papers and notes on methodology

Analysis of brain lipids by high performance thin-layer chromatography and densitometry

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Abstract We have devised a high performance thin-layer chromatography (HPTLC) densitometry method to resolve the major lipid classes of brain tissue. We used DEAE-Sephadex column chromatography to separate the total lipid into neutral and acidic lipid fractions. The lipid fractions were then spotted on separate HPTLC plates and chromatographed in one dimension using two solvent systems. Quantitation was by in situ densitometry with absolute amounts of the lipid classes determined from co-chromatographed standards. An internal standard was also used to improve the precision. The individual lipid classes of rat whole brain, human brain gray and white matter, rat and bovine myelin, and bovine oligodendroglia were quantitated. Human brain phosphatidylethanolamine plasmalogen was also quantitated. Sensitivity was increased by using the cupric acetate charring reagent, which we found to be more sensitive than the conventional sulfuric acid-dichromate reagent. Total lipid (less than 400 μg) was quantitated from 5 mg of tissue wet weight. The limit of detection, on HPTLC, for the individual lipid classes was below 20 ng.—**Macala, L. J., R. K. Yu, and S. Ando.** Analysis of brain lipids by high performance thin-layer chromatography and densitometry. *J. Lipid Res.* 1983, **24:** 1243–1250.

Supplementary key words DEAE-Sephadex chromatography • phospholipids • plasmalogen • acidic lipids

The heterogeneity of the lipid classes in brain has required a variety of separation and quantitative techniques for their analysis. A simple, sensitive, and reliable method for the quantitation of total brain lipids has not been developed. We have found one-dimensional HPTLC, in conjunction with in situ densitometry, to be a suitable method.

In situ densitometry of thin-layer chromatograms was first used by Privett et al. (1). They separated glycerides by TLC and quantitated them after charring with sulfuric acid. Many methods have subsequently been developed to analyze certain, but not all, of the major lipid classes. Most methods are applicable either to the neutral lipid classes (2–8) or the phospholipid classes (9–19), while others include some, but not all, classes of both the neutral and phospholipids (20, 21). In addition some methods involve preliminary column chromatography of the total lipid into three or more fractions which are then analyzed separately (5, 18, 22), three or more solvent systems for TLC (4, 16, 20, 22), or chemical pretreatment of the TLC plate (14, 18).

Vitiello and Zanetta (23) separated the major phospholipid classes on HPTLC, along with cerebrosides and sulfatides, but did not separate the neutral lipid classes. Ando et al. (24, 25) developed a HPTLC method which separates the major neutral and phospholipid classes, with the exception of PI and PS. Also, sulfatides, which were not found in the tissues they analyzed, co-migrate with PE in their system. A novel feature of the method is the use of an internal standard in determining the absolute amount of each lipid class.

We have modified the chromatographic procedure of Ando et al. (24, 25) to resolve LPC, SPM, PC, PE, PI, PS, C, TG, CE, FA, CB, and SULF using HPTLC. Combined with densitometry, this procedure affords a

Abbreviations: TLC, thin-layer chromatography; HPTLC, high performance thin-layer chromatography; LPC, lysophosphatidylcholine; SPM, sphingomyelin; PC, choline phospholipids; PE, ethanolamine phospholipids; PS, serine phospholipids; C, cholesterol; TG, triacylglycerols; CE, cholesteryl esters; FA, fatty acids; CB, cerebrosides; SULF, sulfatides; IS, internal standard; PI, inositol phospholipids. Acidic lipids refer to PI, PS, SULF, and FA; all other lipids are collectively referred to as non-acidic lipids.

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rapid, sensitive, and accurate means of analyzing the lipid composition of brain tissues, cells, and subcellular fractions. A preliminary report has appeared (26).

EXPERIMENTAL PROCEDURE

Materials

Phospholipid, triglyceride, cholesteryl ester, cholesterol, fatty acid, and glycolipid standards were variously obtained from Analabs (North Haven, CT) and Sigma Chemical Co. (St. Louis, MO). With the exception of triolein (TG), cholesteryl oleate (CE), and oleic acid (FA), the fatty acid composition of the lipid classes was heterogeneous, being from biological sources. Each standard lipid was examined on HPTLC (as described herein) and was greater than 98% pure. DEAE-Sephadex (A-25) was from Pharmacia Fine Chemicals (Piscataway, NJ) and 10 × 20 cm HPTLC plates (Silica Gel 60, 0.20-mm layer thickness) were from E. Merck (Darmstadt, Germany). All reagents and solvents were of analytical reagent grade. Chloroform and methanol were redistilled before use. Human brain tissues were obtained, by autopsy, from a 50-year-old male who died without any known neurological diseases. Sixty-day-old Sprague-Dawley rats were used. Bovine myelin and rat myelin were isolated (27) and supplied to us by Drs. W. T. Norton and R. W. Ledeen, respectively. Bovine oligodendroglia were also isolated (28) and supplied to us by Dr. Norton.

Standard lipid mixtures

Each standard lipid was weighed and dissolved in a specific volume of chloroform–methanol 1:1. Four different concentrations of standard lipid mixtures were then prepared, containing 1.0, 3.0, 5.0, and 10.0 μg of each of the following lipids, LPC, SPM, PC, PE, CB, C, TG, and CE, per 5 μl of chloroform–methanol 1:1. Similar standard lipid mixtures were prepared for the acidic lipids (PI, PS, SULF, and FA). To all standard lipid mixtures, oleyl alcohol (as an internal standard) was added to a final concentration of 1 μg/μl.

Homogenization and extraction

Whole rat brain and human gray and white matter samples were weighed and then homogenized with 10 volumes of chloroform–methanol 1:1. The homogenate was filtered using a Buchner funnel with fritted disc (medium porosity). The residue was washed three times with 5 volumes of chloroform–methanol 1:1. The combined filtrates were adjusted to a final concentration of chloroform–methanol–water 30:60:8 (v/v) by adding appropriate amounts of methanol and water.

Lyophilized bovine and rat myelin samples were dissolved, with sonication, directly in chloroform–methanol–water 30:60:8.

The protein content of bovine oligodendroglia was estimated by the method of Lowry et al. (29). The cells were then lyophilized and the lipids were extracted, with sonication, with chloroform–methanol–water 30:60:8.

DEAE-Sephadex column chromatography

The samples were then applied to 1-ml bed volume DEAE-Sephadex (A-25) columns (30). The columns were further eluted with 10 ml of chloroform–methanol–water 30:60:8 and the non-acidic lipids were collected in this fraction. The acidic lipids were then eluted with 10 ml of chloroform–methanol–0.8 M aqueous sodium acetate 30:60:8 (v/v). Both fractions were dried with a stream of N_2 and then under vacuum for about 10 min to remove residual solvents.

The acidic lipid fractions were further treated, to remove the sodium acetate, prior to HPTLC. Three ml of chloroform–methanol 2:1 and 0.6 ml of water (31) was added to each sample. The fractions were centrifuged (Model HN-S; International Equipment Co., Needham Heights, MA) for 5 min at 800 *g* and the upper aqueous phase was removed and discarded. A volume of chloroform–methanol–water 3:48:47, equal to the volume of upper phase removed, was added; the tubes were vortexed, centrifuged, and the upper phase was removed. For tissue wet weights below approximately 50 mg, a third washing step was needed to eliminate any interference with mobility, from salts, on HPTLC. The fractions were again dried using N_2 and vacuum.

HPTLC

HPTLC plates were prewashed with chloroform–methanol–water 60:35:8 (v/v) to eliminate interference from material, intrinsic to the plate, which otherwise migrated with cholesteryl ester and charred with the cupric acetate reagent. They were then activated at approximately 100°C for 15 min and cooled in a vacuum desiccator.

Test plates were run (as described below) for both the non-acidic and acidic lipid fractions to determine the amount of IS (oleyl alcohol) to add and to check each fraction for the absence of any material migrating with the same R_f as the IS. A volume of IS (1 μg/μl) was added so that the concentration of each lipid class would be within the range of 1.0–10.0 μg/5 μl.

The isolated non-acidic lipids were spotted on a plate with appropriate lipid standards and the isolated acidic lipids were spotted on a separate plate with acidic lipid standards. Each concentration of standard lipid mixture was spotted in duplicate on each plate. Each lane was

spotted 1 cm above the bottom edge of the plate and was 5 mm in width with 3-mm spacing between lanes. The spotting volume was 5 μl for all lanes. A maximum of 20 lanes was spotted on a single plate.

Plates were chromatographed, in the 10-cm dimension, in TLC tanks (6 × 12 × 3.75 in; Analabs, North Haven, CT) lined with filter paper. Solvent systems (approximately 70 ml total) were equilibrated several hours before use. The non-acidic lipid fractions were chromatographed, using chloroform–methanol–acetic acid–formic acid–water 35:15:6:2:1 (v/v) until the solvent front ascended to about 4.5 cm above the bottom edge of the plate. Acidic lipids were chromatographed to about 6 cm using the same solvent system. Following development, excess solvent was evaporated in a fume hood for 15 min and then in a vacuum desiccator for 15 min. Both non-acidic and acidic lipids were then chromatographed using hexanes–diisopropyl ether–acetic acid 65:35:2 (v/v) until the solvent front ascended to the top of the plate. Excess solvent was evaporated as described above.

Following chromatography the lipids were charred for densitometry. Plates were either sprayed with a 0.25% sodium dichromate (w/v)–15% sulfuric acid (v/v) reagent and heated for 30 min at 120°C or dipped into a 3% cupric acetate (w/v)–8% phosphoric acid (v/v) solution (22) and heated at 180°C for 15 min (32) or at 130°C for 30 min. All plates were heated on an aluminum block (20 × 20 × 2.5 cm) set on a hot plate (130°C), or set in a hot air oven (180°C). Plates were generally scanned immediately after heating, or stored at −20°C and scanned within 24 hr.

Densitometry

Plates were scanned from the origin to the solvent front, using a Shimadzu CS-910 scanning densitometer, and the integrated areas were computed by a Shimadzu CR1A data processor (Shimadzu Scientific Instruments, Inc.; Columbia, MD). Plates were scanned at 350 nm (tungsten source) in the reflectance mode. The beam dimensions were 4.0 mm × 0.2 mm and the scan speed was 24 mm/min. Each lane was scanned three times and the average values for the three scans were determined.

Quantitation

The ratio of the density (μV-sec) of each lipid class to the density (μV-sec) of the internal standard was calculated for both the standard and sample lanes on each plate. The density ratios for the different standard amounts (1.0, 3.0, 5.0, and 10.0 μg) were used to obtain standard curves for each lipid class. The ratios of the density of each lipid class in the sample lane to the density of the internal standard, in the same lane, were used to determine the absolute amounts of each lipid class from their respective standard curves. The total lipid for each sample was calculated as a summation of the absolute values for each lipid class.

The plasmalogen content of the ethanolamine phospholipids was also quantitated in situ. After spotting the samples and standards (as described above), the plate was set for 20 min in a TLC tank saturated with HCl vapors. This cleaves the plasmalogen into free aldehyde and lysophosphatidylethanolamine (33). The plate was then dried in a fume hood for 15 min, followed by drying in a vacuum desiccator for 15 min. After TLC (as described above), the lanes were scanned to determine the amount of unhydrolyzed diacyl PE remaining. This amount was subtracted from the total PE previously quantitated to determine the amount of plasmalogen.

RESULTS

HPTLC plates, with their corresponding densitometric chromatograms are shown for the non-acidic lipid fractions (**Fig. 1**) and the acidic lipid fractions (**Fig. 2**) of rat whole brain.

Standard curves for each non-acidic lipid class (**Fig. 3**) and each acidic lipid class (**Fig. 4**) indicate that each lipid class had a different densitometric response, which was nonlinear over the range of 1.0–10.0 μg.

Data for each lipid class, expressed as a percent of total lipid, and total lipid, expressed on an absolute basis, are shown in comparison to data reported by other investigators (**Table 1** and **Table 2**).

The precision of the method, when the same sample is quantitated on different HPTLC plates and for different amounts of tissue wet weight, is shown by the data for rat whole brain lipids. Total lipids were extracted from a single rat whole brain. The extract was adjusted to a final concentration of chloroform–methanol–water 30:60:8. Aliquots corresponding to 100, 50, 20, 10, and 5 mg of tissue wet weight were taken, each in triplicate. After isolating the non-acidic and acidic lipids, each sample was spotted once on three different HPTLC plates and the individual lipid classes were quantitated (nine determinations for each tissue wet weight). The precision for all determinations is also shown (N = 45) (Table 1).

The precision when samples were spotted on the same HPTLC plate is shown by the data for human brain gray matter (two samples, each spotted twice; N = 4) and white matter (three samples each spotted once; N = 3) and bovine and rat myelin lipids (one sample of each spotted three times; N = 3) (Table 2).

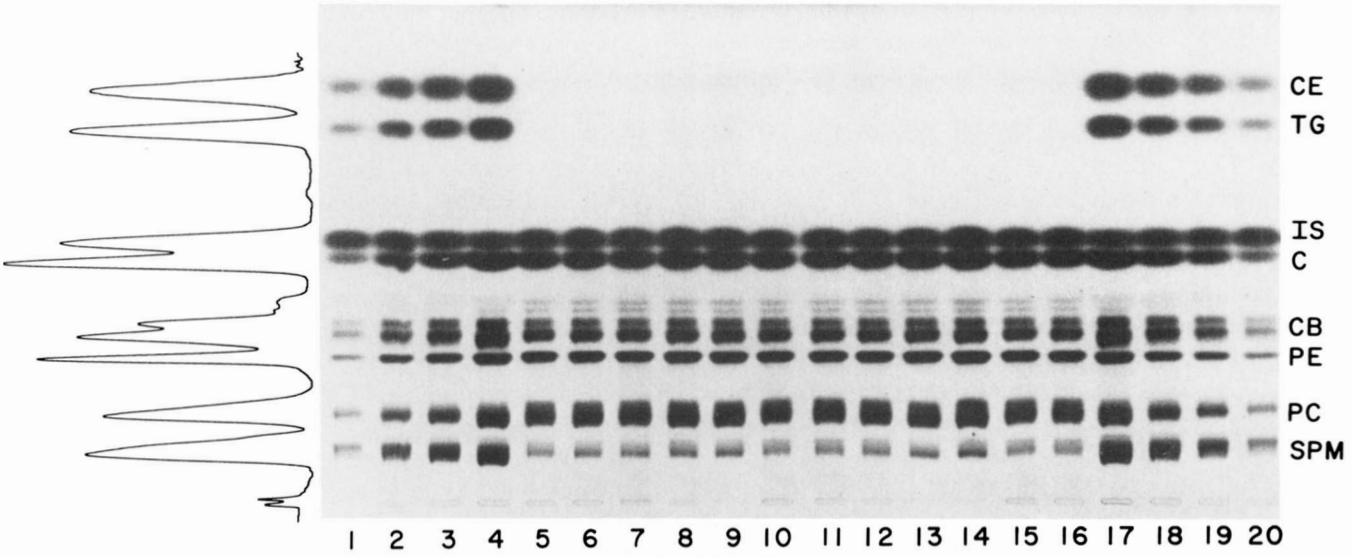

Fig. 1. HPTLC chromatogram of rat brain non-acidic lipids, with densitometric chromatogram of lane 18. Lanes 1 and 20, 1 μg of each standard lipid; 2 and 19, 3 μg of standard; 3 and 18, 5 μg of standard; 4 and 17, 10 μg of standard; 5–16, rat whole brain (approximately 21 μg of total non-acidic lipid per lane). The first development, with chloroform–methanol–acetic acid–formic acid–water 35:15:6:2:1 (to about 4.5 cm) separated sphingomyelin (SPM), choline phospholipids (PC), ethanolamine phospholipids (PE), and cerebrosides (CB). Lysophosphatidylcholine (which is not shown on this plate) migrated below SPM. The second development, with hexanes–diisopropyl ether–acetic acid 65:35:2 (to the top of the plate), separated cholesterol (C), internal standard (IS), triglycerides (TG), and cholesteryl esters (CE). The charring reagent was 3% cupric acetate in 8% phosphoric acid.

DISCUSSION

The difficulty of resolving and quantitating the major lipid classes is evident from the numerous methods in the literature and from the lack of suitability of any one method for analyzing all the major lipid classes. We have modified the chromatographic and densitometric procedure of Ando et al. (24, 25) to quantitate, simply and rapidly, the major lipid classes, plasmalogen, and total lipid in brain.

Using the solvent systems reported by Ando et al. (24, 25) sulfatides co-migrate with PE and PI co-migrates with PS. To resolve these, we initially separated the total lipid extract into non-acidic and acidic lipid

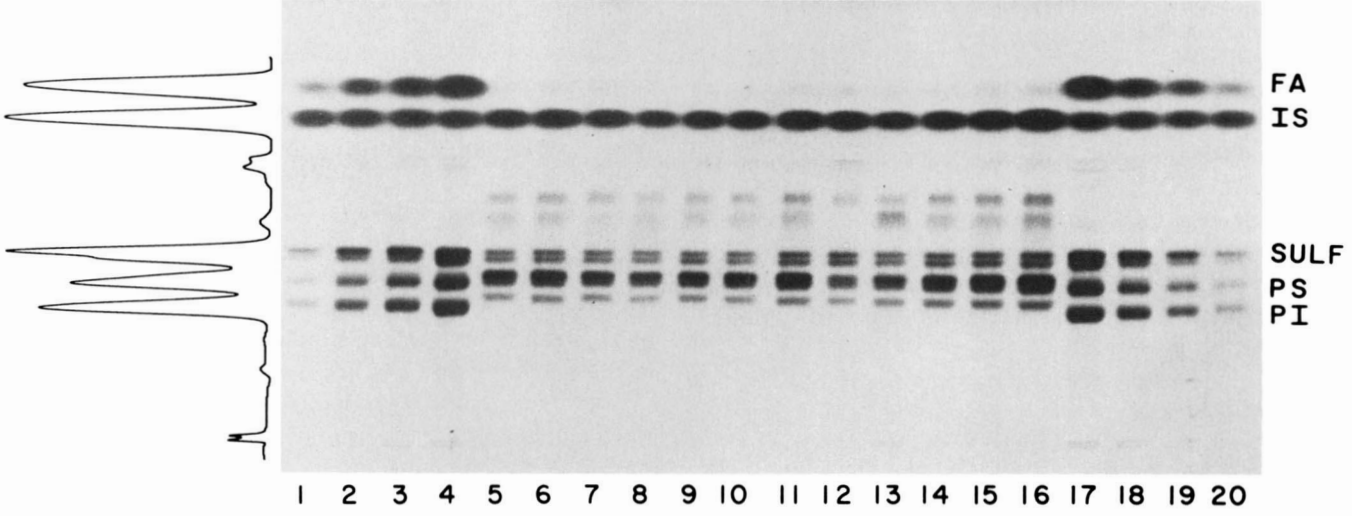

Fig. 2. HPTLC chromatogram of rat brain acidic lipids, with densitometric chromatogram of lane 18. Lanes 1 and 20, 1 μg of each standard lipid; 2 and 19, 3 μg of standard; 3 and 18, 5 μg of standard; 4 and 17, 10 μg of standard; 5–16, rat whole brain (approximately 12 μg of total acidic lipid per lane). The first development, with chloroform–methanol–acetic acid–formic acid–water 35:15:6:2:1 (to about 6 cm) separated inositol phospholipids (PI), serine phospholipids (PS), and sulfatides (SULF). The second development, with hexanes–diisopropyl ether–acetic acid 65:35:2 (to the top of the plate), separated the internal standard (IS) from fatty acids (FA). The charring reagent was 3% cupric acetate in 8% phosphoric acid.

TABLE 1. Analysis of rat whole brain lipids[a]

	Tissue Wet Weight (mg)						
	100 (N = 9)	50 (N = 9)	20 (N = 9)	10 (N = 9)	5 (N = 9)	All (N = 45)	Literature (Ref. 27)
Cholesterol	18.4 ± 0.5 (8.7)[b]	18.4 ± 0.4 (6.8)	19.1 ± 0.4 (5.9)	18.4 ± 0.4 (6.5)	18.9 ± 0.4 (7.2)	18.6 ± 0.2 (6.9)	22.2 ± 0.3
Sphingomyelin	2.7 ± 0.1 (9.5)	2.6 ± 0.1 (8.4)	2.6 ± 0.1 (9.4)	2.8 ± 0.1 (8.7)	3.0 ± 0.1 (10.0)	2.8 ± 0.0 (10.1)	4.3 ± 0.4
Choline phospholipids	22.4 ± 0.4 (6.1)	21.7 ± 0.4 (6.0)	22.5 ± 0.5 (6.3)	22.4 ± 0.7 (8.9)	24.2 ± 0.6 (7.9)	22.6 ± 0.3 (7.8)	24.1 ± 0.2
Ethanolamine phospholipids	25.1 ± 0.4 (5.2)	23.5 ± 0.3 (3.8)	23.0 ± 0.4 (5.8)	23.8 ± 0.4 (5.8)	25.3 ± 0.4 (4.1)	24.1 ± 0.2 (6.1)	19.8 ± 0.5
Inositol phospholipids[c]	2.4 ± 0.1 (8.0)	2.6 ± 0.1 (8.3)	2.4 ± 0.0 (6.2)	2.3 ± 0.1 (8.0)	2.1 ± 0.1 (16.5)	2.4 ± 0.0 (12.1)	2.7 ± 0.4
Serine phospholipids	14.0 ± 0.8 (17.8)	16.3 ± 0.4 (7.3)	15.8 ± 0.4 (6.6)	15.6 ± 0.4 (7.2)	11.3 ± 1.0 (26.5)	14.6 ± 0.4 (17.9)	7.0 ± 0.2
Cerebrosides	11.2 ± 0.2 (4.8)	11.0 ± 0.2 (4.9)	11.2 ± 0.2 (4.8)	11.4 ± 0.2 (4.2)	11.6 ± 0.2 (4.6)	11.3 ± 0.1 (4.9)	11.9 ± 0.2
Sulfatides	3.7 ± 0.1 (7.0)	3.8 ± 0.1 (4.6)	3.3 ± 0.1 (8.2)	3.3 ± 0.1 (6.5)	3.7 ± 0.1 (11.8)	3.6 ± 0.0 (9.6)	3.6 ± 0.1
Total lipid (mg/gww)	75.3 ± 1.9 (7.7)	74.8 ± 1.5 (5.8)	74.1 ± 1.3 (5.3)	76.5 ± 1.2 (4.6)	70.8 ± 1.4 (6.1)	74.3 ± 0.7 (6.3)	84.5

[a] Individual lipid classes are expressed as a percent of total lipid ± SEM (HPTLC values) or ± the mean deviation (literature values).
[b] Values in parentheses are coefficients of variation.
[c] Monophosphoinositide.

fractions by DEAE-Sephadex column chromatography (30). The two fractions are then spotted on separate HPTLC plates, separating sulfatides (acidic) from PE (zwitterionic). Additionally, the two fractions are developed to different heights with the first solvent system. Extending the development of the acidic lipid fraction to 6 cm separates PI and PS.

Sensitivity was also improved by using the cupric acetate reagent (22), in conjunction with HPTLC. We found that the cupric acetate reagent was about three times more sensitive than the sulfuric acid-dichromate reagent, when heating was at 130°C for 30 min. When the lipids were charred with the cupric acetate reagent, at 180°C for 15 min, 20 ng of PC gave a signal-to-noise ratio of about 10 in the reflectance mode. The transmission mode gave the same result. With the present method less than 1 μg of each lipid class was easily quantitated and as little as 400 μg of total lipid was analyzed (in triplicate). Because of the increased sensitivity of the cupric acetate reagent the method could be adapted to analyze much less total lipid. In addition to improved sensitivity we found some differences between the absolute amounts of the lipid classes of human white matter, when charred with the sulfuric acid-dichromate reagent, at 120°C for 30 min, as reported by Ando et al. (24, 25) and the cupric acetate reagent (22), at 180°C for 15 min (32). The absolute values for PE, PC, PI, SPM, and SULF were lower with the cupric acetate reagent, while PS, C, and CB were about the same with both reagents. The absolute values of the individual lipid classes, when charred with the cupric acetate reagent, were generally in better correspondence to the literature values than were the values we obtained with the sulfuric acid-dichromate reagent.

Several reports have appeared using the cupric acetate reagent to char lipids at 130°C (8, 34). We compared the absolute amounts of the lipid classes, for all samples in Table 2, when they were charred at 130°C for 30 min and at 180°C as originally reported by Fewster, Burns, and Mead (22). PC, PS, C, and CB generally gave higher absolute amounts at 180°C than at 130°C, while SPM, PE, PI, and SULF generally were the same at both temperatures. The percent distribution of the individual lipid classes when charred at 180°C generally corresponded better to the literature values than the percent distribution when charring was done at 130°C.

In addition to the total lipids, we also quantitated ethanolamine plasmalogen by one-dimensional HPTLC. Two-dimensional TLC of plasmalogen, using $HgCl_2$ for hydrolysis of the alk-1-enyl groups, was reported by Owens (35). Horrocks (33) also used two-dimensional TLC but with HCl vapors as the hydrolyzing agent. One-dimensional TLC, following hydrolysis with HCl in methanol, was reported by Eng and Noble (36). In

TABLE 2. Lipid composition[a] of CNS tissues, myelin, and cells

	Brain (Human)				CNS Myelin					
	Gray Matter		White Matter		Bovine		Rat		Oligodendroglia Bovine	
	HPTLC N = 4	Lit.[b]	HPTLC N = 3	Lit.[b]	HPTLC N = 3	Lit.[b]	HPTLC N = 3	Lit.[c]	HPTLC	Lit.[d]
Cholesterol	17.5 ± 0.6	22.0	23.3 ± 0.5	27.5	22.8 ± 0.4	28.1	23.5 ± 0.2	25.9	11.5	14.3
Sphingomyelin	4.5 ± 0.1	6.9	7.0 ± 0.1	7.7	7.0 ± 0.1	7.1	1.9 ± 0.0	3.5	4.0	3.6
Choline phospholipids	24.8 ± 0.7	26.7	8.8 ± 0.2	12.8	8.2 ± 0.1	10.9	15.0 ± 0.1	12.0	36.4	31.2
Ethanolamine phospholipids (plasmalogen)	24.5 ± 0.5 (60.1 ± 0.7)	22.7 (38.8)	19.6 ± 0.3 (84.3 ± 0.3)	14.9 (75.2)	17.2 ± 0.4	17.4	26.3 ± 0.1	16.2	17.8	15.9
Inositol phospholipids[e]	2.7 ± 0.1	2.7	1.2 ± 0.0	0.9	1.1 ± 0.0	0.8	0.9 ± 0.0	1.2	8.6	5.0
Serine phospholipids	15.2 ± 0.4	8.7	12.5 ± 0.4	7.9	18.4 ± 0.7	6.5	10.0 ± 0.3	6.2	7.0	4.5
Cerebrosides	6.6 ± 0.4	5.4	20.0 ± 0.0	19.8	18.9 ± 0.5	24.0	17.6 ± 0.2	23.9	12.4	16.9
Sulfatides	4.2 ± 0.3	1.7	7.7 ± 0.1	5.4	6.7 ± 0.2	3.6	4.9 ± 0.1	6.6	2.3	3.4
Total lipid[f]	73.0 ± 1.6	59	199.0 ± 2.8	156	790.1 ± 9.2	753	766.5 ± 10.0	708	172.4	169.5

[a] Individual lipid classes are expressed as a percent of total lipid ± SEM (where indicated).
[b] Ref. 37.
[c] Ref. 27.
[d] Ref. 28.
[e] Monophosphoinositide.
[f] Values are expressed as mg/g wet weight (human brain gray and white matter); mg/g dry weight (bovine and rat myelin); and μg/mg protein (bovine oligodendroglia).

each case plasmalogen content was determined after scraping the appropriate bands off the TLC plate. We have adapted our HPTLC-densitometry procedure to include the analysis of plasmalogen in situ.

Our values for plasmalogen, as a percent of total PE, are 84.3% for human brain white matter and 60.1% for human brain gray matter, corresponding to literature values (37) of 75.2% and 38.8%, respectively. It should be mentioned that the gray matter value for PE plasmalogen reported by Norton (37) may be too low. O'Brien and Sampson (38) found that PE plasmalogen represented 44–56% of the total PE in adult human gray matter. Our estimation of PE plasmalogen content is in close agreement to theirs. Although we quantitated only PE plasmalogen, the method could be extended to include other plasmalogens.

To determine the absolute amount of each lipid class, external standards were co-chromatographed with the samples. Errors in quantitation, due to differences between the lipid classes in charring density, carbon percent, diffusion due to different R_f values, and densitometric response, are minimized (5). Within each lipid class the degree of unsaturation of the fatty acid moieties results in different degrees of charring and stan-

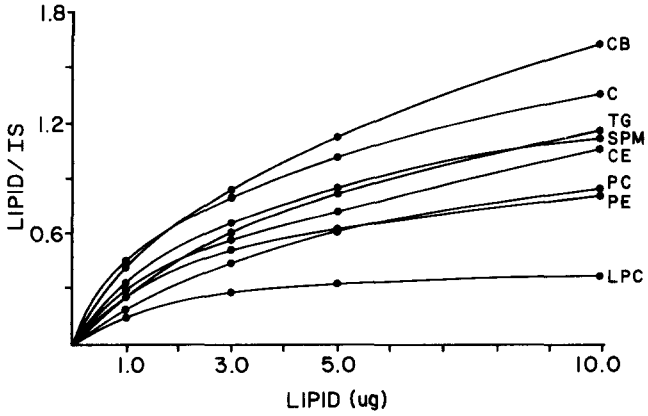

Fig. 3. Standard curves for the non-acidic lipid classes.

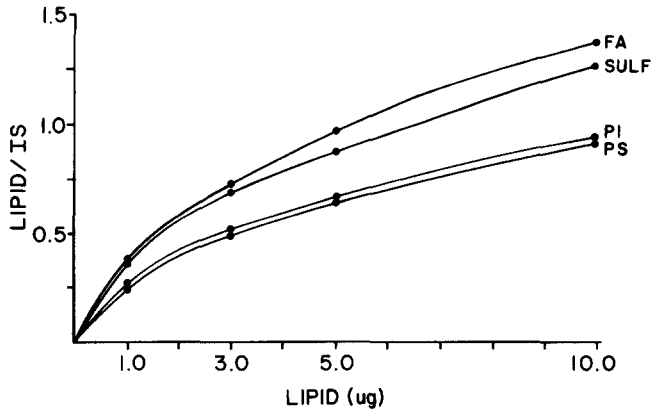

Fig. 4. Standard curves for the acidic lipid classes.

dards need to be selected to account for these differences. Hedegaard and Jensen (17) suggested that the selection of standards derived from natural sources gives a better approximation than synthetic standards. Kabara and Chen (7) also recognized the effect of unsaturation on charring density and recommended the use of an external standard as being sufficiently precise in analyzing most biological samples. Using the cupric acetate charring reagent, Shand and Noble (32) concluded that significant differences in the extent of charring of the phospholipids as a result of differences in the unsaturated fatty acid content are unlikely to be encountered in practice. They also reported that the degree of unsaturation had no significant effect on the extent of charring of the neutral lipids that they analyzed. Therefore, choosing standards from biological sources appears reasonable for determining the absolute amounts of each lipid class.

When compared to literature values, the data we obtained by densitometry for the individual lipid classes are in close agreement. Cholesterol as a trend was slightly lower, whereas serine phospholipids were about two times higher than the literature values. Our values for total lipid were higher than the literature values, with the exception of rat whole brain, which was lower, and bovine oligodendroglia, which was about the same. The higher values for total lipid may reflect a greater recovery by our method, due to the small number of manipulations made on the samples. The non-acidic lipid fraction was applied directly to HPTLC, following elution from the column. Recovery should be complete unless there is adsorption on the column. The consistency of the data for the individual non-acidic lipid classes, for each tissue wet weight from rat whole brain, suggests that the recovery is complete. There may be some loss of acidic lipid, by adsorption on the column, or in the partitioning step to remove the salts. The lower values for PI, PS, and total lipid, for the 5-mg wet weight sample, compared to the other tissue wet weights, suggests this. It is also possible that because of the small amount of total lipids in the 5-mg wet weight sample, the small amount of solvent (25 μl) used to redissolve them after partitioning, results in some loss.

The addition of an internal standard to both the samples and the external standards minimizes differences in quantitation due to variation in spotting volumes, thereby improving the precision of the analysis and allowing a small volume (5 μl) to be spotted. The precision of our method compares to those that have been previously reported. The coefficient of variation for multiple scans of the same lane on HPTLC is similar to that obtained by Bitman and Wood (34). The standard errors of the mean for samples spotted in multiple on the same plate (Table 2) are comparable to those reported by others (12, 14, 17, 21, 22). In addition, we have determined the precision of the method (Table 1) when the same sample is spotted on different plates. For the individual lipid classes, the range of the coefficient of variation was 4.9% (cerebrosides) to 17.9% (serine phospholipids). The determination of the interplate precision allows comparisons to be made between different samples analyzed over a period of time.

In addition to quantitating the major lipid classes normally found in brain, several lipid classes (cholesteryl esters, free fatty acids, and triacylglycerols), which are not found in normal adult brain, but may increase in certain pathological conditions, were resolved and could be quantitated. Also, lysophosphatidyl choline, which is found in some pathological conditions, was resolved and detected, but did not char sufficiently to be quantitated in the range of our standards (1–10 μg).

In conclusion, we have developed a method to analyze the major lipid classes in brain, including plasmalogen. The method is simple and rapid. It requires only a single column chromatography step to separate the total lipids into non-acidic and acidic fractions, followed by one-dimensional HPTLC using only two different solvent systems. The same technique, in situ densitometry, is used to quantitate each lipid class, using external standards and an internal standard. The range of the SEM for samples spotted in multiple, on the same plate, is ±0.0 to ±0.7. The range of the SEM for samples spotted on different plates is ±0.0 to ±1.0. Approximately 1.0 μg of each lipid class could be quantitated and a triplicate analysis could be done with less than 400 μg of total lipid. The procedure should be useful in determining the lipid composition of small amounts of tissues or in samples where the lipid content is low.

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REFERENCES

1. Privett, O. S., M. L. Blank, D. W. Codding, and E. C. Nickell. 1965. Lipid analysis by quantitative thin-layer chromatography. *J. Am. Oil Chem. Soc.* **42:** 381–393.
2. Skipski, V. P., A. F. Smolowe, R. C. Sullivan, and M. Barclay. 1965. Separation of lipid classes by thin-layer chromatography. *Biochim. Biophys. Acta.* **106:** 386–396.
3. Freeman, C. P., and D. West. 1966. Complete separation of lipid classes on a single thin-layer plate. *J. Lipid Res.* **7:** 324–327.
4. Downing, D. T. 1968. Photodensitometry in the thin-layer chromatographic analysis of neutral lipids. *J. Chromatogr.* **38:** 91–99.
5. Privett, O. S., K. A. Dougherty, and J. D. Castell. 1971. Quantitative analysis of lipid classes. *Am. J. Clin. Nutr.* **24:** 1265–1275.

6. Hojnacki, J. L., R. J. Nicolosi, and K. C. Hayes. 1976. Densitometric quantitation of neutral lipids on ammonium sulfate-impregnated thin-layer chromatograms. *J. Chromatogr.* **128:** 133–139.
7. Kabara, J. J., and J. S. Chen. 1976. Microdetermination of lipid classes after thin-layer chromatography. *Anal. Chem.* **48:** 814–817.
8. Bitman, J., D. L. Wood, and J. M. Ruth. 1981. Two-stage, one-dimensional thin-layer chromatographic method for separation of lipid classes. *J. Liq. Chromatogr.* **4:** 1007–1021.
9. Skipski, V. P., R. F. Peterson, and M. Barclay. 1964. Quantitative analysis of phospholipids by thin-layer chromatography. *Biochem. J.* **90:** 374–378.
10. Skipski, V. P., M. Barclay, E. S. Reichman, and J. J. Good. 1967. Separation of acidic phospholipids by one-dimensional thin-layer chromatography. *Biochim. Biophys. Acta* **137:** 80–89.
11. Adams, G. M., and T. L. Sallee. 1970. A method for the one-dimensional thin-layer chromatographic separation of serum phospholipids. *J. Chromatogr.* **49:** 552–554.
12. Chapelle, S. M. 1979. Rapid quantitative analysis of phospholipid erythrocytes by densitometric thin-layer chromatography. *Clin. Chim. Acta.* **92:** 11–18.
13. Kolins, M. D., E. Epstein, W. H. Civin, and S. Weiner. 1980. Amnotic fluid phospholipids measured by continuous-development thin-layer chromatography. *Clin. Chem.* **26:** 403–405.
14. Painter, P. C. 1980. Simultaneous measurement of lecithin, sphingomyelin, phosphatidylglycerol, phosphatidylinositol, phosphatidylethanolamine, and phosphatidylserine in amniotic fluid. *Clin. Chem.* **26:** 1147–1151.
15. Touchstone, J. C., J. C. Chen, and K. M. Beaver. 1980. Improved separation of phospholipids in thin-layer chromatography. *Lipids.* **15:** 61–62.
16. Gentner, P. R., M. Bauer, and I. Dieterich. 1981. Thin-layer chromatography of phospholipids: separation of major phospholipid classes of milk without previous isolation from total lipid extracts. *J. Chromatogr.* **206:** 200–204.
17. Hedegaard, E., and B. Jensen. 1981. Nano-scale densitometric quantitation of phospholipids. *J. Chromatogr.* **225:** 450–454.
18. Fine, J. B. and H. Sprecher. 1982. Unidimensional thin-layer chromatography of phospholipids on boric acid-impregnated plates. *J. Lipid Res.* **23:** 660–663.
19. Pappas, A. A., R. E. Mullins, and R. H. Gadsden. 1982. Improved one-dimensional thin-layer chromatography of phospholipids in amniotic fluid. *J. Clin. Chem.* **28:** 209–211.
20. Pernes, J. F. 1980. Lipids: thin-layer chromatographic separation in twelve fractions by three successive unidirectional developments on the same plate. *J. Chromatogr.* **181:** 254–258.
21. Nutter, L. J., and O. S. Privett. 1968. An improved method for the quantitative analysis of lipid classes via thin-layer chromatography employing charring and densitometry. *J. Chromatogr.* **35:** 519–525.
22. Fewster, M. E., B. J. Burns, and J. F. Mead. 1969. Quantitative densitometric thin-layer chromatography of lipids using copper acetate reagent. *J. Chromatogr.* **43:** 120–126.
23. Vitiello, F. and J. P. Zanetta. 1978. Thin-layer chromatography of phospholipids. *J. Chromatogr.* **166:** 637–640.
24. Ando, S., K. Kon, and Y. Tanaka. 1980. High-precision TLC-densitometry of membrane lipids. *In* Membrane Fluidity: Biophysical Techniques and Cellular Regulation. M. Kates and A. Kuksis, editors. Humana Press, Inc., Clifton, NJ. 43–55.
25. Ando, S., K. Kon, Y. Tanaka, S. Nagase, and Y. Nagai. 1980. Characterization of hyperlipidemia in Nagase analbuminemia rat (NAR). *J. Biochem.* **87:** 1859–1862.
26. Macala, L. J., and R. K. Yu. 1982. High-performance thin-layer chromatography densitometry of brain lipids. *Trans. Am. Soc. Neurochem.* **13:** 147.
27. Norton, W. T., and S. E. Poduslo. 1973. Myelination in rat brain: changes in myelin composition during brain maturation. *J. Neurochem.* **21:** 759–773.
28. Farooq, M., W. Cammer, D. S. Snyder, C. S. Raine, and W. T. Norton. 1981. Properties of bovine oligodendroglia isolated by a new procedure using physiologic conditions. *J. Neurochem.* **36:** 431–440.
29. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
30. Ledeen, R. W., R. K. Yu, and L. F. Eng. 1973. Gangliosides of human myelin: sialosylgalactosylceramide (G7) as a major component. *J. Neurochem.* **21:** 829–839.
31. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
32. Shand, J. H., and R. C. Noble. 1980. Quantification of lipid mass by a liquid scintillation counting procedure following charring on thin-layer plates. *Anal. Biochem.* **101:** 427–434.
33. Horrocks, L. A. 1968. The alk-1-enyl group content of mammalian myelin phosphoglycerides by quantitative two-dimensional thin-layer chromatography. *J. Lipid Res.* **9:** 469–472.
34. Bitman, J., and D. L. Wood. 1981. Quantitative densitometry in situ of lipids separated by thin-layer chromatography. *J. Liq. Chromatogr.* **4:** 1023–1034.
35. Owens, K. 1966. A two-dimensional thin-layer chromatographic procedure for the estimation of plasmalogens. *Biochem. J.* **100:** 354–361.
36. Eng, L. F., and E. P. Noble. 1968. The maturation of rat brain myelin. *Lipids.* **3:** 157–162.
37. Norton, W. T. 1976. Formation, structure, and biochemistry of myelin. *In* Basic Neurochemistry. G. J. Siegel, R. W. Albers, R. Katzman, and B. W. Agranoff, editors. Little, Brown and Company, Boston, MA. 74–99.
38. O'Brien, J. S., and E. L. Sampson. 1965. Lipid composition of the normal human brain: gray matter, white matter, and myelin. *J. Lipid Res.* **6:** 537–544.