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APPLICATION OF A THIN-LAYER CHROMATOGRAPHY–FLAME-IONIZATION DETECTION SYSTEM FOR THE DETERMINATION OF COMPLEX LIPID CONSTITUENTS

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SUMMARY

A simple and rapid method for the determination of relative amounts of complex lipid constituents was developed using a thin-layer chromatography–flame ionization detection (TLC–FID) system. The methanolysis products from a sphingoglycolipid (ceramide monohexoside) were separated on Chromarod SII (silica gel) with double development to distances of 6 and 10 cm from the start using chloroform–methanol–15 *N* ammonia solution (60:10:1) and *n*-hexane–diethyl ether (50:2). The components separated on the rod were automatically scanned with a hydrogen flame-ionization detector (Iatroscan). The relative responses of methyl glucoside and methyl galactoside to sphingosine and a fatty acid methyl ester were in the same proportions as the molecular ratio.

The methanolysis products of sphingomyelin were also separated by the same system. Methanolytes of glyceroglycolipids and glycerophospholipids were separated into individual components with triple development using ethyl acetate–formic acid (70:2) twice and *n*-hexane–diethyl ether (50:20) to distances of 5 and 10 cm, respectively, from the start.

INTRODUCTION

Previous studies showed a Chromarods thin-layer chromatographic–flame-ionization detection (TLC–FID) system to be useful for the separation of lipid components[1–5]. Other investigators have also described the application of this system to the determination of lipids[6–16].

In the last 10 years, much progress has been made in the study of the structure and function of glycosphingolipids, and their biological importance has become apparent. The development of a simple and rapid procedure for the determination of these polar lipid constituents may greatly facilitate the study of these molecules.

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This paper describes the application of a TLC–FID system to the determination of the relative amounts of the various constituents comprising neutral glycosphingolipids (sugars, fatty acids and sphingosine) and the separation of the constituents of glyceroglycolipids and phospholipids.

EXPERIMENTAL

Apparatus and experimental conditions

The equipment consisted of an Iatroscan TH-10 TLC–FID analyser (Iatron Lab., Tokyo, Japan) equipped with a 7000AS Chromatogram Processer (System Instruments, Tokyo, Japan) and a Hitachi 056 two-pen recorder (Hitachi, Tokyo, Japan). Silica gel SII (5 μm particle size) Chromarods were used for separation. The Iatroscan was operated under the following conditions: flow-rate of hydrogen, 160 ml/min; flow-rate of air, 2000 ml/min; scanning speed, 30 sec per scan; chart-drive speed, 120 mm/min; and range of recorder, 50 mV (chromatogram).

Materials

Dihydroxysphingosine (Sph), methyl 12-hydroxystearate (h-$C_{18:0}$-Me), methyl lignocerate ($C_{24:0}$-Me), sphingomyelin (SPM, from bovine brain), ceramide monohexoside (CMH, from bovine brain), phosphatidylcholine (PC, from bovine brain) and digalactosyl diglyceride (DGDG, from spinach leaves) were purchased from Sardary Research (Ontario, Canada) and methyl glucoside (Glc-Me) from Sigma (London, U.K.). Methyl galactoside (Gal-Me) and glycerol were obtained from Wako (Osaka, Japan) and methyl stearate ($C_{18:0}$-Me) and cholesterol from Tokyo Kasei Kogyo (Tokyo, Japan). A mixture of Sph, Glc-Me, Gal-Me, $C_{18:0}$-Me, h-$C_{18:0}$-Me and $C_{24:0}$-Me was prepared at the concentrations specified in Table I and used as a standard mixture.

The methanolysates of SPM, CMH, PC and DGDG were prepared by the following process. A 5-mg amount of each lipid sample was treated with 2 ml of 5% hydrochloric acid–methanol (Kokusan Kagaku, Tokyo, Japan) in a screw-cap tube at 100°C for 3 h. After cooling, the solvent was evaporated and dried over sodium hydroxide *in vacuo*. The residue was dissolved in chloroform–methanol (1:2). The phosphorylcholine fraction was extracted from the methanolysis product of PC.

Organic solvents were of the highest quality commercially available and were used without further purification.

Method

The rods were activated by passing them through the TLC–FID analyser just before sample application. Each rod was spotted with 1 μl of sample solution containing 20–30 mg/ml of a mixture of the methanolysates indicated above. The rods were developed with a solvent mixture at room temperature (20–25°C), then dried at 120°C for 5 min and scanned with the Iatroscan. The developing solvents were (a) chloroform–methanol–15 *N* ammonia solution (60:10:1), (b) *n*-hexane–diethyl ether (50:20) and (c) ethyl acetate–formic acid (70:2).

TABLE I

STANDARD METHANOLYSIS PRODUCT MIXTURES OF CEREBROSIDE

Abbreviations: Glc-Me = methyl glycoside; Gal-Me = methyl galactoside; Sph = sphingosine; $C_{18:0}$-Me = methyl stearate; h-$C_{18:0}$-Me = methyl 12-hydroxystearate; $C_{24:0}$-Me = methyl lignocerate.

Sample	*Molecular ratio*					
	Glc-Me	*Gal-Me*	*Sph*	*$C_{18:0}$-Me*	*h-$C_{18:0}$-Me*	*$C_{24:0}$-Me*
A		1.00	1.00			1.08
B		1.00	0.50			0.53
C		1.00	0.33			0.36
D		1.00	0.25			0.26
E		1.00		0.49	0.48	
F		1.00		0.66	0.65	
G		1.00		0.98	0.96	
H		1.00		2.06	2.03	
I		1.00				0.48
J		1.00				0.65
K		1.00				0.96
L		1.00				2.05
M	1.00			0.48	0.47	
N	1.00			0.68	0.67	
O	1.00			0.98	0.96	
P	1.00			2.02	2.00	
Q	1.00					0.47
R	1.00					0.63
S	1.00					0.93
T	1.00					1.95

RESULTS AND DISCUSSION

Determination of sphingoglycolipid constituents

The methanolysis products of sphingoglycolipids such as ceramide monohexoside and ceramide dihexoside, *i.e.*, fatty acid methyl esters, methyl glycosides and spingosines, were resolved on a Chromarod SII by a double development technique. The first development was made with solvent (a) to a distance of 6 cm from the start. After being dried *in vacuo* for 10 min, the rods were developed for 10 cm with solvent (b). Methyl glycoside and sphingosine were separated with the first solvent and non-hydroxy fatty acid methyl esters and hydroxy fatty acid methyl esters with the second. The chromatogram for this is shown in Fig. 1.

Standard mixtures A–D (Table I) were separated on Chromarod SII and the constituents were determined with an Iatroscan. The relationship between the peak-area ratios and molecular ratios of methyl galactoside to methyl lignocerate and to dihydroxysphingosine are shown in Fig. 2. The following linear relationships between peak-area ratios (y) and the molecular ratios (x) of methyl galactoside to methyl lignocerate and dihydroxysphingosine were found to be, respectively

$$y = 0.17x$$
$$y = 0.24x \qquad (1)$$

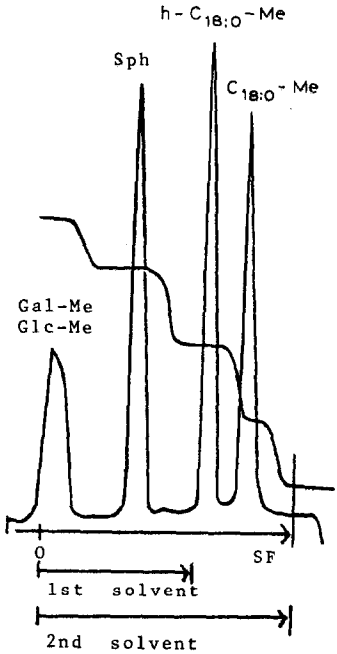


Fig. 1. Typical chromatographic separation of a mixture containing methyl glucoside (Glc-Me), methyl galactoside (Gal-Me), dihydroxysphingosine (Sph), methyl 12-hydroxystearate (h-$C_{18:0}$-Me) and methyl stearate ($C_{18:0}$-Me) on a Chromarod SII. The rod was developed in chloroform–methanol–15 *N* ammonia solution (60:10:1). When the solvent front reached a point 6 cm from the origin, development was terminated and the rod was placed in a vacuum desiccator. The rod was redeveloped in *n*-hexane–diethyl ether (50:20) to a distance 10 cm from the origin. SF = Solvent front.

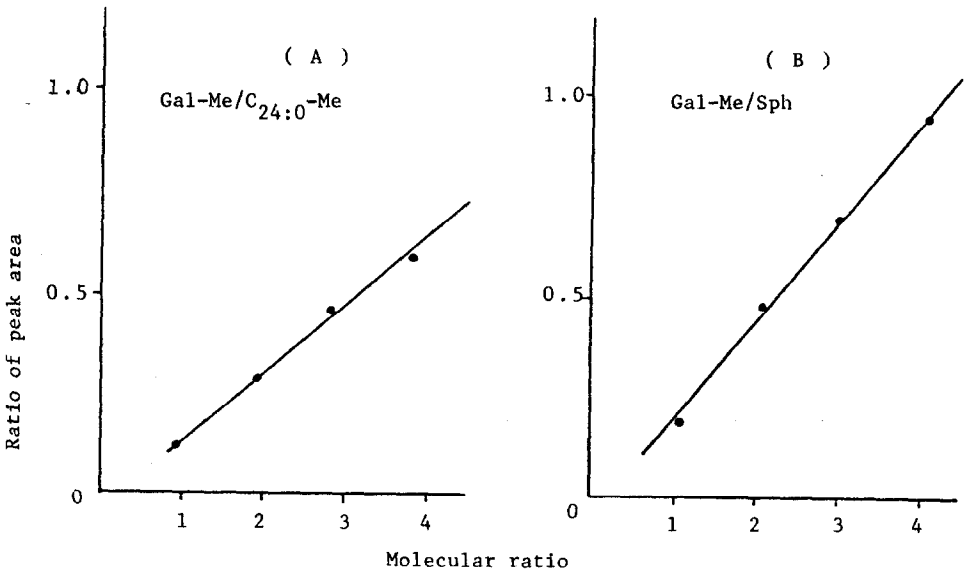


Fig. 2. Profiles of flame-ionization detector responses for methyl galactoside, methyl lignocerate and sphingosine base. (A) Relationships between Gal-Me and $C_{24:0}$-Me. (B) Relationship between Gal-Me and dihydroxysphingosine. Data points represent mean values obtained from ten rods. Relative standard deviations were within 10% for each point.

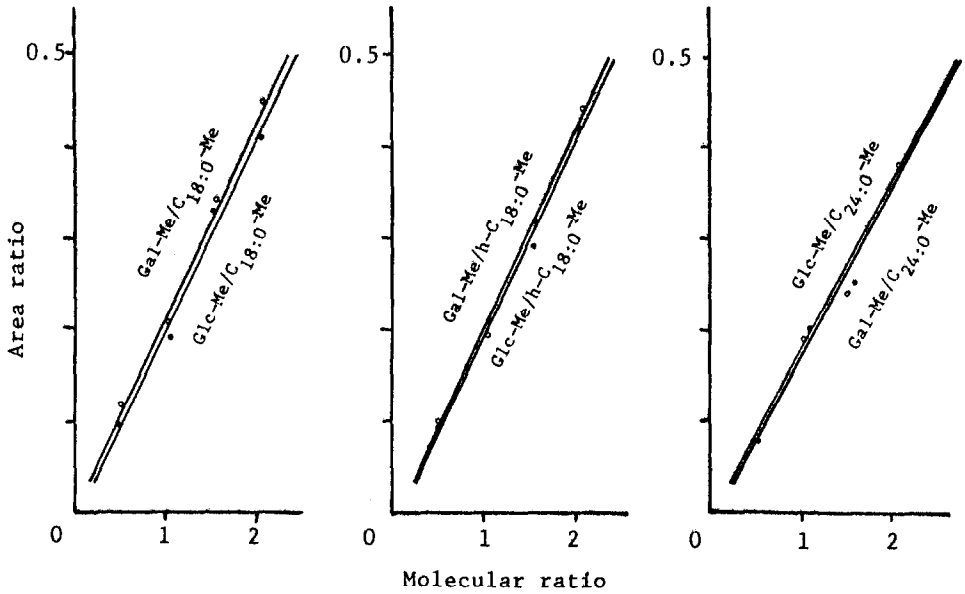


Fig. 3. Relationships between peak-area ratios and molecular ratios for methyl glycosides and fatty acid methyl esters. Data points represent mean values obtained from ten rods. Relative standard deviations were within 10% for each point.

The results of the analysis of standard mixtures E–T (Table I) are shown in Fig. 3. The following relationship between the peak-area ratios (y) and the molecular ratios (x) of methyl glycoside (methyl galactoside and methyl glucoside) to fatty acid methyl esters (methyl stearate and methyl 12-hydroxystearate) was found to be

$$y = 0.2x \tag{2}$$

The slopes of the two straight lines for glucose and galactose and also those for methyl stearate and methyl 12-hydroxystearate are almost identical. These results demonstrate that the sensitivities of glucose and galactose are almost the same, and that those of non-hydroxy fatty acid methyl esters and hydroxy fatty acid methyl esters are also approximately the same. The peak-area ratio of methyl galactoside to methyl lignocerate was smaller than that to methyl stearate (eqns. 1 and 2). The reason for this is considered to be the differences in molecular weights. Although the fatty acids contained in natural sphingoglycolipid are a mixture of homologues of different carbon chain lengths, as in the case of sphingosine, the content of fatty acids and sphingosine homologues can be expressed in moles calculated from stearic acid (or ligunoceric acid) and dihydroxysphingosine content, using the graphs in Figs. 2 and 3.

A chromatogram of the methanolysates of ceramide monohexoside from bovine brain is shown in Fig. 4. The molecular ratios of methyl glycoside (Gly-Me) to fatty acid methyl esters (FA-Me) and to Sph were calculated from this chromatogram (Table II). Table II shows that the molecular ratio of methyl glycoside to the fatty acid methyl esters is 0.9 on substituting stearic acid for fatty acid, and 1.0 on sub-

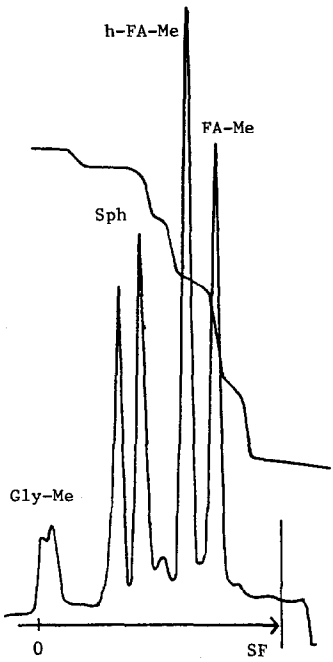


Fig. 4. Chromatogram of the methanolysis products of natural cerebroside (bovine brain). Mobile phase as in Fig. 1. Abbreviations: h-FA-Me, hydroxy fatty acid methyl ester; FA-Me, non-hydroxy fatty acid methyl ester; Gly-Me, methyl glycoside.

stituting lignoceric acid for fatty acid. The molecular ratio of methyl glycoside to sphingosine on substituting dihydroxysphingosine for sphingosine was 1.0. These results confirm that the molecular ratio of glycose, sphingosine and the fatty acid of ceramide monohexoside from bovine brain was 1:1:1. This coincides with the theoretical valuc for ceramide monohexoside. Table II also shows that the reproducibility of the peak-area ratio was within 10% (C.V.).

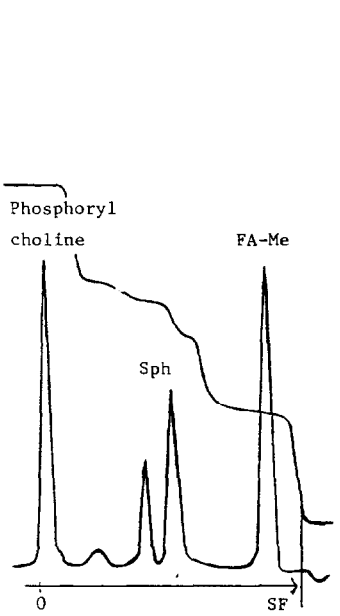
TABLE II

COMPARISON OF PEAK-AREA RATIO WITH MOLECULAR RATIO FOR CEREBROSIDE METHANOLYSATES

The results were obtained from five rods. Relative amounts of methanolysates of natural ceramide monohexoside determined by the TLC–FID method.

Parameter	*Gly-Me/FA-Me*	*Gly-Me/Sph*
Average peak-area ratio	0.17	0.23
Coefficient of variation (%)	8.4	9.6
Molecular ratio	0.85* ($C_{18:0}$) 1.0** ($C_{24:0}$)	1.0

* Calculated from methyl stearate.
** Calculated from methyl lignocerate.



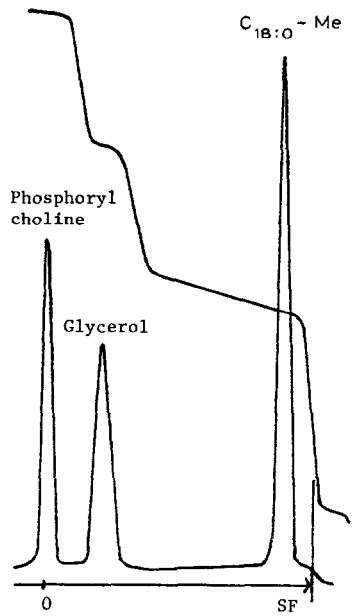


Fig. 5. Chromatogram of sphingomyelin (bovine brain) methanolysates. Mobile phase as in Fig. 1.

Fig. 6. Typical chromatographic separation of a mixture containing phosphorylcholine, glycerol and methyl stearate ($C_{18:0}$-Me) on a Chromarod SII. The rod was developed 5 cm with ethyl acetate–formic acid (70:2) twice and with *n*-hexane–diethyl ether (50:20) to 10 cm from the origin.

Determination of other complex lipid constituents

The methanolysis products of sphingomyelin were separated on a Chromarod SII with the above solvent systems. The methanolysis products of glyceroglycolipid and glycerophospholipid were also separated with triple development, with solvent (c) twice and solvent (b), to distances of 5 and 10 cm, respectively, from the start. Chromatograms of the methanolysates of sphingomyelin and glycerophospholipid are shown in Figs. 5 and 6, respectively. The molecular ratio of these constituents can easily be calculated in the same way as for ceramide monohexoside.

Up to now, the method for determining the molecular ratio of glycose, glycerol, fatty acids and sphingosine, etc., of the constituents in complex lipids such as glycolipids and phospholipids consisted in decomposing the lipids by chemical or enzymatic means; each product was then separately determined by a suitable chemical method[17–19]. It has now been found that each complex lipid constituent can be quickly determined using the TLC–FID system.

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