notes on methodology

Simultaneous fractionation of four placental neutral glycosphingolipids with a continuous gradient

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Summary We describe a method for isolating milligram quantities of the four neutral glycosphingolipids, glucocerebroside, lactosylceramide, triaosylceramide, and globoside, from human placental tissue. This procedure is carried out on a silicic acid column eluted with a continuous chloroform–methanol gradient (19:1 to 4:1); the four glycosphingolipids elute as separate fractions with no need for further separation. The method is simple, rapid, and yields sufficient material to use as analytical standards for several hundred runs. The lipids have been identified by NMR spectroscopy. Placental tissue is freely available in most centers and is an excellent untapped source for these compounds. Given that lactosylceramide is not commercially available and that triaosylceramide (ceramide trihexoside) cannot be obtained in a reliable state, this technique represents an effective solution to this dilemma.—**Strasberg, P., A. Grey, I. Warren, and M-A. Skomorowski.** Simultaneous fractionation of four placental neutral glycosphingolipids with a continuous gradient. *J. Lipid Res.* 1989. **30:** 121–127.

Supplementary key words NMR • placenta • dimethylsulfoxide • glucocerebroside • lactosylceramide • triaosylceramide • globoside

Laboratories throughout the world are becoming increasingly interested in the quantitation, characterization, and analysis of the glycosphingolipids (GSL). These compounds have long been recognized as the storage compounds in the majority of the lysosomal storage diseases (1, 2); they are present in the blood associated with the major lipoprotein fractions and with the membranes of erythrocytes, lymphocytes, platelets, neutrophils, and leukocytes (3–8). The carbohydrate portion of the GSL is responsible for blood group specificities, and serves as receptor for various hormones, lectins, and toxins (6). Elevated plasma levels or altered cellular compositions of GSL can indicate lysosomal storage disease, hypercholesterolemia, leukemia, etc.

Therefore it is strange that it is currently difficult to buy suitable neutral GSL standards for use in TLC, HPLC, GLC, etc. Glucocerebroside (GL-1a, GluCer) can be obtained from Supelco (Bellefonte, PA) or Sigma (St. Louis, MO) in milligram quantities, but most workers needing greater amounts prefer to isolate it themselves (9); globoside (GL-4a, $GbOse_4Cer$) is available from Supelco. Ceramidetrihexoside (GL-3a, $GbOse_3Cer$) is sold by Supelco, but is impure and not ready for use. Realistically, it must be isolated or "borrowed." Lactosylceramide (GL-2a, LacCer), once available from Miles-Yeda (Israel), can no longer be purchased and must be obtained from other laboratories or isolated when required.

Many techniques have been used over the past 20 to 30 years for extracting and isolating glycosphingolipids from tissues. Generally one begins with the extraction method of Folch, Lees, and Sloane Stanley (10) of the tissue lipids using chloroform and methanol, followed by filtration and the addition of water or saline to form a biphasic system. The neutral glycosphingolipids partition into the lower phase (10). This fraction is then exposed to a variety of procedures to remove contaminants. These include mild alkali-catalyzed methanolysis (11), DEAE-Sephadex chromatography (5), Florisil columns (12), silicic acid column chromatography (3, 4, 11, 12), Sephadex-G25 column chromatography (12), Sephadex LH20 chromatography (13), droplet counter current chromatography (7), and TLC (6, 8, 11, 14).

Silicic acid columns have been used in essentially two different ways: *1*) to obtain a total neutral GSL fraction, using acetone–methanol 9:1 (11) and *2*) using stepwise, linearly increasing amounts of methanol in chloroform to separate GSL into fractions with different polarities (12). Invariably, however, the final purification has always been achieved by preparative TLC (3–8, 11–14).

Herein we describe a method for the rapid simple isolation and simultaneous separation of the four neutral GSL from human placenta. Our technique involves the use of a continuous chloroform–methanol gradient 19:1 to 4:1 on one silicic acid column, which yields 1 to 3 mg of GL-1a to -4a, each in different fractions. Whereas our method does certainly provide GL-1a and -4a, it is especially relevant for isolation of GL-2a and -3a in sufficient quantities for several hundred sets of analyses. NMR spectroscopy was used to confirm the structures of the isolated GSL.

Abbreviations: NMR, nuclear magnetic resonance; GSL, glycosphingolipid; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; HPLC, high performance liquid chromatography.

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MATERIALS AND METHODS

Extraction of glycosphingolipids from placenta

Fresh or frozen placenta tissue was homogenized in 10 mM sodium phosphate buffer (pH 6) for 3 min at high speed in a Waring commercial blender (15). After centrifugation (8000 *g* for 30 min at 0°C), 933 g of pellet was stirred in 1 liter of chloroform–methanol 2:1 overnight at room temperature. This was resuspended with a Polytron and stirred overnight again in an additional 2.5 liters of chloroform–methanol 2:1.

The suspension was filtered through a glass fiber filter retained in a Buchner funnel, resulting in a 2800-ml filtrate. An 800-ml upper phase was discarded. Four hundred ml of water was added to the lower phase and the mixture was left at 4°C to separate.

The lower phase (1300 ml) was dried on a rotary evaporator, dissolved in a minimum volume of chloroform–methanol 98:2, and applied to a 100-g silica Vance and Sweeley column (11) poured in chloroform. This column was first eluted with 1 liter of chloroform; this step serves to elute neutral lipids (11) including ceramide. The neutral GSL were subsequently eluted with 1 liter of acetone–methanol 9:1 (11). This fraction was similarly dried on a rotary evaporator, redissolved in 5 ml chloroform–methanol 98:2, and applied to a 5-g silica column prepared in the same solvent.

Up to this point, we have been describing the usual Folch et al. extraction procedure (10) for tissue lipid extraction and subsequent purification. Traditionally, this second column has been eluted in steps, resulting in GSL fractions composed of various mixtures that have to be rechromatographed on other columns. We chose to elute the GSL with a continuous chloroform–methanol gradient, composed of 200 ml each of chloroform–methanol 19:1 and 4:1 in the first and second bottles, respectively, with the added proviso that the weights of the two starting solvents were equal. One hundred sixty fractions (2.5 ml each) were collected and the contents of every third tube was analyzed by TLC. In order not to dissolve the gradient maker and tubing itself, we used specially designed glass gradient-maker bottles (30 cm high, with a base 7 cm in diameter). The first bottle had two outlets at the base, the second, one. The bottles were joined with solvent resistant hard plastic tubing going from inside the outlet of bottle 1 to inside the inlet of bottle 2; these connecting inlets and outlets were then joined on the outside with Tygon tubing in the usual manner. This prevented leakage and provided a guide to the smaller tubing inside. A similar type of tubing arrangement led from the outlet of bottle 1 to the top of the column.

Thin-layer chromatography

Aliquots from every third fraction from column two were examined by TLC (see Fig. 1) in chloroform–methanol –water 14:6:1, versus GSL standards: GL-1a, glucocerebroside, prepared in our laboratory from Gaucher spleen as previously described (9, 16); GL-2a, lactosylcerebroside, purchased from Miles-Yeda, GL-3a, prepared in our laboratory from kidney (17); and GL-4a, globoside, purchased from Supelco. Fractions 9 to 12, 21 to 30, 33 to 39, and 48 to 57 were pooled to represent GL-1a, -2a, -3a, and -4a, respectively. Plates were sprayed with 50% sulfuric acid and charred at 100°C to locate the GSL bands.

Chemical purification of GL-2a and -3a

Fractions 21 to 30 (GL-2a) were combined, dried under nitrogen, dissolved in a minimum volume of methanol, and applied to a 4.5-cm Cellex-D column that had been poured in methanol, in order to eliminate any contaminating sulfatides. The column was washed with 5 ml of methanol and the GL-2a was eluted with 10 ml methanol containing 5% acetic acid. Under these conditions, sulfatides would have remained behind on the column.

Fractions 33 to 39 (GL-3a) were combined, dried under nitrogen, and dissolved in 4 ml of methanol. A volume of 0.44 ml of 1 N NaOH in water was added and the alkaline hydrolysis was carried out at 37°C overnight to destroy any contaminating phospholipids (11).

All four glycolipid fractions were then run with standards on TLC (see Fig. 2).

HPLC identification of the purified GSL

Aliquots of each pooled, treated fraction were benzoylated and run on HPLC as previously described (see Figs. 3 and 4) (18, 19).

Orcinol quantitation

The amount of glycosphingolipid in each fraction was quantitated by the orcinol procedure (20).

NMR spectroscopy

All NMR spectra were obtained at 300°K in DMSO-d6 on a Bruker AM 500 MHz NMR spectrometer. Samples were dried and dissolved in DMSO-D6 with TMS as the internal reference standard. Spectra were obtained over a spectral range of 4500 Hz with a 3.6 sec acquisition time and were rerun after a D20 exchange (see Fig. 5).

RESULTS AND DISCUSSION

When the neutral GSL fraction derived from human placenta was fractionated on a Silica column using a continuous chloroform–methanol gradient (see Methods) the four neutral GSLs eluted separately in subsequent fractions, starting with GL-1a and proceeding to GL-4a, as the ratio of chloroform to methanol was gradually increased (**Fig. 1**). There was no evidence of a band representing cer-

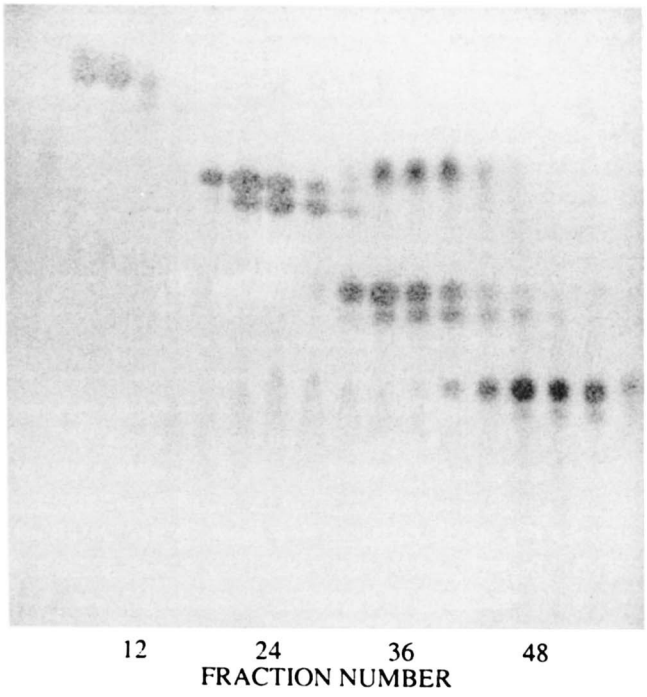

Fig. 1. TLC analysis of individual placental GSL fractions from a silicic acid column. Aliquots of the 2.5-ml fractions from the gradient-chloroform-methanol-eluted silicic acid column were run on TLC in chloroform-methanol-water, 14:6:1 as described in the Methods and the plates were sprayed with 50% H_2SO_4. Numbers 12, 24, 36, and 48 represent individual fractions; every third sample was run so the unmarked lanes represent fractions 3, 6, 9, 15, etc.

amide migrating faster than the GSL in the early fractions (Fig. 1). Any impurities in the GL-2a or GL-3a fractions were then removed by treating with Cellex-D (to remove sulfo-containing GSL) or alkaline hydrolysis (to destroy phospholipids), respectively.

Fig. 2 demonstrates that when the GL-2a and GL-3a fractions were purified and free of nonneutral-GSL contaminants, all four GSL ran on TLC similarly to the available standards. The identities of these GSLs were determined by comparing their migrations on HPLC to those of authentic standards (Figs. 3 and 4). **Fig. 3** is an HPLC chromatogram of the individual purified fractions of GSL; **Fig. 4** shows the pattern of the combined fractions. The use of these two figures shows that the individual fractions are not cross-contaminated (21). An early peak which elutes with the solvent front before GL-1a comigrated with the position of benzoylated ceramide (22). However, ceramide comes off the Vance and Sweeley column in the chloroform wash, and should not have later been continuously eluted from the column with GL-1a, -2a, -3a, and -4a; it did not appear on any TLC plates. The peak also appeared in runs involving blanks and GSL standards. Therefore, we must conclude that it is not present in these fractions. It should be noted that GSL are present in tissues as hydroxylated and nonhydroxylated forms, and contain a variety of fatty acid side chains (12), so that, depending on the source of the tissue and the chromatography procedure, the different species of a single type of glycosphingolipid may elute as separate bands. Hence, one may note one or two bands for GL-2a, and one to three bands for GL-3a. For example, our GL-3a standard was derived from Fabry kidney and runs in three adjacent bands on TLC (Fig. 2) and shows three major peaks on HPLC (Fig. 4). The GL-3a from human placenta has only the first two bands; it is not surprising for lipids from different tissues to contain different types of fatty acids and numbers of hydroxyl groups. Some tissues contain galactosyl- as well as glucosylceramide. We have found that, on HPLC, galactosylceramide elutes as two peaks; only one corresponds to one of the GL-1a peaks, so that a mixture of galactosyl- and glucosylceramide elutes as three distinct peaks (18). Our HPLC chromatogram did not give evidence for the presence of galactosylceramide in this preparation.

The NMR spectra for the GSL GL-1a, GL-2a, GL-3a, and GL-4a displayed common features showing resonances in the 1.8–0.8 ppm region consistent with the presence of two long chain fatty acid components (i.e., in-chain $(CH_2)_n$ at 1.2–1.3 ppm, two terminal methyl triplet resonances at 0.846 and 0.842 ppm, $J = 7$ Hz). The triplet resonance at 2.013 ppm ($J = 7.3$ Hz) is consistent with a methylene group adjacent to an amide or ester carbonyl group, while a mul-

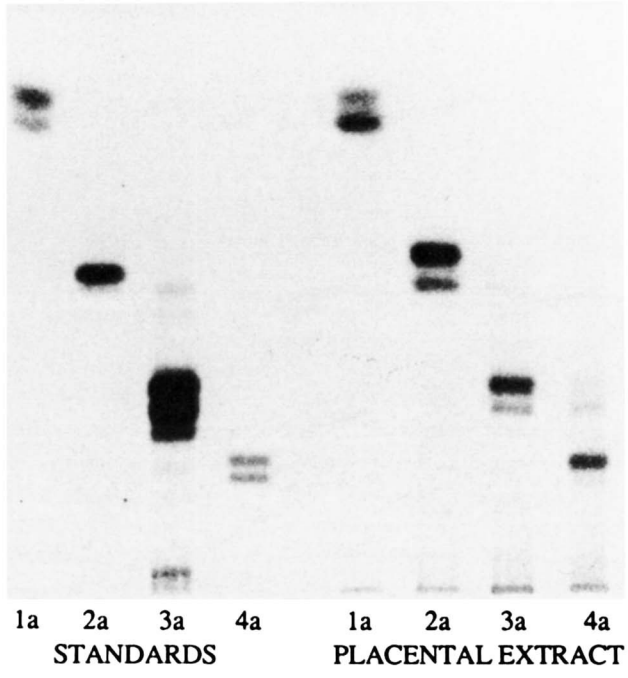

Fig. 2. Pooled placental GSL fractions from gradient elution. Individual fractions were pooled and run on TLC as outlined in the Methods and in Fig. 1 versus GSL standards. Numbers 1, 2, 3, and 4 represent aliquots of pooled fractions 9–12, 21–30, 33–39, and 48–57, respectively.

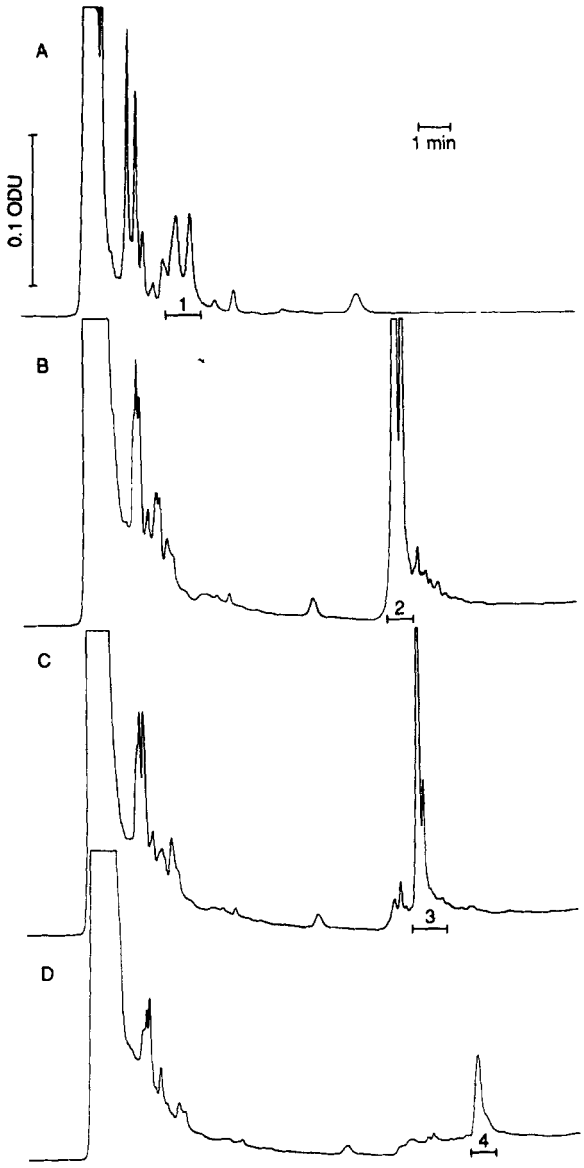

Fig. 3. HPLC analysis of individual placental GSL fractions. Aliquots of the fractions labeled 1, 2, 3, and 4 in Fig. 2 were perbenzoylated and run separately on HPLC, as A, B, C, and D, respectively.

tiplet at 2.08–1.90 ppm suggests a $-CH_2-C = C$ moiety. The site of unsaturation, *trans*-CH = CH-, displayed resonances that appeared as multiplets at 5.53 ppm ($J_{4,5\ trans}$ = 15.0 Hz, $J_{3,4\ vicinal}$ = 7 Hz) and 5.35 ppm ($J_{4,5\ trans}$ = 15.0 Hz, $J_{5,6\ vicinal}$ = 6.9 Hz), respectively (see refs. 23 and 24).

The region of interest for the carbohydrate components was found between 5.28 and 3.00 ppm. The D_2O exchange with the concomitant loss of all exchangeable OH/NH groups, provided simplified spectra from which several equivocal conclusions could be drawn (**Fig. 5** and **Fig. 6**). Firstly, the OH groups were found in the 5.28–4.00 ppm region with typical couplings of $J_{H,OH}$ of 3–4 Hz and 4.5–6.0 Hz for axially and equatorially oriented OH groups, respectively. All carbohydrate OH resonances on C_2, C_3, and C_4 appeared as doublets except for those on C_6 which appeared as triplets. Secondly, the anomeric hydrogen doublet resonances for alpha and beta anomers were found to lie between 4.7 and 4.8 and 4.5 and 4.0 ppm, respectively. Thirdly, all other resonances with an exchanged OH group were simplified by the loss of all $J_{H,OH}$ couplings. This is especially useful for identifying H_4 of galactose which bears small vicinal coupling constants with H_3 and H_5 and usually appears as a distinctively narrower resonance when the 4-OH has been exchanged.

The data with the corresponding assignments are reported with their chemical shifts and coupling constants in **Table 1**. The exchangeable hydrogen resonances NH and OH are shown in the second and third columns. Their integrated numbers correspond to 1, 2, 3, and 4 carbohydrate units for GL-1a (6 observed, 6 expected), GL-2a (9 observed, 9 expected), GL-3a (12 observed, 12 expected), and GL-4a (15 observed, 15 expected), respectively, which include two extra resonances for an amide NH and a CHOH group in the lipid portion.

The nonexchangeable doublets in the 4.05–4.8 ppm region are assigned to the anomeric H_1 resonances as designated in the fourth and fifth columns. The galactose H_4 assignments are also listed here and are supported by the

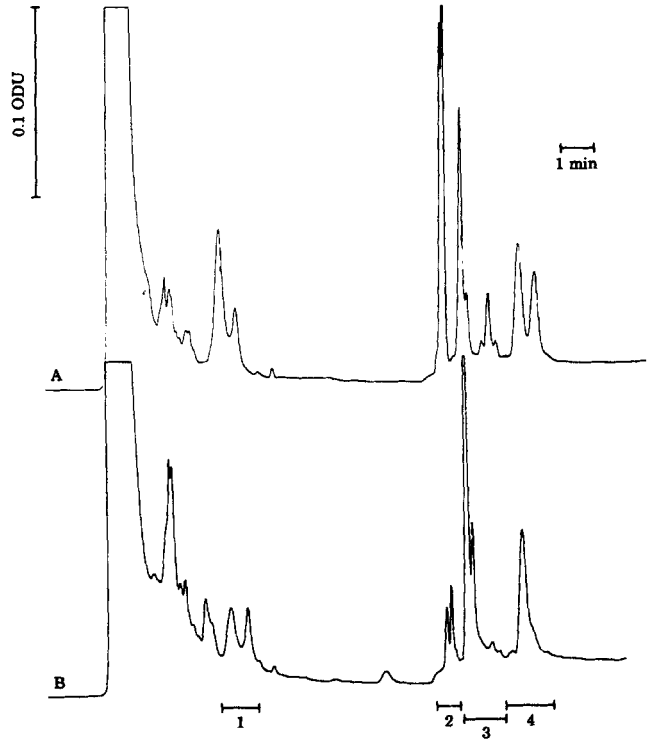

Fig. 4. HPLC analysis of pooled placental GSL fractions. Aliquots of the fractions labeled 1, 2, 3, and 4 in Fig. 2 were perbenzoylated and compared on HPLC to perbenzoylated standards. A and B refer to standard and tissue GSL respectively.

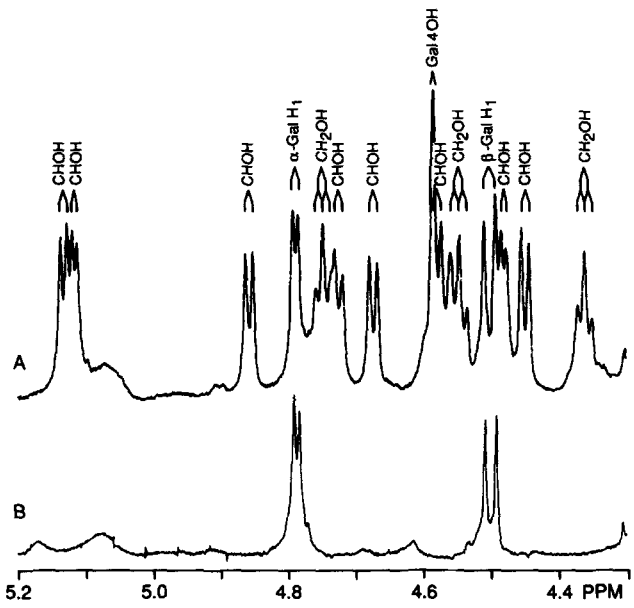

Fig. 5. NMR spectra of purified glycosphingolipid fractions. A. The sugar hydroxyl and anomeric region of the 500 MHz ^1H NMR spectra of GL-4a in Me_2SO-d-6. B. Repeated with D_2O exchange showing the disappearance of exchangeable OH and NH resonances.

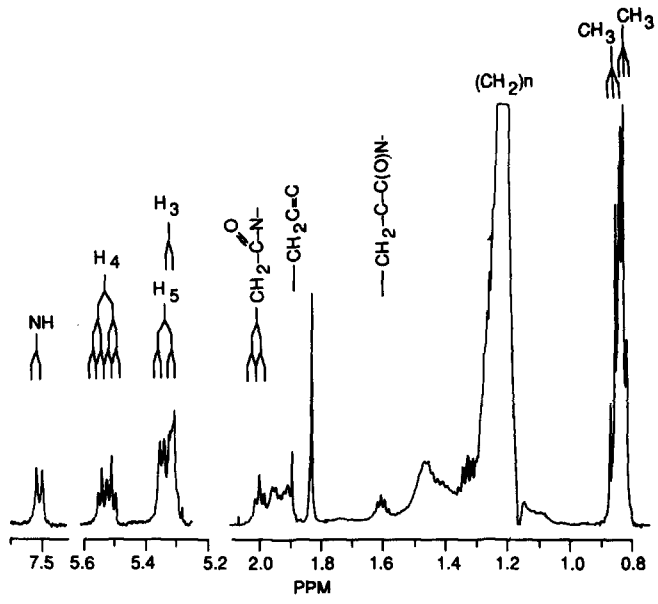

Fig. 6. NMR upfield and downfield expansion of GL-4a assignment described in text.

TABLE 1. NMR spectroscopy of glycosphingolipids

	Exchangeable Resonances[a]		Assignments[b]			Dabrowski[c]
GL-1a	7.469(9.1,d), 4.897(4.8,d), 4.847(5.6,d),	4.997(3.0,d) 4.876(5.0,d) 4.470(5.8,t)	β-Glc(1,1)Cer	H1	4.067(7.8,d)	4.10(7.2)
GL-2a	7.475(9.1,d), 5.073(4.0,d), 4.764(3. ,d), 4.633(5.2,t), 4.490(4.6,d)	5.111(3.8,d) 4.849(5.5,d) 4.653(<1 ,d) 4.543(6.0,t)	β-Gal(1,4) β-Glc(1,1)Cer β-Gal(1,4)	H1 H1 H4	4.186(7.2,d) 4.150(8.0,d) 3.600(2.3,m)	4.22(7.3) 4.17(7.7) 3.64
GL-3a	7.498(9.0,d), 5.147(br), 4.820(br), 4.633(br), 4.60(5 ,br), 4.44(t?,br)	5.24(br) 4.861(<2,br) 4.633(br) 4.633(br) 4.56(t?,br) 4.38(d,br)	α-Gal(1,4) β-Gal(1,4) β-Glc(1,1)Cer β-Gal(1,4) α-Gal(1,4)	H1 H1 H1 H4 H4	4.771(3.7,d) 4.237(7.6,d) 4.147(7.8,d) 3.770(3.3,m) 3.610(3.8,m)	4.81(4.0) 4.27(7.7) 4.16(8.1) 3.81 3.76
GL-4a	7.481(9.2,d), 5.116(3.6,d), 4.749(5.4,t), 4.725(5.6,d), 4.585(<1 ,d), 4.567(5-7,d), 4.481(3.8,d), 4.362(5.1,t)	5.132(4.7,d) 4.857(5.5,d) 4.731(5.6,t) 4.674(5.7,d) 4.579(5.8,d) 4.546(5.5,t) 4.450(5.7,d)	α-Gal(1,4) β-GalNAc(1,3) β-Gal(1,4) β-Glc(1,1)Cer α-Gal(1,4) β-Gal(1,4) β-GalNAc(1,3)	H1 H1 H1 H1 H4 H4 H4	4.789(3.7,d) 4.502(8.4,d) 4.246(7.5,d) 4.171(7.0,d) 3.972(3.0,m) 3.799(2.9,m) 3.608(4.4,m)	4.81(3.6) 4.52(8.1) 4.26(7.7) 4.16(7.7) 3.98 3.82 3.64

NMR spectra were obtained at 300°K in DMSO-d6 on a Bruker AM 500 MHz NMR spectrometer as outlined in Methods.

[a]Chemical shifts in PPM (coupling constant J in Hz, Multiplicity); d, doublet; t, triplet; br, broad; m, multiplet.

[b]Glc, glucose; Gal, galactose; GalNAc, 2-N-acetyl-galactosamine; Cer, ceramide.

[c]Data from Dabrowski et al. (24).

TABLE 2. Yield of glycosphingolipids from placenta

GSL	nmol	mg
GL-1a	580	0.5
GL-2a	2600	2.5
GL-3a	1120	1.3
GL-4a	1310	0.6

Glycosphingolipids were extracted from 933 g of placental tissue and isolated and purified as described in the text.

sharpening of those resonances when D_2O was added. The data from Dabrowski, Hanfland, and Egge (24) are given in column six for comparison.

These NMR data are consistent with the following structures:

GL-1a — Gluco-β-1-R
GL-2a — Galacto-β-(1,4)-Gluco-β-1-R
GL-3a — Galacto-α-(1,4)-Galacto-β-(1,4)-Gluco-β-1-R
GL-4a — 2-NAc-Galacto-β-(1,3)-Galacto-α-(1,4)-Galacto-β-(1,4)-Gluco-β-1-R

where R = $-CH_2NHC(=O)(CH_2)_{20}CH_3$ with a $H-C-CH=CH(CH_2)_{12}CH_3$ / OH group on the adjacent carbon.

The actual amount of GSL isolated from 933 g of placental pellet is outlined in **Table 2**, being essentially 0.5, 2.5, 1.3, and 0.6 mg for GL-1a, -2a, -3a, and -4a, respectively. A repeated isolation yielded essentially the same values. Whereas all 4 GSLs can be isolated via this procedure, this is of particular importance in the cases of lactosylceramide and ceramide trihexoside because these GSLs cannot be obtained easily; there is virtually no commercial source for lactosylceramide (as of 1987), and the ceramide trihexoside commercially available (Supelco) is not reliable in terms of purity.

This procedure provides the researcher with enough GL-2a and GL-3a to perform up to 200 HPLC (1 to 10 nmol per day) or TLC (several μg at most in standard lane) sets of analyses using these compounds as standards. Placentas are usually freely available in most centers and the fact that this is human tissue is an added benefit. The method is rapid, reproducible, and following the universal Folch et al. and Vance and Sweeley initial treatment of lipid samples, uses only one simple column requiring no more than common inexpensive laboratory equipment.

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