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Note

Analytical and preparative separation of glucosylceramide and galactosylceramide by borate-impregnated silica gel chromatography

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Monohexosylceramides (MHCs), galactosylceramide (GalCer) and glucosylceramide (GlcCer) are the simplest glycolipids and the biosynthetic precursors common to elongation of the carbohydrate chains in all glycolipid series. GalCer can be extended enzymatically to glycolipids belonging to galaseries and to GalCer sulphate. GlcCer can be elongated to all other glycolipid series via lactosylceramide[1]. The relative distributions of GalCer and GlcCer appear to be characteristic for tissues, cell types and animal species (for a review, see ref. 2). For instance, MHCs of adult mammalian brain and Gaucher's spleen consist almost exclusively of GalCer and GlcCer, respectively, whereas in many other tissues the two classes coexist.

In order to separate GlcCer and GalCer, chromatography on a borate-impregnated thin-layer plate[3], prepared from a silica gel slurry in a borax solution, has been employed. More recently, high-performance liquid chromatography (HPLC) of perbenzoylated MHC derivatives was adopted for the separation[4]. These procedures are suitable for analytical purposes rather than preparative separations. However, the necessity for preparative separation of MHCs in tissues is often encountered, such as for the composition analysis of the lipid moieties in each MHC class.

This paper describes an improved thin-layer chromatographic (TLC) and a preparative separation method for GlcCer and GalCer. With the use of these procedures, relative amounts of the two classes in MHCs in various tissues were determined.

EXPERIMENTAL

Materials

Precoated TLC plates (silica gel 60, 20 × 20 cm) and silicic acid (Iatrobeads 6RS-8060) were obtained from Merck (Darmstadt, F.R.G.) and Iatron (Tokyo,

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Japan), respectively. Other reagents were of analytical-reagent grade. Human renal cell carcinoma tissues were obtained at operation and dissected into tumour and uninvolved portions. From the cancer tissues or from the other tissues (see Results and Discussion), an MHC fraction was obtained as described previously[5]. MHC from bovine milk was kindly donated by Snow Brand Milk Products (Tokyo, Japan). Standard glycolipids were prepared in this laboratory. Tritium labeling of GlcCer and GalCer was carried out as described by Schwarzmann[6].

Modified thin-layer chromatography

Proportions of components of solvent mixtures are expressed by volume. In place of a laboratory-made borate-impregnated silica gel plate[3], a precoated silica gel plate was employed. The plate was sprayed with 1% (w/v) aqueous borax (sodium tetraborate), air dried and activated at 110°C in an oven before use. The plate was developed with chloroform–methanol–water–15 *M* ammonia solution (280:70:6:1) (solvent A)[3] or chloroform–methanol–pyridine (60:40:2) (solvent B). Glycolipids were revealed with orcinol–sulphuric acid reagent.

Borate-impregnated silica gel column chromatography

Iatrobeads were suspended in three volumes of 3% (w/v) aqueous borax with vigorous stirring, filtered and dried at 110°C in an oven. A slurry of the silica gel in chloroform–methanol–pyridine–water (70:30:2:1, solvent C) saturated with borax was packed under normal pressure into a glass tube to make a column of 41 × 1.0 cm I.D. Approximately 50 mg of an MHC mixture dissolved in the minimum volume of the same solvent were applied to the column. The column was eluted in a stepwise manner with five column volumes each of solvent C and chloroform–methanol–water (50:50:5, solvent D). The eluates were collected (2 ml per fraction), each concentrated *in vacuo* and monitored by TLC as described above using solvent B for separation of GalCer and GlcCer. The separated MHC was combined, evaporated for the solvent and subjected to Folch's partition[7] to remove the salt.

Analytical methods

The compositions of monosaccharide, fatty acid and long-chain base were determined as described previously[8] using a Shimadzu GC-8A gas chromatograph equipped with a capillary column (25 m × 0.3 mm I.D.) coated with HR-1. The ratio of GalCer and GlcCer in the mixture was also measured by densitometry of the glycolipid spots on the TLC plate with a Shimadzu GS-910 chromatogram scanner as described previously[9].

RESULTS AND DISCUSSION

Improved thin-layer chromatography

When MHCs were developed with two solvent systems containing ammonia solution (solvent A, Fig. 1A) or pyridine (solvent B, Fig. 1B) on the borate-impregnated plates, much better resolution of GlcCer and GalCer with their subcomponents was achieved with the latter solvent. MHC fractions from the same tissue of different animal species and different tissues of the same species were examined by TLC (Fig. 1C). Pig spleen contained only GlcCer, whereas equine spleen contained a small

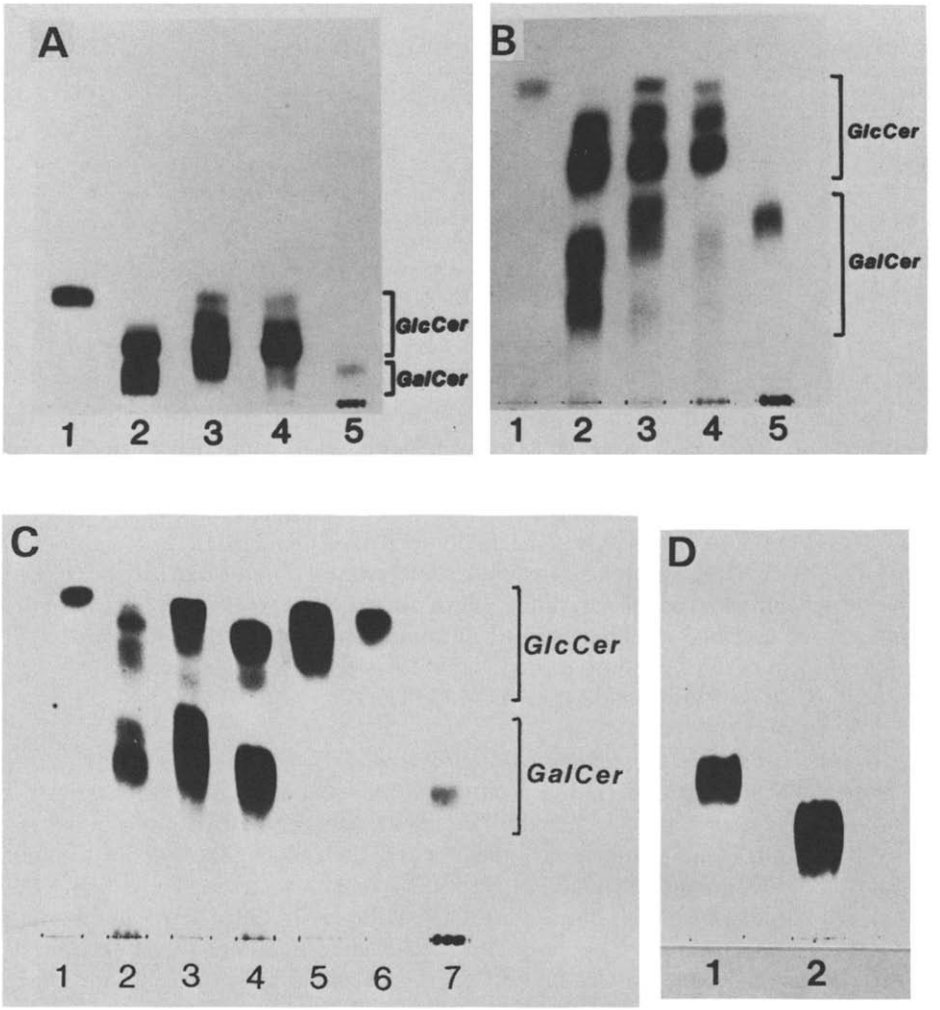

Fig. 1. Borate-impregnated TLC of various MHC with previous and present solvents. A, solvent A[3]; B, C and D, solvent B. Plates A and B: 1 = pig spleen; 2 = equine kidney; 3 = rat kidney; 4 = dog kidney; 5 = cat brain. Plate C: 1 = pig spleen; 2 = pig erythrocyte; 3 = pig serum; 4 = pig intestine; 5 = equine spleen; 6 = bovine milk; 7 = cat brain. Plate D: 1 = upper band of equine kidney GalCer separated by borate-impregnated silica column chromatography; 2 = lower band of the GalCer.

amount of GalCer in addition to GlcCer. For kidney, MHCs from the horse and rat consisted of the two glycolipid classes with a higher content of GlcCer (Fig. 1B), whereas dog contained largely GlcCer (Table I). These results were consistent with observations on rat[10] and dog[11] kidney obtained by carbohydrate analysis of the MHC fractions. An MHC pattern characteristic of tissues was observed in pig (Fig. 1C, Table I). Pig erythrocytes, serum and intestine afforded similar compositions of GlcCer and GalCer, whereas the spleen MHC was exclusively GlcCer. MHC from adult cat brain was composed of exclusively GalCer (Fig. 1), like brain MHC from many other animal species.

TABLE I

RELATIVE CONTENTS OF GlcCer AND GalCer IN VARIOUS TISSUES

Relative contents were determined by densitometric analysis of glycolipid spots on the TLC plate.

Tissue	*GlcCer (%)*	*GalCer (%)*	*Tissue*	*GlcCer (%)*	*GalCer (%)*
Equine kidney	68	32	Pig intestine	66	34
Rat kidney	66	34	Pig spleen	100	0
Dog kidney	90	10	Equine spleen	94	6
Pig erythrocytes	61	39	Bovine milk	100	0
Pig serum	57	43	Cat brain	0	100

On the other hand, when human kidney tumour was measured for the relative contents of the two MHC classes, GlcCer was increased in comparison with that in uninvolved tissue (Table II). In the GlcCer fraction a faster moving component composed mainly of lignoceric and behenic acids (71% of total acids) and sphingenine (95% of total long-chain bases) was markedly increased (Table III). As a significant elevation of PAPS: GalCer sulphotransferase level brought about a marked increase in GalCer sulphate in renal cell carcinoma[5], a decrease in GalCer relative to GlcCer in the tumour may be ascribed to enhanced utilization of GalCer for sulphatide synthesis.

The improved TLC method did not separate two dihexosylceramides, lactosylceramide and galabiosylceramide (Gal-α1–4-GalCer).

Borate-impregnated silica gel column chromatography

When an MHC mixture (50 mg) of equine kidney was subjected to separation on a borate-impregnated silica column, the best separation of the two glycolipids was attained by elution with 5 volumes each of solvent C and solvent D (data not shown). The recoveries of GlcCer and GalCer were 95% and 85%, respectively, using the tritiated glycolipids. Although elution with ten volumes of chloroform–methanol–pyridine (85:15:2) containing no aqueous borate gave a complete separation of GlcCer, the recovery of GalCer thereafter was poor. In instances where slight

TABLE II

MONOHEXOSYLCERAMIDE COMPOSITION IN HUMAN RENAL CELL CARCINOMA

Case[a]	*GlcCer (%)*[b]	*GalCer (%)*
1 U	56(20)	44
1 T	93(86)	7
2 U	89(44)	11
2 T	92(74)	8
3 U	82(29)	18
3 T	93(74)	7

[a] U = Uninvolved tissue; T = tumour tissue.

[b] Values in parentheses indicate percentages of faster moving GlcCer in total GlcCer.

TABLE III

LONG-CHAIN BASE COMPOSITION OF GlcCer AND GalCer FROM EQUINE AND RAT KIDNEY

Kidney	*Glycolipid*	*Composition (%)*[a]			
		d18:1	*d18:0*	*t18:0*	*t20:0*
Equine	GlcCer	1	11	25	63
	GalCer (whole)	38	19	10	33
	GalCer (upper)	86	10	2	2
	GalCer (lower)	3	1	39	57
Rat	GlcCer	8	43	36	13
	GalCer	85	19	5	N.D.

[a] The composition was determined by gas chromatography. d = Dihydroxy base; t = trihydroxy base. N.D. = Not detected.

coelution of two glycolipids (less than 10% of GlcCer with major GalCer) occurs, this overlapping could be prevented by increasing the elution volume of the first solvent mixture, or rechromatography of the overlapped fractions was sufficient for complete separation. As GalCer of equine kidney gave two subfractions with upper and lower bands, the lipid compositions of the subfractions were analysed. The lower components contained fairly large amounts of trihydroxy long-chain bases compared with those in the upper components (Table III), while the fatty acid composition in rat MHC was similar in the two glycolipid classes which could not be separated into subfractions by the column; GalCer contained more dihydroxy bases and 2-hydroxy acids than GlcCer. Thus, in addition to the interaction between borate ion and the galactose moiety of the MHC mixture, the lipid moiety containing *cis*-diol groups also interacts with borate ion, as was observed previously[12].

In conclusion, the improved borate-treated silical gel chromatography on a precoated plate and a column gave a clearer and simpler separation of GlcCer and GalCer for subsequent determination and preparation, respectively.

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