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Note

Thin-layer chromatography of neutral glycosphingolipids: an improved simple method for the resolution of GlcCer, GalCer, LacCer and Ga_2Cer

KIYOKO OGAWA*, YUKO FUJIWARA, KUMIKO SUGAMATA and TOSHIAKI ABE

Department of Pediatrics, Teikyo University School of Medicine, Kaga, Itabashi-ku, Tokyo 173 (Japan)

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The kidney with Fabry's disease has shown accumulation of galabiosylceramide (Galα1–4Gal–Cer; Ga_2Cer)* and globotriaosylceramide (Gb_3Cer) [1,2]. The presence of Ga_2Cer in the dihexosylceramide (CDH) fraction in normal human kidney [3,4], neutrophils [5], meconium [6] and porcine pancreas [7] has also been reported.

Thin-layer chromatography (TLC) is very useful for the characterization and separation of glycosphingolipids. However, it has so far been impossible to separate Ga_2Cer from lactosylceramide (Galβ1–4Glc–Cer; LacCer) by TLC without sample derivatization. Although the separation of these two types of CDH has been achieved by TLC using acetylated derivatives [8], the simultaneous separation of CDH and other glycolipids having a longer sugar chain has been impossible. On the other hand, a borate-impregnated TLC plate has been utilized to separate glucosylceramide (GlcCer) from galactosylceramide (GalCer) [9]. However, the simultaneous analysis of CMH and other lipids has also been impossible using the pre-treated plate.

In the course of our studies of glycosphingolipidosis, a simplified and rapid method was required for the simultaneous analysis of these glycosphingolipids, including GlcCer, GalCer, Ga_2Cer, LacCer, Gb_3Cer and Gb_4Cer. In this paper we describe a novel TLC developing system which permitted the simple separation of the above six glycosphingolipids.

*Glycosphingolipid nomenclature is based on the system of Makita and Taniguchi, in H. Wiegandt (Editor), New Comprehensive Biochemistry, Vol. 10, Elsevier, Amsterdam, 1985, p. 1.

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EXPERIMENTAL

Glycosphingolipids dissolved in chloroform–methanol (2:1, v/v) were applied to a pre-coated thin-layer plate (HPTLC, silica gel G 60, 10 cm long; Merck, Darmstadt, F.R.G.). The plates were run at room temperature in a paper-lined tank with the following systems: S1 = 1-propanol–15 *M* ammonia solution–water [75:5:25 (S1-a) and 80:5:15 (S1-b), v/v/v], S2 = 2-propanol–15 *M* ammonia solution–methyl acetate–water [75:5:0:25 (S2-a), 75:5:5:25 (S2-b) and 75:10:5:15 (S2-c), v/v/v], S3 = chloroform–methanol–water (65:25:4, v/v/v), S4 = chloroform–methanol–5 *M* ammonia solution (65:35:5, v/v/v). After spraying with 50% sulphuric acid the spots were rendered visible by heating at 110°C.

Extraction of total lipids and purification of the neutral glycosphingolipid fraction from human kidney was performed as described previously [10]. Briefly, tissue extracted with chloroform–methanol (2:1 and 1:1, v/v) was subjected to column chromatography on DEAE-Sephadex A-25 (Pharmacia, Uppsala, Sweden). After acetylation of the neutral lipid fraction from the DEAE-Sephadex column, column chromatography with Iatrobeads 6RS-8060 (Iatron, Tokyo, Japan) was performed to separate glycosphingolipids from other lipids, followed by deacetylation with methanolic sodium hydroxide solution and desalting. Each lipid was isolated from the neutral glycolipid fraction by preparative TLC. The sugar composition of the isolated glycosphingolipids was determined as the trimethylsilylmethylglycosides by gas–liquid chromatography (GLC) with an OV-101 capillary column.

Standard glycosphingolipids were obtained from the following tissues: GlcCer, Gaucher's disease spleen; GalCer, human brain; LacCer, human erythrocyte and human lung; Gb_3Cer, Fabry's disease heart and human erythrocyte; Ga_2Cer, Fabry's disease kidney; and Gb_4Cer, human erythrocyte. Ga_2Cer from porcine pancreas and Galβ1–6Gal–Cer from *Monodonta labio* were generous gifts from Professor S. Handa, Tokyo Medical and Dental University, and Prof. A. Hayashi, Kinki University, respectively.

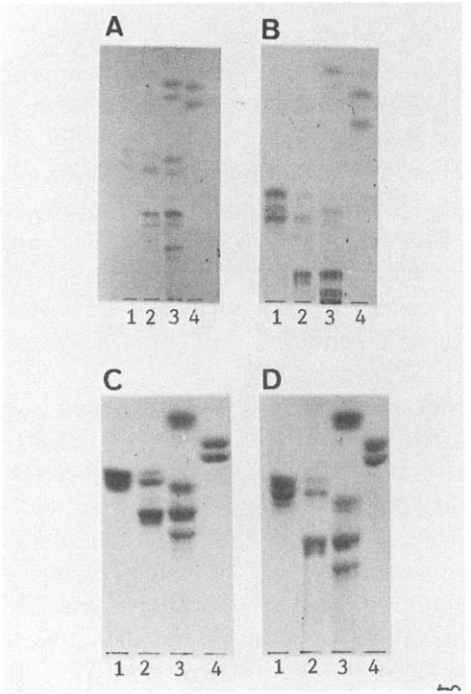
RESULTS

Separation of GlcCer and GalCer

Although GlcCer and GalCer have the same migration rate on TLC when developed with the neutral solvent system S3 (Fig. 1A), the alkaline developing systems S4 (Fig. 1B) and S1 (Fig. 1C) permitted their separation. Using these alkaline systems, however, the resolution of LacCer and Ga_2Cer was incomplete. Among the alkaline developing solvents, S1 appeared to be superior to S4 with regard to both the sharpness of each band and the simultaneous separation of several other glycolipids having longer carbohydrate moieties.

Separation of Ga_2Cer and LacCer

We found that solvent system S2-a (Fig. 1D) was more effective than S1 (Fig. 1C) for the separation of Ga_2Cer and LacCer. Further addition of methyl acetate



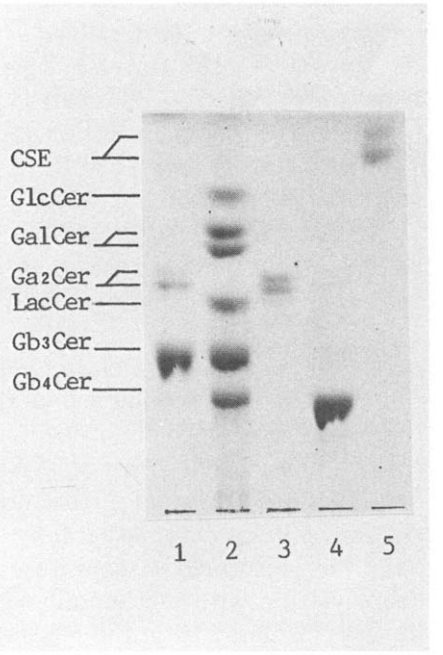


Fig. 1. Thin-layer chromatographic patterns of neutral glycosphingolipids developed with various solvent systems. Lane 1, Ga_2Cer from porcine pancreas; lane 2, Ga_2Cer (above) and Gb_3Cer (below) from Fabry kidney; lane 3, GlcCer, LacCer, Gb_3Cer, Gb_4Cer (from top to bottom); lane 4 GalCer. Solvent system: (A), S3; (B) S4; (C) S1-a; (D) S2-a. After spraying with 50% sulphuric acid, the spots were rendered visible by heating at 110°C.

Fig. 2. TLC separation of neutral glycosphingolipids including Ga_2Cer and LacCer. Lane 1, Fabry's disease kidney, Ga_2Cer (above) and Gb_3Cer (below); lane 2, GlcCer, GalCer, LacCer, Gb_3Cer, Gb_4Cer (from top to bottom); lane 3, pancreas Ga_2Cer; lane 4, Forssman glycolipid; lane 5, CSE. Solvent system, S2-b.

to the developing solvent enhanced the complete separation of Ga_2Cer from LacCer. As shown in Fig. 2, the double spot of Ga_2Cer migrated faster than LacCer when developed with solvent system S2-b.

This developing system also allowed the simultaneous separation of the six glycosphingolipids GlcCer, GalCer, LacCer, Ga_2Cer, Gb_3Cer and Gb_4Cer with a single development. Using this system, digalactosylceramide from *Monodonta labio*, which has a Galβ1–6 Gal linkage, migrated between LacCer and Gb_3Cer as well as developed with solvent system S3. Sulphatide moved faster than GlcCer despite its polarity.

The R_F values of glycosphingolipids on TLC with the various solvent systems are summarized in Table I. The proportions of the components in solvent system S2 were critical for obtaining a complete separation of Ga_2Cer and LacCer. The most successful system, especially for separating CDH, was S2-b. However, in order to analyse other glycosphingolipids simultaneously, such as Gb_3Cer and Gb_4Cer, in addition to CDH, solvent system S2-c appeared to be more suitable

TABLE I

R_F VALUES OF NEUTRAL GLYCOSPHINGOLIPIDS ON TLC WITH DIFFERENT SOLVENT SYSTEMS

n.d. = not determined.

Glycosphingolipid	Solvent system						
	S1-a	S1-b	S2-a	S2-b	S2-c	S3	S4
CSE	n.d.	n.d.	0.80	0.82	n.d.	n.d.	n.d.
			0.76	0.77			
GlcCer	0.57	0.56	0.65	0.69	0.54	0.67	0.70
			0.63			0.63	0.65
GalCer	0.52	0.48	0.58	0.60	0.44	0.65	0.63
	0.48	0.45	0.54	0.56	0.39	0.59	0.54
Ga_2Cer	0.44	0.40	0.48	0.50	0.31	0.45	0.27
	0.42	0.37	0.44	0.48	0.28	0.40	0.23
LacCer	0.41	0.36	0.42	0.45	0.25	0.43	0.31
	0.39	0.33	0.40		0.23	0.39	0.25
Gb_3Cer	0.34	0.23	0.31	0.33	0.13	0.25	0.08
						0.22	
Gb_4Cer	0.29	0.15	0.15	0.24	0.07	0.15	0.03
						0.12	

than the former, with which Gb_3Cer and Gb_4Cer migrated too closely. The best resolution pattern was obtained with newly prepared developing solvent.

Glycosphingolipid composition of human kidney

The neutral glycolipid composition of human kidney was determined by TLC densitometry using solvent system S2-c. The results obtained with our system were similar to those of Mårtensson [4] (Table II). In order to establish whether a sufficient separation of Ga_2Cer and LacCer was achieved with our system, preparative TLC was performed to isolate each glycolipid of kidney. As shown in Fig. 3, nine glycosphingolipids (bands 1–9) were obtained from human kidney. The

TABLE II

DISTRIBUTION OF NEUTRAL GLYCOSPHINGOLIPIDS IN HUMAN KIDNEY

Glycosphingolipid	Determined by TLC densitometry (%)	Ref. 4 (mg/g dry tissue)	Calculated from ref. 4 (%)
GlcCer	1.7	0.19	5.5
GalCer	4.3		
Ga_2Cer	7.3	0.38	11.0
LacCer	5.4		
Gb_3Cer	29.4	0.98	28.4
Gb_4Cer	51.8	1.90	55.0

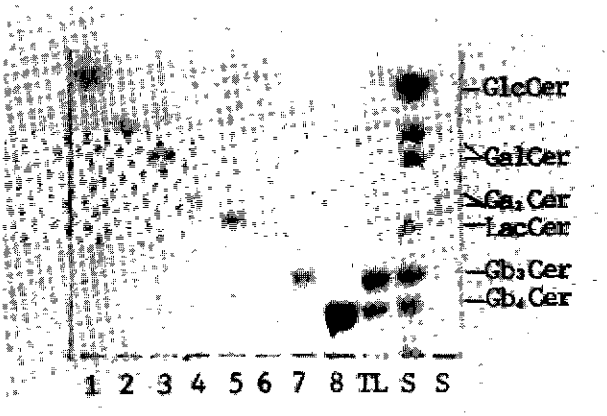


Fig. 3. Neutral glycosphingolipids isolated from normal human kidney by preparative TLC using solvent system S2-c. Bands 1–8 were isolated from the kidney neutral glycolipid fraction by preparative TLC using solvent system S2-c. TL, total lipid fraction from human kidney; S, standard glycolipids.

TABLE III

CARBOHYDRATE COMPOSITION OF BANDS 1–6 ISOLATED FROM HUMAN KIDNEY

Band	Molar ratio, Glc Gal
1	1 0
2	0.19·1*
3	0.27:1*
4	0.05:1*
5	0.15:1*
6	1·1.11**

*Gal taken as 1.0.
**Glc taken as 1.0.

sugar compositions of the isolated mono- and dihexosylceramides (bands 1–6) determined by GLC are summarized in Table III.

Treatment of bands 4 and 5 with α-galactosidase (green coffee beans; Sigma, St. Louis, MO, U.S.A.) converted them into new glycolipids with the same mobilities as GalCer, whereas β-galactosidase (Jack beans; Sigma) digested band 6 to GlcCer. From these results, both bands 4 and 5 were identified as Ga_2Cer and band 6 as LacCer. Hence our developing solvent gave an adequate separation of Ga_2Cer from LacCer.

DISCUSSION

TLC is a very simple and useful technique for glycosphingolipid analysis, but the separation of GlcCer from GalCer or Ga_2Cer from LacCer by the common TLC procedure has never been successful. In this work we have demonstrated an excellent developing system (2-propanol–15 *M* ammonia solution–methyl acetate–water) which permitted the simultaneous separation of GlcCer, GalCer,

Ga_2Cer and LacCer on TLC together with Gb_3Cer and Gb_4Cer in a very simple way. This developing system, devised for separating Ga_2Cer and LacCer, did not require derivatization of the sample or pre-treatment of the TLC plate. It can also be expected to be very useful for identifying the products of the glycosidase digestion of glycolipids.

Kean [9] previously reported the complete separation of GlcCer and GalCer by means of a borate-impregnated TLC plate. In his work a slight separation was observed even with a non-borate-impregated plate when developed with an alkaline solvent. Our work, however, revealed that a pre-coated HPTLC plate, instead of a manually prepared plate, allowed the complete separation of two types of CMH with an alkaline developing solvent containing 1- or 2-propanol.

When developed with an alkaline solvent system, acidic glycolipids such as sulphatide and ganglioside showed a faster migration rate than when developed with a neutral solvent. This phenomenon can be explained by neutralization of the negative charge by the alkaline solution. For this reason, GM3 ganglioside co-migrated with Ga_2Cer using our solvent system (data not shown).

It was found that our developing system could be applied to isolate Ga_2Cer and LacCer by using preparative TLC in a simple way. Our preliminary study also showed that this solvent system could be effective in the resolution of Ga_2Cer and LacCer using silicic acid column chromatography.

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