with equal amounts of cold GM3, GM2 and GM1 to render 300 nmol per injection with a specific activity of 1000 cpm/nmol sialic acid.

Gangliosides	n		Recovery (%)
[^{3}H]Cer-GM1	4		99–101
Monosialogangliosides (human infant brain)	4		96–105
Human brain gangliosides (total fraction of human adult brain)	4		95–100
GT1b	2		93
Total	14	Mean ± S.D	98 ± 4.3

Separation of the monosialogangliosides from TE671

Gangliosides were isolated from the human medulloblastoma permanent cell line TE671, and the monosialoganglioside fraction was separated by the HPLC procedure. The major components of this fraction were gangliosides GM3 and GM2 (ca. 85%). Therefore, the initial isocratic step of the gradient (shown in Fig. 2B) was prolonged by 10 min. Each fraction was assayed by TLC with subsequent immunostaining (Fig. 5). The C-50 MAb revealed two different antigenic regions, containing 3′-isoLM1 (fractions 33–41) and sialyllacto-hexaosylceramide (fractions 42–46), respectively (ganglioside structures are shown in Fig. 4). It was also revealed by FAB-MS that the gangliosides with N-acetylneuraminic acid eluted earlier (fractions 33–36) than the corresponding gangliosides with N-glycolylneuraminic acid (fractions 34–41).

Separation of GalNAc-GM1 from GalNAc-isoGM1

Two monosialogangliosides GalNAc-GM1 and GalNAc-isoGM1 (Fig. 4) were obtained by formic acid hydrolysis of GalNAc-GD1a. Assay of the mono-sialoganglioside fraction by TLC in chloroform–methanol–0.25% aqueous potassium chloride (5:4:1, v/v) revealed two components with R_{GM1} values of 0.81 and 0.73, respectively. A complete separation of the components was obtained by HPLC (Fig. 6). FAB-MS showed that the faster moving component was GalNAc-isoGM1 (fractions 35–49) and that the other was GalNAc-GM1 (fractions 51–61).

Separation of disialogangliosides from human erythrocytes

Gangliosides were isolated from human erythrocytes and separated by anion-exchange chromatography. The disialoganglioside fraction consisted of

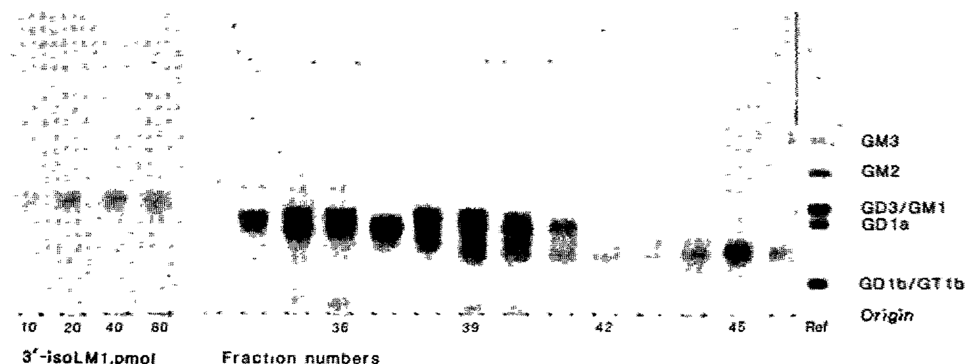


Fig. 5. HPLC separation of monosialogangliosides from a human medulloblastoma permanent cell line TE671 grown as xenograft in nude mice. Gangliosides were visualized by immunostaining with the C-50 MAb after separation by TLC with chloroform–methanol–2.5 *M* ammonia (5:4:1, v/v) (the extraction and immunostaining procedures are described in Experimental). Fractions 34–41 contained ganglioside LM1 and fractions 42–46 contained ganglioside sialyllactohexaosylceramide.

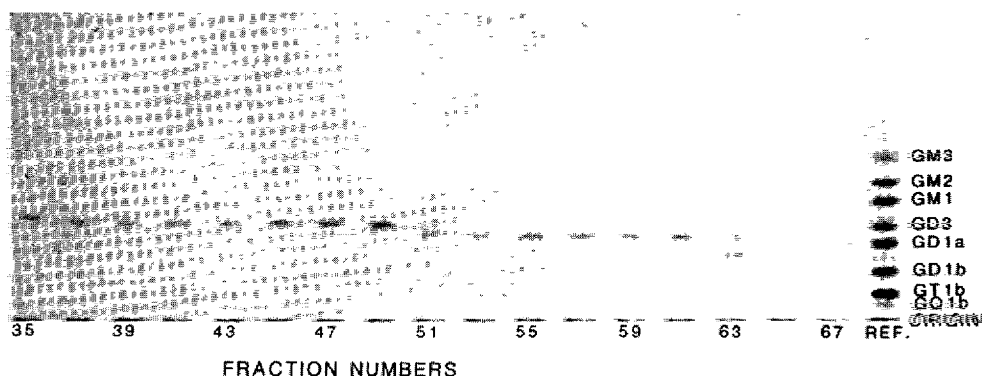


Fig. 6. Monosialogangliosides GalNAc-isoGM1 and GalNAc-GM1, obtained after acidic hydrolysis of GalNAc-GD1a and isolated and separated by HPLC. Every second fraction between fractions 35 and 67 was assayed by TLC with chloroform–methanol–0.25% potassium chloride (5:4.1, v/v) as developing solvent and visualized by the resorcinol reagent. Fractions 35–49 and 51–61 were pooled for structural confirmation by FAB-MS. Fractions 35–49 contained ganglioside GalNAc-isoGM1 and fractions 51–61 contained ganglioside GalNAc-GM1.

three major gangliosides, namely GD3, GD1a and LD1. The LD1 ganglioside had a retention time of 35–36 min, and was thus well separated from the GD3 and GD1a peaks (retention times of 16–18 and 22–32 min, respectively) also present in the sample.

DISCUSSION

TLC is an excellent tool for analytical separation of gangliosides. For preparative purposes this separation procedure has two drawbacks. First, only a certain number of bands can be scraped from the plates. Second, individual gangliosides are not quantitatively recovered, and they are heavily contaminated with gel material. Owing to the development of highly inert column packing materials, providing reproducible separations, HPLC has become the method of choice for efficient preparative separation of gangliosides.

Gazzotti et al. [6] presented an HPLC method with a LiChrosorb-NH_2 column and achieved resolution of 1–50 nmol portions of bovine brain gangliosides within 80 min, which is satisfactory for analytical purposes, but for semi-preparative amounts (1–5 μmol) their system required a very high flow-rate: 39 ml/min. This is beyond the capacity of standard HPLC solvent-delivery systems and leads to high consumption of solvent. By comparison, up to 5 μmol of ganglioside sialic acid could be separated within 180 min on the semi-preparative column in our procedure, at a flow-rate of 2.0 ml/min and a solvent consumption of 420 ml.

Anion-exchange chromatography, for the separation of gangliosides into monovalent fractions [3–5], enhances the capacity of subsequent straight-

phase HPLC. In a previous paper [4] we described the separation of gangliosides by Spherosil-DEAE-dextran. As this resin is not commercially available we have in this paper elaborated a solvent system for an equivalent separation on DEAE-Sepharose.

2-Propanol–hexane–water is used as eluting solvent in most reports concerning ganglioside separation by straight-phase HPLC [7–10,12–14]. We, however, prefer to use chloroform–methanol–water, as it provides better resolution and more readily dissolves the gangliosides. The separation and identification of the gangliosides 3′-isoLM1 and VI^3-NeuGc(NeuAc)-$nLcOse_6$-Cer is an example that shows the capacity of this method (Fig. 5). A better example of the resolution of the present system is the separation of GalNAc-GM1 from GalNAc-isoGM1. On TLC their migration was very close, but HPLC led to a complete separation of the two isomers (Fig. 6).

Glycosphingolipids lack significant UV absorption, and therefore many investigators [10,12] use precolumn derivatization to introduce a UV-absorbing aromatic chromophore. Such derivatizations are partly irreversible and thus excluded from preparative methods. We have used TLC with subsequent staining of the sialic acid or enzyme-linked immunosorbent assay (ELISA) techniques, to monitor the elution of gangliosides. This method requires only negligible portion of the sample, but will give information about the homogeneity and migration of the gangliosides eluted from the column.

Using the combination of TLC and ELISA with a large library of monoclonal antibodies possessing high epitope specificity, we have been able to detect novel gangliosides in normal tissues and tumours [16,20]. Migration of the ganglioside fraction on the TLC plate will also give important information that will allow modification of the solvent gradient for a second separation of the actual ganglioside fraction. In this way, gangliosides that constitute less than 0.1% of total gangliosides have been isolated in pure form and characterized by FAB-MS and GC of permethylated sugars [16]. It is not necessary to purify gangliosides to carbohydrate homogeneity when their structure or metabolism is studied, but it is essential to have pure gangliosides or to know exactly the structure of the contaminants when the biological function of certain gangliosides is studied [24]. This new HPLC procedure is an important tool in the achievement of this goal.

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