Preparative isolation of cerebrosides (galactosyl and glucosyl ceramide)

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Summary An improved method for isolating cerebrosides from natural sources is described. The method is particularly suited to large scale work and can be adapted to the isolation of sphingolipids that are less polar than the gangliosides. It is based on the use of sodium sulfate to absorb the water from chloroform–methanol tissue extracts, the use of triiodide to cleave the ether linkage of plasmalogens, and the use of alkaline methanolysis to cleave the ester linkages of the glycerolipids. The final separation of the lipids is done with a silica gel column.

Supplementary key words glucocerebroside · galactocerebroside

The currently available methods for isolating sphingolipids are readily carried out on a small scale. However when one attempts to scale up the procedures somewhat, several of the steps become tedious or impractical in most laboratories. Solvent volumes become unpleasantly large for handling, evaporations consume much time, partitioning of solvents becomes difficult if emulsions form, chromatographic steps require large columns and solvent handling systems, and the cost of solvents becomes quite noticeable.

The size of an adsorption column is determined by the weight of the polar components in the sample mixture, that is, the weight of desired lipid plus the weight of the lipids that are more polar than the desired lipid. In the case of sphingolipid isolations, it is customary to reduce the weight of the latter fraction by use of alkaline methanolysis, which converts the glycerolipid esters to the nonpolar methyl esters and the water-soluble glycerides. The latter are removed by liquid/liquid partitioning. However the alkenyl ether linkage of the plasmalogens is stable to alkali and the lyso ethers remain with the polar lipid fraction. This increases the size of the subsequent chromatographic operation. Another difficulty with the methanolysis step is the need for anhydrous conditions; these are commonly achieved on a small scale by simply evaporating the lipid extract to dryness.

The above evaluation of the difficulties of large

Abbreviation: TLC, thin-layer chromatography.

scale work led to the following approaches. The alkenyl ether linkage was cleaved by reaction with iodine (1, 2). The water in the initial lipid extract, arising from the original tissue, was absorbed by anhydrous sodium sulfate, which is readily removed by filtration. Conditions of extraction were chosen to give reasonably complete extraction and fast filtration with a relatively small volume of solvent. Reexamination of the load capacity of silica gel led to the finding that a satisfactory separation could be achieved with a smaller column and with less solvent than previously believed necessary.

The procedure described below was designed for 500 g batches of brain or Gaucher spleen, but its advantages apply to smaller samples. Suggestions are offered for modifications of the method for laboratories lacking some of the equipment used here.

Materials and equipment

Chloroform, methanol, benzene, and hexane were redistilled ACS reagents. The column adsorbent was silica gel 60, 70/230 mesh, #7734 (EM Laboratories, Elmsford, N. Y.). The iodinolysis reagent was made by dissolving 4 g of iodine and 2 g of NaI in 100 ml of absolute ethanol.

Mixing and partitioning were carried out in 2-gal flint glass bottles on a sturdy rotatory table mixer (Model G-2 laboratory rotator, New Brunswick Scientific, New Brunswick, N. J.). Lyophilization of the cerebroside solutions was carried out with a mechanically cooled lyophilizer operating at −85°C (FD-ULT-1, Thermovac Corp., Copiague, L.I., N. Y.). Ordinary mechanically cooled lyophilizers do not trap benzene adequately since they are not cold enough.

Chromatography was performed in a 3.1 × 120 cm glass column, fitted with a sintered glass disc at the bottom and a 28/15 ball joint at the top. Solvent was supplied by a pair of chromatography pumps (Waters Associates, Framingham, Mass.) connected to the column via 1/16″ Teflon tubing terminating in a Cheminert tubing fitting. The fitting was connected to the ball joint of the column through a Teflon adapter, held onto the column by a special clamp. The adapter was threaded internally at the top end with a ¼-28 tap to accomodate the Cheminert fitting. The lower end was machined out with a ball end mill to accommodate the column's ball joint. A small hole was drilled to connect the top and bottom ends of the adapter.

Fractions from the column were collected in 32-oz bottles held in a modified fraction collector (3). The tissue was homogenized in a Chemixer with a 6-liter flask (The VirTis Co., Gardiner, N. Y.).

Thin-layer chromatography was performed with silica gel plates, developed with chloroform–methanol–water 24:7:1. The spots were visualized with ninhydrin, iodine vapor, phospholipid spray (4), and charring spray (5).

Procedure

Brain was extracted by cutting 500 g of fresh or frozen pig brain into small pieces with a knife, then homogenizing it for 15 min with 500 ml of chloroform and 1500 ml of methanol. To the mixture was added 100 g of "analytical grade" Celite and 500 ml of chloroform, and the homogenizer was run at a lower speed for 15 min more. The suspension was then filtered through a 2-l sintered glass Buchner funnel, coarse porosity. Most of the mixture had to be filtered with only a partial vacuum in order to minimize evaporation of solvent. The homogenizing flask and funnel were then rinsed with 1000 ml of chloroform–methanol 95:5. The rinse also served to complete the transfer of the filtrate from the filter flask into the 2-gal bottle, which was used for the next step.

Iodinolysis was carried out by adding 10-ml portions of triiodide solution, mixing after each addition. Each successive portion was added as soon as the reddish color faded. When the color persisted up to 10 min, small portions of sodium bisulfite were promptly added to reduce the excess iodine and restore the original color. About 60 ml of triiodide solution and 200 mg of bisulfite were used. When spleen was the lipid source, the reddish color of the filtrate obscured the iodine color, but the latter was readily detectable when portions of the entire mixture were sucked up into a Pasteur pipet.

Next, water was removed by mixing the filtrate with 750 g of sodium sulfate for 1 hr in the same bottle. To this mixture was added a hot solution of 20 g of NaOH pellets in 130 ml of methanol; the bottle was capped and the contents were mixed for 1 hr. This procedure cleaves the phospholipid esters.

To neutralize the alkali, 28.6 ml of acetic acid was added, followed by 100 g of Celite. The mixture was shaken 10 min and filtered as above, aided by a rinse of 500 ml of chloroform–methanol 95:5. The filtrate volume was about 3800 ml, less than theoretical due to evaporative losses and retention in the filter cakes.

The inorganic materials and glyceride degradation products were removed by adding 2350 ml of 1% NaCl to the filtrate in a 2-gal bottle. The partition system was adjusted to approximate the system of Folch, Lees, and Sloane Stanley (6) by adding 1800

ml of chloroform, which brought the bottle contents to the top. After a brief mixing, the liquids were left to separate in the dark overnight. The above steps were readily carried out in one working day.

The next morning the upper layer was found to be rather cloudy but when it was removed by suction transfer to another bottle its cloudiness was seen to be greatly diminished.

The lower phase was washed by mixing with 500 ml of methanol, which yielded a clear solution, and then with 500 ml of 1% NaCl. This too was left overnight for separation, but fairly good separation was obtained in much less time. The upper layer was quite clear after suction transfer and the lower layer was fairly clear, with small amounts of waxy material near the interface.

The lower phase, together with the interface material and some of the upper phase, was then evaporated to a small volume in a 2000-ml flask with a rotary evaporator, using additions of benzene to help remove water. (Water removal is complete when a further addition to benzene produces a single-phase distillate.) Complete evaporation was avoided because of the great danger of splashing toward the end. About 200 ml of chloroform–methanol 2:1 was added to dissolve most of the residue, 125 g of silica gel was added, and the solvent was removed completely. To avoid spattering at this stage, only minimal heat and a partial vacuum were used at first. The drying was then completed with full vacuum and a 37°C bath.

The column was packed with a slurry of 210 g of silica gel in hexane, then the lipid–silica gel mixture was added as a slurry in chloroform–methanol 98:2. An ultrasonic bath proved helpful in transferring the lipid–powder slurry to the column. After the upper portion of the packing settled, the transfer was completed with 100-, 50-, and 50-ml portions of the same solvent. Now the nonpolar lipids were eluted by pumping 3350 ml of the same solvent at a flow rate of 18 ml/min (2.38 ml/min/cm^2). The cerebrosides were then eluted with 3350 ml of chloroform–methanol 90:10, collection being made in 600 ml fractions.

Examination of the fractions by spotting 0.02 ml portions on TLC plates showed that the cerebroside fractions contained only the expected spots. The cerebroside-containing fractions were pooled, evaporated to a small volume under vacuum while displacing the solvents with benzene, and finally lyophilized. The yield of galactocerebroside from pig brain was about 6.5 g (1.3% of the brain).

The fractions coming out of the column just before the cerebrosides showed no spots under these conditions, while the cerebroside spots were intense. Partial separation of the nonhydroxy and hydroxy cerebrosides was accomplished with the above column system. Examination of the total lipids from brain before application to the column showed only three lipids of major significance that were more polar than the cerebrosides: sulfatide, alkyl glycerophosphoethanolamine, and sphingomyelin. A minor unknown spot was also visible just below the sphingomyelin; it reacted very strongly with ninhydrin and phospholipid sprays, but was pale with the charring spray, indicating it was really a minor component.

When the above procedure was applied to human Gaucher spleen for the isolation of glucocerebroside, the polar lipids migrating on TLC plates were found to be rather minor compared to the cerebroside. The column was thus able to handle more cerebroside than in the case of brain. Because of the variability in the cerebroside content of Gaucher spleens, one should run a small test column before committing the entire extract in order to avoid overloading the column. The ratio of silica gel to tissue weight used in the procedure for brain (335:500) should be increased in the test run, and the total polar lipids eluted with chloroform–methanol 40:60 instead of 90:10. This test gives the weight of polar lipids and allows one to choose a suitable weight of silica gel for the remainder of the sample. Apparently one can use a load ratio of 1 g polar lipid to 30 or 35 g of silica gel, and an elution volume of 10 ml/g of adsorbent.

Glucocerebroside is somewhat more insoluble in chloroform–methanol than galactocerebroside. Because of this, the procedure of drying the crude lipids on part of the silica gel is not quite effective in preventing clogging of the column. It is therefore recommended that the spleen lipids be dried on *half* instead of 37% of the total silica gel used for the column. The yield of glucocerebroside from our latest Gaucher spleen was 5.1% of the tissue.

Gaucher spleen that has been stored in formalin should be ground and washed with water before being extracted.

Discussion

The purity of the cerebrosides isolated by this procedure was tested only by TLC. The product was white and was ninhydrin-negative and phosphate-negative when tested at a heavy load on TLC plates. Since there was little that was unconventional about our procedure, it may be presumed that the cerebroside purity is comparable to that of other preparations. Brain contains fatty acid esters of cerebroside, which enhance the yield of cerebroside after

methanolysis. Brain also contains 1-alkyl-2-acyl-3-galactosyl glycerol, and the lyso compound resulting from methanolysis is probably a trace contaminant in the final product.

The sulfatide and other polar lipids remaining on the column could be isolated by elution with more polar solvent mixtures. One could increase the yield of cerebroside by solvolytic cleavage of the C-O-S linkage in sulfatide (7).

Some of the steps in the procedure could be carried out reasonably readily with smaller equipment. The lyophilization step ought not to be omitted as attempts to evaporate cerebroside solutions under vacuum (especially glucocerebroside) almost invariably result in bad losses. A chromatographic pump is not essential for the column step, and compressed air can be used to drive the solvent. However bubble formation is a serious complication in this method, and one ought to use a lower flow rate and a wider column.

According to Siggia and Edsberg (1), alkyl vinyl ethers are converted to 2-iodo-1-alkoxy-1-methoxy-ethanes by the iodine reaction; that is, there is no cleavage of the ether linkage. Such a reaction with plasmalogens, followed by alkaline methanolysis, might be expected to yield a polar lipid. However our TLC data indicate that the plasmalogens were converted to a nonpolar lipid. It might be more accurate to call the reaction "iodinolysis" rather than "addition of iodine."

It should be noted that we obtained complete alkaline methanolysis of the ester lipids by the use of NaOH in the presence of hydrated sodium sulfate. While some procedures for alkaline methanolysis call for the use of sodium methoxide or specially dried methanol, there is evidently no need for the extra efforts at achieving dry conditions.

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