methods

A procedure for fractionation of sphingolipid classes by solid-phase extraction on aminopropyl cartridges

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Abstract Solid-phase extraction (SPE) methods are easy, rapid, and reliable. Their growing popularity is in part due to their operational simplicity and cost reduction in solvents, and partly because they are easier to automate. Sphingolipids are implicated in various cellular events such as growth, differentiation, and apoptosis. However, their separation by small SPE cartridges has attracted limited attention. Here we describe an SPE procedure on aminopropyl cartridges that by sequential elution allows the separation of a lipid mixture into free ceramides, neutral glycosphingolipids, neutral phospholipids (sphingomyelin), and a fraction containing the acidic phospholipids and phosphorylated sphingoid bases, phosphoceramides and sulfatides. Individual components are obtained in high yield and purity. We applied the procedure to obtain data on separation of [^{3}H]myristic acid-labeled sphingolipids from fish gills, and from human melanoma tumor tissue. Individual lipids in the SPE fractions were identified by chromatography on several high-performance thin-layer chromatography (HPTLC) systems. The chromatographic behavior of free sphingoid bases is also reported.—Bodennec, J., O. Koul, I. Aguado, G. Brichon, G. Zwingelstein, and J. Portoukalian. **A procedure for fractionation of sphingolipid classes by solid-phase extraction on aminopropyl cartridges. *J. Lipid Res.* 2000.** 41: **1524–1531.**

Supplementary key words ceramides • sphingosine • dihydrosphingosine • phytosphingosine • neutral glycosphingolipids • sphingomyelin • ceramide 1-phosphate • sphingosine 1-phosphate • sulfatides • solid-phase extraction

Sphingolipids, ceramides, and sphingoid bases are involved in numerous cellular events such as differentiation, growth, and apoptosis (1–7). Studies with these compounds usually require tedious methods that often involve preparative thin-layer chromatography or liquid–solid cartridge chromatography or both. Solid-phase extraction (SPE) is a technique that has elicited growing interest because it is rapid and reliable, requires less solvent, and can be easily automated (8). This technique has been used for extraction of lipid classes such as gangliosides (9), prostaglandins, and related eicosanoids (10) from biological samples. SPE with silica gel and aminopropyl-bonded matrix has been used in the fractionation of complex lipid mixtures, specifically for purification of neutral lipids (reviewed in refs. 11–13) and for recovery of some phospholipid classes (14, 15). Despite a growing interest in the study of sphingolipid metabolism, however, no detailed SPE procedure is available to sequentially separate free ceramides, neutral glycosphingolipids, and sphingophospholipids in a given sample. The sequential separation of different sphingolipids in the same sample is particularly important because their intracellular concentrations are interdependent, and they share structural, metabolic, and regulatory relationships (6, 16–18). We describe a procedure that allows simultaneous separation of these sphingolipids in a sample without any loss of resolution or recovery. Other molecules of interest in the lipid mixture, neutral lipids and phospholipids, are also recovered. The efficacy and the degree of separation were determined in two ways: *a*) by monitoring radioactivity in lipids labeled with [^{3}H]myristate and *b*) by visualization of lipid spots on high-performance thin-layer chromatography (HPTLC) plates. The solid-phase extraction procedure was applied to separate lipids extracted from fish gills, and to purify free ceramides, neutral glycosphingolipids, and sphingomyelin from human melanoma tumors.

Abbreviations: CDH, ceramide dihexoside; CE, cholesteryl esters; CerIII, ceramide type III; CerIV, ceramide type IV; CerP, ceramide containing phytosphingosine and normal fatty acids; CMH, ceramide monohexoside; CTH, ceramide trihexoside; C-1-P, ceramide 1-phosphate; DG, diglycerides; DHS, dihydrosphingosine; DPG, diphosphatidylglycerols; FA, fatty alcohol; FFA, free fatty acids; globo, globosides; LAV, lower acceptable volume; LPC, lysophosphatidylcholine; mFFA, methylated FFA; MG, monoglycerides; NL, neutral lipids; OH-FFA, α-hydroxy FFA; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PHS, phytosphingosine; PI, phosphatidylinositol; PS, phosphatidylserine; S-1-P, sphingosine 1-phosphate; Sph, sphingosine; SPE, solid-phase extraction; SPH, sphingomyelin; Sulf, cerebroside sulfate; TG, triglycerides; UAV, upper acceptable volume.

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MATERIALS AND METHODS

Materials

All solvents were of analytical grade and were purchased from SdS (Peypin, France) or Carlo Erba (Milan, Italy). Lipid standards (cholesterol, cholesteryl oleate, methyl oleate, oleic acid, α-hydroxy palmitic acid, monoolein, diolein, triolein, oleyl alcohol, ceramides type III with sphingosine and normal fatty acids, ceramides type IV containing sphingosine and α-hydroxy fatty acids), sphingoid bases (sphingosine, phytosphingosine, DL-*erythro*-dihydrosphingosine), sphingosine 1-phosphate, ceramide 1-phosphate, cerebroside sulfate, and bovine serum albumin (fatty acid free) were from Sigma (Saint Quentin Fallavier, France). Ceramides containing phytosphingosine and normal fatty acid were from Coletica (Lyon, France). Mono-, di-, and trihexosylceramide and globoside standards were isolated from melanoma cells according to the procedure of Saïto and Hakomori (19) and purified by high-performance liquid chromatography (HPLC) (20). Cadmium acetate and HPTLC plates (precoated silica gel 60 without fluorescence indicator) were from E. Merck (Darmstadt, Germany). Aminopropyl-bonded (LC-NH_2) silica gel cartridges (100- and 500-mg matrix) were from Supelco (Bellefonte, PA) or Macherey-Nagel (Hoerdt, France; Chromabond, 100 mg). Radiolabeled [9,10-^{3}H(N)]myristic acid (specific activity, 24 Ci/mmol) was purchased from Isotopchim (Ganagobie-Peyruis, France), and [4-^{14}C]cholesterol (specific activity, 58.4 mCi/mmol) was from Amersham (Les Ulis, France). Myristic acid was conjugated in our laboratory to bovine serum albumin according to published procedures (21).

Labeling and lipid extraction from fish gills

Lipids from fish gills labeled with [^{3}H]myristic acid were used to obtain data on the lipid recovery in each fraction because previous experiments showed that a high proportion of free ceramide species containing phytosphingosine and α-hydroxy fatty acids was present (22). Moreover, these lipids were efficiently labeled with [^{3}H]myristic acid (23), making them a good choice with which obtain data on lipid recovery when compared with lipids from human melanoma tissues, in which phytosphingosine is present in trace amounts. The gills were obtained from Mediterranean sea bass *Dicentrarchus labrax* and labeled as previously described (23). Briefly, gills were cut at their base and washed in seawater. Gills were incubated for 2 h in vitro in 4 mL of filtered sea water containing 10 μCi of [^{3}H]myristic acid conjugated to bovine serum albumin (21). At the end of incubation, gills were rinsed with unlabeled seawater, and the lipids were extracted according to the procedure of Folch et al. (24). The lipid extracts were washed according to Chapelle et al. (25) but the pH was adjusted to between 5 and 6 in order to retain part of sphingosine 1-phosphate (S-1-P) in the lower phase. The lower phase was used for separation of lipids by the SPE procedure described herein, and the fractions monitored by different TLC methods.

SPE separation of total lipids

An aliquot of the radioactive lipid extract from fish gills was mixed with an unlabeled lipid standards mixture containing 20 μg of each of the following: triacylglycerol (TAG), 1,2- and 1,3-diolein, ceramide type III, ceramide type IV, ceramide with phytosphingosine linked to normal fatty acid, cholesterol, cholesteryl oleate, oleic acid, methyl oleate, oleyl alcohol, α-hydroxy palmitic acid, neutral glycosphingolipids (mixture of ceramide monohexoside [CMH], ceramide dihexoside [CDH], ceramide trihexoside [CTH], and globosides), sphingomyelin, phytosphingosine, sphinganine, and sphingosine. The combined mixture was dried down and resuspended in 200 μL of chloroform and loaded on a 100-mg LC-NH_2 cartridge preconditioned with 2 mL of hexane. Cartridges (500 mg) were preconditioned with 5 mL of hexane. The sample was allowed to adsorb to the matrix by percolation through the cartridge by gravity. The 100-mg cartridge was washed with solvent A consisting of 1.4 mL of ethyl acetate–hexane 15:85 (v/v) at a flow rate of 0.3 mL/min by applying negative pressure. The 500-mg cartridges were washed in 2 mL of the same solvent. This volume was shown to be the minimal (or lower) acceptable volume (LAV) to ensure good elution of lipids. However, this volume could be increased up to 5 mL, a maximal value before any overlapping phenomenon was observed with the lipid species normally eluted in the next fractions. These upper acceptable volumes (UAV) can be used and are particularly useful when applying high amounts of lipids on the 500-mg cartridges (up to 15 mg of material). Solvent system A (ethyl acetate–hexane 15:85 [v/v]) eluted neutral lipids in a single fraction 1, without eluting ceramides, monoglycerides (MG), and free fatty acids (normal and α-hydroxy fatty acids). The second wash was with 1.6 mL (3-mL LAV and 4-mL UAV for 500-mg cartridges) of chloroform–methanol 23:1 (v/v) (solvent B), which eluted free ceramides and the monoacylglycerols (fraction 2). A portion of free sphingosine and sphinganine was also eluted with this solvent. The third solvent mixture (solvent C) applied was made of 1.8 mL (2-mL LAV and 3-mL UAV for 500-mg cartridges) of diisopropyl ether–acetic acid 98:5 (v/v), which eluted normal and α-hydroxy free fatty acids and trace amounts of free sphingoid bases in fraction 3. The fourth wash was performed with 2 mL (4-mL LAV and 11-mL UAV for 500-mg cartridges) of acetone–methanol 9:1.35 (v/v) (solvent D), which eluted neutral glycosphingolipids and the remaining free sphingoid bases in fraction 4, but left their phosphorylated derivatives still bound to the cartridge. Neutral phospholipids (sphingomyelin) (fraction 5) were eluted from the LC-NH_2 cartridge with 2 mL (3-mL LAV and 4-mL UAV for 500-mg cartridges) of solvent sytem E, consisting of chloroform–methanol 2:1 (v/v). Finally, sphingosine 1-phosphate, ceramide 1-phosphate, and sulfatides were obtained by washing the cartridge with 2 mL (4 mL for 500-mg cartridges) of chloroform–methanol–3.6 M aqueous ammonium acetate 30:60:8 (v/v/v) (solvent F, fraction 6).

The fractions eluted from the aminopropyl cartridge were dried down under nitrogen and redissolved in 3 mL of hexane–isopropanol 3:2 (v/v). An aliquot was taken to determine the radioactivity in the fractions. The uneluted radioactivity in the LC-NH_2 cartridge was determined by removing the silica gel sorbent matrix from the cartridge, mixing it in a counting vial with 3 mL of ethanol–water 1:1 (v/v) and 8 mL of the scintillation cocktail (Packard, Downers Grove, IL) (26). The radioactivity was measured in a Packard Tricarb 460 β radiospectrometer as described previously (23). The retained radioactivity was expressed as a percentage of total radioactivity loaded onto the cartridge.

The procedure was established with 100-mg cartridges from Supelco or Macherey-Nagel, and the effluents were collected in tubes positioned in a Visiprep vacuum manifold apparatus (Supelco, Saint Quentin Fallavier, France) equipped with disposable Teflon solvent guides. After application of the sample on the preconditioned cartridge, the different solvent mixtures were sequentially applied to elute different lipid classes. Similar protocols were used for 500-mg cartridges with an adjustment of solvent volumes as detailed above.

Extraction of sphingolipids from melanoma tumors

The metastatic melanoma tumors were freshly isolated from human liver and immediately treated for lipid extraction according to Folch et al. (24). The total lower phase was evaporated to dryness and subjected to alkaline methanolysis to remove glycerolipids. For this purpose, the lower phase was dried and the residue was resuspended in 8 mL of chloroform–0.6 N NaOH in

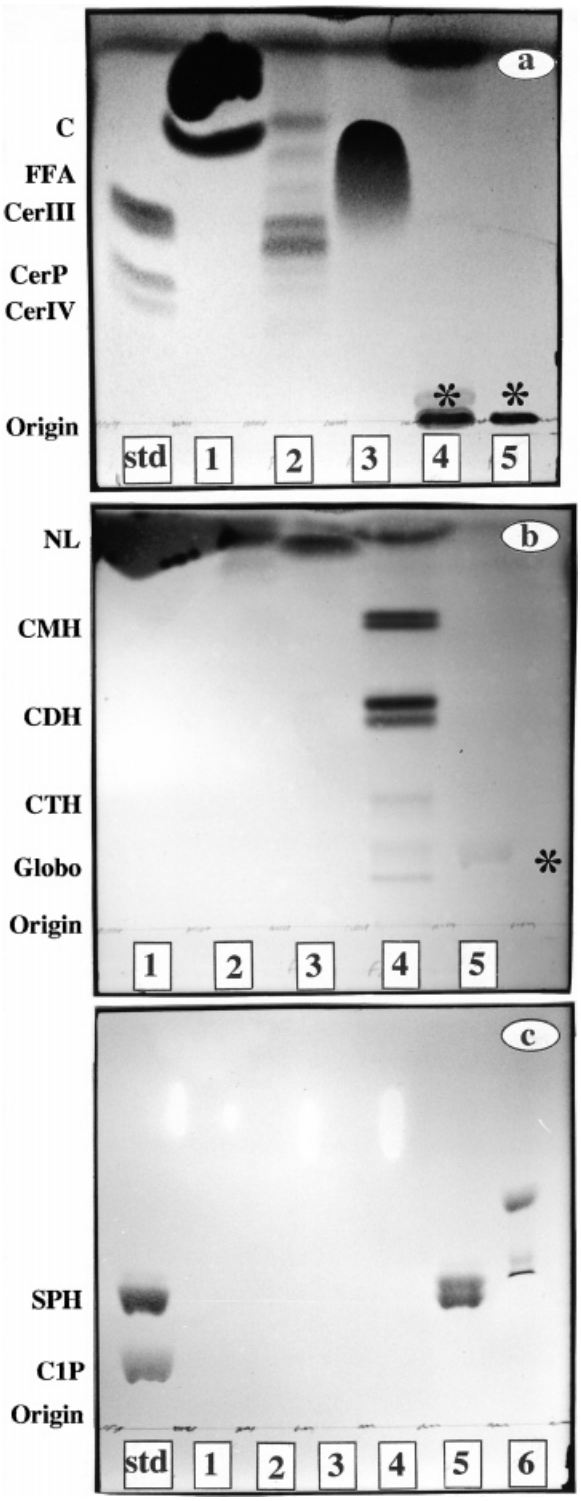

Fig. 1. HPTLC profile of sphingolipids from melanoma tumor tissue. The alkaline methanolysis-treated lower phase lipids from melanoma were loaded on an LC-NH_2 cartridge (500 mg). SPE fractions were obtained after successive elution (see procedure in text) of the cartridge, and spotted on HPTLC plates developed in various solvent systems to resolve different sphingolipid classes. Plate (a) was developed in chloroform–methanol 50:3.5 (v/v) to resolve free ceramide species, sprayed with Dittmer and Lester reagent to visualize phospholipids, and then heated at 180°C for 5 min to char. Asterisks (*) indicate Dittmer and Lester reagent-positive spots before heating the plate. Plate (b) was developed in chloroform–methanol–water 65:25:4 (v/v/v) for separation of neutral glycosphingolipids and visualized with orcinol reagent. The asterisk (*) indicates sphingomyelin (SPH), which is slightly revealed when heating the plate because of the presence of sulfuric acid in orcinol reagent. Plate (c) was developed in chloroform–methanol–20% ammonia 65:35:7.5 (v/v/v) and sprayed with Dittmer and Lester reagent. Different ceramide standards (std) were spotted alongside in (a) and SPH and C-1-P standards in (c) as indicated. Lane numbers 1 through 6 refer to fractions 1 through 6 eluted from the SPE cartridge.

methanol 1:1 (v/v) and kept at room temperature for 1 h. At the end of hydrolysis, 3.2 mL of distilled water was added and mixed thoroughly. The upper phase obtained after centrifugation was removed and the lower phase was washed with theoretical upper phase (chloroform–methanol–water, 3:48:47 by volume) (8). The lower chloroform phase containing the sphingolipids was evaporated to dryness under nitrogen. About 10-mg equivalent aliquots of lipids in 500 μL of chloroform were loaded onto a preconditioned LC-NH_2 (500-mg) cartridge. The lipids were eluted with the different solvents described above for SPE separation. The volumes of solvents were the maximal volumes given in the SPE procedure description (see above) and named UAV in order to show that, even with such high volumes of solvents, an efficient separation and recovery of lipids could be obtained. The fractions were dried down and spotted on HPTLC plates, and the plates were developed in chloroform–methanol 50:3.5 (v/v) for separation of free ceramides, or in chloroform–methanol–water 65:25:4 (v/v/v) for separation of neutral glycosphingolipids. The alkali-stable phospholipid (sphingomyelin) was developed in chloroform–methanol–20% ammonia 65:35:7.5 (v/v/v) and the spots were visualized immediately after spraying with Dittmer and Lester reagent (**Fig. 1c**) (27). The plates were sprayed with orcinol reagent to visualize neutral glycosphingolipids (Fig. 1b) and with Dittmer and Lester reagent followed by charring at 180°C for 5 min to visualize free ceramides (see Fig. 1a).

Determination of quality and efficiency of separation by the SPE procedure

The quality and efficiency of the SPE procedure were assessed by chromatography of each fraction on HPTLC plates. Authentic standards were chromatographed alongside the samples. Several TLC systems and staining procedures were used to separate individual lipids. Additional procedures were used to determine the extent of contamination (described below).

Measurement of recovery and cross-contamination of fractions by individual lipids: use of radioisotopic controls. To determine the extent of cross-contamination between the different fractions, [^{3}H]myristate-labeled lipids from fish gills (see above) were applied to LC-NH_2 (100-mg) cartridges and eluted as described in the procedure above. The eluted fractions were then mixed with known lipid standards, and separated into neutral and polar lipids (see **Fig. 2**). Neutral lipids were separated by the 3D-TLC procedure described by us (22). This allows the separation of different ceramide species, MGs, free fatty acids (FFA), fatty alcohol, cholesterol, 1,2- and 1,3-diacylglycerols, triacylglycerol, methyl fatty acids, and cholesteryl esters from each other. In this system polar lipids (phospholipids and neutral glycosphingolipids) and free sphingoid bases remain at the origin (Fig. 2a). The latter (polar lipids) are separated according to a different 2D-TLC procedure (28), which resolves all major phospholipids from each other and from neutral glycosphingolipids: CMH, CDH, CTH, and globosides (see Fig. 2b).

S-1-P, ceramide 1-phosphate (C-1-P), sphingomyelin, and sulfatides were separated from each other by one-dimensional TLC

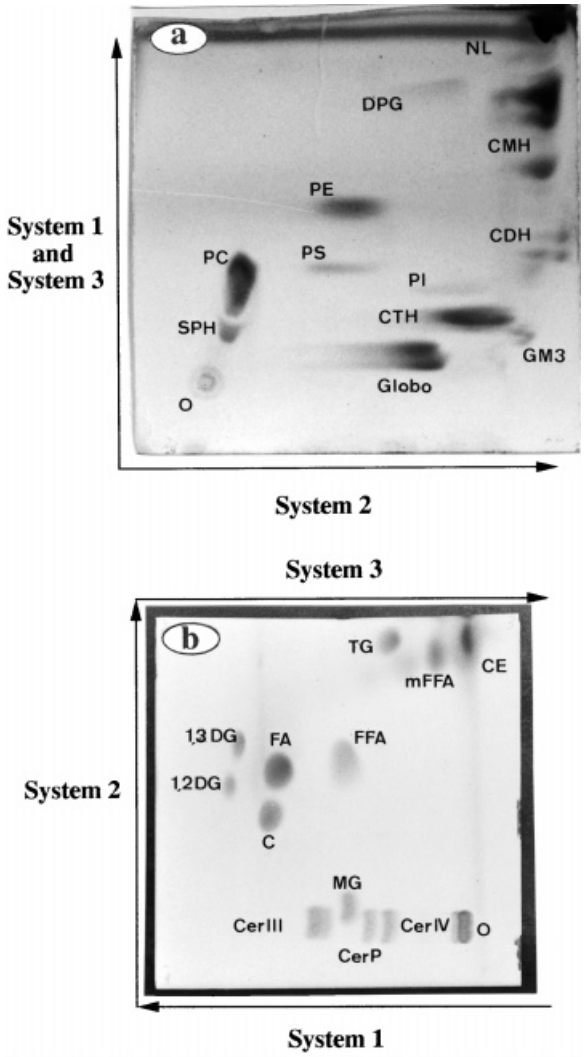

Fig. 2. Typical profile of a lipid mixture on separation by 2D- or 3D-HPTLC. These systems were used to check the recovery and cross-contamination between lipids in each fraction. (a) Profile of polar lipids on a 2D-HPTLC plate according to our published procedure (28). The plate was run consecutively in two directions as indicated, with different successive solvent systems: system 1, diisopropyl ether; system 2, tetrahydrofuran–acetone–methanol–deionized water 50:20:40:6 (v/v/v/v); system 3, chloroform–acetone–methanol–acetic acid–deionized water 50:20:10:15:5 (v/v/v/v/v). O, Origin. (b) Profile of neutral lipids on a 3D-HPTLC plate (22). The following solvent systems were used successively: system 1, chloroform–methanol 50:3.5 (v/v); system 2, petroleum ether–ethyl ether–acetic acid 40:60:0.1 (v/v/v); system 3, hexane–ethyl ether–acetic acid 80:20:1 (v/v/v). O, Origin. Abbreviations of individual lipids are given in text. Phospholipids, neutral glycosphingolipids, and free sphingoid bases remain at the origin during this 3D-HPTLC separation.

developed in chloroform–methanol–20% ammonia, 65:35:7.5 (v/v/v) (29). This TLC procedure was performed after alkaline hydrolysis (further described in the section relating the extraction of sphingolipids from melanoma tumors) of each eluted fraction to remove glycerophospholipids, which can overlap with these sphingolipids when developing the plate in the solvent used herein, a phenomenon that would have resulted in some errors in the determination of radioactivity in sphingolipids. After alkaline hydrolysis, the fractions were further acidified to recover sphingolipids in the chloroform phase that were loaded onto HPTLC plates. After development the plates were air dried and exposed to iodine vapors, or sprayed with Dittmer and Lester reagent for visualization of S-1-P, C-1-P, and sphingomyelin (27).

The lipid spots identified on the HPTLC plates were individually scraped into vials for determination of radioactivity (23). Total radioactivity in given spots of lipid X recovered across all the fractions was determined and used as the reference 100% for that lipid X. The radioactivity measured in a given lipid spot in each fraction was, therefore, recalculated as a percentage of the overall reference number for that lipid. This percentage is the possible extent of contamination across the fractions, assuming that most of the lipid is eluted in one fraction. This is expressed for lipid X as follows:

$$\% \text{ Recovery} = (\text{dpm recovered in lipid X from fraction a/total dpm recovered in all lipid X spots in all fractions}) \times 100$$

Similar calculations were done for all the detectable lipid spots. At the end of SPE elution, the silica gel sorbent was removed from the cartridge to determine the radioactivity retained in the matrix, and data were expressed as a percentage of the total radioactivity initially loaded onto the cartridge.

Estimation of cholesterol leakage. With our protocol, most of the cholesterol elutes in a fraction just prior to the elution of free ceramides. However, a small portion of cholesterol is observed in the fraction containing free ceramides (see Fig. 1a). We therefore undertook a study of cholesterol leakage in the ceramide fraction. A known amount of [4-^{14}C]cholesterol was mixed with increasing amounts of unlabeled cholesterol (see **Fig. 3**). The mixtures were individually loaded onto separate LC-NH_2 (100-mg) SPE cartridges preconditioned and eluted according to the procedure described above. The radioactivity in each fraction was determined to assess the recovery of cholesterol. Cholesterol contamination in ceramide and other fractions was expressed as a percentage of the total radioactivity of cholesterol recovered in all the different fractions combined.

Estimation of sphingoid base recovery. As free sphingoid bases were shown to elute in three different fractions (fractions 2, 3,

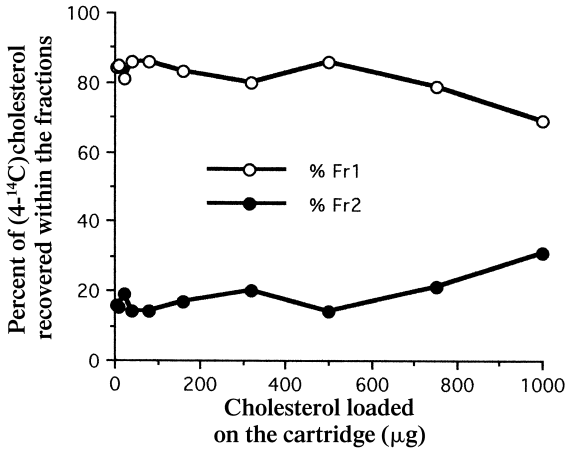

Fig. 3. Increasing amounts of unlabeled cholesterol were mixed with a definite amount of [4-^{14}C]cholesterol. The different mixtures were individually loaded onto separate 100-mg LC-NH_2 cartridges and treated as described in text, and radioactivity recovered in each fraction was measured. Cholesterol breakthrough in fraction 2 was expressed as the percentage of total radioactivity recovered in all the different fractions combined.

and 4) an estimation of the recovery of sphingosine, dihydrosphingosine, and phytosphingosine in each of these fractions was performed. To do this, 30 μg of these different sphingoid bases was loaded onto a preconditioned LC-NH_2 (100-mg) cartridge and eluted as described above. The fractions were spotted on an HPTLC plate and developed in chloroform–methanol–2 N ammonia 40:10:1 (v/v/v) (30). Ninhydrin reagent was used to visualize the spots, followed by scanning densitometry at 520 nm with a dual-wavelength Shimadzu (Kyoto, Japan) CS 930 scanner.

Quantification of sphingoid bases was also performed by colorimetry of their cadmium acetate derivatives according to the procedure described by Esser (31). Briefly, the HPTLC plate was immersed for a short time in a solution of 0.5% ninhydrin in acetone (w/v). The plate was heated at 70°C for 105 min, and the differently colored ninhydrin-positive spots were immediately scraped into tubes containing 0.5% cadmium acetate in methanol (w/v; 0.4 ml). The contents were mixed thoroughly and allowed to stand at room temperature for 2 h. The tubes were centrifuged and the supernatant was used to determine absorption at 494 nm. Recovery of a sphingoid base was calculated as the percentage of the total quantity of that sphingoid base in all the different fractions.

RESULTS

We developed the SPE procedure with [^{3}H]myristic acid-radiolabeled fish gill lipids mixed with known standards to separate lipid components on an LC-NH_2 cartridge (100 mg). Fractions 1–6 obtained from the cartridge were spotted on HPTLC plates and separated into components as shown in Fig. 1. **Table 1** indicates that most neutral lipids were eluted in the first fraction with ethyl acetate–hexane 15:85 (v/v). Fraction 1 contains cholesteryl esters, methyl fatty acids, triglycerides, cholesterol, and 1,2- and 1,3-diglycerides. These are mostly undetectable in the latter fractions. However, a small amount of cholesterol is eluted in fraction 2. This is also visible in Fig. 1. The data obtained from Fig. 3 indicate that about 15% of cholesterol is eluted in fraction 2 as a leakage phenomenon. In contrast to neutral lipids, free fatty acids (normal and α-hydroxy) are not eluted in fraction 1, but are retained on the cartridge. The fatty acids are eluted from the cartridge with diisopropyl ether–acetic acid 98:5 (v/v) in fraction 3 (Fig. 1a). An efficient elution and recovery of ceramides in fraction 2 is indicated by the fact that different types of free ceramides are recovered only in fraction 2 and are not detected in other fractions as demonstrated by charring the plate (Fig. 1a and Table 1). Monoacylglycerols were completely recovered in the fraction containing ceramides. Similarly, the different neutral glycosphingolipids (CMH, CDH, CTH, and globoside) are fully recovered in fraction 4 as shown in Fig. 1b and Table 1. None of these glycosphingolipids was detected in any other fractions (Fig. 1b and Table 1), proving an efficient recovery of glycosphingolipids with acetone–methanol 9:1.35 (v/v). All sphingomyelin was detected in fraction 5 as determined by reaction with Dittmer and Lester reagent (Fig. 1c). Fraction 6 is a unique fraction containing the phosphorylated and sulfated derivatives with S-1-P, C-1-P, and sulfatides as shown in Table 1 but traces of C-1-P and

TABLE 1. Recovery (percent) of radiolabeled lipids in the different fractions

	Fraction					
Lipid	1	2	3	4	5	6
Cholesteryl ester	98 ± 1.2	—	—	—	—	—
Methyl fatty acids	97.5 ± 2.0	—	—	—	—	—
Triacylglycerols	98.2 ± 1.1	—	—	—	—	—
Fatty alcohol	97.2 ± 2.5	—	—	—	—	—
Cholesterol	85 ± 3.5	14 ± 0.6	—	—	—	—
1,2-DAG	93 ± 2.5	6.2 ± 0.3	—	—	—	—
1,3-DAG	95 ± 3.0	4.7 ± 0.4	—	—	—	—
Cer III	—	99 ± 1.0	—	—	—	—
Phyto-Cer	—	96.5 ± 2.1	—	—	—	—
Cer IV	—	98.2 ± 1.3	—	—	—	—
MG	—	98 ± 2.0	—	—	—	—
FFA	—	—	96.5 ± 2.5	—	—	—
OH-FFA	—	—	89.6 ± 3.2	8.2 ± 0.9	—	—
CMH	—	—	—	98.9 ± 1	—	—
CDH	—	—	—	98 ± 1.8	—	—
CTH	—	—	—	91.5 ± 3.1	7.6 ± 0.8	—
Globo	—	—	—	95.3 ± 2.3	4.2 ± 0.9	—
SPH	—	—	—	—	98 ± 2.0	—
S-1-P	—	—	—	—	7.3 ± 0.8	90.9 ± 3.9
C-1-P	—	—	—	—	2.2 ± 0.4	96.3 ± 2.4

Gills from fish were labeled with [^{3}H]myristic acid and lipids were extracted as described in the text. The lipids were loaded on an LC-NH2 (100 mg, Supelco) cartridge and eluted successively in fractions 1 through 6. Equal volume aliquots of fraction were mixed with authentic lipid standards and subjected to two- and three-dimensional thin-layer chromatography to resolve polar and neutral lipid species as mentioned in the text. The spots were scraped and the radioactivity was determined. The radioactivity measured in a given lipid spot in a fraction was calculated as the percentage of the total dpm recovered in that lipid across all the fractions. Hence the data on distribution /recovery are expressed as percent of the total for each lipid. The values are mean ± SEM of four separate experiments. Recoveries of less than 0.5% are indicated by a '—'. Similar results were obtained with LC-NH2 Cartridges (100 mg) from Macherey-Nagel. Standard mixtures were mixed with radioactive lipids to ensure visualization of lipid spots before determination of radioactivity.

TABLE 2. Recovery (percent) of free sphingoid bases in different eluted fractions

	Fraction 2		Fraction 3		Fraction 4	
Type of Base	Method A	Method B	Method A	Method B	Method A	Method B
Sphingosine	74.7 ± 5.7	89.2 ± 8.1	6.05 ± 1.5	1.5 ± 0.45	19.26 ± 4.97	9.3 ± 1.1
Dihydrosphingosine	53.18 ± 3.31	65.1 ± 7.5	14.89 ± 1.88	4.4 ± 1.6	31.93 ± 5.12	29.8 ± 4.3
Phytosphingosine	11.53 ± 3.97	5.7 ± 2.3	2.74 ± 0.5	1.8 ± 0.6	85.7 ± 9.64	92.5 ± 7

A mixture of commercial sphingoid base standards was applied to an LC-NH_2 cartridge (100 mg) and eluted in fractions 2 through 4 as described in text. The fractions were dried down and applied to an HPTLC plate to resolve different sphingoid bases. The sphingoid base spots were visualized with ninhydrin, and quantitated by colorimetry (method A) or densitometry (method B) as described in text. The values are means ± SEM of six experiments for method A and four experiments for method B. The fraction numbers are given in text.

S-1-P were detected with sphingomyelin (SPH). Gangliosides such as GM3, which partition between the upper and lower phase during the Folch extraction were also shown to elute in this fraction 6 (not shown). Fractions 2, 3, and 4 contain the three different free sphingoid bases (**Table 2**). Most sphingosine is eluted with free ceramides in fraction 2, while the main part of phytosphingosine is recovered with neutral glycosphingolipids in fraction 4. Dihydrosphingosine is uniformly distributed among these three fractions. Small amounts of free sphingoid bases were degraded to unknown compounds migrating faster than sphingosine on HPTLC plates (not shown). This phenomenon could be prevented by using freshly purified solvent.

We also applied the SPE method to separate sphingolipids from melanoma tumors, and were able to isolate purified neutral glycosphingolipids in fraction 4. This was the only fraction that was orcinol positive on the HPTLC plate (see Fig. 1b). The chromatogram indicates that CMH and CDH are the major neutral glycosphingolipids in these tumors, and spots comigrating with CTH and globoside are minor components. Free ceramides and sphingomyelin from the tumor were obtained in fractions 2 and 5 as shown in Fig. 1a and c, respectively. Because S-1-P is much more soluble in aqueous/methanolic phase than in the organic solvents when alkaline conditions are used (32) and is partitioned in the upper phase, our SPE fractionation of the lower phase therefore excluded it. The identity of the two Dittmer-positive spots in fraction 6 (see Fig. 1c) is not known, although the alkali-stable nature suggests they may be acidic ether-containing phospholipids.

About 0.1% of the initial total load of radioactivity on the LC-NH_2 cartridge remained uneluted and was retained by the cartridge matrix at the end of the elution procedure, showing the strikingly high efficiency of this SPE procedure for lipid recovery and isolation.

DISCUSSION

The SPE procedure described here allows the separation of major sphingolipid classes present in a Folch-derived lower phase. Although the free ceramide species elute along with monoacylglycerols and a portion of cholesterol, the former can be eliminated by alkaline treatment and the latter (cholesterol) can be efficiently removed by passing fraction 2 onto a second LC-NH_2 cartridge (not shown). Solvent system 1 has been used by Kaluzny et al. (13) for elution of diacylglycerols from aminopropyl cartridges by SPE. In their study, cholesterol was fully recovered by using a solvent system of lower polarity (ethyl acetate–hexane 5:95 [v/v]) than the solvent system we have used for elution of cholesterol and neutral lipids. However, we were unable to elute cholesterol completely with this solvent system. Increasing the concentration of ethyl acetate in hexane (15:85, v/v) also failed in the total elution of cholesterol because about 15% of it was still found in the ceramide fraction (Fig. 3 and Table 1). This can be removed from the fraction as outlined above. The reasons for the difference can only be conjectural, and may include differences in the manufacturing process of SPE cartridge matrix, their preconditioning, and differences in the succession of various solvents (33). However, in our hands, cartridges (100-mg LC-NH_2) from two different manufacturers (Supelco and Macherey-Nagel) did not significantly affect our results. The composition of solvent system A proved optimal at 15% ethyl acetate in hexane (15:85, v/v) and increasing the concentration of ethyl acetate in hexane beyond this percentage lowered the cartridge performance with ceramides leaking in fraction 1.

The solvent mixture described by Kaluzny et al. (13) for the elution of all neutral lipids (except free fatty acids) from LC-NH_2 cartridges, that is, chloroform–isopropanol 2:1 (v/v), has been widely used (14, 34, and reviewed in ref. 11). In this system, free ceramides have been reported to be completely recovered (22, 35). In preliminary experiments with this solvent system, we found that a portion of CMH (nearly 20%) coeluted with free ceramides in fraction 2. This CMH leakage was prevented by our solvent system B of chloroform–methanol 23:1 (v/v), and at the same time ensured an optimal recovery of free ceramide species. Therefore we chose this solvent system rather than the mixture of chloroform–isopropanol 2:1 (v/v). Such a CMH leakage was not reported by Kim and Salem (14) when using standard cerebrosides and eluting their neutral lipids with chloroform–isopropanol 2:1 (v/v).

Elution of fatty acids (normal and α-hydroxy fatty acids) was made possible by solvent C, that is, diisopropyl ether–acetic acid 98:5 (v/v). Although our solvent system C closely resembles the solvent used by Kaluzny (i.e., acetic acid in diethyl ether to 2:98 [v/v]) in their study (13, reviewed in ref. 11), we had to increase the concentration of

acetic acid, because α-hydroxy free fatty acids did not elute at 2% acetic acid in the solvent system, and eluted with our solvent system D along with neutral glycosphingolipids. However, increasing the concentration of acetic acid to 5% in our solvent system C ensured elution and good recovery of α-hydroxylated fatty acids, and retention of neutral glycosphingolipids on the cartridge and their later recovery in a fraction with acetone–methanol 9:1.35 (v/v).

Few studies have focused on the isolation of neutral glycosphingolipids with small SPE tubes. In the studies by Kim and Salem (14) cerebrosides were eluted along with neutral phospholipids in the same fraction on LC-NH_2 cartridge, and no mention was made of attempts to separate neutral glycosphingolipids from phospholipids. No information on the purification of other neutral glycosphingolipids is available anywhere else. In fact, most of the studies undertaken to separate neutral glycosphingolipids from phospholipids by SPE have used unbonded silica gel cartridges (reviewed in refs. 11 and 36–38), and have used acetone or acetone–methanol 9:1 (v/v) to elute neutral glycosphingolipids. However, the recovery and purity of recovered lipids in the fraction are often not reported. We preferred aminopropyl-bonded silica gel matrix to unbonded silica gel because of its greater relative affinity to free fatty acids on appropriate conditioning. This property allowed a better separation of free ceramides from fatty acids instead of being coeluted as seen with the classic silica gel cartridges. This property is particularly useful after alkaline methanolysis of lipid samples, during which many more free fatty acids are released.

SPH is also obtained in fraction 5 by solvent system E along with other neutral phospholipids such as lysophosphatidylcholine (LPC), phosphatidylcholine (PC), and phosphatidylethanolamine (PE) (not shown). These phospholipids can be easily removed by alkaline treatment prior to the SPE or later separated by TLC. No neutral glycosphingolipids are detectable in this fraction. Sphingolipid metabolic products S-1-P, C-1-P, and sulfatides are retained on the matrix and are recovered with acidic phospholipids such as phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidic acid (PA), and phosphatidylglycerol (PG) in solvent system F. This system was better suited for further manipulation of the fractions in contrast to the system used by Kim and Salem (14) to elute acidic phospholipids from LC-NH_2. In our hand, their system consisting of hexane–2-propanol–ethanol–0.1 M ammonium acetate–formic acid 420:350:100:50:0.5 (by volume) containing 5% phosphoric acid was difficult to evaporate. Our attempts to separate S-1-P, C-1-P, and sulfatides from each other were unsuccessful. However, isolation of S-1-P needs careful attention because it partitions into the aqueous upper phase at alkaline pH (32, 39), so that the conditions of extraction and the following steps in the process determine the degree of its presence in the lower phase and later application onto an LC-NH_2 cartridge.

Our SPE procedure allows simultaneous separation of the major sphingolipid classes in the Folch lower phase, from neutral lipid classes and phospholipids. This is an advantage because the latter two classes can then be further processed by conventional published procedures (13, 15, 34). Thus, with our procedure, most of the neutral lipids are eluted in a single fraction with solvent system A. If needed, this fraction containing cholesteryl esters, methyl fatty acids, triglycerides, cholesterol, 1,2- and 1,3-diacylglycerides can be separated into individual components on an LC-NH_2 cartridge according to the Kaluzny procedure or on a silica gel SPE cartridge according to Hamilton and Comaï (40). Similarly, the phospholipids PI and PS remaining on the aminopropyl cartridge after elution of SPH with solvent system E can be separately recovered from the LC-NH_2 cartridge by the procedure described by Pietsch and Lorenz (34). Although it is possible to fractionate phospholipids on a silica gel cartridge by the procedure described by Suzuki (15), the drawback of S-1-P, C-1-P, and sulfatide overlapping should be kept in mind.

In our hands, up to 15 mg of total lipids could be applied onto a 500-mg LC-NH_2 cartridge without leakage between the different eluted fractions. We routinely used 500-mg cartridges for separation of 10 mg of lipids in order to purify free ceramides, neutral glycosphingolipids, and SPH for further analysis. The smaller 100-mg LC-NH_2 cartridges are particularly useful for a low amount of lipids (e.g., as radiolabeled samples), or from a small tissue sample. We demonstrated that, when radiolabeled fish lipids were used on 100-mg cartridges, only 0.1% of the initial radioactivity loaded onto the cartridge was lost in the frits and the matrix. Up to 4 mg of lipid samples could be separated on these 100-mg LC-NH_2 cartridges. The procedure described in this report, therefore, can be a useful alternative to existing procedures for isolation of sphingolipids of interest. In summary, the SPE procedure described here is simple and can be used by itself for isolation of individual sphingolipids, or in combination with additional published separation techniques for other lipid classes.

O.K. was partially supported by grants from the U.S. National Multiple Sclerosis Society (PP0581) and The Solving the Mystery of Autism Foundation. This work was facilitated by a professor exchange award (O.K.) from the Office of International Relations of the University Claude Bernard Lyon-I.

Manuscript received 25 February 2000 and in revised form 16 May 2000.

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