Quantitative determination of the neutral glycosyl ceramides in human blood

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ABSTRACT A method is described for the qualitative and quantitative estimation of four neutral glycosyl ceramides from human plasma and erythrocytes. Total lipids extracted from 50 ml of plasma or packed erythrocytes were separated by silicic acid chromatography into neutral lipids, a fraction of mixed glycolipids that was eluted with acetone–methanol 9:1, and phospholipids. After mild alkali-catalyzed methanolysis to remove contaminants from the crude fraction of glycolipids, individual glycosyl ceramides were isolated by preparative thin-layer chromatography. The oligosaccharide portions of these lipids were characterized by cleavage with methanolic hydrogen chloride and gas chromatography of the *O*-trimethylsilyl methyl glycosides. It was possible to study the composition of the carbohydrate and sphingolipid base fractions in the same gas chromatographic analysis. With mannitol as an internal standard for gas chromatographic estimation of glucose, concentrations of each of the glycosyl ceramides were determined with a precision of about 10%.

Recoveries of the lipids from plasma varied with the complexity of the oligosaccharide moiety and ranged from 94% with glucosyl ceramide to 71% with globoside. Concentrations of the four glycosyl ceramides in plasma and in erythrocytes were determined for samples from young, healthy males. Amounts of glycolipid as low as 0.1 μmole can be determined conveniently by this procedure.

KEY WORDS glycosyl ceramides · cerebroside · dihexoside · trihexoside · globoside · plasma · erythrocytes · silicic acid chromatography · acetone–methanol elution · mild alkali-catalyzed methanolysis · thin-layer chromatography · methanolysis · mannitol · trimethylsilyl methyl glycosides · gas–liquid chromatography · precision · recovery · levels

In 1951 KLENK AND LAUENSTEIN reported the discovery of a galactosamine-containing glycolipid in a fraction of stroma from human erythrocytes (1). Yamakawa and Suzuki found the same substance, which they called globoside, in washed stroma from fresh human erythrocytes (2) and also described the isolation of a related glycolipid (hematoside), containing neuraminic acid instead of galactosamine, from horse erythrocytes (3). Globoside, shown recently by Yamakawa, Nishimura, and Kamimura (4) to be *N*-acetylgalactosaminyl-(1 → 3)-galactosyl-(1 → 4)-galactosyl-(1 → 4)-glucosyl-ceramide, is the predominant constituent of a mixture of glycosyl ceramides in human erythrocytes. Two closely related glycolipids that occur with it are lactosyl ceramide (5) and a trihexosyl ceramide with galactose and glucose in a 2:1 molar ratio (6).

Cerebroside has long been known to occur in the lipid fraction from human serum, but until recently little information was available about the possible occurrence of other glycosyl ceramides in serum. In 1958 Svennerholm and Svennerholm described a quantitative method for the determination of plasma cerebroside and obtained preliminary evidence for the presence of more complex glycolipids in their extracts (7). Chromatographic procedures for the isolation of individual glycosyl ceramides were subsequently described (8) and used for a detailed study of the glycolipids from 17 liters of human serum (9). Glucosyl ceramide and a dihexosyl ceramide with equimolar amounts of glucose and galactose were present in approximately equal quantities in the mixture, along with much lesser proportions of a trihexosyl ceramide and globoside or a related aminoglycolipid with the same molar ratio of glucose, galactose, and galactosamine (1:2:1).

Abnormalities of glycolipid metabolism that occur in several of the sphingolipidoses such as Gaucher's disease,

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; TMSi, trimethylsilyl; gal, galactose; glu, glucose.

Fabry's disease, and metachromatic leukodystrophy have led to a general interest in the composition, metabolic behavior, and physiological function of the glycosyl ceramides in human blood. Previous efforts to purify the individual glycosyl ceramides included a variety of chromatographic steps on cellulose (10), silicic acid (9, 11), alumina (12), and Florisil (10, 11). Crude glycolipids from serum were separated into four fractions by preparative TLC on silica gel (9). Although suitable for isolations of glycolipids from large pooled volumes of plasma or erythrocytes, previously described procedures were ineffective for the routine determination of these lipids in small aliquots of blood from individual subjects. In the present report we present the details of a method for the separation and quantitative determination of neutral glycosyl ceramides of human blood that requires at most 50 ml of plasma or packed erythrocytes. A preliminary account of this work was given recently (13).

METHODS

Extraction of Lipids

Freshly drawn, heparinized blood or outdated ACD (acid–citrate–dextrose) blood (see Materials) was centrifuged at 650 *g* for 1 hr, after which the supernatant plasma and the buffy coat were removed with a pipet. Erythrocytes were resuspended in an equal volume of isotonic saline and centrifuged at 650 *g*. After another wash with saline, 50 ml of packed erythrocytes and 50 ml of plasma were frozen separately for subsequent extraction of the lipids.

Thawed plasma or the plug of erythrocytes was mixed with 300 ml of methanol and 600 ml of chloroform was added. The mixture was stirred with a magnetic bar for 15 min at room temperature and filtered, and the residue on the filter paper was washed with 100 ml of chloroform–methanol 2:1. The combined extracts from plasma were mixed with 200 ml of water and the biphasic mixture was set aside in the cold room overnight. The residue from red cells was further extracted at a gentle reflux temperature with 450 ml of chloroform–methanol 2:1 for 2 hr. After filtration, the residue was washed once more with 50 ml of chloroform–methanol 2:1. Combined extracts from the erythrocytes were mixed thoroughly with 325 ml of water and the layers were allowed to separate overnight in the cold room. Upper aqueous methanolic layers were removed by aspiration and the lower layers were evaporated to dryness in vacuo in tared flasks.

Column Chromatography

4 g of silicic acid, previously activated for at least 12 hr at 80°C, were removed from an oven and suspended immediately in diethyl ether. The slurry was poured into a small column (1 cm I.D. × 30 cm) with closed stopcock. After the silicic acid had settled into a packed bed, the ether was allowed to percolate through the column and the adsorbent was washed with 15 ml of chloroform. A solution, in a few milliliters of chloroform, of crude total lipids from plasma was applied to the column. Neutral lipids were eluted with 100 ml of chloroform, after which crude total glycosyl ceramides were obtained by elution with 200 ml of acetone–methanol 9:1. Phospholipids were recovered from the column, if desired, by final elution with 100 ml of methanol. A mixture of glycosyl ceramides was obtained from erythrocytes in the same manner, except that in addition we washed the column with 10 ml of ethyl acetate between the chloroform and acetone–methanol steps to remove an interfering pigmented fraction.

Mild Alkali-Catalyzed Methanolysis

The crude mixture of glycosyl ceramides was subjected to mild alkaline methanolysis as described previously (14), according to the method of Dawson (15) as modified by Hübscher, Hawthorne, and Kemp (16). A solution of lipids in 1 ml of 0.6 N methanolic NaOH and 1 ml of chloroform was allowed to stand for 1 hr at room temperature, after which we added 1.2 ml of 0.5 N methanolic HCl, 1.7 ml of water, and 3.4 ml of chloroform. The well-mixed biphasic system was centrifuged to recover the glycolipids in the lower layer. After this extract had been washed twice with equal volumes of methanol–water 1:1 the solvents were removed at 50°C under a stream of nitrogen.

TLC Separation of Individual Glycosyl Ceramides

The preparative TLC system has been described by Svennerholm and Svennerholm (9). It was important to heat the plates coated with silica gel for at least 2 hr at 90°C just prior to applying the solution of mixed glycolipids as a streak on the plate. The tank contained a paper liner and solvent mixture (chloroform–methanol–water 100:42:6) that was added to the tank 2 to 4 hr before TLC. Individual lipids were located on the developed plate by spraying with a 0.2% solution of 2′,7′-dichlorofluorescein (17). The lipids were recovered from silica gel (0.1–0.3g) by elution with 50 ml of chloroform–methanol–water 100:50:10. TLC on borate–silica gel plates was carried out as described by Kean (18) and Young and Kanfer (19).

Methanolysis

Methanolyses of the glycolipids were carried out in methanolic HCl, prepared by bubbling gaseous HCl through methanol at room temperature. Reaction mixtures containing 3 ml of 0.5–1.5 N HCl were heated at

80°C for 18–24 hr in screw-capped tubes fitted with Teflon liners. After cooling, the tubes were opened and 0.3 μmole (150 μl) of mannitol and 0.2 ml of water were added. The stock solution of mannitol, containing 2 μmoles/ml, was a solution in 99% methanol. Methyl esters of fatty acids were removed into hexane by three extractions with equal volumes, and HCl was removed subsequently by percolation of the aqueous methanolic HCl mixture through 1 g of Amberlite CG-4B resin (OH phase) packed in a small column (0.5 cm I.D. × 21 cm). Methyl glycosides and sphingolipid bases were recovered completely if the resin was washed with 35 ml of methanol. Solvents were removed in vacuo and the residue was transferred in a small volume of methanol to a capped vial; methanol was removed from this solution under a stream of nitrogen.

GLC

The TMSi derivatives of methyl glycosides were prepared from the dry residue above by the addition of 150 μl of pyridine–hexamethyldisilazane–trimethylchlorosilane 10:4:2 (20). After about 15 min an aliquot of the reaction mixture was injected onto a column containing 2% SE-30 or OV-1 (silicone phases from Applied Science Laboratories Inc., State College, Pa.) at 160°C according to previously described methods for the GLC of these derivatives from glycolipids (21). After elution of TMSi mannitol in 15–20 min, peaks for the TMSi derivatives of sphingosine and related compounds were observed as the temperature of the GLC column was increased at 10°C/minute to 200°C. Areas of the GLC peaks were obtained by planimetry.

MATERIALS

Outdated and fresh blood were obtained at the Pittsburgh Blood Bank. Methanol for methanolyses was dried by distillation from zinc turnings, but otherwise reagent grade solvents were used without further purification. Column packings for GLC were prepared in this laboratory as described previously (21, 22). Mixed glycosyl ceramides were applied in narrow bands to TLC plates with a Radin-Pelick streaking device from Applied Science Laboratories Inc. Silicic acid (200–325 mesh Unisil) was purchased from Clarkson Chemical Company, Inc., Williamsport, Pa., and Silica Gel G for TLC was obtained from Brinkmann Instruments Inc., Westbury, N. Y. Glucosyl ceramide was purified from the spleen of a patient with Gaucher's disease according to well-established procedures. Digalactosyl ceramide and trihexosyl ceramide (gal/glu 2:1) were isolated from a section of formalin-fixed kidney of a patient with Fabry's disease as previously outlined (23). Globoside was isolated from human erythrocytes by the procedure described in Methods. Crude galactosyl ceramide from The Wilson Laboratories, Chicago, Ill., was purified by silicic acid and Florisil chromatography. D-Glucose was a generous gift from Dr. Ronald Bentley; the sample was from Batch 41 from the National Bureau of Standards, Washington, D.C. Commercial anhydrous D-galactose was recrystallized from hot aqueous alcohol as described by Wolfrom and Thompson (24), mp 165–168°C. Mannitol from Nutritional Biochemicals Corporation, Cleveland, Ohio, was recrystallized from pyridine. A criterion of purity used for each of the purified carbohydrates was the absence of spurious peaks on GLC of a sufficient amount of the TMSi derivative to give a major peak that was off-scale on the recorder.

RESULTS

Isolation of Glycosyl Ceramides

The extraction of glycolipids from plasma presented no difficulties. Total lipids were obtained in the usual way by extraction with chloroform–methanol 2:1 at room temperature, and water-soluble contaminants were removed from the extract according to a procedure described by Folch, Lees, and Sloane Stanley (25). Glycosyl ceramides were then recovered from the residue of crude total lipids.

Comparisons were made of yields of total lipids and glycolipids from erythrocytes by the same procedure and a newer one described by Rose and Oklander (26), who claimed that a mixture of chloroform and isopropanol gave an extract with lesser contamination by pigments. Although preliminary results suggested that equal recoveries were achieved by the two methods of extraction, we observed that the isopropanol solvent extracted an unknown, UV-absorbing material that interfered to some extent with TLC of the glycolipids. Extraction of the erythrocytes at room temperature with chloroform–methanol 2:1 was therefore used routinely in these studies, but one extraction did not suffice to remove all of the glycosyl ceramides. A second extraction obtained at reflux temperature contained measurable quantities of all four neutral glycosyl ceramides; further extractions of the residue failed to yield glycolipid.

In a study of the composition of milk lipids, Smith and Lowry described a procedure in which acetone was used to elute glycolipids preferentially from silicic acid–diatomaceous earth columns (27). Similarly, Vorbeck and Marinetti made use of mixed acetone and chloroform as a selective eluting agent to recover glycosyl diglycerides from silicic acid (28). Since one of the main problems in the isolation of glycolipids from plasma or red cells was an unfavorable ratio of these lipids to the phospholipids, these important observations afforded

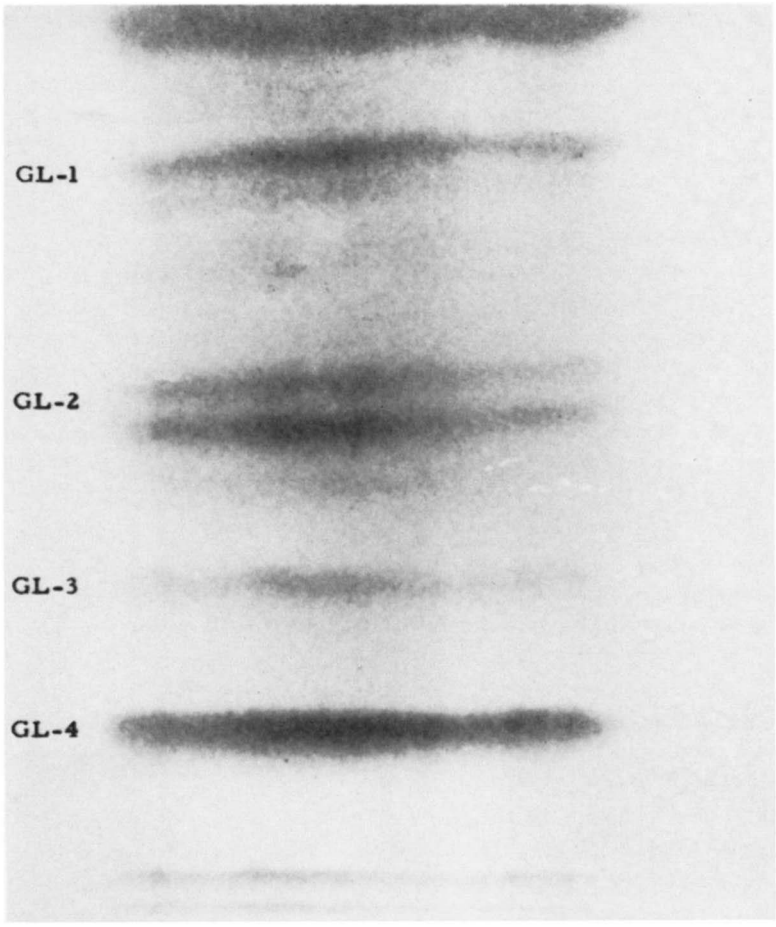

FIG. 1. Thin-layer chromatogram of the glycosyl ceramides from normal human plasma. The bands are defined as GL-1 for cerebroside, GL-2 for dihexosyl ceramide, GL-3 for trihexosyl ceramide, and GL-4 for globoside. The lipids were made visible by exposure to iodine vapor; the amounts of glycolipids in the bands were not directly proportional to the intensity of absorbed iodine.

us a simple basis for the rapid separation of trace proportions of glycolipids in a single chromatographic step. It was necessary to modify the solvent somewhat by the addition of methanol, however, since acetone alone eluted glucosyl ceramide, lactosyl ceramide, and the trihexoside but was not sufficiently polar to elute globoside from silicic acid.

Initially, the mixture of glycolipids eluted with acetone or acetone–methanol was contaminated by a phosphorus-containing lipid with an R_f on TLC that was intermediate between those of cerebroside and lactosyl ceramide. This phospholipid was probably phosphatidyl ethanolamine; it was not eluted by these solvents when the silicic acid had been dried at 90°C for at least 12 hr just prior to its use in the column chromatographic step. To be certain that glycolipids were not partially removed from the column with ethyl acetate in the isolation of glycolipids from erythrocytes, and that small amounts were not retained by the silicic acid after elution with acetone–methanol 9:1, the ethyl acetate and methanol fractions were subjected to methanolysis and GLC. Since peaks for TMSi methyl glycosides of glucose and galactose were not observed under these conditions, we concluded that the glycosyl ceramides were wholly recovered in the acetone–methanol fraction.

Several minor contaminants that cochromatographed with the glycolipids on preparative TLC were removed by mild alkaline methanolysis of the mixed lipid fraction eluted from silicic acid with acetone–methanol. Recoveries of glycolipids after this step were not studied in detail, but a sample of trihexosyl ceramide from Fabry kidney was recovered in quantitative yield and we presumed that the other glycosyl ceramides would survive equally well.

Individual glycosyl ceramides were well separated into zones on preparative TLC plates, as shown by the reproduction in Fig. 1. Adequate control of the R_f values and resolution of bands on these plates were dependent on the thorough drying of the plates and use of tanks to which solvent had been added 2–4 hr previously.

When the plates were developed in solvent added to the tank more than 12 hr prior to TLC, all of the glycolipids migrated to positions closer to the solvent front and the bands were less clearly separated from each other. Under such conditions complete separations of the glycolipids into individual types were seldom achieved.

Methanolysis and Recovery of Methyl Glycosides

High ratios of galactose/glucose, attributed to incomplete cleavage of the glycosidic bond of glucose to sphingosine, were always observed when methanolyses were conducted in 0.5 N (or less concentrated) methanolic HCl for periods less than 24 hr. Similarly, methanolyses of more than 1 mg of glycolipid in 3 ml of the reagent were usually accompanied by high ratios even after 24 hr with 1 N methanolic HCl. Low gal/glu ratios, believed to result from selective secondary reactions of the methyl galactosides, had previously been observed when HCl more concentrated than 1 N was employed in the methanolysis procedure (21). However, methanolysis of 1 mg or less of any of the four neutral glycosyl ceramides in 3 ml of 0.75 N methanolic HCl for 24 hr at 80°C gave consistently high yields of the methyl glycosides of glucose and galactose, and the molar ratios of these sugars were close to those predicted by the glycolipid structure. Yields were evaluated in these studies by comparing the GLC peaks from the glycolipids with those obtained from standard amounts of glucose and galactose subjected to the same procedure.

Low gal/glu ratios also resulted whenever HCl and methanol were removed together by evaporation of the methanolysate under a stream of nitrogen. To eliminate this source of error in the GLC determinations of the sugars, we removed HCl by means of a small column containing a weak anion exchange resin, from which the methyl glycosides and sphingolipid bases were completely eluted with 35 ml of methanol. The products were then stable to evaporation of the solvent by any convenient method.

GLC Determination of Methyl Glycosides and Sphingolipid Bases

The TMSi derivatives of methyl glucosides and methyl galactosides were readily separated by GLC on a nonpolar silicone column, as described previously (21, 29). These were the only carbohydrate components observed from isolated mono-, di-, and trihexosides but globoside also yielded methyl glycosides of galactosamine, the TMSi derivatives of which were eluted from SE-30 or OV-1 just before the TMSi methyl galactosides, as shown in Fig. 2. The trimethylsilylation reagent also converts sphingosine and related bases to TMSi derivatives that are volatile but require higher GLC column temperatures than the TMSi sugars (30, 31). We found in this investigation that qualitative identifications of the sphingolipid bases could be made in the same analysis by increasing the column temperature after elution of TMSi mannitol, as indicated in Fig. 2. Three peaks for the *O*-trimethylsilyl derivatives of sphingosine and *O*-methyl sphingosines were observed, as previously reported (30); other bases, though not present in these particular lipids, would have retention times of the same general magnitude (32).

Individual glycosyl ceramides were identified by their R_f values on the preparative TLC plates and also by the gal/glu ratios calculated from GLC data. Direct com-

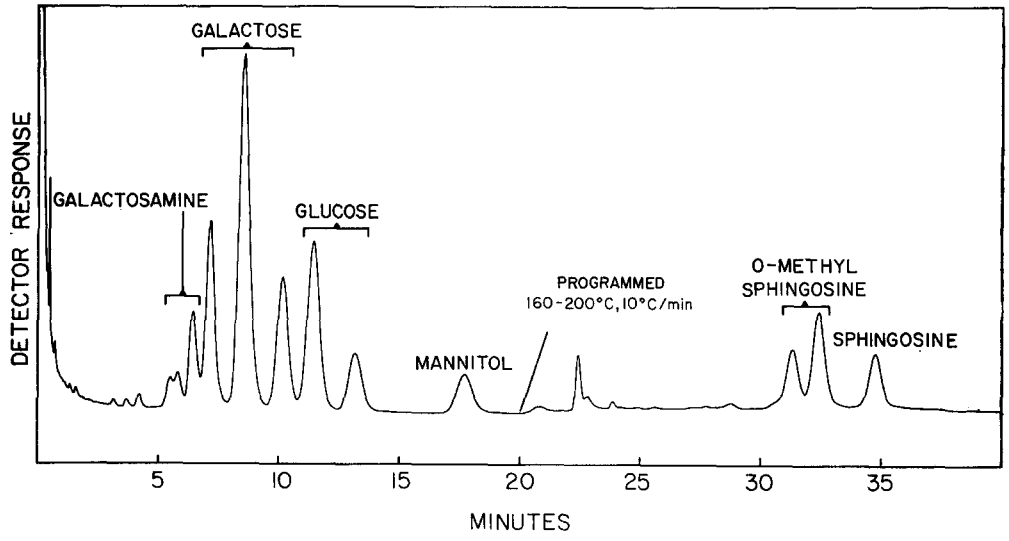

FIG. 2. Gas chromatogram of TMSi methyl glycosides and TMSi sphingosine bases from globoside of human erythrocytes, plus added TMSi mannitol; 2% SE-30 at 160°C until TMSi mannitol was eluted, then temperature increased to 200°C.

TABLE 1 CONVERSION FACTORS FOR GLC ESTIMATION OF GLUCOSE AND GALACTOSE IN METHANOLYSIS MIXTURES

Preparation	Gal/Glu*	Mannitol/Glu*
1	1.00	1.22
2	0.97	1.27
3	1.00	1.25
Mean	0.99	1.25
SD	0.02	0.02

Equimolar aliquots of carbohydrate solutions were subjected to methanolysis for 18 hr at 70°C with 0.5 N methanolic HCl; mannitol was added and HCl removed by ion exchange; estimation of TMSi derivatives took place on 2% SE-30 at 160°C.

* Ratios of observed areas on GLC chromatograms.

TABLE 2 PRECISION IN THE GLC ESTIMATION OF GLOBOSIDE

Aliquot	Gal/Glu	Glu
		μmole
1	2.04	0.138
2	1.98	0.139
3	2.00	0.145
Mean	2.01	0.141
SD	0.03	0.004

Aliquots of a globoside solution were subjected to methanolysis in 0.75 N methanolic HCl for 24 hr at 80°C; mannitol was added and HCl removed by ion-exchange; estimation of TMSi derivatives took place on 2% SE-30 at 160°C.

parisons of the areas from gas chromatograms provided sufficiently accurate molar ratios since equimolar amounts of the glucose and galactose standards gave equal total areas on GLC after they were subjected to methanolysis, as shown by the data in Table 1. Apparently these two sugars behaved identically during conversion to methyl glycosides and the yields of TMSi derivatives were the same; they would be expected to elicit the same response in a flame ionization detector since they are stereoisomeric compounds. Yields of the TMSi methyl glycosides of galactosamine were always low, as found in previous work (21).

Since each of the four glycolipids from plasma or erythrocytes contained one glucose residue per molecule,[1] it was possible to base quantitative measurements of these glycolipids on the GLC determination of glucose recovered after methanolysis. The TMSi derivative of mannitol was particularly well suited as an internal standard in this determination although it was necessary to use a factor of 1.25 (Table 1) to correct the results for differences in relative response of the detector.

The reproducibility of this method for the determination of glycosyl ceramides is indicated by the data in Table 2. When aliquots of a solution of globoside were subjected to methanolysis and GLC of the TMSi methyl glycosides, the observed over-all precision was about equal to the limits usually found in measuring areas of GLC peaks.

Precision and Recovery in Plasma and Red Cell Glycolipid Analyses

Results are presented in Table 3 for triplicate analyses of the glycosyl ceramides from 50 ml of packed human erythrocytes from a single donor. Similarly, Table 4 contains the results of analyses of these glycolipids in triplicate samples (50 ml) of plasma from the same donor. Remarkably similar standard deviations of about 10% were observed with all the glycolipids except glucosyl ceramide from plasma or from erythrocytes, for which the SD was somewhat higher. These results demonstrated the adequate sensitivity of the method for analyses of as little as 0.08 μmole in a 50 ml aliquot of plasma or red cells.

To determine average recoveries of the glycosyl ceramides from plasma, we added known amounts of glucosyl ceramide, digalactosyl ceramide, a trihexoside (gal/gal 2:1), and globoside to two 50 ml aliquots of plasma that had been analyzed previously for endogenous glycolipids. The results, summarized in Table 5, clearly indicated that the recoveries were not the same with the various classes of neutral glycosyl ceramides. The recovery of glucosyl ceramide (94%) was highest and that

TABLE 3 PRECISION IN GLYCOLIPID ANALYSES* OF HUMAN ERYTHROCYTES

Aliquot	Cerebroside	Dihexoside	Trihexoside	Globoside
	μmole/50 ml			
1	0.26	0.61	0.47	2.68
2	0.36	0.60	0.48	2.86
3	0.28	0.50	0.41	2.38
Mean	0.30	0.57	0.44	2.64
SD	0.05	0.06	0.04	0.24

* Triplicate analyses of 50 ml aliquots from one donor.

TABLE 4 PRECISION IN GLYCOLIPID ANALYSES* OF HUMAN PLASMA

Aliquot	Cerebroside	Dihexoside	Trihexoside	Globoside
	μmole/50 ml			
1	0.57	0.36	0.08	0.21
2	0.39	0.30	0.08	0.17
3	0.47	0.30	0.10	0.18
Mean	0.48	0.32	0.09	0.19
SD	0.09	0.03	0.01	0.02

* Triplicate analyses of 50 ml aliquots from one donor.

[1] The trace of galactosyl ceramide that might exist in plasma, of course, has no glucose units. The amount of galactose observed in analyses of this fraction were not included in calculations of the levels of cerebroside in plasma.

TABLE 5 RECOVERY OF ADDED GLYCOLIPIDS FROM HUMAN PLASMA

	Cerebroside	Dihexoside	Trihexoside	Globoside
	μmoles			
Observed total glycolipid	1.13	0.49	0.49	0.47
Endogenous glycolipid	0.36	0.25	0.12	0.23
Added glycolipid recovered	0.77	0.24	0.37	0.24
Amount of added glycolipid	0.82	0.30	0.46	0.34
% Recovery	94	80	81	71

Known quantities of glucosyl ceramide, digalactosyl ceramide, trihexosyl ceramide (gal/glu 2:1), and red cell globoside were added to two 50 ml aliquots of human plasma. Endogenous glycolipids were determined on two other aliquots from the same blood.

of globoside (71%) was lowest, suggesting that losses of glycolipid at one or more stages in the isolation were dependent to a large degree on the size of the oligosaccharide moiety.

Analyses of Human Plasma and Erythrocytes

Freshly drawn blood from four healthy Caucasian males, aged 21–25 yr, was separated into plasma and erythrocytes as described, and 50 ml aliquots of each fraction were analyzed for concentrations of individual glycolipids. The major constituent of the glycosyl ceramide fraction in plasma was (gluco)cerebroside, as shown in Table 6; the average concentration of this lipid was about 0.8 mg/100 ml, calculated from the observed molar yield of glucose and without correction for losses in isolation. In all of the samples of plasma analyzed, the presence of galactosyl ceramide in this fraction was suggested by small quantities of methyl galactosides that were observed in the GLC chromatograms, as shown by the typical recording in Fig. 3. The proportions of glucose and galactose indicated that the fraction was more than 90% glucosyl ceramide. Although the TLC zones for this lipid and the dihexosyl ceramide appeared to be completely separated, the alternative possibility of a slight contamination of the cerebroside fraction by dihexoside cannot be excluded. Attempts to separate a galactosyl ceramide from this fraction, using a borate TLC system (18, 19), gave unsatisfactory results since TMSi methyl galactosides still appeared on the GLC chromatogram from glucosyl ceramide, and TMSi glucosides were obtained from the TLC fraction that should have been galactosyl ceramide.

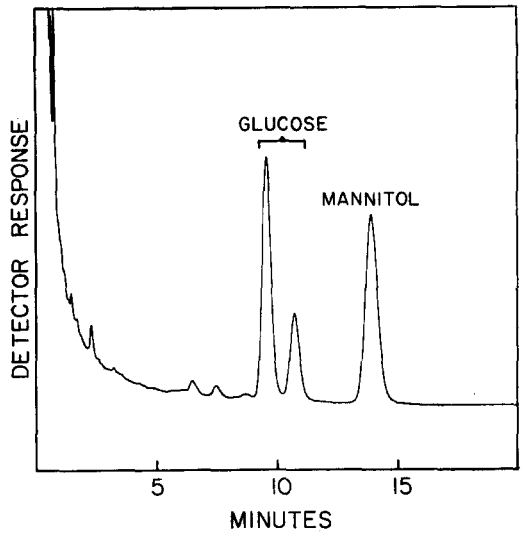

FIG. 3. Gas chromatogram of TMSi methyl glycosides from the cerebroside fraction of human plasma, with added TMSi mannitol; 2% SE-30 at 160 °C.

The dihexosyl ceramide from plasma was assumed to be lactosyl ceramide since it contained equimolar amounts of glucose and galactose (Table 6) but the exact nature of this glycolipid has not been established. Its concentration of about 0.5 mg/100 ml was lower than that of glucosyl ceramide and higher than those of the more complex glycolipids. These more polar fractions were judged to be trihexosyl ceramide and globoside on the basis of gal/glu ratios and TLC mobility but again, exact chemical structures have not been determined. Their concentrations were relatively constant and about equal (0.25 mg/100 ml) in the samples of normal plasma that have been examined to date. As indicated in Table 6, the gal/glu ratios of plasma globoside were somewhat low; this result is inexplicable at the present time.

TABLE 6 CONCENTRATIONS OF GLYCOSYL CERAMIDES IN NORMAL HUMAN PLASMA

Donor	Age	Cerebroside	Dihexoside		Trihexoside		Globoside	
			Concn	Gal/Glu	Concn	Gal/Glu	Concn	Gal/Glu
		μmole/50 ml	*μmole/50 ml*		*μmole/50 ml*		*μmole/50 ml*	
JG	21	0.57	0.30	0.99	0.14	2.04	0.13	1.83
PS	24	0.50	0.40	0.93	0.09	2.09	0.11	1.80
JN	24	0.41	0.24	1.04	0.13	1.93	0.08	1.78
Mean		0.49	0.31	0.99	0.12	2.02	0.11	1.80

TABLE 7 CONCENTRATIONS OF GLYCOSYL CERAMIDES IN NORMAL HUMAN ERYTHROCYTES

Donor	Age	Cerebroside	Dihexoside		Trihexoside		Globoside	
			Concn	Gal/Glu	Concn	Gal/Glu	Concn	Gal/Glu
		μmole/50 ml	*μmole/50 ml*		*μmole/50 ml*		*μmole/50 ml*	
JG	21	0.19	1.22	1.13	0.57	1.94	3.75	2.13
JN	24	0.20	0.62	1.09	0.53	2.00	3.50	2.08
DV	24	0.19	0.46	1.12	0.71	1.89	3.41	1.97
Mean		0.19	0.77	1.11	0.60	1.94	3.55	2.06

Globoside is decidedly the predominant glycolipid in human erythrocytes, as shown in Table 7. On the basis of the observed molar ratios of glucose, galactose, and galactosamine and the absence of fucose and glucosamine, we presumed that this glycolipid is the classic one described earlier (1, 2) and currently referred to by Yamakawa et al. as globoside I (4). Relatively little variation was noted in the concentration of this lipid, which averaged about 9 mg/100 ml, and values obtained with fresh and outdated blood were in the same range.

Concentrations of the other glycosyl ceramides in erythrocytes are summarized in Table 7. Although there was no doubt about the identities of the di- and trihexosyl ceramides, the gal/glu ratios of these glycolipids from erythrocytes were always high for the dihexoside and low for the trihexoside. These findings have been attributed to slightly incomplete separation of the two lipids on the TLC plates. The reason for lower chromatographic efficiency with this mixture, as compared with the glycolipids from plasma, is not known.

DISCUSSION

Evidence for the occurrence of four neutral glycosyl ceramides in human plasma was reported by Svennerholm and Svennerholm in 1962 (12). A preparative method for the isolation of these substances was subsequently developed and preliminary chemical studies were described by these authors (8, 9). In the present investigation the gal/glu ratios of the four lipids were the same as those reported previously (9), but the quantities isolated from 50 ml of plasma were not sufficient for positional analyses of the glycosidic bonds or for studies of the arrangement of hexose units in the oligosaccharide moieties. Although the exact chemical structures of the dihexosyl ceramide, trihexosyl ceramide, and aminoglycolipid from plasma have not yet been firmly established, it is reasonable to expect that they will be shown to be identical with lactosyl ceramide (33, 34), the trihexoside previously found in normal (35, 36) and Fabry kidney (23), and globoside I, respectively.

The isolation of these glycolipids from a small aliquot of plasma posed a formidable obstacle to development of a quantitative method at first, but it was possible to avoid elaborate steps in purification when it became apparent that acetone–methanol 9:1 was excellent for the selective desorption of glycosyl ceramides from thoroughly dried silicic acid. High yields of a fraction that consisted almost entirely of glycolipids were thus obtained by the simple schema involving sequential elution of neutral lipids, glycolipids and, finally, phospholipids from a silicic acid column. Each of the four glycosyl ceramides were then easily obtained with the preparative TLC system previously described in some detail by Svennerholm and Svennerholm (9). Separations of the lipids were achieved on a scale that was well suited for subsequent analysis of the lipid by methanolysis and GLC of the TMSi methyl glycosides. Unfortunately the recoveries of glycolipids were not uniformly high. It appeared that the recovery of a glycolipid, while reasonably reproducible, was a function of the complexity of its oligosaccharide group, a result that had previously been noted by the Svennerholms (9) who attributed this to losses on TLC. Other procedures such as gradient elution from a silicic acid column have not been attractive alternatives, however, because of the increased time for an analysis and requirements for greater sample size. The concentrations that have been obtained by this method are therefore low to the extent that the individual glycolipids are lost during TLC; corrections for these losses have not been made in reporting the concentrations in Tables 6 and 7.

The selection of a solvent system for the extraction of glycolipids from red blood cells was based on past experiences of Ways and Hanahan (37) with the recovery of phospholipids and those of Yamakawa, Irie, and Iwanaga (11) with the isolation of glycosyl ceramides from stroma. Since it was technically impractical to determine over-all recovery from the red cells, we assumed that the concentrations of glucose observed by methanolysis and GLC would reflect the same manipulative losses that were found with plasma.

The results obtained in several laboratories led to the conclusion that methanolysis is the method that most consistently gives quantitative yields of hexose from glycosyl ceramides; the methyl glycosides that are products of this reaction were previously shown to be conveniently analyzed by GLC of the TMSi derivatives

(21). Complete methanolysis of any of the glycosyl ceramides is a slow process in comparison with rates of hydrolysis in aqueous acid, but this disadvantage cannot be overcome by using high concentrations of HCl in the reagent. With the GLC method of analysis, 1.5 N or higher concentrations of HCl in methanol gave low gal/glu ratios, probably because of secondary changes in the galactosides (21). As debated in a recent review on problems associated with the liberation of sugars from glycolipids (38), it is possible that some free galactose is actually formed during methanolysis and that the yields of this form are directly proportional to the HCl concentration. Kishimoto and Radin recently reported that significant amounts of methyl chloride and water are formed in methanolic HCl, especially at the elevated temperatures used for methanolyses (39). Perhaps sufficient water is present when the HCl concentration is high to cause partial hydrolysis of the methyl galactosides. Although various parameters in the methanolysis reaction are not easily balanced, the conditions described in this investigation gave nearly optimal results.

When carefully purified samples of glucose and galactose were subjected to methanolysis, ion-exchange chromatographic removal of the HCl, and preparation of the TMSi ethers, GLC gave identical areas from equimolar amounts of the two sugars. This result was not observed by Penick and McCluer (29) who reported a glu/gal ratio of 1.10 for equimolar amounts of methyl glucopyranoside and methyl galactopyranoside. An explanation for this discrepancy is the possibility that the monohydrate of methyl galactopyranoside synthesized by Penick and McCluer was compared with an anhydrous glucose derivative, since the molar ratio of glu/gal for equal weights would then be 1.10. A factor of 1.45 for the ratio of glu/gal, reported by Bolton, Clamp, Dawson, and Hough (40), is considerably different and we cannot account for it. Differences in the relative response of the detector to TMSi mannitol and TMSi methyl glycosides were approximately accountable for by the differences in molecular weights of these substances. The ratio of 1.25 for the areas of equimolar amounts of TMSi mannitol and of TMSi methyl glucosides compared favorably with the value of 1.30 reported by Penick and McCluer (29). This factor was used in the calculation of glucose levels from GLC data as shown below.

$$\mu\text{moles glucose} = \frac{\text{area of glucose peaks}}{\text{area of mannitol peak}} \times 1.25 \times \mu\text{moles mannitol added}$$

Relatively little individual variation has been noted in the concentrations of glycosyl ceramides in circulating human plasma from normal subjects. We found the levels of trihexoside and globoside to be measurably higher than those reported by Svennerholm and Svennerholm (9) and those of dihexoside lower. It is possible that the discrepancies are due primarily to different relative losses during preparative TLC, but other manipulative losses of globoside during isolation from several liters of plasma might have been greater than those encountered in the present procedure with much smaller amounts of material.

A substance that we presume to be identical with Yamakawa's globoside I accounted for about 70% of the total red cell glycolipid measured in this procedure. Other globosides from human erythrocytes, referred to by Yamakawa as globosides II and III, have been shown to contain glucosamine, fucose, and sialic acid in addition to glucose, galactose, and galactosamine (4). These more complicated glycolipids were not found in the present study, presumably because they were selectively partitioned into the aqueous methanolic phase during the "Folch wash" of total lipids. The presence of lactosyl ceramide in substantial amounts in human erythrocytes was originally reported by Makita and Yamakawa (41) and has been confirmed by the present study of red cell glycolipids. It was surprising to find a trihexosyl ceramide (gal/glu 2:1) in the erythrocytes since to our knowledge this lipid has never been reported in the glycolipid fractions studied by Yamakawa. Gatt (6) recently made a passing reference to its occurrence in red cells, however. The concentration of this glycosyl ceramide was nearly as high as that of lactosyl ceramide in the red cell, and it was considerably more prominent a component here than in the plasma. Glucosyl ceramide was clearly a trace component of the red cell glycolipids; this is the first report of its occurrence in the human erythrocyte. There was no indication that this cerebroside might be a mixture with galactosyl ceramide since TMSi methyl galactosides were not visible on GLC chromatograms.

The procedure described here for the isolation and estimation of glycosyl ceramides in plasma and erythrocytes is sufficiently sensitive for routine work with 50 ml aliquots. By appropriate decreases in the size of the TLC plate, the silicic acid column, and the volume used in preparing TMSi derivatives, it should be possible to obtain quantitative data on as little as 10 ml of sample. The method might therefore be extended to studies of the glycolipids in blood from infants and small animals. Glycolipids have recently been reported to be constituents of leukocytes (42); we hope that the procedure may be adapted for analyses of glycosyl ceramides in this fraction of blood as well. The investigation of blood levels of glycosyl ceramides in glycolipid lipidoses is an obvious application; the concentrations of these lipids in several patients with Fabry's disease will be reported in a separate communication.

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