Journal of Chromatography, 636 (1993) 221–229
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 879

Separation and analysis of amino alcohol-containing diacylglycerophospholipids and their hydrolytic metabolites

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(First received October 29th, 1992; revised manuscript received January 13th, 1993)

ABSTRACT

A simple radiometric procedure is presented for the separation and determination of amino alcohol-labelled zwitterionic phospholipids (phosphatidylcholine and phosphatidylethanolamine) and their hydrolytic metabolites. The protocol allows the determination of all potential amino alcohol-containing metabolites, in a range of at least 0.2–500 nmol, in the absence or presence of non-ionic (Triton X-100) and ionic (sodium deoxycholate, hexadecyltrimethylammonium bromide) detergents. The discontinuous assay is based on the determination of both water-soluble and lipid-soluble metabolites, *i.e.*, on lipid extraction and thin-layer and ion-exchange chromatography. In addition, simplified and less time-consuming modifications of the procedure have been developed for specifically monitoring phospholipase C and D activities in the course of enzyme purification. The validity of the methods is documented by employing various model phospholipases.

INTRODUCTION

The zwitterionic phospholipids PC and PE are major constituents of mammalian biomembrane diacylphosphoglycerides. As hydrolytic cleavage of these phospholipids has been shown to be implicated in signal transduction [1], there is now growing interest in methods for the determination of their metabolites. In general, phospholipid metabolism in membrane turnover and in particular its involvement in the regulation of various biological processes can be studied by stereospecifically labelling different moieties of these membrane constituents. Investigating the metabolism of a specific phospholipid species, however, requires labelling of the polar head group. Hydrolysis of amino alcohol-labelled phosphatidylcholine (PC) or phosphatidylethanolamine (PE), for example, may lead to radioactive [lysophosphatidylcholine (LPC), glycerophosphonylcholine (GPC), choline phosphate (CP) and choline (C) or lysophosphatidylethanolamine (LPE), glycerophosphorylethanolamine (GPE), ethanolamine phosphate (EP) and ethanolamine (E)] and non-radioactive metabolites (glycero-3-phosphate, free fatty acids (FFA), diacylglycerol, monoacylglycerol and glycerol). In this study, we developed simple methods for separating these radioactively labelled compounds from each other and for their determination by liquid scintillation spectrometry. The protocols were applied to amounts of up to 0.5 μmol of each compound and are easily performed with standard laboratory equipment.

EXPERIMENTAL

Chemicals and biological materials

All reagents and solvents were of analytical-reagent grade and, with the exception of those named below, were obtained from Sigma

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0021-9673/93/$06.00

(Deisenhofen, Germany). Organic solvents and precoated silica gel 60 thin-layer chromatographic plates (20 × 5 × 0.025 cm^3) were obtained from Merck (Darmstadt, Germany). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoryl[N-methyl-^{14}C]choline (52 mCi/mmol), 1-palmitoyl-2-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphorylcholine (55 mCi/mmol)1-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphorylcholine (56 mCi/mmol), 1,2-dioleoyl-*sn*-glycero-3-phosphoryl[2-^{14}C]ethanolamine (55 mCi/mmol), phosphoryl[methyl-^{14}C]-choline (55 mCi/mmol), [methyl-^{14}C]choline chloride (55 mCi/mmol) and [2-^{14}C]ethanolamine hydrochloride (54.1 mCi/mmol), were purchased from Amersham Buchler (Braunschweig, Germany). Glycero-3-phosphoryl[N-methyl-^{14}C]-choline and glycero-3-phosphoryl[2-^{14}C]ethanolamine were prepared by mild alkaline hydrolysis [2] of the parent diacylphosphoglycerides and purified by ion-exchange chromatography [3]. Phosphoryl[2-^{14}C]ethanolamine was prepared by incubating 1,2-dioleoyl-*sn*-glycero-3-phosphoryl[2-^{14}C]ethanolamine with *Bacillus cereus* phospholipase C (pH 8.0, 5 m*M* $CaCl_2$) and the water-soluble phosphorylethanolamine product purified by ion-exchange chromatography [4]. For enzyme assays, the radioactive phospholipids were adjusted to the specific radioactivity required (*ca.* 2000 dpm/nmol for standard assays), by adding the corresponding unlabelled phospholipid and subsequently measuring the radioactivity and phosphorus. Lipase (EC 3.1.1.32; *Rhizopus arrhizus*; 14 000 U/mg) and phospholipases C (EC 3.1.4.3; *Bacillus cereus*; 2000 U/mg) and D (EC 3.1.4.4; cabbage; 200 U/mg) were purchased from Boehringer (Mannheim, Germany). Porcine pancreatic phospholipase A_2 purified to homogeneity (1300 U/mg) was kindly provided by Professor Dr. G.H. de Haas (Department of Enzymology and Protein Engineering, Free University, Utrecht, Netherlands). The ion-exchange resin Dowex 1-X4 (200–400 mesh) was obtained from Serva (Heidelberg, Germany) and Bio-Rex 63 (100–200 mesh) from Bio-Rad (Munich, Germany).

Ion-exchange chromatography

Anion-exchange chromatography was performed with Dowex 1-X4 in Pasteur pipettes containing a small plug of glass-wool in the tips just below the shank. The resin was used in either the acetate or in the hydroxide form. The acetate form, in columns filled to a height of 2 cm (bed volume *ca.* 0.7 ml), was prepared with 3 ml of 1 *M* acetic acid followed by 3 ml of water. The hydroxide form, in columns filled to a height of 4 cm (bed volume *ca.* 1.4 ml), was prepared with 5 ml of 200 m*M* glycine–NaOH (pH 12) followed by 3 ml of 20 m*M* glycine–NaOH (pH 12).

Cation-exchange chromatography was performed in Pasteur pipettes filled with Bio-Rex 63 to a height of 4 cm (bed volume *ca.* 1.4 ml). This resin was converted into the H^+ form with 3 ml of 1 *M* HCl followed by 3 ml of water.

The columns can be reused indefinitely. To regenerate them, they are washed with 3 ml of methanol–1 *M* HCl (9:1, v/v) followed by the washing procedures described above for the respective resins.

Chemical analysis

Inorganic phosphorus was determined after ashing [5] as described by Chen *et al.* [6].

Polyacrylamide gel electrophoresis

Protein purity was assessed by sodium dodecyl sulphate (SDS) polyacrylamide slab gel electrophoresis (PAGE) according to the method of Laemmli [7], using Bio-Rad Mini-PROTEAN II system slab gradient gels (0.75 mm thick resolving gels with a linear gradient of 4–20% polyacrylamide and stacking gels of 2.6% polyacrylamide). The Pharmacia electrophoresis calibration kit was used as a molecular mass reference. The gels were stained with Coomassie Brilliant Blue G-250 [8] and silver [9]. The purity of the lipolytic enzymes used was established by applying 2 μg of purified enzyme. With the silver stain 0.5 ng of bovine serum albumin (corresponding to contamination of 0.25%) was still detectable.

Assays of phospholipases

Phospholipase A_1, A_2, C and D activities were determined as described in detail in Tables III and IV.

Recommended procedure for analysing phosphatidylcholine- and hydrolytic choline-containing products

Extraction. Incubation of samples (250 μl) is terminated by adding 1.5 ml of chloroform–methanol (1:2, v/v), 0.5 ml of chloroform, 0.5 ml of dilutte HCl (the concentration depends on the buffer used in the assay mixture and is previously assessed for acidification to *ca.* pH 3). After vortex mixing and centrifugation (2 min at 500 *g* in a laboratory centrifuge) the chloroform phase is quantitatively recovered and the aqueous phase is washed three times with 0.5-ml portions of chloroform. The combined chloroform phases are washed with 0.5 ml of water and all the aqueous phases are combined. Aliquots of the combined and washed chloroform phase are used for counting and for thin-layer chromatography. The combined aqueous phase is neutralized (with NaOH) and aliquots are taken for counting and for anion- and cation-exchange chromatography.

Ion-exchange chromatography (for separating GPC, CP and C). An aliquot of the aqueous phase is applied to Dowex 1-X4 (acetate form); the column is washed three times with 0.5-ml portions of water. The flow-through and washes contain GPC + C. CP is subsequently eluted with 3 ml of 1 *M* acetic acid and collected in a counting vial. After addition of 7 ml of Pico-Fluor 30 (Packard), CP is counted in a liquid scintillation spectrometer. The flow-through containing GPC + C is divided into two portions of equal volume (one for counting and the other for cation-exchange chromatography). For the latter, the flow-through of the Dowex column is applied to a Bio-Rex column and this column washed with 2 ml of water. This flow-through contains GPC and is counted after addition of 7 ml of Pico-Fluor 30 in a liquid scintillation spectrometer. C is eluted with two 1-ml portions of methanol–1 *M* HCl (9:1, v/v) into a counting vial and counted in a liquid scintillation spectrometer after adding 7 ml of Pico-Fluor 30. Contamination of the ion-exchange columns by PC and LPC can be removed by washing the resins three times with 1-ml portions of methanol–1 *M* HCl (9:1, v/v).

Thin-layer chromatography (for separation of PC and LPC). An aliquot of the combined chloroform phases is dried under vacuum. The residue is taken up in 100 μl of chloroform–methanol (2:1, v/v) and applied to silica gel G thin-layer plates for thin-layer chromatography, together with reference substances (PC and LPC). The thin-layer chromatographic plates are developed in chloroform–methanol–acetic acid–water (50:30:8:2, v/v) [10]. After scanning the radioactivity, the areas corresponding to PC and LPC are transferred to counting vials and counted for radioactivity, after addition of 2 ml of water and 7 ml of Pico-Fluor 30.

Recommended procedure for analysing phosphatidylethanolamine- and hydrolytic ethanolamine-containing products

Extraction. The reaction is terminated and the sample extracted as described for the PC substrate.

Anion-exchange chromatography (for separating GPE, EP and E). An aliquot of the aqueous phase is applied to Dowex 1-X4 (acetate form) and washed three times with 0.5-ml portions of water (the eluent contains GPE + E). EP bound to Dowex is eluted with 3 ml of 1 *M* acetic acid into a counting vial, and after addition of 7 ml of Pico-Fluor 30 is counted in a liquid scintillation spectrometer. The effluent containing GPE + E is made alkaline by adding 0.5 ml of 200 m*M* glycine–NaOH (pH 12) (buffer A) and aliquots are taken for counting and the second anion-exchange chromatography. The latter is applied to Dowex (OH^- form) and washed with 2 ml of 20 m*M* glycine–NaOH (pH 12) (buffer B). The flow-through contains E and is counted in a liquid scintillation spectrometer (after adding 7 ml of Pico-Fluor 30). GPE adsorbed to Dowex under these conditions is eluted with three 1-ml portions of 1 *M* acetic acid (into a counting vial) and counted for radioactivity (after adding 7 ml of Pico-Fluor 30) in a liquid scintillation spectrometer. Contamination of the Dowex columns by PE and LPE may be removed by washing the resins three-times with 1-ml portions of methanol–1 *M* HCl (9:1, v/v).

Thin-layer chromatography (for separation of PE and LPE). An aliquot of the combined chloroform phases is dried under vacuum. The residue is dissolved in 100 μl of chloroform–methanol (2:1, v/v) and applied to silica gel G

thin-layer plates for thin-layer chromatography, together with reference substances (PE and LPE). The thin-layer chromatographic plates are developed in chloroform–methanol–water (100:30:3, v/v/v). Areas corresponding to PE and LPE are transferred into counting vials and the radioactivity is counted, after addition of 2 ml of water and 7 ml of Pico-Fluor 30.

Truncated modifications for analysing for choline or ethanolamine

Time-saving modifications of the methods developed for PC and PE metabolites have been developed for specifically determining choline or ethanolamine in order to monitor, for example, phospholipase C and D activities during enzyme purification. Phospholipase C may be assessed with PC and phospholipase D with either PE or PC substrates. Each enzyme reaction is terminated by adding 1.5 ml of chloroform–methanol (1:2, v/v), followed by vortex mixing vigorously and adding 0.5 ml of chloroform.

For phospholipase C determination (assay PLC-I), 0.45 ml of NaOH or HCl (concentrations as required for neutralization) and 50 μl of 0.5 *M* glycylglycine–NaOH buffer (pH 7.5; to stabilize pH) are added. After phase separation, an aliquot (*e.g.*, 1 ml = 60%) of the aqueous layer (total volume 1.66 ml) is subjected to ion-exchange chromatography on Dowex 1-X4 (acetate form). After sample application, the resin is washed with water (2 ml), to remove potentially contaminating hydrolytic PC products such as GPC and C. Subsequently, the specific phospholipase C metabolite (CP) applied to the column (60%) is quantitatively eluted under acidic conditions (3 ml of 1 *M* acetic acid).

For monitoring phospholipase D activity with PE as a substrate (PLD-I), 0.5 ml of 200 m*M* glycine–NaOH (pH 12) is added. After phase separation, an aliquot (*e.g.*, 1 ml = 60%) of the aqueous phase (total volume 1.66 ml) is subjected to ion-exchange chromatography on Dowex (OH^- form). After sample application, the resin is washed with 20 m*M* glycine–NaOH (pH 12) (2 ml). Under these conditions, the specific phospholipase D metabolite (E) applied to the column (60%) is eluted quantitatively, whereas PE, LPE, GPE and EP are not eluted at all. This latter assay (PLD-I), however, does not work with PC substrates because CP under these conditions does not bind to Dowex in the OH^- form. Alternatively, employing PC as a substrate (assay PLD-II), 0.45 ml of NaOH or HCl (concentrations required for neutralization) and 50 μl of 0.5 *M* glycylglycine–NaOH buffer (pH 7.5; to stabilize pH) are added. After phase separation, an aliquot (*e.g.*, 1 ml = 60%) of the aqueous layer is subjected to ion-exchange chromatography on Bio-Rex (H^+ form). The resin is washed with water (2 ml) and the phospholipase D-specific metabolite (C) is eluted under acidic conditions [2 ml of methanol–1 *M* HCl (9:1, v/v)].

RESULTS AND DISCUSSION

For dissolving PC, PE and their metabolites, various buffers have been used, such as glycine–NaOH (pH 8.0), Tris–HCl (pH 8.0) and sodium acetate (pH 5.0), at final concentrations of 50 m*M* and with final volumes of up to 0.5 ml, without or with (acyl-containing compounds) ultrasonication, and without or with detergent, including up to 2 μmol of non-ionic (Triton X-100) or ionic (sodium deoxycholate or hexadecyltrimethylammonium bromide) detergents. The separation of the amino alcohol-containing phospholipids and their respective amino alcohol-containing metabolites has been accomplished with both non-radioactive and radioactively labelled compounds, up to 500 nmol, and is based on well proven methods.

First, the lipid-soluble diacylphosphoglycerides and monoacylphosphoglycerides are quantitatively separated from their water-soluble metabolites by lipid extraction, as outlined below. These lipids were separated according to standard procedures by thin-layer chromatography, the appropriate solvent mixture depending on the nature of the polar head group.

The water-soluble products were quantitatively recovered in the aqueous phase. After neutralizing the aqueous phase, they were separated from each other by ion-exchange chromatography.

For separating choline-containing metabolites, the neutralized aqueous phase was applied first

to an anion-exchange resin (Dowex 1-X4, acetate form) and the flow-through subsequently to a cation-exchange resin (Bio-Rex, H^+ form). Whereas GPC under these conditions is not bound to either of the exchangers, CP is quantitatively bound to the anion exchanger and C to the cation exchanger. Both are eluted under acidic conditions. For separating ethanolamine-containing metabolites, the neutralized aqueous phase was subjected first to an anion-exchange resin (Dowex 1-X4) in the acetate form and the flow-through, under alkaline conditions, subsequently to an anion-exchange resin (Dowex 1-X4) in the OH^- form. Whereas E under these conditions is not bound to either form, EP is quantitatively bound to the acetate and GPE to the OH^- form, respectively. Both are eluted under acidic conditions.

As assessed by phosphorus determination (data not shown) and/or radiometry (Tables I and II), each of the radioactive or non-radioactive amino alcohol-containing standards (applied in the range 0.5–500 nmol) was completely recovered in its corresponding chromatographic fraction. As a rule, quantitative recoveries in ion-exchange chromatography were obtained within the first 75% of the elution volumes of the respective fractions (data not shown). Hence the minor contaminations of standards in other fractions (Tables I and II) were undoubtedly due to hydrolytic impurities in the commercially available substances. Non-ionic and ionic detergents, of up to 2 μmol, did not interfere with the assays (data not shown).

As already mentioned above, the protocols are based on and combine proven standard chromatographic methods which have been modified for optimum separation and quantitative recovery of the amino alcohol-labelled PC, PE and their respective metabolites. Whereas standard procedures have been modified slightly for lipid extraction into chloroform phases [11] and lipid separation (diacylphosphoglycerides from monoacylphosphoglycerides) by thin-layer chromatography [10,12], separation of the water-soluble PC and PE metabolites represents simplified but specific applications of the general principles of ion-exchange chromatography. Ion exchangers have already been applied for separating (although not all) water-soluble hydrolytic products of PC, specifically for the assessment of phospholipases C and D (reviewed in refs. 13–16) or

TABLE I

DISTRIBUTION PATTERNS OF STANDARDS OF CHOLINE-LABELLED PC AND ITS METABOLITIES IN THIN-LAYER AND ION-EXCHANGE CHROMATOGRAPHY

PC and hydrolytic PC metabolites were separated from each other and determined as described in the text. Data from triplicate determinations are given as means ± S.D.

Standard applied		Amount recovered in fraction (%)				
Species	Amount (nmol)	PC	LPC	GPC	CP	C
PC	5	97.5 ± 2.2	2.2 ± 1.1	0.2 ± 0.2	0.3 ± 0.1	0.4 ± 0.1
	500	96.4 ± 2.4	3.5 ± 0.9	0.1 ± 0.3	0.4 ± 0.2	0.5 ± 0.2
LPC	5	0.4 ± 0.4	98.1 ± 1.9	0.2 ± 0.2	0.8 ± 0.2	0.4 ± 0.1
	500	0.2 ± 0.1	97.8 ± 2.4	0.2 ± 0.2	0.4 ± 0.2	0.4 ± 0.1
GPC	5	0 ± 0	0 ± 0	98.6 ± 0.5	0.3 ± 0.2	1.4 ± 0.1
	500	0 ± 0	0 ± 0	98.8 ± 0.9	0.6 ± 0.2	1.8 ± 1.1
CP	5	0 ± 0	0 ± 0	0.2 ± 0.1	98.7 ± 1.5	1.0 ± 0.3
	500	0 ± 0	0 ± 0	0.2 ± 0.2	98.4 ± 1.9	1.4 ± 0.6
C	5	0 ± 0	0 ± 0	0.5 ± 0.5	0.4 ± 0.3	98.8 ± 2.1
	500	0 ± 0	0 ± 0	0.2 ± 0.3	0.3 ± 0.2	99.1 ± 1.4

TABLE II

DISTRIBUTION PATTERNS OF STANDARDS OF ETHANOLAMINE-LABELLED PE AND ITS METABOLITIES IN THIN-LAYER AND ION-EXCHANGE CHROMATOGRAPHY

PE and hydrolytic PE metabolites were separated from each other and determined as described in the text. Data from triplicate determinations are given as means ± S.D.

Standard applied		Amount recovered in fraction (%)				
Species	Amount (nmol)	PE	LPE	GPE	EP	E
PE	5	98.5 ± 1.2	1.6 ± 0.4	0.1 ± 0.2	0.2 ± 0.1	0.3 ± 0.1
	500	97.9 ± 1.6	2.0 ± 0.7	0.1 ± 0.2	0.2 ± 0.2	0.3 ± 0.2
LPE	5	0.2 ± 0.6	97.9 ± 2.6	0.3 ± 0.2	0.4 ± 0.3	0.3 ± 0.2
	500	0.3 ± 0.4	97.3 ± 2.9	0.2 ± 0.1	0.3 ± 0.2	0.2 ± 0.1
GPE	5	0 ± 0	0 ± 0	98.2 ± 2.4	0.4 ± 0.3	1.2 ± 0.4
	500	0 ± 0	0 ± 0	97.8 ± 2.9	0.7 ± 0.4	1.3 ± 1.1
EP	5	0 ± 0	0 ± 0	0.4 ± 0.3	97.6 ± 2.0	2.0 ± 1.1
	500	0 ± 0	0 ± 0	0.5 ± 0.3	98.0 ± 2.3	1.2 ± 0.5
E	5	0 ± 0	0 ± 0	0.7 ± 0.6	0.2 ± 0.1	98.1 ± 1.9
	500	0 ± 0	0 ± 0	0.4 ± 0.3	0.4 ± 0.2	97.8 ± 1.5

TABLE III

PC METABOLITES FORMED IN THE PRESENCE OF VARIOUS PHOSPHOLIPASES

PC with a specific radioactivity of 1991 dpm/nmol was used. After incubation, radioactive PC and radioactive products were measured as described in the text. All reaction products determined in these experiments with PC substrate formed linearly with time up to the time periods employed. Data from triplicate determinations (corrected for non-enzymic hydrolysis which did not exceed 3.6%) are given in per cent of radioactivity, as means ± S.D., recovered in the respective fractions.

Enzyme	Substrate remaining	Metabolite			
	(PC)	LPC	GPC	CP	C
Phospholipase A_1[a]:					
Without detergent	59.8 ± 2.1	26.8 ± 2.1	11.8 ± 0.6	0.2 ± 0.2	0.4 ± 0.1
With detergent	73.2 ± 3.9	20.2 ± 2.0	4.9 ± 0.1	0.3 ± 0.2	0.4 ± 0.2
Phospholipase A_2[b]	64.1 ± 2.5	26.7 ± 1.9	4.0 ± 0.8	0.2 ± 0.2	0.8 ± 0.2
Phospholipase C[c]	0 ± 1.9	0 ± 0	0.1 ± 0.2	98.8 ± 4.1	0.1 ± 0.2
Phospholipase D[d]	58.2 ± 3.1	0 ± 0	0.1 ± 0.1	0.2 ± 0.1	41.2 ± 3.3

[a] For assays of phospholipase A_1 activity, the reaction mixture (total volume 250 μl), containing 25 m*M* Tris–HCl (pH 8.0), 10 m*M* $CaCl_2$, 1.5 μg of *Rhizopus arrhizus* lipase and 127 μM PC, without or with 0.1% (w/v) Triton X-100, was incubated at 37°C for 60 min.

[b] For assays of phospholipase A_2 activity, the reaction mixture (total volume 250 μl), containing 25 m*M* Tris–HCl (pH 9.0), 10 m*M* $CaCl_2$, 0.1 μg of porcine pancreatic phospholipase A_2 and 52 μM PC, was incubated at 37°C for 30 min.

[c] For assays of phospholipase C activity, the reaction mixture (total volume 250 μl), containing 20 m*M* glycine–NaOH (pH 8.0), 5 m*M* $CaCl_2$, 2 μg of *Bacillus cereus* phospholipase C and 46.5 μM PC, was incubated at 37°C for 30 min.

[d] For assays of phospholipase D activity, the reaction mixture (total volume 250 μl), containing 20 m*M* sodium acetate (pH 5.0), 10 m*M* $CaCl_2$, 10 μg of cabbage phospholipase D and 57.4 μM PC, was incubated at 37°C for 15 min.

glycerophosphocholine phosphodiesterase [17] activities.

Different modes of elution have been used to determine specific PC or PE metabolites. Gradient elution of water-soluble hydrolytic PC metabolites from ion exchangers [3], although more accurate, is far more laborious, time consuming and not well suited for analysing large numbers of samples. Similarly, the stepwise group elution of water-soluble C-containing metabolites from an ion-exchange resin followed by volume reduction and further separation on charcoal [18] is not appropriate for multi-sample experiments. As only two compounds are involved, a simple and rapid method using stepwise elution from ion-exchange resins has been reported for the separation of ^{32}P-labelled choline phosphate from AT^{32}P [19]. This method, however, was not applicable to the separation of all the water-soluble PC metabolites used in our studies. Thin-layer chromatography as an alternative procedure for the separation of water-soluble C-containing compounds [3,18,20], is simple and rapid and has multi-sample capacity. Nevertheless, our method appears to be superior to thin-layer chromatography, simply because the latter requires more time for volume reduction and sample application to the plates. The same considerations also hold true for the separation of water-soluble PE metabolites by thin-layer chromatography [4]. Our procedures allow direct quantitative analysis of at least twelve aqueous samples within 30 min. Hence the protocols presented here for the separation of the water-soluble PC and PE metabolites avoid volume reduction and are extremely easy to perform with, excellent quality of separation, recovery, speed of analysis and cost of materials.

The validity of the procedures presented was demonstrated by incubating various purified and electrophoretically homogeneous (except for cabbage phospholipase D) lipolytic enzymes with PC (Table III) and PE substrates (Table IV) and determining the metabolites formed. The enzyme assays were designed so as to lead to a high accumulation of metabolites rather than to stay in the appropriate kinetic range of substrate hydrolysis. Phospholipases C and D and their expected positional specificities are correctly assessed with respect to the metabolites obtained (Tables III and IV). Likewise, phospholipases A were shown to catalyse the deacylation of

TABLE IV

PE METABOLITES FORMED IN THE PRESENCE OF VARIOUS PHOSPHOLIPASES

PE with a specific radioactivity of 1972 dpm/nmol was used. After incubation, radioactive PE and radioactive products were measured as described in the text. Formation of reaction products in these experiments with PE substrate was not linear with time. Data from triplicate determinations (corrected for non-enzymic hydrolysis) are given in per cent, as means ± S.D., recovered in the respective fractions.

Enzyme	Substrate remaining (PE)	Metabolite			
		LPE	GPE	EP	E
Phospholipase A_1[a]	55.3 ± 4.3	32.1 ± 2.3	13.9 ± 2.2	0.3 ± 0.3	0.2 ± 0.2
Phospholipase A_2[b]	2.1 ± 3.4	67.3 ± 0.4	24.1 ± 4.0	1.9 ± 1.0	1.5 ± 0.4
Phospholipase C[c]	10.2 ± 1.8	0 ± 0	0 ± 0.1	80.9 ± 3.6	0.2 ± 0.3
Phospholipase D[d]	42.0 ± 3.3	0 ± 0	0.2 ± 0.2	0 ± 0.2	58.8 ± 3.4

[a] For assays of phospholipase A_1 activity, the reaction mixture (total volume 250 μl), containing 25 m*M* Tris–HCl (pH 8.0), 10 m*M* $CaCl_2$, 7 μg of *Rhizopus arrhizus* lipase and 27 μ*M* PE, was incubated at 37°C for 60 min.

[b] For assays of phospholipase A_2 activity, the reaction mixture (total volume 250 μl), containing 25 m*M* Tris–HCl (pH 9.0), 10 m*M* $CaCl_2$, 0.1 μg of porcine pancreatic phospholipase A_2 and 31 μ*M* PE, was incubated at 37°C for 30 min.

[c] For assays of phospholipase C activity, the reaction mixture (total volume 250 μl), containing 20 m*M* glycine–NaOH (pH 8.0), 5 m*M* $CaCl_2$, 2 μg of *Bacillus cereus* phospholipase C and 28 μ*M* PE, was incubated at 37°C for 30 min.

[d] For assays of phospholipase D activity, the reaction mixture (total volume 250 μl), containing 20 m*M* sodium acetate (pH 5.0), 10 m*M* $CaCl_2$, 10 μg of cabbage phospholipase D and 29 μ*M* PE, was incubated at 37°C for 30 min.

TABLE V

COMPARISON OF STANDARD ASSAYS AND RAPID TESTS FOR PHOSPHOLIPASE C AND D ACTIVITIES

Reactions catalysed by phospholipases C (substrate 50.3 μM PC) and D (substrate 43.2 μM PE) were performed as described in Tables III and IV, respectively. The corresponding metabolites were analysed by the standard assays (SA) and rapid tests (PLC-I and PLD-I) as described in the text. Data from triplicate determination (corrected for non-enzymic hydrolysis) are given in dpm, as means ± S.D. recovered in the respective fractions.

Enzyme	Test	Metabolite			
		CP	(%)	E	(%)
Phospholipase C	SA	12110 ± 697	(100 ± 6)	ND[a]	
	PLC-I	7182 ± 341	(59 ± 3)	ND	
Phospholipase D	SA	ND		6008 ± 311	(100 ± 5)
	PLD-I	ND		3542 ± 199	(59 ± 3)

[a] ND = Not determined.

diacylphospholipids with the expected positional specificity, but all preparations employed here were shown also to catalyse the deacylation of lysophospholipids. *Rhizopus arrhizus* lipase analyses showed GPC and GPE formation from PC (Table III) and PE (Table IV), respectively, confirming earlier reports on an intrinsic lysophospholipase activity of a lipase catalysing phospholipid hydrolysis [10]. As seen from the disappearance of GPC relative to LPC formation (Table III), this lysophospholipase activity was almost abolished by Triton X-100, whereas phospholipase A_1 activity (based on the sum of LPC and GPC formation) was only reduced (by *ca.* 47%). Partial inhibition of phospholipase A_1 and complete inhibition of lysophospholipase activities of the *R. arrhizus* enzyme was confirmed by using identical assay conditions but substrates with a different stereospecific label. For example, hydrolysis of PC (112 μM) fatty acid-labelled in the *sn*-1-position was inhibited by *ca.* 44%, and hydrolysis of LPC (98 μM) fatty acid-labelled in the *sn*-1-position by about 98%. It may well be possible that hydrolysis of the *sn*-2-acyl-lysophospholipids generated during phospholipase A_1-catalysed hydrolysis of diacylphospholipids proceeds in two steps, *i.e.*, via acyl migration to the *sn*-1-position and subsequent hydrolysis of the *sn*-1-acyl ester bond. This possibility remains to be clarified.

Apparently *sn*-1-directed lysophospholipase activity (GPC and GPE formation from PC,1-acyl-LPC and PE, respectively) was also observed in the electrophoretically pure (contaminations of more than 0.25% would have been detectable by SDS-PAGE) phospholipase A_2 used here (Tables III and IV). Pancreatic phospholipase A_2-associated lysophospholipase activity is extremely low ($v_{max} \approx 0.08$ U/mg [21]) and detectable in purified bee venom phospholipase A_2 as well. Phospholipase A_2-associated lysophospholipase activity is also shown with 1-acyl-lysophospholipid susbstrates, and presumably due to acyl migration followed by enzymatically catalysed deacylation from the *sn*-2-position [21].

In their modified forms (see truncated modifications), our methods are particularly suitable for monitoring phospholipase C or D activities during purification of these enzymes. The specific phospholipase C metabolite (CP) and the specific phospholipase D metabolite (C or E) are quantitatively eluted (Table V).

ACKNOWLEDGEMENTS

The authors are grateful to Drs. Henk van den Bosch and Kay Nakamaye for critical discussions and advice.

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