Rapid separation and quantification of lipid classes by high performance liquid chromatography and mass (light-scattering) detection

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Abstract The major lipid classes in animal tissues, varying in polarity from cholesteryl esters to lysophosphatidylcholine, can be separated and accurately quantified by high performance liquid chromatography on a short 3-μ silica column and using a mass (light-scattering) detector. Sample sizes of 0.2 to 0.4 mg are optimum and the analysis is completed in only 20 min. The column is reactivated and ready for the next analysis after a further 10 min. After acid treatment, the plasmalogen forms of phospholipids can be determined. Applications of the procedure to the analysis of rat liver, heart, erythrocytes, and plasma lipids are described. —**Christie, W. W.** Rapid separation and quantification of lipid classes by high performance liquid chromatography and mass (light-scattering) detection. *J. Lipid Res.* 1985. **26:** 507–512.

Supplementary key words mass detector • neutral lipids • phospholipids • plasmalogens • heart • liver • erythrocytes • plasma

The full potential of the resolving power of high performance liquid chromatography (HPLC) in the separation and analysis of the simple and complex lipid classes from animal tissues has yet to be realized because of the non-availability commercially of a universal detector for lipids. Moving-wire flame-ionization detectors held great promise, but until recently they had not been produced commercially for a number of years. Nonetheless, Privett and colleagues have demonstrated some excellent separations with such a system (1–5). A 'mass detector' (not to be confused with mass spectrometry) or 'evaporative analyzer' has been described (6) in which the solvent emerging from the HPLC column is evaporated in a stream of compressed air; the solute does not evaporate and passes in the form of minute droplets through a light beam, which is reflected and refracted. The amount of scattered light is measured and bears a relationship to the weight of material in the HPLC eluant. A commercial detector, based on this principle, is available at a cost comparable to that of other optical detectors. An application of this detector to the separation of molecular species of triacylglycerols has been described (7). Other investigators have used a custom-made mass-detector, which they term a 'light-scattering detector,' for the same purpose (8, 9).

An HPLC method is here described that utilizes the mass detector for the rapid separation and quantification of the main lipid classes in animal tissues, ranging from cholesteryl esters at one end of the polarity spectrum to lysophosphatidylcholine at the other. A ternary solvent-proportionating system is used, together with a 3-μ silica adsorbent in a short (10-cm) column.

MATERIALS AND METHODS

Cholesteryl palmitate, triolein, tripalmitin, cholesterol, dipalmitin, 2-monopalmitin, dolichol, cerebrosides (from bovine brain), cardiolipin (bovine heart), phosphatidic acid (dipalmitoyl) (free acid, Na^+ and Ca^{++} salts), phosphatidylglycerol (dipalmitoyl), phosphatidylethanolamine (bovine liver), phosphatidylinositol (soybean), phosphatidylserine (bovine brain), phosphatidylcholine (egg yolk), phosphatidyl-N,N-dimethylethanolamine (dipalmitoyl), phosphatidyl-N-monomethylethanolamine (dipalmitoyl), sphingomyelin (bovine brain), lysophosphatidylethanolamine (egg yolk), and lysophosphatidylcholine (egg yolk) were obtained from Sigma Chemical Co. (Poole, Dorset, UK) and were 98–99% grades. All solvents were Analar or HPLC grades (Fisons Ltd., Loughborough, Leics., UK). The purity of each was assessed by the final HPLC procedure. Only components that appeared to be 98% pure or better were used for weighed standards.

Tissues

A liver was removed from a 250-g female Wistar rat following cervical dislocation. Blood was withdrawn from a 750-g male Wistar rat by aortic cannulation of the pentobarbital-anaesthetized animal; 8% sodium citrate in 0.9% sodium chloride solution was added as anticoagulant. The plasma was removed following centrifugation and the erythrocyte layer was washed twice with saline solution. The still-beating heart was plunged into cold saline,

Abbreviations: HPLC, high performance liquid chromatography.

rapidly chopped with scissors, and rinsed with fresh saline. The lipids were extracted at once with chloroform–methanol 2:1 (v/v) from all tissues.

HPLC system

A Spectra-Physics Model 8700 solvent delivery system was used (Spectra-Physics Ltd., St. Albans, UK), and consisted of a programmable ternary solvent-proportionating valve on the low-pressure side of a single pump; a rheodyne valve injector was equipped with a 10-μl loop. The ACS 750/14 Mass Detector (Applied Chromatography Systems Ltd., Luton, Beds., UK) was connected to an air compressor capable of delivering filtered air at 50 psi at 5 litres/min. The internal air pressure was set at 27 psi, and the 'evaporator set' (an arbitrary parameter that fixes the temperature in the evaporator) was adjusted to 40. For all the quantitative work described here, a 10 cm × 5 mm (i.d.) column packed with Spherisorb™ 3-μ silica (Hichrom Ltd., Reading, Berks., UK) was used; in much of the exploratory work, a 25 cm × 5 mm (i.d.) column together with a 5 cm × 5 mm guard column packed with Hypersil™ 5-μ silica (HPLC Technology, Macclesfield, Cheshire, UK) was used.

The three vessels on the ternary solvent mixer contained the following: A, isooctane–tetrahydrofuran 99:1 (v/v); B, isopropanol–chloroform 4:1 (v/v); C, isopropanol–water 1:1 (v/v). All solvents were filtered through a 10-μm stainless steel frit, and were degassed with helium (10). The solvent program employed is shown in **Table 1**. The first 20 min of the elution scheme was designed to separate the lipid components, while the last 10 min regenerated the column prior to injection of the next sample. As the programmer only permitted seven timed steps, the final one was timed manually. The flow rate was 2 ml/min throughout. Lipid samples (0.2 to 0.4 mg) were passed through an 0.5-μ filter and injected onto the column in 5 μl of chloroform–isooctane 1:1 (v/v).

TABLE 1. Ternary gradient elution system required for the elution of lipid classes and reactivation of the column

Time[a]	% Solvent		
	A	B	C
min			
0	100		
1	100		
5	80	20	
5.1	42	52	6
20	32	52	16
20.1	30	70	
25	100		
30	100		

[a]A linear gradient was produced between the compositions specified at each time interval.

Lipid components were quantified by electronic integration using an LDC Model 308 integrator (Laboratory Data Control Ltd, Stone, Staffs., UK).

Determination of plasmalogens

A portion of the heart lipid extract was dissolved in 4 ml of chloroform–methanol 2:1 (v/v) and concentrated hydrochloric acid (0.1 ml) was added. After 30 min, water (1 ml) was added, and the chloroform layer was separated and taken to dryness in a stream of nitrogen. The plasmalogen content was determined by comparing the analyses before and after this treatment.

RESULTS

The use of the mass detector for HPLC analysis imposes two constraints on the elution solvents used, i.e., they must be sufficiently volatile to evaporate under conditions that do not vaporize the solute, and they cannot contain inorganic ions. In order to separate natural lipid classes of widely differing polarity in a single step, it is necessary to use gradient elution, starting with a solvent of low polarity and ending with a solvent mixture containing an appreciable amount of water. A solvent of medium polarity, miscible both with hydrocarbons and with water, is required to mediate the transfer from one extreme to the other. Isopropanol was found to be the solvent most suited for the purpose. In preliminary experiments with a 25-cm Hypersil™ 5-μ silica column, gradients of isopropanol in isooctane gave satisfactory separations of the simple (neutral) lipids, while gradients of water in isooctane–isopropanol mixtures gave usable resolutions of individual phospholipids, provided that the flow rate was maintained at about 2 ml/min. At slower flow rates, peak broading or double peaks were obtained with pure phospholipid standards, possibly because of competing adsorption/partition effects over a relatively long time scale. Similar solvent combinations have been used by other workers specifically for phospholipid separations (11–14). However, phosphatidylcholine and sphingomyelin were poorly resolved, and very high pressures were required to maintain the flow rate. Dilution of isopropanol in reservoir B with other solvents was therefore tried; the necessity of maintaining a single phase with isooctane, isopropanol, and water acted as a constraint, and the addition of 20% chloroform was found to relieve both problems.

After an analysis, it was necessary to reequilibrate the column by removing as much as possible of the bound water, prior to injecting the next sample. The capacity of the column to resolve cholesteryl esters and triacylglycerols was the best test of whether this had been achieved. Re-

equilibration occurred only slowly when isooctane or isooctane–isopropanol mixtures were pumped through the column, but was achieved rapidly when 1% tetrahydrofuran (or dioxan or dimethoxypropane) was present in isooctane. If the column was inadvertently reequilibrated for too long, it was advisable to bleed in a little of solvent B, then to reequilibrate with solvent A for the correct time, before proceding to the next analysis.

Three-micron adsorbents offer the advantages of high resolution and short analysis times. A short Spherisorb™ 3-μ silica column, which was claimed to contain few residual mineral ions that could retard the elution of phospholipids, was tried. Much better resolution was immediately attained than with the longer column; less water was required to elute the phospholipids, lower operating pressures were needed (3,700 psi at maximum), and the column was very rapidly reequilibrated. This column was used for all the quantitative analyses.

When the solvent gradients were optimized, the temperature in the mass detector was set to its optimum value by adjusting the 'Evaporator Set' control until a minimum noise level was obtained over the whole gradient (as recommended by the manufacturer). The precise temperature, which this represented, is not known. At higher temperature settings, some loss of free fatty acids by evaporation was observed.

The type of separation that is achieved with the solvent gradients described and mass detection is illustrated by **Fig. 1**, where a rat liver lipid extract is the example. There is no solvent peak as is seen with many other detectors, and the recorder base-line was entirely stable throughout the analysis, in spite of the abrupt changes in solvent composition at various points in the elution scheme. Cholesteryl esters and triacylglycerols were clearly resolved, diacylglycerols formed a shoulder (sometimes better resolved) on the cholesterol peak (dolichol cochromatographed with cholesterol), and unesterified fatty acids were eluted just ahead of the phospholipids. Phosphatidylglycerol, diphosphatidylglycerol (cardiolipin), and ceramide eluted together, but phosphatidylethanolamine, phosphatidylinositol, and phosphatidylcholine were eluted as sharp peaks followed by sphingomyelin (two bands). Pure lipid standards were chromatographed individually to confirm retention times. A variety of other lipids were cochromatographed with the lipid extract to determine where they would elute and these are indicated on the figure. Phosphatidic acid alone caused difficulty; the free acid and sodium salts eluted as broad humps with phosphatidylserine, while the calcium salt emerged in a similar manner with phosphatidylinositol. At physiological concentrations, phosphatidic acid would probably not be detected. It should be noted that only 0.2 to 0.4 mg of lipid in total is required for an analysis, the separation was complete in 20 min, and only a further 10 min of elution was needed to reequilibrate the column.

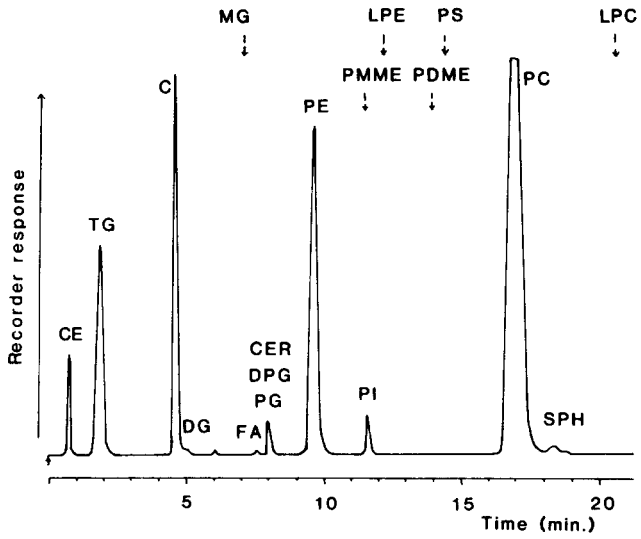

Fig. 1. Separation of rat liver lipids (0.35 mg) by HPLC with mass detection. Elution conditions are described in the Materials and Methods section. Abbreviations: CE, cholesteryl esters; TG, triacylglycerols; C, cholesterol; DG, diacylglycerols; FA, unesterified fatty acids; CER, cerebrosides; DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PC, phosphatidylcholine; SPH, sphingomyelin. The following compounds were not present at detectable levels, but their retention times were determined and are indicated: MG, monoacylglycerols; PMME, phosphatidylmonomethylethanolamine; LPE, lysophosphatidylethanolamine; PDME, phosphatidyldimethylethanolamine; PS, phosphatidylserine; LPC, lysophosphatidylcholine.

With the 3-μ column, the triacylglycerols were sometimes found to elute as a double peak, masking the cholesteryl esters. This could be prevented by injecting the sample in 5 μl or less of a relatively low-polarity solvent such as chloroform–isooctane 1:1 (v/v), in addition to carefully timing the reequilibration of the column; stopping the solvent flow for 2 min immediately prior to injecting a sample also helped.

Application of the method to lipid samples from rat heart, erythrocytes, and plasma is shown in **Fig. 2**; simple inspection confirms the important compositional features. For example, heart tissue contained relatively little neutral lipid and high proportions of phospholipids; cardiolipin was prominently featured. The erythrocytes contained mainly cholesterol, phosphatidylethanolamine, and phosphatidylcholine, while the plasma lipids comprised largely cholesteryl esters, triacylglycerols, cholesterol, and phosphatidylcholine.

2,6-Di-*tert*-butyl-*p*-cresol (BHT) could safely be added to lipid extracts as an antioxidant as it was found to evaporate in the mass detector and did not affect the base line of the recorder.

To test the value of the method for quantification purposes, standard mixtures of pure lipid classes were made up and injected onto the column in 5 μl of solvent. The integrator response was determined and related to the amount of each lipid injected. Results for the more abun-

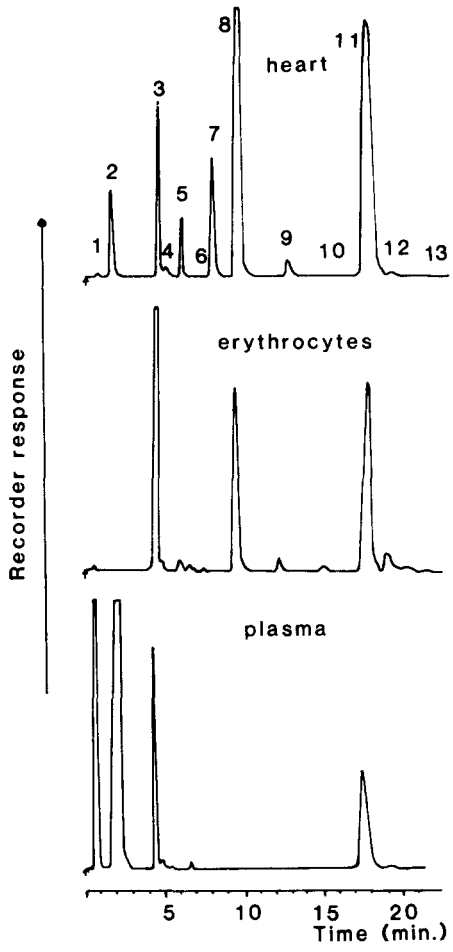

Fig. 2. Separation of rat heart, erythrocytes, and plasma lipids (0.25–0.35 mg). Elution conditions are described in the Materials and Methods section. Abbreviations: 1, cholesteryl esters; 2, triacylglycerols; 3, cholesterol; 4, diacylglycerols; 5, unidentified; 6, unesterified fatty acids; 7, diphosphatidylglycerol (predominantly); 8, phosphatidylethanolamine; 9, phosphatidylinositol; 10, phosphatidylserine; 11, phosphatidylcholine; 12, sphingomyelin; 13, lysophosphatidylcholine.

dant lipid classes are shown in **Fig. 3**. For most lipid classes, the detector response was approximately linear in the range 50 to 200 μg but tended to tail off rapidly below 10 μg. The calibration curve for triacylglycerols was close to that for cholesteryl esters, and there was no difference in the response to tripalmitin and triolein. The calibration curves for most of the phospholipids were close to those for phosphatidylethanolamine and phosphatidylcholine; only the lines for lysophosphatidylcholine and sphingomyelin were appreciably below this range, possibly because these compounds eluted as broader (or double) peaks. Two weeks after the initial calibrations, that for cholesteryl palmitate was repeated and had not changed. Charlesworth (6) obtained calibration curves similar in shape to those found in this study.

The lipid extracts from rat heart, liver, erythrocytes, and plasma were analyzed by the HPLC procedure and quantified using the calibration lines. Where this was not possible (e.g., with unidentified components), the calibration line for the component eluting nearest was used. The results are shown in **Table 2**. Each analysis was repeated five times and the reproducibility of the procedure can be gauged from the standard errors of the means, which were often less than 1% of the mean values for major components (>20% of the total) and less than 3% of the mean value for the remaining substantial constituents (5–20% of the total). The data for rat heart phospholipids resembled those reported by others (15), as did those for the liver (16, 17). Similarly, the results for the total lipids of erythrocytes were close to published values (18). The rat from which the plasma sample was taken was old, fat, and hardly typical, but the results serve as a further example of a tissue of distinctive composition that could be handled by the method.

The principal disadvantage of the method appeared to be that minor phospholipid components, i.e., less than 0.5% of the total, might not be seen during routine analyses, because of the shape of the detector response curves. They could, however, generally be detected by increasing the detector sensitivity. For example, phosphatidylserine was present at low levels in the liver and heart tissue, but was not picked up by the integrator under the standard conditions.

After treating the heart lipids with acid to destroy the plasmalogens, the proportion of each lipid that was unchanged was calculated, following reanalysis, by using the cholesterol content, determined earlier, as an internal standard; the plasmalogen content was calculated by difference. By this means, the phosphatidylethanolamine-plasmalogen and phosphatidylcholine-plasmalogen were found to be 16.22 ± 0.481% (n = 3) and 10.93 ± 0.345%

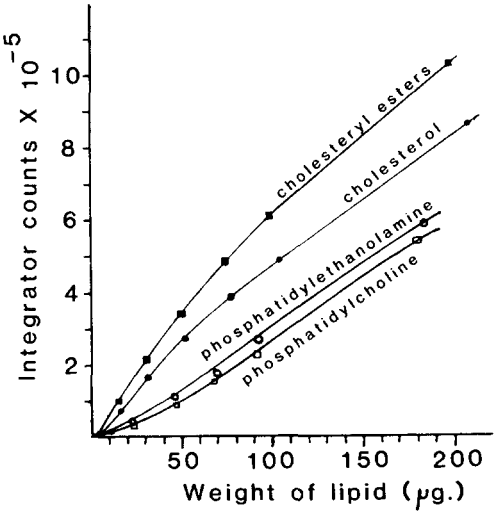

Fig. 3. Calibration curves of weight of individual lipids injected onto the column against detector response (integrator counts). Each point represents the mean of five determinations; ■, cholesteryl esters; ●, cholesterol; ○, phosphatidylethanolamine; □, phosphatidylcholine.

TABLE 2. Lipid composition of lipid classes in rat heart, liver, erythrocytes, and plasma determined by the HPLC method with mass detection

Lipid Class	Tissue			
	Heart	Liver	Erythrocytes	Plasma
	wt % of total			
Cholesteryl esters	0.22 ± 0.042	1.47 ± 0.102		16.04 ± 0.300
Triacylglycerols	3.77 ± 0.231	6.66 ± 0.151		48.65 ± 0.707
Cholesterol	4.06 ± 0.159	5.40 ± 0.130	30.23 ± 0.342	5.65 ± 0.102
Diacylglycerols	0.65 ± 0.074		0.38 ± 0.092	0.54 ± 0.055
Unidentified	1.54 ± 0.181		0.96 ± 0.062	
Unesterified fatty acids		0.32 ± 0.031		1.66 ± 0.102
Diphosphatidylglycerol[a]	12.30 ± 0.403	4.54 ± 0.045		
Phosphatidylethanolamine	33.44 ± 0.209	19.91 ± 0.080	20.75 ± 0.205	
Phosphatidylinositol	3.69 ± 0.055	4.43 ± 0.077	3.42 ± 0.141	
Phosphatidylserine			3.11 ± 0.455	
Phosphatidylcholine	38.57 ± 0.203	55.18 ± 0.126	32.03 ± 0.143	24.13 ± 0.586
Sphingomyelin	1.76 ± 0.502	2.09 ± 0.060	8.22 ± 0.170	1.99 ± 0.071
Lysophosphatidylcholine			0.89 ± 0.154	1.35 ± 0.060

Results are expressed as means ± SEM of five replicate analyses.
[a]Also contains phosphatidylglycerol and cerebrosides.

(n = 3), respectively, of the total lipids, values similar to those recorded for heart muscle in a number of species (19). Methods that are similar in principle have been used by other workers (20–22).

DISCUSSION

The results of this study have demonstrated that HPLC can be applied to the separation and quantification of a wider range of lipid classes than has hitherto been possible. The procedure is rapid, and would be capable of some degree of automation by adding an autoinjection system or by computer analysis and calculation of the results. The separation was optimized for samples of a specific size (0.2 to 0.4 mg), and for the main range of lipid classes encountered in animal tissues, but could undoubtedly be modified if enhanced resolution of particular components were desired.

Three recent technological innovations have made these separations possible: the commercial availability of the mass detector, the development of ternary solvent-programming HPLC systems, and use of 3-μ silica particles. The mass detector has proved simple and rugged in use. It is capable of at least a 10-fold increase in sensitivity, but would have to be carefully calibrated in the low mass range. The physics of the detection process have been discussed elsewhere (6, 9). After an initial warm-up period for the detector, the recorder base-line needed little adjustment over a full day of use. Although the detector is destructive, it is a simple matter to install a stream splitter between the end of the column and the detector to collect fractions for analysis by other means.

The retention times of the most abundant lipid components did not change significantly during this study, although phosphatidylinositol and phosphatidylserine were migrating about 30 sec later towards the end. Approximately 400 injections were made onto the column, of which 200 used the complete ternary elution system. Little loss of resolution has been observed. The maximum pressure required to carry out elution rose to over 5,000 psi after about 300 injections, apparently because of particulate blockage to which 3-μ columns are prone. The problem was resolved simply by reversing the column. In routine use, it might be advisable to insert a filter, capable of removing particles greater than 0.5 μm in diameter, between the injection valve and the column. A silica precolumn between the pump and the injection valve, to saturate the eluting solvent with silica, might prolong column life.

Most methods for the analysis of lipids require an initial fractionation into simple (neutral) lipids and phospholipids prior to the further separation of each (23). The only published separations comparable to those reported here are those of Privett and colleagues (1–5), where the separation and regeneration of the column took much longer than was necessary here; they used their own design of flame-ionization detection system, not a commercial instrument. The elution scheme described here could probably also be used with such a detector. Innumerable separations of phospholipids by means of HPLC have been described and some may be superior to those described in this report. However, the author knows of no other single procedure capable of resolving and accurately quantifying lipids varying so widely in polarity in such a short time, nor of any that is so satisfying aesthetically.

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