Phosphatidylcholine assay by phosphate determination after dry ashing

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Introduction

Phospholipids (PLs) are essential and major constituents of biologic membranes. They include phosphatidylcholine (PtdCho), phosphatidylinositol, phosphatidylserine, sphingomyelins, cephalins, and plasmalogens. PtdCho is the most abundant in mammalian membranes. PtdCho makes up about 40–70% of mammalian membrane PLs and represents a significant storage form for choline.

A typical PL assay involves the following steps: (1) Extraction of total lipids from biological samples. This extraction can be done using the Bligh-Dyer extraction procedure.[1] (2) Separation of PLs from total lipids and resolution of individual PLs. This is usually accomplished by thin layer chromatography (TLC). To aid in the identification of PLs, PL standards are spotted alongside biological samples, and the bands that co-chromatograph with the standards are used in the assay procedure. (3) Extraction or elution of the PLs of interest from silica. PLs may be efficiently extracted from silica with $CHCl_3$:MeOH (1:2, vol/vol). However, some assay procedures that employ acid digestion may not require this step. (4) Liberation of the phosphate ion from the PL moiety. This can be done by either wet or dry ashing and is necessary when phosphate assays are used. The chromogenic assay of Hundriesen et al.[2] does not require this step because the chromogenic reagent reacts directly with the phospholipid. (5) Assay procedure to quantitate the phosphate ions.

Materials and methods

(1) Chloroform:methanol:water (1:2:0.8 vol/vol/vol): to extract lipids from tissue samples.

(2)Chloroform:methanol (1:1,vol/vol): to resuspend the standards and samples after drying for application to TLC plate.

(3) Chloroform:methanol (1:2, vol/vol): to elute the standards and samples from silica gel.

(4) Acetone: to pre-develop TLC plates. This step eliminates possible

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interference that neutral lipids, including triglycerides, might have on the separation of the PLs present in the samples.

(5) Chloroform:methanol:acetic acid:formic acid:water (70:30:12:4:2, vol/vol): to develop the TLC plates.

(6) Silica gel TLC plates (Baker Si250-PA): to separate the different phospholipids.

(7) Phosphatidylcholine (dipalmitoyl): reference standards (Sigma Chemical Co., St. Louis, MO USA). The stock solution containing 2 μmoles/ml $CHCl_3$:MeOH (2:1, vol/vol), was prepared by dissolving 14.68 mg phosphatidyl choline in 10 ml $CHCl_3$:MeOH (2:1, vol/vol). The standard solution contained one part of the stock solution and nine parts ($CHCl_3$:MeOH (2:1, vol/vol).

(8) 10% ascorbic acid: This was prepared by dissolving 10 g ascorbic acid in 100 mL distilled water and stored in the refrigerator. This solution is stable for about 7 weeks.

(9) 0.42% ammonium molybdate: 420 mg of $(NH_4)_6Mo_7O_{24}{\cdot}4\ H_2O$) is dissolved in 100 mL of 1N H_2SO_4 (2.86 mL concentrated H_2SO_4 diluted to 100 mL with H_2O)

(10) Reagent C: 1 part 10% ascorbic acid and 6 parts 0.42% ammonium molybdate are mixed for each assay.

(11) 10% $Mg(NO_3)_2$: 10 g $Mg(NO_3)_2$ are dissolved in 100 mL absolute ethanol.

(12) 1N HCl: 25 mL of concentrated HCl are diluted to 300 mL with distilled water.

Apparatus

(1) Drying oven capable of heating up to 140° C.

(2) Vacuum concentrator (SpeedVac Concentrator, Savant Instruments, Inc., Farmingdale, NY USA).

(3) Spectrophotometer capable of reading at 820 nm.

(4) Developing and iodine staining chambers for TLC.

(5) Refrigerated centrifuge.

(6) Heating block capable of heating to 110° C.

(7) Hot plate capable of heating up to 350° C.

(8) Pyrex tubes.

(9) Marbles used to cap the test tubes and minimize evaporation during the hydrolysis of pyrophosphate.

Procedures

Lipid extraction from tissue. 500 μL of the extraction medium, chloroform:methanol:distilled water (1:2:0.8 vol/vol/vol, where the 0.8 water fraction includes the water content of the sample), is added to each test tube containing the samples. Tissue samples may be homogenized in this mixture with a glass homogenizer or with motor-driven homogenizers suitable for use with flammable liquids. The test tubes are vortexed and incubated for 1 hour at −20° C. The test tubes are vortexed 4 times during the incubation period. After incubation, tubes are centrifuged in a high speed centrifuge at 3,000 RPM (2300 g) for 5 minutes. The supernatants are drawn off from the pellets, put into fresh tubes and the pellet washed with 250 μL extraction medium, vortexed, and spun as before. Supernatant is drawn off and added to previous supernatant. To separate the phases, 200 μL chloroform and 200 μL distilled water are added to the pooled supernatant; the samples are vortexed and spun at 3,000 RPM for 5 minutes. The upper aqueous layer is aspirated and discarded while the lower organic phase is dried in a vacuum concentrator.

Separation of PtdCho from total PLs. PtdCho is separated from the neutral lipids and other phospholipids using thin layer chromatography (TLC). The dry organic phase is resuspended in 30 μL chloroform:methanol (1:1 vol/vol) and spotted on a TLC plate. Each tube is washed with 20 μL of

$CHCl_3$:MeOH in the same ratio and applied to the previous spots. Duplicate PtdCho standards (0, 20, 40, 60, 80, and 100 nmoles) are also spotted on the TLC plates. Because biological samples often contain large amounts of neutral lipids, the plates are pre-developed in acetone, which carries the neutral lipids to the top of the plate, leaving the phospholipids at the origin. This pre-development stage is necessary to provide an optimum separation of PtdCho from other phospholipids. After the plate is air-dried, it is then transferred into a developing chamber to separate the different PLs. The developing system[3] of chloroform:methanol:acetic acid:formic acid:water (70:30:12:4:2, vol/vol) separated PtdCho ($R_f = 0.61$), phosphatidylinositol (PtdIns) ($R_f = 0.67$), phosphatidylserine (PtdSer) ($R_f = 0.83$), sphingomyelin ($R_f = 0.36$), lysophosphatidylcholine ($R_f = 0.26$), and cardiolipin ($R_f = 0.97$), but not phosphatidylglycerol (PtdGly) ($R_f = 0.90$) and phosphatidylethanolamine (PtdEtn) ($R_f = 0.90$). This system is, however, better than $CHCl_3$:MeOH:H_2O (65:30:4, vol/vol/vol), which does not permit an optimum separation of PtdCho, PtdSer, and PtdIns. After the development is complete (i.e., when the developing solvent is 1 inch from the top of the plate) the plates are taken out, allowed to dry, and then stained in an iodine chamber. Next the plates are taken out and each band that co-chromatographed with the PtdCho standards is scraped off into a test tube.

Elution procedure. This step removes PtdCho from the silica gel to prevent any effect it (silica gel) might have on the assay results. An experiment was performed to determine which of several commonly used organic solvents is best for elution of PtdCho from silica gel *(Table I)*. The removal of PtdCho from the silica gel using the different solvents was determined by spiking the standards with a known amount of ^{14}C-PtdCho before the TLC and counting the organic solvent for eluted radioactivity. Solvents tested were $CHCl_3$:MeOH (1:2), $CHCl_3$:MeOH (2:1), $CHCl_3$:MeOH (1:1), chloroform, butanol, and methylene chloride. $CHCl_3$:MeOH (1:2) was found to yield the highest recovery of samples after a single elution with 500 μL of solvent mixture (Table 1). When three washes with $CHCL_3$:MEOH (2:1) were used, recovery of label was typically 80–85%.

For this assay, 500 μl $CHCL_3$:MeOH (1:2) was added to each tube, vortexed, and centrifuged at 2000 RPM for 5 minutes. The supernatant was transferred into fresh Pyrex tubes and the pellets washed as before. After a third elution with 500 μL of the solvent mixture, the samples in the combined solvent washes were dried in a vacuum concentrator and used in the assay procedure.

Assay procedure. 25 μL of 10% $MgNO_3$ is added to each tube and dried overnight at 140° C in a drying oven. The tubes are heated in aluminum heating blocks on a hot plate at 300–350° C for 3–5 minutes (or until the brown fumes disappear). The use of a hot plate allows the treatment of multiple samples at one time and also standardizes the heating of all the

Table 1 Recovery of PtdCho from silica
PtdCho standards containing ^{14}C-PtdCho were chromatographed on TLC plates and the recovery of the original radioactivity determined after a single elution with test solvents.

Organic solvent	% Recovery
Chloroform:methanol (1:2, vol/vol)	48.76%
Chloroform:methanol (1:1, vol/vol)	46.14%
Chloroform:methanol (2:1, vol/vol)	28.35%
Chloroform	2.09%
Butanol	2.92%
Methylene chloride	3.41%

The recovery with chloroform:methanol (1:2) after three elutions was typically 80–85%.

samples. The original procedure used hot flame to drive off nitrates that are present; however, based on previous experiments (data not shown), the use of a hot plate at a high temperature gives the same result as when a hot flame is used. After the tubes are cooled, 0.3 mL 1N HCl is added to each tube. The tubes are capped with marbles to minimize evaporation and incubated in a heating block at 110° C for 15 minutes to hydrolyze any pyrophosphate formed during the ashing procedure. Next, 0.7 mL reagent C is added to each tube and incubated at 37° C for 1 hour.[4] The absorbance of the samples are then read at 820 nm. The data are plotted as absorbance versus standard concentrations and sample phospholipid concentrations may be calculated from a linear regression of the data.

Discussion

Most assay procedures for PLs used in clinical laboratories involve the conversion of PL phosphorus to inorganic phosphorus. This conversion usually involves acid digestion using acids such as perchloric and/or sulfuric acid. However, these acids must be used with care because severe burns and explosion may result if they are not handled properly. Therefore, we have chosen an assay that produces the same or better sensitivity as the acid digestion procedures but does not require either perchloric or sulfuric acid. We have compared this method, which converts PL phosphorus to inorganic phosphorus by a dry ashing procedure, with an assay procedure using a small volume of perchloric acid and a procedure that forms a chromogenic complex with PLs without acid digestion.

The perchloric acid assay[5] for phospholipid phosphate is widely used. An advantage of this procedure is that assay results are not affected by the presence of silica because the silica is digested by the acid. This eliminates the elution procedure. However, this is a hazardous procedure because of the explosive potential of concentrated perchloric acid preparations.

The direct formation of a chromogenic complex by the method of Hundriesen et al.[2] eliminates acid digestion, but two problems were encountered in the use of this assay. First, the procedure was developed for analysis of total PLs in human milk where phospholipid concentrations are much higher than in plasma. Thus, this assay produces a linear response only over the concentration range of 62.5–312.5 nmoles phosphorus, well above the plasma PtdCho concentrations. Because this assay eliminates the use of perchloric acid, we attempted to increase the sensitivity of the assay by (1) reducing the volume of the reagents, thus keeping the sample volume constant, and (2) increasing the concentration of the chromogenic reagent. These attempts, however, only increased the sensitivity slightly, and the standard curve still did not include PL concentrations expected for plasma PLs. The second problem encountered was the interference of silica gel with the assay. When PtdCho was eluted from the silica, it was impossible to get a solution completely free of silica. Thus when the chromogenic solution was added, the silica adsorbed some of the molybdate-phosphate complex. Thus, the presence of silica in the eluates reduced the absorbance compared to that of silica-free samples and further reduced the sensitivity of the assay.

The third procedure that was tried and is recommended for PL analysis is a modified microdetermination of phosphorus[4] after dry ashing. This procedure does not require acid digestion, and it is not affected by the presence of small amounts of silica. The effect of silica on the sensitivity of this assay was tested by performing the assay on two identical sets of standards. One set of standards was applied on TLC plates, eluted from the silica gel and then put through the assay procedure. The second set of standards was not put through TLC but was subjected to the same assay procedure. The results of this comparison are illustrated in *Figure 1*. On comparing the assay results from the two sets of standards, no significant

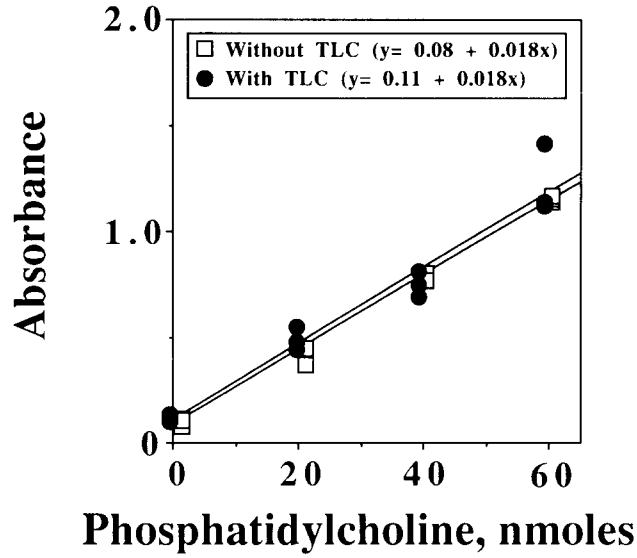

Figure 1 Effect of silica on assay. PtdCho standards were assayed after elution from TLC plate (with TLC) or directly (without TLC).

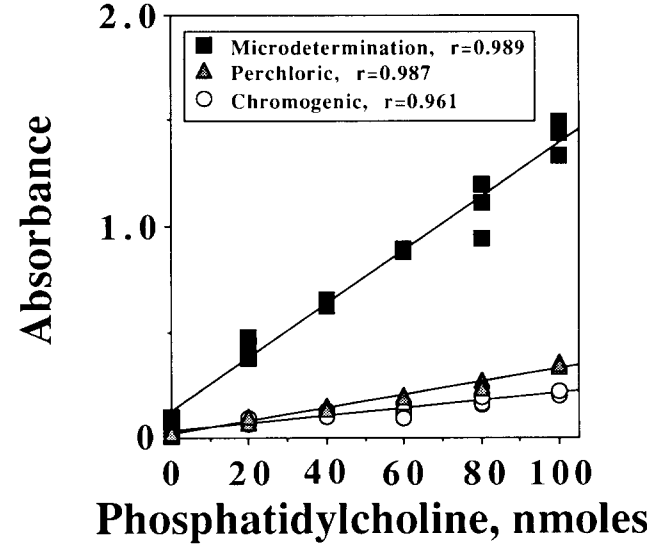

Figure 2 Comparison of three assays. Three sets of PtdCho standards were chromatographed on TLC plates, eluted, and assayed by one of the three test methods.

difference was observed. Hence, silica or TLC has no significant effect on the sensitivity of the microdetermination of phosphorus assay procedure.

The absorbance of the chromogenic complexes formed with increasing amounts of PtdCho standard with each of the three assay procedures is reported in *Figure 2*. Three sets of standards of 0, 20, 40, 60, 80, and 100 nmoles of PtdCho were chromatographed in triplicate on TLC plates as described and the PtdCho eluted with chloroform:methanol (2:1). Each set of standards was then assayed in either the microdetermination,[4] chromogenic,[2] or perchloric acid[5] assays *(Figure 2)*. The responses of all three procedures was linear over this range of PtdCho concentration and the correlation coefficients were 0.96 or better. The precision of the microdetermination assay procedure was similar to that of the perchloric acid procedure and was much lower than that of the chromogenic assay over the same standard concentrations *(Table 2)*.

Table 2 Assay variability
The coefficient of variation (COV = mean/SD) was calculated for each set of three standards from 20–100 nmoles

	Microdetermination			Chromogenic			Perchloric acid		
Conc.	Mean	SD	COV	Mean	SD	COV	Mean	SD	COV
20	0.427	0.047	0.110	0.073	0.014	0.195	0.094	0.009	0.093
40	0.635	0.014	0.021	0.112	0.014	0.120	0.145	0.009	0.060
60	0.884	0.008	0.009	0.113	0.019	0.164	0.189	0.015	0.077
80	1.085	0.132	0.122	0.169	0.019	0.112	0.248	0.020	0.082
100	1.424	0.081	0.057	0.213	0.011	0.050	0.346	0.013	0.038
Mean COV			0.064			0.128			0.070

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