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Note

Separation of diacyl and plasmalogen phospholipids of rat brain synaptosomal membranes on chromarods

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Plasmalogen phospholipids are important components of central nervous system membranes[1], red blood cells[2] and many other vertebrate tissues[3]. Such phospholipids are difficult to separate and quantify as they chromatograph with their diacyl form. Separation of the two species involves mild acid hydrolysis and extensive thin-layer chromatography (TLC).

Separation of polar lipids on silica-coated quartz rods coupled with a flame ionization detector (FID) has already been reported for some lipid mixtures[4,5]. We wish to report a simple and fast method for quantifying synaptosomal lipids containing both diacyl- and 1-alk-1[1]-enyl, 2-acyl-phosphatidylethanolamine. The present report is an adaptation of the reactional two-dimensional TLC system of Horrocks[6] replacing up to three TLC stages and quantitation of separated phospholipids by inorganic phosphorus determination. The application of this method to complex lipid extracts of biological origin containing such plasmalogen phospholipids is demonstrated.

EXPERIMENTAL

Synaptosomal membranes were isolated from rat brain[7] and total lipids extracted with 20 volumes of chloroform–methanol (2:1) followed by 20 volumes of chloroform–methanol–28% aqueous ammonia (35:5:2). Extracts were evaporated to dryness at 40°C under nitrogen and dissolved in 300 μl chloroform, containing 10 mg/ml nonadecane as an internal standard, to give a final phospholipid concentration of *ca.* 5 mg/ml. Chromarods (type S, Technical Marketing, Mississauga, Canada) were stored in 10 *N* sulphuric acid and washed at least four times in distilled water prior to use. The rods were dried 5 min at 110°C and then activated by passage through the FID (Iatroscan Model TH10 obtained from Technical Marketing). Lipid extracts (1 μl) were applied to each chromarod using a 1-μl syringe (Precision Sampling, Baton Rouge, LA, U.S.A.). The rods were developed 20 min in light petroleum (b.p. 50–110°C)–diethyl ether (85:15) to separate neutral lipids and the nonadecane internal standard. The rods were air dried 5 min, then dried 5 min at 70°C and transferred to the FID scanning frame. Rods were scanned to the beginning of the phospholipid band which remained at the origin of the chromarod.

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Half the rods were then placed in a glass frame and exposed to hydrochloric acid fumes for 5 min by positioning the frame over concentrated hydrochloric acid in a TLC tank in such a way that the phospholipid band was approximately 4–5 cm from the level of the acid. The rods were dried 5 min at 70°C and then reactivated by scanning the upper portion of the rod a second time. Phospholipids were separated by development for 40 min in chloroform–methanol–water (80:35:3). The rods were air dried 5 min, then oven dried 5 min at 70°C and scanned in full.

Chromarods were scanned through the FID at 30 sec per rod utilizing Gear No. 40. The hydrogen flow-rate was 80 ml/min and the air flow-rate 2 l/min. The FID was used in combination with a Hewlett-Packard (Avondale, PA, U.S.A.) 3390A reporting integrator. This integrator was also used to plot chromatograms at a chart speed of 10 cm/min, peak width 0.01, threshold 3 and attenuation 6 utilising a full scale deflection of 64 mV (Fig. 1).

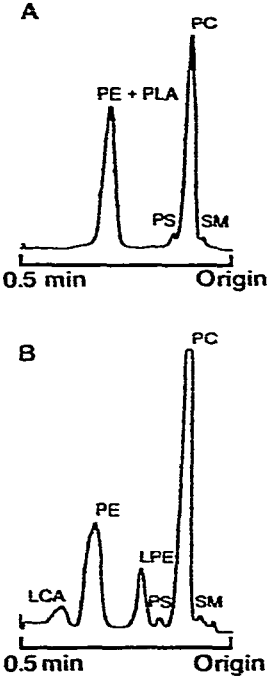

Fig. 1. Phospholipid separation from a sample of rat brain synaptosomal membrane on Chromarod S rods. The sample in Fig. 1B was exposed to HCl fumes prior to development on the chromarod as explained in the text. LCA = Long-chain aldehydes; PE = phosphatidylethanolamine; PLA = ethanolamine plasmalogen; LPE = lysophosphatidylethanolamine; PS = phosphatidylserine; PC = phosphatidylcholine; SM = sphingomyelin.

The FID response to each lipid was determined from purified standards. The concentration of each standard was confirmed by phosphorus analysis[8].

RESULTS AND DISCUSSION

Following acid hydrolysis of the phospholipid mixture two new peaks appear

on the chromatogram: lysophosphatidylethanolamine and long chain aldehydes. The phosphatidylethanolamine peak was also reduced in size relative to phosphatidylcholine. The phospholipids were hydrolysed for up to 10 min but no increase in the plasmalogen: phosphatidylethanolamine ratio was observed after the first few minutes of hydrolysis. This chromatographic system is capable of separating neutral lipids, phosphatidylethanolamine, lysophosphatidylethanolamine, phosphatidylserine, phosphatidylcholine and sphingomyelin within 3 hours of lipid extraction (Fig. 1A and B).

Conventional methods for separation of lipid mixtures containing plasmalogens are considerably more time consuming. Phospholipids are separated from neutral lipids and then subjected to 2-dimensional TLC. The total phosphatidylethanolamine fraction may then be acid hydrolysed and the resulting mixture of aldehydes, phosphatidylethanolamine, and lysophosphatidylethanolamine separated by further TLC[2]. Alternatively the two-dimensional reactional TLC of Horrocks[6] may be employed where phospholipids are acid-hydrolysed after separation in the first dimension. These TLC methods have been well characterized but require larger quantities of membrane lipid, involve several TLC stages, loss of lipid during recovery from TLC plates and chemical analysis for inorganic phosphorus.

Because of rod variability response factors were calculated for each lipid on each rod (Fig. 2). The standard deviation of FID response to 10 μg nonadecane was 13.7% when data from all 10 rods were used but was 3–5% when a single rod was examined. For this reason regression equations were routinely calculated for each lipid type on each rod (Fig. 2). Values of $r \geqslant 0.99$ indicate that these analyses are highly reproducible within the lipid concentrations tested.

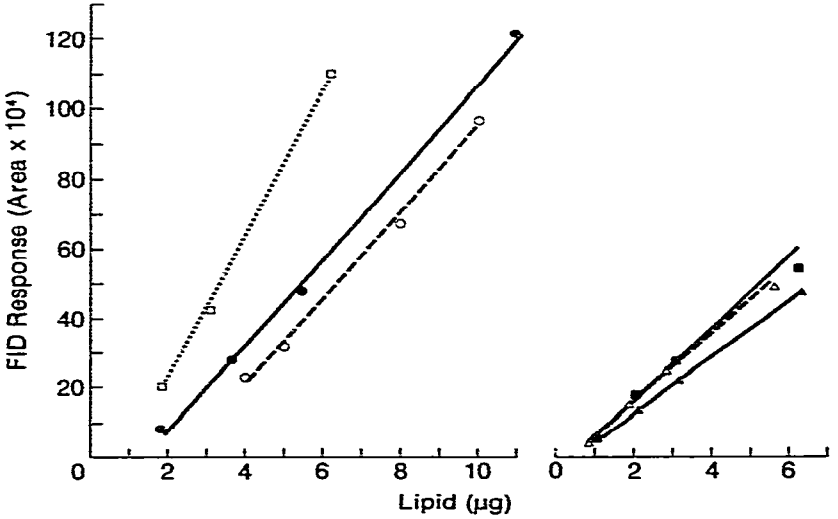

Fig. 2. Linearity of the response of different neutral lipids and phospholipids. □, cholesterol; △, phosphatidylcholine; ■, sphingomyelin; ●, phosphatidylethanolamine; ▲, lysophosphatidylethanolamine; ○, nonadecane. The following regression equations were determined for each line and in each case $r \geqslant 0.99$ for $n = 10$. □, $y = 20.92x - 20.87$; △, $y = 10.86x - 5.8$; ■, $y = 10.94x - 6.38$; ●, $y = 12.36x - 17.59$; ▲, $y = 8.23x - 4.07$; ○, $y = 12.47x - 29.25$.

TABLE I

POLAR LIPID COMPONENTS IN SYNAPTOSOMAL MEMBRANES OF WEANLING RATS

Values represent the average ± s.d. for three separate synaptosomal preparations from weanling rats. Each sample was analysed in triplicate.

Lipid	*nmoles lipid/mg synaptosomal protein*
Phosphatidylcholine	284 ± 26.6
Sphingomyelin	79 ± 6.7
Phosphatidylethanolamine	198 ± 14.3
Plasmalogen (ethanolamine)	168 ± 10.9
Phosphatidylserine	<50
Cholesterol	480 ± 32

Quantitative application of this method of analysis is illustrated for syaptosomal membrane prepared from brains of three groups of weanling rats (Table I). The speed and reproducibility of this method of analysis indicates that it has quantitative advantages for routine accurate determination of naturally occurring polar lipid constituents present in complex lipid extracts of biological origin.

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