Analysis of lipids by high performance liquid chromatography: Part I

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Overview

High performance liquid chromatography (HPLC) methods are described to isolate and quantitate major categories of lipids in animal tissues. For convenience, a minimum of two complete HPLC systems are recommended. One system will be used with a mobile phase that contains a salt solution and is dedicated to the separation of major classes of phospholipids. The other system can be used for all other separations with either silica or reverse phase columns and totally organic mobile phases. In this section, HPLC procedures will be outlined for the isolation of major phospholipid classes and neutral lipids and for the quantitation of cholesterol. In Part II, HPLC procedures will be outlined for the separation and quantitation of individual molecular species of phospholipids. The sequence of the major procedures described is illustrated in *Figure 1*. Although a number of alternative procedures are available for quantitating lipids, the HPLC methods described will permit the quantitation of mass as well as preserve the acyl group structure of individual molecules for other kinds of analysis. Most particularly, in the case of phospholipids these methods may be used to determine changes in overall molecular composition as well as changes in portions of molecules that are selectively utilized (and remodeled) in metabolic reactions.

I. Preparation of samples for chromatography

Materials

1. Organic solvents for lipid extraction and for subsequent HPLC are "HPLC-grade" and are used without further purification.
2. All glassware is either disposable or "acid-washed" (in a sulfuric acid-dichromate solution). All samples are routinely collected and stored in screw-top culture tubes with teflon-lined caps.

Procedure

Lipids are generally extracted from tissues according to the method of Folch *et al.*[1] To protect samples from degradative (oxidative) changes, samples are kept under N_2 at all times. With the use of N_2, the addition of

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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.

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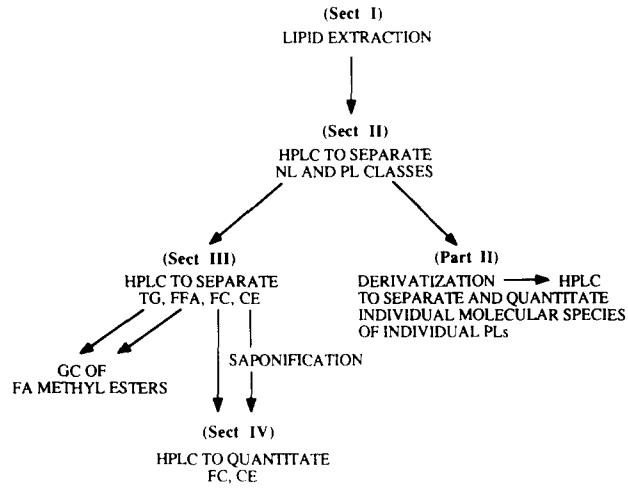

Figure 1 A flow diagram to illustrate the major steps of the procedures outlined. The "Sect" designations relate to sections of the text. The methods under Part II will follow in another issue. Abbreviations: NL, neutral lipids; PL, phospholipids; TG, triglycerides; FFA, free-fatty acids; FC, free cholesterol; CE, cholesteryl esters.

an antioxidant to samples is unnecessary, and antioxidants may interfere with detection at 205 nm (see below). To quantitate individual components, extractions are performed as follows:

1. A weighed amount of tissue, kept at 0°C, is homogenized in water. A known volume of the homogenate (generally, 0.5–2 ml) is transferred to a screw-top culture tube to which is added 19 vol of chloroform: methanol (premixed, in a ratio of 2:1).
   The amount of the tissue homogenate extracted may be as much as 100 mg (wet weight of tissue) or as little as 5–10 mg to obtain sufficient lipid for subsequent analysis. The lipids in 100 mg of tissue (using liver) are the maximum amount that can be resolved using the initial HPLC system described below (Sect II).
2. Internal standards are added at this point to correct for losses. Depending on the lipids to be quantitated, standards will consist of a known amount (in 10–100 μl of chloroform) of:
   a. stigmasterol, for free cholesterol;
   b. stigmasterol acetate, for cholesteryl esters;
      (each added in an amount that is anticipated to be present in the sample)
   c. trieicosenoin (tri-20:1), for triglycerides;
   d. hepatadecanoic acid (17:0), for free fatty acids; and
   e. dipalmitoleoyl phosphatidylcholine (di-16:1PC), for phosphatidylcholines.
      (each added in an amount that is anticipated to be 10-20% of the amount in the sample).
   All of these standards are kept at −20°C, in separate screw-top tubes. (Each is generally made up and stored in multiple aliquots to avoid repeatedly sampling from the same tube which can result in evaporation of the solvent and concentration of the standard.)
3. Samples are flushed with N_2, capped, and mixed well.
4. 0.9% NaCl, equal to 20% of the volume of chloroform:methanol, is then added.
5. Samples are mixed and centrifuged (1000 × *g* for 10 min) to separate the phases.
6. The upper methanol-water phase is discarded and the lower chloroform phase is transferred to a second screw-top culture tube and evaporated (under N_2) to dryness.

7. Fresh chloroform is then added, the tube is flushed with N_2 and tightly capped.

II. Separation of major phospholipid classes

The HPLC procedure that is described will result in the separation of all major phospholipid classes and the separation of these phospholipids from neutral lipids (as a group).[2,3]

HPLC equipment

For these procedures there are no specific considerations or requirements for specific components except those that are described below: (In particular, there is no need for any gradient-making equipment, and the use of fraction collectors has not proven to be useful.)

Injectors. It is desirable to fit all injectors with a 200 μl sample loop (which is large enough to accommodate the sample and yet small enough to avoid mixing of the sample with the mobile phase in the injector loop). A screen-type filter (2 μ) is installed between the injector and column.

Detectors. A high quality variable wavelength instrument is most desirable. Flow cells should be small (with a capacity of 8 μl or less).

Recorders. If components of a sample are to be collected, a recorder is usually necessary for real-time monitoring of the column effluent.

Integrators. It is necessary to add a programmable integrator to a system (preferably with printer-plotter capability) for quantitation of peak areas.

HPLC conditions

Mobile phase. Hexane-isopropanol-ethanol-25 mM potassium phosphate (pH 7.0)-acetic acid (376:485:100:56.2:0.2 to 0.6).

Stationary phase. LiChrospher Si-100 column (25 cm × 4.0 mm, 5 μm particle size) (E. Merck, Darmstadt, FRG).

Flow rate. 1 ml/min.

Detection. 205 nm (but 195–214 nm is adequate).

Special considerations

Selection of the stationary phase: The LiChrospher column listed is effective in achieving the separations shown. Other silica columns may not necessarily produce the same favorable results.

Procedure

1. Preparation of the mobile phase: add in sequence
   a. 55 ml of water
   b. 1.2 ml of 1 M potassium phosphate buffer (pH 7.0)
   c. 0.2 to 0.6 ml acetic acid (see below)
   d. 485 ml isopropanol
   e. 376 ml hexane
   f. 100 ml absolute ethanol

   The components are mixed, allowed to stand overnight, and then filtered to remove precipitated potassium phosphate. (Filter under vacuum using a 0.45 μ [or finer] filter that is compatible with organic solvents.)
2. Preparation of the stationary phase: Before initial use, the column is

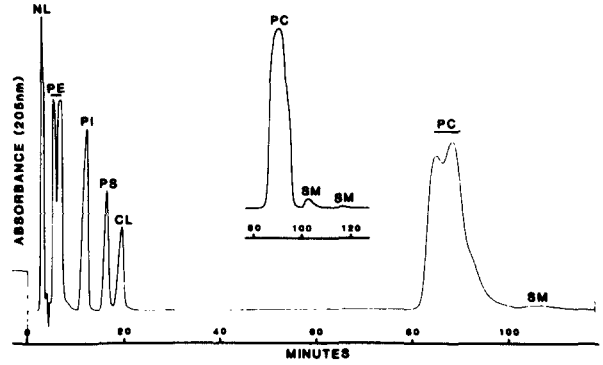

Figure 2 HPLC separation of the major classes of phospholipids in a whole lipid extract of rat liver. Chromatography was performed with a LiChrospher Si-100 column, a mobile phase of hexane-isopropanol-ethanol-25 mM potassium phosphate (pH 7.0)-acetic acid (376:485:100:56.2:0.6), and a flow rate of 1.0 ml/min. Neutral lipids elute as a group. Insert shows HPLC with an increased sample size to demonstrate the entire area that sphinogomyelin will elute (marked with SM from start to end). Abbreviations: NL, neutral lipids; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; CL, cardiolipin; PC, phosphatidylcholine; SM, sphingomyelin. (Lysolecithin has a retention time that is 2.6 times greater than PC and is not shown.)

washed with mobile phase at ~0.5 ml/min for 2–3 days. The column, stored in mobile phase, is ready for immediate use at any time thereafter. This column can be expected to last for several years with filter changes (see above) every 1–3 months.

3. The sample is dried under N_2 and resolubilized in 100 μl of mobile phase. The entire sample is generally injected. Retention times are shown in *Figure 2* for a rat liver extract.

Special considerations

1. Retention times can be decreased by adding more water to the complete mobile phase (after filtration). With an additional 1.5 ml of water per 100 ml of mobile phase, the phosphatidylcholine peak will elute at ~25–35 min (*Figure 3*) instead of from 80–100 min with the mobile phase used in *Figure 2*.
2. Changes in the amount of acetic acid will result in changes in the retention time of cardiolipin (an increase in acetic acid increases cardiolipin retention). At a low concentration of acetic acid, cardiolipin will generally elute between phosphatidylinositol and phosphatidylserine (*Figure 3*) and at a higher concentration, after phosphatidylserine (*Figure 2*). The concentration of acetic acid that is used will depend on the individual characteristics of a column.
3. Small changes in the relative proportions of the organic solvent components of this system will have virtually no effect on the elution pattern of lipids.
4. If this procedure is to be used to simply quantitate the total amount of each phospholipid class (i.e., not used for further phospholipid analysis), the di-16:1PC internal standard is not added and the phospholipids that are collected from the column are analyzed for phosphorus. (Before phosphorus can be measured, however, it is necessary to re-extract each of the collected fractions by the Folch method and wash the lower phase two times with methanol-NaCl (1:1) to ensure that the phosphate from the mobile phase has been entirely removed.)
5. The detection of lipids in the 200-nm range is principally a function of the number of fatty acid double bonds. It is, therefore, not possible to quantitate by integration of peak areas the components of a sample that have a different fatty-acid composition. Poor detection of phospholipids that are highly saturated may pose a problem. However, this is ordinarily only of concern in the case of sphingomyelin that, as shown in the insert in *Figure 2*, will elute over a relatively large area.

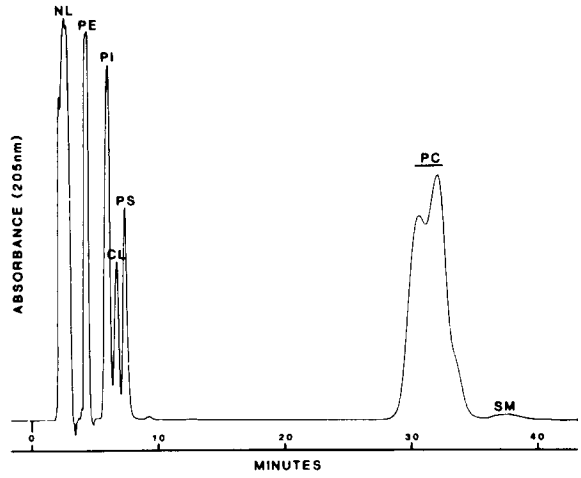

Figure 3 HPLC separation of major classes of phospholipids in a whole lipid extract of rat liver. Chromatography was performed as in *Figure 2*, but to decrease retention times 1.5 ml water was added per 100 ml of complete mobile phase. To reposition cardiolipin between phosphatidylinositol and phosphatidylserine, the mobile phase was also changed to contain less acetic acid (0.275 ml instead of the 0.6 ml used for the HPLC in *Figure 2*). Abbreviations are the same as in *Figure 2*.

III. Separation of major neutral lipid classes

The HPLC system that is described is used to separate cholesteryl esters, triglycerides, free-fatty acids, and free cholesterol. The collected fractions may be then individually analyzed to determine total mass and fatty acid composition by gas chromatography (GC) or by additional HPLC procedures (see below).

HPLC conditions

Mobile phase. Hexane-tetrahydrofuran-acetic acid (500:20:0.1).

Stationary phase. LiChrospher Si-100 column (see above).

Flow rate. 1 ml/min.

Detection. 205 nm.

Special considerations

The LiChrospher column used for the phospholipid class separation above cannot be conveniently dried and, thus, a second LiChrospher column should be used for separating neutral lipids.

Procedure

1. Before initial use, this column is washed with chloroform for several hours and then with the mobile phase until there is no further decrease in absorbance at 205 nm (i.e., until all of the chloroform has been washed from the column). Columns are stored in the mobile phase and are ready for use ~30 min after start-up.
2. The neutral lipid fraction that was collected above is dried under N_2 and extracted by the method of Folch et al., (to remove the potassium phosphate from the preceding chromatography).
3. The sample is dried, solubilized in ~100 μl of hexane (or complete mobile phase), and injected for HPLC. The pattern of elution is illustrated for rat liver neutral lipids in *Figure 4*.

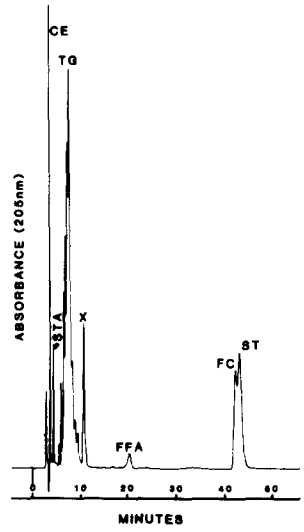

Figure 4 HPLC separation of the neutral lipids of rat liver, obtained from the HPLC shown in *Figure 2* or *Figure 3*. Chromatography was performed with a LiChrospher column, a mobile phase of hexane-tetrahydrofuran-acetic acid (500:20:0.1), and a flow rate of 1.0 ml/min. The stigmasterol standards that were added to the initial lipid extract are detected just after the cholesterol peaks. Abbreviations: CE, cholesteryl ester; STA, stigmasterol acetate; TG, triglyceride; FFA, free-fatty acids; FC, free cholesterol; and ST, stigmasterol. The peak labeled X is unidentified and is a presumed contaminant.

IV. Quantitation of cholesterol

HPLC conditions

Mobile phase. Methanol.

Stationary phase. Ultrasphere ODS column (25 cm × 4.6 mm, 5 μm particle size) (Beckman/Altex, San Ramon, CA, USA. Other ODS columns may be substituted).

Flow rate. 1 ml/min.

Detection. 205 nm.

Procedure

1. The cholesteryl esters and free cholesterol together with their stigmasterol internal standards (obtained from HPLC in Sect III) are dried under N_2. These fractions can be pooled and saponified together (see below) if it is not necessary to separately quantitate cholesteryl ester and free cholesterol amounts.
2. The steryl ester fraction is saponified in 2 ml of 1M KOH (made up in 93% ethanol) with heating at 80°C for 4 h. After cooling, 5 ml of water is added and the sterols are extracted (3 times) into 5 ml of hexane. The combined hexane extracts are dried under N_2. (One drop of acetic acid is added to the hexane to neutralize any residual base.)
3. The free sterols are resolubilized in methanol.
4. Each sterol fraction is quantitated by reverse phase HPLC and integration of peak areas in conjunction with known concentrations of authentic cholesterol and stigmasterol standards (*Figure 5*). (For the conditions described, 10 μg of cholesterol will produce a detector response of ~0.15 absorbance units.)

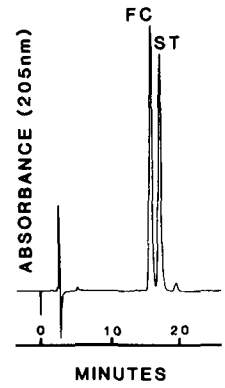

Figure 5 HPLC of the sterols obtained from chromatography of the neutral lipid fraction of rat liver (*Figure 4*). HPLC was performed with a reverse phase ODS column (Ultrasphere), a mobile phase of methanol, and a flow rate of 1.0 ml/min. Stigmasterol (ST), added as an internal standard to the initial lipid extract, was used to determine the amount of free cholesterol (FC) in the sample.

Special considerations

If the acyl group composition of the cholesteryl esters needs to be determined, the steryl ester fraction is not saponified but is transesterified with sodium methoxide and the resulting methyl esters are then quantitated by GC.[4] In this case, cholesteryl heptadecanoate is used instead of stigmasterol acetate as an internal standard.

V. Quantitation of triglycerides and free-fatty acids

Triglycerides and free-fatty acids obtained by HPLC in Section III are quantitated as fatty acid methyl esters by capillary GC. A detailed discussion and illustrations of the chromatography are provided by Patton *et al.*[4]

Discussion

The HPLC procedures described have been shown to result in the quantitative recovery of all major phospholipids and neutral lipids from animal tissues.[2] These procedures will have their greatest utility in studies in which it is desirable to isolate the fatty acid components of different classes of lipids or when radiolabeling is used to measure the turnover of particular lipids. With these methods it is possible to measure radioactivity and mass directly from the same sample and to isolate lipids with radiolabels at different portions of the same molecule. These procedures may be used in conjunction with the molecular species analysis of phospholipids (see Section II) to isolate samples for the identification of specific substrates and products of enzyme reactions, to trace the transport of individual molecules, and to more precisely characterize membranes of different origins.[3,5]

Acknowledgment

This work was supported by The General Medical Research Service of the Veterans Administration and National Institutes of Health Grant AM 28640.

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