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PURIFICATION OF HUMAN MILK GANGLIOSIDES BY SILICA GEL CHROMATOGRAPHY AND ANALYSIS OF TRIFLUOROACETATE DERIVATIVES BY GAS CHROMATOGRAPHY

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SUMMARY

Two of the main gangliosides in human milk were purified by silica gel (230—400 mesh) column chromatography. The gangliosides were identified as G_{D_3} and G_{M_3} by methanolysis (2 *M* hydrochloric acid; 60 or 85°C) and gas chromatography of trifluoroacetate derivatives on a fused-silica capillary column. The molar ratios of galactose, glucose and sialic acid were 1:1:2 and 1:1:1, respectively, and the sequence in both gangliosides comprised sialic acid—galactose—glucose—ceramide, as indicated by the time course of cleavage of individual components during methanolysis at 60°C.

INTRODUCTION

Human milk fat contains 98% triglycerides. Most of the polar lipids like phospholipids [1] and cerebrosides [2] are found in the fat globule

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membranes. In bovine milk, gangliosides are found almost exclusively in fat globule membranes [3], and it is likely that gangliosides in human milk are located in similar structures since a sialic acid-containing oligosaccharide has been identified among oligosaccharides liberated from human milk fat globule membrane glycosphingolipids [4].

We have previously reported the presence in human milk of three major gangliosides [5], which together amount to 11 mg/l. In the present study, milk gangliosides were purified by silica gel column chromatography and the structures of two of the gangliosides were studied by gas chromatography (GC).

EXPERIMENTAL

Chemicals and reference compounds

Analytical-grade chloroform and methanol were obtained from Merck (Darmstadt, F.R.G.). Trifluoroacetic anhydride was from Koch-Light Labs. (Colnbrook, U.K.). Methanolic hydrochloric acid was prepared by bubbling dry hydrogen chloride gas (Messer Griesheim, Dusseldorf, F.R.G.) into HPLC-grade methanol until saturation, and diluting to 2 *M* hydrochloric acid with methanol. Fluorescamine was obtained from Supelco (Bellefonte, PA, U.S.A.) and orcinol ferric chloride from Sigma (St. Louis, MO, U.S.A.). Purified G_{M1}, G_{D1a} and G_{T1b} gangliosides (bovine brain) were obtained from Supelco. Bovine brain ganglioside mixture (type III), lactosylceramide, galactosylceramide, glucosylceramide, N-acetylneuraminic acid, N-glycolylneuraminic acid, meso-inositol, sialyllactose and methyl ester of fatty acid 17:0 were purchased from Sigma.

Milk samples

Human milk was obtained from the Milk Bank, Ullevål Hospital (Oslo, Norway). Milk samples from ten healthy women, two to ten months post partum, were stored at −20°C. Milk fat was pooled after centrifugation (35 000 *g*; 20 min; 4°C). By wet weight, the yield was 62 g of fat per litre of milk. This milk fat preparation contained 40% water, measured by weighing dried aliquots.

Isolation of milk gangliosides

Gangliosides were isolated by extraction and solvent partition, essentially as described by Svennerholm and Fredman [6]. Briefly, milk fat was homogenized with 3 vols. distilled water followed by the addition of 10.6 vols. of methanol and 5.3 vols. of chloroform. The mixture was stirred for 1 h, Hyflo Supercell (Johns-Mansville, Denver, CO, U.S.A.) was added (1 g/100 ml) and stirring was continued for another 30 min before filtration through a glass-sintered funnel. Gangliosides were recovered in the upper (aqueous) phases from three successive solvent partitions of the total lipid extract [6]. The pooled upper phases were evaporated in a rotary evaporator and the material was suspended in chloroform—methanol—water (60:30:4.5). Undissolved protein material was precipitated and discarded. The supernatant was evaporated and dialysed against water. This crude ganglioside preparation was taken to dryness and dissolved in chloroform—methanol—water (65:25:1).

Whenever dried ganglioside fractions were dissolved in organic or aqueous solvents, it was essential for quantitative dissolution to ultrasonicate the material in a water bath ultrasonicator (Bandelin Sonorex, RK 102H) for 30—60 s.

Silica gel column chromatography

Silica gel 60 (230—400 mesh; Merck), prewashed in sequence with chloroform—methanol—water (4:1:0, 65:25:1 and 65:25:4), was packed into two columns (I and II).

Silica gel column I (3 cm I.D., gel bed height 3—10 cm) was run at a flow-rate of 5—10 ml/min. After equilibration with 10 vols. chloroform—methanol—water (65:25:1), the crude ganglioside preparation derived from 10—40 g of milk fat (wet weight) (1—4 mg of gangliosides) was applied in a volume of 2—5 ml. Less polar lipids were eluted with 10 vols. of equilibration solvent. The solvent was changed to chloroform—methanol—water (65:25:4) and gangliosides were collected in fractions of 1—2 bed vols. (20—60 ml fractions). The elution was monitored by the use of high-performance thin-layer chromatography (HPTLC).

Pooled ganglioside fractions (0.5—2 mg of gangliosides) from silica gel column I were dissolved in 1—3 ml of chloroform—methanol—water (65:25:4) and applied on silica gel column II (1 cm I.D., gel bed height 20—30 cm). The column was equilibrated and eluted with chloroform—methanol—water (65:25:4) at a flow-rate of 0.3—0.4 ml/min. Fractions of 0.3—0.5 bed vols. (5—10 ml fractions) were collected.

High-performance thin-layer chromatography

HPTLC was carried out on silica gel 60 HPTLC precoated glass-plates (Merck) by development in chloroform—methanol—0.25% calcium chloride in water (60:35:8) in a filter-lined tank. In some experiments, the solvent system used was chloroform—methanol—2.5 *M* ammonium hydroxide in water (60:35:8). For visualization of gangliosides, the plates were sprayed with resorcinol [7] and placed face down on a clean glass plate preheated to 100°C. Densitometry was performed by single-wavelength zig-zag scanning of HPTLC lanes at 580 nm (beam dimension 0.4 × 0.4 mm) in the reflection mode, using a Shimadzu CS-930 dual-wavelength TLC scanner. The amounts of individual gangliosides were calculated from a standard curve obtained for purified G_{M1} (0.05—3.0 μg) on the same plate.

Gas chromatography

Gangliosides (1—20 μg) were heated with methanolic 2 *M* hydrochloric acid at 85°C for 18—20 h and derivatized with trifluoroacetic anhydride [8]. Partial methanolysis was performed at 60°C for 15—60 min. Samples (in 10% trifluoroacetic anhydride) were injected onto a fused-silica capillary column (splitless mode; 25 m × 0.2 mm I.D.) of either BP-1 [dimethylsilicone, bonded phase; Scientific Glass Engineering (S.G.E.), Australia] or BP-5 (5% phenylmethyl silicone, bonded phase; S.G.E.). A Hewlett-Packard 5710A gas chromatograph with a 18740B capillary column control, a flame ionization detector and a 3390A electronic recorder—integrator was, after 2 min at 90°C, programmed at 8°C/min to 290°C.

The GC method allowed simultaneous determination of sugars and fatty acids [8] by injection of derivatized material corresponding to ca. 0.5 μg of ganglioside. Quantitation of glucose, galactose and fatty acids was based on known molar response factors [8]. A molar response factor for sialic acid of 0.89 relative to galactose was estimated from GC analysis of gangliosides G_{M1}, G_{D1a} and G_{T1b}.

Neuraminidase digestion

Gangliosides (5 μg) were dissolved in 45 μl of 11.1 m*M* sodium acetate buffer, pH 5.4, and 5 μl (5 mU) of *Clostridum perfringens* neuraminidase (type VIII, EC 3.2.1.18; Sigma) were added. After incubation for 22 h at 37°C, the solvent was evaporated by a stream of nitrogen and the residue was dissolved in chloroform—methanol (2:1) for HPTLC analysis.

RESULTS AND DISCUSSION

Ganglioside isolation

HPTLC analysis of the three major human milk gangliosides (hmG), hmG I, hmG II and hmG III is shown in Fig. 1. The mobility of hmG I was identical with that of bovine brain G_{D3}, while hmG II and hmG III migrated similar to G_{M2} and G_{M3}, respectively (Fig. 1; ref. 9). The mobilities of the human milk gangliosides relative to bovine brain gangliosides were similar in two solvent systems: chloroform—methanol—0.25% calcium chloride (60:35:8) and chloroform—methanol—2.5 *M* ammonium hydroxide (60:35:8).

The upper phases after solvent partition of the milk fat extract were analysed by HPTLC. According to densitometric measurements, the total amount of gangliosides obtained by three solvent partitions was ca. 0.33 mg/g

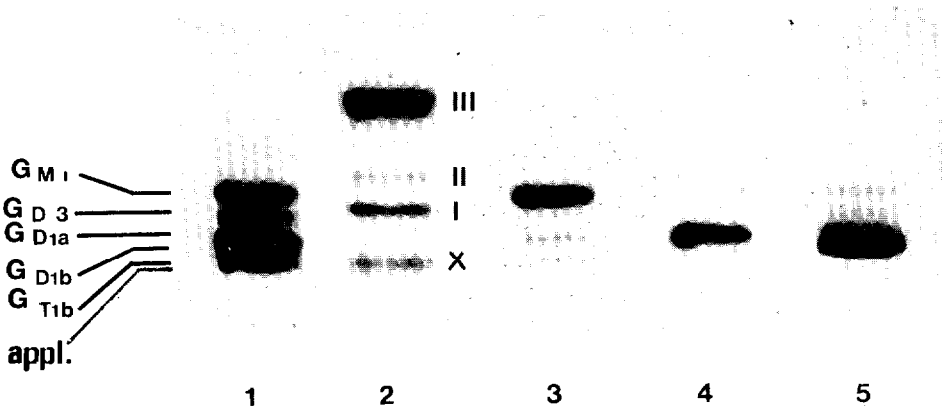

Fig. 1. HPTLC analysis of human milk gangliosides visualized with resorcinol [7]. The HPTLC plate was developed twice in chloroform—methanol—0.25% calcium chloride (60:35:8). Between the two runs, the plate was dried for 1 h at room temperature. Lane 1, bovine brain gangliosides; lane 2, crude human milk ganglioside preparation; lane 3, G_{M1}; lane 4, G_{D1a}; lane 5, G_{T1b}. The relative amounts of human milk gangliosides were 20–25% hmG I, 2—3% hmG II and 75—85% hmG III as measured by densitometry in ten independent HPTLC experiments. Component X (lane 2) stained yellow with resorcinol and thus did not contain sialic acid.

of milk fat (dry weight). The amount of gangliosides recovered after the next two purification steps, protein precipitation and dialysis, was ca. 0.16 mg/g of milk fat, which indicated a 50% loss during these two steps. Svennerholm and Fredman [6] reported a ganglioside recovery of > 95% after protein precipitation and dialysis of pooled upper phases from solvent partition of brain extracts. The lower yield of human milk gangliosides may partly be due to a different protein composition of the pooled upper phases from human milk compared to those from brain extracts.

Purification of milk gangliosides

Chromatography on silica gel column I removed most of the less polar lipids and the protein material in the crude ganglioside preparation, although trace amounts of protein may still be present. Most of the hmG III was collected in the first three to five fractions, while hmG I was eluted in the subsequent six to seven fractions. HmG II was eluted together with both hmG III and hmG I. Fractions enriched in either hmG I or hmG III were pooled and chromatographed on silica gel column II. By this procedure, preparations of hmG I and hmG III containing < 1% of other gangliosides were obtained. Less polar lipids were not detected by HPTLC of the purified gangliosides hmG I and hmG III stained with iodine. Trace amounts of non-ganglioside contaminations with an HPTLC mobility intermediate to hmG I and hmG III were still present as

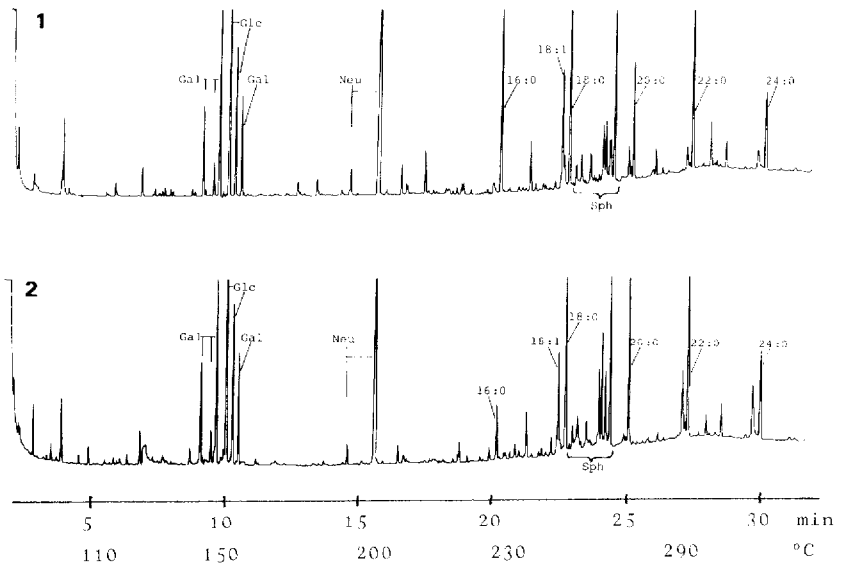

Fig. 2. Profiles obtained by GC analysis of human milk gangliosides hmG I and hmG III. Ganglioside samples were methanolysed (2 *M* hydrochloric acid; 85° C; 18—20 h) and trifluoroacetylated [8] before injection into a fused-silica capillary column (splitless mode; 25 m × 0.2 mm I.D.) of BP-5. The temperature was, after 2 min, programmed from 90 to 290° C at 8° C/min. Profile 1, hmG I; profile 2, hmG III. Trifluoroacetylated methyl glucosides are indicated as follows: Gal, galactose; Glc, glucose; Neu, neuraminic acid (methoxy-). Fatty acids are designated by the number of C atoms before the colon, and the number of C—C double bonds after. Sph, unidentified constituents of sphingosine base origin.

judged by weak positive staining with fluorescamine and orcinol ferric chloride. The overall recovery of pure gangliosides after silica gel chromatography of crude ganglioside preparation was 40—50% for hmG I and > 50% for hmG III.

HPLC as described by Kundu and Scott [10] did not improve the separation of milk gangliosides (Lægreid and Kolstø Otnaess [11]).

Ganglioside analysis

Profiles obtained by GC analysis of the purified milk gangliosides hmG I and hmG III are shown in Fig. 2. HmG I and hmG III contained galactose and glucose in equimolar amounts, whereas the contents of sialic acid were 1.7 and 1.1 mol, respectively (Table I). Hexosamines were not detected in either ganglioside. The sugar compositions recorded for hmG I and hmG III were close to those known for gangliosides G_{D3} and G_{M3}, respectively. Partial methanolysis of hmG I and hmG III showed that sialic acid was cleaved off in less than 15 min. Galactose was released faster than glucose ($t_{½}$ = 10 min and 22 min, respectively). This indicated sugar sequences as in G_{D3} and G_{M3}, with sialic acid linked to galactose—glucose—ceramide.

TABLE I

COMPOSITION OF HUMAN MILK GANGLIOSIDES DETERMINED BY METHANOLYSIS AND GAS CHROMATOGRAPHY OF TRIFLUOROACETATE DERIVATIVES ON A FUSED-SILICA CAPILLARY COLUMN

Molar values relative to galactose = 100.

Component	hmG I	hmG III
Sugars		
Galactose	100	100
Glucose	114	101
Sialic acid	165	107
*Fatty acids**		
14:1	1.5	0.3
14:0	0.7	trace
15:0	1.0	trace
16:1	2.1	1.4
16:0	17.9	4.6
17:0	0.7	0.3
18:1	7.9	8.7
18:0	23.9	22.7
19:0	0.7	0.3
20:1	2.9	1.8
20:0	8.5	12.8
21:1	1.9	trace
22:1	1.5	6.0
22:0	22.7	22.5
23:0	3.2	2.8
24:1	3.3	6.5
24:0	7.7	7.9
26:0	0.5	—
Total fatty acids	107	97

*See Fig. 2 for fatty acid designation.

In addition to identified sugars and fatty acids, the GC profiles revealed a number of unknown peaks, which probably were sphingosine bases or by-products, known to be formed during anhydrous methanolysis of sphingosine bases [12]. The characteristic pattern of unknown peaks was largely the same for hmG I and hmG III, and similar profiles were obtained upon GC of G_{M1}, G_{D1a} and G_{T1b} (data not shown).

Since G_{D3} and G_{M3} are sensitive to *Clostridium perfringens* neuraminidase (both are converted to lactosylceramide), hmG I and hmG III were treated with this enzyme and analysed by HPTLC. After treatment, both hmG I and hmG III were devoid of sialic acid (resorcinol-negative spots) and their HPTLC migration was similar to lactosylceramide (Fig. 3). In agreement with the results obtained by GC analysis, this suggested that hmG I and hmG III were both sialidated lactosylceramides. Based on the results obtained by HPTLC, GC analysis and enzyme hydrolysis, hmG I was identified as G_{D3} and hmG III as G_{M3}.

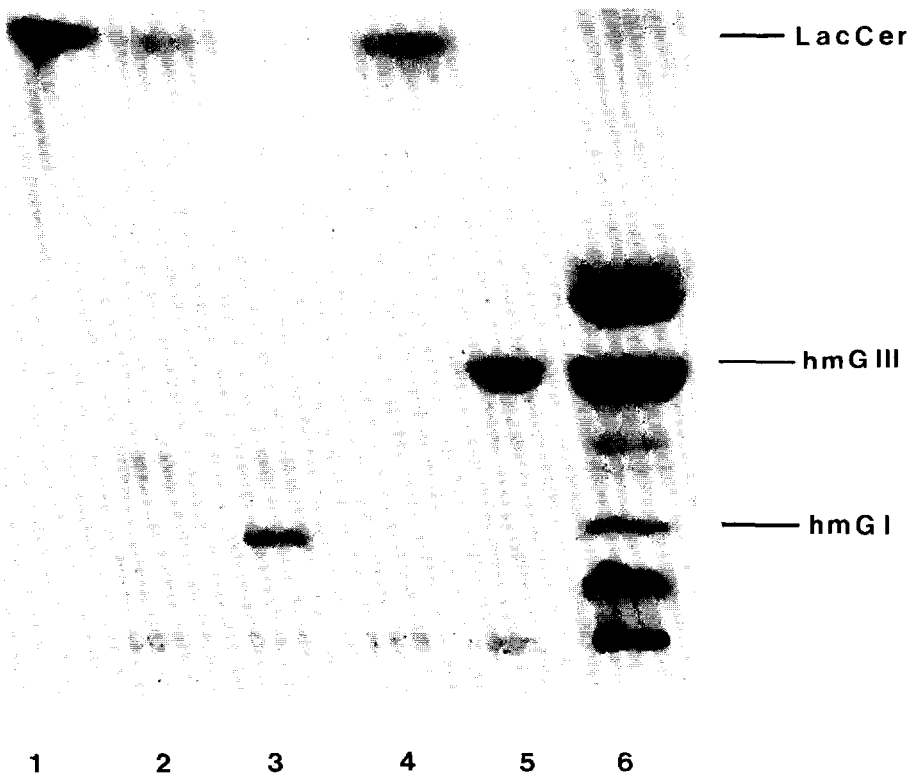

Fig. 3. A 5-μg volume of each of the human milk gangliosides hmG I and hmG III was incubated for 22 h at 37° C with or without 5 mU of *Clostridium perfringens* neuraminidase in a total volume of 50 μl of 10 m*M* sodium acetate buffer (pH 5.4). After evaporation of the solvent by nitrogen, the residues were dissolved in chloroform—methanol (2:1) and applied to an HPTLC plate, which was developed once with the same solvent as in Fig. 1 and stained with orcinol ferric chloride. Lane 1, lactosylceramide; lane 2, hmG I incubated with neuraminidase; lane 3, hmG I incubated without neuraminidase; lane 4, hmG III incubated with neuraminidase; lane 5, hmG III incubated without neuraminidase; lane 6, crude human milk ganglioside preparation.

The identification of the human milk ganglioside hmG III as G_{M3} is in agreement with preliminary GC analysis [13]. However, the preparation of hmG I analysed in the same study contained impurities which lead to erroneous GC results. The preparations of hmG I and hmG III analysed in the present study had been purified by two successive silica gel columns and contained < 1% of other gangliosides and only trace amounts of non-ganglioside impurities.

The ganglioside composition of human milk is characterized by the predominance of G_{M3} (75—85% of total gangliosides), which together with G_{D3} constitutes > 95% of the total gangliosides. The predominance of G_{M3} is a feature of most extraneural tissue [14]. Similar high proportions of sialyllactosylceramides as in human milk (> 90% G_{M3} + G_{D3}), have been reported for human tissues, e.g. liver [15], kidney [16] and small intestine mucosa [17]. Other tissues and cells, e.g. intestinal muscular layers [17], skeletal muscle [18], erythrocytes [19], granulocytes [20] and lymphocytes [21], contain higher proportions of gangliosides with a more complex core of neutral sugars.

In addition to G_{D3} and G_{M3}, only one minor ganglioside, hmG II, was detected by staining the chromatograms with resorcinol. HmG II may be identical with G_{M2} [9], as judged by the similar chromatographic behaviour with a bovine milk ganglioside identified by others as G_{M2} [3, 22]. So far, we have not been able to purify sufficient quantities of this ganglioside for analysis by GC. By a sensitive HPTLC immunoassay, trace amounts of G_{M1} (0.1% of the total gangliosides) were detected in human milk [9] and further analysis with sensitive techniques may reveal other minor ganglioside components.

APPENDIX

The ganglioside nomenclature is according to Svennerholm [23].

G_{M1} = Galβ1—3GalNAcβ1—4(NeuNAcα2—3)Galβ1—4Glcβ1—1Cer
G_{M2} = GalNAcβ1—4(NeuNAcα2—3)Galβ1—4Glcβ1—1Cer
G_{M3} = NeuNAcα2—3Galβ1—4Glcβ1—1Cer
G_{D3} = NeuNAcα2—8NeuNAcα2—3Galβ1—4Glcβ1—1Cer
G_{D1a} = NeuNAcα2—3Galβ1—3GalNAcβ1—4(NeuNAcα2—3)Galβ1—4Glcβ1—1Cer
G_{D1b} = Galβ1—3GalNAcβ1—4(NeuNAcα2—8NeuNAcα2—3)Galβ1—4Glcβ1—1Cer
G_{T1b} = NeuNAcα2—3Galβ1—3GalNAcβ1—4(NeuNAcα2—8NeuNAcα2—3)Galβ1—4Glcβ1—1Cer

where Gal = galactose, Glc = glucose, GalNAc = N-acetylgalactosamine, NeuNAc = N-acetylneuraminic acid and Cer = ceramide.

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