papers and notes on methodology

High performance liquid chromatography preparation of the molecular species of GM1 and GD1a gangliosides with homogeneous long chain base composition

Sandro Sonnino, Riccardo Ghidoni, Giuliano Gazzotti, Günther Kirschner,* Giovanni Galli, and Guido Tettamanti**

Study Center for the Functional Biochemistry of Brain Lipids, Department of Biological Chemistry, School of Medicine, University of Milano, Milano, Italy; Fidia Research Laboratory,* Abano Terme, Italy; and Chair of Applied Biochemistry, School of Pharmacy,** University of Milano, Milano, Italy

Abstract A semi-preparative, analytical high performance liquid chromatographic (HPLC) procedure is described for the isolation of molecular species of GM1 and GD1a gangliosides containing a single long chain base, C18 or C20 sphingosine, C18 or C20 sphinganine, each in its natural *erythro* or unnatural *threo* form. The *threo* forms were obtained from 2,3-dichloro-5,6-dicyanobenzoquinone/$NaBH_4$-treated gangliosides. The ganglioside molecular species separated by HPLC were analyzed for carbohydrate, fatty acid, and long chain base composition. In particular, long chain bases were submitted to gas–liquid chromatographic–mass spectrometric analyses as their trimethylsilyl (TMS) or N-acetyl-TMS derivatives, and chain length, presence or absence of C_4–C_5 double bond, and C-3 steric configuration were ascertained. The final preparations of individual molecular species of GM1 and GD1a gangliosides were more than 99% homogeneous in their saccharide moiety, contained a single long chain base (homogeneity higher than 99%), and had a fatty acid composition primarily of stearic acid (92 to 97%). All the individual molecular species of GM1 and GD1a gangliosides were also prepared in radioactive form by selective tritiation at C-3 of the long chain base. Their specific radioactivity ranged from 1.3 to 1.45 Ci/mmol. The availability of these molecular species of gangliosides is expected to facilitate studies aimed at ascertaining the role played by the hydrophobic portion in the functional behavior of gangliosides.—**Sonnino, S., R. Ghidoni, G. Gazzotti, G. Kirschner, G. Galli, and G. Tettamanti.** High performance liquid chromatography preparation of the molecular species of GM1 and GD1a gangliosides with homogeneous long chain base composition. *J. Lipid Res.* 1984. **25:** 620–629.

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Gangliosides are normal components of the plasma membranes of mammalian cells and are particularly abundant in neuronal membranes (1, 2). They are constituted by a hydrophobic portion, the ceramide, which is inserted in the lipid core of the membrane, and a hydrophilic portion, the sialic acid-containing oligosaccharide, which protrudes from the outer membrane surface. The oligosaccharide moieties of the gangliosides have different chemical structures constituting the reference basis for ganglioside separation and their recognition as individual entities (1, 2). The ceramide moiety of the most common gangliosides from adult mammalian and avian brain has a more homogeneous fatty acid composition, with a prevalence of stearic acid, but differs in the long chain base composition. The most abundant long chain bases in these gangliosides are the C18 and C20

Abbreviations: The paper follows the ganglioside nomenclature of Svennerholm (23) and the IUPAC-IUB recommendations (24). GM1, II^{3}αNeuAc-GgOse$_4$Cer ↔ Galβ1 → 3GalNAcβ1 → 4(NeuAcα2 → 3)Galβ1 → 4Glcβ1 → 1′Cer; GD1a, II^{3}αNeuAc,IV^{3}αNeuAc-GgOse$_4$Cer ↔ NeuAcα2 → 3Galβ1 → 3GalNAcβ1 → 4(NeuAcα2 → 3)Galβ1 → 4Glcβ1 → 1′Cer; long chain bases: C18 sphingosine, 2D-amino-octadec-4-ene-1,3-D-diol; C20 sphingosine, 2D-eicos-4-ene-1,3-D-diol; C18 sphinganine, 2D-amino-octadecan-1,3-D-diol; C20 sphinganine, 2D-aminoeicosan-1,3-D-diol; DDQ, 2,3-dichloro-5,6-dicyanobenzoquinone; 3-keto-ganglioside, oxidized ganglioside at the C-3 of sphingosine; TLC, thin-layer chromatography; HPTLC, high performance thin-layer chromatography; HPLC, high performance liquid chromatography; GLC, gas–liquid chromatography; TMS, trimethylsilyl ether; NeuAc, N-acetylneuraminic acid; MS, mass spectrometry.

compounds, in their *erythro* form, with or without a double bond at the C_4–C_5 position.

The chemical features of the hydrophobic portion of lipid constituents play an important role in membrane organization and function. This has been proved for phospholipids and simple glycolipids (3, 4). Reports have already appeared that stressed the importance of the lipid portion of gangliosides in determining their physicochemical properties (5) and some of their biosynthetic projections (6). An additional interesting aspect is the role played in membrane organization by precise steric configurations of the sphingolipid components. A major problem, for instance, is whether the C-3 hydroxyl group of long chain base is involved in stereospecific hydrogen bonding. Such questions can be answered by comparing the properties of natural sphingolipids, in their *erythro* form, with those of unnatural analogues having a *threo* configuration. Simple glycosphingolipids have been already prepared in their *threo* and *erythro* diastereoisomers (7–9), and it is important to extend the availability of these molecular species to gangliosides.

The present investigation was undertaken with the aim: *a*) to separate natural gangliosides, homogeneous in their oligosaccharide portion, in each of the molecular species containing C18 and C20 long chain base in the unsaturated and saturated form; *b*) to prepare for each of the above molecules containing the natural *erythro* form of long chain base, the unnatural *threo* isomer; and *c*) to prepare each of these molecular species in an isotopically radioactive form. The study was carried out on pure GM1 and GD1a gangliosides, prepared in order to contain stearic acid as the most abundant fatty acid. The methodological approach we developed is an application of reversed phase HPLC under conditions suitable for resolving ganglioside species on the basis of length, double bond occurrence, and C-3 stereoisomerism of long chain base. The ganglioside species containing C18 and C20 unsaturated long chain bases were obtained directly from the starting gangliosides, where they were preponderant; the species containing C18 and C20 saturated long chain bases were obtained after catalytic hydrogenation of the starting gangliosides; the species containing the *threo* forms of C18 and C20 long chain bases were obtained after chemical oxidation of the starting ganglioside at the 3-position of sphingosine with 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ), followed by reduction with sodium borohydride. This procedure is known to involve steric inversion at the C-3 position of sphingosine (7).

EXPERIMENTAL

Materials

Commercial chemicals were of analytical grade or of the highest grade available. Common solvents were redistilled before use and water for routine use was freshly redistilled in a glass apparatus. Toluene was dehydrated by metallic sodium before use. HPLC-grade reagents were used for HPLC. Silica gel 100 for column chromatography (0.063–0.2 mm, 70–230 mesh, ASTM), silica gel precoated thin-layer plates (HPTLC, Kieselgel 60, 250 μm thick, 10 × 10 cm), 2,3-dichloro-5,6-dicyanobenzoquinone, Triton X-100, trimethylsilylimidazole, platinum dioxide, and sodium borohydride were purchased from Merck GmbH, Darmstadt, West Germany; Sephadex G-25 fine was from Pharmacia, Uppsala, Sweden; reagents for gas–liquid chromatography were from C. Erba, Milan, Italy; glucose, galactose, N-acetylgalactosamine, and m-inositol were from Fluka, Buchs, Switzerland; N-acetylneuraminic acid was from Sigma Chemical Co., St. Louis, MO; dialysis tubing was from A. Thomas, Philadelphia, PA; Dowex 2-×8 (200–400 mesh), prepared in acetate form according to Svennerholm (10), was from Bio-Rad Laboratories, Richmond, CA; and [^{3}H]sodium borohydride (6500 Ci/mol) was from the Radiochemical Center, Amersham, Great Britain.

Preparation of standard long chain bases

Galactosylceramide was isolated from calf brain according to Radin (11). The *erythro* and *threo* forms of C18 sphingosine were obtained and purified from galactosylceramide following the procedure of Iwamori, Moser, and Kishimoto (7).

The *erythro* and *threo* forms of C18 sphinganine were obtained from the corresponding unsaturated compounds by reduction with platinum dioxide–H_2 under the same conditions used for hydrogenation of gangliosides (see below).

Preparation of gangliosides

GM1 and GD1a gangliosides, extracted from calf brain according to Tettamanti et al. (12), were purified and structurally characterized as described by Ghidoni et al. (13). During silica gel 100 column chromatographic purification of both GM1 and GD1a, the central fractions of the respective eluted peaks were collected. These fractions contained the ganglioside species having the highest percentage of stearic acid. The final purity of GM1 and GD1a preparations, referred as conventionally used to the oligosaccharide portion, was over 99%. These ganglioside preparations could be dissolved in water at concentrations up to 250 mg/ml to yield clear and colorless solutions.

Production of gangliosides containing the *threo* form of sphingosines

GM1 and GD1a gangliosides (50 mg each) were oxidized with DDQ and then reduced with sodium borohydride under the conditions described by Ghidoni et al.

(14). This treatment caused partial inversion of the *erythro* to the *threo* form of sphingosines and produced a mixture of the ganglioside species containing both diastereoisomers. DDQ/$NaBH_4$-treated gangliosides were obtained in a final yield of about 50% and had a saccharide composition and TLC behavior identical to that of the corresponding natural gangliosides. Noteworthy, DDQ/$NaBH_4$-treated gangliosides contained only C18 and C20 sphingosines, since DDQ treatment did not affect the ganglioside species containing saturated long chain bases, and the oxidized gangliosides (3-keto-derivatives) were separated from the nonoxidized species prior to reduction (14).

Hydrogenation of gangliosides

Ganglioside hydrogenation was carried out by conventional techniques (15) using the catalyst and the experimental conditions established as optimal in preliminary experiments. Briefly, GM1 and GD1a gangliosides (natural or DDQ/$NaBH_4$-treated compounds, 10–25 mg each) dissolved in 96% aqueous ethanol (1 mg/ml) were treated with hydrogen gas (pressure: 1.05 atm) for 36 hr at 20–22°C in the presence of platinum dioxide (0.5 mg/mg ganglioside) as catalyst, under constant stirring. At the end of the reaction the mixture was filtered through paper in order to remove the catalyst, evaporated to dryness, and dialyzed against 500 vol of redistilled water (overnight, 0–4°C). The solution was then lyophilized and stored at −20°C. The type of long chain base and fatty acid formed after ganglioside hydrogenation was determined by GLC–MS of the corresponding derivatives. The GLC–MS data obtained were consistent with the introduction into the sphingosine moiety of a molecule of hydrogen in the C_4–C_5 position. In addition, unsaturated fatty acids (i.e., C18:1, 0.2% of total fatty acid content in the starting natural ganglioside) were hydrogenated. The yield of the hydrogenation reaction was better than 99%. Hydrogenated gangliosides had a saccharide composition and TLC behavior identical to those of the corresponding parent compounds.

Labeling of gangliosides at the C-3 position of long chain base

GM1 and GD1a gangliosides were tritiated at the C-3 position of the long chain base and purified as described by Ghidoni et al. (14). The radiochemical purity of the labeled gangliosides was greater than 99% and the specific activity was 1.30 Ci/mmol for GM1 and 1.45 Ci/mmol for GD1a.

Separation and preparation by HPLC of ganglioside species homogeneous in their long chain base moiety

Semi-preparative method. Up to 5 mg of GM1 and GD1a gangliosides (natural or DDQ/$NaBH_4$-treated gangliosides) were dissolved with 25 μl of redistilled water in a microtube and introduced in a syringe loading sample injector (Model 7125, Rheodyne Inc., Cotati, CA) equipped with a 50 μl loop. The microtube was washed with 25 μl of redistilled water and the wash was added to the previous sample in order to minimize loss of material. Gangliosides were then chromatographed on a semi-preparative reversed phase Spherisorb-S5 OD S2 column (10 × 250 mm; Phase separations LMD, Queensferry, U.K.) using a Gilson HPLC apparatus (Model 303, Paris, France). The following experimental conditions were employed: temperature, 18–20°C; solvent system, acetonitrile–5 mM sodium phosphate buffer, pH 7.0, in the volume ratio of 3:2 for GM1 and of 1:1 for GD1a; flow rate, 7.5 ml/min for both gangliosides. The elution profile was monitored by using a Gilson UV detector (Mod. Holochrome, Paris, France) at 195 nm. The HPLC apparatus was connected with a Gilson computer-assisted automatic fraction collector (Mod. 201, Paris, France) which was programmed for collecting separately eluted fractions, or peaks, on the basis of UV signals. A complete cycle of analysis took about 60 min for GM1 and 40 min for GD1a. In one working day four or five cycles of analyses could be performed using a 30-min washing with the elution solvent between cycles. This enabled processing a total of 20–25 mg of ganglioside per day.

Each eluted fraction was collected. (Generally the peaks of the same ganglioside species from different cycles of experiments were pooled.) The fractions were evaporated to dryness at 30°C, dissolved in a small volume (3–4 ml) of redistilled water, dialyzed overnight against 500 vol of redistilled water under constant stirring (0–4°C, two or three changes), submitted to high speed centrifugation (40,000 rpm, 30 min, 18–20°C) in order to remove contaminating insoluble materials released by the HPLC column, and finally lyophilized. Further purification was attained by precipitation with cold acetone, as described by Ghidoni et al. (13). A portion of the final purified material was used for analyses. When radioactive GM1 and GD1a were used, 1–2 mCi of tritiated compound was chromatographed and the elution profile was monitored with an HPLC radioactivity monitor (Berthold, Mod. LB 503) equipped with a 120-μl solid scintillator cell.

Analytical method. One to 10 μg of ganglioside, dissolved in 25 μl of redistilled water, was chromatographed on an analytical reversed phase Spherisorb S5 OD S2 column (4.5 × 250 mm; Phase separations LMD, Queensferry, U.K.) using the same HPLC apparatus described for the semi-preparative method. The experimental conditions were: 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, 1.0 ml/min for both GM1 and GD1a. The elution profile

was recorded as described for the semi-preparative method. One complete analysis took about 40 min for GM1 and 20 min for GD1a.

Radiochemical purity and specific radioactivity of labeled gangliosides

The radiochemical purity of labeled gangliosides was determined as follows. A sample of each ganglioside species (about 10^5 dpm) was mixed with 1 nmol of the corresponding unlabeled product, spotted on a TLC plate, and chromatographed. The plate was dried, submitted to fluorography by spraying with a surface radioautography enhancer (EN^3HANCE^{TM} spray, New England Nuclear, Boston, MA), and exposed overnight at −80°C to an X-O-Mat SO 282 Kodak Film. TLC plates were scanned radiochromatographically using a Berthold TLC linear analyzer LB 282 equipped with an Apple II data system. The specific radioactivity of radiochemically pure ganglioside species was determined by assaying radioactivity in a liquid scintillator counter (Packard Tri-Carb, 460) using 5 ml of emulsifier (Instagel, Packard) and by measuring sialic acid by the colorimetric method (see Analytical procedures).

Analytical procedures

Carbohydrate and fatty acid analysis. Dried samples (0.1 mg) of gangliosides were methanolyzed in screw-capped tubes according to Zanetta, Breckenridge, and Vincendon (16). After fatty acid methyl esters were extracted with n-hexane, methyl glycosides were analyzed by GLC as the corresponding per-O-trifluoroacetyl derivatives (for neutral sugars), or per-N,O-trifluoroacetyl derivatives (for amino sugars), according to Zanetta et al. (16). Fatty acid analysis was accomplished by GLC. For this purpose the dry residue, obtained after evaporation of the n-hexane phase, was dissolved in 20 μl of CS_2 and 2–3 μl was injected directly on the GLC column (see below).

Long chain base analysis. Long chain bases were analyzed by GLC as the trimethylsilyl ethers according to the method of Carter and Gaver (17), with some modification (13). The *erythro* and *threo* forms of long chain bases were separated by GLC as the corresponding N-acetyl, trimethylsilyl ethers, according to Gaver and Sweeley (18). With regard to this latter analysis it should be remembered that the aqueous acidic conditions used for methanolysis are able per se to produce partial steric inversion of the *erythro* to the *threo* forms of long chain bases provided the glycolipid contains sphingosine(s) rather than sphinganine(s) (19, 20). In preliminary experiments we confirmed both the absence of the *threo* diastereoisomers when this procedure was used to analyze ganglioside preparations containing only sphinganines (hydrogenated gangliosides), and the occurrence of artifactual *threo* diastereoisomers when ganglioside preparations containing sphingosines were analyzed. On this basis we adopted the following strategy for determining the exact long chain base composition of the various ganglioside preparations, with special concern for *erythro* and *threo* configuration: *a*) GLC analysis of TMS derivatives, applied to the ganglioside preparation, for determining the amount of and the proportion between unsaturated and saturated long chain bases; *b*) GLC analysis of N-acetyl-TMS derivatives, applied to the same compound but previously submitted to full hydrogenation, for determining the presence and proportion of C-3 diastereoisomers in the starting compound.

Gas–liquid chromatography. GLC of derivatives of individual monosaccharides, fatty acids and long chain bases was carried out on a DANI Mod. 2001 gas chromatography apparatus (Milan, Italy) with a 25-m SE-52 capillary column, using in all cases a N_2 flow of 1.5 ml/min. The individual monosaccharides were identified and quantified by temperature programming at 10°C/min from 60°C to 250°C and introducing an initial isotherm at 60°C for 2 min; the fatty acids were analyzed by using a program at 10°C/min from 180°C to 280°C; the TMS derivatives of long chain bases were analyzed isothermally at 230°C; the N-acetyl-TMS derivatives were analyzed isothermally at 240°C.

Gas–liquid chromatography and mass spectrometry. GLC–MS analyses of long chain bases were performed on a Varian Mat 112 S gas chromatograph–mass spectrometer coupled with a PDP 11/34 data system. Operating conditions were as follows: glass capillary column, same as described for long chain base GLC analysis; temperature, 240°C; helium flow rate, 2 ml/min; transfer line, separator, and ion source temperature, 270°C; filament current, 60 μA; electron energy 70 eV; accelerating voltage, 3.5 KV; resolution, 1000. The analyses were carried out on standard *erythro* and *threo* C18 sphingosine and sphinganine and on natural or treated gangliosides, hydrolyzed and derivatized for long chain base analysis as described above.

Thin-layer chromatography. TLC of natural or treated 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 $CaCl_2$ 60:35:8 (by vol); time, 1 hr. Spots were detected as described by Ghidoni et al. (13).

Radio-GLC analysis of long chain bases. A sample of labeled molecular species of GM1 or GD1a (about 10^5 dpm) was mixed with a known amount (1 mg) of the corresponding unlabeled product and analyzed for long chain base. The determinations were performed by the GLC procedures specified above and peaks corresponding to the various derivatives of long chain bases were radioscanned. A Model 9GV C.Erba (Milan, Italy) chromato-

graph connected with a Nuclear Chicago flow counter, Model 4998, was employed.

Colorimetric methods. Ganglioside-bound sialic acid was determined by the method of Warren (21) after acid hydrolysis of the sample in 0.05 M H_2SO_4 (1 hr at 80°C) and purification of liberated sialic acid by ion exchange chromatography on a Dowex 2-×8 (acetate form) column (10). Pure N-acetylneuraminic acid was used as the standard.

RESULTS

HPLC semi-preparative resolution of gangliosides into individual molecular species

Natural GM1 and GD1a were separated, by the HPLC semi-preparative method described in the Experimental section, into four fractions, A, B, C, and D (**Fig. 1-I**). Fractions B and D were quantitatively minor components and had a partial overlapping with fractions A and C, respectively. Fractions A and C were completely cleared of contaminating fraction B and D, respectively, by HPLC rechromatography under the same conditions. Hydrogenated GM1 and GD1a provided, under the same conditions, two fractions having the same retention times as fractions B and D (Fig. 1-II). Fractions A, B, C, and D from GM1 had the same saccharide composition and TLC behavior as the starting natural GM1. Fractions A, B, C, and D from GD1a maintained the saccharide composition and TLC behavior of the original GD1a. This excludes the occurrence of any degradation of the ganglioside molecule during HPLC.

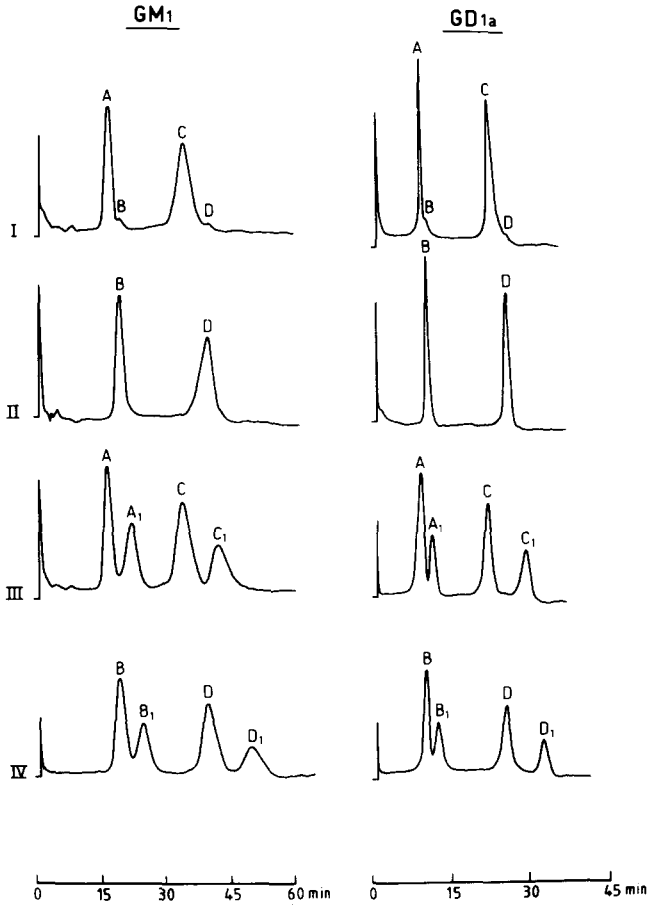

Fig. 1. Semi-preparative HPLC fractionation of GM1 and GD1a gangliosides. I, Natural gangliosides; II, natural gangliosides hydrogenated by PtO_2/H_2 treatment; III, DDQ/$NaBH_4$-treated gangliosides; IV, DDQ/$NaBH_4$-treated gangliosides followed by hydrogenation with PtO_2/H_2. Quantity of ganglioside injected in each column: 4 mg. For details and symbols (A, A_1, etc.) see the Results section.

DDQ/$NaBH_4$-treated GM1 and GD1a were separated by the HPLC semi-preparative method into four distinct fractions A, A_1, C, C_1, with no overlapping between adjacent fractions (Fig. 1-III). The retention times of fractions A and C closely resembled those of the corresponding fractions obtained from natural GM1 and GD1a. DDQ/$NaBH_4$-treated GM1 and GD1a, after PtO_2/H_2 hydrogenation, could be separated by the same procedure into four resolved fractions, B, B_1, D, and D_1 (Fig. 1-IV). The retention times of fractions B and D were close to those of the corresponding fractions obtained from natural GM1 and GD1a. The saccharide composition and TLC behavior of all the fractions obtained from the treated GM1 and GD1a preparations were the same as those of starting GM1 and GD1a, respectively. Therefore there was no degradation of DDQ/$NaBH_4$-treated gangliosides as a result of HPLC.

In all cases the retention times on repetitive assays were highly reproducible and each purified fraction provided, after HPLC re-chromatography, a peak which practically overlapped the original starting one.

The fractions obtained by HPLC from natural, DDQ/$NaBH_4$-treated, and hydrogenated gangliosides were dialyzed and purified as described in the Experimental section. Each of the final fractions provided, after HPLC re-chromatography, a single peak.

Long chain base and fatty acid composition of the individual molecular species of gangliosides separated by HPLC

The identification of the long chain bases present in the individual HPLC fractions was attained by GLC and MS analyses. The results of GLC analyses carried out on the HPLC fractions obtained from GM1 are reported in **Fig. 2** (fractions A, A_1, C, C_1) and **Fig. 3** (fractions B, B_1, D, D_1). Analysis of the TMS derivatives of long chain bases of fractions A and A_1 showed the same peak which corresponded to that of standard C18 sphingosine (Fig. 2) (the *erythro* and *threo* forms were undistinguishable under these experimental conditions); the peak for fractions C and C_1 was attributed to C20 sphingosine. The nature

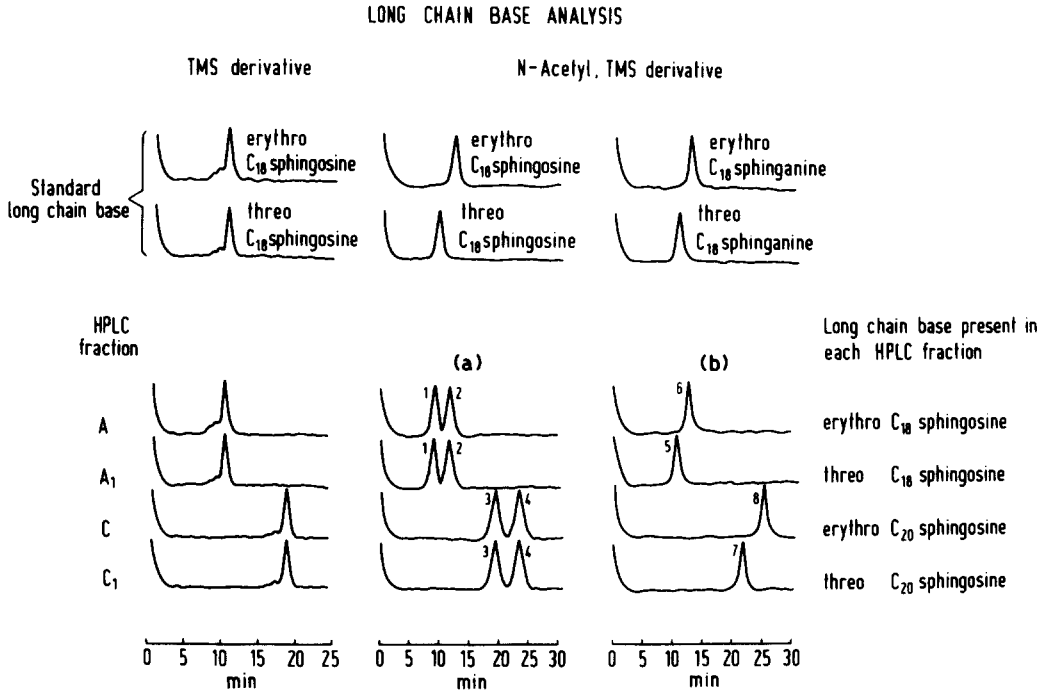

Fig. 2. Long chain base compositional analysis of the different HPLC fractions obtained from GM1 ganglioside. HPLC fractions containing C18 and C20 sphingosines: fractions A, A_1, C, C_1. Long chain bases were analyzed by GLC as their TMS derivatives and as their N-acetyl-TMS derivatives. This latter analysis was accomplished on: a) the original fractions; b) the same fractions after hydrogenation by PtO_2/H_2 treatment. TMS-derivative analysis was used for establishing chain length and presence or absence of the C_4–C_5 double bond. Owing to occurrence of artifactual C-3 steric inversion during derivation, N-acetyl-TMS derivative analysis, carried out on previously hydrogenated compounds, was used only for establishing C-3 steric configuration in the original compounds. For details and symbols (A, A_1, etc.) see the Results section. Fractions A and C were prepared from original GM1; fractions A_1 and C_1 were from DDQ/$NaBH_4$-treated GM1. Identical results were provided by the analogous HPLC fractions obtained from GD1a ganglioside.

of the long chain base moiety contained in these peaks was assessed by N-acetyl-TMS derivative analysis assisted by MS. Under these conditions, fractions A and A_1 provided both peaks (peaks 1 and 2 of Fig. 2) which corresponded to those of standard *threo* and *erythro* C18 sphingosine, respectively. This was due to partial steric inversion at C-3 occurring during aqueous acidic methanolysis (20). The mass spectra of peaks 1 and 2 were virtually identical. They had a M-15 at 470 m/e and corresponded to those, already published (18), of *threo* and *erythro* C18 sphingosines. Fractions C and C_1 also provided two peaks (peaks 3 and 4 of Fig. 2) which gave identical mass spectra. These spectra (reproduced in **Fig. 4a**) resembled very closely those of *threo* and *erythro* C18 sphingosines, but showed fragments systematically having molecular weights of 28 daltons higher and an M-15 at 498 m/e. They can be attributed to C20 sphingosine and correspond to isomers of the same long chain base. In analogy with the behavior of the *threo* and *erythro* forms of C18 sphingosine under the same experimental conditions, peak 3 was assigned to *threo* C20 sphingosine and peak 4 was assigned to *erythro* C20 sphingosine. In order to establish the C-3 stereoisomerism of the long chain bases present in the original A, A_1, C, C_1 fractions, each of these fractions was submitted, prior to N-acetyl-TMS derivation, to full hydrogenation as described in the Experimental section. This treatment transformed C18 and C20 sphingosines into C18 and C20 sphinganines and overcame artifactual steric inversion at C-3 during derivation. As shown in Fig. 2b, fractions A and A_1 each provided one peak (peaks 6 and 5, respectively) which had identical mass spectra with an M-15 at 472 m/e corresponding to those (18) of *erythro* and *threo* C18 sphinganines, respectively. This indicates that the long chain base in fraction A had an *erythro* configuration and in fraction A_1 a *threo* configuration. Fractions C and C_1 also each produced one peak (peaks 8 and 7, respectively). The mass spectra of these peaks were identical, had an M-15 at 500 m/e (Fig. 4b), and were attributed to *erythro* and *threo* C20 sphinganines, respectively. Therefore the long chain bases in fractions C and C_1 had an *erythro* and *threo* configuration, respectively.

As shown in Fig. 3, fractions B and B_1 provided, after TMS derivation of long chain bases, identical peaks which

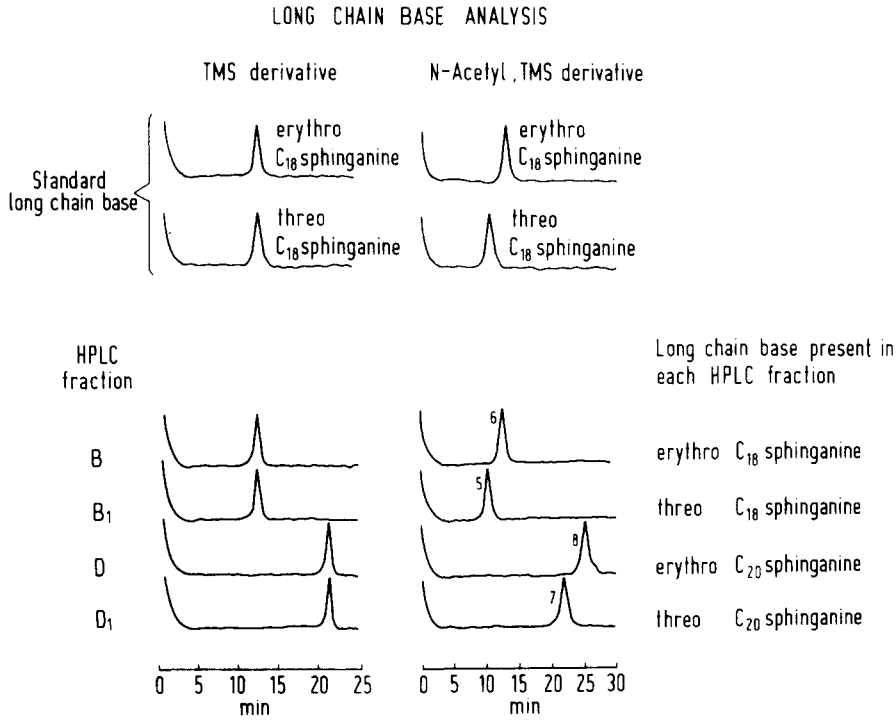

Fig. 3. Long chain base compositional analysis of the different HPLC fractions obtained from GM1 ganglioside. HPLC fractions containing C18 and C20 sphinganines: fractions B, B_1, D, D_1. Long chain bases were analyzed by GLC as: a) their TMS derivatives for establishing chain length and presence or absence of the C_4–C_5 double bond, and b) their N-acetyl-TMS derivatives for establishing also steric configuration of C-3. For details and symbols (B, B_1, etc.) see the Results section. Fractions B and D were prepared from GM1 ganglioside hydrogenated by PtO_2/H_2 treatment; fractions B_1 and D_1 were from DDQ/$NaBH_4$-treated GM1 followed by hydrogenation with PtO_2/H_2. Identical results were provided by the analogous HPLC fractions obtained from GD1a ganglioside.

corresponded to that of standard C18 sphinganine; fractions D and D_1 also provided the same peak which was attributed to C20 sphinganine. GLC analysis of the N-acetyl-TMS derivatives of long chain bases and MS studies of the peaks demonstrated that fraction B contained *erythro* C18 sphinganine (peak 6 of Fig. 3) and fraction B_1 contained *threo* C18 sphinganine (peak 5 of Fig. 3). Under the same experimental conditions, both fractions D and D_1 provided one peak each (peaks 8 and 7 of Fig. 3, respectively) which were assigned after MS analysis (Fig. 4b) to *erythro* and *threo* C20 sphinganine, respectively.

All HPLC fractions provided upon GLC analysis of long chain bases a single peak. The only exception was GLC of N-acetyl-TMS derivatives of unsaturated long chain bases where two peaks appeared as the result of artifactual steric inversion at C-3. The small peaks preceding those of C18 and C20 sphingosines in GLC analysis of long chain base TMS derivatives likely correspond to the 3-O methyl derivative easily generated under the derivation conditions used (17, 18). Considering the high sensitivity of the detection system used in GLC analysis, this indicates that the homogeneity of each of the purified HPLC fractions, as judged by long chain base composition, was on the order of 98–99%.

The long chain base composition of the HPLC fractions obtained from GD1a was exactly the same as that of the corresponding fractions obtained from GM1. The fatty acid composition of the different GM1 and GD1a species differing in the long chain base moiety and separated by HPLC is reported in **Table 1.** Data on original GM1 and GD1a gangliosides are also included. C18:0 is the most abundant fatty acid (always over 92% of the total fatty acids). However, the ganglioside species carrying C18 long chain bases had an enriched content of C18:0 (up to 97%) as compared to the species carrying C20 long chain bases (92%). Conversely, the content of C20:0 fatty acid was 2–3 times higher in the species carrying C20 long chain bases than in those carrying the C18 homologues.

Separation of ganglioside species carrying a single long chain base by the HPLC analytical method

The analytical method had a resolution power and reproducibility comparable to that of the semi-preparative

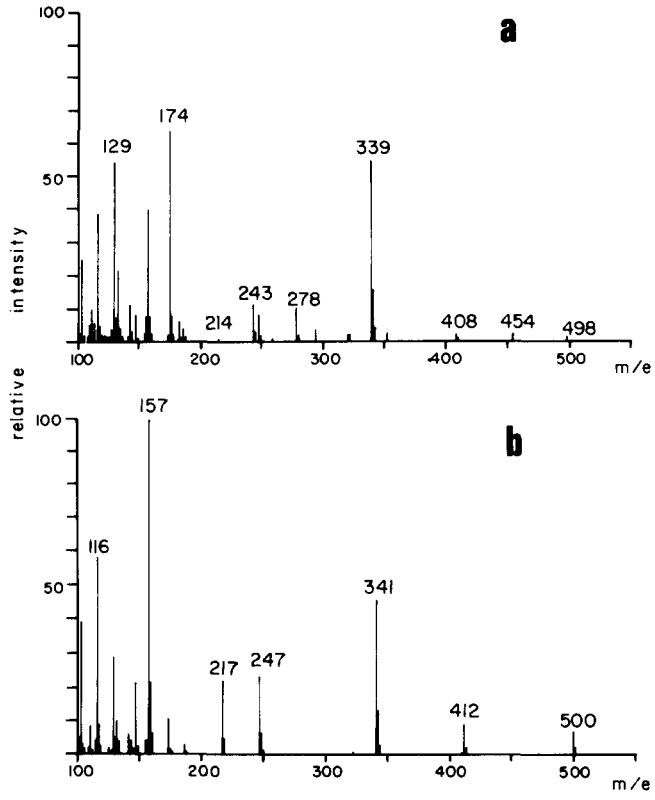

Fig. 4. Mass spectra of peak 4 (1,3-di-O-trimethyl-silyl-N-acetyl C20 sphingosine) (a) and of peak 8 (1,3-di-O-trimethyl-silyl-N-acetyl C20 sphinganine) (b), referred to in Fig. 2. Mass spectra of peaks 3 and 7, referred to in Fig. 2, were identical to those of the above peaks 4 and 8, respectively.

method. Of course the retention times of the various separated compounds were shorter than those of the semi-preparative method. The retention times of GM1 *erythro* C18 sphingosine and GD1a *erythro* C18 sphingosine were 12.5 min and 6.25 min, respectively. The relative retention times, referred to that of GM1 *erythro* C18 sphingosine (considered as 1.00) were 1.27 for GM1 *threo* C18 sphingosine (peak A_1), 2.05 for GM1 *erythro* C20 sphingosine (peak C), 2.60 for GM1 *threo* C20 sphingosine (peak C_1), 1.12 for GM1 *erythro* C18 sphinganine (peak B), 1.40 for GM1 *threo* C18 sphinganine (peak B_1), 2.30 for GM1 *erythro* C20 sphinganine (peak D), and 2.96 for GM1 *threo* C20 sphinganine (peak D_1). The relative retention times referred to that of GD1a *erythro* C18 sphingosine (considered as 1.00) were 1.23 for GD1a *threo* C18 sphingosine (peak A_1), 1.84 for GD1a *erythro* C20 sphingosine (peak C), 2.37 for GD1a *threo* C20 sphingosine (peak C_1), 1.09 for GD1a *erythro* C18 sphinganine (peak B), 1.39 for GD1a *threo* C18 sphinganine (peak B_1), 2.09 for GD1a *erythro* C20 sphinganine (peak D), and 2.67 for GD1a *threo* C20 sphinganine (peak D_1).

Preparation of the ganglioside molecular species carrying a single long chain base in radioactive form

The above HPLC procedure was applied to preparation of the different molecular species of GM1 and GD1a with a single long chain base labeled with ^3H in the C-3 position. The molecular species containing C18 and C20-*erythro*- and *threo*- sphingosines were prepared directly from the original tritiated ganglioside; the molecular species containing C18 and C20-*erythro*- and *threo*-sphinganines were obtained after hydrogenation of the original tritiated ganglioside. In all cases the UV peaks corresponding to the HPLC fractions exactly overlapped the peaks of radioactivity. Upon radio-GLC analysis, the only recorded peaks of radioactivity corresponded to the long chain base constituent of the individual ganglioside species. These species displayed on TLC plates a single spot after

TABLE 1. Fatty acid composition of the molecular species of GM1 and GD1a gangliosides separated by HPLC

Ganglioside Molecular Species	Fatty Acid Composition, %					
	C16:0	C16:1	C18:0	C18:1	C20:0	C22:1
GM1, natural	0.6	0.2	94.6	0.2	3.2	1.2
GM1-(*erythro* C18 sphingosine)	0.5	0.1	97.2	0.1	1.6	0.5
GM1-(*erythro* C18 sphinganine)	0.6		97.4		2.0	
GM1-(*erythro* C20 sphingosine)	0.3		92.7	0.2	5.1	1.7
GM1-(*erythro* C20 sphinganine)	0.3		93.1		6.6	
GD1a, natural	1.7	0.4	93.3	0.5	2.6	1.5
GD1a-(*erythro* C18 sphingosine)	1.2	0.2	96.4	0.2	1.2	0.6
GD1a-(*erythro* C18 sphinganine)	1.3		96.7		2.0	
GD1a-(*erythro* C20 sphingosine)	0.6	0.2	91.8	0.4	5.2	1.8
GD1a-(*erythro* C20 sphinganine)	0.9		92.4		6.7	

Data are reported which refer to the ganglioside species containing the *erythro* forms of long chain bases. Results obtained with the ganglioside species containing the *threo* forms of long chain bases were quite similar.

fluorography, which overlapped the spot visualized by colorimetric procedures. Radiochromatoscanning also showed a single peak of radioactivity corresponding to the TLC spot. The above analyses indicate a radiochemical purity better than 98% for each HPLC fraction. The specific radioactivities of the various molecular species of GM1 and GD1a gangliosides were virtually the same as those of the starting ganglioside (reported in the Experimental section).

DISCUSSION

Gangliosides, particularly those from adult mammalian and avian brain, contain C18 and C20 long chain bases, with a preponderance of the forms unsaturated at C_4–C_5. Considering the pathway of sphingosine biosynthesis (22) and the observations made on other mammalian sphingolipids (20), the long chain bases of gangliosides are assumed to have an *erythro* configuration. Since our aim was to develop a methodology capable of resolving ganglioside molecular species on the basis not only of the length and presence of C_4–C_5 double bond, but also of C-3 stereoisomerism, we performed our studies on GM1 and GD1a gangliosides using the natural, and hydrogenated, compounds as the source of the "*erythro*" molecular species, and the DDQ/$NaBH_4$-treated substances as the source of the "*threo*" species.

The HPLC procedure we developed for separation of GM1 and GD1a molecular species having a homogeneous long chain base composition provides well-resolved fractions in a rapid and reproducible way. The excellent performance of the procedure is based mainly on the presence of phosphate buffer in the solvent system. This achievement was the result of a long series of pilot experiments. It is worth noting that the procedure did not cause any chemical modification to the ganglioside molecule. With the present methodology, a maximum of 5 mg of ganglioside can be processed through HPLC. In one working day up to 20–25 mg can be conveniently isolated. This can be considered successful from the preparative point of view, since most of studies requiring ganglioside species with homogeneous long chain base composition could be accomplished without spending too much time in preparation. Enhancement of the amount of processable ganglioside in a single cycle of operation is mainly a problem of ganglioside solubility in the solvent volume which can be applied to the column and in the eluting solvent. As generally used in HPLC, we also employed absorbance at 195 nm for monitoring the elution of gangliosides from the column. This limits the choice of the organic solvents for elution to those that are transparent at 195 nm. The availability of alternative approaches for monitoring ganglioside elution from the column could remove this limitation and improve the preparative features of the HPLC procedure. Efforts in this direction are being made in our laboratory.

The different molecular species of GM1 and GD1a obtained by HPLC are very close to being pure and homogeneous substances. In fact, they are more then 99% homogeneous in their carbohydrate and long chain base moiety and from 92% to 97% homogeneous in their fatty acid moiety. The availability of individual gangliosides pure in their oligosaccharide portion opens the door to investigations on the functional role of gangliosides. In fact, the molecular basis for the specific interactions among gangliosides and several ligands is believed to be in the different chemical features and reactivity of the different oligosaccharide moieties. The present availability of up to eight different species of a single ganglioside, each of them with a unique long chain base, and also in radioactive form, is expected to have a complementary role in improving and expanding the research strategies on ganglioside function. Particularly, the use of these gangliosides will facilitate inspection of the specific role played by the hydrophobic portion in the interactions of gangliosides with a variety of molecules, with special reference to those that act as natural partners in the biological membranes, phospholipids, cholesterol, and proteins. Some basic problems concerning ganglioside involvement in trans-membrane passage of external signals and in connecting intracellular architecture with membrane structural components will possibly be investigated.

In conclusion, the procedure described here, based on application of reversed phase HPLC, is suitable for separating and preparing the molecular species of gangliosides GM1 and GD1a containing a single long chain base, C18 or C20 sphingosine, C18 or C20 sphinganine, each of them in its *erythro* or *threo* form. In principle the procedure should be applicable, with some adaptations, to similar fractionation of other gangliosides.

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