Quantitative isolation of total glycosphingolipids from animal cells

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SUMMARY The quantitative isolation of total glycosphingolipids from crude lipid extracts without contamination from other lipid classes is described. The method consists of (*a*) acetylation of total lipids with pyridine and acetic anhydride, (*b*) separation of acetylated glycolipids from nonglycolipids on a magnesia–silica gel (Florisil) column, and (*c*) deacetylation of glycolipid in chloroform–methanol–sodium methoxide. This method is useful for determination of microgram quantities of glycolipids derived from less than 1 ml of packed cells.

SUPPLEMENTARY KEY WORDS glycolipids · acetylation · acetic anhydride · pyridine · magnesia–silica gel (Florisil) · 1,2-dichloroethane (DCE) · acetone · deacetylation

A METHOD for quantitative isolation and determination of total glycosphingolipids is increasingly demanded since the physiological and pathological variations in both the quantity and the type of cellular glycolipids have been known to be significant. The partition method of Folch, Arsove, and Meath (1) or its modification (2) is the most convenient method for separation of higher gangliosides, and chromatography on Florisil columns (3) offers the most convenient method for separation of cerebroside and sulfatide from phospholipid. Skipski, Smolowe, and Barclay (4) have used chromatography on thin layers of a mixture of silica gel and magnesium silicate. Gray (5) has developed a two-dimensional chromatography system using chloroform–methanol–water 65:25:4 for one direction, and tetrahydrofuran–methylal–methanol–water 10:6:4:1 for the other direction. By either of these methods neutral glycolipids with shorter carbohydrate chains (ceramide mono- to tetrasaccharides) were separated from phospholipids, but gangliosides (sialosylglycolipids) and neutral glycolipids with longer carbohydrate chains (ceramide penta- to octasaccharides) (6–12) were impossible to separate from some phospholipids. Alkaline degradation of phospholipids followed by separation of glycolipids (13, 14) has often been used; however, sphingomyelin and plasmalogens are not degraded and are still present in the glycolipid fraction. The mercuric ion-catalyzed hydrolysis for removal of plasmalogen (15) cannot be used in the presence of sialosyl or fucosyl lipids.

Methods employing preparation of acetate derivatives of glycolipids followed by separation of the acetate from phospholipid have been used for preparation of blood-group glycolipids and other glycolipids having long carbohydrate chains (6–9). This note describes quantitative isolation of total glycosphingolipids as acetates without contamination from other lipid classes. The method is useful for determination of microgram quanti-

Abbreviations: DCE, 1,2-dichloroethane.

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ties of glycolipids derived from 10^7–10^8 cells as well as for mass preparation of glycolipids from 100 g of tissue.

Materials and Methods. Spleen, liver, and kidney from rats of the Buffalo strain, Morris hepatomas, and fibroblastic cells (BHK, C13/21) were used. All glycolipid samples were prepared in our laboratory; ceramide and phospholipids were purchased from Applied Science Laboratories Inc., State College, Pa.

(*a*) *Procedure 1.* The packed cells (0.2–0.5 ml) or a small piece of tissue (less than 1 g) were homogenized with 10 ml of chloroform–methanol 2:1 and centrifuged. The cell residue was again homogenized with chloroform–methanol 1:2 and centrifuged. The two extracts were combined and evaporated to dryness under a stream of nitrogen. The residue (crude lipid extract) was dried in vacuo over phosphorous pentoxide, and then dissolved in 0.3 ml of dried pyridine and 0.2 ml of acetic anhydride. The mixture was allowed to stand at room temperature for 18 hr. A large volume (about 50 ml) of dry toluene was added to the reaction mixture and the solvents were completely evaporated in a rotary evaporator. The dry residue was dissolved in hexane–DCE 1:4 (v/v) and put onto a column (0.7 × 10 cm, capacity 5 g) of magnesia–silica gel (Florisil, 50–100 mesh; Floridin Co., Tallahassee, Fla., distributed by Fisher Scientific Co.), which was prepared with the same solvent. The column was eluted successively with 15 ml each of the following solvents (Table 1): hexane–DCE 1:4, DCE alone, DCE–acetone 1:1 (or DCE–methanol 9:1), and DCE–methanol–water 2:8:1. All the acetylated glycolipids were eluted by DCE–acetone 1:1 or DCE–methanol 9:1, leaving all the phospholipids on the column. The solution of acetylated glycolipids was evaporated to dryness in vacuo. The residue was transferred to a small tube, dissolved in 100–200 μl of chloroform–methanol 2:1, and 25–50 μl of 0.5% sodium methoxide in methanol was added. After 30 min the reaction mixture was neutralized with ethyl acetate, evaporated to dryness, emulsified in water with slight warming, and dialyzed in ice water overnight. When emulsifying in water was difficult, an addition of the "theoretical upper phase" (2) was helpful. The dialysis was not necessary in the case of washed cultured fibroblastic cells. The dialyzed solution was lyophilized or evaporated under a stream of nitrogen and the residue was dissolved in a suitable quantity of chloroform–methanol. The resultant solution is ready for analysis of glycolipids by thin-layer chromatography, by an isotope-dilution method, or by conventional chemical methods.

(*b*) *Procedure 2.* The crude lipid extract of procedure 1 was dissolved in 6 ml of chloroform–methanol 2:1, and 1 ml of water was added; the mixture was then shaken and centrifuged. The lower phase was extracted eight times with the theoretical upper phase (chloroform–methanol–0.1% NaCl 1:10:10) (10). The upper phases were combined, evaporated to a small volume after addition of water, dialyzed in ice water overnight, and lyophilized (Fr. 1). The lower phase was treated with $^1/_5$ volume of dry Sephadex G 25 and shaken for 10 min in order to eliminate water. The mixture was then filtered, and the Sephadex on the filter was washed with chloroform–methanol 2:1. The filtrate and washings were treated in the same way as in procedure 1. The glycolipid acetate was deacetylated and analyzed without dialysis. The analysis was made with or without combining Fr. 1.

TABLE 1 SEPARATION OF GLYCOSPHINGOLIPIDS

Solvent for Elution	Substances Eluted (Alcoholic Hydroxyl Group in Acetylated State)	Recovery
1,2-Dichloroethane(DCE)	cholesterol	not measured
DCE–acetone 1:1 fraction	ceramide, cerebroside, lactosylceramide, ceramide trihexoside, globoside, ceramide pentahexoside (Le^a-active), ceramide octasaccharide (Le^b-active), hematosides, monosialoganglioside	95–100%
	disialosylhematoside, trisialoganglioside	80–85%*
DCE–methanol–water 2:8:1 fraction	cardiolipin, lecithin, phosphatidylethanolamine, phosphatidylserine, sphingomyelin, phosphatidic acid	not measured

A mixture of 17 lipids as above was treated by procedure 1; glycolipids were about 30–50 μg for each substance, and phospholipids and cholesterol were about 100–200 μg for each substance.
* Increased up to 95% by procedure 2.

Results and Discussion. Procedure 1 is suitable for analysis of all kinds of neutral glycolipids, hematosides, and monosialosylgangliosides. Recovery of higher gangliosides containing sialosylsialosyl residues was not quantitative using procedure 1; however, recovery was quantitative using procedure 2.[1] No detectable structural change nor loss of any of the glycolipids has been observed as a result of acetylation and deacetylation under the conditions described. Loss of glycolipid during dialysis is negligible at an ice-cold temperature and at higher concentrations of glycolipid. The conditions for deacetylation have been studied using acetylated gangliosides and globoside. Methanolic ammonia, methanolic barium methoxide, methanolic sodium methoxide, aqueous or methanolic sodium hydroxide, and chloroform–methanolic sodium methoxide were compared. The last reagent, with the given conditions, gave the most satisfactory results. Methanolic sodium hydroxide and methanolic ammonia occasionally gave multiple

[1] Sulfatide was separated with 95–100% recovery by either procedure 1 or 2.

spots on thin-layer chromatograms. The method cannot be used for isolation of glycolipids containing base-sensitive linkage, e.g., a ganglioside having *N-O*-diacylneuraminic acid (16).

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