Separation of lipid classes by solid phase extraction

Hye-Yong Kim1 and Norman Salem, Jr.

Section of Analytical Chemistry, LCS, DICBR, NIAAA, Building 10, Room 3C-102, 9000 Rockville Pike, Bethesda, MD 20892

Summary  A rapid and reliable method for the separation of lipid classes is described using aminopropyl disposable columns. This method is a modification to an existing procedure that allows the separation of both neutral and acidic phospholipid fractions and a high recovery of the latter. Acidic phospholipids were eluted with a mixture of hexane-2-propanol-ethanol-0.1 M ammonium acetate-formic acid 420:350:100:50:0.5 containing 5% phosphoric acid after neutral phospholipids had been eluted with methanol. It was verified that extremely high recoveries of cholesterol (CH), triglycerides (TG), free fatty acids (FFA), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidic acid (PA), sphingomyelin (SM), and cerebrosides were obtained with this method. In addition, there appeared to be no preferential losses or degradation of any particular molecular species as the fatty acid distribution of bovine brain PS and the molecular species profile of plant PI were unaltered by the procedure. Depending on the tissue, this method may yield fractions containing pure lipid classes and/or simple mixtures of lipid classes of similar polarity. These fractions may then be more easily separated by thin-layer chromatography or high performance liquid chromatography for a complete lipid class analysis.


Supplementary key words  phospholipids • neutral lipids • thermospray LC-MS

Many studies in lipid biochemistry require the preparation of various neutral or polar lipid classes for further analysis. The most desirable method will be a rapid, convenient, and inexpensive method that provides for a high yield and the purity of each lipid class and, at the same time, maintains the integrity of the original lipid compositional profile. Among currently available techniques are preparative TLC, silicic acid or alumina chromatography, or preparative HPLC (1, 2). However, both column chromatography and TLC techniques are time-consuming and oxidation of polyunsaturates often occurs during the TLC process. In addition, chromatographic techniques require the use of large volumes of organic solvents, and equipment that may not be present in many research laboratories. Furthermore, it is difficult to obtain pure lipids by column chromatography or preparative HPLC techniques when a complex mixture of lipids is present in the mixture applied.

A very useful solid phase extraction method using Bond Elut aminopropyl disposable columns has been reported previously (3). The method could separate neutral lipid, free fatty acid, and phospholipid fractions. However, no fractionation of phospholipid classes was performed and attempts to reproduce this method in our laboratory indicated that the acidic phospholipids could not be eluted from the columns with the indicated solvents.

In this report, a method to elute these acidic phospholipids and separate them from neutral phospholipids as well as other lipid classes is described. Fatty acid and molecular species profiles were also examined in order to determine whether any selective losses occur during this procedure. In addition, the elution behavior of some glycolipids was examined using this procedure.

MATERIALS AND METHODS

Materials

The phospholipid standards were obtained from Avanti Polar Lipids, Inc. (Pelham, AL) and cholesterol, cerebrosides, free fatty acid, and triglyceride standards were obtained from Supelco Chemical Co. (Bellefonte, PA). Radioactive lipid standards were obtained as follows: [1,2,3H(N)]cholesterol, [9,10-3H(N)]triolein, 1-stearoyl-2-[1-14C]arachidonyl phosphatidylcholine, [glycerol-1-14C(U)]di-palmitoylphosphatidic acid, and [1-14C]docosahexaenoic acid were obtained from New England Nuclear (Boston, MA); [N-methyl-14C]sphingomyelin (bovine), 1-stearoyl-2-[1-14C]arachidonoyl phosphatidylcholine, 1-acyl-2-[1-14C]arachidonoyl phosphatidylethanolamine, and 1,2-dioleoyl-phosphatidylserine were obtained from Amersham Corp. (Arlington Heights, IL) and used without further purification. Bond Elut aminopropyl disposable solid phase columns were purchased from Analytichem International (Harbor City, CA). The solid phase extraction apparatus used in this study was obtained from Supelco Chemical Co. Radioactivity was measured using a Beckman model LS8200 liquid scintillation counter.

Preparation of rat plasma

Adult rats (Sprague-Dawley) fed a standard laboratory chow diet were decapitated and the blood was drawn into a tube with acid citrate dextrose as the anticoagulant. After centrifugation at 250 g for 15 min at room temperature, the supernatant was removed. This platelet-rich plasma was again centrifuged at 650 g for 20 min and the supernatant was subjected to lipid extraction according to the method of Bligh and Dyer (4).

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid; TG, triglyceride; SM, sphingomyelin; FFA, free fatty acid; CH, cholesterol; LC-MS, liquid chromatography–mass spectrometry; TLC, thin-layer chromatography; HPLC, high performance liquid chromatography; GC, gas chromatography.

1To whom correspondence should be addressed.
Solid phase extraction procedure

Extraction of neutral lipids was performed using a Bond Elut-aminopropyl disposable cartridge column (3 ml) according to a reported procedure (3) with 0.01 μCi of various radiolabeled lipids in the presence of 50 μg of the corresponding nonlabeled lipid. The cartridge column was equilibrated by rinsing twice with 2 ml of hexane using the Supelco extraction apparatus. Lipids dissolved in 100 μl of chloroform were loaded onto the column and the chloroform was pulled through. Thereafter, the column was eluted with 4 ml of a chloroform-2-propanol 2:1 mixture (neutral lipid fraction). Then, 4 ml of ether containing 2% acetic acid followed by 4 ml of methanol was applied to elute free fatty acids and neutral phospholipids, respectively. Finally, acidic lipids were eluted using 4 ml of a mixture of hexane-2-propanol-ethanol-0.1 M ammonium acetate in water-formic acid 420:350:100:50:0.5 containing 5% phosphoric acid. Between each elution step, care was taken not to dry the column. The flow rate was adjusted to approximately 3-5 ml/min. Each fraction collected was dried under a stream of nitrogen and subjected to liquid scintillation counting after with Aquasol (NEN). Since the acidic phospholipid fraction contained phosphoric acid, this fraction was dried under nitrogen for 10 min to evaporate the hexane and then extracted with 1 ml of chloroform three times after adding 1 ml of water. The chloroform extracts were combined, dried under nitrogen and subjected to liquid scintillation counting. In addition, experiments, unlabeled lipids alone (50-500 μg) were separated according to the same procedure for HPLC, GC, and thermospray LC-MS analyses.

HPLC analysis

Separation of phospholipid classes was performed using an Axxiom-Si column (5 pm, 4.6 mm x 25 cm) (Thomson Instruments, Springfield, VA) and a mixture of hexane-2-propanol-ethanol-0.1 M ammonium acetate in water-formic acid 420:350:100:50:0.5 as the mobile phase. The solvent was delivered by a Beckman 114M pump at a flow rate of 0.8 ml/min. Separation was monitored by UV absorbance at 205 nm using a Knauer UV detector (Thomson Instruments). Under these conditions, separation of PI and PS was achieved within 10 min. For analysis of cerebrosides, an Altex Ultrasil column (5 pm, 4.6 mm x 25 cm) (Beckman, San Ramon, CA) was used with a solvent mixture of methanol-2-propanol-hexane-0.1 M ammonium acetate in water 210:125:15:25.

Fatty acid analysis

Standard bovine brain PS obtained from Avanti Polar Lipid was transmethylated before and after solid phase extraction using BF3-methanol (14% w/v) at 100°C for 20 min according to Morrison and Smith (5). After extraction three times with hexane, the extracts were dried under nitrogen and redissoved in hexane. Fatty acid methyl esters were injected into an HP5880A gas chromatograph equipped with a flame ionization detector. A DB-FFAP capillary column (0.25 mm i.d. x 30 m) with a 0.25 μm film thickness (J&W Scientific, Folsom, CA) was employed. Oven temperature was programmed from 130°C to 175°C at 4°C/min and then to 210°C at 10°C/min. Injector and detector temperatures were 240°C. Hydrogen was used as carrier gas with a linear velocity of 54 cm/sec. Fatty acid methyl esters were identified by their retention times in comparison to those of commercial standards.

Thermospray LC-MS analysis

An Extrel ELQ-400 quadrupole mass spectrometer (Pittsburgh, PA) was used with a Vestec thermospray interface (Vestec, Houston, TX). Samples were injected into an Axxiom-Si column using the conditions described above. Mono- and diglyceride ions as well as head group ions of phospholipids were detected with the electron-emitting filament on as previously described (6-8). The vaporizer and source temperatures were 146°C and 300°C, respectively.

Table 1. Recoveries of various lipid classes after solid phase extraction

<table>
<thead>
<tr>
<th>Fraction</th>
<th>CH</th>
<th>FFA</th>
<th>PC</th>
<th>PE</th>
<th>SM</th>
<th>PI</th>
<th>PS</th>
<th>PA</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHCl3-2-propanol 2:1</td>
<td>102</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.7</td>
</tr>
<tr>
<td>Ether-acetic acid 100:2</td>
<td>0.6</td>
<td>102.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Methanol</td>
<td>-</td>
<td>-</td>
<td>100.2</td>
<td>97.4</td>
<td>97.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mobile phase'-phosphoric acid 100:5</td>
<td>-</td>
<td>-</td>
<td>3.8</td>
<td>95.6</td>
<td>107.0</td>
<td>101.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1Approximately 10 nCi of various radiolabeled lipids was extracted in the presence of 50 μg of the corresponding nonlabeled lipid.

2Percent recovery with respect to the reference radioactivity measured for a nonextracted duplicate. Figures represent the average of two samples except PS and PI (three determinations were carried out) for which the standard deviation was less than 2% (PI, 95.6 ± 1.9; PS, 107.0 ± 0.58). The blanks indicate a recovery of less than 0.5%.

3Hexane-2-propanol-ethanol-0.1 M ammonium acetate in water-formic acid 420:350:100:50:0.5.
RESULTS AND DISCUSSION

As reported previously (3), various lipid classes can be separated using solid phase extraction on an aminopropyl column. In addition to PC and PE, SM and cerebrosides also eluted in the methanol fraction. The recovery of these lipids was greater than 95% and cross contamination with other fractions was minimal as shown in Table 1. Although recovery of the radioactivity in the free fatty acid fraction (ether fraction) was about 100%, degradation of polyunsaturated fatty acids was observed when the acidic-ether fraction was dried completely under nitrogen. Thus, when *14C-labeled 22:6n-3 obtained by complete drying of the ether fraction was reapplied in chloroform to a solid phase extraction column, about 50% of the radioactivity was observed in the methanol fraction. When the ether fraction was washed twice with water prior to drying and reapplied onto the column, 97% of radioactivity was recovered in the ether fraction. Therefore, in order to prepare free fatty acids for further analysis, it is recommended that the ether fraction be washed with water in order to remove ether peroxides and acetic acid prior to evaporation so that decomposition is prevented. Since radiolabeled standards of cerebrosides were not available, recovery of this lipid was determined using normal phase HPLC with UV detection at 205 nm. As shown in Fig. 1, cerebrosides eluted as multiple peaks since they contain hydroxylated and nonhydroxylated fatty acids. They were recovered only in the methanol fraction and the recovery was nearly 100%.

However, washing with methanol did not elute acidic phospholipids from these columns. This required acidic solvents in which these phospholipids can be solubilized. Therefore, a mixture of hexane-2-propanol-ethanol-0.1 M ammonium acetate in water-formic acid 420:350:100:50:0.5 plus 5% phosphoric acid was used for this purpose. Neither the solvent mixture without phosphoric acid nor methanol containing 5% phosphoric acid was able to elute the acidic phospholipids. The recovery of PA, PS, and PI using this solvent was greater than 95% (Table 1). This was confirmed for PS and PI by normal phase HPLC as shown in Fig. 2. It was of interest to note

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Before Extraction (n = 3)</th>
<th>After Extraction† (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18:0</td>
<td>43.46</td>
<td>45.36 ± 1.85</td>
</tr>
<tr>
<td>18:1n-9</td>
<td>26.98</td>
<td>29.73 ± 0.59</td>
</tr>
<tr>
<td>18:1n-7</td>
<td>3.70</td>
<td>3.36 ± 0.59</td>
</tr>
<tr>
<td>20:1n-9</td>
<td>2.38</td>
<td>2.42 ± 0.16</td>
</tr>
<tr>
<td>20:1n-7</td>
<td>0.50</td>
<td>0.45 ± 0.02</td>
</tr>
<tr>
<td>20:3n-6</td>
<td>0.20</td>
<td>0.18 ± 0.01</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>1.10</td>
<td>1.01 ± 0.04</td>
</tr>
<tr>
<td>22:1n-9</td>
<td>0.88</td>
<td>0.42 ± 0.05</td>
</tr>
<tr>
<td>22:4n-6</td>
<td>1.44</td>
<td>1.35 ± 0.03</td>
</tr>
<tr>
<td>22:5n-6</td>
<td>1.56</td>
<td>1.49 ± 0.03</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>2.46</td>
<td>2.17 ± 0.06</td>
</tr>
<tr>
<td>24:0</td>
<td>0.32</td>
<td>0.32 ± 0.03</td>
</tr>
<tr>
<td>Total PUFA</td>
<td>6.76</td>
<td>6.20 ± 0.03</td>
</tr>
</tbody>
</table>

*Bovine brain PS (50 μg) was transmethylated using BF₃-methanol and the fatty acid distribution was analyzed by GLC.
†Data are expressed as the mean ± SD.
that after solid phase extraction, the PS peak on HPLC became sharper than the unextracted standard PS, presumably due to acidification. Recovery based on the peak area was 95% for PI, and 107% for PS. The higher than theoretical recovery for PS was presumably due to a small amount of irreversible adsorption to the normal phase silica column for the nonacidified reference PS.

Since a highly acidic solvent system was employed to elute the acidic phospholipids, polyunsaturates may be selectively lost by this procedure. In addition, it was possible that the solid phase extraction process may itself preferentially retain certain species resulting in changes in fatty acid distribution. Therefore, the fatty acid profile before and after extraction was compared for bovine brain PS (Table 2). Only a minor decrease in polyunsaturates was observed. However, it should be noted that recovery from alternative methods such as TLC usually leads to greater loss and also introduces greater variability.
As reported earlier (6-8) diglyceride ions were detected at 
phospholipid molecular species distribution is thermo-
spray LC-MS responding to the characteristic head group ion for PI was detected at 
species. Although molecular ion peaks were not detected, 
m/z 16:0-18:1, 18:0-18:3 (or 18:1-18:2), di-18:1 (or 18:0-18:2), 
and 18:0-18:1, respectively, with 16:0-18:2 as the major 
species. Although molecular ion peaks were not detected, 
the characteristic head group ion for PI was detected at 
m/z 198. This observation and the HPLC retention be-
behavior indicated that these were all PI species. The two 
spectra obtained before and after the solid phase extrac-
tion were virtually identical. These data indicate that the 
solid phase extraction procedure used here does not cause 
aberrations in the molecular species distributions.

In order to ensure that this method was applicable to 
complex lipid mixtures found in biological matrices, an 
extraction with radiolabeled standards of representative 
lipid classes was carried out in the presence of rat plasma 
lipids. The extract obtained from 1.6 ml of rat plasma was 
applied to each solid phase column. The approximate 
quantities of lipids in the plasma extract were estimated 
as follows: total lipid, 4 mg; free cholesterol, 500 μg; 
cholesteryl ester, 1.0 mg; TG, 900 μg; total PL, 1.8 mg (9). 
As shown in Table 3, cross contamination of the various 
fractions was minimal. Total recovery of radioactivity was 
also greater than 95% for the lipid standards tested.

As demonstrated in this report, a rapid, efficient, and 
reliable method for the isolation of acidic and neutral 
phospholipids as well as other lipid classes is now available 
using a solid phase extraction. This method is compatible 
with subsequent analyses of fatty acid or molecular spe-
cies distribution in biological mixtures as it maintains the 
original lipid compositional profile with high recoveries.

Since currently available methods impose difficulties, we 
believe this method will prove very useful in lipid research.

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**TABLE 3. Recovery of radioactive lipids separated by solid phase 
extraction in the presence of rat plasma**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>% Recovery</th>
<th>TG</th>
<th>FFA</th>
<th>PC</th>
<th>PS</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHCl₃-2-propanol 2:1</td>
<td>97</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Ether–acetic acid 100:2</td>
<td>1</td>
<td>98</td>
<td>−</td>
<td>98</td>
<td>5</td>
</tr>
<tr>
<td>Methanol</td>
<td>−</td>
<td>2</td>
<td>103</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Approximately 1.2 μCi of each radiolabeled lipid was applied in 100 μl chloroform solution of rat plasma extract containing approximately 4 mg of total lipid.

*Percent recovery with respect to the reference radioactivity measured for a nonextracted duplicate. The figures represent the average of two samples. The blanks indicate a recovery of less than 1%.