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Note

Purification of the peracetylated glycosphingolipids of the Gala series (galactosyl- and galabiosylceramides)

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The presence of galabiosylceramides in the ceramide dihexoside fraction of the neutral glycolipids has been reported in humans in Fabry's kidney disease [1], in neutrophils [2] and meconium [3]. On thin-layer chromatography, lactosyl- and galabiosylceramides have the same migration rate [4]. In the course of our study of neutral glycolipids of human thyroid [5], the preliminary analysis of glycolipid fractions suggested such a mixture in the ceramide dihexoside. The separation of these two ceramides was achieved by McCluer et al. [6] by high-performance liquid chromatography of derivatized glycosphingolipids. In an attempt to separate these two compounds with a simplified method, we have compared the results obtained with the ceramide dihexoside fraction, with or without peracetylation, on silica gel high-performance thin-layer plates with and without borate impregnation. The investigation was extended to the cerebroside fraction in order to define the best possible procedure.

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EXPERIMENTAL

Thin-layer chromatography

Silica gel 60 glass plates for high-performance thin-layer chromatography (HTPLC) and silica gel 60 plastic-backed plates were obtained from Merck (Darmstadt, F.R.G.).

The solvents were of analytical-reagent grade (Merck). Three solvent systems were used: (1) chloroform–methanol–water (65:25:4); (2) dichloroethane–methanol (98:2); (3) chloroform–methanol–water–28% ammonia solution (40:10:0.9:0.15).

The plates were run at room temperature for 20 cm with solvents 1 and 2 and for 10 cm with solvent 3 in 20 × 20 × 10 cm chambers (Desaga, Heidelberg, F.R.G.) lined with filter-paper.

Purification of the neutral glycolipids

Neutral glycolipids from human thyroids were extracted and characterized using the procedure described earlier [5]. Briefly, after removal of thyroglobulin, the tissue was homogenized and the lipids were extracted into chloroform–methanol–water (1:2:0.7) [7]. After desalting on a Sephadex G-25 column [8] and removal of the acidic lipids on DEAE-Sephadex [9], the non-acidic lipids were acetylated and applied to a Florisil column according to Saito and Hakomori [10]. The neutral glycolipids were eluted with dichloroethane–acetone (1:1). After deacetylation with 1% sodium methoxide in methanol, the salts were removed on a Sephadex G-25 column. The glycolipids were separated on a silicic acid column by a gradient of methanol in diisopropyl ether as described elsewhere [5]. Elution was monitored on silica gel 60 high-performance thin-layer plates developed in solvent 1 and detected with orcinol–sulphuric acid reagent at 120°C.

Isolation of the neutral glycolipids

The different compounds of the mono- and dihexosylceramide fractions recovered from the silicic acid column were isolated by TLC using two methods. Glucosylceramide from Gaucher spleen (Sigma, St. Louis, MO, U.S.A.), galactosylceramide from rat brain and lactosylceramide from human liver were used as standards.

Separation following peracetylation [10]. The acetylated fractions were applied to a silica gel 60 plate (Merck) developed with solvent 2. Part of the plate was sprayed with orcinol–sulphuric acid reagent in order to localize the spots and the glycolipids were recovered from the scraped gel by sonication in chloroform–methanol (1:1). The glycolipids were deacetylated as described above.

Separation on borated silica gel plates according to Kean [11]. Plastic-backed silica gel 60 plates (Merck) were dipped in a 1% methanolic solution of sodium tetraborate. After removal of the solvent, the plates were dried and kept at room temperature. They were then used for separation of the deacetylated glycolipids in solvent 3. Part of each developed plate was sprayed with orcinol–sulphuric acid reagent at 120°C and the gel corresponding to the located spots was recovered. Each compound was eluted and purified as described above.

Gas—liquid chromatography

The samples were hydrolysed with either 0.5 *M* hydrochloric acid in dry methanol for 18 h at 80°C (for fatty acid and carbohydrate analysis) or with methanol—HCl—water (83:8.6:9.4) (for long-chain base analysis) and subjected to gas—liquid chromatography on a Packard 427 chromatograph as described previously [12].

Carbohydrate analysis. The carbohydrates were analysed as trifluoroacetate derivatives [13]; inositol (Merck) was added to the samples as an internal standard and analysis was carried out on a 3% SP 2401 on 100–200 mesh Supelcoport (Supelchem, Paris, France) glass column (200 × 0.2 cm I.D.) with nitrogen as the carrier gas. The injection port was heated at 200°C, the flame-ionization detector at 250°C and the column from 100 to 210°C at 2°C/min. Identification and quantification were accomplished by comparison with known standard sugars under the same conditions.

Fatty acid analysis. After acid hydrolysis, fatty acid methyl esters were extracted with *n*-hexane and fractionated on a Florisil (100–120 mesh) column into non-hydroxy and hydroxy fatty acid methyl esters [14]. $C_{21:0}$ fatty acid methyl ester (Sigma) was added as an internal standard to both fractions to quantify the relative proportions of non-hydroxy and hydroxy fatty acids. Analyses were performed on a 3% OV-1 on 100—200 mesh Supelcoport column (200 × 0.2 cm I.D.) (Supelchem). The column was heated from 175 to 310°C at 3°C/min. The fatty acid methyl esters were identified by comparison with standard hydroxy and non-hydroxy fatty acids (Applied Science Europe, Oud-Beijerland, The Netherlands). In some instances, the hydroxylated fatty acids were also identified after silylation according to Bouhours and Glickman [15].

Long-chain base analysis. Long-chain bases were extracted from the methanolysate in diethyl ether after addition of sodium hydroxide [16], silylated [17] and the trimethylsilyl (TMS) derivatives were analysed on a 3% OV-1 on 100—200 mesh Supelcoport column. The column was heated from 230 to 290°C at 3°C/min. Identification was performed by comparison with standard long-chain bases (Serva, Heidelberg, F.R.G.).

RESULTS AND DISCUSSION

The pattern of neutral glycolipids of human thyroid revealed the presence of lipids co-migrating with ceramide monohexoside (CMH), ceramide dihexoside (CDH), ceramide trihexoside (CTH) and globoside. Their complete analysis will be reported elsewhere [5].

The deacetylated glycolipid fractions eluted from the silicic acid column were homogeneous on thin-layer plates and their carbohydrate composition was determined by GLC. The molar ratios obtained were Gal:Glc = 0.9:1 for CMH and 2:1 for CDH and CTH. These results suggested a mixture of glucosyl- and galactosylceramides in CMH, and also a mixture of lactosyl- and galabiosyl-ceramides in CDH. An additional point of interest was the presence of both sphingosine and phytosphingosine, as well as hydroxy and non-hydroxy fatty acids in these fractions. Therefore, several methods were used in an attempt to isolate the different compounds present in CMH and CDH.

TABLE I

CARBOHYDRATE AND LONG-CHAIN BASE COMPOSITION OF THE DIFFERENT BANDS SEPARATED BY THIN-LAYER CHROMATOGRAPHY ON SILICA GEL 60, UNLESS STATED OTHERWISE

For the solvent systems, see Experimental. Means of three separate analyses with a range of variation of 3—5%.

Type	Component*	Ceramide monohexoside							Ceramide dihexoside: CDH (human thyroid)			
		CMH (human thyroid)					Acetylated CMH (rat brain)		Non-acetylated		Acetylated	
		Silica gel 60		Borate-impregnated silica gel 60								
		UB**	LB**	UB	IB**	LB	UB	LB	UB	LB	UB	LB
Carbohydrates (molar ratios)	Gal	0.4	1.2	0	0.1	1	1	1	1.1	2.7	2	0.9
	Glc	1	1	1	1	0.1	0.2	0.1	1	1	0.2	1
Long-chain bases (%)	d 18:1	78	76	98	44	95	N.D.***	N.D.	91	95	85	99
	t 18:0	22	24	2	56	5	N.D.	N.D.	9	5	15	1

*Gal = galactose; Glc = glucose; d 18:1 = C_{18} sphingosine; t 18:0 = 4D-hydroxysphinganine.
**UB = upper band; IB = intermediate band; LB = lower band.
***N.D. = Not determined.

TABLE II

FATTY ACID DISTRIBUTION OF THE DIFFERENT BANDS SEPARATED BY THIN-LAYER CHROMATOGRAPHY

Means of three separate analyses with a range of variation of 3–5%. Each band is expressed as a percentage of the total, normal (N) or hydroxylated (OH) fatty acids

Fatty acid	Ceramide monohexoside														Ceramide dihexoside CDH (human thyroid)							
	CMH (human thyroid)										Acetylated CMH (rat brain)				Non-acetylated				Acetylated			
	Silica gel 60				Borate-impregnated silica gel 60						UB		LB		UB		LB		UB		LB	
	UB*		LB*		UB		IB*		LB													
	N 96%	OH 4%	N 26%	OH 74%	N 92%	OH 8%	N 67%	OH 33%	N 48%	OH 52%	N 55%	OH 45%	N 95%	OH 5%	N 85%	OH 15%	N 88%	OH 12%	N 82%	OH 18%	N 89%	OH 11%
16.1	2	—	10	—	1	—	2	—	3	—	7	—	4	—	15	—	8	—	14	—	12	—
16·0	5	—	20	—	14	—	16	—	17	—	32	—	19	—	31	—	26	—	37	—	36	—
18·1	2	—	10	—	7	—	4	—	5	—	26	—	12	—	15	—	14	—	10	—	12	—
18·0	2	—	10	8	7	—	7	13	10	4	20	1	14	—	12	23	15	20	14	14	12	14
20·0	12	20	12	15	12	9	6	24	8	8	1	2	3	12	4	15	6	10	2	6	3	8
22·0	31	—	20	25	33	21	22	20	20	25	4	28	12	25	8	10	11	13	8	15	9	12
23·0	—	—	—	—	6	—	8	—	5	—	1	—	2	—	1	—	2	—	2	—	2	—
24.1	21	40	5	25	7	49	18	28	15	25	2	29	9	50	2	33	4	41	5	30	5	41
24.0	25	40	15	28	13	21	18	16	16	37	6	40	25	13	11	18	14	16	8	35	9	25

*UB = upper band, IB = intermediate band, LB = lower band

Each band of the doublet migrating on TLC as CMH and CDH in solvent 1 was analysed by GLC. As can be seen in Tables I and II, all bands contained galactose and glucose, although the lower band of each doublet seemed to be enriched in galactose. The variations in migration could not be accounted for by clear differences in the ceramide portion. Kean [11] reported the separation of gluco- and galactocerebrosides by means of borate TLC. His method was applied, using borate-impregnated HPTLC plates, to the cerebrosides of human thyroid. As shown in Fig. 1, we were able to separate three bands. The upper band co-migrated with authentic glucosylceramide from Gaucher spleen and contained only glucose, C_{18} sphingosine (Table I) and a small amount (8%) of hydroxy fatty acids (Table II). The intermediate band contained glucose, an equal amount of C_{18} sphingosine and phytosphingosine and a significant proportion (33%) of hydroxy fatty acids. This band appears to be still heterogeneous. The lower band that co-migrated with galactosylceramide from

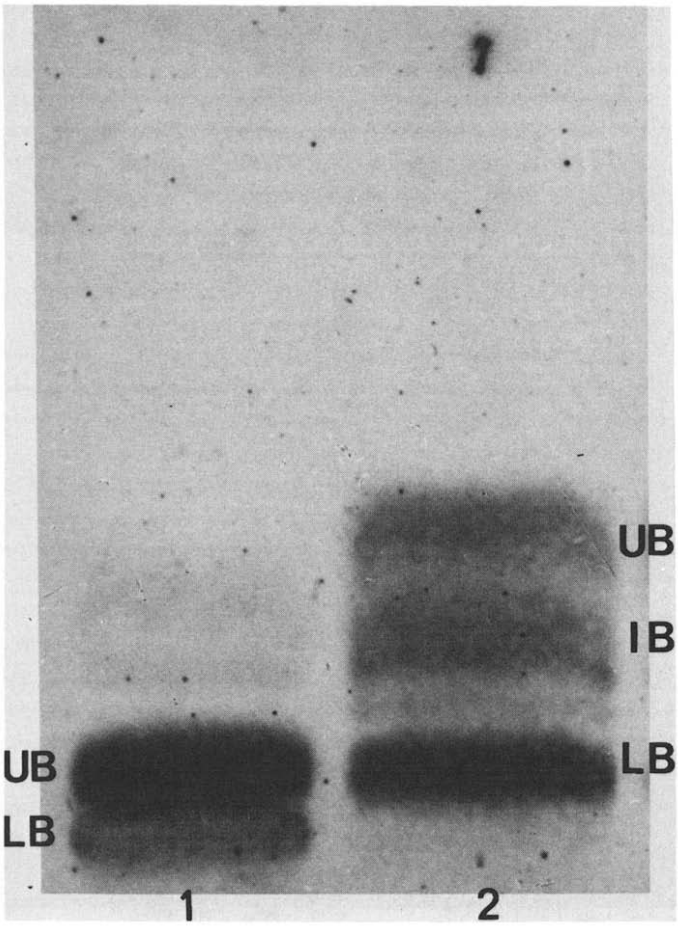

Fig. 1. Thin-layer chromatography of neutral glycolipids on borate-impregnated silica gel 60. Lane 1, rat brain CMH; lane 2, human thyroid CMH. UB, upper band; IB, intermediate band; LB, lower band. Solvent system 3: chloroform—methanol—water—28% ammonia solution (40:10:0.9 0.15).

rat brain (Fig. 1) consisted of galactose, C_{18} sphingosine and a large amount (52%) of hydroxy fatty acids. The separation obtained with cerebrosides of human thyroid was consistent with the results obtained by Karlsson et al. [18] for bovine kidney cerebrosides by means of column chromatography on borate-impregnated silica gel G.

A second method of separation involved TLC after peracetylation of the cerebroside fraction. As shown in Fig. 2, a double band was obtained with the cerebrosides extracted from both human thyroid and rat brain. Because of the small amount of material available, the analysis of the bands eluted from silica gel was carried out on rat brain cerebrosides. The results revealed that both bands contained galactose and the upper band seemed to be enriched in hydroxylated fatty acids (Table II). Therefore, the use of borate-impregnated silica gel appears to be the best method, yielding a satisfactory separation of subfractions from cerebrosides.

As reported by Kean [11], TLC was ineffective in separating lactosyl- and galabiosylceramides, whether the plates were prepared in the presence of borate (data not shown) or not. However, following peracetylation, two bands were obtained by TLC (in solvent 2) of the ceramide dihexoside fraction extracted from human thyroid, in contrast to authentic lactosylceramide, which gave only one band, as shown in Fig. 2. The fast-migrating band yielded only galactose, as would be expected from pure galabiosylceramide, whereas the lower band had the carbohydrate composition of lactosylceramide. Moreover, all the phytosphingosine found in the CDH fraction was in the upper band.

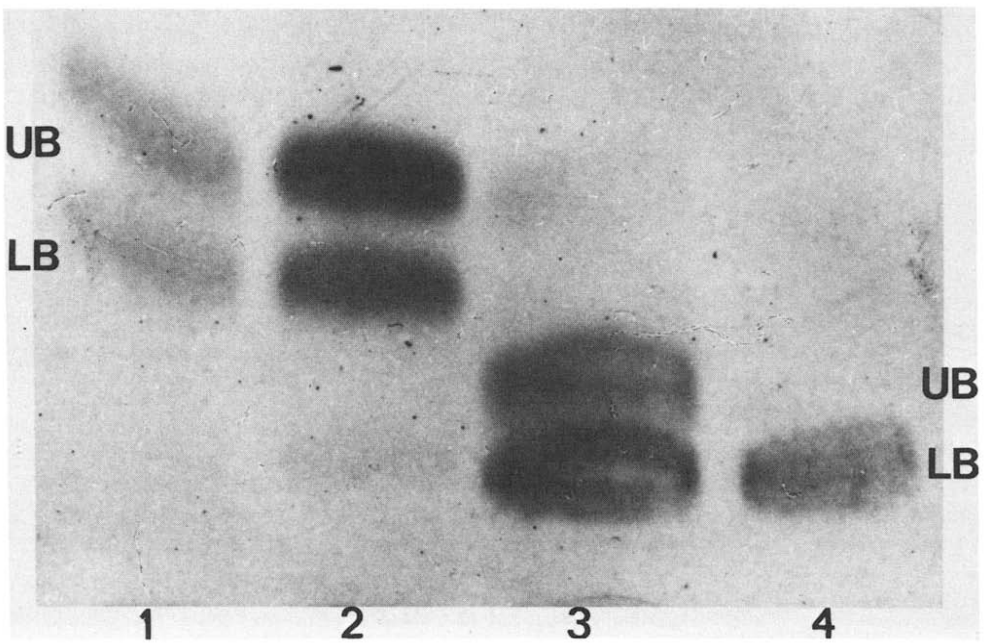

Fig. 2. Thin-layer chromatographic separation of peracetylated neutral glycolipids. Lane 1, human thyroid CMH, lane 2, rat brain CMH; lane 3, human thyroid CDH; lane 4, standard CDH (human liver). UB, upper band; LB, lower band. Solvent system 2 dichloroethane–methanol (98:2)

Both contained some hydroxylated fatty acids. Hence lactosyl- and galabiosylceramides are readily separated by TLC after peracetylation. An alternative procedure using column chromatography on Florisil with gradient elution is currently being investigated in our laboratory, which should avoid any contamination of the samples when eluting the glycolipids from thin-layer plates. In any case, the method described in this paper allows the reliable purification of galabiosylceramide from a ceramide dihexoside fraction in a very simple way compared with the sophisticated procedure described by McCluer et al. [6], involving high-performance liquid chromatography of perbenzoylated glycosphingolipids.

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