Analytical and Preparative High-Performance Liquid Chromatography of Gangliosides

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Analytical and preparative procedures are described for high-performance liquid chromatography (HPLC) fractionation of gangliosides without previous derivatization. These procedures make use of a reversed-phase Lichrosorb RB-8 or µBondapak RP-18 column, and of a mixture of acetonitrile and 5 mM phosphate buffer, at fixed or varying volume ratios, as solvent system. Peak elution from the column is monitored by flow through reading of absorbance at 195 nm. Under all the described conditions HPLC is capable of resolving all common gangliosides and of separating each of them into four molecular species containing C18-sphingosine, C18-sphinganine, C20-sphingosine, or C20-sphinganine.

The analytical method has been successfully applied to fractionation of ganglioside mixtures from calf brain and to verification of homogeneity of single-ganglioside preparations. It is suitable for quantitative purposes, with high sensitivity (detection limit, 0.1 n mole) and precision (SD less than 10% of mean values in the concentration range 0.1–50 n moles). The semipreparative method, which provides successive cycles of analysis in a fully automated way, enables the preparation in 2–4 days of 100-mg amounts of each molecular species starting from single gangliosides, like GM1 and GD1a. The preparative method makes use of acetonitrile-phosphate buffer–tetrahydrofuran as eluting solvent, and requires the addition to the starting ganglioside of the corresponding radioactive compound as tracer. This procedure, applied to GM1 ganglioside, is devised for processing up to 50 mg of ganglioside per analysis.

Key words: ganglioside HPLC, ganglioside molecular species, ganglioside separation, ganglioside preparation

Abbreviations used: This paper follows the ganglioside nomenclature of Svennerholm [1964] and the IUPAC-IUB Recommendations [1977], J. Biol. Chem., (1982), 257: 3347–3351): GM2, II(NeuAc-GgOse)2Cer; GM1, II(NeuAc-GgOse)Cer; Fuc-GM1, IV(NeuAc, IV3(NeuAc)-GgOse)Cer; GD1a, IV(NeuAc, IV3(NeuAc)-GgOse)Cer; GT1b, IV3(NeuAc, IV3(NeuAc)-GgOse)Cer; GQ1b, IV3(NeuAc, IV3(NeuAc, IV3(NeuAc)-GgOse)Cer; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; SD, standard deviation.

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INTRODUCTION

Gangliosides are normal components of mammalian cell plasma membranes [Ledeen and Yu, 1978], where they are assumed to play a role in receptor phenomena and biotransduction of membrane-mediated information [Svennerholm et al., 1980; Fishman, 1982]. Gangliosides are constituted of a hydrophilic portion, the sialic acid-containing oligosaccharide, which protrudes from the outer membrane surface, and a hydrophobic portion, the ceramide, which is inserted into the lipid core of the membrane. The oligosaccharide portion offers a variety of different chemical structures, which contribute to and determine the binding potential and specificity of the membrane. The ceramide moieties of the most common gangliosides have rather homogeneous fatty acid compositions, with prevalence of stearic acid, but differ in long-chain base composition. The most abundant long-chain bases are the C18 and C20 compounds, with or without a double bond at the C4–C5 position. The chemical features of the hydrophobic portion have been recognized as important factors in defining the contribution given by gangliosides to the functional architecture of the membrane [Yohe et al., 1976; Maggio et al., 1981].

Gangliosides are extracted from tissues as mixtures of many species that differ in their oligosaccharide or ceramide portions. Several procedures have been devised for fractionating ganglioside mixtures into individual entities [Ledeen and Yu, 1982; Kundu, 1981]. These procedures mainly consist of thin-layer chromatography (TLC) and column chromatography on silica gel or sialic acid, in possible combination with ion exchangers. They generally separate gangliosides on the basis of their different oligosaccharide portions, but do not resolve the species having identical oligosaccharide but different ceramide portions. In the last decade high performance liquid chromatography (HPLC) has been introduced in glycolipid methodology and successfully applied to analysis of cerebrosides and higher neutral glycosphingolipids [Jungalwala et al., 1977; Ullman and McCluer, 1977; Suzuki et al., 1980]. One of the advantages of this approach is its potential ability to resolve glycosphingolipid molecular species differing only in their ceramide portions [Suzuki et al., 1976, 1977]. HPLC was first applied to ganglioside analysis by Bremer et al. [1979], who succeeded in separating and quantifying the different monosialogangliosides contained in human liver and plasma. It should be noted that all the mentioned HPLC procedures require transformation of the glycosphingolipids into their perbenzoyl or O-acetyl-N-p-nitrobenzoyl derivatives prior to analysis. Therefore they are suitable for analytical but not for preparative purposes.

In the last few years efforts have been made in our laboratory to obtain HPLC fractionation of gangliosides without preliminary derivatization. The aim of our studies was to set up analytical and preparative HPLC procedures capable of separating natural gangliosides in the molecular forms that are homogeneous in both their oligosaccharide and ceramide portions. A recent investigation [Sonnino et al., 1984] reported a reversed-phase HPLC method for preparing the molecular species of GM1 and GD1a gangliosides with homogeneous long-chain base composition. The present article describes general procedures for HPLC analysis of gangliosides, and for HPLC preparation of fully homogeneous molecular species of the different gangliosides.
MATERIALS AND METHODS

Chemicals

Commercial chemicals were of analytical grade or of the highest grade available. Common solvents were redistilled before use, and water for routine use was redistilled in a glass apparatus. HPLC-grade reagents were used for HPLC. Silica gel 100 for column chromatography (0.063–0.2 mm, 70–230 mesh, ASTM) and silica gel precoated thin-layer plates (HPTLC, Kieselgel 60, 250μm thick, 10 × 10 cm) were purchased from Merck GmbH; N-acetylenuraminic acid (NeuAc) from Sigma Chemicals Co; Sephadex G-25 fine from Pharmacia; Dowex 2-X8, prepared in acetate form [Svennerholm, 1957], from Bio-Rad Labs; dialysis tubing from A. Thomas and (3H)sodium borohydride (6,500 Ci/mole) from the Radiochemical Center, Amersham.

Preparation of the Total Ganglioside Mixture and of Individual Gangliosides

The total ganglioside mixture was prepared from calf brain according to Tettamanti et al [1973]. The individual gangliosides GM2, GM1, Fuc-GM1, GD1a, GD1b, Fuc-GD1b, GT1b, and GQ1b were purified from the ganglioside mixture and structurally characterized as described by Ghidoni et al [1980]. During silica gel 100 column chromatographic purification of all gangliosides, the criterion was followed of collecting the central fractions of the corresponding eluted peaks, which carried the ganglioside species having the highest percentage of stearic acid. The final purity of all ganglioside preparations, referred, as conventionally used, to the oligosaccharide portion, was over 99%. In all of them stearic acid covered more than 92% of the fatty acid content. These ganglioside preparations could be dissolved in water at concentrations up to 250 mg/ml, yielding clear and colorless solutions. The molecular species of GM1 ganglioside homogeneous in the long-chain base moiety (GM1-C20 erythrosphingosine; GM1-C20 erythrosphinganine; GM1-C18 erythro-sphingosine; GM1-C18 erythrosphinganine) were prepared and structurally characterized according to Sonnino et al [1984]. Gangliosides GM1 and GD1a were hydrogenated in order to fully transform unsaturated long-chain bases into saturated ones according to Sonnino et al [1984]. Ganglioside GM1 was (3H)-labeled at the level of terminal galactose (specific radioactivity, 1.52 Ci mmole) by the method of Suzuki and Suzuki [1972] as perfected by Ghidoni et al [1974].

HPLC Separation of Ganglioside Molecular Species

Analytical method. A 0.1- to 50-nmole portion of pure gangliosides GM2, GM1, Fuc-GM1, GD1a, GD1b, Fuc-GD1b, GT1b, and GQ1b, or 20–100 nmoles of calf brain total ganglioside mixture as bound NeuAc, was dissolved in 25–40 μl of redistilled water in a microtube and introduced into a syringe-loading sample injector (Model 7125 Rheodyn Inc.) equipped with a 100-μl loop. The microtube was washed with 25–40 μl of redistilled water, and the washing was added to the previous sample in order to minimize loss of material. Gangliosides were then chromatographed on a reversed-phase Lichrosorb RP-8 (5 μm, 150 × 4.6 mm, Merck, Darmstadt) with a Gilson HPLC apparatus, Mod. 303. Chromatography of the total ganglioside mixture was carried out at 18–20°C with the solvent system acetonitrile–5 mM sodium phosphate buffer, pH 7.0, in a volume ratio that was maintained at the constant value of 1:1 for the first 8 min, then continuously enhanced to 3:2 in the following 6 min,
following the gradient program illustrated in Figure 1, and finally kept at 3:2 till the end of the run. The flow rate was 0.5 ml/min and the elution profile was monitored by flow-through detection of U.V. absorbance at 195 nm (Gilson UV detector, Mod. Holochrome). Peak identification was accomplished by the use of pure standard gangliosides chromatographed under the same experimental conditions. A complete analysis took about 45 min. Separation of the molecular species of the individual pure gangliosides for analytical purposes was accomplished under the above experimental conditions with the only difference that the volume ratio between acetonitrile and 5 mM sodium phosphate buffer was constant throughout the chromatographic run. The volume ratio was 3:2 for GM2, GM1, and Fuc-FM1; 11:9 for GD1a, GD1b, and Fuc-GD1b; 1:1 for GT1b and GQ1b. The time required for complete analysis ranged from a minimum of 5.9 min for GQ1 to a maximum of 16.2 min for GM2. The wavelength at which gangliosides provided maximum UV absorbance (195 nm) was preliminarily determined with ganglioside solutions of concentrations ranging from $10^{-6}$ M to $10^{-4}$ M, and a UVIDEC-505 double-beam spectrophotometer (Jasco Int. Co.)

![Fig. 1. Application of the HPLC analytical method to the separation of the gangliosides present in a calf brain extract; the resolution of the gangliosides constituting a mixture of standards; the separation of the molecular forms of individual standard gangliosides. In all chromatographies, the proportions between acetonitrile and 5 mM phosphate buffer changed during the run, following the trend indicated in the upper graph of the figure. Column: Lichrosorb RP-8; flow rate: 0.5 ml/min; peak detection by flow-through reading of absorbance at 195 nm; amount of injected ganglioside (as sialic acid): 5–15 nmoles. For details see Materials and Methods.](image-url)
Automatic semipreparative method. An automatic semipreparative method was devised and applied to analysis of gangliosides GM1 and GD1a. The following experimental conditions were employed: Gilson, Mod 303, HPLC apparatus provided with two pumps, A and B; μBondapak RP-18 column (10-μm diameter, 250 mm × 10 mm, Water Associates Inc., Milford, ME; temperature, 18–20°C; solvent system, acetonitrile–5 mM sodium phosphate buffer, pH 7.0 in the volume ratio of 7:3 for GM1 and 3:2 for GD1a; flow rate, 3.0 ml/min for both gangliosides; elution profile monitored by continuous recording (Gilson UV detector, Mod Holochrome) at 195 nm. The HPLC apparatus was connected with a Gilson computer-assisted automatic fraction collector Mod 201, programmed for collecting separately eluted fractions on the basis of UV signals, and for automatic repetitive cycles of analyses. Each cycle of analysis was operated as follows. Pump A drew 1 ml of aqueous ganglioside solution (2.5 mg/ml) and injected the sample into the column in the given time of 30 sec. At the end, chromatography was initiated by the action of pump B. Eluted peaks were automatically collected on the basis of U.V. absorbance signals and established times of elution (see Fig. 5). After elution of the last peak, the column was washed with the eluting solvent for 15 min, pump B was stopped, and pump A initiated a second cycle of analysis. A complete cycle of fractionation took about 79 min for GM1 and 42 min for GD1a. When hydrogenated GM1 and GD1a were used, the washing time was shortened to 10 min and 8 min, respectively.

Preparative method. This method was set up and applied to ganglioside GM1. A 50-mg quantity of GM1 ganglioside, mixed with 0.5 μCi of tritiated compound, was dissolved in 250 μl of redistilled water in a microtube and introduced into a syringe-loading sample injector (Mod 7125, Rheodyn Inc.) equipped with a 300-μl loop. The microtube was washed with 50 μl of redistilled water and the washing was added to the previous sample. Ganglioside was then chromatographed on a reversed-phase μBondapak column (10 μm, 250 mm × 10 mm, Water Associates Inc., with a Gilson HPLC apparatus, Mod 303. The following conditions were employed: temperature, 18–20°C; solvent system, acetonitrile–5 mM sodium phosphate buffer (pH 7.0 )/tetrahydrofuran in the volume ratio of 9:7:4; flow rate, 7.5 ml/min. The elution profile was monitored with a computer-assisted HPLC radioactivity monitor (Bert-hold, Model LB 503) equipped with a 120 μl solid scintillator cell. Hydrogenated GM1, mixed with an hydrogenated sample of radiolabelled compound, was submitted to HPLC fractionation under the same experimental conditions.

Compositional Analysis of Gangliosides

The carbohydrate, fatty acid, and long-chain base composition of the molecular species of each ganglioside, obtained by HPLC, were determined by gas-liquid chromatography (GLC) and GLC-MS analysis as previously described [Ghidoni et al, 1980; Sonnino et al, 1984].

Thin-Layer Chromatography

TLC of gangliosides was carried out on HPTLC silica gel precoated plates under the following conditions: temperature, 18–20°C; solvent system, chloroform–methanol–0.3% aqueous CaCl₂, 50:42:11 (by vol); run time, 1 hr; detection of the spots by treatment with an Erhlich spray reagent and heating at 120°C [Chigorno et al, 1982].
Colorimetric Methods

Ganglioside-bound sialic acid was determined by the method of Warren [1959] after acid hydrolysis of the sample in 0.05 M H$_2$SO$_4$ (1 h at 80°C) and purification of liberated sialic acid by ion exchange chromatography on a Dowex 2-X8 (acetate form) column [Tettamanti et al., 1973]. Pure N-acetylneuraminic acid was used as the standard.

RESULTS

HPLC Analytical Separation of Gangliosides

The total ganglioside mixture from calf brain was described as containing [Chigorno et al, 1982] four major gangliosides—GD1a, GT1b, GM1, and GD1b (which cover 38%, 16%, 14% and 9.5% of total bound NeuAc, respectively)—and a number of minor species. Of these some were identified as GQ1b, Fuc-GM1, Fuc-GD1b, and GM2, none of which exceeding 2% of total NeuAc.

The calf brain total ganglioside mixture was submitted to HPLC fractionation according to the gradient-based analytical method described in the experimental section. Pure standard GM2, GM1, Fuc-GM1, GD1a, GD1b, Fuc-GD1b, GT1b, and GQ1b were processed in parallel, either individually or mixed together (Fig. 1). Each standard ganglioside was separated into four fractions, two of which were the most abundant and two present in small amounts. Compositional analyses performed on the four fractions from all individual gangliosides showed, in agreement with previous data [Sonnino et al, 1984], that the two major fractions corresponded to the ganglioside molecular species containing C18 and C20 sphinganines, and the two minor species to those containing C18 and C20 sphingosines. The molecular species containing C18 long-chain bases were eluted before those containing C20 long-chain bases. In all gangliosides the molecular species carrying unsaturated long-chain bases constituted 90–95% of the total. The elution time and the percentage of phosphate buffer in the eluting system appeared to be inversely proportional to the sialic acid content and the overall polarity of the ganglioside molecule.

The HPLC profile of the standard gangliosides mixture displayed 16 well-separated peaks, which are just the sum of the two main peaks (C18 and C20 sphingosine-containing molecular species) of each of the constituting gangliosides. The small peaks corresponding to the molecular species containing saturated long-chain bases were almost completely undistinguishable, due to their overlapping with some of the much greater peaks of the species containing unsaturated long-chain bases. The profile of the total ganglioside mixture from calf brain clearly showed the peaks of the C18 and C20 sphingosine-bearing molecular species of major gangliosides GD1a, GT1b, GM1, and GD1b. In addition the peaks corresponding to GQ1b, Fuc-GM1 and Fuc-GD1b could be recognized. The main peaks of GM2 overlapped with the peaks of the minor species of GM1, and could not be identified.

As already reported in the experimental section when individual gangliosides were to be submitted to HPLC fractionation for analytical purposes, elution was accomplished with acetonitrile and phosphate buffer at fixed volume ratios. An example of HPLC analytical separation by this procedure, referring to ganglioside GM1, is given in Figure 2. The elution pattern was identical to that illustrated in Figure 1, with the four peaks, named A, B, C, D, which correspond to the molecular species containing C18 sphingosine, C18 sphinganine, C20 sphingosine, and C20 sphinganine.
TABLE I. Retention Times (min) of the Four Molecular Species of Different Gangliosides
Separated by the HPLC Analytical Method

<table>
<thead>
<tr>
<th>Ganglioside</th>
<th>C18-sphingosine</th>
<th>C18-sphinganine</th>
<th>C20-sphingosine</th>
<th>C20-sphinganine</th>
</tr>
</thead>
<tbody>
<tr>
<td>GQ1b</td>
<td>3.5</td>
<td>4.1</td>
<td>5.0</td>
<td>5.9</td>
</tr>
<tr>
<td>GT1b</td>
<td>5.9</td>
<td>6.5</td>
<td>9.0</td>
<td>9.9</td>
</tr>
<tr>
<td>Fuc-GD1b</td>
<td>3.9</td>
<td>4.3</td>
<td>6.2</td>
<td>7.0</td>
</tr>
<tr>
<td>GD1b</td>
<td>4.6</td>
<td>4.9</td>
<td>7.3</td>
<td>8.0</td>
</tr>
<tr>
<td>GD1a</td>
<td>5.6</td>
<td>6.2</td>
<td>8.5</td>
<td>9.6</td>
</tr>
<tr>
<td>Fuc-GM1</td>
<td>6.9</td>
<td>7.6</td>
<td>10.4</td>
<td>11.6</td>
</tr>
<tr>
<td>GM1</td>
<td>7.9</td>
<td>8.8</td>
<td>12.3</td>
<td>13.9</td>
</tr>
<tr>
<td>GM2</td>
<td>9.2</td>
<td>10.3</td>
<td>14.1</td>
<td>16.2</td>
</tr>
</tbody>
</table>

For details see Materials and Methods. On repeated analyses the retention times remained fairly constant: SD values were always less than 2% of the mean values.

sphinganine, respectively. The retention times of the molecular species obtained from the different standard gangliosides are reported in Table I. In general under these experimental conditions the baseline appeared more flat and linear than when using elution on a gradient, and higher reproducibility could be attained.

Gangliosides, dissolved in acetonitrile–phosphate buffer mixtures, provided an absorbance maximum at 195 nm. The relationship between UV responses at 195 nm of the different molecular species of gangliosides (as areas of the corresponding peaks) and amounts of injected ganglioside was studied by using standard gangliosides. A linear relationship was observed to occur between peak areas of the individual molecular species and amounts of injected ganglioside in the range from 0.1–0.2 nmoles to 40–50 nmoles, depending on the ganglioside employed and the molecular species involved. Examples of these linear relationships are given in Figure 3a and b. Figure 3a refers to the C18 sphingosine, which contains molecular species of gangliosides GT1b, GD1a, GM1, and Fuc-GM1; it shows that the response at 195 nm
Fig. 3. Application of the HPLC analytical method to ganglioside quantification. Relationship between peak area (mm$^2$) and amount of injected ganglioside (nmoles). Influence of the oligosaccharide composition, especially sialic acid and fucose content, studied with the individual molecular species of ganglioside GM1 containing C18-sphingosine, C18-sphinganine, C20-sphingosine, and C20-sphinganine. Same experimental conditions as in Figure 2. Solvent system: acetonitrile-5mM phosphate buffer, pH 7.0, at the volume ratio of 3:2. Peak areas were calculated by a computer-assisted integrator.

increased with the increasing sialic acid content in the ganglioside molecule. The same relationship was observed with the C20 sphingosine (which contains molecular species of the same gangliosides. Figure 3b, which refers to standard GM1-C18 erythroosphingosine, GM1-C18 erythroosphinganine, GM1-C20 erythroosphingosine, and GM1-C20 erythroosphinganine, shows that the response at 195 nm was higher with the species carrying C20 than C18 long-chain bases, and with the species carrying sphingosines than sphinganines. On repetitive analyses SD values of responses were lower than 5% of mean values for ganglioside amounts between 0.1 and 50 nmoles, but became much higher for ganglioside amounts lower than 0.1 n mole. This means that the analytical HPLC method can be also used for quantitative purposes. Figure 2 shows the results of application of the analytical HPLC method to quantification of the individual molecular species of GM1 ganglioside: The percentage value (± SD) of GM1-C18 erythroosphingosine, GM1-C18 erythroosphinganine, GM1-C20 erythroosphingosine, and GM1-C20 erythroosphinganine were 51.4±3.9%, 2.1±0.32%, 44.1±3.7%, and 2.4±0.33%, respectively.

The combination of excellent resolution and high sensitivity makes the analytical method applicable to verification of the purity of individual ganglioside preparations. An example of this application is given in Figure 4, which shows the results of HPLC analysis of a GM1 preparation to which 1% of GD1a was added prior to analysis. The small peaks of the GD1a molecular species were clearly visible, in contrast with the huge peaks of the GM1 molecular species.

Semipreparative Automatic HPLC Separation of Gangliosides

Figure 5 illustrates a semipreparative automatic fractionation of GM1 and GD1a ganglioside on HPLC. Fractions B and D, corresponding to the minor species containing C18 and C20 sphinganines, had a partial overlapping with fractions A and C, respectively. Therefore the system was programmed to avoid collection of the last eluted portion of fractions A and C, and fractions B and D were discarded. On the other hand fraction B and D could be obtained by using the same semipreparative
procedure and preparations of GM1 and GD1a that were fully hydrogenated before HPLC fractionation.

We submitted 200 mg of natural GM1 and GD1a, and 200 mg of hydrogenated GM1 and GD1a to 70 successive cycles of HPLC fractionation by the semipreparative automatic method. The entire analysis lasted 4 days for GM1 and 2 days for GD1a. After analysis the following amounts of individual molecular species were obtained: GM1-C18 erythrosphingosine, 102 mg; GM1-C20 erythrosphingosine, 70 mg; GD1a-C18 erythrosphingosine, 77 mg; GD1a-C20 erythrosphingosine, 94 mg; GM1-C18 erythrosphinganine, 108 mg; GM1-C20 erythrosphinganine, 72 mg; GD1a-C18 erythrosphinganine, 81 mg; GD1a-C20 erythrosphinganine, 99 mg. The recovery ranged from 85% to 90%. The individual molecular species thus obtained, upon chemical analysis and analytical HPLC, turned out to be over 99% pure.

**HPLC Preparative Separation of GM1 Molecular Species With the Use of Radioactive Tracer**

A higher solubility of gangliosides in the HPLC set could be obtained by introducing tetrahydrofuran in the eluting solvent system. Of course under these conditions UV reading could not be used to monitor ganglioside elution, owing to the strong absorbance of tetrahydrofuran. Hence the necessity of a radioactive tracer for monitoring elution.

A 50-mg amount of natural GM1 ganglioside with 100 μCi of tritiated compound were submitted to HPLC preparative fractionation. Then 50 mg of fully hydrogenated GM1, with 100 μCi of the corresponding labeled compound, was also processed under the same conditions. As shown in Figure 6, natural GM1 provided two broad peaks (a and b), which corresponded to the molecular species containing C18 sphin-
Fig. 5. Application of the HPLC automatic semipreparative method to the separation of the molecular species of ganglioside GD1a (upper graph) and GM1 (lower graph). Column: μBondapak RP-18; solvent system: acetonitrile−5 mM phosphate buffer, pH 7.0, in the volume ratio of 7:3 and 3:2 for GM1 and GD1a, respectively; flow rate: 30 ml/min. Injection time, waiting time before collection, collecting time, and rinse time between successive runs are indicated. The small peaks preceding the elution of peaks A correspond to contaminants present in the ganglioside preparations.

Fig. 6. Application of the HPLC preparative method with radioactive tracer to the preparation of the molecular species of GM1 containing C18-sphingosine, C18-sphinganine, C20-sphingosine, and C20-sphinganine. Column: μBondapak RP-18; solvent system: acetonitrile−5 mM phosphate buffer−tetrahydrofuran in the volume ratio of 9:7:4; flow rate: 7.5 ml/min; peak detection by flow through measurement of radioactivity with a Berthold HPLC radioactivity monitor LB-503. 1: Natural GM1: 50 mg containing 100 μCi of [3H]-labeled compound. 2: Hydrogenated GM1: 50 mg containing 100 μCi of the corresponding [3H]-labeled compound.
gosine and C20 sphingosine. Owing to the relatively low sensitivity of the detecting system the peaks of the sphinganine-containing species could not be detected. Hydrogenated GM1 also provided two well-resolved peaks (c and d) partially overlapping with peak a and b, respectively, and corresponding to the molecular species carrying C18 and C20 sphinganine. In both peaks a and b the overlap started from the half-height of the descending line after the peak maximum. Moreover, the relative position of the peaks proved to remain constant on repeated runs. Therefore, when processing natural GM1, peaks a and b were collected till the beginning of the overlap. But peaks c and d, obtained from hydrogenated GM1, were collected entirely. The recovery of GM1-C18 sphingosine, GM1-C20 sphingosine, GM1-C18 sphinganine, and GM1-C20 sphinganine, was 27 mg, 18 mg, 29 mg, and 20 mg, respectively. Each molecular species upon chemical analysis and analytical HPLC turned out to be over 99% pure.

DISCUSSION

The present investigation shows that gangliosides can be submitted to HPLC fractionation without previous derivatization. The experimental design described here is based on two conditions that are critical for success; the use of rather apolar reversed-phase columns, like Lichrosorb RP-8 or µBondapak RP-18, and the use of a solvent system containing an aqueous buffer. The analytical procedures conventionally used for ganglioside fractionation separate the different gangliosides on the basis of their different oligosaccharide portions [Ledeen and Yu, 1982; Kundu, 1981]. The potential superiority of HPLC over these systems resides in its capability of separating gangliosides on the basis of polarity differences contributed by both the oligosaccharide and the ceramide portions. In particular, referring to the brain gangliosides of mammalian and avian species, which contain mostly stearic acid as the fatty acid moiety, each ganglioside can be separated into four molecular species containing C18 sphingosine, C18 sphinganine, C20 sphingosine, or C20 sphinganine. This was proved with a number of pure gangliosides—GM2, GM1, Fuc-GM1, GD1a, GD1b, GT1b and GQ1b—and likely applies to other gangliosides. According to our experience the application of HPLC for analysis of brain ganglioside mixtures has to follow a precise strategy. First, the solvent system, a mixture of acetonitrile and phosphate buffer, should be initially pumped with the highest possible proportion of buffer (about 50%), in order to separate the most polar gangliosides, and then with a gradient increasing of the percentage of acetonitrile (up to 60%) in order to better separate the less polar gangliosides. Also, a moderately apolar column should be used, such an Lichrosorb RP-8. Under these conditions it was possible to obtain good resolution for gangliosides between GM2 and GQ1b in 45 min. We expect to be able to separate GM3, which was not used in our study, from GM2 as well. In the case of calf brain, it was readily possible to separate the following species: GM1, Fuc-GM1, GD1a, GD1b, Fuc-GD1b, GT1b, GQ1b, each of them in the two preponderant molecular forms, ie, containing C18 and C20 sphingosine. However, there are some problems. Since the proportions among the different gangliosides markedly varied, it happened that a molecular species containing sphinganine of an abundant ganglioside overlapped a species containing sphingosine of a minor ganglioside. Thus the minor ganglioside GM2 in calf brain could not be identified, owing to overlapping of its major peaks with those of C18 or C20 sphinganine containing species of GM1. Furthermore, gangliosides that have a polar-
ity higher than GQ1b are expected to be difficult to resolve and to elute very close to the initial solvent peak. A possible solution to these problems, which are shared by TLC procedures, would be to perform fractionation of gangliosides by two HPLC runs, one on a column and with a solvent system more suitable for highly polar gangliosides and another under conditions appropriate to less polar gangliosides. This approach is actually under investigation. When solvent systems, such as aceto-nitrile-phosphate buffer are used, the elution of ganglioside peaks can be monitored by flow-through recording of absorbance at 195 nm. The response at 195 nm was not equal for all gangliosides and for the individual molecular species of a single ganglioside. At the same ganglioside concentration the response increased with increasing number of sialic acid residues; it was higher for the species containing C20 than C18 long-chain bases and higher for the species containing sphingosine than sphinganine. However, in all cases UV responses related linearly with ganglioside amounts ranging from 0.1 to 50 nmoles, with good precision. The sensitivity of estimates—from 0.1 nmoles—was of the same order as that (0.05 nmole) given by benzoylated gangliosides on HPLC [Bremer et al, 1979], much higher than that provided by colorimetric determination of ganglioside-bound NeuAc [Warren, 1959], and comparable to that of densitometric procedures commonly employed for ganglioside quantitation on TLC plates [Chigorno et al, 1982]. Therefore the HPLC analytical method appears to be fairly suitable for quantitation of separated ganglioside molecular species, provided that standard curves are determined for each ganglioside specie. Of course this latter condition may constitute a limitation to a wide application of the method to analysis of brain ganglioside mixtures. A field where HPLC procedures appear to constitute a new and powerful tool in ganglioside methodology is analysis and medium-scale preparation of the molecular species of a given ganglioside with homogeneous long-chain base composition. For instance, detection of other ganglioside contaminations in a ganglioside preparation can easily reach 5%, and even less on a qualitative basis. Therefore HPLC can be extremely useful for purifying individual species of gangliosides to be employed for very selective and specific assays, such as binding of toxins or hormones to gangliosides.

The HPLC automatic procedure described here can be employed for preparing 100-mg amounts of a single-ganglioside molecular specie, with standard laboratory HPLC apparatuses, and with assistance by professional operators limited to occasional check-up. Continuous reproducibility and good standards of performance are guaranteed by maintaining pressure and flow rate at the levels reported in the experimental section. Higher pressures and flow rates are suggested only for single runs [Sonnnino et al, 1984].

The preparation of large amounts of ganglioside molecular species by common laboratory HPLC equipment requires the use of solvents, such as tetrahydrofuran, that make it impossible to monitor elution by UV absorbance recording. In this case addition of radiolabeled compound as a tracer can solve the problem. Therefore all the gangliosides that can be radiolabeled on the oligosaccharide moiety [Suzuki and Suzuki, 1972; Ghidoni et al, 1974; Carubelli et al, 1984] can be submitted to preparative fractionation by this method. The possible use of ganglioside molecules radiolabeled in the long-chain base moiety for this purpose is under current investigation.

In conclusion, application of HPLC procedures for separation of nonderivatized gangliosides into molecular species that carry an individual long-chain base opens a
new chapter in ganglioside methodology, which will surely be improved and expanded in the near future. The availability of these molecular species, homogeneous in both their oligosaccharide and ceramide portions, is expected to greatly contribute to a better understanding of the role played by gangliosides in membrane structure and physiological function.

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