Separation of derivatized glycosphingolipids into individual molecular species by high performance liquid chromatography

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Summary The high performance liquid chromatography separation of the perbenzoyl derivatives of the neutral glycosphingolipids (GlcCer, LacCer, $GbOse_3Cer$, $GbOse_4Cer$, and $GgOse_3Cer$) and the *p*-bromophenacyl and 2,4-dinitrophenyl hydrazide derivatives of the gangliosides (G_{M4}, G_{M3}, G_{M2}, G_{M1}, G_{D1a}) into individual molecular species on a C18 reversed-phase column is described. Peaks were identified by comparing their relative retention times to the relative retention time of the corresponding glycosphingolipid of known molecular species composition. As little as 5 to 10 pmol of each molecular species of neutral glycosphingolipids and 3 to 5 pmol of the gangliosides can be detected. The effects of changes in the proportion of acetonitrile, methanol, and water in the mobile phase and of column temperature on the molecular species separation are described. A procedure for the tentative identification of glycosphingolipid molecular species based on their relative retention times is presented.—**Kadowaki, H., K. E. Rys-Sikora, and R. S. Koff.** *Separation of derivatized glycosphingolipids into* individual molecular species by high performance liquid chromatography. *J. Lipid Res.* 1989. **30:** 616–627.

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Abbreviations: GlcCer, Glcβ1-1ceramide; LacCer, Galβ1-4Glcβ1-1ceramide; $GbOse_3Cer$, Galα1-4Galβ1-4Glcβ1-1ceramide; $GbOse_4Cer$, GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1ceramide; $GgOse_3Cer$, GalNAcβ1-4Galβ1-4Glcβ1-1ceramide; $GgOse_4Cer$, Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1ceramide; G_{M4}, NeuAcα2-3Galβ1-1ceramide; G_{M3},NeuAcα2-3Galβ1-4Glcβ1-1ceramide; G_{M2}, GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1ceramide; G_{M1}, Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1ceramide; G_{D1a}, NeuAcα2-3Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1ceramide; HPLC, high performance liquid chromatography; HPTLC, high performance thin-layer chromatography; LC–MS, liquid chromatography–mass spectrometry; GLC, gas–liquid chromatography; RRT, relative retention time. The molecular species abbreviations suggested by Breimer, Karlsson, and Samuelsson (27) are utilized throughout. For example, in the notation d18:1–18:0, the d18:1 represents the long chain base sphingosine (1,3 dihydroxy-2-aminooctadecene) and 18:0 represents the fatty acid (octadecanoic acid); d18:1–h24:1 represents a molecular species containing sphingosine (d18:1) and the hydroxy fatty acid (2-hydroxytetracosenoic acid).

The quantitative analysis of glycosphingolipid classes is frequently performed by normal phase HPLC following derivatization of the glycosphingolipids with an ultraviolet-absorbing chromophore. The introduction of the chromophore into the molecules not only increases the sensitivity of the analysis but also permits quantitation of the glycosphingolipid classes by simply integrating the area under the peaks. Procedures have been reported for converting the carbohydrate and fatty acid hydroxyl groups of the neutral glycosphingolipids to the benzoyl ester (1), and for benzoylating not only the hydroxyl groups but also the amide nitrogens of both the sugar and the ceramide (2). Similarly, the hydroxyl groups and amide nitrogens of the acidic glycosphingolipids (gangliosides) can also be benzoylated (3, 4) or the carboxyl group of sialic acid can be converted to the *p*-bromophenacyl ester (5) or to the 2,4-dinitrophenylhydrazide (6). In addition those neutral and acidic glycosphingolipids that do not contain hydroxy fatty acids can be converted to the O-acetyl-N-*p*-nitrobenzyl derivatives (7, 8).

While there is considerable interest in determining the molecular species composition of glycosphingolipids in brain and various other tissues, there are only two reports describing the molecular species separation of derivatized glycosphingolipids. These studies were limited to the most common neutral glycosphingolipid classes and one ganglioside G_{M3} (7, 9). However, no systematic study of the factors affecting the molecular species separation of derivatized glycosphingolipids has been reported.

In this report we have extended those earlier studies to more complex glycosphingolipids and a wider range of derivatives. In particular, we have examined the reversed-phase molecular species separation of the major glycosphingolipids after derivatization by the most commonly used derivatization procedures, i.e., the perbenzoyl derivatives of the neutral glycosphingolipids (2), and of the *p*-bromophenacyl (5) and 2,4-dinitrophenylhydrazide (6) derivatives of the gangliosides. We have also examined how the column temperature and the proportion of acetonitrile in a methanol–water mobile phase affect the resolution of the molecular species. Moreover, we demonstrate that, in most cases, the long chain base and fatty acid composition of a particular peak can be determined by the retention time of the peak.

EXPERIMENTAL PROCEDURES

Materials

Analytical and HPLC grade solvents were obtained from Fisher Scientific (Medford, MA); *p*-bromophenacylbromide from Pierce (Rockford, IL) was recrystallized as previously described (10). 2,4-Dinitrophenylhydrazine hydrochloride was prepared according to the method of Miyazaki et al. (6) from dinitrophenylhydrazine (Sigma, St. Louis, MO) and methanolic-HCl (Supelco, Bellefonte, PA). Unisil was from Clarkson (Williamsport, PA) and HPTLC plates were from EM Science (Cherry Hill, NJ). Glucosylceramide (GlcCer) and G_{D1a} were obtained from Sigma, and globotriaosylceramide ($GbOse_3Cer$), globotetraosylceramide ($GbOse_4Cer$), and bovine brain gangliosides were from Supelco. Lactosylceramide (LacCer) was prepared from human red blood cells. Red blood cell neutral glycosphingolipids were isolated according to the method of Gross and McCluer (1) and converted to LacCer by enzymatic cleavage of the total neutral glycosphingolipids as previously described (11). G_{M3} was isolated from human and dog erythrocytes (PelFreeze, Rogers, AR) and G_{M4}, G_{M2}, and G_{M1} were isolated from human brain as previously described (12). Neuraminidase (*Vibrio cholerae*) was obtained from Calbiochem-Behring (La Jolla, CA), and β-galactosidase (jack beans), α-galactosidase (green coffee beans), and β-N-acetylhexosaminidase (jack beans) were obtained from Sigma.

Instruments

HPLC was performed with a Shimadzu (Columbia, MD) LC-6A liquid chromatograph equipped with a Rheodyne (Cotati, CA) model 7125 syringe-loading sample injector, a Shimadzu SPD-6A variable wavelength UV detector, and a Shimadzu C-R3A Chromatopac integrator. Molecular species separations were performed using a 5-μ particle, 2 × 250 mm Ultrasphere ODS column (Beckman, Fullerton, CA) which was maintained at 30°C with a Rainin (Woburn, MA) column temperature controller.

Preparation of $GgOse_3Cer$

Gangliotriaosylceramide ($GgOse_3Cer$) was prepared from bovine brain gangliosides. Gangliosides (10 mg) were incubated with 2.0 ml of 0.05 N sulfuric acid at 80°C for 2 hr. The resulting hydrolyzate was neutralized with sodium hydroxide and 10 ml of chloroform–methanol 2:1 was added to make a Folch et al. partition condition (13). After vigorous mixing the two solvent phases were separated by centrifugation. The neutral glycosphingolipids (whose major component was $GgOse_4Cer$) were recovered in the lower-phase. The lower-phase was dried under nitrogen and the terminal galactose of $GgOse_4Cer$ was cleaved by reaction with β-galactosidase as described below, and then the solvent system was adjusted to the Folch partition condition. The lower-phase was dried under nitrogen, redissolved in 10 ml of chloroform–methanol 96:4, and applied to a Unisil (100–200 mesh) column (0.8 × 3 cm). The column was eluted with a stepwise gradient of increasing proportions of methanol in chloroform from 4% to 20% methanol in 2% intervals of 20 ml each. An aliquot of each fraction was monitored

by HPTLC developed with chloroform–methanol–water 60:35:8 and visualized by spraying with orcinol–sulfuric acid reagent (14) and heating at 130°C. The fractions containing $GgOse_3Cer$ were combined.

Conditions for enzymatic hydrolysis of glycosphingolipids

Neuraminidase. Gangliosides (up to 1 mg) were incubated for 2 hr at 37°C with 0.2 units of neuraminidase in 0.2 ml of 0.05 M acetate buffer (pH 5.5) containing 0.025 M calcium chloride. When G_{M3} was the substrate, the resulting LacCer was recovered in the Folch partition lower-phase. When G_{D1a} was the substrate, the resulting G_{M1} was recovered in the Folch partition upper-phase.

β-galactosidase. $GgOse_4Cer$ or LacCer (up to 1 mg) was mixed with 20 mg of sodium taurodeoxycholate in methanol and dried under nitrogen. The sample was incubated for 16 hr at 37°C with 4 units of β-galactosidase in 1.0 ml of 0.1 M citrate buffer (pH 4.0). The resulting $GgOse_3Cer$ or GlcCer was recovered in the Folch partition lower-phase.

α-galactosidase. $GbOse_3Cer$ (up to 1 mg) was mixed with 1 mg of sodium taurodeoxycholate in methanol and dried under nitrogen. The sample was incubated for 16 hr at 37°C with 1 unit of α-galactosidase in 0.5 ml of 0.2 M citrate buffer (pH 5.0). The resulting LacCer was recovered in the Folch partition lower-phase.

β-N-acetylhexosaminidase. $GbOse_4Cer$ or $GgOse_3Cer$ (up to 1 mg) was mixed with 15 mg of sodium taurodeoxycholate in methanol and dried under nitrogen. The sample was incubated for 16 hr at 37°C with β-N-acetylhexosaminidase (15 units for $GbOse_4Cer$ and 30 units for $GgOse_3Cer$) in 1.0 ml of 0.1 M citrate buffer (pH 4.0). The resulting $GbOse_3Cer$ or LacCer was recovered in the Folch partition lower-phase.

Derivatization of glycosphingolipids

Neutral glycosphingolipids (GlcCer, LacCer, $GbOse_3Cer$, $GbOse_4Cer$, $GgOse_3Cer$) were converted to O- and N-benzoyl derivatives by reaction with benzoylchloride in pyridine as previously described (2). Gangliosides (G_{M4}, G_{M3}, G_{M2}, G_{M1}, G_{D1a}) were converted to the *p*-bromophenacyl ester of the carboxyl groups of N-acetylneuraminic acid by reaction with *p*-bromophenacyl bromide in dimethylformamide as previously described (5). Gangliosides (G_{M3}, G_{M1}, G_{D1a}) were also converted to the 2,4-dinitrophenylhydrazide by reaction with 2,4-dinitrophenylhydrazine hydrochloride and dicyclohexylcarbodiimide in dimethylformamide as previously described (6).

Identification of molecular species

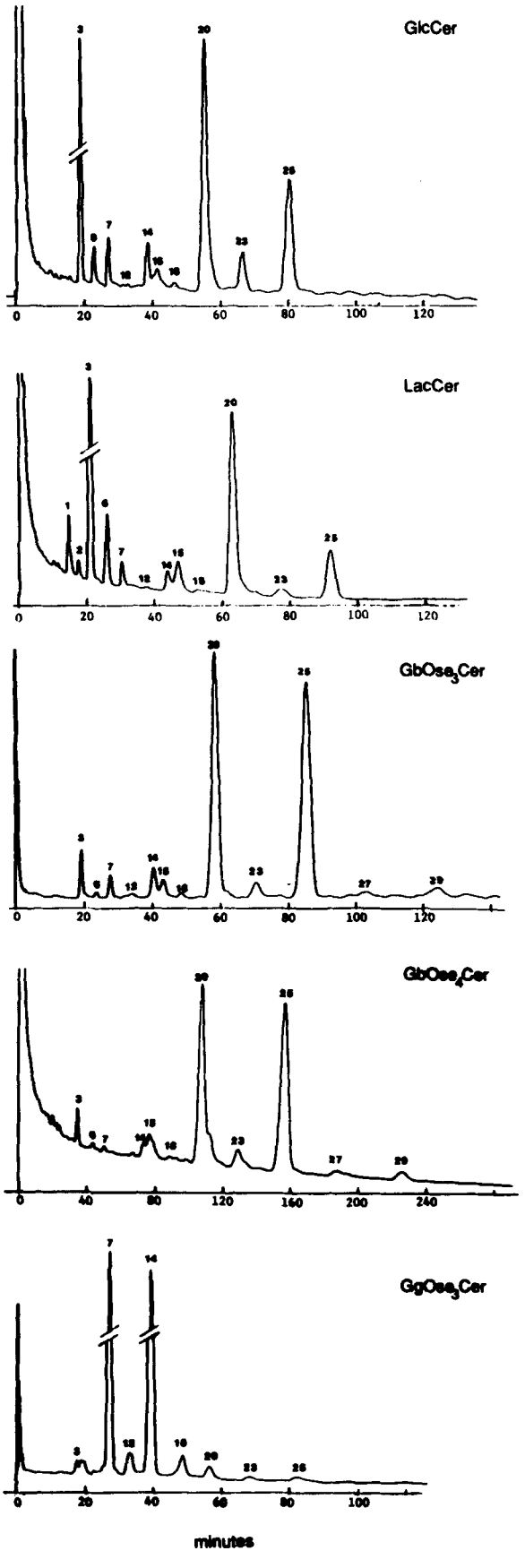
The LacCer molecular species were identified by comparison of their retention times to the retention times of LacCer obtained from G_{M3} of known molecular species composition by the cleavage of N-acetylneuraminic acid with neuraminidase as described above. We have previously determined the molecular species composition of underivatized G_{M3} by GLC and HPLC analysis of the hydrolysis products and by LC–MS of the intact molecular species (12). GlcCer molecular species were identified by comparison of their retention times to the GlcCer produced by β-galactosidase cleavage of the LacCer of known molecular species composition described above. Likewise, $GbOse_3Cer$, $GbOse_4Cer$, and $GgOse_3Cer$ were converted to LacCer by enzymatic hydrolysis and their molecular species were identified by comparison of their retention times to those of the LacCer of known composition described above. $GbOse_3Cer$ was converted to LacCer by cleavage with α-galactosidase; $GbOse_4Cer$ by cleavage with β-N-acetylhexosaminidase and α-galactosidase; and $GgOse_3Cer$ by cleavage with β-N-acetylhexosaminidase. The molecular species of the derivatized monosialogangliosides were identified by comparison of their relative retention times (RRTs) to the RRTs of the corresponding gangliosides whose molecular species compositions have been previously determined (12). G_{D1a} molecular species were identified by comparison of their retention times to G_{M1} following cleavage of the terminal N-acetylneuraminic acid of G_{D1a} with neuraminidase.

RESULTS

Separation of glycosphingolipid molecular species

The molecular species separation of the O- and N-benzoyl (perbenzoyl) derivatives of the major neutral glycosphingolipids (GlcCer, LacCer, $GbOse_3Cer$, $GbOse_4Cer$, and $GgOse_3Cer$) is presented in **Fig. 1.** The molecular species separation of the *p*-bromophenacyl esters of the gangliosides, G_{M4}, G_{M3}, G_{M2}, G_{M1}, and G_{D1a} is presented in **Fig. 2** and of the 2,4-dinitrophenylhydrazides of G_{M3}, G_{M1}, and G_{D1a} in **Fig. 3.**

For each class of neutral glycosphingolipids the greater the number of carbohydrates the longer the retention time of any particular molecular species, hence, different flow rates were required in order to obtain chromatograms with comparable retention times (Fig. 1). For the more complex (longer carbohydrate chain) neutral glycosphingolipids, such as $GbOse_4Cer$, the molecular species resolution was not as good as for the simpler (shorter carbohydrate chain) glycosphingolipids. This is probably due to the relatively diminished influence of the acyl chains compared to the greater influence of the 15 benzoyl groups (12 benzoyl groups on the hydroxyl groups of the carbohydrates, one on the hydroxyl group of the long chain base, and one each on the amide nitrogen of ceramide and N-acetylgalactosamine). As also shown in Fig. 1, the use of a slower flow rate (0.5 ml/min), which allows for the near



equilibrium partitioning of the derivatized $GbOse_4Cer$ between the mobile phase and the stationary phase, increases the relative influence of the acyl groups and thus improves the resolution but greatly increases the elution time. No such relationship between the number of carbohydrate moieties and retention time was observed for the gangliosides (Figs. 2 and 3) since, with the exception of G_{Dla}, the number of chromophores per molecule is the same. (Both *p*-bromophenacyl bromide and 2,4-dinitrophenylhydrazine hydrochloride derivatize only the sialic acid carboxyl group of the gangliosides.)

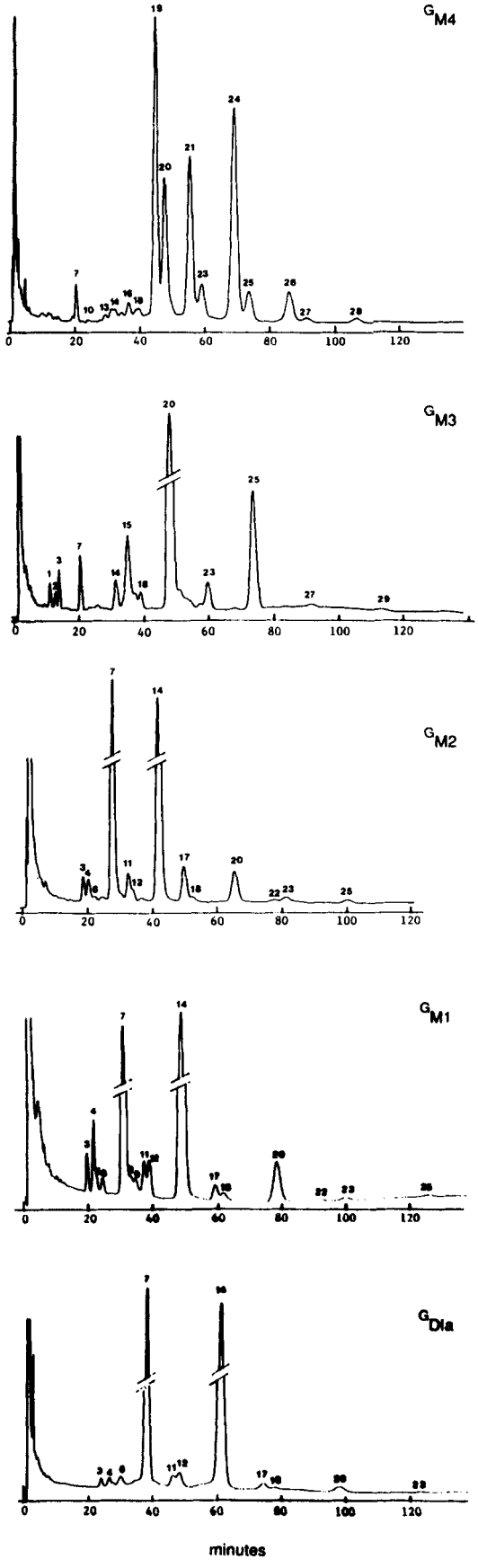
Molecular species composition and the RRTs

The molecular species composition and the RRTs of the neutral glycosphingolipid peaks are shown in **Table 1,** and the molecular species composition and the RRTs of the *p*-bromophenacyl and 2,4-dinitrophenylhydrazide derivatives of the gangliosides are shown in **Table 2** and **Table 3,** respectively. The RRT is calculated by dividing the retention time of each peak by the retention time of the d18:1-18:0 molecular species. Although there are differences in the absolute retention times of a given molecular species among the various neutral glycosphingolipid classes, the RRTs of the perbenzoyl derivatives of the neutral glycosphingolipids are the same for all the neutral glycosphingolipid classes regardless of the number of carbohydrates (Table 1). Similarly, for both the *p*-bromophenacyl esters and the 2,4-dinitrophenylhydrazides, the RRTs of all the ganglioside classes are the same for a given derivative, even though the RRTs differ between the different types of derivatives (Tables 2 and 3).

Effect of acetonitrile on the separation of glycosphingolipid molecular species

Under the conditions employed here (methanol–water) there is overlap of those molecular species that contain the same long chain base but have fatty acids that differ by two methylene groups and one double bond such as d18:1-22:0 and d18:1-24:1, peak 20. A pair of molecular species of this type can be resolved by replacing a portion of the methanol in the mobile phase with acetonitrile. An example of how increasing the proportion of acetonitrile in the mobile phase alters the relative positioning of the

Fig. 1. Molecular species separation of perbenzoylated neutral glycosphingolipids. GlcCer (2.00 nmol), LacCer (1.65 nmol), $GbOse_3Cer$ (0.98 nmol), $GbOse_4Cer$ (1.17 nmol), and $GgOse_3Cer$ (0.71 nmol) were separated into individual molecular species by HPLC. The mobile phase was methanol. The flow rate, which varied depending upon the number of carbohydrates, was: GlcCer, 0.5 ml/min; LacCer, 0.6 ml/min; $GbOse_3Cer$, 0.7 ml/min; $GbOse_4Cer$, 0.5 ml/min; and $GgOse_3Cer$, 0.8 ml/min. The column effluent was monitored at 230 nm. The detector sensitivity for GlcCer and $GbOse_3Cer$ was 0.04 absorbance unit full scale (AUFS), and for the others was 0.08 AUFS. The peak numbers shown in the chromatograms correspond to the peak numbers in Table 1.



molecular species of the 2,4-dinitrophenylhydrazides of G_{M3} is shown in **Fig. 4.** The addition of increasing amounts of acetonitrile improves the separation of those pairs of molecular species, such as d18:1-22:0 and d18:1-24:1, d18:1-23:0 and d18:1-25:1, and d18:1-24:0 and d18:1-26:1. However, as can also be seen in Fig. 4, the d18:1-20:0 (peak 14) and d18:1-24:2 (peak 15) gradually merge as the acetonitrile concentration increases. The more acetonitrile in the mobile phase, however, the longer the retention time of any given molecular species. Therefore, in order to obtain a comparable retention time, it is necessary to increase the flow rate or decrease the amount of water.

DISCUSSION

Glycosphingolipids are relatively minor components of most tissues, yet they have been implicated in many cell surface-related functions, such as the regulation of cellular differentiation, development, and growth (15–17) as well as being the receptors for a variety of molecules (18–21). Studies of glycosphingolipid function or metabolism and studies of the glycosphingolipid molecular species composition of those cell types which can only be obtained in small quantities have been hindered by the lack of accurate and sensitive methods of analysis. There are currently several procedures for the analysis of glycosphingolipid molecular species. In the first procedure, the fatty acid and long chain base compositions of the purified glycosphingolipid classes are determined by HPLC of the long chain base (22, 23) and GLC of the fatty acids after acid hydrolysis. The disadvantages of this method of ceramide analysis are that acid hydrolysis results in poor recoveries and chemical modification of the long chain base including stereochemical isomerization, and hydroxyl group and double bond migrations as well as the formation of numerous unspecified artifacts (22). In addition, the long chain base and fatty acid analyses are not as sensitive as the analysis of the glycosphingolipid classes, and when there is more than one type of long chain base the actual ceramide molecular species composition cannot be unambiguously determined.

Another method of glycosphingolipid molecular species analysis which has undergone rapid development in re-

Fig. 2. Molecular species separation of *p*-bromophenacyl esters of gangliosides. G_{M4} (1.16 nmol), G_{M3} (0.74 nmol) isolated from dog erythrocytes, G_{M2} (0.60 nmol), G_{M1} (1.69 nmol), and G_{DIa} (0.95 nmol) were separated into individual molecular species by HPLC. The mobile phase for G_{M4}, G_{M3}, and G_{M2} was methanol-water 96:4 (v/v), and for G_{M1} and G_{DIa} was methanol-water 94:6 (v/v). The flow rate for G_{M4} and G_{M3} was 0.3 ml/min, for G_{M2} was 0.2 ml/min, and for G_{M1} and G_{DIa} was 0.3 ml/min. The column effluent was monitored at 261 nm and the detector sensitivity was 0.02 AUFS. The peak numbers shown in the chromatograms correspond to the peak numbers in Table 2.

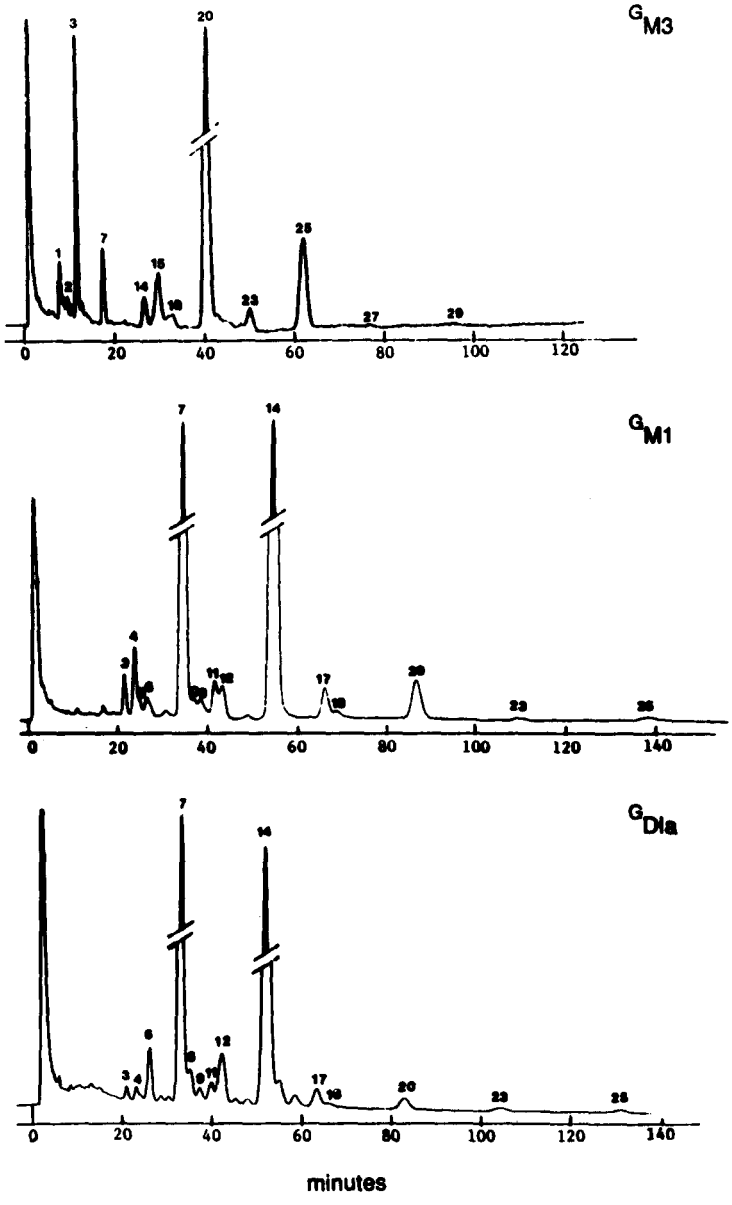


Fig. 3. Molecular species separation of 2,4-dinitrophenylhydrazide derivatives of gangliosides. G_{M3} (0.60 nmol) isolated from human erythrocytes, G_{M1} (2.18 nmol) and G_{DIa} (0.62 nmol) were separated into individual molecular species by HPLC. The mobile phase for G_{M3} was methanol-water-acetic acid 96:4:0.02 (v/v/v), and for G_{M1} and G_{DIa} was methanol-water-acetic acid 94:6:0.02 (v/v/v). The flow rate for G_{M3} was 0.3 ml/min, and for G_{M1} and G_{DIa} was 0.2 ml/min. The column effluent was monitored at 342 nm and the detector sensitivity for G_{M3} was 0.01 AUFS, and for G_{M1} and G_{DIa} was 0.02 AUFS. The peak numbers shown in the chromatograms correspond to the peak numbers in Table 3.

cent years is mass spectrometry (MS). This procedure involves the preliminary separation of the derivatized or underivatized glycosphingolipid classes into individual molecular species on a reversed-phase column and then either collecting the peaks for later MS analysis or introducing the column effluent directly into the MS (LC-MS) (12). Although this procedure is very helpful for identifying the molecular species in a given peak, the quantitative analysis of glycolipid molecular species by LC-MS has not been reported. However, Evans and McCluer (24) have reported the quantitative analysis of neutral glycosphingolipid classes by LC-MS. Even if these MS techniques were applied to the quantitative analysis of molecular species, the method would still require relatively large amounts of material (at least 1 nmol) compared to that usually available from class analysis. Moreover, when

TABLE 1. Relative retention times of benzoylated neutral glycosphingolipid molecular species

Peak No.[a]	Molecular Species	GlcCer	LacCer	$GbOse_3Cer$	$GbOse_4Cer$	$GgOse_3Cer$	Mean ± SD
1	d18:1-14:0		0.500				0.500
2	d18:1-15:0[b]		0.592				0.592
3	d18:1-16:0	0.707	0.707	0.698	0.697	0.699	0.702 ± 0.004
6	d18:1-17:0	0.848	0.860	0.836	0.867		0.852 ± 0.012
7	d18:1-18:0	1.000	1.000	1.000	1.000	1.000	1.000
12	d18:1-19:0	1.199	1.195	1.215		1.208	1.204 ± 0.008
14	d18:1-20:0	1.426	1.426	1.438	1.445	1.440	1.435 ± 0.008
15	d18:1-24:2[c]	1.532	1.525	1.539	1.506		1.526 ± 0.012
18	d18:1-21:0, d18:1-23:1	1.704	1.696	1.724		1.710	1.708 ± 0.010
20	d18:1-22:0, d18:1-24:1	2.038	2.038	2.076	2.095	2.054	2.060 ± 0.022
23	d18:1-23:0, d18:1-25:1	2.448	2.465	2.492	2.516	2.468	2.478 ± 0.024
25	d18:1-24:0, d18:1-26:1	2.932	2.950	3.013	3.056	2.968	2.984 ± 0.045
27	d18:1-25:0			3.623	3.648		3.636
29	d18:1-26:0			4.364	4.405		4.385

For each of the points in the table (except the last column), the values are the mean of three chromatograms for each glycosphingolipid class. The coefficient of variation was less than 5%. The last column "Mean ± standard deviation (SD)" is the mean ± SD of the values listed in the table, not the mean ± SD of all the individual observations.

[a]From chromatograms shown in Fig. 1.

[b]Peak was identified by the RRT (Fig. 5).

[c]Major component of the peak.

TABLE 2. Relative retention times of *p*-bromophenacyl derivatives of gangliosides

Peak No.[a]	Molecular Species	G_{M4}	G_{M3}	G_{M2}	G_{M1}	G_{D1a}	Mean ± SD
1	d18:1-14:0		0.529				0.529
2	d18:1-15:0[b]		0.610				0.610
3	d18:1-16:0, d16:1-18:0[c]		0.669	0.673	0.679	0.660	0.670 ± 0.007
4	d18:2-18:0			0.750	0.734	0.718	0.734 ± 0.013
5	d16:0-18:0				0.747		0.747
6	d18:1-17:0			0.814	0.794	0.812	0.807 ± 0.009
7	d18:1-18:0	1.000	1.000	1.000	1.000	1.000	1.000
8	d20:2-18:0				1.048		1.048
9	d16:0-20:0				1.076		1.076
10	d18:1-h19:0[b]	1.160					1.160
11	d18:0-18:0			1.171	1.176	1.218	1.188 ± 0.021
12	d18:1-19:0			1.222	1.219	1.264	1.235 ± 0.021
13	d18:1-h20:0[b]	1.433					1.433
14	d18:1-20:0, d20:1-18:0[c]	1.523	1.526	1.505	1.495	1.522	1.514 ± 0.012
15	d18:1-24:2[d]		1.699				1.699
16	d18:1-h21:0[b]	1.768					1.768
17	d18:0-20:0, d20:0-18:0[c]			1.772	1.769	1.839	1.793 ± 0.032
18	d18:1-21:0, d18:1-23:1	1.880	1.879	1.902	1.831	2.019	1.902 ± 0.063
19	d18:1-h22:0, d18:1-h24:1	2.183					2.183
20	d18:1-22:0, d18:1-24:1, d20:1-20:0[c]	2.323	2.316	2.288	2.254	2.385	2.313 ± 0.043
21	d18:1-h23:0, d18:1-h25:1	2.686					2.686
22	d20:0-20:0			2.664	2.716		2.690
23	d18:1-23:0, d18:1-25:1	2.858	2.850	2.812	2.771	3.018	2.862 ± 0.084
24	d18:1-h24:0, d18:1-h26:1	3.322					3.322
25	d18:1-24:0, d18:1-26:1, d20:1-22:0[c], d20:1-24:1[c]	3.530	3.524	3.472	3.426		3.488 ± 0.042
26	d18:1-h25:0	4.117					4.117
27	d18:1-25:0	4.367	4.379				4.373
28	d18:1-h26:0	5.103					5.103
29	d18:1-26:0		5.407				5.407

For each of the points in the table (except for the last column), the values are the mean of three to four chromatograms for each ganglioside class. The coefficient of variation was less than 3%. The last column "Mean ± SD" is the mean ± SD of the values listed in the table, not the mean ± SD of all the individual observations.

[a]From chromatograms shown in Fig. 2.

[b]Peaks were identified by their RRTs (Fig. 5).

[c]Molecular species identified in glycosphingolipids isolated from brain.

[d]Major component of the peak.

TABLE 3. Relative retention times of 2,4-dinitrophenylhydrazide derivatives of gangliosides

Peak No.[a]	Molecular Species	G_{M3}	G_{M2}	G_{M1}	G_{D1a}	Mean ± SD
1	d18:1-14:0	0.459				0.459
2	d18:1-15:0[b]	0.552				0.552
3	d18:1-16:0, d16:1-18:0[c]	0.663	0.652	0.641	0.641	0.649 ± 0.009
4	d18:2-18:0		0.708	0.707	0.704	0.706 ± 0.002
6	d18:1-17:0		0.807	0.800	0.793	0.800 ± 0.006
7	d18:1-18:0	1.000	1.000	1.000	1.000	1.000
8	d20:2-18:0			1.067	1.059	1.063
9	d16:0-20:0			1.105	1.128	1.117
11	d18:0-18:0		1.188	1.201	1.203	1.197 ± 0.007
12	d18:1-19:0		1.271	1.249	1.280	1.267 ± 0.013
14	d18:1-20:0, d20:1-18:0[c]	1.552	1.574	1.570	1.574	1.568 ± 0.009
15	d18:1-24:2[d]	1.733				1.733
17	d18:0-20:0, d20:0-18:0[c]		1.884	1.897	1.906	1.896 ± 0.009
18	d18:1-21:0, d18:1-23:1	1.919	2.019	1.965	1.971	1.969 ± 0.035
20	d18:1-22:0, d18:1-24:1, d20:1-20:0[c]	2.355	2.485	2.471	2.482	2.448 ± 0.054
23	d18:1-23:0, d18:1-25:1	2.907	2.961	3.055	3.108	3.008 ± 0.078
25	d18:1-24:0, d18:1-26:1, d20:1-22:0[c], d20:1-24:1[c]	3.616	3.643	3.881	3.907	3.762 ± 0.133
27	d18:1-25:0	4.410				4.410
29	d18:1-26:0	5.552				5.552

For each of the points in the table (except for the last column), the values are the mean of three chromatograms for each ganglioside class. The coefficient of variation was less than 3%. The last column "Mean ± SD" is the mean ± SD of the values listed in the table, not the mean ± SD of all the individual observations.

[a]From chromatograms shown in Fig. 3.

[b]Peak was identified by the RRT (Fig. 5).

[c]Molecular species identified in glycosphingolipids isolated from brain.

[d]Major component of the peak.

peaks contain more than one component, it is difficult to quantitate or even identify minor components in the presence of one or more major components.

One of the benefits derived from previous work identifying molecular species by LC-MS was the development of HPLC procedures to separate the underivatized glycosphingolipid classes into their individual molecular species (12). However, quantitative analysis by separation of underivatized glycosphingolipids suffers from the disadvantage of requiring relatively large amounts of material because detection in the 205 nm region is relatively insensitive, and the baseline is very noisy. Moreover, quantitative analysis is particularly difficult because the extinction coefficient of the various molecular species varies depending on the number and location of double bonds.

With the introduction of reliable procedures for derivatizing the glycosphingolipids, developed primarily to facilitate the analysis of glycosphingolipid classes, it has become possible to quantitate the molecular species of each class with relative ease by simply integrating the area under the peaks as they elute from the reversed-phase column. Ullman and McCluer (2) demonstrated that the absorbance of perbenzoylated neutral glycosphingolipids at 230 nm was linear up to at least 30 nmol and the recovery after derivatization was 80–90%. Likewise, Nakabayashi, Iwamori, and Nagai (5) demonstrated that the absorbance of the *p*-bromophenacyl esters of the gangliosides at 261 nm was linear up to 50 nmol of sialic acid and the recovery was 100 ± 1.5%, and Miyazaki et al. (6) showed that the absorbance of the 2,4-dinitrophenylhydrazide derivatives of gangliosides at 342 nm was linear up to 1.6 nmol and the recovery was 93–101%. Since only the chromophore absorbs at their respective wavelengths, it is possible to obtain the molar ratio of each molecular species by digital integration of the peaks eluted from a reversed-phase column. The molar extinction coefficient of the benzoyl group (1×10^4 at 230 nm) (7, 8) is greater than that of the 2,4-dinitrophenylhydrazide group (7×10^3 at 342 nm) (6) and nearly as great as that of the *p*-bromophenacyl group (2.3×10^4 at 261 nm) (5), but at 230 nm (benzoyl) there is greater baseline noise which makes it impractical to use a detector sensitivity greater than 0.04 absorbance units full scale (AUFS). On the other hand, at 261 nm (*p*-bromophenacyl) or at 342 nm (2,4-dinitrophenylhydrazide) a detector sensitivity of up to 0.005 AUFS is possible. Thus, as little as 5 to 20 pmol of each molecular species of neutral glycosphingolipids (depending on the number of benzoyl groups) and 3 to 5 pmol of gangliosides can reliably be quantitated.

The success of this procedure assumes that the peaks eluting from the column contain only one component. Because of the large number of molecular species present in

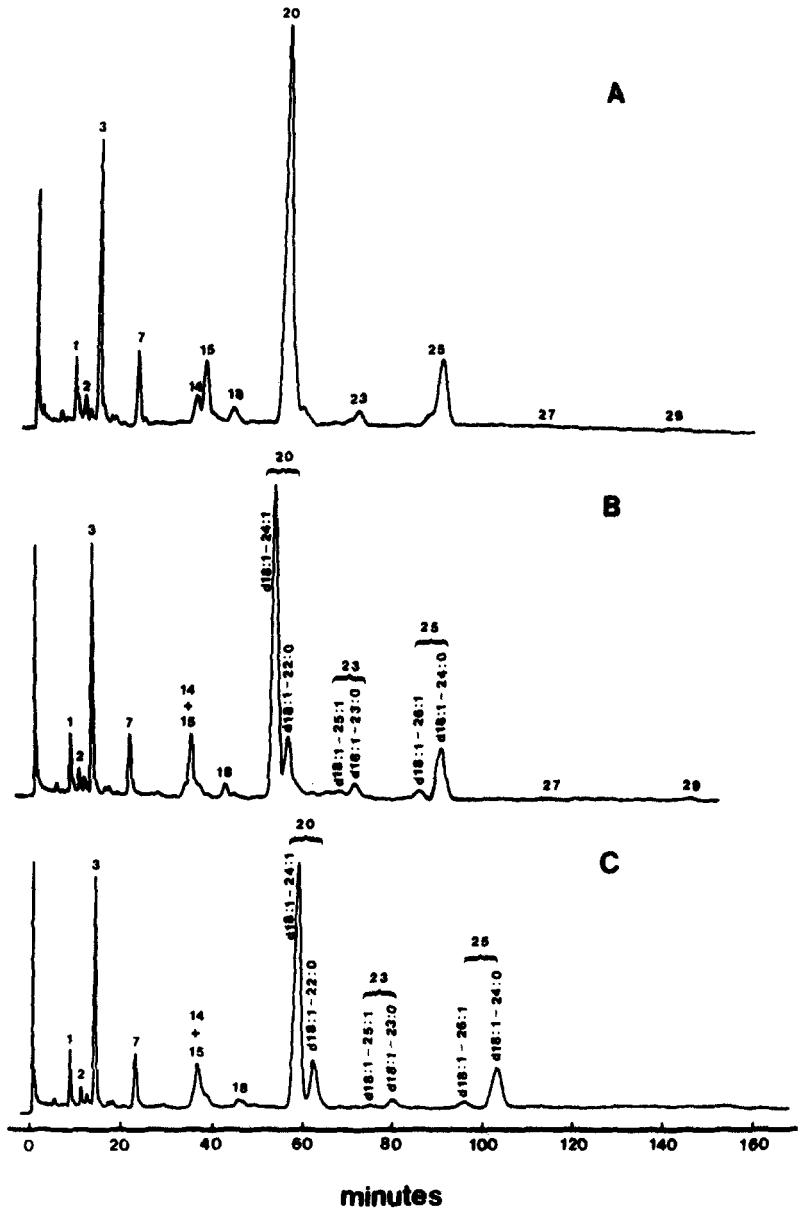


Fig. 4. Molecular species separation of 2,4-dinitrophenylhydrazide derivatives of G_{M3} with various concentrations of acetonitrile in the mobile phase. G_{M3} (1.20 nmol), isolated from human erythrocytes, was separated into individual molecular species. The ratios of methanol–acetonitrile–water–acetic acid in the mobile phase were: A, 72:24:4:0.02; B, 48:48:4:0.02; and C, 24:72:4:0.02. The flow rate for A was 0.3 ml/min, and for B and C was 0.4 ml/min. The column effluent was monitored at 342 nm and the detector sensitivity was 0.02 AUFS. The peak numbers shown in the chromatograms correspond to peak numbers in Table 3.

tissue glycosphingolipids, however, it is not possible to resolve all the glycosphingolipid molecular species under a single set of chromatographic conditions. Hence, for any given set of conditions, there will be overlap of some molecular species. With the system used in Figs. 1, 2, and 3, i.e., methanol–water, there is overlap of those molecular species that have the same long chain base and a fatty acid which differs by two carbon units and one double bond (i.e., d18:1–22:0 and d18:1–24:1). This is because the presence of a single double bond decreases the retention time to exactly the same extent that two methylene groups increase the retention time (25). Likewise, those pairs of molecular species that contain a long chain base and fatty acid such that the total number of carbons and the total number of double bonds is the same, such as d18:1–20:0 and d20:1–18:0, are also not resolved.

Two methods have been shown to alter the relative elution time of these pairs of glycosphingolipid molecular species, i.e., changing the temperature of the column and adding acetonitrile to the mobile phase. Hirabayashi et al. (26), using underivatized bovine brain galactosylceramide with methanol as the mobile phase, demonstrated that changing the temperature of the column resulted in differences in the relative positioning of the molecular species owing to differences in the temperature dependence of their partition coefficients. As the temperature was lowered the retention time of all the molecular species increased, but the retention time of those molecular species that contain a double bond increased more slowly than the more saturated molecular species. Therefore, we also examined the influence of column temperature on the molecular species separation of the 2,4-dinitrophenylhydrazides of G_{M3}. In contrast to the results of Hirabayashi et al. (26), no differences in the relative retention times of the derivatized molecular species were observed at various temperatures between 21°C to 37°C with methanol–water–acetic acid 96:4:0.02 as the mobile phase (data not shown). Two possible explanations for the discrepancy between the results of Hirabayashi et al. (26) and this study are that the critical temperature of the derivatives is outside the range tested or that with the underivatized species there is an interaction between the carbohydrate and fatty acid which is disrupted by derivatization.

A second procedure for resolving pairs of molecular species, such as d18:1–22:0 and d18:1–24:1, is to add acetonitrile to the mobile phase (9). Acetonitrile forms a complex with the double bonds of the fatty acid thereby making the unsaturated molecular species more soluble in the mobile phase, thus the more unsaturated molecular species elute faster. Depending on the molecular species composition of a given glycosphingolipid class, or depending on which particular molecular species one is interested in studying, different proportions of acetonitrile would be required. A comparison of the RRTs obtained in the absence and then in the presence of acetonitrile can also aid in identifying peaks by showing which contain double bonds (relatively shorter RRT in the presence of acetonitrile) and which do not (no change in RRT). The addition of acetonitrile does not, however, affect the resolution of those pairs that contain a long chain base and fatty acid such that the total number of carbons and the total number of double bonds is the same (d18:1–20:0 and d20:1–18:0).

As shown in Table 1, for a particular molecular species all the benzoylated neutral glycosphingolipid classes have the same RRT. Likewise, the *p*-bromophenacyl (Table 2)

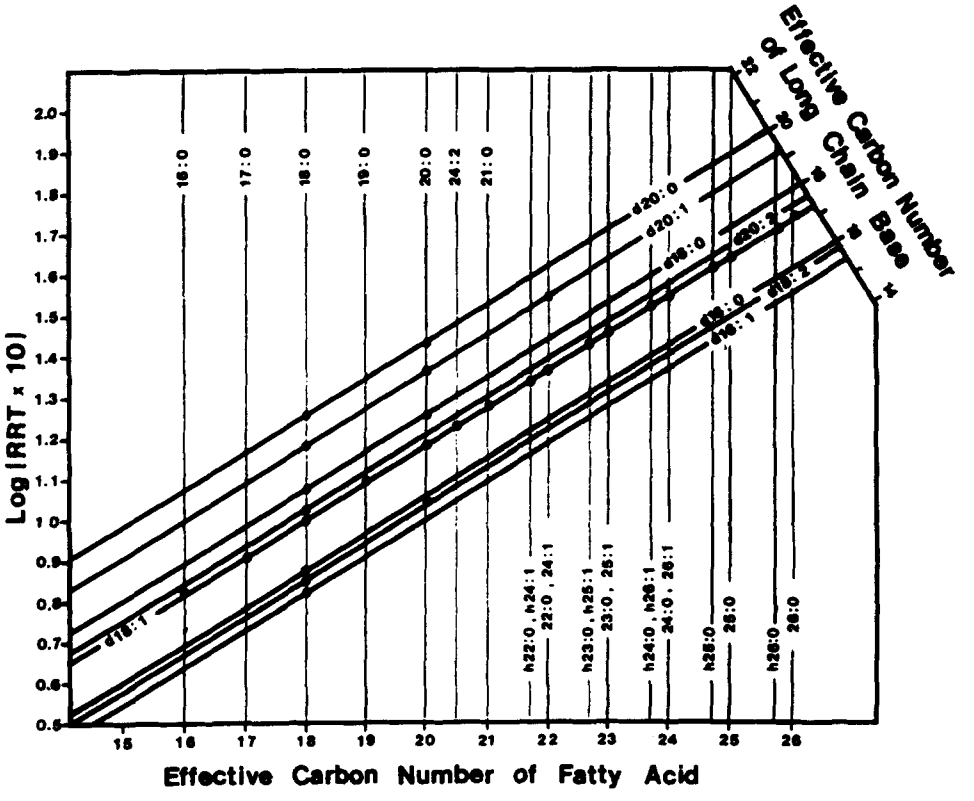


Fig. 5. The effect of fatty acid and long chain base composition on the elution pattern of *p*-bromophenacyl esters of ganglioside molecular species. The RRT calculated for each molecule (Table 2) was plotted as the log (RRT × 10) against the carbon number of the fatty acid. Oblique lines connect points that have the same long chain bases. The intersection of oblique and perpendicular lines provides the RRT of individual molecules.

or 2,4-dinitrophenylhydrazide (Table 3) derivatives of all the gangliosides have the same RRT. Thus, when the molecular species composition of any one of the glycosphingolipid classes can be identified, the molecular species composition of the other members of the group (i.e., perbenzoylated neutral glycosphingolipids or *p*-bromophenacyl or 2,4-dinitrophenylhydrazide derivatives of gangliosides) can also be identified solely by their RRT. Moreover, the molecular species composition of any peak of derivatized glycosphingolipids can be predicted from their position on a plot of effective carbon number of the fatty acid and the long chain base versus the log of the RRT as previously described for underivatized glycosphingolipids (12). As shown in **Fig. 5** for the *p*-bromophenacyl esters of the gangliosides, when the log (RRT × 10) of the individual molecular species (y axis) is plotted against the effective carbon number of the fatty acids (x axis), a series of parallel oblique lines is obtained such that each of the oblique lines represents a particular long chain base, i.e., d18:1, d20:1, d18:0, etc. Likewise, those molecular species that contain a particular fatty acid fall on a line parallel to the y axis and the effective carbon number of the fatty acid can be read on the x axis. For instance, a hydroxy fatty acid has an effective carbon number 0.3 units less than the corresponding nonhydroxy fatty acid; 24:1 and h24:1 have effective carbon numbers of 22 and 21.7, respectively. Of greater importance, for any given peak in a chromatogram if a line parallel to the x axis is drawn through the log (RRT × 10) of that peak, the identity of the possible components of the peak is determined by those points where the three lines (RRT, fatty acid, and long chain base) intersect. If a similar plot was performed for the same sample rechromatographed in a mobile phase containing 72% acetonitrile, a similar but slightly different figure would emerge. For instance, 24:1 fatty acid which has an effective carbon number of 22 in methanol–water would have an effective carbon number of 21.8 in 72% acetonitrile and would therefore plot lower on the oblique lines. Consequently, a line through the RRT of a given peak would now yield a considerably reduced set of possible molecular species, and in many cases, a single molecular species.

The methodology described in this report is useful for the quantitation of molecular species of the various glycosphingolipid classes using the very small quantities obtained from the class analysis. It is of greatest utility when the molecular species composition of a large number of samples must be determined, and a rapid and reliable quantitative procedure is required. It can also be useful for tentative identification of the molecular species composition of samples which have not previously been examined. However, absolute identification of the molecular species is not possible because certain types of molecular species cannot be resolved, i.e., pairs of the type d18:1–20:0 and d20:1–18:0. Absolute identification must be obtained by other methods such as MS or fatty acid analysis, providing sufficient material can be obtained. Because the number of possible molecular species in a given peak is limited as determined by the RRT, it is only necessary to determine the fatty acid composition of the peak in order to positively identify all the molecular species.

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