Microanalysis of brain lipids: multiple two-dimensional thin-layer chromatography

S. Pollet, S. Ermidou, F. Le Saux, M. Monge, and N. Baumann

Laboratoire de Neurochimie INSERM U. 134, Hôpital de la Salpêtrière, 75634 Paris Cedex 13, France

Summary The techniques described allow the quantitation of cholesterol, cerebrosides, sulfatides, ethanolamine phospholipids, phosphatidylcholine, -serine, -inositol, and gangliosides on 500 μg of lipid. Lipid extraction required sonication. Separation of the lipid classes was performed on TLC glass plates (10 × 10 cm) coated with silica gel HPTLC 60 F 254 Merck using one multiple two-dimensional chromatography. Most of the methods used for quantitation were standard methods that had been scaled down. The procedure was applied to study regional differences in the central nervous system, to analyze cell membranes or subcellular particles, or to analyze pathological biopsies in the central and peripheral nervous systems.

Abbreviations: TLC, thin-layer chromatography; NANA, *N*-acetylneuraminic acid; PE, ethanolamine phospholipids; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidic acid; Sph, sphingomyelin; P, phosphorus.

Supplementary key words lipid analysis · biopsies

Cellular and subcellular analyses of lipids in small areas of brain or in biopsied peripheral nerves require the use of scaled-down techniques. Numerous procedures have been published. Some, although very complete (1–4), necessitate a large amount of starting material (around 1 g); others have been scaled down (5–8) but need several thin-layer chromatographic separations, often combined with column chromatography. Some procedures have been adapted for studying the lipid composition of other organs and therefore have limitations relative to microanalysis of brain samples (9). The method we report utilizes one multiple dimensional thin-layer chromatography and combines techniques already available. It gives a simple, accurate, and reproducible quantitative estimation of all the major lipid classes in a small amount of sample (around 500 μg of lipid).

MATERIAL AND METHODS

Chemicals were purchased from Merck. Lipid standards were either prepared in the laboratory by standard procedures (10) or purchased from Koch-

Light or Applied Science Laboratories. The methods were tested on fragments of brains from mice. All solvent and reagent combinations were volume–volume.

Lipid extract

Fresh material was homogenized briefly in chloroform–methanol 1:1 (20 ml/g) with a Polytron PCU_2 homogenizer. Lyophilized samples must be suspended in chloroform–methanol–water 70:30:4, 1 ml for each 20 mg (dry weight). For amounts less than 100 mg dry weight or 250 mg wet weight, extraction was performed in 5 ml of solvent. Extraction was by six 30-sec sonications (Sonifier B-12 Branson) at 4°C with 30 sec between each sonication. The residue was easily pelleted after centrifugation at 2,000 rpm for 10 min (International Clinical Centrifuge). The lipid extract was diluted to a known final volume. When enough material was available, an aliquot could be evaporated to dryness to determine the weight of the total lipid extract. Foaming was controlled by adding some drops of isoamyl alcohol (11) or isobutanol.

Separation of lipids

Separation was performed on TLC glass plates (10 × 10 cm) coated with silica gel HPTLC 60 F 254 Merck. Approximately 500 μg of total lipid extract was spotted 2 cm from the bottom left corner of the plate. It was not necessary to activate the plate before spotting. The procedure was as follows: *1*) The plate was developed in chloroform–methanol–water 70:30:4 from the bottom to the top of the plate. *2*) The chromatoplate was rotated counterclockwise 90 degrees and placed in the second solvent system, chloroform–methanol 2:8; the solvent was allowed to run ⅔ the distance of the plate. *3*) In the same direction, the plate was then developed in chloroform–methanol 2:1 from the bottom to the top of the plate. *4*) Finally, the plate was rotated clockwise back to its original position and developed in chloroform–methanol 2:1. It was important to dry the plate well (on the bench) between each development. A drier can be used for a few minutes.

Lipids were detected by iodine vapor. The position of the various lipids was previously characterized by comparison with lipid standards and by specific sprays. After complete sublimation of the iodine from the plate, the areas of the individual lipids were scraped off for quantification of the lipids.

Quantification of lipids

Most of the methods used were standard, but were scaled down and slightly modified. When enough material was available, quantitative analyses of cholesterol, P, hexoses, and NANA were performed on the total lipid extract. These results, compared to those obtained after TLC, allowed the estimation of the overall recovery of lipids. If the amount of material was too low, only a determination of NANA was done on the total lipid extract to quantify gangliosides. The hexoses in the gangliosides were determined after TLC. In most cases, the standard addition method was used: each assay was performed with a constant amount of lipid, to which were added increasing amounts of the standard substance to be determined. For determinations done directly on the silica gel (hexoses and phosphorus), this method could not be used. For all determinations after TLC, it was necessary to take into account the influence of silica gel. Therefore spots of silica gel of the same size as the spots of different lipids were sampled. Assays were also performed on these spots.

Cholesterol determination. Cholesterol was determined by a slight modification of the method of Searcy and Bergquist (12). After TLC, the cholesterol areas were extracted by stirring with 1 ml of chloroform–methanol 2:1 for 2 min. Aliquots of this extract (and possibly of the total lipid extract) corresponding to about 5 μg of cholesterol were removed and analysis was carried out in the presence of 10, 20, and 30 μg of cholesterol standard (standard solution 200 μg/ml in chloroform–methanol 2:1). The quantity of chloroform–methanol 2:1 in the assay solution must not be more than 0.25 ml for a final volume of 2.05 ml of the assay mixture, because of a problem of miscibility between organic and aqueous phases. Under these conditions, evaporation of the sample to dryness was not useful. A solution of 1.2 ml of $FeSO_4$ (1 g in 200 ml of acetic acid)–acetic acid 9:1 and 0.6 ml of concentrated H_2SO_4 were added. In order to avoid further precipitation with concentrated H_2SO_4, it was better not to use, as previously described (12), a saturated solution of $FeSO_4$. The test tubes were then allowed to stand 30 min in the dark. After removal, the absorbance at 460 nm was stable for 10 min. Therefore the number of analyses must be limited for each experiment.

Phospholipid determination. After TLC, areas corresponding to phospholipids were scraped into Pyrex tubes. Dry mineralization was then performed in a 220 V, 300 W electric rack at 500°C for 30 min. This procedure was adequate for at least up to 3 μg of P in each tube. The determination of P was according to the method of Dawson (13). For phospholipid spots containing less than 0.1 μg of P (generally PI and PA), the residue was resolubilized in 0.3 ml of 72% $HClO_4$–water 1.5:1. Ammonium molybdate solution (0.8% in doubly distilled water) (0.5 ml) and

0.085 ml of ANSA reagent–water 2:1 were added. (The ANSA reagent was made up of 28.5 g of $Na_2S_2O_5$, 6 g of Na_2SO_3, and 0.5 mg of 1,2,4-aminonaphtolsulfonic acid in doubly distilled water up to 250 ml). Test tubes had to be vigorously shaken between each reagent addition. After 20 min, at 100°C the samples were centrifuged (International Clinical Centrifuge) at 2,000 rpm for 2 min. The absorbance was measured at 820 nm. In the same way, standard determinations were carried out with 0.1, 0.2, and 0.3 μg of P (a standard solution KH_2PO_4 in doubly distilled water was used, 4 μg P/ml); these samples were heated at 500°C for 30 min. For phospholipid spots containing between 0.1 μg and 1 μg of P (generally PS, Sph), the same procedure was used but all the volumes were multiplied by 2 (final volume 1.77 ml) and phosphorus standards of 0.2, 0.4, and 0.6 μg were used. For phospholipid spots containing more than 1 μg of P (essentially PE, PC), all the volumes were multiplied by 5 and the standards consisted of 1, 2, and 3 μg of P. When possible, aliquots of total lipid extract corresponding to about 0.1 μg of P were evaporated to dryness in Pyrex tubes. Mineralization was performed in the presence of 0.2, 0.4, and 0.6 μg of P from the standard KH_2PO_4 solution. P determination was carried out as described above (final volume of 1.77 ml).

Galactose determination. Hexoses were quantified according to slight modifications of the method of Neskovic et al. (14) and Svennerholm (15).

After TLC, sugars were assayed directly on silica gel with 1 ml of sulfuric–orcinol reagent. This reagent consisted of 2 mg of orcinol (recrystallized from benzene) per ml conc. H_2SO_4–water 1:0.5. The reagent was chilled (>0°C) for 1 hr before it was used. Undiluted concentrated H_2SO_4 could not be used because it resulted in a diminished absorbance. The test tubes were then warmed to 80°C for 20 min. After centrifugation (2,000 rpm for 2 min), the absorbance was measured at 505 nm. Standards contained 1, 2, and 3 μg of galactose (20 μg galactose per ml in chloroform–methanol) and were evaporated to dryness. Aliquots of the lipid extract, equivalent to about 0.8 μg of hexoses were evaporated to dryness in the presence of 1, 2, and 3 μg of galactose from the standard solution. Hexoses were then determined as described above.

Ganglioside determination. Gangliosides were estimated according to modified methods of Svennerholm (16) and Miettinen and Takki-Luukkainen (17). Spots corresponding to gangliosides were scraped off the plate and extracted with 2 × 0.5 ml of water. To 0.4 ml of this extract in a test tube was added 0.4 ml of resorcinol reagent. This reagent consisted of 10 ml of a 3% resorcinol solution in water, 80 ml of concentrated HCl, 0.25 ml of 0.1 M $CuSO_4$, and water up to 100 ml. The test tubes were heated to 100°C for 30 min. After cooling, 0.8 ml of a mixture of butyl acetate–*n*–butanol 85:15 was added to each tube. Absorbance of the organic phase was measured at 580 nm. Standards of 2.5, 5, and 7 μg of NANA (50 μg NANA/ml in water) were also analyzed. Determinations were also carried out on the total lipid extract. Aliquots corresponding to about 1 μg of NANA were sampled and NANA was determined in the presence of 2.5, 5, and 7.5 μg of standard as described above.

RESULTS

Lipid extract

Different conditions of extraction involving time of sonication, amount of solvent, and number of extractions were tested. The best results were obtained by sonicating fresh tissue for six 30-sec periods in chloroform–methanol 1:1 (20 ml/g) without solvent changes between each sonication. With three 30-sec sonications, 97% of the total lipids were obtained. Lyophilized samples could be used but the presence of water was needed in the extraction media, i.e., chloroform–methanol–water 70:30:4. These results were identical to those obtained by homogenization of the fresh tissue in chloroform–methanol 2:1 and extraction of lipids by stirring for three 30-min periods at 4°C.

Separation of lipids by TLC

The system used was two-dimensional with multiple development (**Fig. 1**). The first migration in chloroform–methanol–water 70:30:4 allowed a separation of most of the lipid classes except one group comprising PC, PS, PI and another containing gangliosides and proteolipids. The second chromatography separated PC from PI + PS. The third system separated sulfatides, cerebrosides, cholesterol, and gangliosides from proteolipids. The fourth chromatography separated PI and PS and improved the separation of sulfatides from PE. Cerebrosides yielded three spots in this system.

Quantification of lipids

Cholesterol determination. Evaporation of the lipid extract to dryness was unnecessary for cholesterol determination. The $FeSO_4$ solution was not saturated and the amount of 36 N H_2SO_4 was increased (0.6 ml instead of 0.4 ml). The absorption spectra showed a maximum at 460 nm and not at 490 nm as previously described (12).

Galactose determination. The orcinol reagent used for galactose determination was slightly different from that used in the method of Neskovic et al. (14), which utilized 1 mg/ml in 36 N H_2SO_4–water 1.4:0.6. The best results were obtained with an orcinol concentration of 2 mg/ml in 36 N H_2SO_4–water 1:0.5.

Ganglioside determination was always carried out on a total lipid solution of a concentration not above 5 mg/ml (concentration of total lipid in the extract) because of the low solubility of gangliosides in chloroform–methanol 1:1. It was important not to heat longer than 30 min at 100°C; after that there was formation of a degradation product that absorbed at 440 nm. Other lipids, especially cerebrosides, did not interfere with ganglioside determination. Higher values for NANA in the total lipid extract could occur from nonganglioside sialic acid related to protein extraction by methanol. However, proteolipids were separated from gangliosides by the TLC procedure.

Phospholipids. The method that was modified the most was the mineralization procedure in the determination of phospholipids. Mineralization of dry material for 30 min to 2 hr at 500°C resulted in the same amount of P in the total lipid extract as mineralization in the presence of 1 ml of concentrated HNO_3, $HClO_4$, or H_2SO_4 for 1.5 hr. Dry mineralization was therefore chosen as the simplest and most adequate method. Total mineralization seemed to be obtained by this technique. This was confirmed by studying standard ethanolamine phospholipids, mouse brain lipid extract, peripheral nerve lipid extract, and phospholipids separated by TLC. These results were linear up to 3 μg of P.

Sensitivity. The minimum amounts of materials that could be determined accurately (absorbance 0.05) were 5 μg for cholesterol, 0.1 μg for P, 0.8 μg for hexose, and 1 μg for NANA.

Applications to mouse and rat brain

These procedures were tested on mouse and rat brains and the results obtained were compared to those found in the literature. For mouse brains, 15 experiments were performed on brains of 50 C57 black mice; only animals of the same brain weight (435 mg $\mp$ 10) were used. Results are reported in **Table 1;** no comparative data exist for cerebrosides, sulfatides, or neuraminic acid content in mouse brain. For rat brains, 12 experiments on three male Sprague-Dawley rats (42 days old) were carried out. Only data that could be compared with literature values are presented in **Table 2.** For rat and mouse brains, results are in good agreement with those previously published.

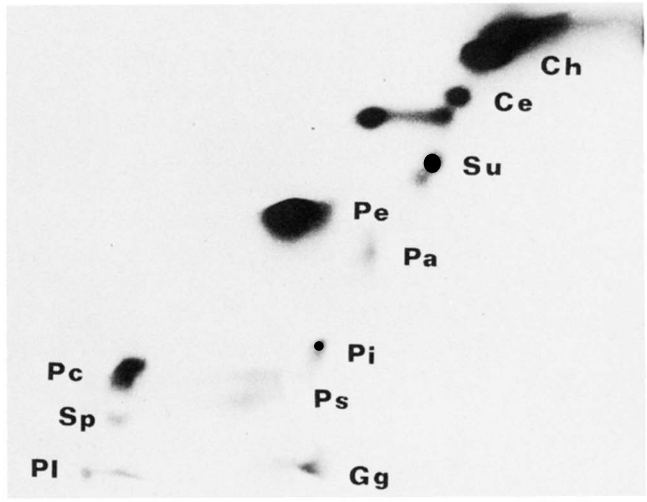

Fig. 1. TLC of total adult mouse brain lipid extract. For conditions, see Methods. Ch, cholesterol; Ce, cerebrosides; Su, sulfatides; Pe, phosphatidylethanolamine; Pa, phosphatidic acid; Ps, phosphatidylserine; Pi, phosphatidylinositol; Pc, phosphatidylcholine; Sp, sphingomyelin; Gg, gangliosides; Pl, proteolipids.

DISCUSSION

Lipid extraction procedures have been well reviewed (18, 19). Extraction with chloroform–methanol appears to be the best general method for extracting wet tissues. Heat and agitation with an ultrasonic vibrator have been reported (19, 20) but not in detail. In this study, different conditions of sonication have been examined. On the other hand, it has been reported that dry tissues (lyophilized) should probably be rehydrated before extraction (19). Lyophilization usually results in a decreased extraction of very polar lipids, especially the gangliosides (0.45 μg NANA/mg wet weight after lyophilization vs. 0.8 μg without lyophilization). Finally, it has been assumed that the protein–lipid complexes that are extracted from the tissues are decomposed by taking the extract down to dryness (21). However, no differences were found between the evaporated and nonevaporated extracts after TLC, assuming that proteolipid complexes stay at the origin of the plates.

Usually, separation of the different lipid fractions is carried out by the use of small silicic acid columns, followed by quantitative examination by TLC of each lipid fraction from the column, sometimes after additional procedures, e.g., hydrolysis (2). Our method necessitates multiple development of a single thin-layer plate. With a 10 × 10 cm plate, it takes about 1 hr to obtain complete development of the chromatogram. The major classes of lipids can be separated, including separation of PS, PI, and gangliosides. Thereafter, without extraction procedures

TABLE 1. Lipid analysis of mouse brain

	Present Study	Data from Previous Studies: Jacque (24) Baumann et al. (25, 26)	Data from Previous Studies: Dawson and Clarke (27)
Total lipid extract (mg/brain)	40 ∓ 1.5	40	
Cholesterol (μg/mg wet weight)	19.1 ∓ 0.5	20.5 ∓ 0.8	
Phosphorus (μg/mg wet weight)	2.05 ∓ 0.09		2.1 ∓ 0.06
PE	0.68 ∓ 0.03		
PC	0.78 ∓ 0.04		
PS	0.15 ∓ 0.02		
PI	0.29 ∓ 0.04		
PA	0.07 ∓ 0.015		
Sph	0.09 ∓ 0.015		
Phosphorus (% of total)			
PE	33		35
PC	37		37
PS	14		13
PI	7		6
PA	3		2.5
Sph	4.5		5
Hexoses (μg/mg wet weight	2.3 ∓ 0.5	2.3 ∓ 0.2	
Hexoses (% of total)			
Cerebrosides	64	78	
Sulfatides	14		
Gangliosides	22	22	
NANA (μg/mg wet weight)	0.82 ∓ 0.04		

Fifteen experiments were performed on 50 adult C57 black mice. Variation between brain weight was less than 2%. Results expressed in μg/mg wet weight are the mean values ∓ SD.

(except for cholesterol and NANA), lipids can be quantified directly.

The higher sensitivities of our quantification techniques are achieved mainly by scaling down the existing colorimetric methods. The direct mineralization procedure for phospholipid determination is very convenient. Because of the relatively high sensitivity of the phosphorus determination, if only phospholipids have to be analyzed, less than 100 μg of lipid extract is needed.

The TLC technique still allows gas–liquid chromatography, i.e., analysis of the fatty acids present in each lipid.

A comparison of our results with those in the literature for mouse and rat brain clearly indicates the accuracy and the reproductibility of our methods.

The techniques described here do not claim to give a detailed analysis of each type of lipid in brain or in peripheral nerve. In the literature one can find methods that may be more sensitive for one particular class of lipids, e.g., phospholipids. Our techniques seem to have the advantage of allowing a rapid and complete qualitative and quantitative analysis of the main lipid classes in a small sample (around 500 μg of lipid). They can be applied to study regional differences in the central nervous system and to analyze cell membranes or subcellular particles (myelin (22), for instance). For the studies of pathological biopsies, our methods can accurately supply information concerning lipid abnormalities, even in peripheral nerve (23).

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TABLE 2. Lipid analysis of rat brain

	Present Study	Data from Previous Studies: Wells and Dittmer (28)	Wuthier (29)	Neskovic et al. (14)	Hauser (30)	Tettamanti (31) Spence and Wolfe (32)
Cholesterol (μg/mg wet weight)	15.2 ∓ 0.6	15.3				
Phospholipids (P) (μg/mg wet weight)	1.92 ∓ 0.08	2.1				
Phosphorus %						
PE	36	38	36			
PC	40	38	37			
PS	10	12	12			
PI	6	4	3			
PA	1	1.8	1.2			
Sph	7	5.5	6			
Cerebrosides %	80	85		77	83	
Sulfatides %	20	15		23	17	
NANA (μg/mg wet weight)	0.68 ∓ 0.08	0.64				0.62

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