Quantitative analysis of plasma neutral glycosphingolipids by high performance liquid chromatography of their perbenzoyl derivatives

M. David Ullman and Robert H. McCluer

Eunice Kennedy Shriver Center for Mental Retardation, Inc., W. E. Fernald State School, Waltham, MA 02154; Department of Neurology, Massachusetts General Hospital, Boston, MA 02114, and Department of Biochemistry, Boston University, Boston, MA 02118

Abstract A quantitative high performance liquid chromatography method for the analysis of neutral glycosylceramides as their perbenzoyl derivatives has been devised. Samples containing more than 2.5 nmol each of mono-, di-, tri-, and tetraglycosylceramide are benzoylated with 10% benzoyl chloride in pyridine at 37°C for 16 hr. The products are separated from excess reagents by solvent distribution and injected onto a pellicular silica gel (Zipax) column (2.1 mm × 50 cm). The derivatives are eluted with a 10 min linear gradient of 2–17% ethyl acetate in hexane at 2 ml/min and absorbance at 280 nm is recorded. The detector response was proportional to the weight of sample used (2–30 nmol) and the lower limit of detection was about 70 pmol. The procedure has been applied to the quantitative analysis of erythrocyte and plasma glycolipids. As little as 0.5 ml of plasma can be used for analysis. The relative standard deviation of repetitive analyses ranged between 2.0% for glucosylceramide to 5.4% for galactosyllactosylceramide.

Supplementary key words cerebrosides · glucosylceramide · lactosylceramide · ceramidetrihexoside · globosides · hematoside · Gaucher's disease · Fabry's disease

Neutral glycolipids are defined as glycosides of N-acyl spingosine that contain neutral monosaccharide units. The major neutral glycolipids (1) of human plasma have been identified as glucosylceramide (Glc-Cer), lactosylceramide (Lac-Cer), galactosyl($\alpha 1 \rightarrow 4$)lactosylceramide (Gal-Lac-Cer) and N-acetylgalactosaminyl($\beta 1 \rightarrow 3$)galactosyl($\alpha 1 \rightarrow 4$)lactosylceramide (GalNAc-Gal-Lac-Cer or globoside). Altered plasma levels of neutral glycolipids in Gaucher's and Fabry's diseases reflect abnormalities in neutral glycolipid metabolism. Neutral glycolipids are known to contribute to the properties of plasma membranes. Their importance in cell–cell recognition, cell division, and differentiation (2–4) have been studied and various blood group antigens have been conclusively identified as glycolipids (5–8). Certain neutral glycolipids have been identified as tumor- and organ-specific antigens. Investigations into the distribution, metabolism, and function of neutral glycolipids would be facilitated by the availability of a convenient and sensitive technique for their quantitative determination.

In the past, quantitative analysis of neutral glycolipids has required extraction, solvent partitioning, column chromatography, alkaline methanolysis, preparative TLC, and subsequent measurement of hexose by destructive colorimetric or GLC methods (1,9). Previous investigations from this laboratory have shown the simplicity and practicality of quantitating microgram quantities of ceramide (10) and galactosylceramides from brain by HPLC of their perbenzoyl derivatives (11, 12). The feasibility of applying perbenzoylation to the analysis of neutral glycolipids has been previously demonstrated (13). This report now describes the quantitative HPLC analysis of plasma neutral glycolipids.

MATERIALS AND METHODS

Chemicals

Solvents used for HPLC were spectral grade (Burdick and Jackson Laboratories, Inc., Muskegon, MI) and were degassed by boiling briefly under reduced pressure. Benzoyl chloride (Eastman Kodak Co., Rochester, NY), pyridine (dried over 4A

Abbreviations: HPLC, high performance liquid chromatography; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; NFA, nonhydroxy fatty acid; HFA, hydroxy fatty acid; Glc-Cer, glucosylceramide; Gal-Cer, galactosylceramide; Lac-Cer, lactosylceramide; Gal-Lac-Cer, galactosyl($\alpha 1 \rightarrow 4$)lactosylceramide; GalNAc-Gal-Lac-Cer or globoside, N-acetylgalactosaminyl($\beta 1 \rightarrow 3$)galactosyl($\alpha 1 \rightarrow 4$)lactosylceramide; G_{M3} or hematoside, N-acetylneuraminosyllactosylceramide; AUFS, absorbance units full scale.

Molecular Sieves) and solvents used for extraction and TLC (Fisher Chemical Co., Fairlawn, NJ) were used without prior distillation. Glc-Cer (Supelco, Bellefonte, PA) and Lac-Cer (Miles Laboratories, Inc., Elkhart, IN) standards were purchased and Gal-Lac-Cer and GalNAc-Gal-Lac-Cer (globoside) were isolated from human red blood cell ghosts with HPLC techniques.[1] Individual neutral glycolipid standards were dissolved in chloroform–methanol 2:1 and actual concentrations were determined by micro-orcinol assays (14). Assumed molecular weights of 727 for Glc-Cer, 889 for Lac-Cer, 1051 for Gal-Lac-Cer, and 1254 for globoside were used. Aliquots of each standard were combined so that the final mixture of standards contained: Glc-Cer (0.176 mg/ml), Lac-Cer (0.200 mg/ml), Gal-Lac-Cer (0.211 mg/ml), and globoside (0.270 mg/ml).

Thin-layer chromatography

Thin-layer chromatography of neutral glycolipids was performed with either silica gel G or GF plates (Analtech, Inc., Newark, DE) with chloroform–methanol–water 65:25:4 as the solvent system. Benzoylated derivatives were chromatographed on silica gel GF plates with benzene–ethyl acetate 9:1 (12). Neutral glycolipids were detected with orcinol spray reagent, and hematoside (G_{M3}) was detected with resorcinol reagent (15).

Isolation of plasma glycolipid fractions

Neutral glycolipids from plasma were isolated by a slight modification of the method of Vance and Sweeley (1). To obtain total lipids, 1 ml of plasma was stirred with 18 ml of chloroform–methanol 2:1 for 15 min. This mixture was filtered through Reeve Angel grade 202 filter paper, retained in a coarse sintered glass filter, into a 100-ml filter flask. The residue was reextracted with 3 ml of chloroform–methanol 2:1 and the combined filtrates were placed in a 20 × 150 mm screw cap culture tube. The filter flask was rinsed with 1.5 ml of chloroform–methanol 2:1. Five ml of 0.88% KCl in water was added to the culture tube and the solvents were mixed thoroughly and then centrifuged to separate the phases. The upper phase was removed and the lower phase was washed with 13 ml of methanol–water 1:1. The upper phase was removed and the lower phase was dried with a stream of nitrogen. The residue was dissolved in 1 ml of chloroform and placed on a column that contained 80 mg of Unisil (Clarkson Chemical Co.,

Inc., Williamsport, PA). The column was eluted with 2 ml of chloroform and then 4 ml of acetone–methanol 9:1 to obtain the neutral glycolipid fraction. The acetone–methanol was evaporated to dryness and the residue was dissolved in 1 ml of chloroform and 1 ml of 0.6 N methanolic NaOH and allowed to stand at room temperature for 1 hr. The mixture was neutralized with 1.2 ml of 0.5 N methanolic-HCl. After the addition of 1.7 ml of water and 3.4 ml of chloroform, the mixture was stirred on a vortex mixer and centrifuged. The upper phase was withdrawn and the lower phase was washed twice with 2 ml of methanol–water 1:1. The lower chloroform phase was dried under nitrogen and benzoylated as described below.

Perbenzoylation conditions

Samples containing 5–160 μl of mixed standard (4.29–137 μg of total neutral glycolipids) or neutral glycolipids from plasma were dried with N_2 in 13 × 100 mm screw cap culture tubes and desiccated in vacuo over P_2O_5 for at least 3 hr. A 0.5 ml portion of freshly prepared 10% (v/v) benzoyl chloride in pyridine was then added. The tubes were briefly flushed with nitrogen, capped tightly, and incubated at 37°C for 16 hr. The tubes were placed in a water bath maintained at room temperature and pyridine was removed with a stream of nitrogen. Three ml of hexane was added to the residues, which were then washed four times with 1.8 ml of alkaline methanol. The alkaline methanol was prepared by the addition of 1.2 g Na_2CO_3 to 300 ml of methanol–water 80:20 (all of the Na_2CO_3 did not dissolve). The lower phases were withdrawn and discarded. Each sample was then washed once with 1.8 ml of methanol–water 80:20, the lower phase was withdrawn and discarded and the hexane was evaporated with a stream of nitrogen. The benzoylated samples thus obtained were each dissolved in 100 μl of CCl_4 and an appropriate aliquot (20–40 μl) was injected onto the HPLC column.

HPLC

HPLC analyses were performed with reciprocating pumps (Model 6000) combined with a solvent programmer (Model 660) and a universal liquid chromatograph injector (Model U6K); the equipment was from Waters Assoc., Milford, MA. The chromatographic column was a 50 cm × 2.1 mm (ID) stainless steel tube packed with a pellicular silica gel packing (Zipax, E. I. DuPont de Nemours, Inc., Wilmington, DE, part no. 820975001) with an average particle size of 27 μm. The column effluent was

---

[1] Ullman, M. D., and R. H. McCluer. Unpublished observations.
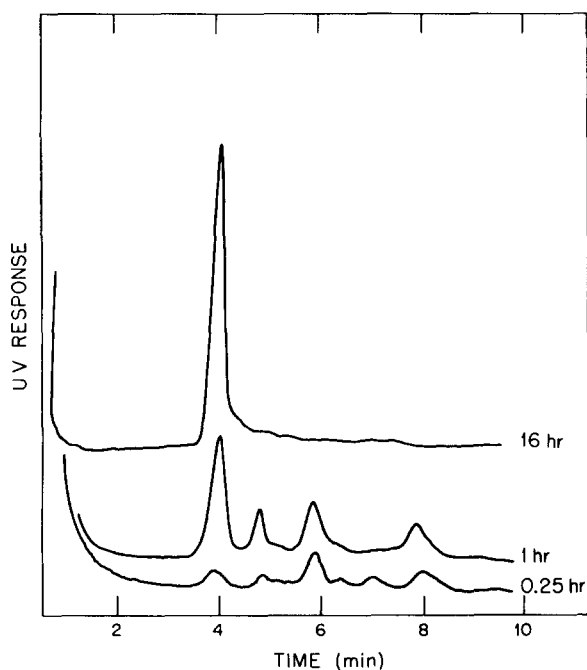
Fig. 1. HPLC of globoside reacted at 37°C with 10% benzoyl chloride in pyridine for various periods of time. Chromatography was with the standard Zipax column but with a 4 min linear gradient of 12–17% ethylacetate in hexane at a flow rate of 2 ml/min. Detection was at 280 nm.

monitored with a variable wavelength UV spectromonitor (Schoeffel Instruments Corp., Westwood, NJ, Model SF 770) and detector output was coupled in series to a single channel computing integrator (Autolab System I, Spectra-physics, Santa Clara, CA), and a stripchart recorder. Separation of perbenzoylated neutral glycolipids was routinely performed with a 10 min linear gradient of 2–17% aqueous ethyl acetate in hexane with a flow rate of 2 ml/min and absorbance was measured at 280 nm. After each gradient run was completed, the gradient was reversed over 2 min and the initial solvent was allowed to flow through the system for at least 4 min. Aqueous ethyl acetate was prepared by mixing dry ethyl acetate and water-saturated ethyl acetate in a ratio of 5:1 (v/v).

RESULTS

Perbenzoylation conditions

The time course of the perbenzoylation of globoside was investigated with 10% (v/v) benzoyl chloride in pyridine at 60°C and the product formed was measured by HPLC and UV absorption. Globoside perbenzoylation was completed by 6 hr but other glycolipids, when treated under those conditions,

showed by-products that were due to either or both incomplete perbenzoylation or degradation of perbenzoylated products. Therefore, the time course of globoside perbenzoylation at lower temperatures was investigated. Satisfactory derivatization was obtained at 20°C and 30°C for 24 hr and at 37°C for 16 hr (**Fig. 1**). Derivatization at 37°C for 16 hr was used as our standard reaction conditions. HPLC of perbenzoylated Glc-Cer, Lac-Cer, Gal-Lac-Cer, and globoside standards showed that in each case one major peak, which contained at least 97% of the total area, was produced (**Fig. 2**).
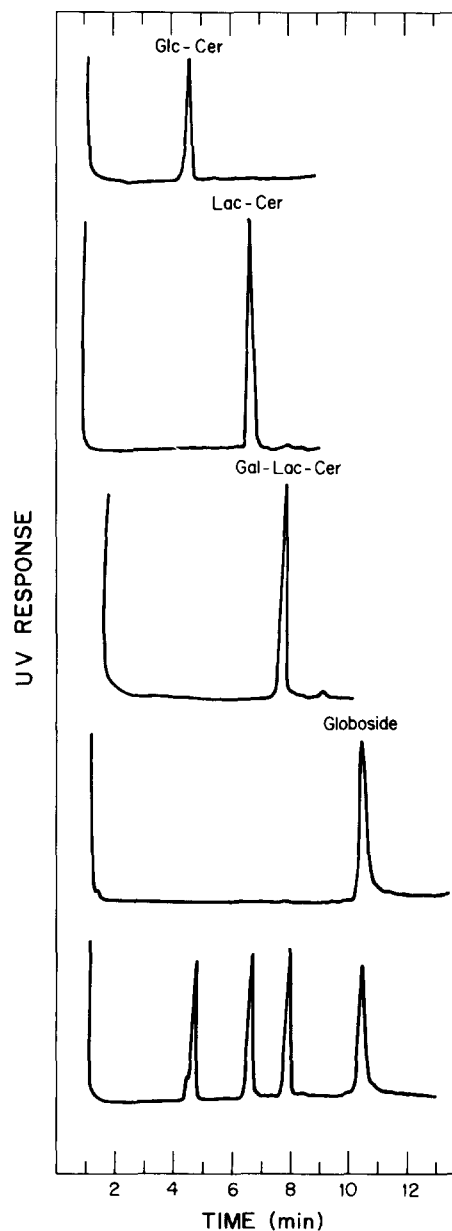

Fig. 2. HPLC of neutral glycolipid standards. Chromatographic conditions were as described under HPLC in the text. Approximately 15 nmol of each standard was used and the detection sensitivity was 0.16 absorbance units full scale.
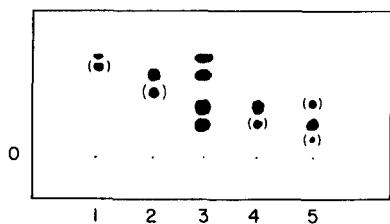
Fig. 3. TLC of products obtained by alkaline methanolysis of perbenzoylated neutral glycolipids. Lane 1, Glc-Cer; Lane 2, Lac-Cer; Lane 3, mixed neutral glycolipid standards untreated; Lane 4, Gal-Lac-Cer; Lane 5, globoside. The solvents employed were chloroform–methanol–water 65:25:4. The plates were viewed under UV light and absorbing spots are marked in brackets ( ). Neutral glycolipids were then visualized with orcinol spray reagent.

Previous investigations of the perbenzoylation of NFA- and HFA-galactosylceramides with benzoyl chloride in pyridine (11) established the structures of the resulting derivatives. Although HFA-Gal-Cer was only O-benzoylated, NFA-Gal-Cer was both O- and N-benzoylated. Further, mild alkaline methanolysis of perbenzoylated Glc-Cer produced a mixture of N-benzoyl and N-acyl derivatives (roughly 60:40, respectively). Analogously the perbenzoylation of oligoglycosylceramides was expected to yield such O- and N-benzoylated derivatives. Neutral glycolipid standards were benzoylated and the derivatives isolated by HPLC. Each was subjected to mild alkaline methanolysis followed by TLC and visualization of products with orcinol spray and with UV light.

Glc-Cer, Lac-Cer, and Gal-Lac-Cer produced two spots and globoside yielded three (**Fig. 3**). In the cases of Glc-Cer, Lac-Cer, and Gal-Lac-Cer, the non-UV-absorbing components comigrated with the appropriate parent standards. It was noted by visual inspection that the relative amount of the UV-absorbing N-benzoyl products formed by mild alkaline methanolysis of the diacylamines decreased in the order Glc-Cer, Lac-Cer, Gal-Lac-Cer, so that the amount of natural product obtained increased in the same order. Theoretically, globoside, which contained an N-acetyl group as well as a long chain amide group, should have yielded four spots on TLC corresponding to: N-fatty acyl, N'-acetyl (the natural product); the N-fatty acyl, N'-benzoyl; N-benzoyl, N'-acetyl; N-benzoyl, N'-benzoyl compounds. After hydrolysis three spots were observed on TLC. Two of these spots absorbed UV light; one had a higher R_f (0.35) and the other a lower R_f (0.13) than the natural product (R_f 0.22) (Fig. 3). The unobserved product (assumed to be the N-benzoyl-N'-acetyl compound based upon expected relative R_f values) may have been formed but was not in high enough concentration to be seen on the TLC plate, or perhaps it did not separate from one
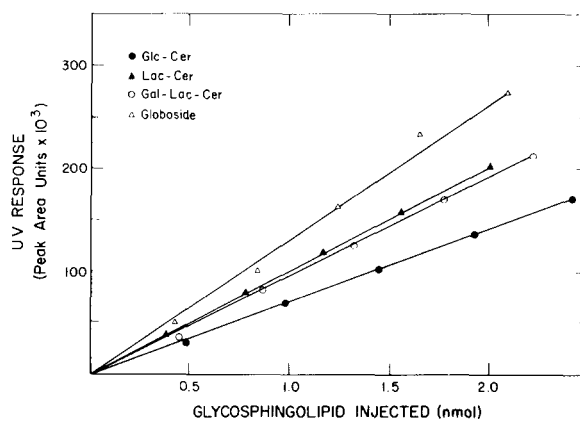
Fig. 4. Linearity of UV response with the amount of benzoyl derivatives injected.

of the other compounds. No further attempt was made to verify the occurence of such a product.

Linearity, sensitivity, and reproducibility

The linearity of recorder response with the amount of derivative injected was demonstrated (**Fig. 4**). The least amount of a neutral glycolipid that could be detected was about 70 pmol. Quantitation of the neutral glycolipids extracted from plasma was performed by comparison with the response of the standards. The relationship between peak area and the quantity of standards benzoylated is shown in **Fig. 5**. The reproducibility of the procedure was determined by the analysis of 9 1-ml aliquots of a single plasma sample. The relative standard deviation ranged between 2.0% for Glc-Cer and 5.4% for Gal-Lac-Cer. The perbenzoyl derivatives are stable for at least 24 hr at 4°C.

Yields

The yields from the perbenzoylation reaction were approximated for each of the neutral glycolipids.
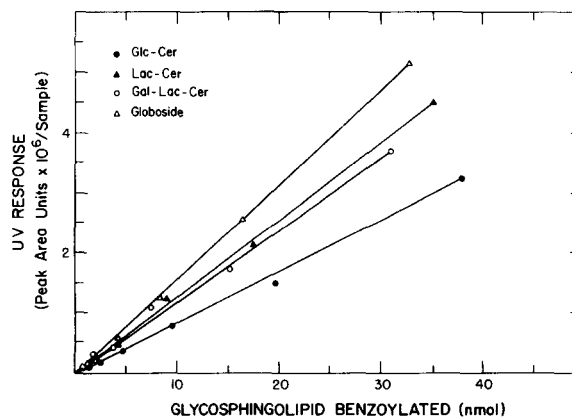
Fig. 5. Linearity of UV response with the amount of neutral glycolipids benzoylated and analyzed by HPLC.

Three assumptions were made: *1*) *N*-benzoyl groups have the same extinction coefficient as *O*-benzoyl groups at 280 nm; *2*) the UV absorptions of benzoyl groups are additive; and *3*) the final products formed are completely benzoylated. Since Glc-Cer contains 6 benzoyl groups, Lac-Cer was assumed to have 9, Gal-Lac-Cer 12, and globoside 15. Therefore the molar absorptivity of Lac-Cer, Gal-Lac-Cer, and globoside relative to that of Glc-Cer was calculated to be 1.2, 2.0, and 2.5, respectively. N-$[1\text{-}^{14}C]$-Stearoyl glucosylspingosine (0.015 μmol: sp act 4.7 $\times$ 10^7 cpm/μmol) was reacted concurrently with individual neutral glycolipid standards in duplicate. The absolute yield of perbenzoylated ^{14}C-labeled Glc-Cer was calculated by determination of the radioactivity recovered in the perbenzoylated Glc-$[^{14}C]$Cer peak collected from HPLC. Yields of the remaining neutral glycolipids were calculated relative to that of Glc-Cer with the above assumed relative molar responses. Yields for Glc-Cer, Lac-Cer, Gal-Lac-Cer, and globoside were found to be 80, 90, 77, and 79% respectively.

Plasma analyses

Neutral glycolipids from plasma of normal subjects and individuals with different diseases were isolated, perbenzoylated, and analyzed by HPLC (**Fig. 6**). The four major constituents were identified by cochromatography with standards. Standard synthetic Lac-Cer eluted just before plasma Lac-Cer, probably because the standard contained stearic acid and dihydrosphingosine and was slightly less polar than the plasma material. The HPLC values obtained for plasma neutral glycolipids compared favorably with published values obtained by TLC–GLC (**Table 1**).

Two minor peaks with retention times between those of Glc-Cer and Lac-Cer were consistently observed in plasma samples. The first minor peak was unidentified, the second minor peak had the same retention time as our HFA-Gal-Cer standard.

Plasma samples from patients with diagnosed
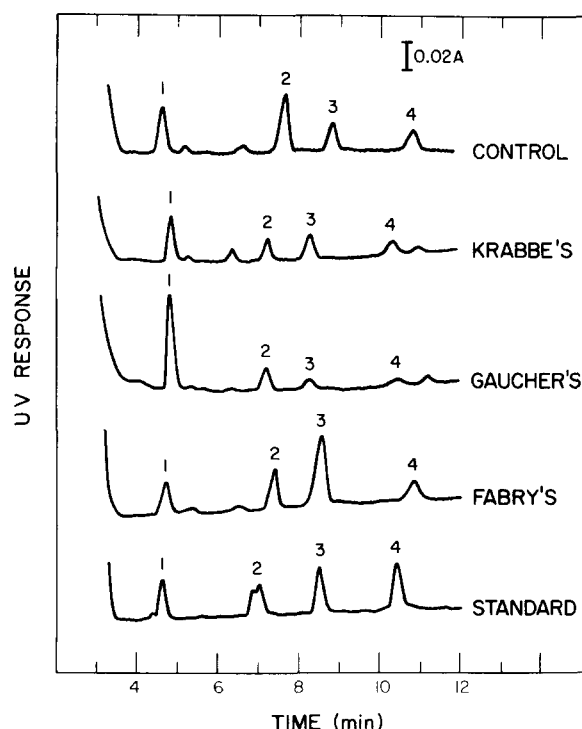
Fig. 6. HPLC analysis of plasma glycolipids. Glycolipid fractions from 1 ml of plasma were isolated, benzoylated, dissolved in 100 μl of CCl$_4$ and 40 μl analyzed by HPLC as described in the text. Peaks are identified as the derivatives of: 1, Glc-Cer; 2, Lac-Cer; 3, Gal-Lac-Cer; 4, globoside.

inborn errors of lipid metabolism were examined. HPLC patterns of plasma neutral glycolipids from patients with Krabbe's, Fabry's, and Gaucher's diseases (16) were obtained (Fig. 6). The chromatograms show, where alterations are known, the expected changes. With the possible exception of slightly lowered Lac-Cer values, normal plasma neutral glycolipid levels were found in the two cases of Krabbe's disease (globoid cell leukodystrophy) examined. Plasma samples from four Gaucher patients contained significantly elevated levels of Glc-Cer and the Fabry's patient showed elevated levels of plasma Gal-Lac-Cer (**Table 2**).

TABLE 1. Normal plasma neutral glycolipid values

Method	Sex	N	Glc-Cer	Lac-Cer	Gal-Lac-Cer	Globoside
				μmol/dl		
TLC–GLC[a]	Both	8–15	0.98 ± 0.09	0.55 ± 0.09	0.21 ± 0.07	0.28 ± 0.08
HPLC	F	6	0.62 ± 0.28 (0.90 ± 0.32)[b]	0.59 ± 0.19	0.26 ± 0.05	0.23 ± 0.06
HPLC	M	4	0.51 ± 0.15 (0.70 ± 0.15)[b]	0.64 ± 0.15	0.17 ± 0.03	0.17 ± 0.06

[a] Reference (1).
[b] Sum of three peaks representing NFA- and HFA- monohexosylceramides.

TABLE 2. HPLC analysis of plasma neutral glycolipids from sphingolipidosis patients

Disorder	Glc-Cer	Lac-Cer	Gal-Lac-Cer	Globoside
			$\mu mol/dl$	
Fabry's	0.48	0.33	0.66	0.24
Fabry's	$(0.78 \pm 0.15)^a$	(0.47 ± 0.08)	(0.76 ± 0.21)	(0.31 ± 0.09)
Gaucher's	0.94	0.31	0.09	0.08
Gaucher's	1.54	0.32	0.16	0.14
Gaucher's	0.97	0.32	0.09	0.10
Gaucher's	1.01	0.19	0.07	0.16
Gaucher's	$(1.98)^b$	(0.52)	(0.20)	(0.37)
Krabbe's	0.45	0.18	0.24	0.26
Krabbe's	0.52	0.38	0.24	0.26
Control				
Male	0.51 ± 0.15	0.64 ± 0.15	0.17 ± 0.03	0.17 ± 0.06
Female	0.62 ± 0.28	0.59 ± 0.19	0.26 ± 0.05	0.23 ± 0.06

[a] Reference 17; n = 10.
[b] Reference 17; one of two individuals.

Interference by other lipids

Hematoside (G_{M3}) is a known constituent of plasma (19). The HPLC peak following globoside (Fig. 6) has been identified as hematoside. The recovery of hematoside with the lower phase lipids was variable with our early partition procedure and interfered to some extent with the measurement of globoside. This problem was eliminated by simply adding an additional methanol–water 1:1 wash of the lower phase from the total plasma lipid extract as described above. No loss of the neutral glycolipids due to the additional wash was observed.

Sulfatide (18) is also a minor component of plasma but was not identified in our preparations. To test whether sulfatide would, through degradation, contribute to the values obtained for monohexosyl ceramides, 8 μg of sulfatide standard was subjected to the benzoylation conditions and analyzed. No peak representing monohexosylceramide or sulfatide was observed.

Analysis of neutral glycolipids from other tissues

Other sources of neutral glycolipids have also been analyzed. The analysis of erythrocyte glycolipids, isolated as described by Vance and Sweeley (1) and analyzed by our standard HPLC perbenzoylation method is shown in **Fig. 7**. The values obtained from one individual (1.6 nmol Glc-Cer, 7.6 nmol Lac-Cer, 14 nmol Gal-Lac-Cer, and 94 nmol globoside in 1 ml of erythrocytes) are in general agreement with quantities previously reported (1). Preliminary studies of neutral glycolipids from other sources (rat kidney and brain) indicate that the procedures reported here will be applicable, although further work will be required to definitively identify the HPLC-detectable components in the glycolipid fractions.
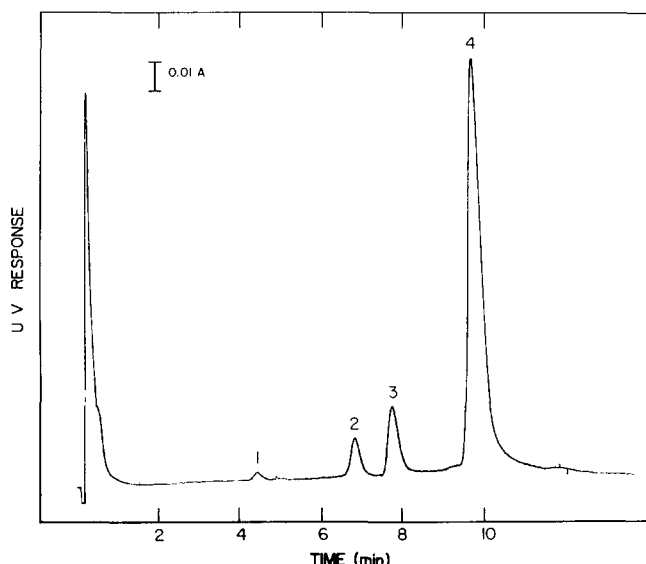

Fig. 7. HPLC analysis of glycolipids from human erythrocytes. The red blood cell neutral glycolipids from 5 ml of normal blood were isolated as described by Vance and Sweeley (1) and analyzed by HPLC as described in the text. Peaks are identified as derivatives of: 1, Glc-Cer; 2, Lac-Cer; 3, Gal-Lac-Cer; 4, globoside.

DISCUSSION

Conditions used for the perbenzoylation of neutral glycolipid mixtures were those that minimized the occurrence of side products. The results showed that the conditions used produced one major peak for each neutral glycolipid standard. Small peaks obtained with Lac-Cer and Gal-Lac-Cer represented less than 3% of the total area in each case. The peak for perbenzoylated globoside trailed slightly and was not

completely symmetrical under any perbenzoylation or chromatographic conditions tested. Although the peak asymmetry may reflect incomplete benzoylation or the presence of degradation products, it may also be due to the molecular heterogeneity of blood neutral glycolipids, which are known to contain a range of fatty acid chain lengths. The presence of small quantities of glycolipids which differ in the structure of their carbohydrate moiety may also contribute to peak asymmetry and the minor peaks seen. Neutral glycolipids other than the four major components are known to be present in blood. Gal-Cer is present in plasma (20) and GlcNAc-Lac-Cer (21) and Gal-GlcNAc-Lac-Cer (Lacto-N-neotetraosyl-Cer or paragloboside) (22, 23) are minor constituents of human red blood cell membranes. Isolation of the minor components and characterization of their perbenzoyl derivatives will be required to identify all of the chromatographic components observed on HPLC.

Although multiple products are obtained after alkaline methanolysis of single perbenzoylated derivatives, the yield of parent compound exceeds 50% for all materials tested except Glc-Cer. Thus isolation of perbenzoylated compounds by HPLC and subsequent alkaline methanolysis can be used as a preparative procedure and the pattern of products obtained will provide information about the number of amide groups present. We are exploring alternative procedures for removal of the benzoyl residues in an attempt to further improve the yields of the parent glycolipids.

The analysis of 1.0 ml of plasma was routinely performed to determine the content of the major neutral glycolipids. Smaller quantities of plasma have been analyzed since an entire sample could be injected and integrated without interference by nonretained compounds. We have performed analyses of the major plasma neutral glycolipids on as little as 0.5 ml of plasma, representing a 20-fold increase in sensitivity over previously reported methods (1). If detection at 230 nm is employed, the sensitivity is about 10-fold greater than reported here, but this requires the use of chromatographic solvents that are transparent at 230 nm. Studies utilizing detection at this low wavelength will be published elsewhere. It should be mentioned that the values obtained for plasma levels of neutral glycolipids are not absolute. It has been reported by Vance and Sweeley (1) that the recovery of neutral glycolipids during their isolation from plasma was 94%, 80%, 81%, and 71% for Glc-Cer, Lac-Cer, Gal-Lac-Cer, and globoside, respectively. We have measured and obtained similar recoveries when the ratio between sample size and

the quantity of Unisil employed is maintained. Our reported values and those reported previously (1, 17, 18) have not been corrected for these losses.

In addition to the increased sensitivity and decreased assay time, HPLC of perbenzoylated neutral glycolipids affords better resolution of plasma glycolipid components than does TLC of the underivatized glycolipids. The poorer resolution of neutral glycolipids by TLC may account in part for the discrepancy observed between our values for Glc-Cer levels in plasma and those published previously (1). It is feasible that Glc-Cer isolated from TLC plates may have contained some Lac-Cer (1) but, more likely, the discrepancies can be attributed to individual variations (17, 24). Examination of the HPLC chromatograms of plasma glycolipids showed three peaks eluted before Lac-Cer. The first is Glc-Cer, the second is unidentified (does not cochromatograph with our standard NFA-Gal-Cer), and the third was tentatively identified as HFA-Gal-Cer. The summed areas of the three peaks provide values for plasma levels of ceramide monohexoside that more closely agree with published values acquired by TLC–GLC analysis.

A difference in the amount of Gal-Lac-Cer in serum lipoproteins of human males and females has been reported (18). We have found a similar difference in total plasma Gal-Lac-Cer. HPLC quantitation of plasma neutral glycolipid levels of patients with inborn errors of metabolism showed, where expected, the appropriate variations from normal values. This technique can, therefore, be used to confirm the diagnosis of suitable enzymopathies or to monitor therapeutic attempts. We also believe the basic HPLC procedure reported here will be generally useful for the analysis of neutral glycolipids from many different tissue sources.∎

REFERENCES

1. Vance, D. E., and C. C. Sweeley. 1967. Quantitative determination of the neutral glycosyl ceramides in human blood. *J. Lipid Res.* **8:** 621–630.
2. Laine, R. A., K. Stellner, and S-I. Hakomori. 1974.

Isolation and characterization of membrane glycosphingolipids. *In*: Methods In Membrane Biology, 2. E. D. Korn, editor. Plenum Press, New York, New York. 205–244.

3. Hakomori, S-I., C. G. Gahmberg, R. A. Laine, and G. Yogeeswaran. 1975. Organization and modification of membrane glycolipids and glycoproteins. *In*: Cellular Membranes and Tumor Cell Behavior. The Williams and Wilkins Co., Baltimore, Maryland. 289–308.

4. Fishman, P. H., and R. O. Brady. 1975. Modification of membrane glycolipids by oncogenic agents. *In*: Modification of Lipid Metabolism. E. G. Perkins and L. A. Witting, editors. Academic Press, Inc., New York, New York. 105–126.

5. Hakomori, S-I. 1970. Glycosphingolipids having blood group ABH and Lewis specificities. *Chem. Phys. Lipids.* **5**: 96–115.

6. Hakomori, S-I., K. Stellner, and K. Watanabe. 1972. Four antigenic variants of blood group A glycolipid: Examples of highly complex, branched chain glycolipid of animal cell membrane. *Biochem. Biophys. Res. Commun.* **49**: 1061–1068.

7. Naiki, M., and D. M. Marcus. 1974. Human erythrocyte P and P^k blood group antigens: Identification as glycosphingolipids. *Biochem. Biophys. Res. Commun.* **60**: 1105–1111.

8. Naiki, M., J. Fong, R. Ledeen, and D. M. Marcus. 1974. Structure of the human erythrocyte blood group P$_1$ glycosphingolipid. *Biochemistry.* **14**: 4831–4837.

9. Warren, K. R., I. A. Schafer, J. C. Sullivan, M. Petrelli, and N. S. Radin. 1976. The effects of *N*-hexyl-*O*-glucosyl sphingosine on normal cultured human fibroblasts: a chemical model for Gaucher's disease. *J. Lipid Res.* **17**: 132–138.

10. Sugita, M., M. Iwamori, J. Evans, R. H. McCluer, J. T. Dulaney, and H. W. Moser. 1974. High performance liquid chromatography of ceramides: application to analysis in human tissues and demonstration of ceramide excess in Farber's disease. *J. Lipid Res.* **15**: 223–226.

11. McCluer, R. H., and J. E. Evans. 1973. Preparation and analysis of benzoylated cerebrosides. *J. Lipid Res.* **14**: 611–617.

12. McCluer, R. H., and J. E. Evans. 1976. Quantitative analysis of brain galactosylceramides by high performance liquid chromatography of their perbenzoyl derivatives. *J. Lipid Res.* **17**: 412–418.

13. Evans, J. E., and R. H. McCluer. 1972. High pressure liquid chromatography of neutral glycosphingolipids. *Biochim. Biophys. Acta.* **270**: 565–569.

14. Balzas, R., B. W. L. Brooksbank, A. J. Patel, A. L. Johnson, and D. A. Wilson. 1971. Incorporation of [^{35}S]sulfate into brain constituents during development and the effects of thyroid hormone on myelination. *Brain Res.* **30**: 273–293.

15. Penick, R. J., M. H. Meisler, and R. H. McCluer. 1966. Thin-layer chromatographic studies of human brain gangliosides. *Biochim. Biophys. Acta.* **116**: 279–287.

16. Stanbury, J. B., J. B. Wyngaarden, and D. S. Fredrickson. 1972. The Metabolic Basis of Inherited Disease. McGraw-Hill Book Company, New York, NY.

17. Vance, D. E., W. Krivit, and C. C. Sweeley. 1969. Concentrations of glycosylceramides in plasma and red cells in Fabry's disease, a glycolipid lipidosis. *J. Lipid Res.* **10**: 188–192.

18. Dawson, G., A. W. Kruski, and A. M. Scanu. 1976. Distribution of glycosphingolipids in the serum lipoproteins of normal human subjects and patients with hypo- and hyperlipidemias. *J. Lipid Res.* **17**: 125–131.

19. Tao, R. V. P., and C. C. Sweeley. 1970. Occurrence of hematoside in human plasma. *Biochim. Biophys. Acta.* **218**: 372–375.

20. Rathke, E., and M. Jones. 1974. Serum cerebrosides in multiple sclerosis. *J. Neurochem.* **22**: 311–313.

21. Ando, S., K. Kon, M. Isobe, Y. Nagai, and T. Yamakawa. 1976. Existence of glucosaminyllactosylceramide (amino CTH-I) in human erythrocyte membranes as a possible precursor of blood group-active glycolipids. *J. Biochem.* **79**: 625–632.

22. Siddiqui, B., and S-I. Hakomori. 1973. A ceramide tetrasaccharide of human erythrocyte membrane reacting with anti-type XIV pneumococcal polysaccharide. *Biochim. Biophys. Acta.* **330**: 147–155.

23. Ando, S., and T. Yamakawa. 1973. Separation of polar glycolipids from human blood cells with special reference to blood group A activity. *J. Biochem. (Tokyo)* **73**: 387–396.

24. Dawson, G. 1972. Glycosphingolipid levels in an unusual neurovisceral storage disease characterized by lactosylceramide galactosyl hydrolase deficiency: lactosylceramidosis. *J. Lipid Res.* **13**: 207–219.