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SEPARATION AND DETERMINATION OF LIPIDS BY ONE-DIMENSIONAL MICRO-THIN-LAYER CHROMATOGRAPHY FOLLOWED BY DENSITOMETRY

WILMA M. FREDERIKS and SIETSKE BROEKHOVEN

Histological Laboratory, University of Amsterdam, Jan Swammerdam Instituut, 1e Const. Huygensstraat 20, Amsterdam (The Netherlands)

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SUMMARY

A method is described for the rapid analysis of a mixture of phospholipids and neutral lipids, which was used for the analysis of extracts obtained from a nuclear fraction isolated from rat liver. The lipids are separated by one-dimensional thin-layer chromatography on microchromatoplates (48 × 24 mm), using three solvents for development. After spraying the plates with phosphoric acid and heating, the amount of carbon from the charred compounds is measured densitometrically. Only 2–10 μg of lipid mixture are needed for the determination of the relative amounts of the separated compounds.

INTRODUCTION

Generally, the total amount of phospholipids and neutral lipids extracted from a nuclear fraction isolated from rat liver is determined by weighing[1] and colorimetric determination of phosphorus[2–4]. About 4% of the dry weight of the nuclear fraction consists of lipids[5–7]. The phospholipid composition is generally determined by two-dimensional thin-layer chromatography (TLC) and determination of phosphorus in the separated compounds[3,5,8]. The total amount of cholesterol and cholesterol esters in the nuclear lipid extract is determined by colorimetric analysis[9]. The amount is usually too small for the separation and determination of the other neutral lipids by TLC; the neutral lipids amount to about 20% to the total lipid content[10,11].

In the method described here, phospholipids as well as neutral lipids are separated by one-dimensional TLC on silica gel on microchromatoplates (48 × 24 mm). After charring with diluted phosphoric acid, the various lipid components are determined quantitatively by densitometry. The method is based on the paper by Althaus and Neuhoff[12], who introduced a procedure of stabilizing the silica gel layer with sodium silicate.

EXPERIMENTAL

Materials

Nuclei were isolated from the livers of Wistar rats weighing 200 ± 25 g.

Silica gel D-O from Camag (Muttenz, Switzerland) was used for the preparation of the microchromatoplates.

Water-glass (sodium silicate solution, sp.gr. 1.36–1.39) was obtained from Brocades (Haarlem, The Netherlands).

Sphingomyelin and phosphatidylinositol were purchased from Supelco (Bellefonte, Pa., U.S.A.), diphosphatidylglycerol from N.B.C. Biochemicals (Cleveland, Ohio, U.S.A.) and a mixture of cholesterol, phosphatidyl ethanolamine, phosphatidylcholine and lysophosphatidylcholine from Applied Science Labs. (State College, Pa., U.S.A.).

The other reagents and solvents were obtained from Merck (Darmstadt, G.F.R.). All solvents used were of pro analisi quality.

Preparation of lipid extracts

Lipids were extracted from a nuclear fraction isolated from rat liver according to a modified method of Widnell and Tata[13]. The lipids were extracted as described by Folch *et al.*[14] and dissolved in chloroform–methanol (2:1) to a concentration of 1.2 mg/ml.

Two-dimensional thin-layer chromatography

The phospholipid composition of a nuclear fraction was determined by two-dimensional TLC according to Rouser *et al.*[15]. The separation was carried out on silica gel H plates (20 × 20 cm). In the first direction, the lipids were separated using chloroform–methanol–25% ammonia solution (65:25:5) then, after drying, the second run was performed with acetone–chloroform–methanol–acetic acid–water (40:30:10:10:5). After detection with iodine vapour, the spots were identified and removed from the plates for determination of phosphorus. The amount of each phospholipid was estimated on the basis of an assumed presence of 25 μg of phospholipid per microgram of phosphorus.

The total amount of neutral lipids is then calculated from the weight of the total lipid extract (as determined on a Cahn balance) and the contribution of phospholipids.

One-dimensional thin-layer chromatography

Preperation of microchromatoplates. The method described by Althaus and Neuhoff[12] was used for the preparation of the microchromatoplates. After suspending 30 g of silica gel in 130 ml of chloroform–methanol (2:1), two cover-glasses (48 × 24 mm) cleaned with chloroform were dipped together in the suspension and, after separation, the silica gel layer was air-dried. The plates were placed in a 50-ml beaker containing an ice-cold water-glass solution (sodium silicate solution–water, 1:20, v/v). This solution was allowed to ascend to the top of the plates, after which the plates were air-dried for 3 h.

Chromatography of phospholipids and neutral lipids. Before application of the chloroform–methanol (2:1) extracts to the plates, the silica gel layer was cleaned by

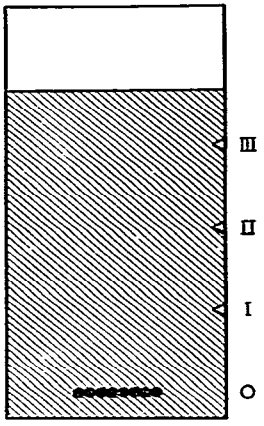

Fig. 1. Microchromatoplate. 0 = start; I = solvent front of chloroform–methanol–water (65:25:4); II = solvent front of chloroform (twice); III = solvent front of *n*-hexane–chloroform (3:1).

a chromatographic run in chloroform–methanol–water (65:25:4) in a direction opposite to that of the run with water-glass.

For the one-dimensional TLC separation of phospholipids and neutral lipids, a stepwise development technique was used. To mark the four chromatographic runs, three notches were placed at positions I, II and III at distances of 10, 20 and 30 mm, respectively, from the start (Fig. 1), and 10 mm of the silica gel layer was scraped from the top of the microplate.

The chromatoplate was placed on a moveable stage and amounts of 2–10 μg of lipids from standard solutions were applied as spots, forming a 10-mm long strip with a maximal width of 1 mm. Use was made of a pipette with a bent tip[16]. The chromatographic runs were performed in a 50-ml beaker containing the chromatoplate, a strip of filter-paper and 1 ml of solvent. The chromatographic chamber was closed with a Petri dish. The first development was performed with the solvent mixture chloroform–methanol–water (65:25:4) until the solvent front had migrated 10 mm. After carefully drying the plate with a stream of nitrogen, a second and third run were carried out in the same direction with chloroform, until the front had migrated 20 mm. The plate was again carefully dried and a fourth development was performed with *n*-hexane–chloroform (3:1), applied to the top of the plate.

The chromatograms were sprayed with the charring reagent (3% copper acetate in 8% phosphoric acid solution[17]). The plates were heated to 120°, until the water had been evaporated, and then to 175°. By means of this treatment, cholesterol and cholesterol esters initially showed a purple colour. After subsequent heating to 200° all of the separated compounds were visible as brown-black bands. After cooling, the plates were fixed on a glass slide to avoid damage during the densitometric determination.

Densitometric determination. A Chromoscan (Joyce Loebl, London, Great Britain) was used for measuring the light absorption of the charred lipids. The instrument was adjusted to an extinction range from 0 to 0.62 unit with a 90° camb. The entrance slit had a width of 0.2 mm and a length of 3 mm.

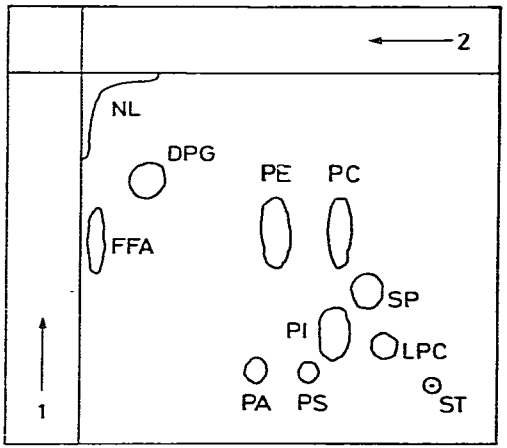

Fig. 2. Two-dimensional TLC of lipids extracted from a nuclear fraction. The indicated spots were detected with iodine vapour. Solvent 1: chloroform–methanol–25% ammonia solution (65:25:5). Solvent 2: acetone–chloroform–methanol–acetic acid–water (40:30:10:10:5). Abbreviations: DPG = diphosphatidylglycerol; FFA = free fatty acids; LPC = lysophosphatidylcholine; NL = neutral lipids; PA = phosphatidic acid; PE = phosphatidylethanolamine; PI = phosphatidylinisitol; PS = phosphatidylserine; SP = sphingomyelin; ST = start.

RESULTS AND DISCUSSION

The results of the two-dimensional separation of lipids extracted from a nuclear fraction are shown in Fig. 2 and the phospholipid distribution in Table I. The amount of neutral lipids relative to the total lipid content was about 20%.

The results of the one-dimensional TLC separation on a micro-scale with a lipid mixture extracted from isolated nuclei are presented in Fig. 3. The observed bands were identified by using a lipid solution of known composition. In Fig. 4 the results are shown schematically. After development with chloroform–methanol–water (65:25:4), the most important phospholipids are readily separated (I). The compo-

TABLE I

PHOSPHOLIPID COMPOSITION OF A NUCLEAR FRACTION, DETERMINED BY THIN-LAYER CHROMATOGRAPHY AND PHOSPHORUS DETERMINATION OF THE SEPARATED LIPIDS

For abbreviations, see Fig. 2.

Phospholipid component	*Content (%)*	*Phospholipid component*	*Content (%)*
PS	2.6	SP	1.7
PI	8.2	PC	57.0
PA	0.2	PE	23.9
LPC	0.4	DPG	6.0

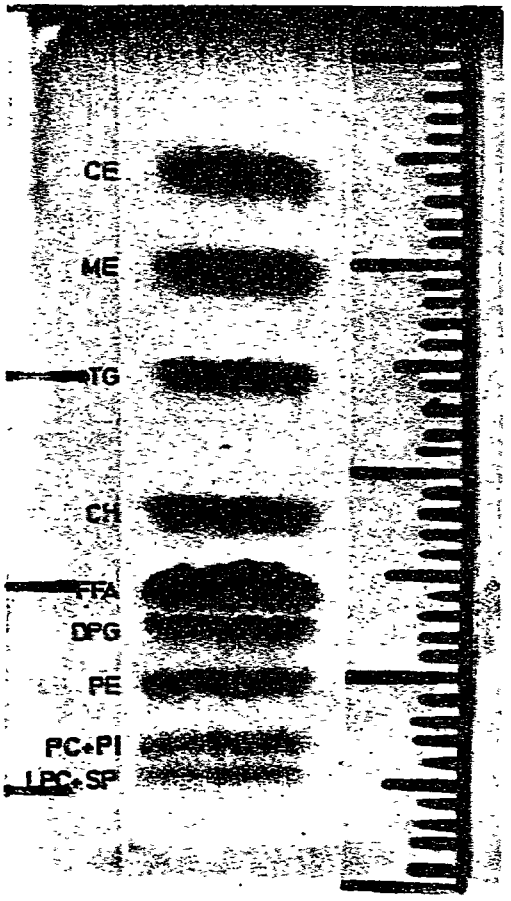

Fig. 3. One-dimensional TLC of lipids extracted from a nuclear fraction, performed as shown in Fig. 1. After the runs, the lipids were detected by charring as described in the text.

nents PC and PI are, however, located at the same position and the same occurs with LPC and SP. The neutral lipids, including the free fatty acids, are localized at the solvent front. After the second and third runs with chloroform to a length of 20 mm above the start, FFA and CH are separated from the other neutral lipids (II). The last development with *n*-hexane–chloroform (3:1) permits the separation of CE, ME and TG.

The quantitative determination of each compound is performed by densitometry of the charred lipids. The peak area is taken as a measure of the amount of carbon in each lipid component and multiplied by a specific correction factor for each compound in order to calculate the composition of the lipid mixture.

These correction factors are determined in experiments with known amounts of pure, commercially available components, and with a phospholipid fraction from isolated nuclei whose composition was established by two-dimensional TLC and determination of phosphorus. The correction factors for the most important lipid components of isolated liver cell nuclei relative to cholesterol are shown in Table II. In our experiments, the correction factors for the neutral lipids are lower than those

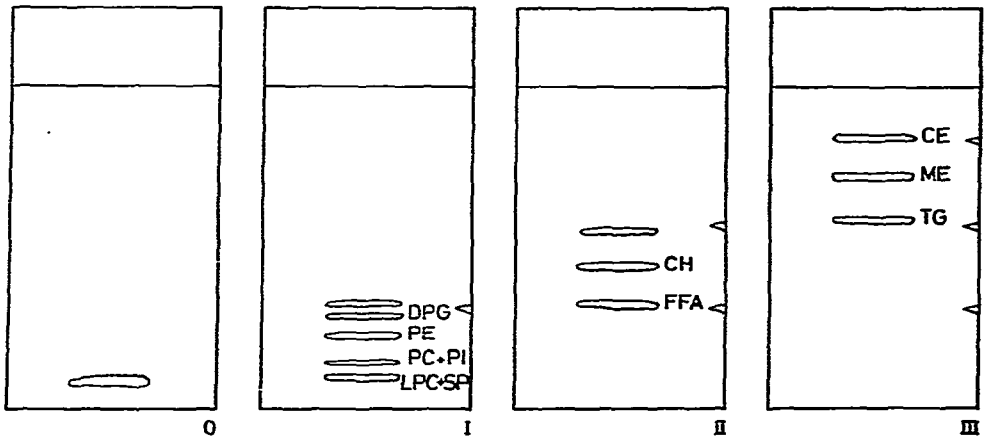

Fig. 4. One-dimensional TLC on microchromatoplates of lipids extracted from a nuclear fraction. 0 = start; I = separation in the solvent chloroform–methanol–water (65:25:4); II = separation in the solvent chloroform; III = separation in the solvent *n*-hexane–chloroform (3:1). For abbreviations, see Fig. 2.

TABLE II

CORRECTION FACTORS FOR THE MOST IMPORTANT LIPID COMPONENTS EXTRACTED FROM A NUCLEAR FRACTION, CALCULATED WITH RESPECT TO CHOLESTEROL = 1.0

For abbreviations, see Fig. 2.

Lipid component	*Correction factor (mean ± s.d.)*	*Lipid component*	*Correction factor (mean ± s.d.)*
CE	0.8 ± 0.1	PE	1.2 ± 0.15
ME	0.8 ± 0.1	PC	2.1 ± 0.2
TG	0.9 ± 0.05	PC + PI	2.0
CH	1.0	LPC	2.5 ± 0.25
FFA	0.8 ± 0.1	SP	1.6
DPG	1.5 ± 0.1		

TABLE III

LIPID COMPOSITION OF A NUCLEAR FRACTION DETERMINED BY ONE-DIMENSIONAL THIN-LAYER CHROMATOGRAPHY FOLLOWED BY DENSITOMETRY

For abbreviations, see Fig. 2.

Lipid component	*Content (%)*	*Lipid component*	*Content (%)*
CE	1.6	DPG	5.6
ME	1.7	PE	16.3
TG	2.8	PC + PI	54.2
CH	8.2	SP	2.3
MG	1.7	PS	2.3
FFA	3.3		

obtained by Van Gent[18], but much higher than the factors which can be calculated from the data of Fewster *et al.*[17].

For the phospholipid components, we found correction factors higher than those obtained by Fewster *et al.*[17], but comparable to those obtained by Van Gent[18] for total blood phospholipids. The differences can be explained by the origins of the investigated lipid components, resulting in different fatty acid contents.

By applying this method, the total lipid composition of lipids extracted from an isolated nuclear fraction was determined for the first time. The results are presented in Table III and agree well with the phospholipid composition, analyzed by two-dimensional chromatography and determination of phosphorus, and with the total neutral lipid content, determined by weighing.

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