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Note

One-dimensional thin-layer chromatographic separation of lipids into fourteen fractions by two successive developments on the same plate

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The investigation of serum neutral lipids, cholesterol ester subfractions and phospholipids has clinical importance in the differential diagnosis of hepatic diseases and lipid metabolism disorders. The analysis of these serum compounds and the investigation of the lipid metabolism are an important application field of thin-layer chromatography (TLC) [1—3].

Numerous papers have been published on the separation of serum lipids using one- and two-dimensional TLC. Sometimes lipids were separated by gradient elution, so compounds with very different polarities could be investigated on the same plate. Multi-development, simultaneous separations with different elution systems have to be used to remove polar and non-polar lipids from each other because they have special migrational properties [4—6].

Recently, high-performance liquid chromatographic [7, 8], overpressured TLC [9, 10] and high-performance TLC [11] techniques have been used for the separation of neutral lipids including cholesterol ester subfractions and phospholipids, but not at the same time or on the same plate.

It has been found that classical TLC methods for the separation of serum lipid components are very time-consuming and the resolution is poor. The complete chromatographic separation of serum lipids, mainly cholesterol esters, on the same plate is so far unsolved. We have developed a one-dimensional TLC separation technique for the separation of phospholipids, neutral lipids and cholesterol ester subfractions on the same plate. Fourteen lipid fractions can be separated within 85 min with two successive developments in the same direction.

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EXPERIMENTAL

Materials

Analytical-reagent grade chemicals and solvents were used. *n*-Hexane was purchased from Mallinckrodt (Paris, KY, U.S.A.), methanol from J.T. Baker (Deventer, The Netherlands) and sodium sulphate, acetone, perchloric acid, ethanol, phosphomolybdic acid and chloroform from Reanal (Budapest, Hungary). The reference lipid standards cholesterol, free fatty acids and triglyceride were purchased from Reanal and phospholipids, non-polar lipid MIX-A and non-polar lipid MIX-B (4-7002, 4-7007) standards from Supelco (Bellefonte, PA, U.S.A.). Solutions were prepared according to a standard procedure [3], resulting in 1 mg/ml reference material concentrations in the solutions. The identification of the gas chromatographic (GC) fatty acid methyl ester peaks was performed using Supelco GC-FAME standards.

Thin-layer chromatography

Pre-coated silica gel plates (Merck No. 5721, 20 × 20 cm, 0.25-mm layer) were used. The plates were activated before using in an oven at 130°C for 30

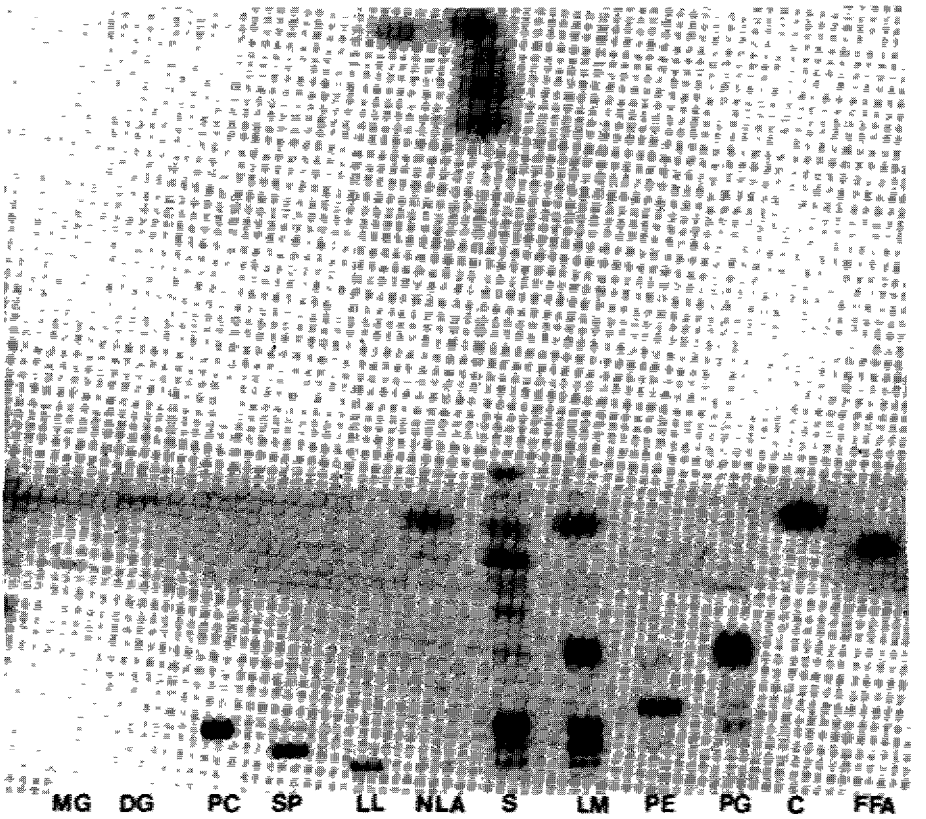


Fig. 1. Thin-layer chromatogram of lipids separated successively with solvent systems I and II. MG = monoglycerides; DG = diglycerides; PC = phosphatidylcholine; SP = sphingomyelin; LL = lysolecithin; NLA = non-polar lipid MIX-A (cholesteryl palmitate, tripalmitin, palmitic acid, cholesterol); S = human serum lipid extract; LM = lipid mixture (LL, SP, PC, PG, C); PE = phosphatidylethanolamine, PG = phosphatidylglycerol; C = cholesterol; FFA = free fatty acid.

min and dried between migrations in a vacuum desiccator at 25°C for 10 min.

Standard and sample solutions (10 μl) were applied with a Hamilton syringe 1.5 cm from the lower edge of the silica gel layer.

The following solvent systems were used: (I) chloroform—methanol—water (65:25:4); (II) *n*-hexane—acetone (100:1); (III) *n*-hexane—acetone (100:3). The plates were developed in a chamber in room temperature, first with solvent system I to a height of 8 cm above the origin (ca. 15 min) according to Pernes et al. [6]. After drying, the plates were developed to 18 cm above the origin with solvent system II (ca. 80 min), then allowed to dry at room temperature.

Spots were detected by spraying with a reagent prepared by dissolving 5 g of phosphomolybdic acid in 100 ml of ethanol and adding 1 ml of perchloric acid, then heating in an oven at 100°C for at least 2 min.

Sample preparation

Blood samples were taken from patients with liver diseases after a 12-h fast. The serum lipids were extracted from 200 μl of serum according to Folch et al. [12]. The samples were kept at −40°C before use. The dried extract was dissolved in 200 μl of chloroform—methanol (1:1) and 10 μl of this solution were spotted at the location marked S in the figures.

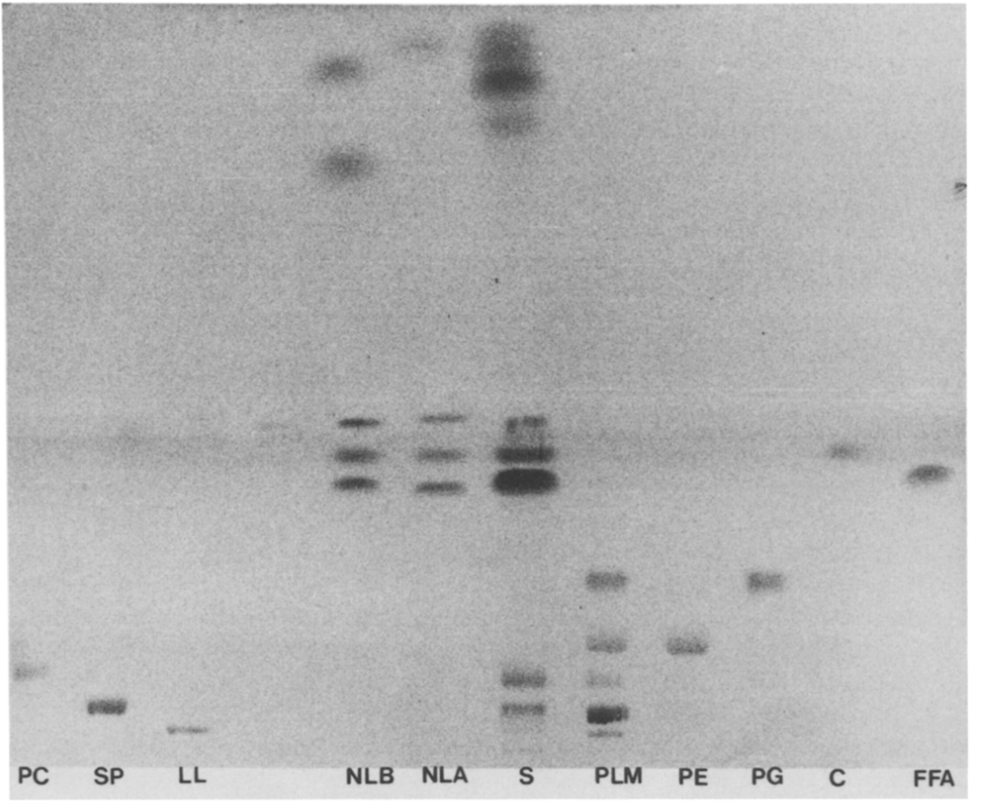


Fig. 2. Thin-layer chromatogram of lipids developed with solvent systems I and II. NLB = non-polar lipid mixture B (cholesterol oleate, methyl oleate, oleic acid, cholesterol); PLM = phospholipids (PC, SP, LL, PE, PG); other abbreviations as in Fig. 1.

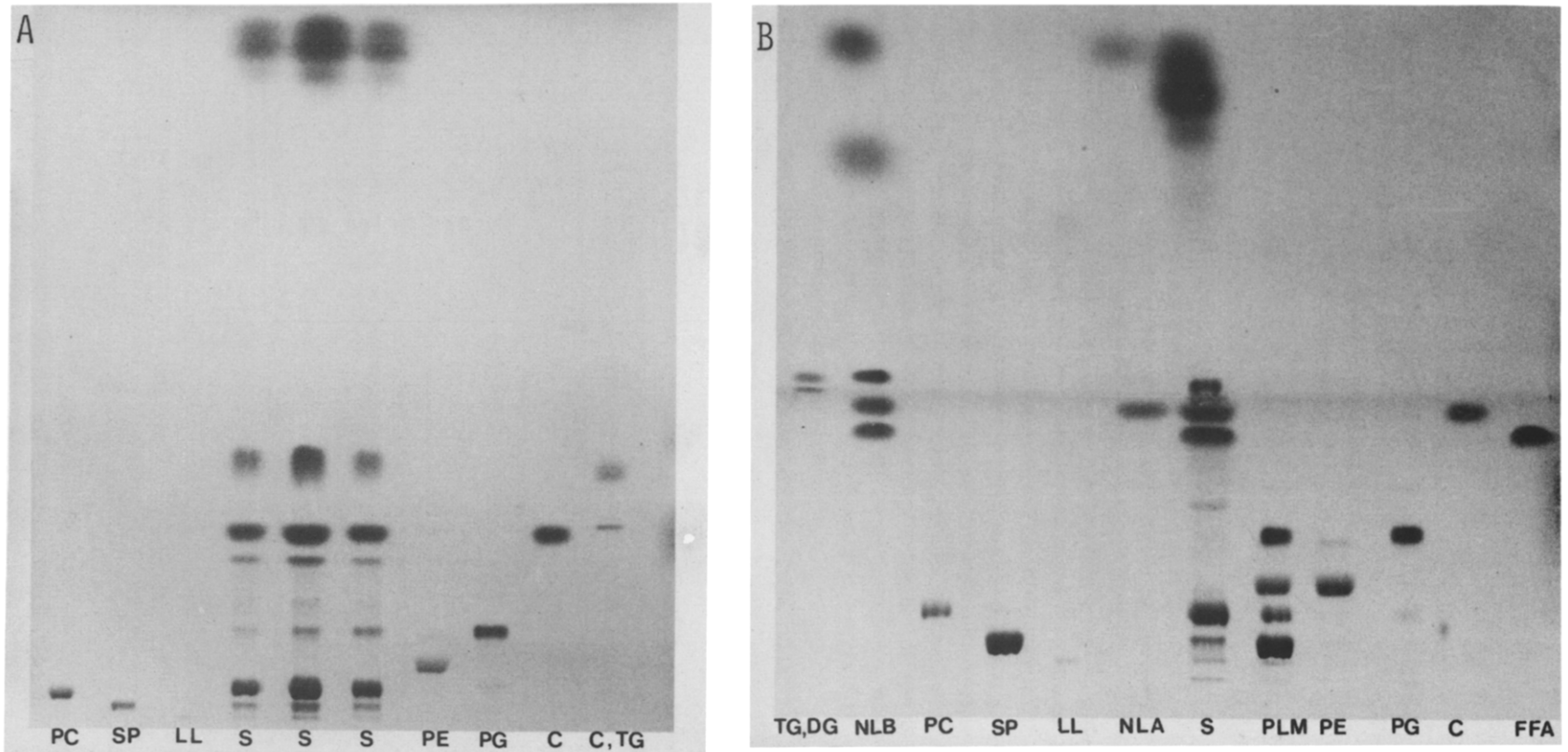


Fig. 3. Thin-layer chromatograms of lipids separated with solvent systems I and III. Abbreviations as in Figs. 1 and 2. In (A) a serum sample without cholesterol ester standard is shown; three, instead of four, cholesterol ester fractions can be seen. In (B) it is shown, by standards, that cholesterol fractions containing saturated and monounsaturated fatty acids show one spot.

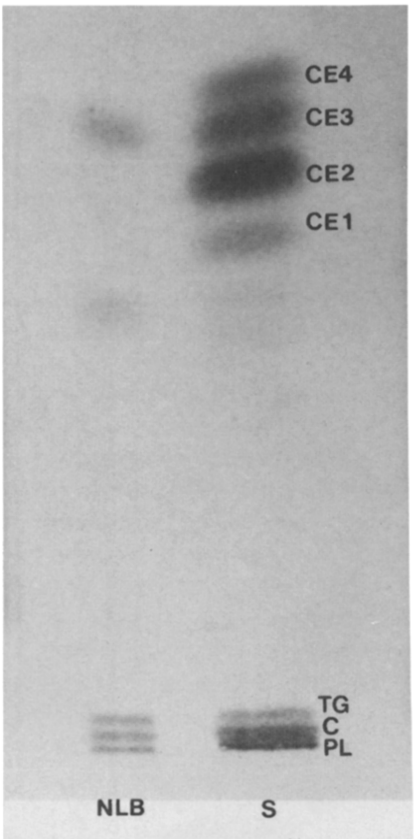
Identification of cholesterol ester subfractions

Cholesterol esters were purified by TLC. The fatty acid contents of separated spots were analysed by GC after methylation [10].

RESULTS AND DISCUSSION

The advantages of development with solvent systems I and II are illustrated in Figs. 1 and 2. If the proportions of the components were varied by increasing or decreasing the amount of acetone (system III) the separation of cholesterol esters becomes worse, because only three fractions could be detected (see Fig. 3). The spots of the cholesterol esters in the serum sample are larger than those for healthy serum. It is a fact that in liver diseases the blood serum contains several cholesterol esters.

This mobile phase separated individual cholesterol ester subfractions according to the sum of the carbon atoms and numbers of double bonds in their



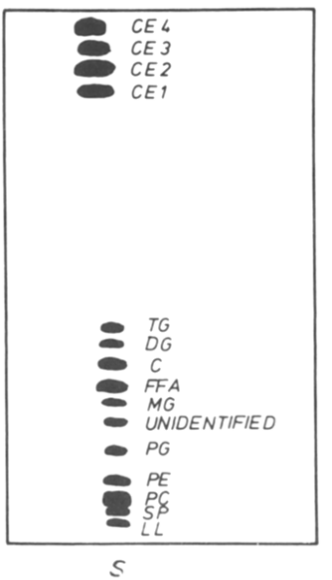


Fig. 4. Thin-layer chromatogram of lipids developed with solvent system II. Abbreviations as in Fig. 1. CE = cholesterol ester; PL = phospholipids. Fraction numbers of cholesterol esters separated by TLC and fatty acid composition· CE4 (16:0), CE3 (16:1, 18:1), CE2 (18:2, 20·2), CE1 (18:4, 20:4)

Fig. 5. Thin-layer chromatogram after development with solvent systems I and II, showing fourteen identified spots. Abbreviations as in Fig. 1.

fatty acid moieties (Fig. 4). It can be seen that the intra-class separation of the cholesterol esters is very good. After the development we can obtain five phospholipid fractions (lysolecithin, sphyngomyelin, lecithin, phosphatidylethanolamine and phosphatidylglycerol) and nine spots for neutral lipids (free fatty acids, free cholesterol, triglyceride, diglyceride, monoglyceride and cholesterol ester subfractions 1, 2, 3 and 4) (Fig. 5). One spot was unidentified.

Our method allows a complete and adequate separation of almost any number of lipid groups and is suitable for quantitative determinations. Earlier Pernes et al. [6] obtained twelve different fractions by three successive unidirectional developments on the same plate, which is the best result until the present study. We have shown that the successive use of two different solvent systems allows the detection of fourteen lipid fractions on the same plate in a short time.

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