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Analysis of complex mixtures of phospholipid classes from cell membranes using two-dimensional thin-layer chromatography and scanning laser densitometry

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ABSTRACT

Increasing recognition of the important roles served by membrane phospholipids in cellular metabolic and signal transduction processes has stimulated interest in examining potential phospholipid abnormalities in patients with psychiatric disorders. This report describes a method, based on several novel modifications of existing techniques, for concurrently analyzing nanomolar amounts of nine phospholipid classes in a single aliquot of membrane extract. With this method, diverse phospholipid classes are first separated by two-dimensional thin-layer chromatography, and then determined using two-dimensional scanning laser densitometry. The method is able to quantitate even small amounts of specific phospholipid classes, corresponding to < 10 ng of lipid phosphorus. The sensitivity of this method allows it to be readily applied to clinical studies involving membranes from cell types that are obtainable only in small quantities.

INTRODUCTION

Cell membrane phospholipids have recently become of interest to investigators studying psychiatric conditions such as bipolar (manic-depressive) disorder and schizophrenia. These membrane components have been reported to have altered composition in erythrocytes (red blood cells, RBCs) from psychiatric patients [1–3], to affect important membrane transport processes [4,5], and (for phosphoinositides) to have a central role in neuronal signal transduction [6–9]. In addition, the antimanic drug lithium interacts directly with membrane phospholipids [10,11], affects phospholipid metabolism [6,9,12,13], and may dampen phosphoinositide-related neuronal signal transduction [14–18]. A variety of specific phospholipid classes have been

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implicated in the studies noted above, including those with headgroups consisting of: (1) choline [phosphatidylcholine (PC), lysophosphatidylcholine (LPC), and sphingomyelin (Sm)]; (2) amino compounds [phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LPE), and phosphatidylserine (PS)]; and (3) inositol [phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP), and phosphatidylinositol 4,5-bisphosphate (PIP_2)].

These various classes of phospholipids have different physicochemical properties. For example, the polyphosphoinositides PIP and PIP_2 are much more polar than the others. In addition, the various phospholipid classes naturally occur in substantially different quantities in the RBC and platelet membrane (ranging from $<1\%$ to $>40\%$ of the total molar phospholipid content). Because of these factors, it is difficult to separate and quantitate the diverse phospholipid classes derived from RBC and platelet membrane sources in any single analytical system.

For our clinical studies of mood disorders in psychiatric patients, it was of interest to measure a comprehensive group of choline, amino, and inositol phospholipids. We were unable to find any published method that could be used to quantitate all of the phospholipids of interest from clinically available amounts of membrane sample. Therefore, we developed a novel method for analyzing nanomolar amounts of nine diverse phospholipid classes in a single aliquot of membrane extract. With this method, which is based on several modifications of existing techniques, the phospholipids being investigated are concurrently separated by two-dimensional thin-layer chromatography (TLC) on high-performance plates, and then quantitated via two-dimensional scanning laser densitometry.

EXPERIMENTAL

Reagents

Phospholipid standards, tris(hydroxymethyl) aminomethane (Tris) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffers, and acid molybdate reagent for phosphorus analysis were purchased from Sigma (St. Louis, MO, USA). Chromatography solvents and other chemicals (analytical-reagent grade) were purchased from Fisher Scientific (Pittsburgh, PA, USA).

Preparation of RBC membranes

Freshly collected venous blood, anticoagulated with sodium heparin, was centrifuged at 1600 *g* and 4°C for 15 min. The RBCs were separated from other blood components, washed twice with ten volumes of ice-cold 110 m*M* $MgCl_2$, and then three times with 172 m*M* Tris buffer (to give pH 7.6 at 4°C). Each wash was followed by centrifugation at 1600 *g* and 4°C for 10 min. The washed cells were then hemolyzed by forceful addition of twenty volumes of ice-cold 11 m*M* Tris buffer (pH 7.6 at 4°C), placed in an ice bath for 10 min to allow the completion of hemolysis, and subsequently centrifuged at 20 000 *g* and 4°C for 10 min. The supernatant was carefully removed, and the sedimented membranes washed four times with twenty volumes of the ice-cold hemolyzing solution. Each wash was followed by centrifugation at 20 000 *g* and 4°C for 10 min. Membranes prepared in this way are flocculent and white in color, with no residual hemoglobin present. After completion of the final wash, the membranes were resuspended in a solution consisting of 320 m*M* sucrose and 5 m*M* HEPES buffer (to give pH 7.8 at 4°C), and stored at −70°C.

Preparation of platelets

A 34-ml volume of whole blood was drawn into a plastic syringe containing 6 ml of acid citrate dextrose anticoagulant (2.5 g of sodium citrate, 1.5 g of citric acid, and 2 g of glucose in 100 ml of water, final volume). The blood was centrifuged at 142 *g* for 20 min (all centrifugations were at 4°C) to obtain platelet-rich plasma (PRP); the PRP was then centrifuged at 570 *g* for 15 min to obtain a platelet pellet. The pellet was washed and resuspended in Ringer's citrate dextrose buffer [110.4 m*M* NaCl, 3.75 m*M* KCl, 1.67 m*M* sodium bicarbonate, 21.2 m*M* sodium citrate, and 0.5% (w/v) dextrose], containing 0.2 m*M* acetylsalicylic acid to prevent platelet aggrega-

tion. The suspension was centrifuged at 208 *g* for 3 min to remove any remaining RBCs, and the suspended platelets were then pelletted by centrifugation at 570 *g* for 10 min. The final platelet pellet was resuspended in HEPES–ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA) buffer [10 m*M* HEPES, 145 m*M* NaCl, 5 m*M* KCl, 1 m*M* $MgSO_4$, 0.5 m*M* sodium hypophosphate, 6 m*M* glucose, and 1 m*M* EGTA (pH 7.4)], containing 0.2 m*M* acetylsalicylic acid, and stored at −70°C [19].

Extraction of membrane phospholipids

Prior to extraction, the frozen, suspended RBC membranes were thawed slowly at room temperature, separated from the sucrose–HEPES solution by centrifugation at 20 000 *g* and 4°C for 15 min, and then washed once with twenty volumes of 10 m*M* HEPES buffer (to give pH 7.8 at 4°C). Phospholipids were obtained by biphasic extraction and partitioning of the washed RBC membrane preparations or thawed platelet suspensions. Erythrocyte membranes (300 μl) or thawed platelet suspension (1–2 ml) were added dropwise to 12 ml of methanol–chloroform–HCl (100:50:1) and stirred for 20 min under a nitrogen atmosphere (all steps from this point were performed under nitrogen). This was followed by addition of 4 ml of water and then 4 ml of chloroform, with subsequent stirring for 20 min. The phases were separated by centrifugation at 1000 *g* for 10 min, and the upper phase was removed and re-extracted with 8 ml of chloroform. The lower phases were combined, and the solvents removed in a rotary evaporator. The residue was quantitatively transferred to a vial using 500 μl of chloroform, which was then evaporated with a stream of nitrogen. The extracted phospholipids were dissolved in 25 μl of chloroform–methanol (1:1) and stored under nitrogen at −70°C. Recovery of total phosphorus from all origins in the membrane using this method was 70–80% (based on comparison of phosphorus content of washed membranes *versus* total lipid extract).

Separation of phospholipids by TLC

High-performance thin-layer chromatography (HPTLC) plates were utilized (rather than standard TLC plates) because of improved separation and because the high-performance plates produce much more uniform background readings during laser densitometry, in which the detector measures transmitted rather than reflected light. These plates consisted of a layer of silica gel adsorbent (200 μm in thickness, 5 μm particle size) and an inert organic binder (LHP-K plates, Whatman, Hillsboro, OR, USA). The plates were 10 cm × 10 cm in size with a pre-adsorbent band on one side for sample application. The HPTLC plates were washed twice with methanol and dried. Then, in order to facilitate resolution of the polyphosphoinositides (see Discussion), the plates were dipped in a solution of 1% potassium oxalate dissolved in methanol–water (3:2) containing 2 m*M* EDTA, and then dried again.

Aliquots of the lipid extracts were used for total lipid phosphorus determinations [20] and for microanalysis of phospholipid classes by TLC according to a modification of the method of Yao and Rastetter [21], which includes a modified form of the charring procedure originally described by Fewster *et al.* [22]. Prior to being developed, the plates were activated by heating to 110°C for 15 min. Samples of lipid extract (approximately 2.5 μl) containing 1.8–2.0 μg of phosphorus were applied to quadruplicate HPTLC plates with a 10-μl Hamilton syringe. The material was applied in successive, superimposed drops, at a location 1.7 cm from the lower edge and 1.7 cm from the side of the plate. The plates were developed at room temperature, in tanks that were lined with filter paper saturation pads (Analtech, Newark, DE, USA), and that had been allowed to saturate with solvent vapors for 60 min. The first dimension was initially performed using chloroform–methanol–4.3 *M* NH_4OH (90:65:20) to resolve the polyphosphoinositides. The solvent front was allowed to ascend to a score line that had been traced by removing a thin line of silica gel parallel to and 0.5 cm below the top edge of the plate. The plates were then dried and developed a second time in the first dimension with chloroform–methanol–concentrated NH_4OH (130:50:10), in order to

separate LPE from PC. Next, the plates were rotated 90°, score lines were traced in the silica gel along the interface between the pre-adsorbent and the silica gel adsorbent, as well as 0.5 cm from and parallel to the top of the rotated plate, and the plates were then developed in the second dimension with chloroform–methanol–acetic acid–water (100:30:35:3) to resolve the remaining phospholipid classes. After being developed in each solvent system, the plates were dried thoroughly before proceeding to the next step.

Densitometry and quantitation

The separated phospholipids were visualized by charring; the plates were saturated with 10% cupric sulfate in 8% phosphoric acid, then heated to 180°C for 10 min [21]. The charred phospholipid spots were scanned in two dimensions with a laser densitometer (LKB Ultroscan XL, Pharmacia-LKB, Bromma, Sweden). The scanning patterns were adjusted to collect from 10 000 to 30 000 readings of optical density, at uniform intervals, from each phospholipid spot and surrounding border of silica gel. The spots for the various classes of phospholipids differed in area and intensity of charring, according to the quantity of phospholipid present. Therefore, readings with the 100-μm-diameter laser light beam were taken using coordinates ranging from 20 μm × 40 μm (*i.e.*, multiply overlapped areas) for the smallest, lightest spots to 80 μm × 160 μm for the larger, darkly charred spots. The data were serially transmitted to a microcomputer (delta binary data format) and stored on magnetic media. The absorbance for each phospholipid class was determined from an algorithmically calculated volume term (LKB GelScan XL software, version 1.3) based on the area of each spot and the measured absorbances within that area, and expressed as absorbance units times area (AU · mm^2). Standards for all of the studied phospholipids were developed and charred in a similar manner.

The linearity of the two-dimensional densitometric quantitation over a range of phospholipid concentrations was assessed under applicable experimental conditions by applying 0.6, 0.9, 1.2 and 1.5 volumes of a lipid extract to chromatography plates, which were then developed and scanned. The correlations between the applied and measured amounts of phospholipids were strong, ranging from 0.976 ($p < 0.025$) for PIP_2 to 0.999 ($p < 0.001$) for PS.

To quantitate the relationship between relative absorbance percentage and molar percentage for the individual phospholipid classes, we measured phospholipids from RBC membranes both with the densitometric technique and by means of phosphorus analysis of the individual phospholipid spots. In the latter case, the spots were visualized with iodine, scraped from the plates, digested with perchloric acid, and then assayed for phosphorus spectroscopically with acid molybdate reagent [20]. The relative absorbance percentage determined densitometrically was similar to the molar percentage based on direct measurement of lipid phosphorus. For the individual phospholipid classes, the differences in observed percentage phospholipid composition were as follows (molar percentage minus absorbance percentage): PC = 1.07%; LPC = 1.56%; Sm = −4.94%; PE = 1.68%; LPE = 6.26%; PS = 0.58%; PI = −0.34%; PIP = −0.38%; PIP_2 = −0.97%.

The parallel densitometric and phosphorus-based analyses described above were also used to calculate the molar absorbancies of the individual phospholipid classes. This factor was then utilized to translate the absorbance values to more precise molar quantities for each class of phospholipids. The molar absorbancy values for the individual phospholipid classes were as follows [in (AU · mm^2)/nmol]: PC = 1.39; LPC = 0.66; Sm = 1.72; PE = 1.61; LPE = 0.59; PS = 1.51; PI = 2.13; PIP = 3.79; PIP_2 = 3.67.

RESULTS

With the method described in this paper, we were able to effectively separate PC, LPC, Sm, PE, LPE, PS, PI, PIP, and PIP_2, on a single chromatography plate, despite the markedly greater polarity of the polyphosphoinositides (PIP and PIP_2) as compared to the other studied phospho-

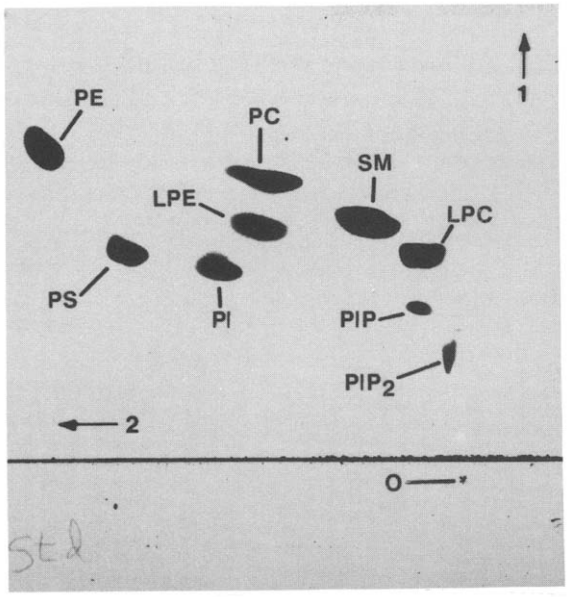

Fig. 1. Charred HPTLC plate showing separation of a phospholipid standard mixture by two-dimensional chromatography. Solvent systems were as described in Experimental. Arrow 1 indicates the direction for the first dimension, and arrow 2 for the second dimension. PC = phosphatidylcholine; LPC = lysophosphatidylcholine; SM = sphingomyelin; PE = phosphatidylethanolamine; LPE = lysophosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PIP = phosphatidylinositol 4-phosphate; PIP_2 = phosphatidylinositol 4,5-bisphosphate; O = origin).

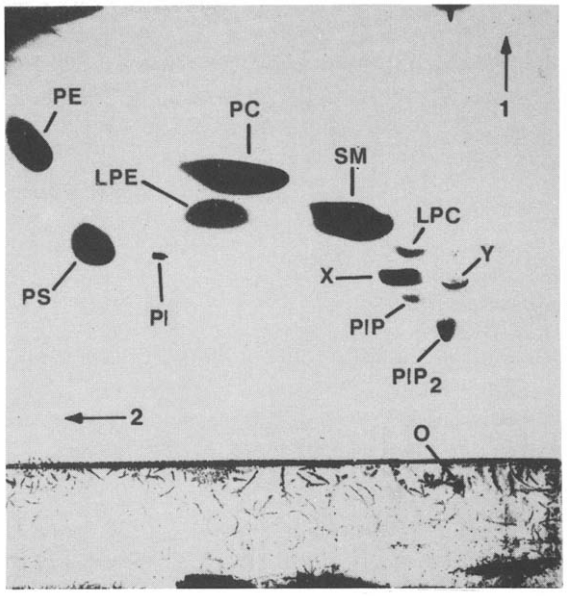

Fig. 2. Charred HPTLC plate showing separation of a complex mixture of lipid classes extracted from RBC membranes, using the same solvent systems and procedures as shown in Fig. 1. The directions for the first and second dimensions are indicated by arrows. X = unknown substance in the extract that contains no phosphorus; Y = an additional unknown density located at the second-dimension origin of X; for other abbreviations, see Fig. 1 legend.

lipids. Fig. 1 is a photograph of an HPTLC plate, showing a separation performed with phospholipid standards. Fig. 2 presents a representative charred HPTLC plate following chromatographic separation of an RBC membrane extract, and Fig. 3 shows a corresponding separation performed with a platelet extract. We were able to quantitate the various phospholipid classes in their natural range of occurrence in RBC membranes (from $<1\%$ to $>25\%$ of the total phospholipid content) and platelets (from $<1\%$ to $>40\%$ of the total phospholipid content), with actual amounts of the minor components corresponding to less than 10 ng of lipid phosphorus. The mean coefficients of variation for determination of phospholipid composition from quadruplicate or triplicate HPTLC plates were 3.73 and 6.26% for the quantitatively major phospholipids (PC, Sm, PE, PS) in RBC and platelet ex-

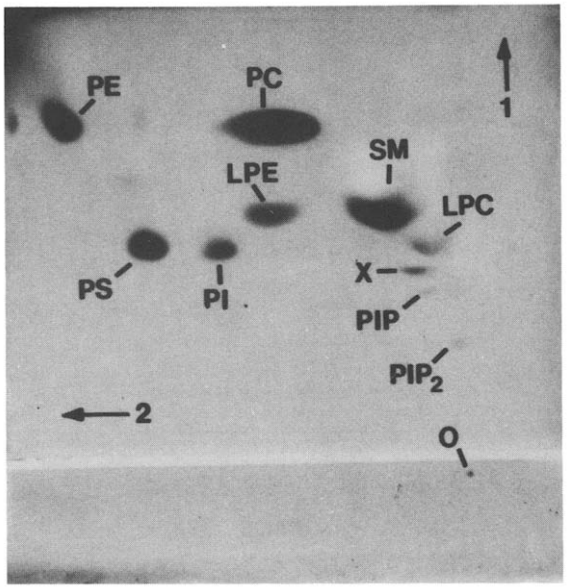

Fig. 3. Charred HPTLC plate showing separation of a complex mixture of lipid classes extracted from platelets, using the same solvent systems and procedures as for standards shown in Fig. 1. The directions for the first and second dimensions are indicated by arrows. X = unknown substance in the extract that contains no phosphorus; for other abbreviations, see Fig. 1 legend.

TABLE I

RELATIVE PHOSPHOLIPID COMPOSITION OF ERYTHROCYTE MEMBRANES FROM CONTROL SUBJECTS

Phospholipid class	Molar percentage of phospholipids (mean ± S.D.)		
	Two-dimensional system[a] (n = 11)	One-dimensional system[b] (n = 12)	Mitchell *et al.* [26] (n = 32)
Phosphatidylcholine	28.7 ± 1.6	28.5 ± 1.9	28 ± 2
Lysophosphatidylcholine	2.43 ± 1.65	–	1 ± 1
Sphingomyelin	24.6 ± 2.6	27.5 ± 2.7	25 ± 3
Phosphatidylethanolamine[c]	30.2 ± 2.3	30.4 ± 1.6	26 ± 2
Phosphatidylserine	12.4 ± 1.2	12.5 ± 1.6	15 ± 2
Phosphatidylinositol	0.62 ± 0.21	2.4 ± 1.1	1.9 ± 0.7
Phosphatidylinositol 4-phosphate	0.20 ± 0.13	–	0.9 ± 0.4
Phosphatidylinositol 4,5-bisphosphate	0.98 ± 0.26		1.5 ± 0.4

[a] Using acidic extraction of washed membranes from hemolyzed RBCs.

[b] Using neutral extraction of intact RBCs; this method does not recover polyphosphoinositides.

[c] Sum of PE + LPE (see text).

tracts, respectively, and 11.4% (RBC) and 11.5% (platelet) for the much less abundant phosphoinositides. These values relating to reproducibility of the assay are closely comparable to those reported by other investigators using nano-scale densitometric analysis [23]. The mean coefficients of variation for replicate extractions and analyses of the same sample on different days were 8.12% for the major phospholipids and 13.2% for the phosphoinositides.

The first column of Table I presents the relative composition of phospholipid classes in RBC membrane extracts from eleven normal control subjects. We compared our two-dimensional method with results obtained after neutral extraction of intact RBCs [24], followed by one-dimensional separation and laser densitometry according to a previously described method [21]; these comparative data are presented in the second column of Table I. There was generally good

TABLE II

RELATIVE PHOSPHOLIPID COMPOSITION OF PLATELET MEMBRANES FROM CONTROL SUBJECTS

Phospholipid class	Molar percentage of phospholipids (mean ± S.D.)	
	Two-dimensional system (n = 5)	Mitchell *et al.* [26] (n = 4)
Phosphatidylcholine	42.6 ± 1.9	36 ± 5
Lysophosphatidylcholine	3.51 ± 1.6	1 ± 1
Sphingomyelin	16.7 ± 1.8	19 ± 4
Phosphatidylethanolamine[a]	24.5 ± 8.0	27 ± 5
Phosphatidylserine	10.2 ± 1.9	12 ± 2
Phosphatidylinositol	2.36 ± 0.87	4.5 ± 0.9
Phosphatidylinositol 4-phosphate	0.11 ± 0.05	0.9 ± 0.4
Phosphatidylinositol 4,5-bisphosphate	0.08 ± 0.05	0.4 ± 0.2

[a] Sum of PE + LPE (see text).

agreement between the two methods for those phospholipids that were measured in both systems, despite the difference in extraction techniques. In addition, the relative amounts of both major and minor phospholipids were generally similar to those reported by other investigators using comparable extraction techniques [25,26] (see third column of Table I), despite the fact that the cells were processed very differently prior to extraction.

The first column of Table II presents the relative composition of phospholipid classes in platelet extracts from four normal control subjects (different from those subjects in the RBC studies). The second column of Table II lists corresponding values obtained from platelet extracts of four normal control subjects from a study by Mitchell *et al.* [26], in which radiolabeling with ^{32}P and quantitation by phosphate assay were utilized. The relative amounts of each of the quantitated phospholipids are generally comparable, again despite significant differences in pre-extraction cell processing.

DISCUSSION

The method described here has several advantages for the study of phospholipids from cell membranes. First, diverse mixtures of phospholipid classes, including strongly polar polyphosphoinositides, can be separated on a single HPTLC plate. Second, the method is highly sensitive and produces satisfactory results with nanomolar quantities of phospholipids. Finally, the various classes of phospholipids that are present in substantially different amounts in membrane extracts can be quantitated and directly compared in the same sample.

Sun and Lin [27] recently described an improved two-dimensional chromatographic procedure similar to the one reported here, and used this to investigate polyphosphoinositides together with major phospholipids. However, their method was not applied to lysophospholipids such as LPC and, moreover, these investigators relied on radiolabeling of the phospholipids for quantitation, an approach that is not readily adaptable to studies of clinically determined alterations in phospholipid classes. Yamamoto *et al.* [28] reported a technique for reflectance imaging densitometry of phospholipids separated two-dimensionally on a TLC plate, that produced high accuracy and reproducibility. Their work provides strong support for the use of two-dimensional densitometric analysis of phospholipid mixtures. However, they did not report the application of their method to the study of either polyphosphoinositides or lysophospholipids. Mitchell *et al.* [26] described a two-dimensional technique for separating all of the commonly encountered major and minor phospholipids, but their method was based on the use of a particular silica gel plate having an organic binder that could not be utilized for charring and subsequent quantitative densitometry. Moreover, the technique described by Mitchell *et al.* [26] requires as much as eight-fold more phospholipid extract than the method described here. The current report is the first, to our knowledge, of two-dimensional, nano-scale densitometric quantitation performed on complex mixtures of major and minor phospholipids including polyphosphoinositides.

To separate the various phospholipid classes found in our RBC membrane and platelet extracts, it was necessary to develop the chromatography plates in three different solvent systems. In the first dimension, the polyphosphoinositides were initially resolved, followed by a second development to separate LPE from PC. The remaining phospholipids were then separated in the second dimension, by means of a third development. We initially attempted these separations using binder-free, conventional TLC plates (silica gel H), because the binders used to stabilize the silica gel layer in commercially produced plates can contain calcium and magnesium, which interfere with the resolution of PIP and PIP_2 due to the formation of salts. However, these plates were unsuitable for densitometric analysis of low concentrations of phospholipids, because the binder-free silica gel layer has slight irregularities in thickness and uniformity that produce significant variability of background readings. There-

fore, it was necessary to utilize HPTLC plates, which give uniform background optical density, but necessarily contain binder to stabilize their fine silica gel particles. This limitation was overcome by pretreating the HPTLC plates with oxalate and EDTA, which produced satisfactory resolution of the polyphosphoinositides.

It should be noted that the values for PE reported in the first column of Tables I and II actually represent a combined total for PE + LPE measured in our system. This was necessary because our extractions were performed using acidic conditions to recover the polyphosphoinositides, which promotes the hydrolysis of ethanolamine plasmalogens (a subclass of PE) to LPE. Because native LPE (not derived from acid hydrolysis of ethanolamine plasmalogens) is present in small amounts compared to total PE [25], we utilized the combined PE + LPE to estimate membrane PE content, as did the other investigators who have reported similar techniques [25,26]. Nevertheless, the general methods described here would be useful to investigators who wanted to measure the small quantities of native LPE directly, provided that the method of extraction was modified. For example, most of the acid could be removed, prior to concentration of the lower phase, by washing with neutral upper phase, and the remaining acid could be neutralized with methanolic ammonia [29].

The sensitivity of this method could allow it to be readily applied in clinical studies involving membranes from cell types that are obtainable only in smaller quantities than are RBCs, such as platelets or cultured fibroblasts. Indeed, we were able to successfully demonstrate the applicability of this method to the quantitation of platelet phospholipids. Given the numerous similarities between platelets and neurons, including the presence of functional receptors, cyclic AMP and polyphosphoinositide second messenger systems, and amine-containing storage granules, the study of platelet phospholipids may shed further light on normal and pathophysiological processes that occur in neurons. This method may therefore represent a promising new tool with which to analyze membrane phospholipids in patients with psychiatric and neurologic disorders. Such investigations could provide a useful additional dimension to clinical studies of mood disorders.

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