Separation of neutral glycosphingolipids and sulfatides by thin-layer chromatography

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ABSTRACT Two one-dimensional systems for separation of glycolipids from total lipid extracts of tissues by thin-layer chromatography are described. System I used, as adsorbent, an alkaline mixture of silica gel without $CaSO_4$ binder (75%) and magnesium silicate (25%), and the lipids were "developed" with three successive solvent mixtures. The separated compounds (from the fastest to the slowest moving) were: ceramide, ceramide monohexosides, sulfatides, ceramide dihexosides, psychosine, ceramide trihexosides, and ceramide *N*-acetylhexosamine trihexosides.

In system II a two-step development was used on an adsorbent consisting of silica gel without $CaSO_4$ binder (80%) and magnesium silicate (20%). The separated compounds were: ceramides, ceramide monohexosides, and ceramide dihexosides. Psychosine and sulfatides as well as ceramide trihexosides and ceramide *N*-acetylhexosamine trihexosides were not separated.

In both systems all neutral lipids moved to the very top of the chromatogram and phospholipids stayed at the origin. Application of systems I and II for separation of glycolipids was demonstrated on total lipid extracts from animal tissues.

KEY WORDS glycolipids · neutral glycolipids · glycosphingolipids · aminoglycolipids · separation · ceramide monohexosides · ceramide dihexosides · ceramide trihexosides · psychosine · sulfatides · ceramides · cerebrosides · ceramide *N*-acetylhexosamine trihexosides · magnesium silicate · thin-layer chromatography

In recent years several systems of TLC for the separation of neutral lipids and phospholipids have been described (1). In most cases they permit qualitative and quantitative analyses of the major classes of neutral lipids and phospholipids on total lipid extracts from different animal or plant tissues. The systematic analysis of the major classes of glycolipids, however, still presents some problems. Although previous investigators (2–11) separated a few classes of glycolipids by applying total lipid extracts from animal tissues on a chromatoplate or on silicic acid-impregnated papers, these systems are not suitable for the study of tissue glycolipids. The main obstacle in the separation of glycolipids on thin-layer chromatoplates is that many phospholipids move together with the glycolipids and interfere with the separation of the latter. Only after all neutral lipids and phospholipids had been removed from lipid extracts were Svennerholm and Svennerholm (12, 13), Gray (14), and Makita (15) able to achieve excellent separation of many classes of glycolipids on thin-layer chromatograms. However, the removal of nonglycolipid material is a rather complicated procedure and requires a relatively large amount of lipid to begin with.

Our principal aim was the development of a TLC system that would permit separation of the major glycolipids, in small amounts of total lipid extracts from tissues, without prior removal of the lipids that usually interfere.

The systems for separation of glycolipids described in this paper were based on the facts that in all polar solvents neutral lipids tend to move on silica gel along with the solvent front, that pyridine and acetone do not move phospholipids from the origin on silica gel, and that magnesium silicate adsorbs phospholipids more strongly than glycolipids.

Two systems were worked out. System I separates ceramides, ceramide monohexosides, ceramide dihexosides, ceramide trihexosides, ceramide *N*-acetylhexosamine trihexosides, psychosine, and sulfatides. System II separates ceramides, ceramide monohexosides, and ceramide dihexosides. Both systems are applicable to the sepa-

Abbreviation: TLC, thin-layer chromatography.

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ration of glycolipids present in various human and animal tissues.

MATERIALS AND METHODS

Solvents, Adsorbents, and Other Chemicals

All solvents were reagent grade and were obtained from Merck & Co., Rahway, N. J., unless otherwise indicated. The following solvents were used: chloroform, washed with water, dried with anhydrous calcium chloride, and redistilled (1% absolute methanol was added as a preservative); methanol, absolute, redistilled under anhydrous conditions; pyridine, redistilled under anhydrous conditions; benzene, thiophene-free, redistilled; acetone, anhydrous, redistilled under anhydrous conditions in the presence of potassium permanganate and potassium carbonate; diethyl ether; and glacial acetic acid. Ammonium hydroxide, reagent grade was purchased from Fisher Scientific Company, New York. Ethanol, absolute, obtained from U.S. Industrial Chemical Co., New York, was redistilled under anhydrous conditions in the presence of potassium hydroxide. Silica gel, fine (D-O type), without $CaSO_4$ binder (Camag, Muttenz, Switzerland, obtained through Arthur H. Thomas Co., Philadelphia, Pa.), and magnesium silicate, Woelm, "for thin-layer chromatography" (M. Woelm, Eschwege, Germany, obtained through Alupharm Chemicals, New Orleans, La.) were used throughout the study. Orcinol from Fisher Scientific Company, and Oil Red O from National Aniline Division, Allied Chemical Corp., New York, were used to detect glycolipids on thin-layer chromatograms.

Reference Lipids and Tissue Extracts

The following glycolipids were used as reference compounds: a mixture of ceramide monohexosides (cerebrosides) isolated from bovine brain, sulfatides (cerebroside sulfates) isolated from bovine spinal cord, pyschosine, and ceramide, all obtained from Applied Science Laboratories Inc. (State College, Pa.); kerasin (galactoside), ceramide dihexoside (cytolipin H), and psychosine (galactosyl sphingosine), synthetic, from Yeda Research and Development Co., Ltd. (Rehovoth, Israel); phrenosin (galactoside) isolated from bovine spinal cord (16, 17); gangliosides from Koch-Light Laboratories, Ltd. (Colnbrook, Bucks, England); ceramide dihexoside, ceramide trihexoside, and ceramide *N*-acetylhexosamine trihexosides (ceramide *N*-acetylgalactosamine trihexoside or mucolipid ML-1) from human kidney, generous gifts from Drs. A. Makita and T. Yamakawa, Department of Chemistry, Institute for Infectious Diseases, University of Tokyo, Tokyo, Japan (15, 18, 19). Analytical data of these glycolipids are given by Makita (15).

The origins of neutral lipids and of phospholipids used in this study have been reported previously (11, 20–22). Lipid extracts from livers, brains, kidneys, spleens, and lungs of adult male albino rats were prepared according to the methods of Folch, Lees, and Sloane Stanley (23). These extracts were washed with 0.2 volume of water (23) or with NaCl solution (24).

Preparation of Plates

In all experiments 200 × 200 mm Pyrex glass plates were used. Adsorbent was applied with an adjustable Desaga applicator, set for 0.5 mm thickness. The composition of the adsorbent and the pretreatment of the plates for the two systems were different. For system I the slurry of adsorbent was prepared by mixing 10 g of magnesium silicate and 30 g of silica gel with 100–110 ml of 0.05 M Na_2CO_3. The plates were allowed to dry in the air for about 45 min and activated in an oven at 150°C for at least 5 hr before use. Activation for several days usually resulted in a better separation of glycolipids. Plates can stay in the oven for as long as 10 days at 150°C without deleterious effect on the efficiency of lipid separation. In system II we prepared the slurry by mixing 8 g of magnesium silicate and 32 g of silica gel and 100–110 ml of water. The plates were activated (at 150°C) in the same way as in system I.

Application of Samples

The samples and reference compounds, dissolved in chloroform–methanol 2:1 or in methanol, were applied to the plates by means of micropipettes or Hamilton syringes, 3.0 cm from the bottom edge. Samples were applied as individual spots or as a series of extremely small spots that formed a band. The amounts of standard compounds applied ranged from 5 to 25 μg, whereas the total lipid extracts from tissue were applied in greater quantities, usually 500–1500 μg.

In order to control the water content in the adsorbent, we cooled the activated plates in a desiccator and applied the samples in a Desaga-Brinkmann Application Box (Model DB, Brinkmann Instruments Inc., Westbury, N.Y.) under dry nitrogen.

Development of Chromatograms

In system I three different solvents were used. The first was acetone–pyridine–chloroform–water 40:60:5:4, which was allowed to run up to a level about 11 cm from the bottom of the plate. The plates were removed from the development tanks, dried in a vacuum oven for 30 min at room temperature, and developed in the second solvent mixture, ethyl ether–pyridine–ethanol–2 M NH_4OH 65:30:8:2. This solvent was allowed to run to 1 cm below the top of the plate. This two-step development assured separation of glycolipids from each other, but free fatty acids overlapped with ceramide *N*-acetylhexosamine trihexosides. In order to remove fatty acids we

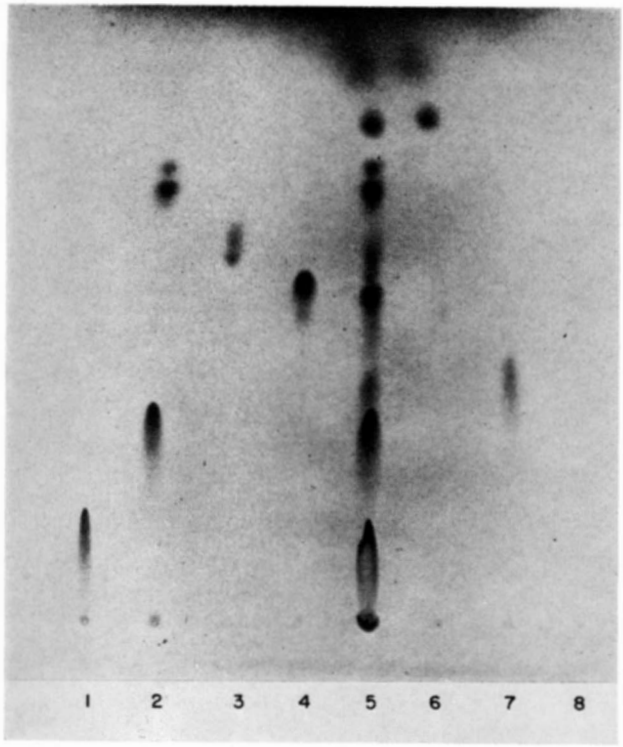

FIG. 1. Separation by TLC system I of reference glycolipids applied individually or as a mixture. The following approximate amounts of lipids were applied. Lane 1, ceramide *N*-acetylhexosamine trihexoside, 8 μg. Lane 2, ceramide monohexosides (two upper spots), 8 μg, and ceramide trihexoside (lower spot), 11 μg. Lane 3, sulfatides (two spots), 5 μg. Lane 4, ceramide dihexoside, 8 μg. Lane 5, mixture of *1–4*, *6*, *7*, and cardiolipin, 5 μg (at the origin of chromatogram). Lane 6, ceramides (two spots), 8 μg. Lane 7, psychosine, 7 μg. In lane 8, oleic acid (10 μg) and 1-monoolein (7 μg) both moved to the front of chromatogram and are not visible. Detection of spots: 40% H_2SO_4 spray.

developed the chromatograms in the third solvent, ethyl ether–acetic acid 100:3, after drying them in a vacuum oven for 30 min to remove pyridine. The third solvent was allowed to run to the top of the plate. It carried the free fatty acids to the upper edge of the plate without essentially affecting the position of different glycolipids on the chromatogram. Since the three-solvent development may take over 8 hr, it is practical to develop the chromatogram with the third solvent overnight (16 hr). This does not interfere with the separation of glycolipids.

In system II, two different developing solvents were used. The first, chloroform–pyridine–water 50:51:5, was allowed to run up to about 19 cm from the bottom of the plate. The plates were dried in a vacuum oven at room temperature for 30 min to remove pyridine, and developed with the second solvent, ethyl ether–acetic acid 100:3, which was allowed to run to the very top of the plates, carrying with it—like the third solvent in system I—free fatty acids. It was again found practical to develop the chromatogram with this second solvent overnight.

Detection of Spots

The following methods were used for the detection of spots on the chromatogram.

(*a*) Orcinol–sulfuric acid spray for sugars (25) (200 mg orcinol was dissolved in 100 ml of H_2SO_4–H_2O 3:1 and stored in the dark under refrigeration; the solution was stable for about a week). The plates were sprayed until the whole surface became obviously moist and then were placed into an oven at 100°C for 15 min. Most of the glycolipids gave violet spots on a white background.

(*b*) "Clorox" and benzidine sprays for sphingolipids (26) [Reagent I: 50 ml of benzene, 5 ml of Clorox bleach (trade name for a commercial bleach; reactive substance sodium hypochlorite), and 5 ml of glacial acetic acid were mixed and immediately sprayed on the plate. Reagent II: 200 mg of benzidine dihydrochloride and 1 small crystal of potassium iodide were dissolved in 20 ml of 50% aqueous ethanol]. After being sprayed with Reagent I, the plates were left at room temperature for 30 min, then dried in hot air (80°C) in the chromatograph-drying hood for 10 min and sprayed with Reagent II. Blue spots appear almost immediately with most sphingolipids.

(*c*) Oil Red O. The plates were sprayed with 0.1% Oil Red O in absolute ethanol. Most lipids gave dark red spots.

(*d*) Sulfuric acid spray. The plates were sprayed with 40–50% H_2SO_4 and heated on a hot plate.

RESULTS AND DISCUSSION

System I

The separation of reference glycolipids and their derivatives, applied individually or as a mixture, is shown in Fig. 1. Cardiolipin, free fatty acid, and monoglyceride were also applied since, in many solvent systems which we tried, these three lipids often interfered with the separation of glycolipids. The following glycolipids were separated (from the top to the bottom of the chromatogram): ceramides (two spots), ceramide monohexosides (two spots), sulfatides (two spots), ceramide dihexosides, psychosines, ceramide trihexosides, and ceramide *N*-acetylhexosamine trihexosides. Cardiolipin stayed at the origin of the chromatogram (Fig. 1, lane 5) or moved only a little. Free fatty acid and monoglyceride (Fig. 1, lane 8) moved with the solvent fronts to the very top of the chromatogram and therefore were not visible. All other phospholipids tested (phosphatidic acid, phosphatidyl glycerol, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol, phosphatidyl choline, lysophosphatidyl choline, and sphingomyelin) stayed at the origin of the chromatogram. Gangliosides also stayed at the origin. All neutral lipids tested (triglycerides, diglycerides, monoglycerides, hydrocarbons, cholesterol, and

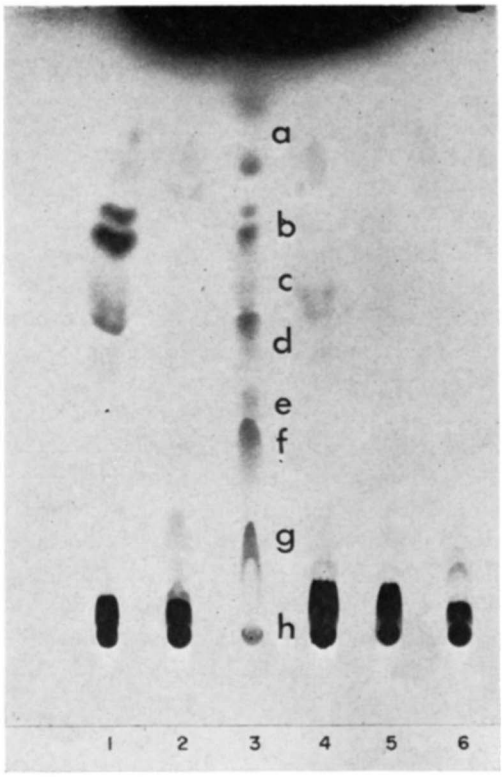

FIG. 2. Chromatogram (system I) of total lipids extracted from different animal tissues. Lane 1, total lipid extract from rat brain, 600 μg. Lane 2, total lipid extract from rat spleen, 600 μg. Lane 3, mixture of reference compounds: *a*, ceramides; *b*, ceramide monohexosides; *c*, sulfatides; *d*, ceramide dihexoside; *e*, psychosine; *f*, ceramide trihexosides; *g*, ceramide *N*-acetylhexosamine trihexosides; *h*, phospholipids (cardiolipin). Lane 4, total lipid extract from rat kidney 600 μg. Lane 5, total lipid extract from rat liver, 600 μg. Lane 6, total lipid extract from rat lung, 600 μg. Detection of spots: 40% H_2SO_4 spray.

cholesteryl fatty acid esters) moved to the top of the chromatogram.

Thus, system I separated all common glycolipids present in animal or human tissues with the exception of gangliosides. On some chromatograms the spots of sulfatides may be very close to that of ceramide dihexosides (Fig. 1, lanes 3–5). By slight alteration of the composition of the second developing solvent—an increase of 2 M NH_4OH from 2 parts to 3 parts—it is possible to improve the separation of these two glycolipids. However, the separation of other glycolipids may be slightly inferior with this developing solvent.

Fig. 2 shows application of system I for the separation of glycolipids present in lipid extracts from different rat tissues. About 600 μg of each lipid extract was chromatographed. The chromatogram of total lipid extract from brain revealed ceramides, ceramide monohexosides, sulfatides and, apparently, a small amount of ceramide dihexosides (Fig. 2, lane 1). The chromatogram of total lipid extract from rat spleen showed ceramides, ceramide dihexosides, and ceramide *N*-acetylhexosamine trihexosides, (Fig. 2, lane 2). The chromatogram of total lipid extract from rat kidney revealed small amounts of ceramides, ceramide monohexosides, ceramide dihexosides, and ceramide *N*-acetylhexosamine trihexosides, and large quantities of sulfatides (Fig. 2, lane 4). The amount of glycolipids in lipid extracts from rat liver and lung was too small to be identified even tentatively. From other chromatograms with larger loads, it is known that liver contains ceramide monohexosides.

Identification of glycolipids in tissue extracts was based on their relative positions on the chromatogram and on color reactions, and is therefore tentative.

System II

System II, essentially a short version of system I, permitted the separation and identification of only ceramides, ceramide monohexosides, and ceramide dihexosides. Fig. 3 shows the separation of reference glycolipids applied individually and as a mixture. It also shows the separation

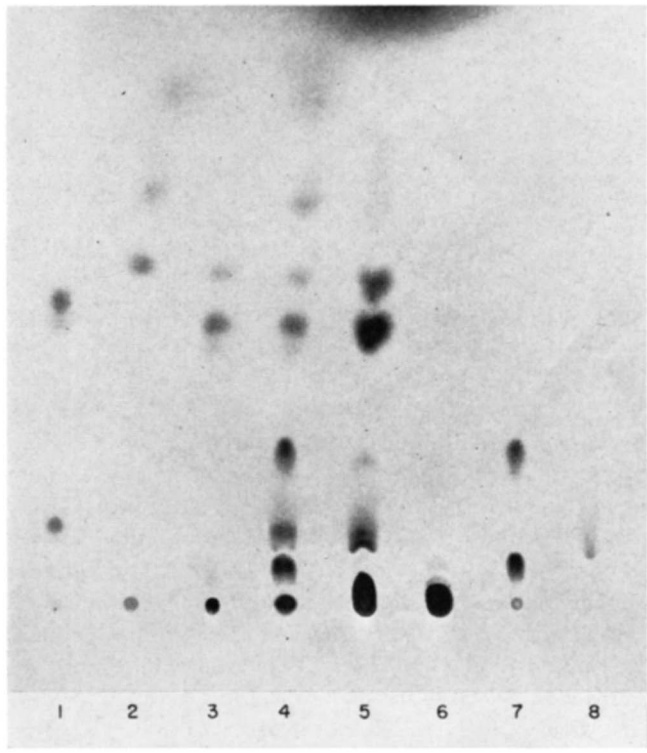

FIG. 3. Separation in system II of reference glycolipids applied individually or as a mixture and of glycolipids present in tissue lipid extracts. The following approximate amounts of lipids were applied. Lane 1, oleic acid, 10 μg, 1-monoolein, 7 μg (both lipids washed out with solvent front), phrenosine (upper spots), 8 μg, and psychosine, 10 μg. Lane 2, ceramides (three upper spots), 10 μg, and kerasine (lower spot), 8 μg. Lane 3, crude mixture of ceramide monohexosides (three upper spots), 12 μg, ceramide *N*-acetylhexosamine trihexoside (faint spot near the origin), 5 μg, and cardiolipin, 6 μg. Lane 4, mixture of *1–3*, *7*, and *8*. Lane 5, total lipid extract from rat brain, 600 μg. Lane 6, total lipid extract from rat lung, 600 μg. Lane 7, ceramide dihexoside (upper spot), 7 μg, and ceramide trihexosides (lower spot), 10 μg. Lane 8, sulfatides, 5 μg. Detection of spots: 40% H_2SO_4 spray.

of glycolipids from total lipid extracts of rat brain and lung. All neutral lipids moved with the solvent front and all phospholipids stayed at the origin. Psychosine and sulfatides, as well as ceramide trihexosides and ceramide *N*-acetylhexosamine trihexosides, have the same R_f values and therefore did not separate. A chromatogram of a tissue extract from rat brain (Fig. 3, lane 5) revealed large amounts of ceramide monohexosides and small amounts of ceramides and ceramide dihexosides. A spot near the origin indicated the presence of sulfatides and (or) psychosine.

The main value of both systems is that they selectively separate glycolipids. All other lipids, as was shown by our experiments with reference phospholipids and neutral lipids, either stay at the origin or move to the top of the chromatogram. Therefore, it is not necessary to remove either neutral lipids or phospholipids before the separation of glycolipids. In other words, these systems permit direct application of total lipid tissue extracts on the chromatoplates. Another advantage of both systems is that reliable results can be obtained with small lipid samples, 1–2 mg. Of course, the size of the sample depends upon the amount of glycolipid present in the lipid extract tested. Application of a 1 mg sample usually revealed all glycolipids present in that sample at a level of 0.2–0.3% or more.

System I (three-step development) assures the separation of the main classes of glycolipids in animal tissues with the exception of gangliosides, for which there are good thin-layer chromatographic systems (26–29). System II (two-step development) permits separation of three classes of neutral glycolipids. However, two of these lipids, ceramide monohexosides and ceramide dihexosides, are apparently the main glycolipids of many animal tissues (12). Moreover, the spots on chromatograms developed in system II show less tailing and can be identified more easily. Therefore, system II should also be useful in many cases, especially where speed and simplicity of procedure are important. Both systems can be used for the identification of glycolipids or qualitative analysis of lipid extracts. Adaptation of these systems for quantitative analysis of glycolipids is being developed in our laboratory.

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