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THIN-LAYER CHROMATOGRAPHIC SEPARATION OF GLYCOLIPIDS IN ANIMAL LIPID MIXTURES

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SUMMARY

A method of thin-layer chromatography was used for the separation of glycolipids from lipid extracts of animal tissues. The technique of two-step development was applied. In the first step glycolipids are separated from phospholipids; the second step makes possible the separation of different glycolipid classes, *viz.* ceramide mono-, tri- and tetrahexosides, sulphatides and monogalactosyl diglycerides. Gluco- and galactolipids were separated on plates partially impregnated with sodium borate. The separation of psychosine, sphingosine and ceramide is also described.

INTRODUCTION

A wide variety of glycolipids is present in lipid extracts from animal tissues. In addition to ceramide monohexosides (cerebrosides) and sulphate esters of cerebrosides (sulphatides), which are abundant in the peripheral and the central nervous system, more complex glycolipids like ceramide di-, tri- and tetrahexosides have been found in many tissues[1]. Complex glycolipids containing sialic acid (gangliosides) are also extracted with chloroform–methanol mixtures. In the partition procedure of FOLCH *et al.*[2] gangliosides are retained in the upper aqueous phase. As thin-layer chromatography (TLC) of gangliosides is not considered in this study, the term "glycolipids" will be confined to the less polar glycolipids recovered in the lower chloroform phase, which contains a mixture of glycolipids and phospholipids of comparable polarities and similar chromatographic mobilities. Therefore, in many tissues, the unfavourable ratio of glycolipids to some common phospholipids makes difficult the direct TLC analysis of glycolipids. The separation of some glycolipids (cerebrosides) may be obtained with one- and two-dimensional systems commonly used in TLC of polar lipids[3–7]. However, in most cases, before a successful TLC analysis can be done, a separation of glycolipids from other lipids by mild alkaline hydrolysis, silicic acid and Florisil column chromatography or by a combination of these procedures is required. These methods have been recently discussed in detail by RENKONEN AND VARO[8].

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A different approach was reported by SKIPSKI *et al.*[9]. In the TLC technique described by these authors the presence of phospholipids and neutral lipids does not interfere with the separation of glycolipids. This advantage was based mainly on the incorporation of pyridine and acetone into the developing solvent mixtures.

In the present study a solvent mixture containing pyridine and acetone was also used as the first solvent in a two-step TLC. This solvent permits the efficient separation of glycolipids from phospholipids. The second development with a solvent mixture of appropriate composition separates different glycolipid classes, *viz.* ceramide, mono-, di-, tri- and tetrahexosides, sulphatides and monogalactosyl diglycerides. The use of chromatoplates partially impregnated with sodium borate permitted the separation of ceramide monoglucosides (glucocerebrosides) from ceramide monogalactosides (galactocerebrosides). The TLC separation of lipids encountered in studies of glycolipid metabolism, *i.e.* sphingosine, psychosine and ceramide, is also described.

MATERIAL AND METHODS

Standard lipids

Ceramide monogalactosides (galactocerebrosides, a mixture of kerasin and phrenosin) and sulphatides were isolated from bovine brain sphingolipids by silicic acid column chromatography[10]. Ceramide monoglucosides (glucocerebrosides) and ceramide dihexosides (lactosides) were prepared from rat spleen by the following procedure. After mild alkaline hydrolysis[11] and acetone precipitation the crude sphingolipids were loaded on the Florisil column (10 mg of lipid/g of adsorbant). Cholesterol and other less polar lipids were washed through with chloroform (ten column volumes) and the glucocerebrosides were subsequently eluted with chloroform–methanol (70:30) (five column volumes). Ceramide dihexosides were recovered in the next fraction eluted with the same solvent (five column volumes). Further purification of ceramide dihexosides was carried out by silicic acid column chromatography[12]. Monogalactosyl diglyceride was isolated from spinach leaf lipids by chromatography on a silicic acid column[13] and purified by TLC on Silica Gel G with chloroform–acetone–methanol–20% aq. ammonia–water (60:40:20:2:2) as developing solvent. Ceramides were obtained after hydrolysis of beef brain cerebrosides[14] and separated into the normal and hydroxy fatty acid containing fractions by TLC on Silica Gel G with chloroform–acetone–ethanol–20% aq. ammonia–water (70:40:6:1:1). Ceramide tri- and tetrahexosides (crude fractions) were prepared by silicic acid column chromatography[12] using stroma from human erythrocytes as starting material. Sphingosine and psychosine were prepared as described previously[15]. Gangliosides were from Koch-Light Laboratories Ltd. (Colnbrook, Bucks., Great Britain) and cardiolipin from Sylvana Chemical Co. (Millburn, U.S.A.). Lipid extracts from rat brain and human serum were prepared by the method of FOLCH *et al.*[2].

Preparation of plates and spotting of samples

Glass plates 20 × 24 cm were coated with a slurry prepared by mixing 54 g of Silica Gel H (Merck, Darmstadt, G.F.R.) and 6 g of finely ground Florisil (Florisil 60/100 mesh, purchased from Serlabo, Paris, France) with 135 ml of water. No attempts have been made to determine the mesh size of this material, but reproducible results were obtained when Florisil was milled in a small electric blender for 5 min.

In our hands this material was more satisfactory than finer-mesh magnesium silicate preparations commercially available. The plates were coated with a fixed-distance (0.5 mm) Desaga spreader and dried in air. For the separation of gluco- and galactocerebrosides (system D, see below) the chromatoplates prepared as described were partially impregnated with sodium borate. The lower part of the layer (5 cm from the bottom) was protected with a metal sheath; then the plates were sprayed with a 1.5% solution of sodium borate and dried in air. The chromatoplates were activated immediately before use by heating at 110° for 30 min. The samples were applied 1.5 cm from the bottom edge as series of spots to give a 1.5-cm-long narrow band.

Development of chromatograms

One-dimensional TLC was performed in chambers (21 × 9 × 23 cm) lined with Whatman No. 3 paper. The solvent mixtures were added 30 min before development and the paper lining was soaked with the solvent. A two-step ascending technique of development has been applied. In all TLC systems described the first solvent was chloroform–acetone–pyridine–20% aq. ammonia–water (20:30:60:2:2). The drying of chromatoplates between the first and the second solvent was carried out in a stream of air for 20 min.

System A. In system A the plate was developed with the first solvent for 10 cm from the bottom. The second solvent, chloroform–acetone–methanol–acetic acid–water (65:35:11:4:1.5) was allowed to run to the top of the plate.

System B. In system B the front of the first solvent was 9 cm above the bottom. After drying the lower part of the adsorbent, a 2.5-cm band starting at the bottom of the plate was scraped off and development proceeded with chloroform–methanol–water (65:25:4) to the top of the plate.

System C. In system C the plate was developed with the first solvent for 8 cm and dried. The adsorbent was scraped from the plate up to a level of 2.5 cm from the bottom and the chromatogram developed with chloroform–acetone–methanol–water (65:30:12:2) to the top of the plate.

System D. In system D the first solvent was allowed to ascend to a level of 8 cm from the bottom of the plate. The chromatogram was dried and the lower part of the layer was removed (2.5 cm from the bottom). The second development was carried out with chloroform–acetone–methanol–acetic acid–water (68:26:12:5:3) up to the top of the plate.

Detection of lipid fractions on thin-layer plates

Orcinol–sulphuric acid spray[16] was used for detection of lipids containing a carbohydrate moiety. For the non-specific detection of lipids by charring, the phosphoric acid–copper acetate reagent[17] was used.

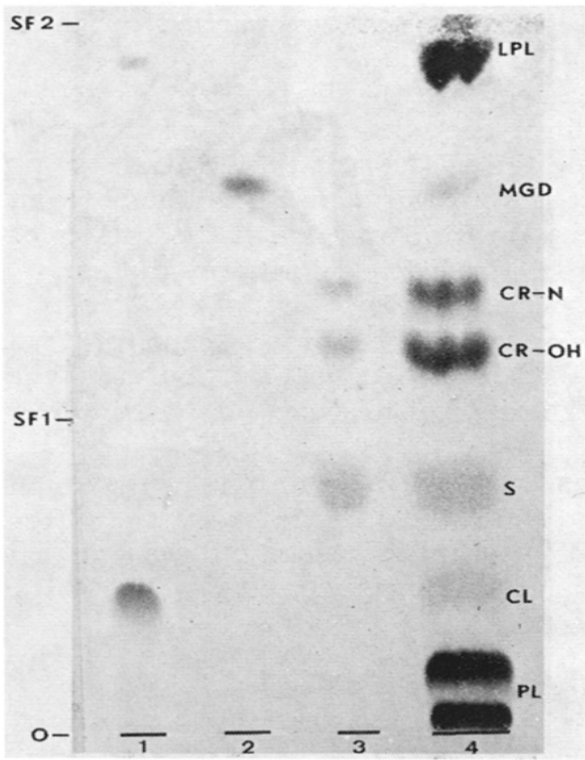
RESULTS AND DISCUSSION

The first solvent, chloroform–acetone–pyridine–20% aq. ammonia–water, which is common for all systems described in this paper, permits a complete separation of glycolipids from phospholipids. Glycolipids, together with cholesterol and other less polar lipids, move near the solvent front; phospholipids stay at the origin

(cardiolipin moves very little) as well as gangliosides, and fatty acids migrate to the area very close to the origin. Of considerable importance for studies involving the use of radioactive precursors is that several non-lipidic compounds (glucose, galactose, glucose- and galactose-1-phosphate, UDP-glucose, UDP-galactose) remain at the origin or move well behind glycolipids.

In system A cerebrosides (kerasin and phrenosin), sulphatides and monogalactosyl diglyceride are clearly separated from each other and from the rest of lipids present in the extracts of animal tissues (Fig. 1). As much as 2.5 mg of total lipids may be chromatographed in this system; when greater amounts were applied, a streaking with the first solvent and bad resolution was observed. This system was successfully applied in our laboratory for rapid quantitative analysis of cerebrosides and sulphatides in brain.

System B was developed primarily for the analysis of neutral ceramide glycosides. Ceramide mono-, di-, tri-, and tetrahexosides were clearly separated as shown in Fig. 2. A rapid qualitative analysis of glycolipids can be carried out on as little as



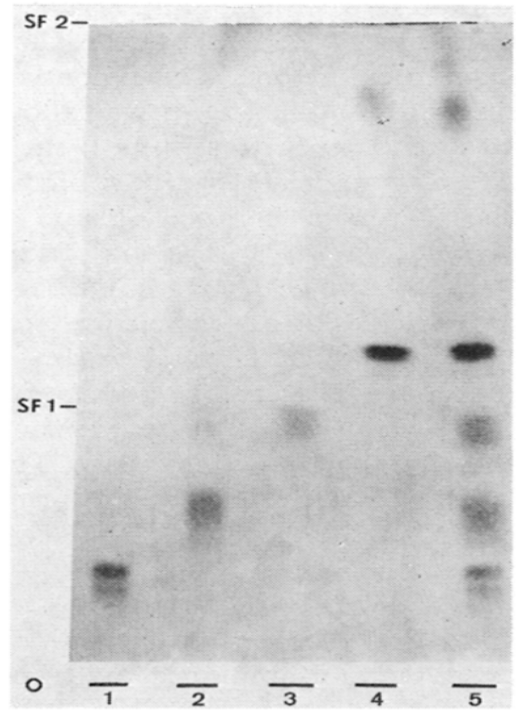
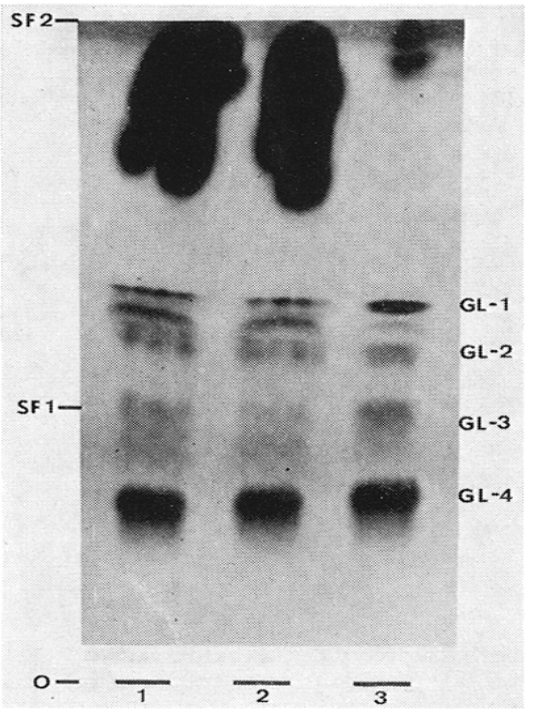


Fig. 1. TLC of lipids extracted from rat brain and of reference lipids in system A. (1) Ceramide with hydroxy fatty acids (upper spot, 40 μg) and cardiolipin (lower spot, 40 μg); (2) monogalactosyl diglyceride (40 μg); (3) galactocerebrosides (upper two spots, 40 μg) and sulphatides (lower spot, 20 μg); (4) lipid extract from rat brain (2 mg of total lipids). Abbreviations: LPL = less polar lipids; MGD = monogalactosyl diglyceride; CR-N = cerebrosides with normal fatty acids, CR-OH = cerebrosides with hydroxy fatty acids; S = sulphatides; CL = cardiolipin; PL = phospholipids; O = origin, SF_1 = first solvent front; SF_2 = second solvent front. Detection: phosphoric acid–copper acetate spray.

Fig. 2. Separation of neutral ceramide glycosides in system B. (1) ceramide tetrahexosides (30 μg); (2) ceramide trihexosides (40 μg); (3) ceramide dihexosides (30 μg); (4) ceramide monohexosides (glucocerebrosides, 40 μg); (5) mixture of 1–4. Abbreviations: O = origin; SF_1 = first solvent front; SF_2 = second solvent front. Detection: phosphoric acid–copper acetate spray.



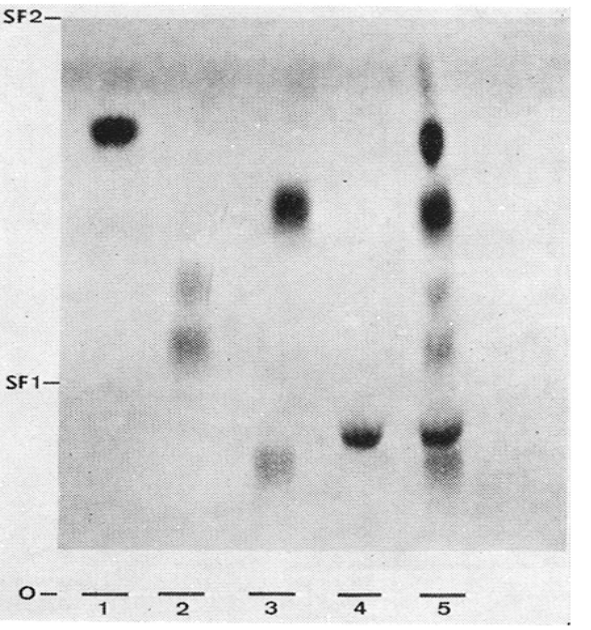


Fig. 3. Separation of human serum glycolipids in system B. (1) and (2): total serum lipids (11 mg, equivalent to 3 ml of serum); (3) mixture of reference compounds (40 μg each). Abbreviations: GL-1 = glucocerebroside; GL-2 = ceramide dihexoside, GL-3 = ceramide trihexoside; GL-4 = ceramide tetrahexoside; O = origin; SF_1 = first solvent front; SF_2 = second solvent front. Detection: phosphoric acid–copper acetate spray.

Fig. 4. TLC of reference lipids in system C. (1) ceramide with hydroxy fatty acids (40 μg); (2) galactocerebrosides (50 μg); (3) sphingosine (upper spot, 40 μg) and psychosine (lower spot, 40 μg); (4) sulphatides (40 μg); (5) mixture of 1–4. Abbreviations: O = origin; SF_1 = first solvent front; SF_2 = second solvent front. Detection: phosphoric acid–copper acetate spray.

3 ml of plasma (Fig. 3). As the glycolipid content of plasma is very small, a large amount of total lipids had to be applied in this case. The application of 10–15 mg of total lipids could be achieved by using thick layers (1 mm) and spotting the sample as a rather large band (1.5 × 1 cm).

System C separates cerebrosides and sulphatides as well as several lipids which may be involved in glycolipid metabolism, *i.e.* ceramides, sphingosine and psychosine (Fig. 4). This system was particularly useful for the rapid separation of labelled lipids during the studies of glycolipid biosynthesis[15]. A more simple TLC method giving a good separation of ceramides, cerebrosides, sphingosine, fatty acids and psychosine was recently reported[18]; however, it is difficult to apply this system in the presence of phospholipids and sulphatides.

In system D the use of plates partially impregnated with sodium borate permitted the separation of gluco-, and galactocerebrosides. TLC of gluco-, and galactocerebrosides on borate-impregnated plates was described previously[19,20], but these techniques are not convenient for analysis of more complex lipid mixtures. With system D a complete resolution of ceramides, monogalactosyl diglyceride, glucocerebrosides, galactocerebrosides, sulphatides and psychosine was obtained (Fig. 5). When a second solvent of somewhat different composition was introduced (chloro-

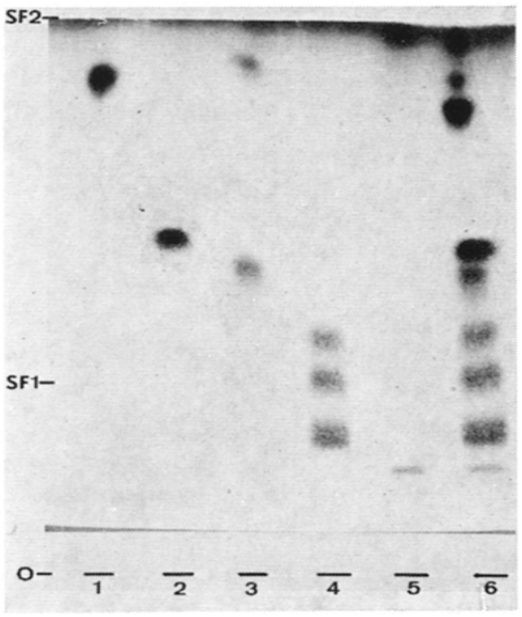


Fig. 5. TLC of reference lipids in system D. (1) ceramide with hydroxy fatty acids (40 μg); (2) monogalactosyl diglyceride (40 μg); (3) ceramide with normal fatty acids (upper spot, 20 μg) and glucocerebrosides (lower spot, 20 μg); (4) galactocerebrosides (upper two spots, 60 μg) and sulphatides (lower spot, 40 μg); (5) psychosine (20 μg); (6) mixture of 1–5. Abbreviations: O = origin; SF_1 = first solvent front; SF_2 = second solvent front. Detection: phosphoric acid–copper acetate spray.

form–acetone–methanol–acetic acid–water, 65:35:11:3:1.5), glucopsychosine was separated from galactopsychosine. Therefore, in the studies where separation of gluco- and galactolipids is important[21], system D may be substituted for system C.

In comparison with the similar TLC procedure for analysis of glycolipids[9] some noteworthy improvements are introduced in the present method: (1) separation of gluco- and galactocerebrosides may be performed simultaneously with other lipids; (2) no additional development is necessary to remove fatty acids which may interfere with the separation of some glycolipids; (3) the systems described provide a convenient separation of labelled glycolipids after the incorporation of radioactive substrates since the risk of contaminating lipid fractions with non-lipidic radioactive material is greatly reduced.

A shortcoming of the method described in this study is that the systems C and D, which separate cerebrosides, sulphatides and related sphingolipids (psychosine, sphingosine and ceramide), are less successful in tissues with an appreciable content of ceramide di-, tri-, and tetrahexosides.

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