papers and notes on methodology

Separation of brain monosialoganglioside molecular species by high-performance liquid chromatography

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Abstract A method for the separation of molecular species of brain monosialogangliosides by high-performance liquid chromatography is described. G_{M4}, G_{M3}, G_{M2}, and G_{M1} were purified from human brain and their individual molecular species were separated on a C18 reversed-phase column. Peaks were identified by mass spectrometry of the intact ganglioside, by gas–liquid chromatography of the fatty acids, and by high-performance liquid chromatography of the long chain bases. A characteristic elution sequence of molecular species permitted their identification based upon their retention times on the reversed-phase column.—**Kadowaki, H., J. E. Evans, and R. H. McCluer.** Separation of brain monosialoganglioside molecular species by high-performance liquid chromatography. *J. Lipid Res.* 1984. **25:** 1132–1139.

Supplementary key words mass spectrometry

The analysis of fatty acids and long chain bases of the ceramide moieties of gangliosides has customarily been performed after acid hydrolysis. This method provides the distribution of fatty acids and long chain bases, but does not give the actual molecular species of the ceramides. This is especially significant for brain gangliosides which contain several long chain bases paired with various fatty acids (1–4), including, in the case of brain G_{M4}, hydroxy fatty acids as well as non-hydroxy fatty acids (5–7).

Recently, HPLC methods have been introduced to separate the molecular species of intact sphingomyelin (8) and hydroxy fatty acid-containing ceramide (9). On the other hand, there has been only one report (10) in which molecular species separation of gangliosides from brain has been attempted by HPLC. The separation of the molecular species was, however, limited to only two major components, such as d18:1 and d20:1 sphingosines paired only with C18:0 fatty acid.

Using human brain monosialogangliosides (G_{M4}, G_{M3}, G_{M2}, and G_{M1}), we have demonstrated the separation of their molecular species by reversed-phase HPLC. Identification of the individual ceramide molecular species in each peak collected from the reversed-phase column was performed by ammonia CI–MS of the intact ganglioside, and following acid hydrolysis by HPLC of the long chain base and GLC of the fatty acid. Quantitation of each peak was by normal-phase HPLC after perbenzoylation of the individual peaks, and recoveries were determined relative to the original unchromatographed sample.

MATERIALS AND METHODS

Chemicals

HPLC grade methanol, and other reagent grade solvents and chemicals were obtained from Fisher Chemical Scientific (Fairlawn, NJ); Iatrobeads 6RS-8060 were from Iatron Industries (Tokyo, Japan); DEAE-Sephadex (25A) was from Pharmacia Fine Chemicals (Piscataway, NJ); LiChroprep RP-18 (25–40 μm) was from Merck (Darmstadt, Germany); and Unisil was from Clarkson Chemical Company (Williamsport, PA).

Abbreviations: HPLC, high-performance liquid chromatography; GLC, gas–liquid chromatography; MS, mass spectrometry; CI, chemical ionization; TLC, thin-layer chromatography. The ganglioside nomenclature used here is according to the system of Svennerholm (22): G_{M3}, II^3NeuAc-LacCer; G_{M2}, II^3NeuAc-GgOse$_3$Cer; G_{M1}, II^3NeuAc-GgOse$_4$Cer; G_{M4}, I^3NeuAc-GalCer. The long chain base abbreviations as suggested by Breimer, Karlsson, and Samuelsson (23) are utilized throughout.

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Preparation of ganglioside fraction

A human whole brain hemisphere (420 g from an 80-year-old female) was extracted twice with 20 volumes of chloroform–methanol–water 4:8:3 (v/v/v), and partitioned by adding water to give a final chloroform–methanol–water volume ratio of 4:8:5.6 as described by Svennerholm and Fredman (11). This resulted in the appearance of two solvent phases and the lower phase was partitioned twice more with water. Crude gangliosides in the combined upper phase were isolated by the procedure described by Williams and McCluer (12) except that a column containing 125 g of C18 reversed-phase packing (LiChroprep) was substituted for the C18 Sep-Pak Cartridge and correspondingly larger volumes of solvents were utilized, since large quantities of material were used. After salt and water-soluble materials were removed from the column with 6 l of water, gangliosides were eluted with 2 l of methanol and the eluate was evaporated to dryness. The following procedures for ganglioside purification were essentially the same as the methods previously reported by Ledeen and Yu (13) except that the LiChroprep reversed-phase column, described above, was used instead of dialysis. Briefly, the dried ganglioside fraction was dissolved in 400 ml of chloroform–methanol–water 30:60:8 and applied to a 200 g DEAE-Sephadex (acetate form) column (7 × 33 cm). After sample application, the column was first washed with 3 l of chloroform–methanol–water 30:60:8. Gangliosides were then removed from the column with 10.5 l of chloroform–methanol–0.8 M ammonium acetate 30:60:8. The solvent was evaporated to a final volume of approximately 500 ml, and adjusted to a final concentration of 0.1 M ammonium acetate in methanol–water 1:1, and then passed through a 125 g LiChroprep reversed-phase column. After the column was washed with 6 l of water, gangliosides were eluted with 1.5 l of methanol and the solvent was evaporated to dryness. The dried residue was treated with 0.25 N sodium hydroxide in methanol at 37°C for 2 hr to destroy ester lipids. After the sample was neutralized with acetic acid, 200 ml of water was added to adjust a final concentration of 0.125 N sodium acetate in methanol–water 1:1, and it was then passed through a LiChroprep reversed-phase column. The gangliosides were eluted with 1.5 l of methanol and the solvent was evaporated to dryness. The gangliosides were dissolved in 200 ml of chloroform–methanol 6:1 and applied on a 100 g Unisil (200–325 mesh) column (2.6 × 51 cm). The column was successively eluted with 1 l of chloroform–methanol 6:1, 1 l of chloroform–methanol 5:1, 3 l of chloroform–methanol–water 10:10:3, and 1.5 l of methanol. The latter two solvent fractions, which contained gangliosides, were combined and the solvent was evaporated to dryness.

Isolation of total monosialogangliosides

The ganglioside fraction was dissolved in 100 ml of methanol and applied to a 100 g DEAE-Sephadex (acetate form) column (7 × 20 cm) to separate monosialogangliosides from other gangliosides. The column was eluted with 2 l of methanol (no gangliosides were detected) and then 8 l of 0.02 M ammonium acetate in methanol (monosialogangliosides plus trace amounts of disialogangliosides were detected). The latter fraction was adjusted to the final concentration of 0.1 M ammonium acetate and methanol–water 1:1, and passed through the LiChroprep reversed-phase column. The monosialogangliosides were eluted from the LiChroprep column with methanol and the solvent was evaporated to dryness.

Isolation of individual monosialogangliosides (G_{M4}, G_{M3}, G_{M2}, and G_{M1})

The monosialoganglioside fraction was redissolved in approximately 1 ml of chloroform–methanol–water 65:35:3 and applied to a 50 g Iatrobead 6RS-8060 column (1.2 × 100 cm). The column was eluted with chloroform–methanol–water 65:35:3 and fractions of 10 ml were collected and gangliosides in each fraction were identified by TLC. TLC was performed on Merck Silica Gel 60 HP–TLC plates with chloroform–methanol–0.25% $CaCl_2$ 55:45:10 as the developing solvent; gangliosides were visualized with resorcinol spray reagent as previously described (14). G_{M4} started to elute immediately after the bed volume of the column and then G_{M3}, G_{M2}, and G_{M1} eluted in succession. Fractions which contained the overlapping end of the G_{M3} peak and the beginning of the G_{M2} peak were reapplied to the Iatrobead column for further separation by elution with the same solvent system. Likewise, the fractions which contained the end of G_{M4} peak and beginning of G_{M3} peak were also reapplied to the column and elute with chloroform–methanol–water 70:30:3. The amounts of pure G_{M4}, G_{M3}, G_{M2}, and G_{M1} obtained were 6.5 mg, 8.1 mg, 8.3 mg, and 74.5 mg, respectively. Any fractions which contained trace amounts of overlapping peaks were discarded and recoveries of each monosialoganglioside following Iatrobead chromatography step were estimated to be over 95% for G_{M4} and G_{M3} and over 90% for G_{M2} and G_{M1} from results of TLC examination of the combined fractions. Pure monosialogangliosides were dissolved in methanol for the analysis of their ceramide molecular species.

Separation of monosialoganglioside molecular species

G_{M4}, G_{M3}, G_{M2}, and G_{M1} were separated into individual molecular species by reversed-phase HPLC on a 4.6 × 250 mm ultrasphere ODS column 5 μm (Altex Sci-

entific, Inc., Berkeley, CA). G_{M4} was eluted with methanol–water 89:11 (v/v), G_{M3} with 87:13, G_{M2} with 84.7:15.3, and G_{M1} with 85.2:14.8. The HPLC was performed with a Waters Associates (Milford, MA) Model 6000A solvent delivery system equipped with a Model U6K injector, or on a Varian (Palo Alto, CA) Model 5020 liquid chromatograph equipped with a Rheodyne (Cotati, CA) Model 7125 syringe loading sample injector. A Schoeffel (Westwood, NJ) variable wavelength detector was set at 205 nm for detection of underivatized gangliosides.

Analytical methods

Each peak collected from a C18 reversed-phase HPLC column was adjusted to a final concentration of 0.1 M potassium chloride in methanol–water 1:1, and applied to a 0.2 g LiChroprep column. The column was washed with 10 ml of methanol–water 1:1 and then gangliosides were eluted with 10 ml of methanol. Aliquots of each reversed-phase peak were taken for quantitation of monosialogangliosides and identification of molecular species. Quantitative analysis of each peak was performed by the normal-phase HPLC on a LiChrosphere SI 4000 column after perbenzoylation as described by Bremer, Gross, and McCluer (15). Identification of molecular species in each peak was performed by MS of the underivatized intact gangliosides with a Finnigan 4500 mass spectrometer equipped with a moving-belt HPLC–MS interface and a Teknivent 56K data system. Ammonia CI was performed with the ion source at 0.8 torr

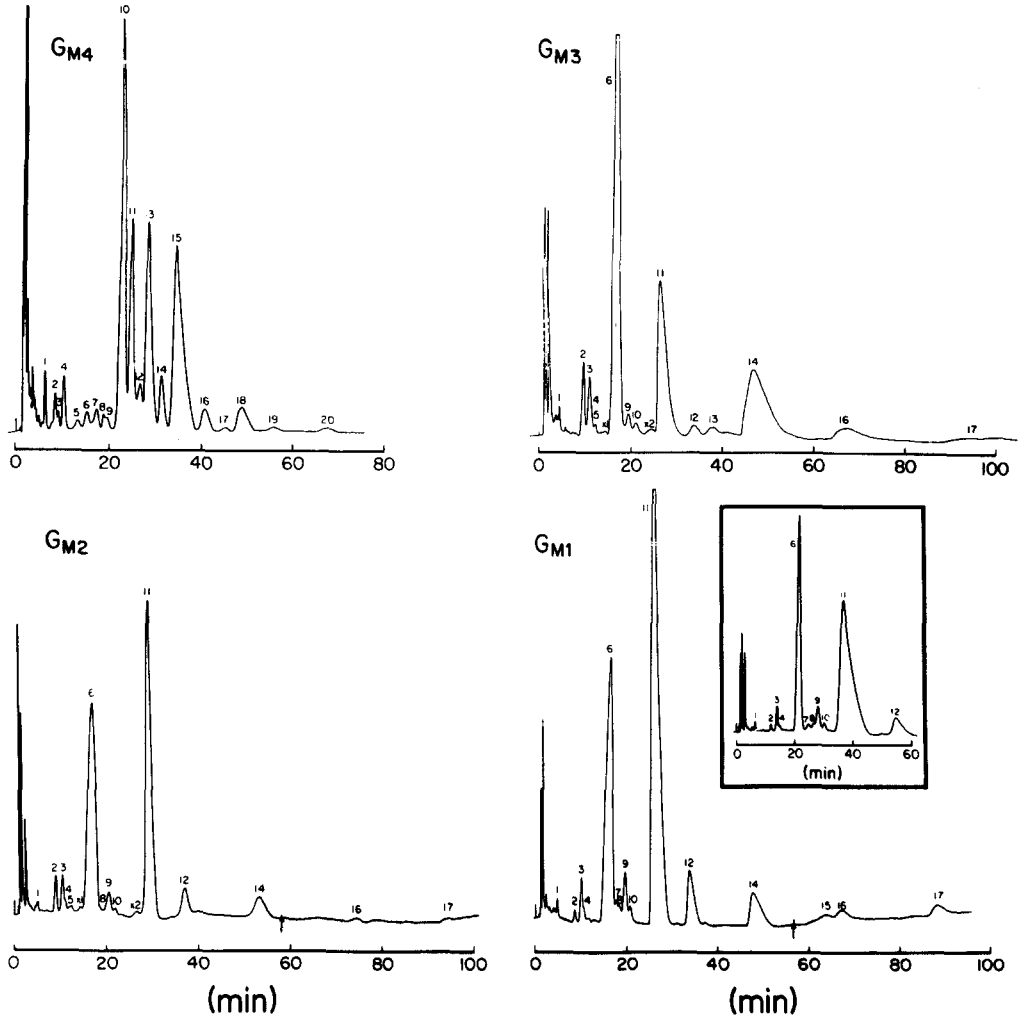

Fig. 1. HPLC separation of the molecular species of human brain G_{M4}, G_{M3}, G_{M2}, and G_{M1} on a C18 reversed-phase column. The chromatograph conditions: G_{M4} (58 μg) was chromatographed with methanol–water 89:11 (v/v) at a flow rate of 1.3 ml/min, at the column temperature of 26°C; G_{M3} (51 μg) with methanol-water 87:13 and 1.5 ml/min at 25°C; G_{M2} (46 μg) with methanol–water 84.7:15.3 and 1.5 ml/min at 28°C; G_{M1} (81 μg) with methanol–water 85.2:14.8 and 1.5 ml/min at 28°C. G_{M1} inset with methanol–water 83.2:16.8 and 1.3 ml/min at 28°C. Detection was by absorption at 205 nm (0.1 absorbance unit full scale and then changed to 0.05 as shown by the arrow of G_{M2} and G_{M1}). Peaks are numbered in sequence of elution and are identified in Tables 1 and 2.

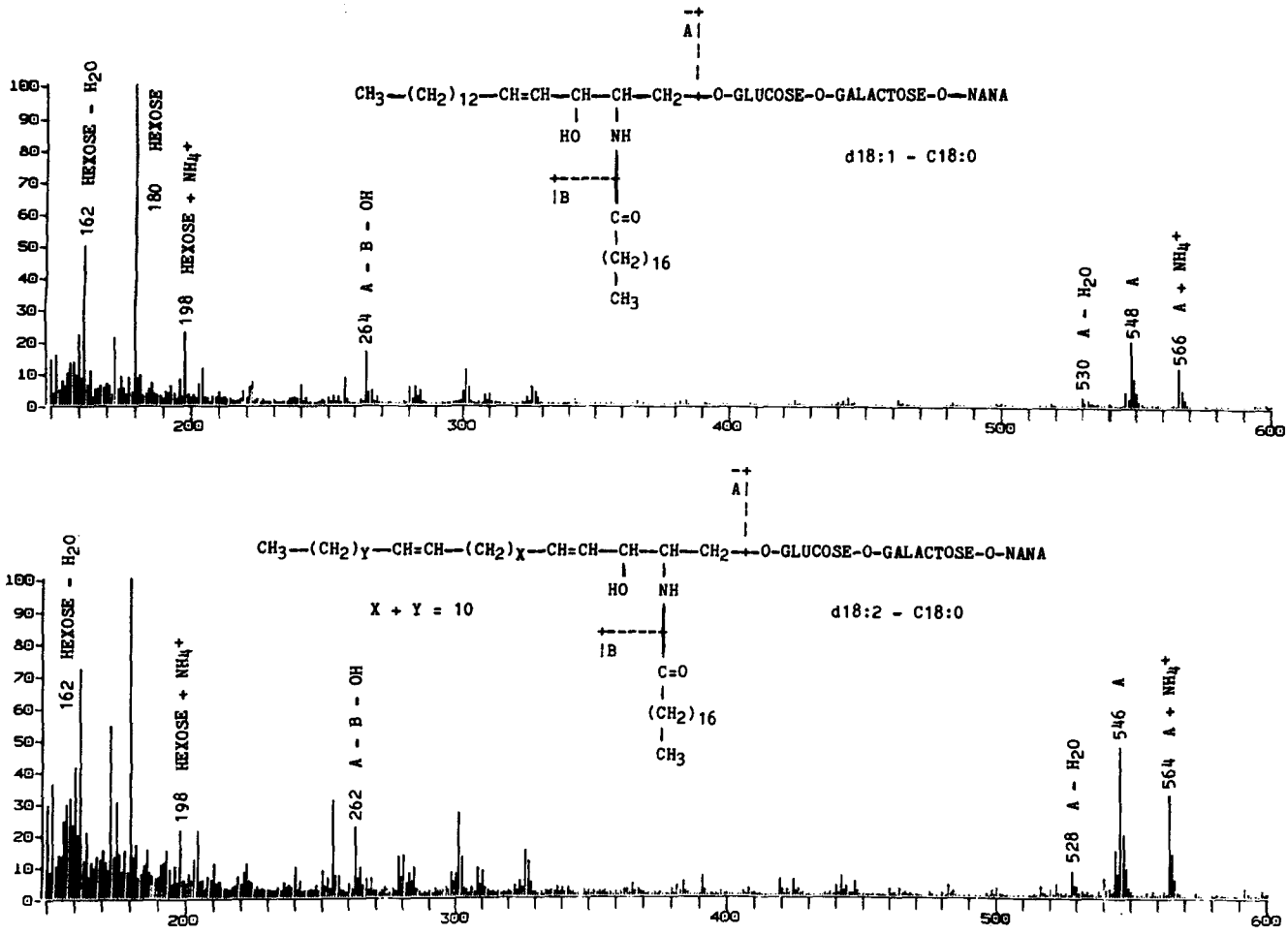

Fig. 2. MS fragmentation patterns of G_{M3}, d18:1–C18:0 (peak 6) and d18:2–C18:0 (peak 3). Ordinate, relative intensity (percent); abscissa, m/z. The intact ganglioside (in methanol) was applied directly to the polyimide moving belt on the LC/MS interface (see Methods for details).

and 150°C. Samples dissolved in methanol were applied directly to the polyimide belt with the evaporator and clean-up heaters at 320°C and 240°C (indicated), respectively. The molecular species of each peak was confirmed by long chain base and fatty acid analyses. Long chain bases of gangliosides were analyzed after acetonitrile–HCl hydrolysis as described by Kadowaki et al. (16). Following acetonitrile–HCl hydrolysis of the gangliosides, fatty acids were extracted with hexane, methylated with 14% boron trifluoride in methanol at 80°C for 10 min, and identified by GLC on an OV-1 column using a Hewlett-Packard 7620-A gas chromatograph equipped with a flame ionization detector with helium as the carrier gas.

RESULTS

Fig. 1 shows chromatograms of the reversed-phase HPLC separation of gangliosides G_{M4}, G_{M3}, G_{M2}, and G_{M1} into their individual molecular species. Peaks were collected and aliquots were taken for the identification of the ceramide composition by direct ammonia CI–MS of the intact ganglioside, and by GLC analysis of the fatty acid with HPLC analysis of the long chain base after acetonitrile–HCl hydrolysis. Representative mass spectra of G_{M3} species are shown in **Fig. 2.** The major ceramide ions in the mass spectra containing various long chain bases paired with C18:0 fatty acid are presented in **Table 1.** The amount of ganglioside in each peak was determined by normal-phase HPLC of the perbenzoylated derivatives.

TABLE 1. Major ceramide ions which contain C18:0 fatty acid identified in the ammonia CI mass spectra of underivatized G_{M3}

	Long Chain Base					
	d16:1	d18:0	d18:1	d18:2	d20:0	d20:1
A[a]	520	550	548	546	578	576
A − H_2O	502	532	530	528	560	558
A − NH_4^+	538	568	566	564	596	594
A − B[a] − OH	236	266	264	262	294	292

[a] MS fragmentation patterns are shown in Fig. 2.

The results from the identification and quantitation for G_{M4} peaks are shown in **Table 2,** and for G_{M3}, G_{M2}, and G_{M1} peaks in **Table 3.** As shown in these tables, recoveries for G_{M4}, G_{M3}, G_{M2}, and G_{M1}, as compared to the original unchromatographed samples, were 92%, 89%, 93% and 93%, respectively. Approximately a 10% loss results from the reversed-phase (LiChroprep) wash which is comparable to the loss observed using the Sep-Pack reversed-phase C18 cartridge reported by Williams and McCluer (12). Since this loss occurs after the molecular species are separated, the loss does not result in a distortion of the ganglioside species distribution.

Approximately half the peaks were determined to be homogeneous, but the separation of all molecular components was not achieved with ceramides that had the same effective methylene numbers (8); such as, d18:1–C20:0 vs d20:1–C18:0, and d18:1–C22:0 vs d18:1–C24:1. Therefore, percentages of each component in the heterogeneous peaks were calculated from the ratio of fatty acids in each peak measured by GLC. In the case of peak 2 of G_{M3}, G_{M2}, and G_{M1}, the ratio of intensity of long chain base ions in their mass spectra was used to determine the proportion of each peak component.

TABLE 2. Distribution of ceramide molecular species from G_{M4}

Peak Number[a]	Molecular Species	G_{M4}
1	d18:0–C16:0	1.46 ± 0.68
2	d18:1–C18:0	0.97 ± 0.59
3	Unidentified	0.31 ± 0.09
4	d18:0–C18:0	0.93 ± 0.58
5	Unidentified	0.71 ± 0.26
6	d18:0–C20:0(OH)[b]	0.56 ± 0.31
7	d18:0–C20:0[b]	0.77 ± 0.45
8	d18:1–C21:0[b]	1.02 ± 0.56
9	Unidentified	0.23 ± 0.19
10	d18:1–C22:0(OH)	2.42 ± 0.35
	d18:1–C24:1(OH)	17.75 ± 2.54
11	d18:1–C24:1	7.79 ± 2.92
12	d18:1–C24:1	0.86 ± 0.72
13	d18:1–C23:0(OH)	10.18 ± 0.41
	d18:1–C25:1(OH)	4.79 ± 0.19
14	d18:1–C25:1	2.84 ± 2.69
15	d18:1–C24:0(OH)	20.84 ± 3.77
	d18:1–C26:1(OH)	3.97 ± 0.72
16	d18:1–C24:0	1.91 ± 0.76
	d18:1–C26:1	1.12 ± 0.45
17	Unidentified	2.66 ± 1.55
18	d18:1–C25:0(OH)	3.30 ± 0.57
19	d18:1–C25:0	2.27 ± 1.44
20	d18:1–C26:0(OH)	2.13 ± 0.80
Remaining peaks[c]		0.17 ± 0.03
Recovery		91.99 ± 5.98

Results are shown as the mean ± SD from triplicate HPLC separation of G_{M4} molecular species (see Fig. 1). Each peak collected from a C18 reversed-phase HPLC column was perbenzoylated and then quantitated by normal-phase HPLC on a LiChrosphere SI 4000 column (15). The amounts of individual molecular species in heterogeneous peaks were calculated from the results of fatty acid analysis (See Methods).

[a] From chromatogram shown in Fig. 1.

[b] Peaks were identified by their retention times.

[c] Remaining peaks = gangliosides between major peaks.

The molecular species of G_{M4} that contained hydroxy fatty acids separated from the components that contained non-hydroxy fatty acids. In addition, we were able to detect and measure d18:0 sphinganine in G_{M4}, even though the total amounts were approximately 3%. A similar amount of d18:0 sphinganine was also found by long chain base analysis of the unfractionated G_{M4}. Peak 12 of G_{M4} was identified as containing C24:1 fatty acid and it may differ from peak 11 only by the position of the double bond.

G_{M3}, G_{M2}, and G_{M1} contained essentially the same ceramide molecular species (Table 3), but their relative amounts were clearly different. Peak 6 (d18:1–C18:0) from G_{M3}, G_{M2}, and G_{M1} may contain small amounts of d20:1–C16:0 but, if present, this minor component was not detected either by MS or long chain base analysis. Although they are minor components, sphingadienines (d18:2 and d20:2) paired with C18:0 fatty acid (peaks 3 and 7) were identified in these monosialogangliosides. The mass spectra of peaks X_1 and X_2 from G_{M3} and G_{M2} showed identical fragmentation patterns as the following peaks 6 and 11, respectively. Further identification of these minor components was not attempted.

DISCUSSION

In this study, we have described a reversed-phase HPLC method that permits separation of the intact molecular species of G_{M4}, G_{M3}, G_{M2}, and G_{M1}. This procedure permitted good separation with as much as 400 μg of monosialogangliosides applied to the HPLC column. The use of larger samples of either G_{M3}, G_{M2}, or G_{M1} resulted in obliteration of the small peaks adjacent to predominant peaks, especially those near d18:1–C18:0. When quantitative analysis by HPLC of the perbenzoylated derivatives of the separated species is to be performed, at least 25 μg of gangliosides is needed. However, if only qualitative information about the molecular species present is desired, as little as 5 μg of gangliosides can be used.

The retention time of a ganglioside molecular species on reversed-phase HPLC depends both on its long chain base and fatty acid composition. In addition, it was noted that the retention times of all peaks are significantly increased at lower column temperatures (more methanol is required to maintain comparable retention times at lower temperatures). However, better resolution of the peaks, especially peaks 4, 7, and 8 of G_{M3}, G_{M2}, and G_{M1}, is obtained with more water in the mobile phase as shown in Fig. 1 (inset of G_{M1}). Similarly, peaks 6 and 7 of G_{M4} were actually heterogeneous and can be resolved by addition of more water to the mobile phase.

TABLE 3. Distribution of molecular species from G_{M3}, G_{M2}, and G_{M1}

Peak Number[a]	Molecular Species	G_{M3}	G_{M2}	G_{M1}
1	d18:1–C14:0	0.32 ± 0.08	0.28 ± 0.06	0.26 ± 0.12
2	d16:1–C18:0	0.51 ± 0.13	0.33 ± 0.07	0.20 ± 0.09
	d18:1–C16:0	2.05 ± 0.52	1.32 ± 0.30	0.15 ± 0.07
3	d18:2–C18:0	1.20 ± 0.98	1.42 ± 0.47	0.77 ± 0.23
4	d16:0–C18:0	0.46 ± 0.12	0.56 ± 0.31	0.18 ± 0.01
5	d18:1–C17:0	0.31 ± 0.08	0.21 ± 0.05	ND[b]
X1	Unidentified	0.22 ± 0.07	0.33 ± 0.07	ND
6	d18:1–C18:0	39.94 ± 4.55	35.93 ± 6.09	25.50 ± 1.58
7	d20:2–C18:0	ND	ND	0.54 ± 0.39
8	d16:0–C20:0	0.37 ± 0.22	0.81 ± 0.43	0.60 ± 0.28
9	d18:0–C18:0	1.39 ± 0.43	2.60 ± 0.65	1.94 ± 0.37
10	d18:1–C19:0	0.75 ± 0.41	0.67 ± 0.47	0.29 ± 0.07
X2	Unidentified	0.53 ± 0.16	0.54 ± 0.12	ND
11	d18:1–C20:0	3.33 ± 0.50	6.37 ± 1.08	7.39 ± 0.98
	d20:1–C18:0	15.15 ± 2.26	33.45 ± 5.67	45.37 ± 6.01
12	d18:0–C20:0	0.10 ± 0.04	0.51 ± 0.07	0.40 ± 0.06
	d20:0–C18:0	0.79 ± 0.32	2.89 ± 0.39	3.60 ± 0.51
13	d18:1–C21:0	0.28 ± 0.10	ND	ND
	d18:1–C23:1	1.70 ± 0.59	ND	ND
14	d18:1–C22:0	2.20 ± 0.60	0.47 ± 0.17	0.52 ± 0.05
	d18:1–C24:1	11.89 ± 3.21	1.26 ± 0.45	0.75 ± 0.07
	d20:1–C20:0	0.59 ± 0.16	1.41 ± 0.51	1.98 ± 0.20
15	d20:0–C20:0	ND	ND	0.30 ± 0.05
16	d18:1–C23:0	0.91 ± 0.13	0.36 ± 0.10	0.26 ± 0.20
	d18:1–C25:1	1.31 ± 0.18	0.43 ± 0.12	0.27 ± 0.21
17	d18:1–C24:0	1.41 ± 1.11	0.49 ± 0.11	0.14 ± 0.10
	d18:1–C26:1	0.53 ± 0.42	0.25 ± 0.05	0.11 ± 0.07
	d20:1–C22:0	0.07 ± 0.06	0.17 ± 0.04	0.15 ± 0.10
	d20:1–C24:1	0.41 ± 0.33	0.32 ± 0.07	0.23 ± 0.16
Remaining peaks[c]		0.40 ± 0.10	0.61 ± 0.44	0.67 ± 0.35
Recovery		89.09 ± 2.91	93.12 ± 15.48	92.54 ± 8.69

Results are shown as the mean ± SD from triplicate HPLC separation of the G_{M3}, G_{M2}, and G_{M1} (see Fig. 1). Each peak collcted from a C18 reversed-phase HPLC column was perbenzoylated and then quantitated by normal-phase HPLC on a LiChrosphere SI 4000 column (15). The amounts of individual molecular species in heterogeneous peaks were calculated from the results of long chain base and fatty acid analyses (see Methods).

[a] From chromatograms shown in Fig. 1.

[b] ND, not detectable.

[c] Remaining peaks = gangliosides between major peaks.

It may be possible to further resolve the components of heterogeneous peaks (such as peaks 10, 13, 15, and 16 of G_{M4} and 13, 14, 16, and 17 of the other gangliosides) by silica bonded silver columns (9) or by addition of a small percent of acetonitrile to the mobile phase (17).

The relative retention times (RRT) of the major molecular species of G_{M4}, G_{M3}, G_{M2}, and G_{M1} are shown in **Table 4.** The RRTs (to the d18:1–C18:0 peak) of any particular ceramide compositions were the same for all of the monosialoganglioside classes studied. Thus, within any class of monosialogangliosides, the order of elution of molecular species was constant and entirely independent of the composition of the component of sugars. A graphic relationship of RRT (actually the log of RRT × 10) for all the monosialoganglioside components is shown in **Fig. 3.** The abscissa provides the effective carbon number of fatty acid and the right hand upper oblique line provides the effective carbon number of the long chain base. A hydroxy group in the 2 position of the fatty acid moiety results in an effective carbon number which is 0.50 of a unit less than that of

TABLE 4. Relative retention times of the molecular species of G_{M4}, G_{M3}, G_{M2}, and G_{M1} from human brain

Molecular Species	RRT	Molecular Species	RRT
d18:1–C14:0	0.278	d18:1–C24:1(OH)	3.056
d16:1–C18:0	0.526	d18:1–C22:0	3.336
d18:1–C16:0	0.526	d18:1–C24:1	3.336
d18:2–C18:0	0.616	d20:1–C20:0	3.336
d16:0–C18:0	0.656	d18:1–C23:0(OH)	3.889
d18:0–C16:0	0.694	d18:1–C25:1(OH)	3.889
d18:1–C17:0	0.723	d20:0–C20:0	4.329
d18:1–C18:0	1.000	d18:1–C23:0	4.457
d20:2–C18:0	1.151	d18:1–C25:1	4.457
d16:0–C20:0	1.192	d18:1–C24:0(OH)	4.917
d18:0–C18:0	1.275	d18:1–C26:1(OH)	4.917
d18:1–C19:0	1.358	d18:1–C24:0	5.900
d18:1–C20:0	1.815	d18:1–C26:1	5.900
d20:1–C18:0	1.815	d20:1–C22:0	5.900
d18:0–C20:0	2.315	d20:1–C24:1	5.900
d20:0–C18:0	2.315	d18:1–C25:0(OH)	6.778
d18:1–C21:0	2.444	d18:1–C25:0	7.656
d18:1–C23:1	2.444	d18:1–C26:0(OH)	9.333
d18:1–C22:0(OH)	3.056		

The relative retention times (RRT) of all peaks were determined by dividing the retention time of each peak by the retention time of d18:1–C18:0.

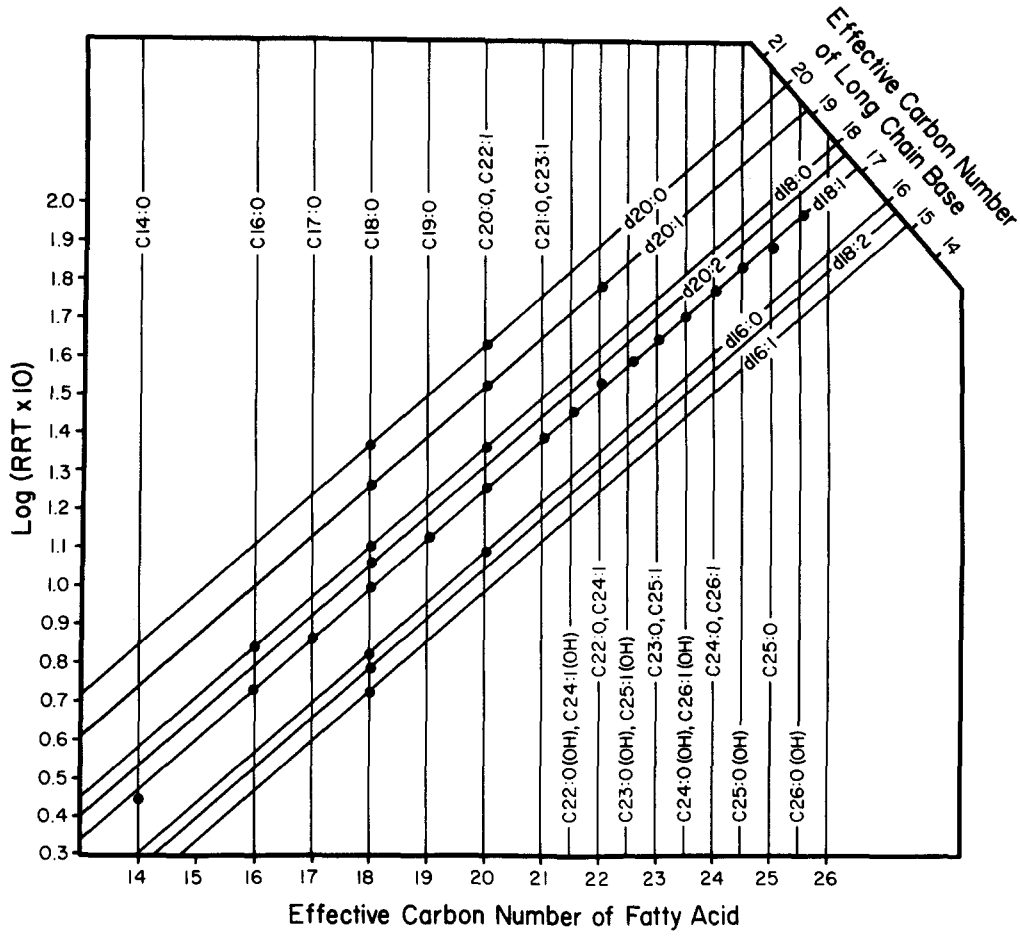

Fig. 3. The effect of fatty acid and long chain base composition on the elution pattern of molecular species of monosialogangliosides. The RRT calculated for each molecule (Table 3) was plotted as the log of the RRT × 10 against the carbon number of the fatty acid. Oblique lines connect points that have the same long chain bases. The intersection of oblique and perpendicular lines provides the RRT of individual molecules.

the corresponding non-hydroxy fatty acid. A constant pattern based on carbon number was also evident for the long chain bases. The effective carbon numbers of d16:1, d18:1, and d20:1 sphingosines are 0.78 carbon unit less than that of corresponding sphinganines. On the other hand, the effective carbon numbers of d18:2 and d20:2 sphingadienines are 2.35 carbon unit less than that of corresponding sphinganines. Therefore, the second double bond of these sphingadienines reduces the effective carbon number twice as much as the first double bond of sphingosines. The total effective carbon number of the ceramide moiety can be obtained by addition of the effective carbon numbers of both the fatty acid and long chain base. Furthermore, it is possible to predict by use of this type of plot the effective carbon number of various molecular species which have not actually been identified.

The gangliosides analyzed in this study were isolated from a single normal human adult brain and generalization from these data about the molecular species composition must be made with caution. The purification of the four individual monosialogangliosides by Iatrobead chromatography involved re-chromatography of overlapping portions of the components and recoveries were estimated to be over 95% for G_{M4} and G_{M3}, and over 90% for G_{M2} and G_{M1}. However, the small quantities discarded from the leading and trailing edge of these peaks could conceivably result in losses of minor molecular species. Nevertheless, the monosialoganglioside preparations subjected to reversed-phase HPLC were highly purified, and the molecular species detected should reflect natural components. All of the molecular species separated by reversed-phase HPLC were subsequently quantitated by normal-phase HPLC of their perbenzoylated derivatives and the presence of other monosialoganglioside classes was not detected in any of the peaks analyzed.

By the direct separation of intact molecular species, it was possible to demonstrate the existence of sphingadienines in gangliosides. The occurrence of sphingadienines in sphingolipids from various sources has been reported by several investigators (18–20). To our knowl-

edge, however, this is the first report of the identification of sphingadienines in human brain gangliosides. Although major long chain bases in brain G_{M3}, G_{M2}, and G_{M1} are known to be d18:1 and d20:1, odd carbon number fatty acids were found paired with only d18:1 sphingosine in these human brain monosialogangliosides. It is of interest to note that the percentage compositions of d20:1 and d20:0 long chain bases increase in the order $G_{M3} < G_{M2} < G_{M1}$. This is reminiscent of the report by Yohe, Roark, and Rosenberg (21) that the d20:1 sphingosine content increases as a function of the number of sialic acid residues in gangliosides. Whether or not the content of the d20 long chain bases in gangliosides also varies as a function of the number of sugar residues requires analysis of additional individual samples. Also of interest is the percentage composition of d18:1–C24:1, which varies in the opposite order $G_{M3} > G_{M2} > G_{M1}$.

In conclusion, we have described reversed-phase HPLC conditions for the separation of intact molecular species of monosialogangliosides. The separation of the molecular species of monosialogangliosides from normal adult human brain allowed the detection of minor species not previously reported and the observation that the percentage composition of d20 long chain bases increases in the order $G_{M3} < G_{M2} < G_{M1}$. This methodology will be useful not only for the analysis of actual molecular species of gangliosides but also for the preparation of ganglioside molecular species for physical, chemical, and biochemical studies.

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