High performance thin-layer chromatography and densitometry of synaptic plasma membrane lipids

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Summary Previously, it has been shown that phospholipids, cholesterol, and glycolipids could be quantitated using the same high performance thin-layer chromatography (HPTLC) method. Here we examined that method in terms of linearity of standards in the nanogram range, recovery of nonacidic and acidic lipids after Sephadex column chromatography, and quantitation of lipids in mouse synaptic plasma membranes (SPM) where lipid content is low. Nonacidic and acidic fractions were separated by Sephadex column chromatography, applied to plates using contact spotting, chromatographed, visualized with cupric acetate, and quantitated using in situ densitometry. Recovery of nonacidic and acidic fractions off the columns was determined with radiolabeled phospholipids. Standards for each lipid class were linear in the nanogram range. Quantitation of SPM lipid classes could be made with as little as 1.5 μg of total lipid. Recovery of the nonacidic fraction after Sephadex column chromatography was approximately 100% whereas the acidic fraction was approximately 91%. Phospholipids, cholesterol, and glycolipids could be determined in nanogram amounts using the same method. This method is an efficient method for examining different lipid classes and in samples where lipid content is low.—**Wood, W. G., M. Cornwell, and L. S. Williamson.** High performance thin-layer chromatography and densitometry of synaptic plasma membrane lipids. *J. Lipid Res.* 1989. **30:** 775–779.

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High performance thin-layer chromatography (HPTLC) and in situ densitometry of lipids have been used to examine lipid content of biological tissue (1–3). Macala, Yu, and Ando (4) have reported on a HPTLC method for analyzing nonacidic and acidic brain lipids that allows quantitation of phospholipids, cholesterol, and glycolipids using the same method. Standard curves were reported for the nonacidic and acidic lipids ranging between 1 μg and 10 μg for individual lipids. The curves for the lipid standards were nonlinear with increasing concentration. It was found that at least 1 μg of each lipid class could be quantitated by densitometry and it was suggested that the method could be adapted to measure lower lipid amounts (4).

Lipid content of whole brain homogenate and myelin were reported by Macala et al. (4) and generally were in agreement with earlier studies that used thin-layer chromatography (5–7). Analysis could be made with a minimum of 400 μg of total lipid. There did appear to be some loss of acidic lipids that may have resulted from the separation of the nonacidic and acidic lipids by Sephadex column chromatography and/or the procedures used to remove salts from the acidic fraction (4).

The method reported by Macala et al. (4) may be appropriate for measuring lipid content where lipid content is low. The purpose of the present paper was to determine whether the HPTLC method reported by Macala et al. (4) could be applied to measure phospholipids, cholesterol, and glycolipids of synaptic plasma membranes (SPM) in the nanogram range using contact spotting (8). In addition, the following issues were addressed: *1*) whether standard curves for each lipid class would be linear in the nanogram range; and *2*) quantifying recovery of the nonacidic and acidic fractions following Sephadex column chromatography.

EXPERIMENTAL PROCEDURE

Materials

Phospholipid, cholesterol, and glycolipid standards were obtained from Supelco (Bellefonte, PA) and Sigma Chemical Company (St. Louis, MO). Solvents were of analytical grade and filtered using Metricel membrane filters (0.2 μm pore size) from Gelman Scientific (Ann Arbor, MI). HPTLC plates (10 × 20 cm, Silica Gel 60) were obtained from CAMAG Scientific (Wrightsville Beach, NC) and DEAE-Sephadex (A-25) was purchased from Sigma Chemical Company. [^{14}C]Phosphatidylcholine ([^{14}C]PC) and [^{14}C]) phosphatidylserine ([^{14}C]PS) were obtained from Amersham Corporation (Arlington Heights, IL).

Membrane preparation and lipid extraction

C57BL/6 male mice 4 months of age were obtained from Harlan Industries (Indianapolis, IN). SPM were prepared as described previously (9–11). Animals were decapitated and brains were dissected. The brain was homogenized in 10 volumes of 0.32 M sucrose, 2 mM HEPES, pH 7.5 (buffer A). All subsequent procedures were performed at 4°C or on ice. The homogenate was sedimented at 1200 *g* for 5 min. The pellet was discarded and the supernatant was further sedimented at 17,000 *g* for 12

Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; Sph, sphingomyelin; HPTLC, high performance thin-layer chromatography; SPM, synaptic plasma membranes.

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min. The pellet was retained, resuspended in buffer A, and again sedimented at 17,000 *g* for 12 min. The pellet was then gently resuspended in buffer A and layered over 7.5% and 12.0% (w/v) Ficoll in buffer A. Gradients were sedimented in a SW28 rotor at 75,000 *g* for 60 min using a L8-80 ultracentrifuge (Beckman Instruments,Inc., Fullerton, Ca). The 7.5 - 12.0% interface was removed, suspended, and synaptosomes were sedimented at 18,120 *g* got 20 min. The pellet was resuspended in 5 mM Tris-HCl (pH8.5), homogenized with three up and down strokes and allowed to lyse for 30 min at 4°C. After lysis was completed, the membranes were sedimented at 40,000 *g* for 20 min. The pellet was resuspended in cold double-distilled water, underlayered with 0.95 M sucrose, 50 mM Tris (pH 7.4) and sedimented at 40,000 *g* for 20 min. The 0.95 M sucrose-water interface was removed and sedimented at 40,000 *g* for 20 min, and the final SPM pellet was resuspended in 1 ml of 50 mM Tris, pH 7.3. Protein was determined by the method of Lowry et al.(12). SPM lipids were extracted in 4 volumes of chloroform-methanol 2:1 (v/v) and centrifuged at 750 *g* for 10 min (13). The lower organic layer was removed and placed in a separate tube. The upper layer was removed and extracted again with 2 volumes of acidified chloroform- methanol 4:1 (v/v) containing 26 mM HCl and centrifuged. The acidic organic layer was then neutralized with NH_4OH and then combined with the neutral organic extract.

Lipid standards

The following lipid standards were used to determine sensitivity and linearity: cholesterol, cerebrosides, phosphatidylethanolamine (PE), phosphatidylcholine (PC), sphingomyelin, sulfatides, phosphatidylserine (PS), and phosphatidylinositol (PI). These standards were plated at five different amounts (25, 50, 100, 150, and 200 ng). Each standard was made up in chloroform-methanol-water 75:25:2 (v/v) to which oleyl alcohol was added as an internal standard (100 ng/10 μl).

Sephadex column chromatography

After lipid extraction, the SPM lipids were applied to a 1-ml bed volume Sephadex (A-25) column to separate the nonacidic and acidic lipids (14). Initially, the column was rinsed with 50 ml of chloroform-methanol-water 30:60:8 (v/v) (solvent A).

A known concentration of SPM lipids was added at a rate of no more than 1 ml/min in 5 ml of solvent A and the nonacidic lipids were collected in this fraction. Acidic lipids were eluted with 15 ml of chloroform-methanol-0.8 M aqueous sodium acetate 30:60:8 (v/v). The nonacidic and acidic fractions were dried under nitrogen and then placed under vacuum. The nonacidic fraction was then brought up in 3 ml of chloroform. The acidic fraction was brought up in chloroform-methanol 2:1 and 0.6 ml of water and centrifuged. This procedure is necessary to remove the sodium acetate. The upper layer was removed, discarded, and an equal amount of chloroform- methanol-water 3:48:47 was added, and the mixture was centrifuged. This step was repeated three times.

Recovery of the nonacidic and acidic fractions was monitored using [^{14}C]PC and [^{14}C]PS (Amersham Corporation). Labeled lipids were run on the Sephadex column using the procedures described above. Samples were dried under nitrogen, brought up in 10 ml of ACS II (Amersham Corporation), and counted on a Beckman LS 6800 scintillation counter.

HPTLC

The HPTLC plates were preconditioned in chloroform-methanol 60:35 (v/v), air-dried for 15 min, and then placed on a heating block at 120°C for 15 min. The plates were then removed and placed in a vacuum oven. Samples were applied to the plates using a contact spotter that has been described previously (8). The contact spotter contains small wells that are covered by a polymer film. A vacuum (30 psi) was applied that resulted in well-defined depressions in each well. Samples were then applied to each well. Both standards and SPM samples were in chloroform-methanol-water 75:25:2 (v/v), oleyl alcohol (100 ng/10 μl) and 1% dodecane. The oleyl alcohol was used as an internal standard and was added to the samples before being placed in the well (4). Dodecane was used to facilitate transfer of the sample from the film to the HPTLC plate (15). The volume of the samples applied to the wells was 10 μl. The temperature of the wells was maintained at approximately 35°C. Following sample application in the wells, the vacuum was released, positive pressure was applied, and the samples were transferred to the HPTLC plate.

Separate HPTLC plates for the acidic and nonacidic lipids were developed using chloroform-methanol-acetic acid-formic acid-water 35:15:6:2:1 (v/v). The solvent front for nonacidic lipids was allowed to ascend to 4.5 cm above the bottom edge of the plate. The solvent front for acidic lipids ascended 6 cm. The plates were air dried for 15 min and then placed under vacuum for 15 min. The plates with nonacidic and acidic lipid were then developed in a solvent system containing hexane-diisopropyl ether-acetic acid 65:35:2 (v/v) and the solvent front was allowed to run to the top of the plate. The plates were removed from the tanks and dried as described above.

Plates were then dipped into a 3% cupric acetate (w/v)-8% phosphoric acid (v/v) solution for 10 sec, air-dried for 5 min, and heated on a hot plate 140°C for 30 min. Plates were then covered with a clear glass plate (10 × 20 cm) and scanned.

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Densitometry

Plates were scanned with a Shimadzu CS-930 scanning densitometer and areas were computed with a Shimadzu DR-2 data processor (Shimadzu Corporation, Kyoto, Japan). The plates were scanned in the reflectance mode at 350 nm with a tungsten lamp. The scanning beam was 10 mm × 0.1 mm. Scanning speed was 1.72 mm/min.

RESULTS

The standard curves of the nonacidic and acidic lipids are shown in **Fig 1.** Linearity was high for all lipids between 25 and 200 ng.

The ratio of lipid to internal standard varied for the various lipids and is related to the amount of unsaturated fatty acids in each lipid (16). PE, cholesterol, and the glycolipids showed the greatest charring as compared to the other lipids.

Recovery differed between the nonacidic and acidic lipids. **Table 1** shows the percentage recovery of [^{14}C]PC and [^{14}C]PS. Recovery of PC was determined on the fraction that came off the Sephadex column in solvent A. The PC fraction showed almost 100% recovery. The recovery of PS was measured after the acidic fraction had been washed three times to remove the sodium acetate. The acidic fraction showed a loss as a result of the steps to remove the sodium acetate. Recovery of PS was approximately 91%. It can be seen in Table 1 that adherence of PC or PS to the Sephadex column after rinsing with solvents A and B, respectively, was minimal for both lipids, although the value for PS was higher as compared to PC.

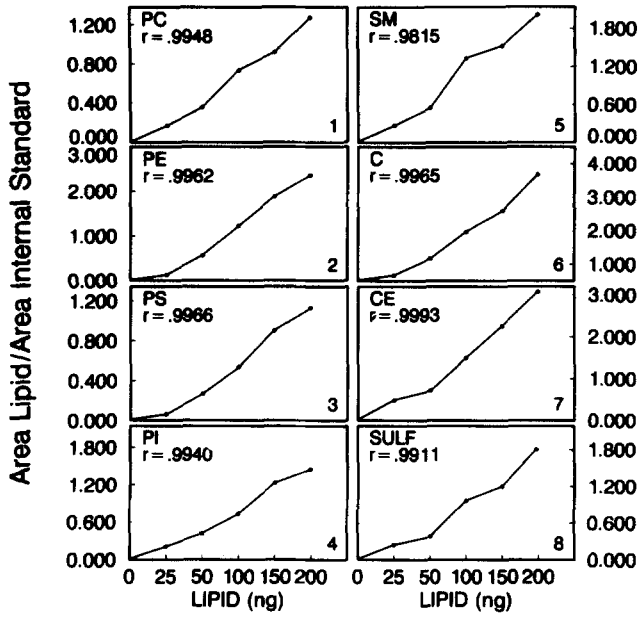

Fig. 1. Nonacidic and acidic lipid standards. Data are expressed as the ratio of the area of each lipid divided by the area of the internal standard. Each point represents the mean of three or four different plates.

TABLE 1. Percent recovery of ^{14}C-labeled PC and PS standards

Phospholipid	% Recovery[a]	% Loss on Sephadex[b]	% Loss during Washes[c]
PC	98 ± 0.33	0.17 ± 0.08	
PS	91 ± 0.42	1.6 ± 0.32	8.22 ± 0.64

[a]Final recovery of phosphatidylcholine and phosphatidylserine. Each value is the mean ± SEM of three experiments.
[b]Percentage of phosphatidylcholine and phosphatidylserine remaining on the Sephadex column after eluting with solvent A or B, respectively.
[c]Percentage of phosphatidylserine lost during the washes to remove the sodium acetate.

SPM lipid content is described in **Table 2** and **Table 3**. Cholesterol, PC, and PE accounted for 82% of the SPM lipid content. PC and PE were the predominant phospholipids.

DISCUSSION

The standard curves for the nonacidic and the acidic lipids were linear over the concentration range of 25–200 ng. Macala et al. (4) reported nonlinearity between 1 and 10 μg using generally the same method. The reliability of the method is increased by the use of samples in the nanogram range, making the method very useful for samples with low lipid content.

Recovery of the nonacidic lipid fraction and the acidic lipid fraction after the Sephadex separation differed between the two fractions. The separation of the nonacidic and acidic lipids results in approximately a 10% loss in the acidic fraction. Previously, it was suggested that the lower values reported for the acidic lipids PS and PI may have resulted from adsorption to the column, steps used to remove sodium acetate, and the small amount of sample used (4). The data in the present study support the obser-

TABLE 2. Mouse synaptic plasma membrane phospholipids

	ng[a]	% Phospholipid[b]	% Total Lipid[c]
PC	427.53 ± 5.55	41.5 ± 0.29	27.57 ± 0.24
PE	400.97 ± 3.21	38.9 ± 0.03	25.83 ± 0.07
Sph	24.13 ± 2.41	2.3 ± 0.34	1.55 ± 0.14
PS	153.17 ± 0.67	15.0 ± 0.01	9.87 ± 0.06
PI	26.40 ± 1.3	2.6 ± 0.20	1.70 ± 0.09

[a]Phospholipids are expressed in nanograms per 10-μl spotting volume. Each value is the mean ± SEM (n = 3).
[b]Individual phospholipids as a percentage of total phospholipid. Each value represents the mean ± SEM (n = 3).
[c]Mean percentage of total lipid ± SEM (n = 3).

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TABLE 3. Cholesterol and glycolipid content in synaptic plasma membranes from mice

	ng[a]	% Total Lipid[b]
Cholesterol	442.67 ± 2.91	28.53 ± 0.02
Cerebrosides	58.00 ± 2.05	4.33 ± 0.49
Sulfatides	18.47 ± 0.73	1.15 ± 0.06

[a]Lipids are expressed in nanograms per 10-μl spotting volume. Each value is the mean ± SEM (n = 3).
[b]Values are percent mean ± SEM of total lipid analyzed (n = 3).

vation that the steps used to remove the sodium acetate are the primary cause of differences in recovery between the nonacidic and acidic lipids. Attempts to use fewer washes resulted in distortion of the acidic lipids on the HPTLC plate. Absorption to the column was greatest for the acidic fraction and little if any adsorption was observed with the nonacidic fraction.

The percent lipid distribution of SPM agrees with results of previous studies (17). We were able to measure approximately 1.5 μg of SPM total extracted lipid. The use of such a small amount was facilitated by using the contact spotting procedure and an internal standard. Samples can be applied to the polymer film in volumes that minimize loss when using very small volumes, e.g., 10 μl. The inclusion of the internal standard in both the samples and external standards further increases the precision of the method by quantifying any loss resulting during the spotting procedures.

The cupric acetate charring reagent has been used in several different studies. Recently, it was reported that the cupric acetate method is less sensitive than molybdenum blue reagent (2). The advantage of the molybdenum reagent was based on the fact that it stains for phosphorus and is not affected by the amount of unsaturated fatty acids in the individual phospholipids. While this is correct, there are some additional issues concerning the two methods that should be considered. Separate lipid standards are necessary for analyzing phospholipids when amounts are low e.g., below 100 ng when using the molybdenum reagent (2). Individual lipid standards are also used with the cupric acetate method and thus, both methods are similar in that regard. An advantage of the cupric acetate method over the molybdenum method is that cholesterol and glycolipids can be determined on the same plate as phospholipids without having to use different procedures. The capacity to analyze different lipids at the same time is important in terms of restricted amount of sample and time required for analysis.

Overall, the method developed by Macala et al. (4) using cupric acetate is a very sensitive and efficient means of analyzing phospholipids, cholesterol, and glycolipids in biological samples. This method is very applicable to quantitation of lipids where total lipid content is low.

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