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Automated separation and quantitation of lipid fractions by high-performance liquid chromatography and mass detection

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ABSTRACT

This report describes an improved separation and quantitation of lipid fractions in a total lipid extract by high-performance liquid chromatography using a modified solvent and gradient system delivered by dual pumps and incorporating a mass detector and autosampler. The detector responses for various lipid fractions (cholesteryl esters, triacylglycerols, free cholesterol, and seven major phospholipid classes) were fitted to a quadratic equation, $y = ax^2 + bx + c$, and quantified after detector calibration by a computer. This new system has the advantage of automation and reproducible separation. The present method was applied to rat liver analysis.

INTRODUCTION

There are numerous publications describing the separation of various lipid fractions by high-performance liquid chromatography (HPLC) [1]. Of these only a few methods were able to quantitate directly the lipid contents using either flame ionization detection [2–4] or light-scattering detection [5–7]. The latter approach was extensively developed by Christie [7] and was shown to be highly reproducible. However, the solvent delivery system in Christie's method consisted of a programmable ternary solvent-proportionating valve on the low-pressure side of a single pump [7]. The program permitted only seven timed steps and required a manually timed eighth step. Hence, the method is not suitable for automation. Using an HPLC system equipped with two pumps capable of delivering solvents by gradient, Breton *et al.* [5] have described an automated binary-solvent HPLC procedure that could separate and quantitate the phospholipid classes.

In this paper we describe a modified solvent and gradient system delivered by dual pumps. Incorporating a mass detector, autosampler and computer, we were able to automate the separation and quantitation of both neutral and polar lipids with highly satisfactory and reproducible results.

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EXPERIMENTAL

Chemicals and reagents

Cholesteryl linoleate (CE), cholesterol (CH), cardiolipin or diphosphatidylglycerol (CL, from bovine heart), phosphatidylethanolamine (PE, from bovine brain), phosphatidylinositol (PI, from bovine brain), phosphatidylserine (PS, from bovine brain), phosphatidylcholine (PC, from bovine brain), sphingomyelin (SM, from bovine brain) and lysophosphatidylcholine (LPC, from egg yolk) were obtained from Sigma (St. Louis, MO, U.S.A.). Triheptadecanoin (TG) and dilinolein (DG) were obtained from Nu-Chek Prep (Elysian, MN, U.S.A.). All solvents (HPLC grade) were supplied by British Drug House (Toronto, Canada), filtered through a Millipore filter (0.5-μm) and degassed prior to use.

Chromatographic column and apparatus

The HPLC system consisted of a Beckman System Gold programmable solvent module 126 and autosampler module 507 (Beckman Instruments, Palo Alto, CA, U.S.A.). The separation was performed on a stainless-steel column (25 cm × 4.5 mm I.D.) packed with 5-μm Ultrasphere Si packing (Beckman, Toronto, Canada). The column was housed in a column oven maintained at 35°C. The quantitation of lipids was assessed with an ACS Model 740/14 mass detector (Applied Chromatography Systems, Macclesfield, U.K.). The output signal from the mass detector via an analogue interface module 406 (Beckman) was integrated by an IBM PS/2 computer (Model 50). The settings on the mass detector were as follows: attenuation range, 16; photomultiplier sensitivity, 5; time constant, 5 s; evaporator set, 40; internal air pressure, 1.86 bar.

Elution system

Three different stock solvents were prepared: solvent A, isooctane (or hexane)–tetrahyrofuran (99:1, v/v); solvent B, isopropanol–chloroform (4:1, v/v); solvent C, isopropanol–water (1:1, v/v). Solvents D and E were prepared by mixing solvents A, B and C at two different ratios, 42:52:6 and 32:52:16 (v/v), respectively. The solvent delivery system for System Gold HPLC is equipped with four solvent lines connected to each pump; lines A1–A4 for pump A and lines B1–B4 for pump B. In this study, we assigned lines A1 and A3 for solvents A and D, respectively, and lines B2 and B4 for solvents B and E, respectively. The flow-rate was 2 ml/min. The elution program, a modified version of Christie's elution scheme [7] is described in Table I. Briefly, samples were injected at time 0 and were eluted with 100% solvent A for 1 min. A linear gradient up to 10% solvent B over the next 4 min was then pumped. At 5 min solvent D was pumped isocratically for 8 min, followed by a linear gradient up to 100% solvent E over the next 14 min after which solvent E was pumped isocratically for another 10 min. At 37 min the elution solvent changed to a mixture containing 70% solvent B and 30% solvent A. At 37.1 min a linear gradient to 100% solvent A for a

TABLE I

BINARY GRADIENT SOLVENT SYSTEM FOR THE ELUTION OF LIPID FRACTIONS

Time (min)	Percentage solvent				Gradient duration (min)
	A	B	D	E	
0	100	–	–	–	
1	90	10	–	–	4.0
5	–	–	100	–	
13	–	–	–	100	14.0
37	30	70	–	–	
37.1	100	–	–	–	4.9
50	100	–	–	–	

duration of 4.9 min was pumped. The column was re-equilibrated with 100% solvent A for an additional 8 min and made ready for the next injection.

Calibration

A standard solution containing 5.0 μg/μl CE, 6.2 μg/μl TG, 4.2 μg/μl CH, 2.0 μg/μl CL, 5.0 μg/μl PE, 2.0 μg/μl PI, 2.0 μg/μl PS, 5.0 μg/μl PC, 2.4 μg/μl SM and 1.1 μg/μl LPC was prepared. Aliquots of 0, 2, 4, 6, 8, 10, 20, 30 and 40 μl of the standard solution were injected twice. The peaks were identified based on the retention time with the authentic standards. A calibration chromatogram of detector response and amount of each lipid was generated (see Fig. 1) and stored in the computer to be utilized later in the development of a calibration curve for each lipid fraction present. To assess the reproducibility and accuracy, an aliquot (10 μl) of the standard solution was injected by the autosampler into the column ten times; the mean, standard deviation and the coefficient of variation (C.V.) of each lipid fraction were calculated. The results for the major lipid fractions are presented in Table II.

Animal sample

Total lipids from a 2-g portion of the liver of a Sprague–Dawley rat fed a regular chow diet were extracted following the method of Folch *et al.* [8]. The lipid extract was dissolved in 2 ml of chloroform.

RESULTS

A typical chromatogram of the standard solution is shown in Fig. 1. All peaks were eluted as single peaks with the exception of SM and LPC which eluted as double peaks. The concentration of the lipids in the standard were prepared to

TABLE II

CALIBRATION REPRODUCIBILITY AND ACCURACY OF INDIVIDUAL LIPIDS IN 10 μl OF THE STANDARD SOLUTION INJECTED TEN TIMES ONTO THE COLUMN

Values are means ± S.D.; values in parentheses are coefficients of variation (%).

Lipid fraction[a]	Area response	Retention time (min)
CE	312.1 ± 3.1 (0.97)	1.65 ± 0.01 (0.55)
TG	198.6 ± 10.8 (5.4)	3.04 ± 0.01 (0.03)
CH	269.8 ± 2.8 (1.1)	6.59 ± 0.02 (0.33)
CL	62.6 ± 1.8 (2.9)	12.83 ± 0.03 (0.20)
PE	287.4 ± 7.1 (2.5)	14.46 ± 0.02 (0.10)
PI	161.3 ± 19.5 (12.1)	23.54 ± 0.09 (0.40)
PS	77.3 ± 7.5 (9.7)	24.76 ± 0.10 (0.42)
PC	281.4 ± 14.7 (5.2)	28.26 ± 0.05 (0.17)

[a] CE = cholesteryl heptadecanoate; TG = triheptadecanoin; CH = cholesterol; CL = cardiolipin; PE = phosphatidylethanolamine; PI = phosphatidylinositol; PS = phosphatidylserine; PC = phosphatidylcholine.

approximate the concentrations of lipids found in rat livers. The calibration curves for the neutral lipids (CE, TG and CH) are given in Fig. 2A and for some phospolipids (CL, PE, PI, PS and PC) in Fig. 2B. The results indicate that the mass responses were not linear for the lipid classes, in agreement with Christie [7]. However, the curves were fitted automatically by the computer to a quadratic

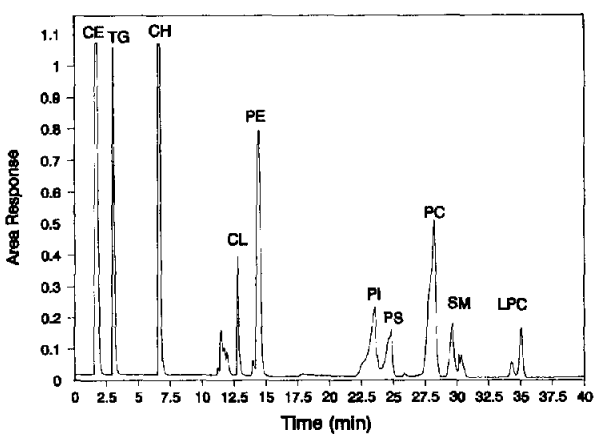

Fig. 1. Separation of lipid standards by automated HPLC with mass detection. The amount injected was 20 μl of the standard solution. The detector response was 1 V full scale. Elution conditions, flow-rate and column are described in the text. Peaks: CE = cholesteryl esters; TG = triacylglycerols; CH = cholesterol; CL = cardiolipin; PE = phosphatidylethanolamine; PI = phosphatidylinositol; PS = phosphatidylserine; PC = phosphatidylcholine; SM = sphingomyelin; LPC = lysophosphatidylcholine.

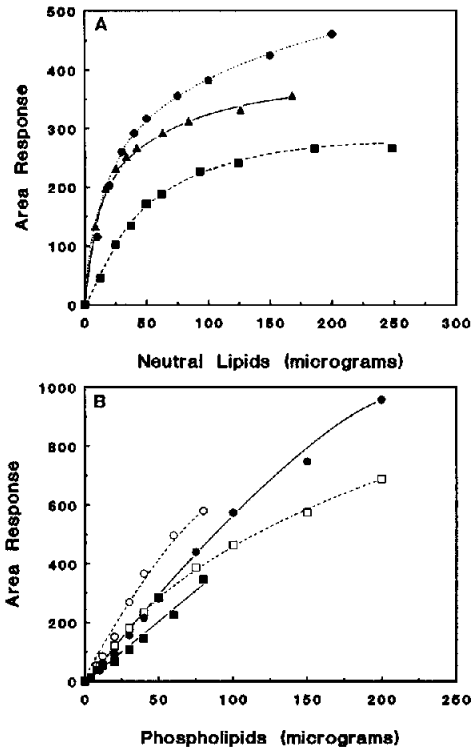

Fig. 2. (A) Calibration curves for neutral lipids (●, CE; ■, TG; ▲, CH). (B) Calibration curves for some phospholipids (□, PE; ○, PI; ■, PS; ●, PC). Each curve was determined from eighteen points and was fitted to a quadratic equation, $y = ax^2 + bx + c$. The coefficients a, b and c were determined by the computer for the best fit.

equation. Based on that curve, the computer calculated and printed the masses of the individual lipids in a given injection. The C.V. values shown in Table II indicate that the reproducibility and accuracy are very good with values less than 1% for the retention times and values around 5% for the area responses. The C.V. values for PI and PS area responses are slightly higher because they elute as broad singlets reducing the accuracy of the integration.

DISCUSSION

Generally, to quantitate major lipid fractions, two vastly different HPLC solvent systems are required. Non-polar solvents are required to separate neutral lipids whereas polar solvents usually containing water to some extent are required to separate the major phospholipid fractions [9–12]. They are usually time-consuming, and constant attention is required. The present study introduces a modified HPLC procedure combining the superior separation of the various lipid

fractions as shown by Christie [7], and the advantage of a computer-driven automated system. We used a dual-pump system in place of the programmable ternary solvent-proportionating valve as described in Christie's method [7], and modified the solvent preparation and elution scheme. The computer controlled the automatic injections and creation of the calibration curves. The curves were then used for the determination of the mass (or concentration) of the various lipids in unknown samples.

Unlike the problem that Christie occasionally encountered with a co-elution, the separation of triacylglycerols and cholesteryl ester in the present study was consistent. Previously, Breton *et al.* [5] have used a binary solvent system and similar HPLC system, but failed to resolve the neutral lipids and several phospholipid subfractions, such as PI and PS. In this study, we show that elution with solvent D isocratically followed by a linear gradient with solvent E could effectively separate PI from PS (Fig. 1). The excellent resolution of different lipid fractions by the present method may be further illustrated by the chromatogram of a rat liver lipid extract (Fig. 3). The sensitivity of the detector was also impressive. It is possible to integrate the two minor PL components, such as PS and LPC, in an injection of a lipid extract from as low as 10 mg rat liver. Moreover, there was no need to stop the flow prior to the injection of the next sample, as the samples were injected automatically every 50 min. Although we did not compare the phosphorus assay and the light-scattering detection assay in this study, there are publications where such comparison has been performed resulting in a satisfactory agreement between them [7,13].

In summary, the present paper introduces a system that may automatically quantitate the different lipid fractions by HPLC. For example, the PC/SM ratio in amniotic fluid may assess the fetal lung maturity [14], the CH/phospholipids

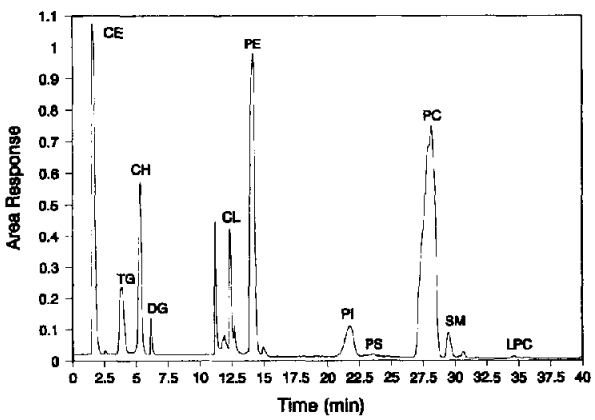

Fig. 3. Separation of rat liver lipids by automated HPLC with mass detection. The amount of lipids injected (0.3 mg) was extracted from approximately 20 mg of rat liver. The elution conditions and peaks are the same as those in Fig. 1.

and PC/PE ratios in membrane lipids reflect the membrane fluidity [15]. The labour time and perhaps the solvent cost normally required to separate lipid fractions by two HPLC systems and by other chromatographic methods are significantly reduced. There are several aspects that could, however, be further improved. Christie [7] has indicated that a smaller 3-μm column provides better resolution, a lower operating pressure (255 bar) and a shorter re-equilibration time. The analysis time of 50 min in our system may be shortened by adopting a smaller 3-μm column. As well, Christie obtained improved resolution of the minor acidic compounds by adding small amounts of organic ions to the aqueous component of the eluent. Namely, he used 0.5–1 mM serine buffered to pH 7.5 with triethylamine. He also indicated that the lifetime of the column was extended using this buffer. We have made well over 1000 injections onto the column with no loss in resolution. We may, however, in the future adopt this buffer. Since the mass detector is destructive, a stream splitter between the column and detector may be installed to collect the fractions for further analysis.

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