Analysis of lipids by high performance liquid chromatography. Part II: Phospholipids

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Overview

In Part I of this series, we described HPLC procedures to fractionate a lipid extract into its major phospholipid and neutral lipid classes. In this paper, we describe procedures for the separation of the phospholipid classes into alkylacyl, alkenylacyl, and diacyl fractions, and the separation of these fractions into their individual molecular species. At the present time, there are no satisfactory procedures to resolve the diradyl forms of intact phospholipids; therefore, these phospholipids must first be hydrolyzed to diglycerides (or ceramide in the case of sphingomyelin) with a lipase and then converted to an appropriate, nonpolar derivative (see below).

I. Enzymatic hydrolysis of phospholipid classes

If the phospholipid class composition is to be quantitated by integration, then an internal standard, dimyristyl phosphatidylcholine (di-14:0PC), is added to each phospholipid class. For a tissue like liver in which diacyl glycerides predominate in each phospholipid class, the di-14:0PC is added to the PC fraction in an amount that approximates the amount of di-16:1PC internal standard that was added to the initial lipid extract (see *Part I*). The di-14:0PC is only used to quantitate the diacyl glycerides. Quantitation of the alkenylacyl and alkylacyl glycerides is accomplished by comparison of their peak areas to the peak areas of the diacyl glycerides (as described in Section IV).

Procedure

1. The individual phospholipid classes that are isolated by HPLC (as described in *Part I*) are extracted according to the method of Folch et al.[1]

Modified in part from Patton, G.M., Fasulo, J.M., and Robins, S.J. (1982). Separation of phospholipids and individual molecular species of phospholipids by high-performance liquid chromatography. *J. Lipid Res.* **23**, 190–196; and Patton, G.M., and Robins, S.J. (1987). HPLC of molecular species of glycerophospholipids in studies of lipoproteins and lipid transport. In *Chromatography of Lipids in Biomedical Research and Clinical Diagnosis*. (A. Kuksis, ed.), pp. 311–347, Elsevier Press, Amsterdam

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The chloroform phase is transferred to a screw-capped culture tube (16 × 125 mm) and dried under N_2.

2. Six units of *Bacillus cereus* phospholipase C (Boehringer-Mannheim, Type I) in 0.5 ml of 50 mM potassium phosphate (pH 7.0) are added to each phospholipid fraction. In addition, 0.25 units of *Bacillus cereus* phosphatidylinositol specific phospholipase C (Boehringer-Mannheim) is added to the phosphatidylinositol fraction, and 0.5 units *Bacillus cereus* sphingomyelinase (Boehringer-Mannehim) is added to the sphingomyelin fraction.
3. Two ml of peroxide-free diethyl ether is added and the culture tube is sealed under N_2.
4. Each tube is vigorously shaken for 1 minute on a vortex mixer after which 4 ml of hexane is added. The tube is again shaken and centrifuged (1,000 g × 10 minutes) to separate the phases.
5. The hexane phase (upper) is removed to a clean culture tube (13 × 100 mm). The hexane extraction is repeated twice; all the hexane phases are combined and dried under N_2 at room temperature.

Notes

1. We do not know whether other phospholipase Cs or other hydrolysis procedures are totally satisfactory for this application.
2. Peroxide-free diethyl ether can be prepared by passing ether over an alumina column. Ether can be maintained peroxide-free by keeping it sealed under N_2.
3. The phospholipase C hydrolysis of cardiolipin (diphosphatidyl glycerol) yields 2 moles of diglycerides/mole of cardiolipin. Thus, the actual molecular species composition of cardiolipin cannot be determined by this procedure.
4. The actual amount of time that each of the phospholipids is exposed to phospholipase C, with the exception of cardiolipin, is apt to be on the order of one hour (during mixing, centrifugation, and transferring samples in the hexane phase). Cardiolipin is soluble in the hexane-ether mixture that is used in the initial extraction and is, therefore, exposed to phospholipase C for a much shorter period than are the other phospholilids. Since cardiolipin is not as good a substrate for phospholipase C as are the other phospholipids, to ensure that hydrolysis has occurred when there is a relatively large amount of cardiolipin, it is advisable to increase the amount of enzyme (4- to 5-fold) or to incubate the sample for at least an hour before extraction.

II. Purification of diglycerides

If the alkylacyl, alkenylacyl, and diacyl glycerides are to be quantitated by integration, it is generally necessary to purify the diglycerides before derivatization to remove a contaminant that interferes with the quantitation of the alkylacyl glycerides.

HPLC conditions

1. Stationary phase	5 μm particle size LiChrospher Si-100 (4.0 × 250 mm)
2. Mobile phase	hexane-tetrahydrofuran-acetic acid (500:50:0.1)
3. Flow rate	1 ml/min
4. Detection	absorbance at 205 nm

Procedure

1. The diglycerides are dried under N_2, dissolved in 100 μl of hexane (or mobile phase), and injected onto the column. The elution pattern for rat

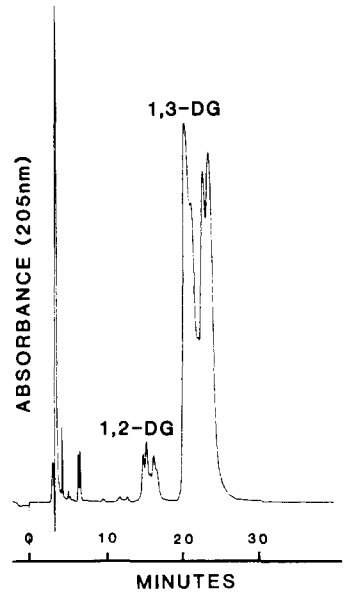

Figure 1 Purification of diglycerides. Diglycerides obtained by phospholipase C hydrolysis of rat liver phosphatidylethanolamine were dissolved in mobile phase and injected onto a 4.0 × 250 mm LiChrospher Si-100 column and eluted with hexane-tetrahydrofuran-acetic acid (500:50:0.1) at a flow rate of 1.0 ml/min. Detection was by absorbance at 205 nm at an attenuation of 1.28 absorbance units full scale (AUFS). 1,2-DG, 1,2-diglycerides and 1,3-DG, 1,3-diglycerides.

liver phosphatidylethanolamine is shown in *Figure 1*. The 1,2 diglycerides (alkylacyl and alkenylacyl) elute first, followed by the 1,3 diglycerides (diacyl). Under the conditions of the phospholipase C hydrolysis described above, the 1,2 diacyl phospholipids undergo an intramolecular rearrangement to 1,3 diglycerides.

2. The 1,2, and 1,3 diglycerides are collected together in a culture tube (13 × 100 mm) and dried under N_2.

III. Benzoylation of diglycerides and ceramide

Two procedures for the benzoylation of diglycerides and ceramide are used in this laboratory. With both procedures, it is essential that the lipids and the benzoylation reagents be totally free of water.

Benzoylation by the method of Blank et al.[2]

1. The diglycerides or ceramide are dried under N_2 at room temperature.
2. Ten mg benzoic anhydride and 4 mg dimethyl aminopyridine in 0.3 ml benzene are added to each tube.
3. The tube is sealed under N_2 and left at room temperature for 1 hour after which the reaction mixture is dried under N_2 at room temperature.

Benzoylation by the method of Ullman and McCluer[3]

1. The diglycerides or ceramide are dried under N_2 at room temperature.
2. 0.5 ml of 10% benzoyl chloride in dry pyridine is added to each tube.
3. The tube is sealed under N_2 and left at room temperature (37°C for ceramide) for 16 hours after which the reaction mixture is dried under N_2 at room temperature.

Purification of the benzoyl derivatives[3]

1. With either benzoylation procedure, add to the dry reaction mixture 3 ml hexane and 2 ml methanol-water (80:20), saturated with Na_2CO_3.

2. Shake vigorously and centrifuge (1,500g × 5 minutes) to separate the phases.
3. Remove and discard the lower methanol-water phase.
4. Wash the hexane phase three more times with 2 ml of the Na_2CO_3 saturated methanol-water and then twice with 2 ml methanol-water (80:20), discarding the methanol-water phases.
5. Remove the washed hexane phase to a clean culture tube (13 × 100 mm) and dry under N_2.

Notes

1. Pyridine is dried by storage over a 4Å molecular sieve that has been heated at 120°C for 16 hours.
2. Na_2CO_3 saturated methanol-water is prepared by dissolving 3.5 g of Na_2CO_3 in a liter of methanol-water (80:20).
3. With the benzoylation procedure of Blank et al., only the free hydoxyl group of ceramide is benzoylated. With the procedure of Ullman and McCleur, both the free hydroxyl group and the amide nitrogen are benzoylated. Consequently, the perbenzoylated ceramide (Ullman and McCluer) has a much greater retention time on a reversed-phase column than ceramide derivatized by the method of Blank et al., and the molar extinction coefficient of the perbenzoylated sphingomyelin is twice that of the internal standard (di-14:0 PC).

IV. Separation of alkenylacyl, alkylacyl, and diacyl benzoyl glycerides[4]

This procedure is used both to prepare pure lipid fractions for subsequent molecular species analysis and to determine the molar ratio of the various diradyl glycerides of a particular lipid class. For quantitation by integration, it is advisable to keep the maximum absorbance less than 0.5 AUFS to ensure that the absorbance is linear with concentration. Since that amount of material generally is not sufficient for molecular species analysis, it is generally necessary to perform this separation twice, once for quantitation of the diradyl glycerides and once for molecular species separation. If an internal standard was added to the intact phospholipid fractions, then it is necessary to correct the area of the diacyl glycerides for the amount of internal standard(s) added. This correction is performed after the diacyl glycerides have been separated into molecular species (Section V).

HPLC conditions

1. Stationary phase	5 μm particle size LiChrospher Si-100 (4.0 × 250 mm)
2. Mobile phase	cyclohexane-hexane-methyl tert.-butyl ether-acetic acid (375:125:10:0.1)
3. Flow rate	1 ml/min
4. Detection	absorbance at 230 nm

Procedures

1. The benzoyl diglycerides are dried under N_2, redissolved in 100 μl of mobile phase, and injected onto the column. The elution pattern of rat brain phosphatidylethanolamine is shown in *Figure 2*. Brain phosphatidylethanolamine is one of the few phospholipids which contains all three types of diradyl glycerides.
2. The fractions are collected in screw-capped culture tubes and dried under N_2.

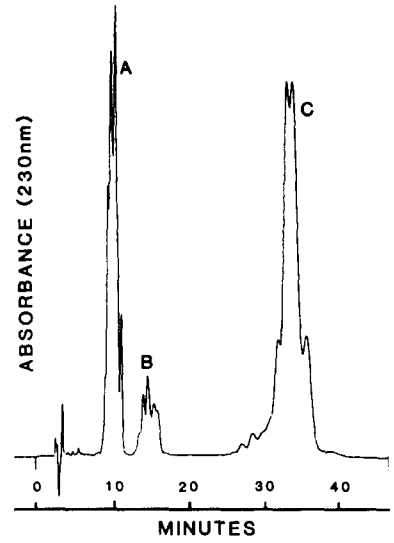

Figure 2 Separation of benzoylated diradyl glycerides. Benzoylated diglycerides derived from rat brain phosphatidylethanolamine were dissolved in 100 μl of mobile phase and injected onto a 4.0 × 250 mm LiChrospher Si-100 column and eluted with cyclohexane-hexane-methyl tert.-butyl ether-acetic acid (375:125:10:0.1) at a flow rate of 1.0 ml/min. Detection was by absorbance at 230 nm at an attenuation of 1.28 AUFS. A, alkenylacyl glycerides; B, alkylacyl glycerides; and C, diacyl glycerides.

Notes

Although there are notable exceptions, phosphatidylethanolamine generally contains almost exclusively alkenylacyl and diacyl glycerides, while phosphatidylcholine generally contains alkylacyl and diacyl glycerides, or only diacyl glycerides. The other glycerophospholipids are almost exclusively composed of diacyl glycerides.

V. Separation of molecular species[5]

HPLC conditions

1. Stationary phase	μm particle size Ultrasphere ODS (2.0 × 250 mm)
2. Mobile phase	methanol-water-acetonitrile (950:40:10)
3. Flow rate	0.3 ml/min
4. Detection	absorbance at 230 nm

Procedure

The benzoyl diglycerides or benzoylated ceramides are dissolved in an appropriate volume of methanol and 100 μl is injected onto the column. The elution pattern of rat liver diacyl phosphatidylcholine is shown in *Figure 3A*. The elution pattern of rat liver sphingomyelin (benzoylated by the method of Blank et al.) is shown in *Figure 4*.

Notes

1. Any C18 reversed-phase column can be used for this application. The only major consideration is the efficiency of the column (i.e., the number of theoretical plates/column). When necessary, better resolution can be obtained by adding an additional column (*Figure 3B*).
2. The precise composition of the mobile phase will depend on the selectivity of the particular columns used and on which particular peaks are of greatest interest. If more water is added to the mobile phase, the *abso-*

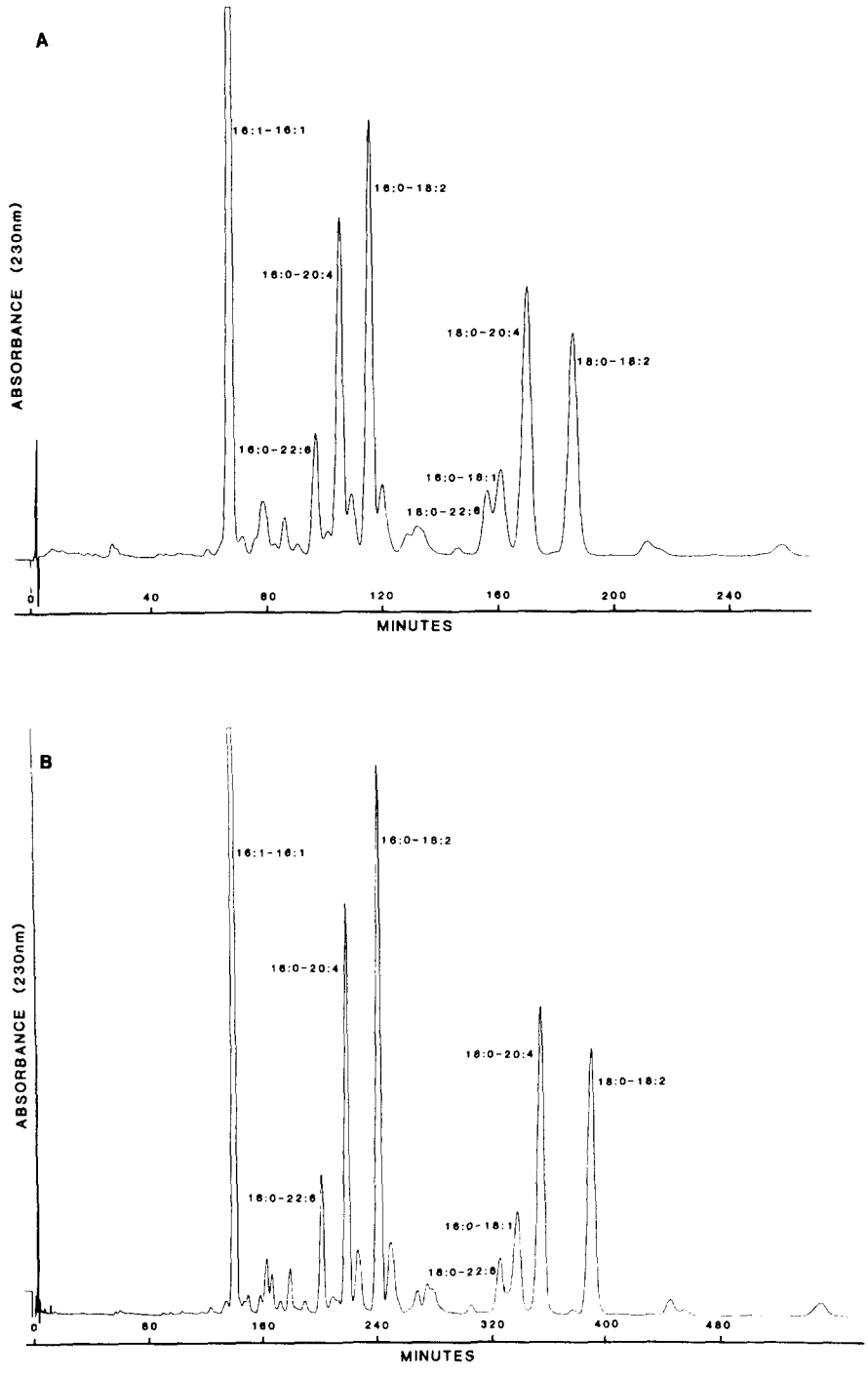

Figure 3 Molecular species separation of benzoylated diacyl glycerides. Benzoyl diacyl glycerides derived from rat liver phosphatidylcholine were dissolved in methanol and injected onto either one (A) or two (B) 2.0 × 250 mm Ultrasphere ODS column(s) and eluted with methanol-water-acetonitrile (950:40:10) at a flow rate of 0.3 ml/min. Detection was by absorbance at 230 mm at an attenuation of 0.08 AUFS. The internal standard (16:1-16:1PC) and major molecular species are labeled. Other molecular species are identified in reference 6.

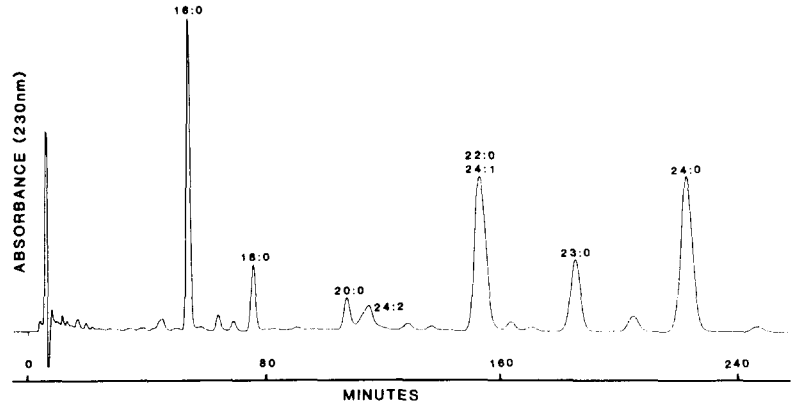

Figure 4 Molecular species separation of benzoylated ceramides. Ceramides derived from rat liver sphingomyelin and derivatized by the method of Blank et al.[2] were dissolved in methanol, injected onto a 2.0 × 250 mm Ultrasphere ODS column, and eluted with methanol at a flow rate of 0.3 ml/min. Detection was by absorbance at 230 nm at an attenuation of 0.08 AUFS. Major molecular species are labeled.

lute retention time of any particular molecular species will increase and the overall resolution will improve. The *relative* retention times of the various molecular species can be manipulated by varying the acetonitrile concentration. Acetonitrile forms a complex with the double bonds of the acyl groups, thereby making the unsaturated molecular species more soluble in the mobile phase and hence causing them to elute earlier. The more double bonds in a molecular species, the greater the effect of the acetonitrile.

3. Since quantitation is by integration, it is advisable to adjust the amount of sample injected so that the maximum absorbance is at or below 0.2 AUFS.
4. The retention time of a given molecular species varies among the diradyl glycerides and ceramides. For example, the relative retention time of 16:0-18:1 is: diacyl (1.00); alkenylacyl (1.46); and alkylacyl (1.75). The retention time (relative to diacyl 16:0-18:1) of the corresponding ceramide (d18:1-16:0) is 0.47 for the monobenzoyl ceramide,[2] and 1.46 for the dibenzoyl ceramide.[3]

Discussion

Lipid extracts of tissues contain natural antioxidants. Thus, these extracts can be stored (under N_2) for prolonged periods without oxidation. In the initial HPLC procedure described (Part I, Section II), these natural antioxidants elute with the neutral lipid fraction which, therefore, can also be stored for prolonged periods. The pure phospholipid fractions, however, contain no natural antioxidants. Thus, as a precaution, small amounts of the antioxidant, BHT, can be added to these fractions if they are to be kept for extended periods. Small amounts of BHT will not interfere with subsequent steps in the fractionation procedure (i.e., hydrolysis or benzoylation) and do not interfere with quantitation at 230 nm.

In a previous publication,[5] we described an HPLC procedure to separate the major molecular species of intact phospholipids. However, the utility of that procedure in metabolic studies is limited by incomplete resolution of individual components, the difficulty of quantitating components, and the inability to resolve the different types of diradyl glycerides. These problems can be solved by derivatizing the purified phospholipid classes with a non-polar chromophore. In this paper, we describe procedures to prepare and fractionate the benzoyl derivatives of phospholipids. Other chromophores may also be used, but very polar chromophores may compromise the separation of the alkylacyl and alkenylacyl glycerides. In addition, it is necessary to avoid derivatization procedures in which the lipids are

exposed to acidic organic solvents, which can result in cleavage of the vinyl ether bond of the alkenylacyl glycerides. Even with derivatized phospholipids, however, it is not always possible to obtain pure molecular species. For most phospholipids, there are only a few (8 to 12) major molecular species but there are often a very large number of minor species (40 to 60) that elute with the major molecular species. Therefore, for the isolation of pure molecular species (especially in studies in which phospholipids have been radiolabeled), it may be necessary to collect the fraction(s) of interest and rechromatograph these fractions using a different mobile phase (such as one that is acetonitrile-based) or a different type of stationary phase.

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