methods

Aminopropyl solid phase extraction and 2 D TLC of neutral glycosphingolipids and neutral lysoglycosphingolipids

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Abstract Methods for isolation of neutral lysoglycosphingolipids (n-lyso-GSLs) such as glucosylsphingosine and galactosylsphingosine normally involve mild alkaline or acid hydrolysis followed by multiple chromatography steps, yielding relatively low recoveries of n-lyso-GSLs and neutral glycosphingolipids (n-GSLs). We now describe a new technique for isolating these compounds using one chromatography step, resulting in quantitative recovery of n-GSLs and n-lyso-GSLs. Lipids are extracted using a modified Folch procedure in which recovery is optimized by reextracting the Folch upper phase with water-saturated butanol. The extract is applied to an aminopropyl solid phase column from which both n-GSLs and n-lyso-GSLs elute in the same fraction. Separation is achieved using a new two-dimensional thin-layer chromatography procedure. The usefulness of this technique for biological samples was tested by examining Glc[4,5-^{3}H]ceramide and Glc[4,5-^{3}H]sphingosine accumulation in metabolically-labeled neurons treated with an inhibitor of lysosomal glucocerebrosidase. Accurate quantification of both lipids was obtained with Glc[4,5-^{3}H]ceramide and Glc[4,5-^{3}H]sphingosine accumulating at levels of 20 nmol/mg DNA and 40 pmol/mg DNA, respectively. This simple and rapid technique can therefore be used for the analysis of lyso-GSLs and GSLs in the same tissue, which may permit the determination of their metabolic pathways in normal and in pathological tissues, such as those taken from Gaucher and Krabbe's disease patients.—Bodennec, J., D. Pelled, and A. H. Futerman. **Aminopropyl solid phase extraction and 2-D TLC of neutral glycosphingolipids and neutral lysoglycosphingolipids. *J. Lipid Res.* 2003.** 44: **218–226.**

Supplementary key words glucosylsphingosine • psychosine • Gaucher disease • Krabbe's disease • Niemann-Pick disease • two-dimensional thin-layer chromatography

Glycosphingolipids (GSLs) are important components of biological membranes where they play both structural and regulatory roles. Their lyso (i.e., *N*-deacylated) derivatives (lyso-GSLs) have also been implicated in various regulatory processes, although since they are found in small amounts in normal tissues, most attention has focused on attempting to understand their roles upon their accumulation in pathological tissues. For instance, the neutral lyso-GSLs (n-lyso-GSLs), glucosylsphingosine (GlcSph), and galactosylsphingosine (GalSph, psychosine), accumulate in tissues from Gaucher (1) and Krabbe's disease patients (2–5), respectively, and in animal models of these diseases (6–9). GalSph and GlcSph modulate various physiological processes, such as calcium release (10–12) and enzyme activities (13–16), and are also cytotoxic at relatively low concentrations (17–19). It has, however, been difficult to distinguish their functions in vivo from those of their parent neutral glycosphingolipids (n-GSLs) [i.e., galactosylceramide (GalCer) and glucosylceramide (GlcCer)]. Moreover, the biosynthetic pathways leading to n-lyso-GSL accumulation are still poorly understood (5, 20), probably in part due to the lack of simple methods that can be used for metabolically-labeling cultured cells in which n-lyso-GSLs are present in the sub-nmol range and n-GSLs in the sub-μmol range (9, 19, 21–24). Moreover, most isolation and analytical procedures for GSLs involve at least one chemical step, such as mild alkaline or acid hydrolysis, which can inadvertently hydrolyze (25) the parent compounds, yielding artifactually high levels of lyso-GSLs.

The most common method of isolating n-lyso-GSLs is by strong cation exchange chromatography, followed by desalting on a C18 reverse phase column (25), a method recently adapted to commercially available solid phase extraction cartridges (22). These purification methods often

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Abbreviations: CBE, conduritol-B-epoxide; CDH, ceramide dihexoside; CTH, ceramide trihexoside; GalCer, galactosylceramide; GlcCer, glucosylceramide; GlcSph, glucosylsphingosine; GalSph, galactosylsphingosine; GSL, glycosphingolipid; HPLC, high performance liquid chromatography; LacSph, lactosylsphingosine; n-GSL, neutral glycosphingolipid; n-lyso-GSL, neutral lysoglycosphingolipid; SM, sphingomyelin; SPC, sphingosylphosphorylcholine; 2D-TLC, two-dimensional thin-layer chromatography.

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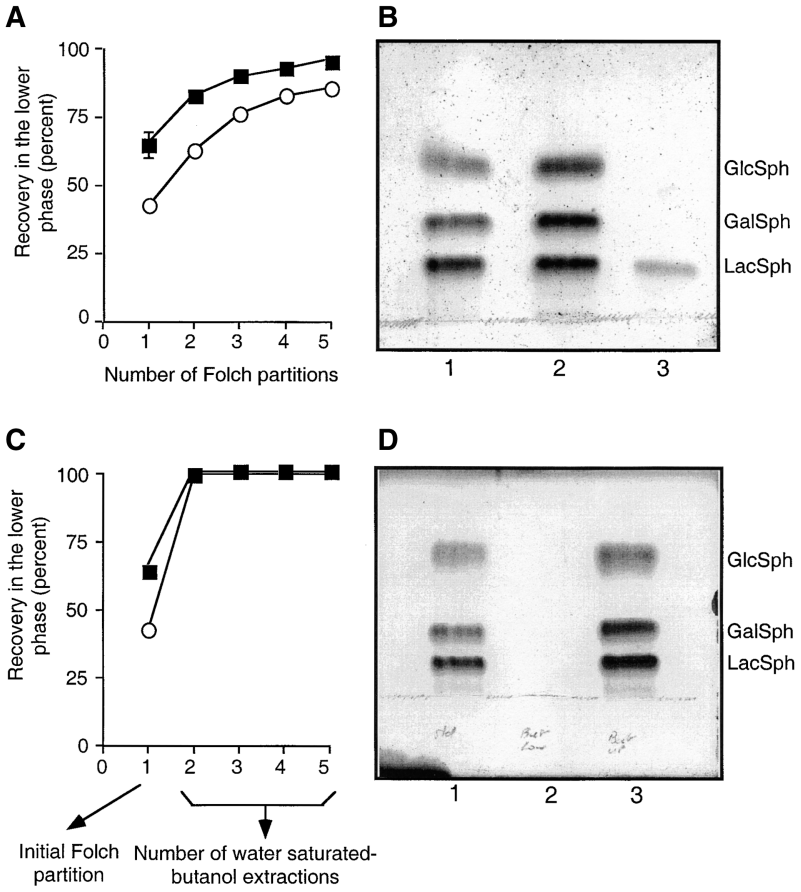

Fig. 1. Recovery of galactosylsphingosine (GalSph) and lactosylsphingosine (LacSph) by liquid extraction. A: GalSph (squares) and LacSph (circles) recovery in the chloroform lower phase after multiple Folch partitions of the aqueous upper phase was compared with (C) extraction of the Folch upper phase by water saturated-butanol. The data are means ± SD for five different experiments. Representative TLC plates of the partitioned lipids are shown in B and D. Lipids were separated on a borated TLC plate using the following developing solvents: chloroform-acetone-methanol-acetic acid-water (50:20:10:15:5, v/v/v/v/v) run on two thirds the length of the plate, followed by chloroform-methanol-deionized water-ammonia (25% v/v) (20:20:2:0.35, v/v/v/v) run on the whole length of the plate. Lipids were visualized using the orcinol reagent. B: authentic standards are shown in lane 1, the lipids recovered in the lower phase after four extractions of the Folch upper phase in lane 2, and the LacSph lost to the upper phase in lane 3. D: Lane 1 shows lipid standards, lane 3 shows the quantitative recovery of the lipids in the pooled butanol-extracted upper phase combined with the initial chloroform phase, and lane 2 the absence of any lyso-glycosphingolipids (GSLs) in the aqueous phase after butanol extraction.

result in low (~45–60%) recovery of n-lyso-GSLs (8, 22, 25). Moreover, chemical treatments such as alkaline methanolysis interfere with the quantitative recovery of n-lyso-GSLs and can indirectly affect their quantification by high-pressure performance liquid chromatography (HPLC), a problem neglected in the past (26, 27). An additional problem is caused by alkali- and acid-labile cerebroside esters (28–34) and plasmalocerebrosides (35, 36), which are major brain constituents (37, 38). Finally, an alkali-labile (39) 3-*O*-acetyl-sphingosine series of myelin n-GSLs has been characterized (39), again raising the possibility of artifactual formation of n-lyso-GSLs when mild alkaline hydrolysis is performed.

In the current study, we describe a simple procedure allowing the simultaneous isolation of n-GSLs and n-lyso-GSLs by solid phase extraction using commercially available aminopropyl cartridges, without the need for prior chemical treatment. We also describe a new two-dimensional thin layer chromatography (2D-TLC) procedure to separate n-lyso-GSLs from n-GSLs, and apply this method to quantify levels of Glc[4,5-^{3}H]Sph and Glc[4,5-^{3}H]Cer in a neuronal model of Gaucher disease.

MATERIALS AND METHODS

Materials

Galactose oxidase (from *Dactylium dendroides*, 220 U/mg), 1-β-D-GlcSph, 1-β-D-GalSph, sphingomyelin (SM), sphingosylphosphorylcholine (SPC), glucosylceramide (GlcCer), galactosylceramide (GalCer), and calf thymus DNA were from Sigma. D-lactosyl-β1-1′-D-*erythro*-sphingosine (LacSph) and lactosylceramide (LacCer) were from Avanti Polar Lipids (Alabaster, AL). Conduritol-B-epoxide (CBE) and a neutral GSL mixture [containing ceramide monohexosides, ceramide dihexosides (CDH), ceramide trihexosides (CTH), and globosides] were from Matreya (Pleasant Gap, PA). Aminopropyl solid phase extraction cartridges (LC-NH2, 100 mg) were from Supelco (Bellefonte, PA). Silica gel 60 TLC plates and sodium tetraborate were from Merck (Darmstadt, Germany). Bisbenzimide dye (Hoescht 33342) was from Molecular Probes (Eugene, OR). L-3-[^{3}H]serine (specific activity of 26 Ci/mmol) and NaB[^{3}]H$_4$ (specific activity of 11 or 25 Ci/mmol) were from Amersham (Little Chalfont, UK). All solvents were of analytical grade and were purchased from Biolab (Jerusalem, Israel).

Radioactive lipids

[4,5-^{3}H]sphinganine (specific activity of 11 Ci/mmol) was synthesized by reduction of D-*erythro*-sphingosine with NaB[^{3}]H$_4$ (11Ci/mmol) (40,41). D-[6-^{3}H]GalSph and D-[6-^{3}H]LacSph were synthesized by oxidation of GalCer and LacCer by galactose oxidase, reduction of the resulting aldehyde by NaB[^{3}]H$_4$ (25 Ci/mmol) (42), alkaline hydrolysis [100°C, 3 h in butanol-10 N KOH (9:1, v/v)] (43), and purification by Unisil column chromatography and TLC (44) to give a final specific activity of 15 Ci/mmol.

Liquid extraction

D-[6-^{3}H]GalSph and D-[6-^{3}H]LacSph (each 10,000 dpm) were added to a glass tube together with 15 μg each of GlcSph, Gal-

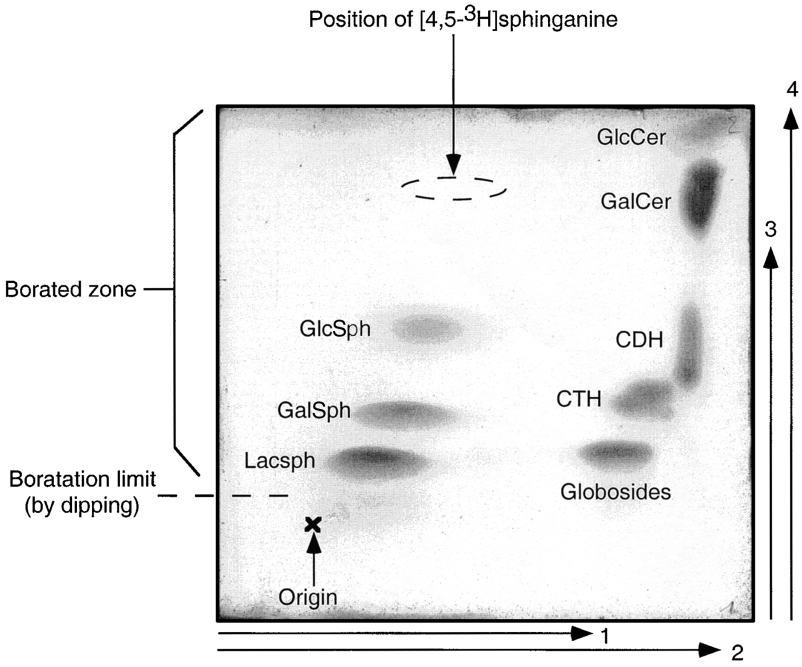

Fig. 2. 2D-TLC of neutral glycosphingolipids (n-GSLs) and their lyso-derivatives. An example of the 2D-TLC separation of GSLs and their lyso-derivatives is shown. Arrow 1 shows the first developing solvent used [tetrahydrofuran-acetone methanol-deionized water (25:10:20:3, v/v/v/v)], which was run two thirds the length of the TLC plate. The same solvent system was used again but run until it was 0.5 cm from the front of the plate (arrow 2). The dashed line indicates the extent to which the TLC plate was dipped in the methanol/1% sodium tetraborate, and the rest of the plate (nonborated zone) was sprayed with borate. Arrow 3 indicates the direction of separation and extent of migration of the third solvent system [chloroform-acetone-methanol-acetic acid-deionized water (50:20:10:15:5, v/v/v/v/v)], and arrow 4 the final solvent system [chloroform-methanol-deionized water-25% ammonia (20:20:2:0.35, v/v/v/v)], which was allowed to migrate the full extent of the TLC plate. GSLs were visualized by orcinol. The position of migration of [4,5-^{3}H]sphinganine, which can leak to some extent into fraction 3 of the aminopropyl column (46), is indicated.

Sph, and LacSph, dried under N_2, re-suspended in 4 ml of chloroform-methanol (2:1, v/v) (45), and phase separation was achieved by addition of 1 ml of deionized water. The aqueous and chloroform phases were separately dried under N_2 and spotted on a borate-impregnated TLC plate. Lipids were separated using chloroform-acetone-methanol-acetic acid-water (50:20:10:15:5, v/v/v/v/v) as the first developing solvent, run on two thirds the length of the TLC plate, and after air-drying, chloroform-methanol-water-ammonia (25% v/v) (20:20:2:0.35, v/v/v/v) was used as the second developing solvent, run the whole length of the TLC plate in the same direction as the first solvent. A good resolution between GalSph and GlcSph was obtained by running the plate in the first solvent system, while the greater polarity of the second solvent system gave a good separation from LacSph. Lipids were visualized with iodine, the n-lyso-GSLs recovered by scraping directly into liquid scintillation vials, to which 1 ml of methanol and 5 ml of Optima gold scintillation cocktail (Packard, Downers Groove, IL) were added, and radioactivity determined in a Packard 2100 β radiospectrometer. The percent recovery of D-[6-^{3}H]GalSph and D-[6-^{3}H]LacSph in the chloroform phase was determined.

Since n-lyso-GSLs were not quantitatively recovered in the chloroform phase, the procedure was modified. First, the aqueous upper phase was re-extracted several times (**Fig. 1**) by addition of theoretical lower phase [chloroform-methanol-water (86:14:1, v/v/v)]. The lower phases were pooled and dried under N_2, as was the upper phase, and lipids were separated, identified, and quantified as above. Secondly, the upper phase was dried under N_2, resuspended in 2 ml of deionized water to which 2 ml of water-saturated butanol (prepared by adding one volume of butanol to one volume of deionized water, the mixture stirred for 30 min at room temperature, followed by phase separation) was added. After vortexing and phase separation, the butanol upper phase was pooled with the chloroform lower phase from the initial partitioning step. The effect of varying the number of water-saturated butanol extraction steps on the recovery of individual n-lyso-GSLs was determined (Fig. 1C), as was the efficiency of the water-saturated butanol extraction using varying amounts of n-lyso-GSLs by adding increasing amounts of GalSph and LacSph to glass tubes containing 10,000 dpm each of D-[6-^{3}H]GalSph and D-[6-^{3}H]LacSph.

Solid phase extraction

The solid phase extraction procedure used herein is modified from a previous study (46) in which n-lyso-GSL isolation was not attempted. Briefly, lipids [either extracted from hippocampal neurons (see below), or standards] dissolved in 200 μl of chloroform-methanol (23:1, v/v) were loaded onto a 100 mg aminopropyl LC-NH2 cartridge that had been pre-equilibrated with 2 ml of hexane, and placed onto a vacuum manifold apparatus [12 port Visiprep (Supelco)]. Neutral lipids (cholesterol, cholesterol esters, triglycerides, diglycerides, monoglycerides, ceramides, fatty alcohols, and methyl fatty acids) (46) were eluted (fraction 1) with 1.6 ml chloroform-methanol (23:1, v/v) at a solvent flow rate of 0.3 ml/min (obtained by applying negative pressure). Free fatty acids were eluted (fraction 2) with 1.8 ml diisopropyl ether-acetic acid (98:5, v/v). GSLs were eluted (fraction 3) using 2.1 ml of acetone-methanol (9:1.35, v/v). Under these conditions, fraction 3 is devoid of other neutral lipids, phospholipids, sulfatides, sphingosine, ceramide-1-phosphate, and gangliosides (46). Neutral phospholipids and SPC were eluted (fraction 4) using 2 ml of chloroform-methanol (2:1, v/v), and acidic phospholipids eluted (fraction 5) using 2 ml chloroform-methanol-3.6 M aqueous ammonium acetate (30:60:8, v/v/v). The fractions were dried under N_2 and resolved by TLC as described above. When SM and SPC where applied on the column, the eluted fractions were separated by TLC using chloroform-methanol-25% ammonia (65;35:7.5, v/v/v) as the developing solvent, and detected after spraying with cupric sulfate and charred at 180°C for 5 min.

The efficiency of the solid phase extraction procedure was determined using D-[6-^{3}H]GalSph and D-[6-^{3}H]LacSph (10,000 dpm each) together with varying amounts of GlcSph, GalSph, and LacSph. The radioactivity remaining on the silica-based matrix after elution was also determined. The extent of cross-contamination of D-[6-^{3}H]GalSph and D-[6-^{3}H]LacSph in the other fractions was calculated after resolving the different fractions eluted from the column by TLC and the radioactivity in each individual n-lyso-GSL

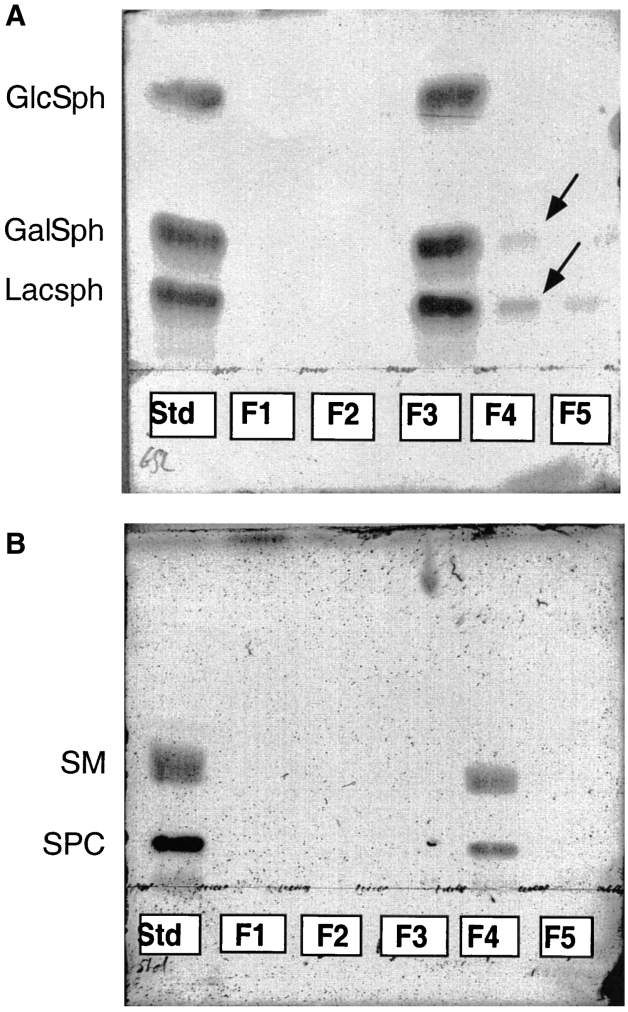

Fig. 3. Fractionation of neutral lyso-glycosphingolipids (n-lyso-GSLs) by aminopropyl solid phase extraction. A: Fifteen micrograms each of LacSph, GalSph, and glucosylsphingosine (GlcSph) were separated on a pre-equilibrated aminopropyl cartridge, eluted, and separated on a borated TLC plate with the developing solvents used in Fig. 1B and 1D, and lipids visualized using the orcinol reagent. The arrows in lane 4 show the small amounts of LacSph and GalSph that are lost to fraction 4 (F4); note that GlcSph was never observed in this fraction. B. Ten micrograms each of sphingomyelin (SM) and sphingosylphosphorylcholine (SPC) were separated on a pre-equilibrated aminopropyl cartridge, eluted, and fractions 1 to 5 (F1 to F5) separated by TLC using chloroform-methanol-25% ammonia (65:35:7.5, v/v/v) as the developing solvent. Lipids were visualized using cupric sulfate and charring the plate at 180°C for 5 min, and identified using authentic standards (Std).

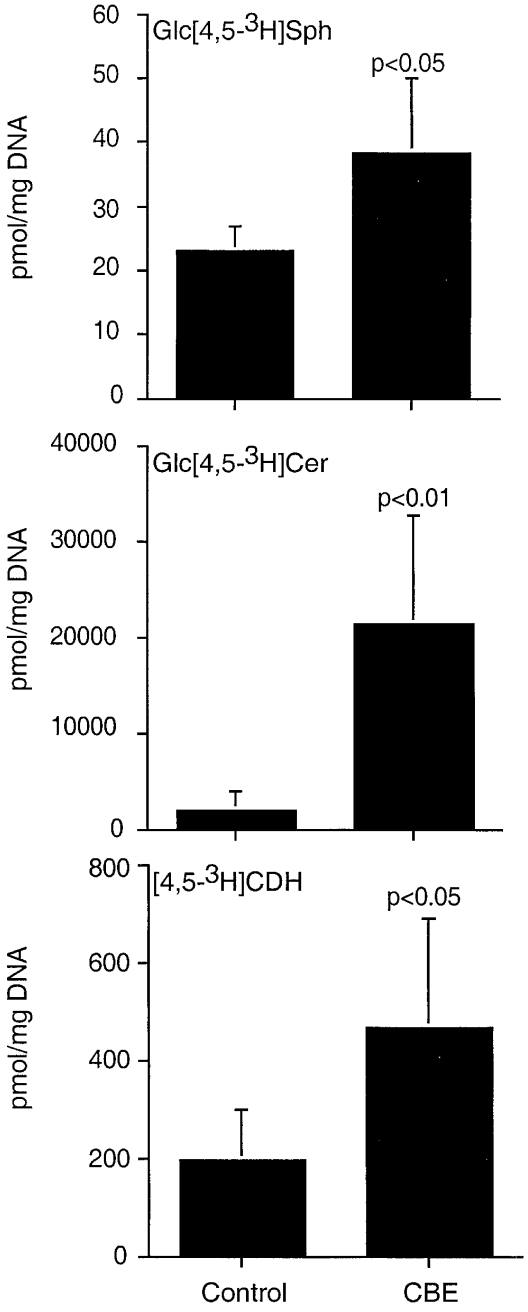

Fig. 4. Analysis of n-lyso-GSLs and n-GSLs in cultured hippocampal neurons. Hippocampal neurons were incubated with or without conduritol-B-epoxide (CBE), metabolically labeled with [4,5-^{3}H]sphinganine, the lipids extracted, fractionated on an aminopropyl column, fraction 3 resolved by 2D-TLC, and Glc[4,5-^{3}H]Sph, Glc[4,5-^{3}H]Cer, and [4,5-^{3}H]ceramide dihexoside (CDH) analyzed. Statistical differences (Student's *t*-test) between control and CBE-treated neurons are indicated. Results are means ± SD from four independent cultures.

spot for each fraction counted. The percentage of cross-contamination was calculated as: percentage contamination in fraction X = (dpm in fraction X/dpm in all the fractions) × 100.

2D-TLC

Fraction 3 from the aminopropyl column was re-suspended in chloroform-methanol (2:1, v/v) and spotted 2 cm from the edge of a 10 × 10 cm TLC plate (**Fig. 2**). The TLC plate was developed in the first direction using tetrahydrofuran-acetone-methanol-deionized water (25:10:20:3, v/v/v/v) run two thirds the length of the TLC plate, which was then air-dried and developed in the same direction with the same solvent system, but run until 0.5 cm from the edge of the plate. After air drying, the plate was briefly dipped (10 s) in a solution of 1% sodium tetraborate in methanol. After air drying, the area of the plate that was not dipped in borate was sprayed with borate, and after air-drying again, the TLC plate was developed in the second direction with chloroform-acetone-methanol-acetic acid-deionized water (50:20:10:15:5, v/v/v/v/v). Migration was stopped when the solvent front reached two thirds of the length of the plate. After air-

TABLE 1. Effect of mild alkaline hydrolysis on the distribution of ^{3}H-radioactivity within n-GSLs and n-lyso-GSLs

	Distribution of ^{3}H-Radioactivity	
Lyso-GSLs and GSLs	No Treatment	Mild Alkaline Hydrolysis
LacSph	2.2	2.0
GalSph	4.5	3.8
GlcSph	1.6	2.0
Globosides	6.7	7.3
CTH	14.9	11.9
CDH	11.5	13.9
GalCer	12.3	15.4
GlcCer	48.5	43.7

CDH, ceramide dihexoside; CTH, ceramide trihexoside; GalCer, galactosylceramide; GlcCer, glucosylceramide; GlcSpH, glucosylsphingosinel; GalSph, galactosylsphingosine; GSL, glycosphingolipid LacSph, lactosylsphingosine; n-GSL, neutral glycosphingolipid; n-lyso-GSL, neutral lyso-glycosphingolipid. After metabolically labeling rat hippocampal neurons with L-3-[^{3}H]serine, samples were either processed directly for the aminopropyl column, or first subjected to alkaline hydrolysis. n-GSLs and n-lyso-GSLs were resolved by two-dimensional thin-layer chromatography (2D-TLC), lipids detected using iodine, ^{3}H-radioactivity within the individual spots analyzed, and the distribution of ^{3}H-radioactivity between individual lyso-GSLs and GSL determined. Results are means of two independent experiments in which the variability between individual values was <20%

drying, the plate was developed in chloroform-methanol-deionized water-25% ammonia (20:20:2:0.35, v/v/v/v), which was allowed to run the full-length of the TLC plate. Lipids were detected using either the orcinol reagent or iodine.

Metabolic labeling with [4,5-^{3}H]sphinganine

Hippocampal neurons obtained from embryonic-day-18 rats (47) were plated on 24 mm glass coverslips (48) and cultured in Neurobasal serum-free medium containing B27 supplement (Life Technologies) and L-glutamine (49, 50). Neurons were incubated with 500 μM CBE immediately after plating. After 6 days, [4,5-^{3}H]sphinganine (0.15 μCi per ml of medium) was added to the cultures, and 24 h later neurons were washed in fresh Neurobasal medium and chased for a further 5 days in the absence of [4,5-^{3}H]sphinganine. CBE was present during the entire labeling and chase periods.

At the end of the chase, neurons were washed with ice-cold PBS and scraped with a rubber policeman into 3 ml of ice-cold methanol, and sonicated. An aliquot was removed for DNA quantification using bisbenzimide (51). ^{3}H-labeled GSLs were extracted by the addition of chloroform to the methanol extract to give a final ratio of 2:1 (v/v) (45), followed by addition of 2.25 ml deionized water. The aqueous and chloroform phases were dried under N_2, and the aqueous phase re-suspended in 3 ml of deionized water and 3 ml of water-saturated butanol. The butanol upper phase was pooled with the initial chloroform phase and dried under N_2. Lipids were re-suspended in 200 μl chloroform-methanol (23:1, v/v) and loaded onto an aminopropyl column, eluted as described above, and recovered. Fraction 3 containing n-GSLs and n-lyso-GSLs was separated by 2D-TLC. The extent of recovery of n-lyso-GSLs from the neuronal tissue was estimated by adding D-[6-^{3}H]GalSph and D-[6-^{3}H]LacSph to a neuronal methanol-extract that had not been metabolically labeled, and subsequently processed identically as for the samples labeled with [4,5-^{3}H]sphinganine.

The possible contamination of the n-GSLs isolated from biological samples with glycerolipids was analyzed by incubating 6-day-old hippocampal neurons with L-3-[^{3}H]serine (10 μCi per ml of medium). Lipids were extracted as above, loaded onto an aminopropyl cartridge, and fraction 3 collected. GSL standards were added to fraction 3, half of which was resolved by 2D-TLC without further treatment, and half of which was subjected to mild alkaline hydrolysis (46) by drying the samples under N_2, re-suspending in 2 ml chloroform-0.6 N NaOH in methanol (1:1, v/v) for 1 h at room temperature, and neutralizing with 10 N HCl. Deionized water (0.8 ml) was added to the mixture and the resulting upper and lower phases dried. The upper phase was extracted twice with water-saturated butanol and the butanol phases pooled with the initial chloroform lower phase. After 2D-TLC separation, lipids were detected with iodine, and radioactivity determined for each n-lyso-GSL and n-GSL. The distribution of ^{3}H-radioactivity within the individual lipids was compared between samples that had or had not been subjected to mild alkaline hydrolysis.

RESULTS

Preliminary experiments demonstrated that only 64% and 42% respectively of GalSph and LacSph were recovered in the chloroform phase of a Folch extract, with the remainder lost to the aqueous phase (Fig. 1A). Re-extraction of the aqueous phase with theoretical lower phase improved the recovery, but even after four reextractions, 15% of LacSph was lost (Fig. 1A, and Fig. 1B, lane 3), and this was not improved by using KCl, NaCl, or $CaCl_2$ instead of deionized water to achieve phase separation. In contrast, recovery was drastically improved by re-extracting the Folch upper phase with water-saturated butanol, with one extraction yielding quantitative recovery of both GalSph and LacSph (Fig. 1C, D). The extraction efficiency using water-saturated butanol remained constant irrespective of the initial amount of GalSph and LacSph used, with 97–98% recovery of GalSph and 93–97% recovery of LacSph using starting amounts between 0.1 ng and 1 μg (not shown). Thus, this method is appropriate for n-lyso-GSL extraction in the mass range in which they are found in cultured cells (23, 24).

When applied to aminopropyl solid phase extraction cartridges, n-lyso-GSLs were recovered in one fraction, namely fraction 3 (**Fig. 3A**), the same fraction as that in which the parent n-GSLs are eluted (46), with only a small amount of GalSph and LacSph lost to fraction 4. The n-lyso-GSLs were well resolved from SPC and SM, which elute in fraction 4 (Fig. 3B), in which the other neutral phospholipids also elute (46). Free long-chain bases, such as sphingosine, dihydrosphingosine and phytosphingosine, are recovered in fraction 2 (46) with a small amount of the free long chain bases found in fraction 3. However, the long chain bases did not interfere with the subsequent analysis of n-lyso-GSLs since they were well-separated by 2D-TLC (Fig. 2). The extent of recovery of n-lyso-GSLs in fraction 3 was essentially constant irrespective of the amount added to the column, with >90% recovered when applying between 10 ng to 50 μg of either GalSph or LacSph and <5% remaining on the cartridge. Precise quantification of GlcSph recovery could not be obtained due to the lack of radioactive GlcSph, but since the recovery of GlcSph in fraction 3 was always complete (Fig. 3A), we assume that GlcSph recovery is at least similar to that ob-

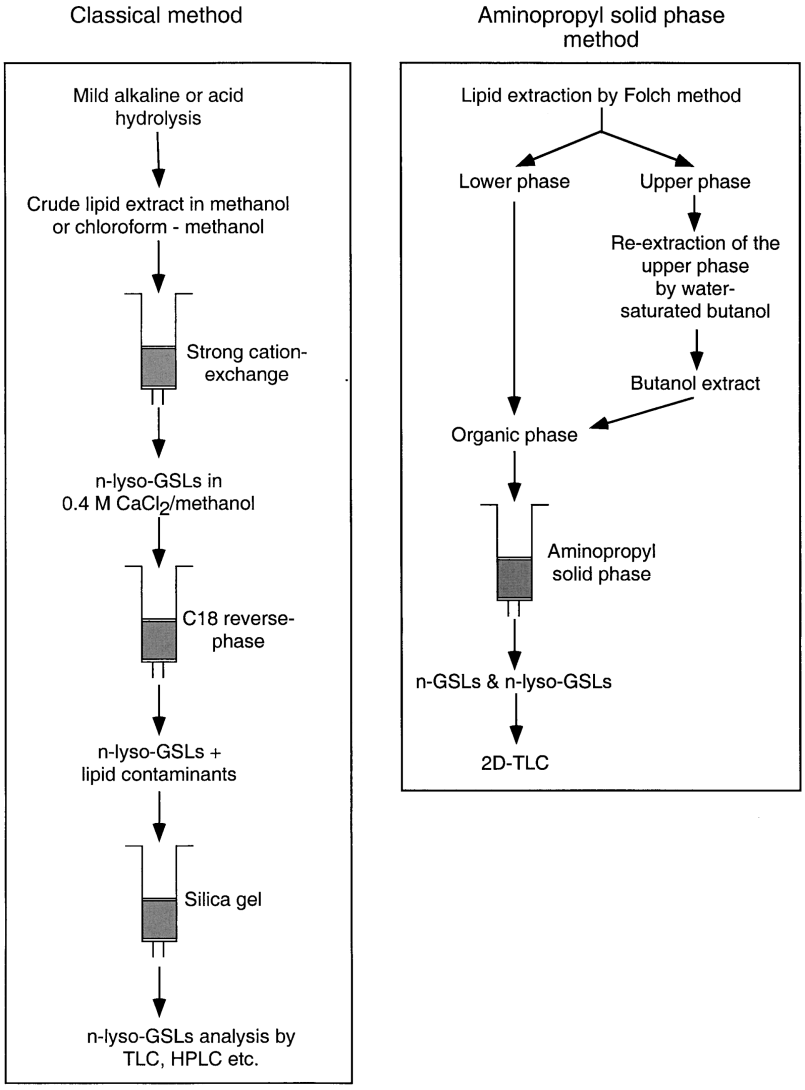

Fig. 5. Schematic diagram of methods used for n-GSL and n-lyso-GSL isolation and analysis. See text for further details.

tained for GalSph and LacSph. Together, these results show that solid phase extraction of n-lyso-GSLs is a highly efficient process for their quantitative recovery in the same fraction as the parent n-GSLs.

To determine the usefulness of this method for n-lyso-GSL analysis in biological samples, rat hippocampal neurons were metabolically labeled with [4,5-^{3}H]sphinganine and incubated with CBE (52), an active-site directed inhibitor of glucocerebrosidase, the enzyme defective in Gaucher disease. Glc[4,5-^{3}H]Cer was ~500-fold higher than Glc[4,5-^{3}H]Sph after CBE-treatment, which resulted in an 11-fold increase in Glc[4,5-^{3}H]Cer levels, similar to that previously reported (47,53), a smaller but nevertheless significant increase (1.7-fold) in Glc[4,5-^{3}H]Sph and [4,5-^{3}H]CDH (**Fig. 4**), and no changes in other [4,5-^{3}H]GSLs compared with untreated controls (not shown). The extent of recovery, estimated by addition of D-[6-^{3}H]GalSph and D-[6-^{3}H]LacSph to neurons that were not metabolically labeled with [4,5-^{3}H]sphinganine, was 85–90%. These results demonstrate that the method described in this study can be applied to biological samples to quantify n-GSLs and n-lyso-GSLs.

Finally, we examined the possible contamination of n-lyso-GSLs isolated from biological samples with glycerolipids by incubating hippocampal neurons with L-3-[^{3}H]serine and examining the distribution of ^{3}H-radioactivity within n-lyso-GSLs and n-GSLs before and after alkaline hydrolysis. Since no change in the distribution of ^{3}H-radioactivity was detected after alkaline hydrolysis (**Table 1**), we conclude that no contamination of individual n-lyso-GSLs by glycerolipids occurs during the solid phase extraction and the 2D-TLC procedure.

DISCUSSION

In the current study, we describe a simple and rapid technique for the isolation and characterization of n-GSLs and n-lyso-GSLs. Compared with other methods (22, 25) that normally use multiple separation and analytical steps, the current method only uses one chromatography step (**Fig. 5**), which results in n-GSL and n-lyso-GSL recovery in one fraction without significant contamination, followed by 2D-TLC for their characterization and quantification (Fig. 2).

The simplicity, rapidity, and the high yield of n-GSL and n-lyso-GSL recovery renders this method preferable to those previously described (Fig. 5), particularly for metabolic labeling studies. Among the advantages of the current method is high n-lyso-GSL recovery due to use of an optimized liquid extraction procedure using water-saturated butanol, which prevents loss of n-GSLs and n-lyso-GSLs to the upper phase. A similar batch extraction approach was successfully used previously to recover sphingosine-1-phosphate (54) from the upper phase of the Bligh and Dyer procedure (55). Another advantage is that glycerolipid degradation by mild alkaline or acid hydrolysis is not required prior to lipid extraction, since complete separation of glycerolipids from GSLs is achieved with the aminopropyl column. Thus, the possible artifactual formation of n-lyso-GSLs from compounds with labile ester or ether bonds is prevented. Moreover, simple extraction procedures, such as the Folch procedure, can be used for the quantitative recovery of other lipids of interest, such as neutral lipids and phospholipids. In previous methods, application of a crude lipid extract in methanol or chloroform/methanol to the initial chromatography column resulted in both the loss of other lipids and their possible interference with subsequent elution steps from the columns.

A further advantage of the method described herein is the use of only one column for n-GSL and n-lyso-GSL separation. This is in contrast to other published methods that use a combination of cation-exchange and reverse phase and silica gel chromatography (Fig. 5) (22), affecting the quantitative recovery of both n-GSLs and n-lyso-GSLs, and resulting in the loss of other lipid classes. Using an aminopropyl solid phase column, all cellular lipids are recovered, and can be stored for further analysis; aminopropyl columns are preferable to silicic acid columns as they are slightly less polar (56), resulting in recovery of n-lyso-GSLs and n-GSLs in the same fraction. Use of different aminopropyl solid phase column sizes, from between 100–500 mg, renders the method useful for separation of different amounts of cellular lipids, ranging between 4–20 mg (46). Of the most importance, n-GSLs and n-lyso-GSLs are recovered in one fraction without significant contamination of other major cellular lipids, although some minor lipids, such as dihydrosphingosine (46), may co-elute with them. However, dihydrosphingosine is well separated from n-GSLs and n-lyso-GSLs using a 2D-TLC system (Fig. 2) in which the first developing solvent was run to two thirds of the length of the TLC plate, which also resulted in optimal separation of GlcSph and GalSph. Glycerolglycolipids, such as galactosylglycerolipid, may also co-elute in fraction 3 of the aminopropyl column, and since these lipids occur at low levels in bacteria and plants (57) but also in some mammalian tissues such as testis and myelin (58, 59), appropriate caution must be taken to assess n-lyso-GSL and n-GSL purity after TLC analysis. One way to avoid potential contamination with glyceroglycolipids is to subject fraction 3 to mild alkaline hydrolysis, although this itself must be performed with caution to avoid artifactual formation of n-lyso-GSLs.

The last advantage of our method is the use of a novel 2D-TLC procedure to completely separate n-GSLs and n-lyso-GSLs from each other and from dihydrosphingosine, and to separate GalSph from GlcSph. The latter has proven difficult by HPLC after derivatization of the free amino group with o-phthalaldehyde (60), although some success has been reported by derivatization with 4-fluoro-7-nitrobenzofurazan (7, 61). To date, no procedure has been described for the separation of these compounds on the same TLC plate, and the solid phase extraction procedure could also be used as a first step to obtain pure n-GSLs and n-lyso-GSLs from biological tissues prior to their subsequent quantification by other analytical methods. The high recovery of n-lyso-GSLs and n-GSLs, and their purification within the same fraction, together with their separation on the same TLC plate, renders this method particularly suitable for metabolic studies. Since this method can also be applied to small amounts of metabolically-labeled tissues, and since the metabolic products and/or precursors (such as ceramide and free sphingoid bases) are recovered in separate fractions (46, 62), we anticipate that it could be used to resolve the metabolic pathways by which n-lyso-GSLs are formed. Moreover, since a similar method has also been applied to recover GSLs from various tissue sources, such as melanoma tumors (46), fish gills (63), and rat mitochondria (64), we also anticipate that it will be useful to determine their levels of accumulation in pathological tissues obtained from patients with lysosomal storage diseases, such a Gaucher, Krabbe's, and Niemann-Pick disease.

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