A Simple HPLC Method for the Simultaneous Analysis of Phosphatidylcholine and Its Partial Hydrolysis Products 1- and 2-Acyl Lysophosphatidylcholine

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ABSTRACT: A simple HPLC method for the simultaneous analysis of phosphatidylcholine (PC), 1-acyl lysophosphatidylcholine (1-acyl LPC), and 2-acyl lysophosphatidylcholine (2-acyl LPC) with refractive index detection is described. The separation of these three compounds was achieved on a Waters (Milford, MA) Spherisorb amino phase column using a mixture of ethanol and an aqueous oxalic acid solution as eluent. The optimal mixture of ethanol to oxalic acid solution was 92:8 (vol/vol). PC and the two regioisomers of LPC eluted within 15 min. The calibration curves were linear in a concentration range from 0.05 to 2.5 mM. Natural PC or LPC eluted in a single peak, despite the diversity in the fatty acid composition. There was no rearrangement between 1-acyl LPC and 2-acyl LPC during analysis or storage in ethanol within 23 h. This method is thus especially suitable for studying reactions on PC and acyl migration in LPC.

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KEY WORDS: Acyl migration, enzymatic phospholipid modification, lysophosphatidylcholine, lysophospholipids, phosphatidylcholine, phospholipids.

Enzymes are of great synthetic importance in phospholipid chemistry, owing to their ability to act specifically on one of the phospholipid molecule ester linkages (1,2). 1,3-specific lipases and phospholipase A_2 have, for instance, been used to modify phosphatidylcholine (PC) specifically in the *sn*-1 and *sn*-2 positions, respectively. The most important reaction is the phospholipase A_2-catalyzed hydrolysis of PC to produce 1-acyl lysophosphatidylcholine (LPC) (3,4), an effective bioemulsifier and important intermediate in the synthesis of PC with defined fatty acid composition. Phospholipase A_2 has also been applied in organic media to synthesize PC with defined fatty acid in the *sn*-2 position starting from 1-acyl LPC and fatty acid (5–8), whereas 1,3-specific lipases have mainly been used to exchange the fatty acid in the *sn*-1 position of PC by transesterification (9–11) and to synthesize 2-acyl LPC (12,13). All of the reactions named above involve LPC either as product, substrate, or intermediate. Ideally there should be only one of the two regioisomers of LPC present in a certain reaction: 1-acyl LPC if the reaction is catalyzed by phospholipase A_2, and 2-acyl LPC if the reaction is catalyzed by an enzyme that is specific for the position *sn*-1. However, the regioisomers of LPC are not stable but may isomerize *via* acyl migration (14). Acyl migration is usually an unwanted side reaction, which renders the interpretation of enzymatic studies more difficult and leads to a decreased yield and less pure products in the synthesis of modified phospholipids and phospholipid derivatives.

To achieve a more complete picture of the enzymatic reaction, the use of an analysis system that discriminates between 1-acyl LPC and 2-acyl LPC without leading to a rearrangement of the two isomers under analysis conditions is advantageous. Furthermore, it is important that the analysis method can be applied to natural phospholipids, containing various fatty acids. Only a few analysis methods fullfill these requirements. NMR spectroscopy has been used for structural determination and quantitative analysis of LPC and PC (14) but is not suitable for the routine analysis of a large number of samples. Another possibility is to use electrospray ionization MS (15). However, since the equipment required for this kind of analysis is not available in most laboratories, there is a need for methods using widely available equipment such as HPLC. Only a few HPLC methods describe the resolution of the two regioisomers of LPC (16–18). Reverse-phase HPLC has been applied for the separation of different molecular species of LPC (16,18). In samples containing LPC with a variety of fatty acids, both 1-acyl LPC and 2-acyl LPC give rise to a number of peaks, which may overlap in complex mixtures. Even PC is separated into different molecular species on reverse stationary phases (19). A possible interference of PC in the method applied to lysophospholipids was not investigated. A method for the separation of PC, phosphatidylglycerol, and their hydrolysis products on an amino phase column with a mixture of methanol, acetonitrile, and buffer as eluent was developed by Grit *et al.* (17). The authors found that the LPC peak was split, and they assumed that the two peaks represented the 1-acyl LPC and 2-acyl LPC, respectively. However, even if this assumption was right, whether the two isomers were present in the sample *a priori* or whether isomerization had taken place during analysis was not investigated.

The method presented in this paper is, to our knowledge, the first HPLC method suitable for the simultaneous quantification of PC, 1-acyl LPC, and 2-acyl LPC that does not lead to a rearrangement of the two regioisomers of LPC under

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analysis conditions. The method is therefore well suited for the analysis of enzymatic reactions on PC and acyl migration studies in LPC. The method is applicable to samples containing natural phospholipid with fatty acids of different chain length and degree of unsaturation.

MATERIALS AND METHODS

Dipalmitoyl L-α-PC and glycerophosphorylcholine (GPC) were purchased from Larodan Fine Chemicals (Malmö, Sweden), L-α-LPC (1-acyl LPC) from egg, L-α-PC from egg (egg PC), and *n*-caproic acid were bought from Sigma (St. Louis, MO). Rapeseed lecithin sludge from Karlshamns (Karlshamn, Sweden) was purified by acetone precipitation and ethanol extraction. 1-Caproyl LPC was synthesized by esterification of GPC with caproic acid in a solvent-free system catalyzed by lipase from *Rhizopus arrhizus* immobilized on EP 100 (20). Lipase from *R. arrhizus* was a gift from Gist-Brocades S.A. (Delft, The Netherlands). Phospholipase A_2 (lecitase 10L, 12,000 IU mL^{-1}) and lipase from *Rhizomucor miehei* (Lipozyme IM) were bought from Novo Industri A/S (Bagsvaerd, Denmark). Amberlite XAD-8 was purchased from Serva Feinbiochemica (Heidelberg, Germany). Oxalic acid was from Merck (Darmstadt, Germany). Ethanol (95%) was bought from Kemetyl (Haninge, Sweden). All other solvents and chemicals used were at least of analytical grade.

Synthesis of 2-acyl LPC. 2-Palmitoyl LPC was synthesized by Lipozyme-catalyzed ethanolysis of dipalmitoyl PC (12). Dipalmitoyl PC (500 mg) was dissolved in 10 mL ethanol (95%), 1 g Lipozyme was added, and the mixture was shaken on an orbital shaker at 170 rpm. The reaction was complete after about 8 h, and the enzyme preparation was filtered off. Ethanol was removed on a rotary evaporator, and the palmitic acid and palmitic acid ethyl ester were removed by extraction with hexane.

HPLC system. The HPLC analysis were carried out with a Beckman System Gold instrument (Beckman Instruments, Palo Alto, CA) consisting of a Programmable Solvent Module 126, an Autosampler 507 (loop volume 100 μL) and a 156 Refractive Index Detector (attenuation 16×). PC, 1-acyl LPC, and 2-acyl LPC were separated on a Waters Spherisorb amino phase column from Hichrom (Reading, United Kingdom). A precolumn from the same manufacturer was placed between the injector and the column. The flow rate was 1 mL/min, and the temperature was ambient. The data were handled by the Beckman System Gold software.

Preparation of the mobile phase. Ethanol (95%) and a freshly prepared 20 mM solution of oxalic acid in water [purified by Millipore (Milford, MA)] were mixed and filtered through a membrane filter of regenerated cellulose with a pore size of 0.45 μm. Air bubbles were removed by applying the vacuum of a water pump.

Preparation of standard solutions. PC and 1-acyl LPC were first dried over phosphorus pentoxide for 2 d and then dissolved in ethanol (95%).

Immobilization of phospholipase A_2. Phospholipase A_2 was immobilized on XAD-8. The support material was washed with ethanol and water and air-dried. The prewashed support material (1 g) was suspended in 18 mL buffer (10 mM Tris/HCl, 5 mM $CaCl_2$, pH 8), 200 μL phospholipase A_2 solution were added, and the mixture shaken on an end-over-end incubator for at least 20 h. The preparation was then filtered, washed with buffer, and dried under reduced pressure.

Phospholipase A_2-catalyzed acidolysis of PC. The phospholipase A_2-catalyzed acidolysis of PC was carried out as described previously (5). The medium contained 10 mM dipalmitoyl PC and 800 mM caproic acid in toluene. The reaction was started by mixing 50 mg enzyme preparation and 1 mL medium. The water activity was adjusted to and maintained at 0.33 during the reaction. The reaction proceeded at 25°C. For HPLC analysis 50-μL samples were withdrawn at appropriate time intervals. The toluene in the sample was first evaporated in a stream of air and then replaced by 500 μL ethanol.

Acyl migration study. The rearrangement between the two regioisomers of LPC was studied in 50 mM Tris/HCl buffer pH 9; 10 μmol 1-acyl LPC or 2-acyl LPC was dissolved in 1 mL buffer and kept at room temperature. Samples (50 μL) were withdrawn in appropriate time intervals, and 450 μL eluent was added for HPLC analysis.

RESULTS AND DISCUSSION

Mobile phase composition. A good separation between PC and its partial hydrolysis products 1- and 2-acyl LPC can be obtained on a Waters (Milford, MA) Spherisorb amino phase column using a mixture of ethanol and an aqueous solution of 20 mM oxalic acid as eluent. A decrease in the volumetric ratio of ethanol to oxalic acid solution resulted in shorter retention times for all three compounds. There was only a minor decrease in resolution between 2- and 1-acyl LPC, when the retention times were shortened. More detailed results are shown in Table 1. The performance was best at a 92:8 (vol/vol) ratio of ethanol to oxalic acid solution. The retention times under these optimal conditions were 8.4, 11.9, and 13.7 for PC, 2-acyl LPC, and 1-acyl LPC, respectively. The corresponding chromatogram is shown in Figure 1.

Calibration. The relationship between the peak area and the concentration of PC and 1-acyl LPC is shown in Figure 2. The response was linear ($R = 0.999$) in the concentration range under investigation (0.05–2.5 mM). The ratio of the

TABLE 1
Influence of the Eluent Composition on the Retention Times of PC, 1-Acyl LPC, and 2-Acyl LPC and on the Resolution of the Two Regioisomers of LPC[a]

Ethanol/oxalic acid solution (vol/vol)	t_{PC} (min)	$t_{2\text{-acyl LPC}}$ (min)	$t_{1\text{-acyl LPC}}$ (min)	$R_{1\text{-acyl,2-acyl LPC}}$
90:10	7.0	9.6	10.8	1.29
92:8	8.4	11.9	13.7	1.43
94:6	9.7	14.3	16.5	1.44
94:4	13.1	20.1	23.7	1.46

[a]PC, phosphatidylcholine; LPC, lysophosphatidylcholine.

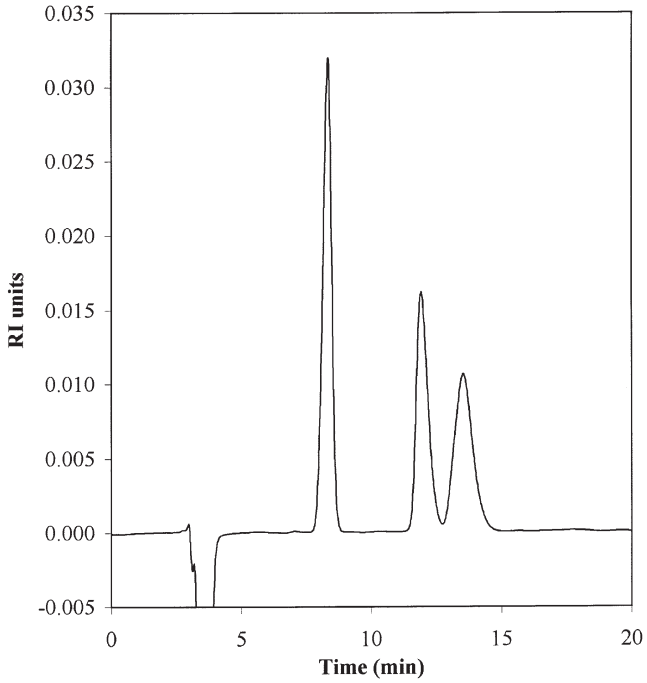

FIG. 1. HPLC profile of phosphatidylcholine (PC), 2-acyl lysophosphatidylcholine (LPC), and 1-acyl LPC. Mobile phase, ethanol/20 mM oxalic acid solution 92:8. Flow rate, 1 mL/min. Temperature, ambient. RI, refraction index.

slopes of the calibration curves of the two compounds was the same as the reciprocal ratio of their molecular weights, as can be expected when using a refractive index detector.

Precision. The precision of the determination was tested by repetitive injection of the same solution containing a given amount of PC and 1-acyl LPC. The results are shown in Table 2. The relative standard deviation was between 0.9 and 1.7% for PC in a concentration range of 0.25 and 2.0 mM and between 0.9 and 4.2% for 1-acyl LPC in the same concentration range.

Stability of the positional isomers under analysis conditions. Isomerization during analysis is a potential risk. Solutions in ethanol with a given amount of 1- and 2-acyl LPC were mixed and analyzed directly after mixing and at different time intervals thereafter. It is shown in Figure 3 that the concentration of both isomers agrees well with the concentration expected from the mixing and that there was no change in the results within 23 h.

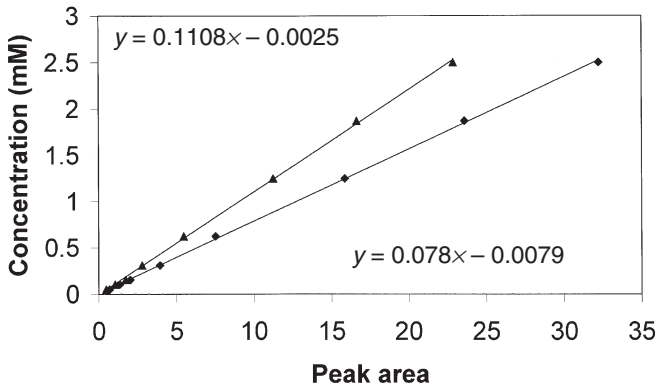

FIG. 2. Standard calibration curve for PC (◆) and 1-acyl LPC (▲). Lines were fitted by linear regression. See Figure 1 for abbreviations.

TABLE 2
Precision of the Analysis[a]

Compound	Concentration (mM)	Area (mm^2)	Standard deviation	CV (%)
PC	0.05	0.763	0.112	14.7
	0.25	3.257	0.053	1.6
	0.5	6.478	0.111	1.7
	1.0	12.923	0.116	0.9
	2.0	25.785	0.445	1.7
1-Acyl LPC	0.05	0.477	0.066	13.9
	0.25	2.309	0.098	4.2
	0.5	4.605	0.102	2.2
	1.0	9.039	0.109	1.2
	2.0	17.190	0.159	0.9

[a]The precision can be described by the coefficient of variation: CV = σ/μ · 100 (σ = standard deviation, μ = mean,). The number of repetitions *n* was 6. See Table 1 for other abbreviations.

There was no 1-acyl LPC present in the 2-acyl LPC preparation, which was synthesized as described above. That means that no acyl migration occurred during synthesis, purification, passage through the HPLC column, or storage in ethanol. However, there was about 3% 2-acyl LPC present in the commercial 1-acyl LPC preparation that had been prepared by phospholipase A_2-catalyzed hydrolysis of egg PC. Given the facts that (i) phospholipase A_2 is absolutely specific to the *sn*-2 position, (ii) 1-acyl LPC is the more stable regioisomer, and (iii) the amount of 2-acyl LPC did not change with time, one may conclude that acyl migration most probably took place during the purification procedure and not during analysis.

Fatty acid composition. PC from egg yolk or lecithin sludge comprises a mixture of different molecular species of

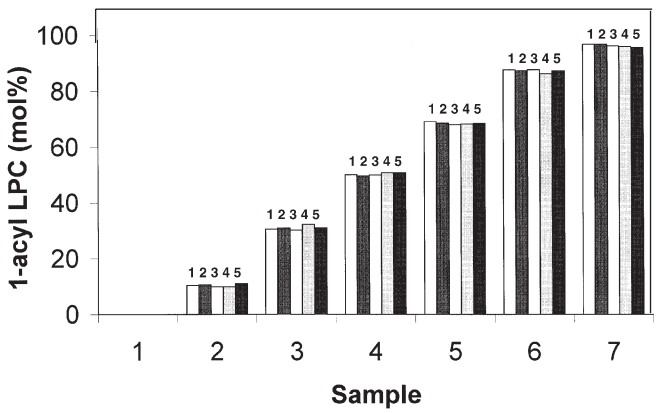

FIG. 3. Stability of samples containing 1-acyl LPC and 2-acyl LPC under analysis conditions. Solutions with known amounts of 1-acyl LPC and 2-acyl LPC were mixed. The total concentration was 10 mM. The different mixtures were analyzed directly after mixing (bar 2) and 6, 12, and 23 h after mixing (bars 3–5). The open bar (bar 1) indicates the concentration of 1-acyl LPC expected to be present. See Figure 1 for abbreviation.

TABLE 3
Fatty Acid Composition (%) of PC from Egg Yolk and Rapeseed Lecithin Sludge[a]

Fatty acid	Egg yolk	Lecithin sludge
C16:0	37.7	7.4
C18:0	12.5	0.7
C18:1	30.7	68.0
C18:2	15.5	21.0
C18:3		2.9
C20:4	3.6	

[a]The analysis was carried out as described by Svensson *et al.* (10). See Table 1 for abbreviations.

PC with an average molecular weight somewhat higher than that of dipalmitoyl PC. The fatty acid composition of PC from egg yolk and PC from rapeseed lecithin sludge is given in Table 3. Both types of natural PC eluted in a single peak about 0.5 min before the dipalmitoyl PC. Thus, it appears that the retention time of PC depends only to a minor extent on its fatty acid composition. However, when the naturally occurring fatty acids present in PC are exchanged against very short ones, species of PC that elute separated from the dipalmitoyl PC peak can be obtained. An example of such a compound is 1-palmitoyl, 2-caproyl-*sn*-glycero-3-phosphorylcholine, which elutes 1.0 min after the dipalmitoyl PC peak with a resolution of 1.1. The dependence of the fatty acid composition on the retention time of LPC was similar to that of PC. Thus, naturally occurring LPC eluted in a single peak, with a retention time similar to that of palmitoyl LPC, whereas species with an unusually short fatty acid had considerably longer retention times. As an example, 1-caproyl LPC eluted after 20 min.

APPLICATIONS

Lipozyme-catalyzed synthesis of 2-acyl LPC. Lipozyme is an enzyme specific for the *sn*-1 position of PC. When dipalmitoyl PC were incubated with Lipozyme in ethanol 2-palmitoyl LPC, palmitic acid and palmitic acid ethyl ester were formed in a reaction called ethanolysis (12). The palmitic acid and palmitic acid ethyl ester eluted with the solvent front and did not disturb the analysis. The other reactants were quantified, and the change in their concentration with time is shown in Figure 4. Dipalmitoyl PC was quantitatively converted into 2-palmitoyl LPC within about 5 h. 1-Palmitoyl LPC was not detected, and the sum of PC and 2-palmitoyl LPC was constant throughout the whole reaction. It can thus be concluded that the enzymatic reaction took place exclusively in the *sn*-1 position and that acyl migration did not take place during the enzymatic reaction.

Acyl migration in LPC. The lipozyme-catalyzed ethanolysis has also been developed as an alternative route to 1-acyl LPC, which can be obtained from 2-acyl LPC upon acyl migration. The HPLC method described in this paper is very well suited to studying the acyl migration in LPC, since there is no rearrangement between the two regioisomers of LPC under analysis conditions. The rearrangement between 1- and 2-acyl LPC can be quite rapid under conditions that favor acyl migration, such as basic pH. An example is given in Figure 5. 1-Acyl LPC and 2-acyl LPC were dissolved separately in 50 mM Tris/HCl buffer pH 9, and the rearrangement between the two regioisomers was followed by HPLC. About 90% of the 2-acyl LPC was converted to 1-acyl LPC within 8 h, and about 10% of the 1-acyl LPC isomer was converted into 2-acyl LPC. Thus, the same equilibrium mixture was obtained starting from either regioisomer. This result agrees well with the data obtained from NMR studies (14).

Phospholipase A_2-catalyzed acidolysis of PC. Phospholipase A_2 can catalyze the fatty acid exchange in the *sn*-2 position of PC by acidolysis (5,21). The reaction proceeds so that dipalmitoyl PC is first hydrolyzed to 1-palmitoyl LPC, which is then reesterified in a second step with caproic acid to yield 1-palmitoyl, 2-caproyl-*sn*-glycero-3-phosphorylcholine. Caproic acid eluted with the solvent front, but all the other reactants involved in this reaction were readily separated and quantitated. The progress curve of the phospholipase A_2-catalyzed

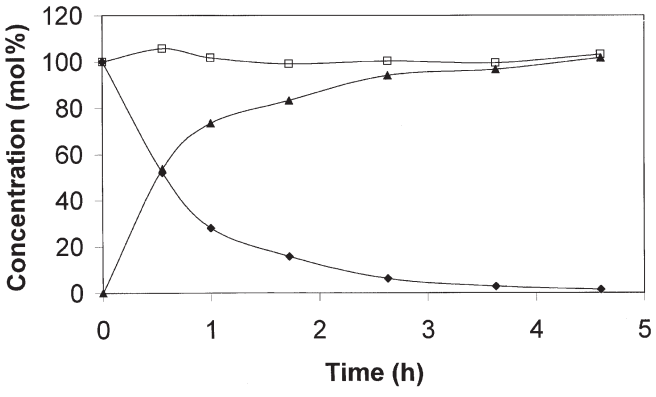

FIG. 4. Lipozyme-catalyzed ethanolysis of PC. Dipalmitoyl PC (500 mg) was dissolved in 10 mL ethanol and incubated with 1 g Lipozyme at 25°C. The mixture was shaken vigorously. (◆) Amount dipalmitoyl PC; (▲) amount 2-acyl LPC; (□) total phospholipid concentration. See Figure 1 for abbreviations.

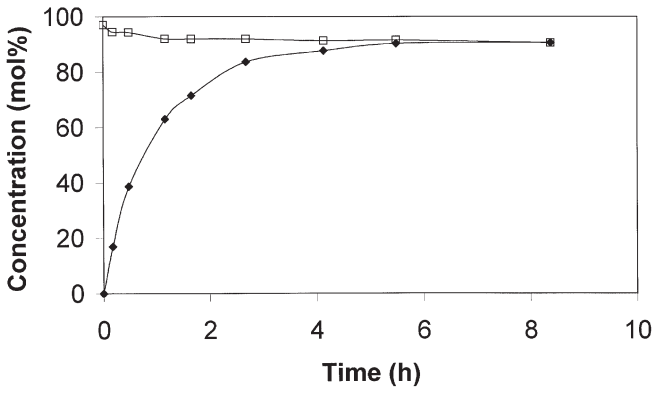

FIG. 5. Acyl migration in LPC. 10 µmol 1- or 2-acyl LPC was dissolved in 1 mL 50 mM Tris/HCl buffer pH 9. The graph shows the remaining amount of 1-acyl LPC present in the solution containing 1-acyl LPC (□) to start with and the amount of 1-acyl LPC formed *via* acyl migration in a solution containing 2-acyl LPC (◆) to start with. See Figure 1 for abbreviations.

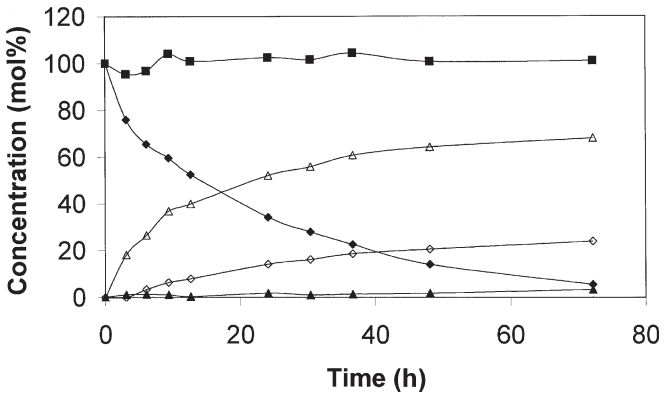

FIG. 6. Phospholipase A_2-catalyzed transesterification of dipalmitoyl PC with caproic acid. The reaction medium consisted of 10 mM dipalmitoyl PC and 800 mM caproic acid. The water activity was adjusted to 0.33. The temperature was 25°C. (◆) Amount dipalmitoyl PC; (△) amount 1-acyl LPC; (◇) amount modified PC; (▲) amount 2-acyl LPC; (■) total phospholipid concentration. See Figure 1 for abbreviations.

acidolysis of dipalmitoyl PC with caproic acid in toluene is shown in Figure 6. About 96% of the dipalmitoyl PC substrate was converted within 75 h. The reaction yielded 24% of the desired product, 1-palmitoyl, 2-caproyl-*sn*-glycero-3-phosphorylcholine, and 68% 1-palmitoyl LPC. Acyl migration was very slow under the given reaction conditions; only 4% 2-palmitoyl LPC was formed during the course of the reaction. The analysis of acidolysis reactions is in other ways rather complicated and normally involves the separation of the reactants on TLC, followed by methylation of the fatty acids and gas chromatographic analysis of the fatty acid methyl esters (10). The acidolysis with caproic acid thus represents a model reaction that can easily be monitored with the method described in this paper.

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