Determination of Lipids in Animal Tissues by High-Performance Thin-Layer Chromatography with Densitometry

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Kidney
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Summary

A high-performance thin-layer chromatographic method with densitometry has been developed, optimized, and used to quantify changes in renal and hepatic lipids after chemical insult. The lipids were extracted from tissues with 2:1 (*v/v*) chloroform–methanol. Petroleum ether (b.p. 40–60°C)–diethyl ether–acetic acid, 80 + 20 + 3 (*v/v*), was used as mobile phase to separate neutral lipids (cholesterol, triacylglycerol, and cholesterol ester); oleic acid methyl ester was used as internal standard. Phospholipids (sphingomyelin, L-α-phosphatidylcholine, L-α-phosphatidylserine, L-α-phosphatidylinositol, cardiolipin, and L-α-phosphatidylethanolamine) were separated with methanol–chloroform–*n*-propanol–methyl acetate–43 mM KCl, 10 + 25 + 25 + 25 + 9 (*v/v*), as mobile phase and with galactosecerebrosides as internal standards.

After chromatography bands were visualized by charring with manganese chloride–sulfuric acid at 110°C. Quantification was performed by scanning densitometry and integration. Application of the optimized method was demonstrated by quantification of renal and hepatic lipids from rats treated with a nephrotoxin. The detection limit was 20 ng lipid and the sample throughput was 20–24 samples per plate.

1 Introduction

Lipids can be identified by microchemical assay [1, 2], or by use of separation techniques such as gas–liquid chromatography (GLC) [3], high-performance liquid chromatography (HPLC), or thin-layer chromatography (TLC). Microchemical assay is time-consuming and not very sensitive–relatively large amounts of lipids are needed for detection. GLC can only be used for analysis of substances that can be volatilized without undergoing decomposition. Lipids must be converted into volatile compounds before use of GLC; fatty acids are, for example, converted into their methyl esters and the free hydroxyl groups are acetylated. The most useful application of GLC in lipid analysis is in the determination of the fatty acid composition of complex lipids.

Lipid molecules do not usually contain chromophores or functional groups which can be readily detected by spectrophotometry, so it is not often possible to monitor the eluent from chromatographic columns on-line. Refractive index (RI), ultraviolet light (UV), evaporative light-scattering detection, flame ionization detection, and mass spectrometry have been used for the determination of lipids [4], but all have a very limited range of application and several of these techniques are expensive. RI can only be used with a mobile phase of constant composition. Few lipids have functional groups that absorb in the UV region of the spectrum and UV detectors must be used at low wavelength where they are very sensitive to refractive index changes and to small amounts of UV-absorbing contaminants. The response of the detector varies with the number of functional groups and with the number of double bonds. Thus, direct quantitation is not possible for phospholipids (PLs) if their degree of unsaturation is unknown [5].

It has been suggested that TLC remains the technique of choice for separation of lipids [6]. It has advantages over column chromatography in that it is more rapid, more sensitive, and more efficient, and many more samples can be applied to a plate. The substances on the plate can be detected in situ and quantified by densitometry [7]. One of the main advantages of using the densitometer in situ is that it eliminates the losses of lipids encountered when the

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were extracted as soon as possible after animals had been killed. When this was not feasible the tissue was stored in a sealed container and frozen as rapidly as possible at –20°C until analysis. All samples were initially homogenized with methanol to prevent incomplete lipid extraction as a result of the formation of paste-like globules of protein [2]. The membrane capsule was removed from the kidney and the tissue was weighed on a glass plate (the weight of each kidney was ca 1 g). The tissue (kidney or liver) was then chopped with a razor blade into small pieces, transferred to 50-mL polypropylene tubes (Alpha Laboratory, East Leigh, Hampshire, UK) containing methanol (5 mL) and homogenized with an Ultra Turrax T8 homogenizer at ca 1500 rpm (IKA Labotechnik, Staufen, Germany) for 2 min. Chloroform–methanol (2:1, *v/v*; 5 mL) was then added, then more chloroform (10 mL) so that the final composition of the extracting solvent was 2:1 (*v/v*) chloroform–methanol, and the ratio of the volume of extracting solvent to that of the tissue was ca 20:1. This mixture was then homogenized at ca 1500 rpm for 30 s.

The volume of tissue sample was computed on the assumption that the tissue had the specific gravity of water, i.e. that the volume of 1 g tissue was 1 mL, in accordance with *Folch* et al. [8]. Samples were well shaken, left for 30 min at 40°C, then centrifuged at 2500 × g for 5 min in a bench-top centrifuge. *Folch* washing of the extract was not applied in the extracting procedure, because in HPTLC separations non-lipid material remains at the origin. Clear supernatant was transferred to an amber glass vial for analysis. For recovery of NL, 25 μL and 50 μL OAME stock solution was added to each kidney and liver sample extract, respectively. Kidney extract (4 μL) or liver extract (2 μL) was applied to the plate such that each lane contained ca 870 ng OAME. For recovery of PL, 20 μL Gal-CB stock solution was added to 0.5 mL kidney or liver extract. Kidney or liver extract (1 μL) was applied to the plate such that each lane contained ca 200 ng Gal-CB. Either OAME (0.435 mg mL^{-1}, 2 μL, i.e. 870 ng OAME) or Gal-CB (0.2 mg mL^{-1}, 1 μL , i.e. 200 ng) was applied to 'standard lanes'. When not in use extracted lipids were kept at –20°C under oxygen-free N_2.

2.5 HPTLC of Lipid Extracts

2.5.1 Sample Application

Compounds were separated on 10 cm × 20 cm silica gel 60 HPTLC plates with 200 μm layers (Merck, Dorset, UK). Samples were applied 10 mm from the edges of the plates by means of a Camag Linomat IV applicator; the band width was 5 mm and the distance between bands 4 mm. Samples were applied at 0.2 μL s^{-1} with the nitrogen (BOC, Morden, London, UK) pressure set at 4.5 bar. Samples are concentrated by the applicator into narrow bands of selectable length. Because mass distribution is uniform over the full length of the bands, densitometric evaluation can be performed by aliquot scanning, which ensures maximum quantitative accuracy.

2.5.2 Chromatogram Development

Plates were developed in a Camag (Muttenz, Switzerland) horizontal chamber simultaneously from opposite sides towards the middle. This enabled the analysis of twice as many samples as is possible by conventional development in a tank. The chamber was kept enclosed and covered with a glass plate during development, to avoid the effects of draughts, evaporation of the most volatile solvents, and edge effects.

2.5.3 Visualization of the Lipids

The developed plates were exposed to iodine vapor in a sealed tank. The separated substances were revealed as brownish spots (as iodine complexes with the double bonds of unsaturated fatty acids). Qualitative identification of lipids was based on comparison of the R_F values of samples and standards, and semi-quantitative analysis was performed by visual comparison of the intensity of staining of the sample with that of standard bands. The lipid bands could be observed after 10 min but were only clearly visible for up to 3 h after exposure to iodine vapor.

Because iodine staining faded quickly, another agent was used for visualization and quantification. Separated bands were visualized by use of a charring reagent comprising manganese chloride (1 g), methanol (150 mL), and concentrated sulfuric acid (10 mL) in deionized water (150 mL) [9]. After development, the plates were dried for 1 min in a fume cupboard at room temperature, then for 10 min in an oven at 110°C, and then immediately immersed for 1 min in a tank filled with reagent. The plates were withdrawn and excess reagent was removed from the back of the plates. They were then placed in an oven on a metal plate to ensure even heating at 110°C for precisely 45 min. Increasing the heating time did not increase peak heights but on scanning the baseline was found to be increased. The bands appeared as a pink color on a white background [10].

2.5.4 Densitometric Quantification of Lipids

A Desaga CD 60 densitometer with the method validation software version 4.3 (Desaga, Heidelberg, Germany) was used for densitometric evaluation. The scanner was linked via an RS 232 interface to a personal computer, from which all commands were passed to the scanner. The scanner transmitted all measurement data in digital form to the computer to create chromatograms. Integration, calibration, and calculation of the results were then performed.

2.6 Statistical Analysis

The means of results from the control and dosed groups were compared. The data are means from four or five separate values, ± S.E.M. (standard error of the mean). For all comparisons, the level of significance was shown by asterisks as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, significantly different from the control. Two-tailed and un-paired

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3.3 Separation of Phospholipids

Because the major PLs in renal tissue are PC, PE, SPM, PS, Car, LPC, and PI [14], only these were studied in this investigation. They were separated by use of methanol–chloroform–*n*-propanol–methyl acetate–43 mM KCl, 10 + 25 + 25 + 25 + 9 (*v*/*v*) as mobile phase [9].

Chromatograms obtained from neutral lipids, phospholipids, and their standards are shown in Figures 1 and 2. Their calibration curves and standard ranges are described in Table 3.

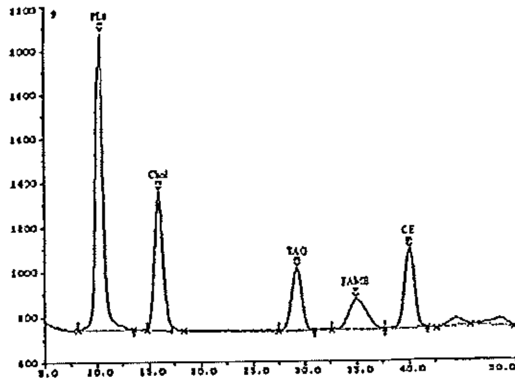

Figure 1

Chromatogram obtained from neutral lipids and their internal standard. (The horizontal axis is the migration distance [mm]).

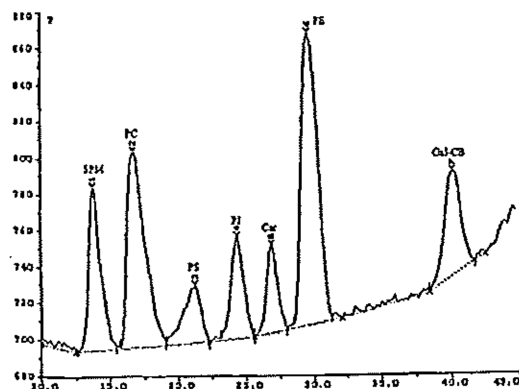

Figure 2

Chromatogram obtained from individual phospholipids and their internal standard. (The horizontal axis is the migration distance [mm]).

The R_F values obtained for the PLs are shown in Table 4. The maximum number of complete separations possible in one run by use of this solvent system was 13 (for a migration distance of 5 cm). The order of migration of the PLs was in agreement with previous reports [15].

3.4 Optimization of Scanning Parameters

Scanning was performed in reflectance mode, with the Hg lamp and signal positive. To determine the optimum wavelength, 400 ng of a PE band was scanned from 210 to 600 nm and peak heights were recorded. As is apparent from Figure 3, the optimum wavelength was 250 nm. The length of the light slit was optimized by recording the signal output from 1000 ng cholesterol for different slit lengths at λ = 250 nm and a slit width of 0.02 mm. As is apparent from Table 5, the best accuracy and high sensitivity were obtained with a slit length of 3 mm. Thus the optimum scanning conditions were wavelength 250 nm, slit width 0.02 mm, and slit length 3 mm. The scanning reproducibility of peak height and peak area for 1000 ng cholesterol on the same plate was 0.25% and 1.02%, respectively.

The integration software used peak detection routines and internal peak detection parameters to acquire peak data for quantification (Figures 1 and 2). Peak area is a more satisfactory analytical parameter than peak height (because it is

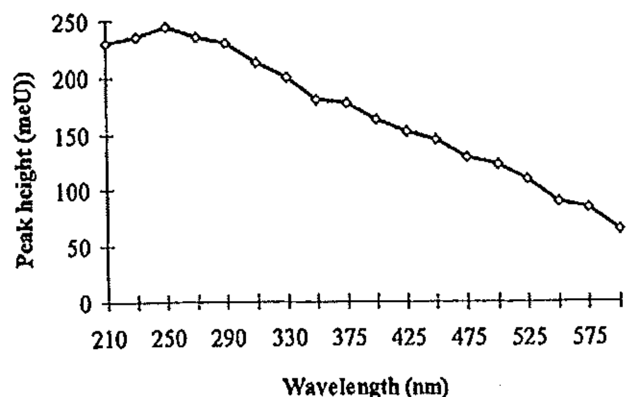

Figure 3

Dependence of peak height on scanning wavelength.

Table 3

Equations of calibration plots and their standard ranges for neutral lipids and phospholipids.

Lipid	Equation	Conc. [mg mL^{-1}]	Standard range [ng]
Cholesterol (Chol)	$y = 2.38x + 0.276$	0.20	200–1000
Triacylglycerol (TAG)	$y = 1.82x - 0.0005$	0.20	200–1000
Cholesterol ester (CE)	$y = 3.94x + 0.0486$	0.10	100–500
L-α-Lysophosphatidylcholine (LPC)	$y = 0.115x + 1.41$	0.05	50–250
Sphingomyelin (SPM)	$y = 0.253x + 8.37$	0.10	100–500
L-α-Phosphatidylcholine (PC)	$y = 0.156x + 11.5$	0.25	250–1250
L-α-Phosphatidyl-L-serine (PS)	$y = 0.243x + 4.06$	0.05	50–250
L-α-Phosphatidylinositol (PI)	$y = 0.34x + 2.06$	0.05	50–250
Cardiolipin (Car)	$y = 0.306x + 1.76$	0.05	50–250
L-α-Phosphatidylethanolamine (PE)	$y = 0.272x + 13.3$	0.25	250–1250

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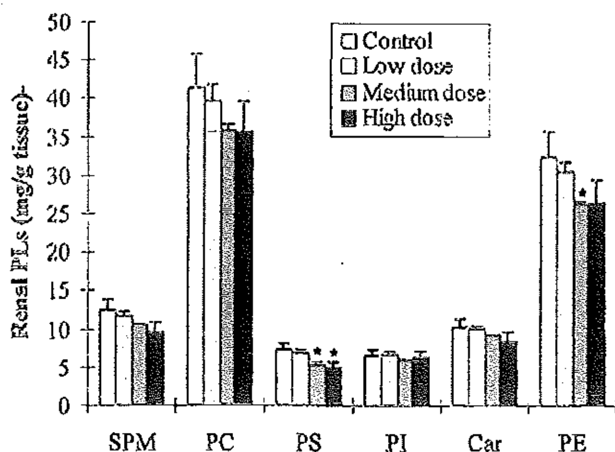

Figure 4

Levels of individual renal phospholipids in rats after treatment for 4 days with 2-bromoethylamine. *$P < 0.05$ compared with the respective control ($n = 5$).

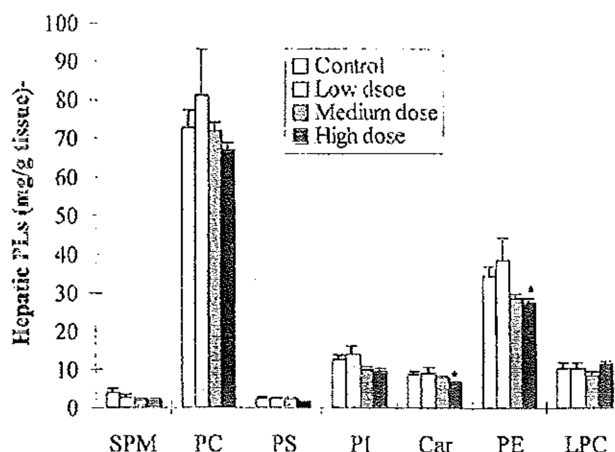

Figure 5

Levels of individual hepatic phospholipids in rats after treatment for 4 days with 2-bromoethylamine. *$P < 0.05$ compared to the respective control ($n = 5$).

The method has also been used to determine the very small amounts of neutral lipids and phospholipids in urine from animals treated with different nephrotoxins [18].

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