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Note

Lipids: Thin-layer chromatographic separation in twelve fractions by three successive unidirectional developments on the same plate

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We were looking for a method capable of measuring a maximum number of lipid fractions in biological samples, in an attempt to obtain a collection of normalized lipid data of acceptable accuracy. Having selected a chromatographic technique, the problem was to try to detect all the fractions on a single chromatogram. With the methods generally used, either the neutral lipids migrate but not the phospholipids [1—9], or the phospholipids migrate but the neutral lipids remain near the solvent front with poor separation in several classes [10—16]. It seemed that no method currently used for neutral lipids can satisfactorily provide simultaneously a good separation between free cholesterol (FC) and diglycerides (DG) and between cholesterol esters (CE) and hydrocarbons such as squalene (SQ), except the procedure described by Chabard et al. [9]. However, their method still does not allow the separation of the main phospholipids. Moreover, it requires a special preparation of plates. In accordance with French and Andersen [7] we preferred to use commercial, stabilized silica gel plates, which are available anywhere, and to try to improve the separation according to the way proposed by Wildgrube et al. [14] using a succession of

solvents. With this technique, we can locate twelve well-separated fractions in three successive migrations in the same direction. At the same time, we quantify each fraction with acceptable accuracy since interference between the different lipids is avoided [15] or immediately detected.

MATERIALS AND METHODS

Solvents

Chloroform, *n*-hexane, and carbon tetrachloride were purchased from Merck (Darmstadt, G.F.R.); methanol and ethanol were from Carlo Erba (Milan, Italy). These solvents were of the best analytical grade available and were used without further purification. The following solvent systems were used: system I, chloroform—methanol—distilled water (65:25:4, v/v); system II, chloroform—*n*-hexane (3:1, v/v); and system III, carbon tetrachloride.

Lipid standards

All reference lipids were obtained from Sigma (St. Louis, Mo., U.S.A.). Standard solutions were prepared by dissolving 100 mg of each individual pure reference compound in 10 ml of chloroform—methanol (1:1, v/v), except squalene which was dissolved in chloroform—methanol (1:1) at 10 g/l. These stock solutions were diluted for use to 1 mg/ml, except for trioleylglycerol (TG) which was diluted to 2 mg/ml and for a mixed solution of cholesteryl linoleic ester (CE) at 2 mg/ml and squalene (SQ) at 1 mg/ml.

All compounds migrated as homogeneous spots on thin-layer chromatographic (TLC) plates except DG which is a mixture of 85% 1,3-dioleylglycerol (1,3DG) and 15% 1,2-dioleylglycerol (1,2DG). In our development system, the sphingomyelin (SP) standard migrated as one spot; however, in other systems [10—12] two spots were observed.

A complete migration standard solution (labeled M on the plates) was a mixture of SQ (1 mg/ml), CE (1 mg/ml), TG (1 mg/ml), 1,3DG (0.85 mg/ml), 1,2DG (0.15 mg/ml), FC (1 mg/ml), oleic acid as non-esterified fatty acid (NEFA; 1 mg/ml), monolinoleylglycerol (MG; 2 mg/ml), phosphatidyl ethanolamine (PE; 1 mg/ml), phosphatidylcholine (PC; 2 mg/ml), SP (1 mg/ml) and lysolecithin (LL; 0.5 mg/ml).

Plates

We used standard TLC plates (Merck No. 5721, 20 × 20 cm, 250 μm).

They were washed with migrating solvent system I to the top of the plate, then air dried at room temperature for 30 min and finally stored in a desiccator. Standard and sample solutions were applied with a capillary pipette (Camag, Muttenz, Switzerland) at 1.5 cm up from the lower edge of the silica gel layer. Twelve drops each of 10 μl were placed on each plate.

Development procedure

Development of the chromatograms was carried out at room temperature in a set of three covered glass tanks (Camag) internally lined with filter paper to saturate the environment. The tanks were filled with solvent to a level of 10 mm at least 4 h before use. The plates were developed to a height of 8 cm

above the origin in solvent system I (ca. 20 min) then to 13.5 cm above the origin in system II (ca. 30 min) and in system III to the upper edge of the plate (ca. 80 min).

Between each migration, the plates were allowed to dry at room temperature for 10 min. Spots were detected either by incubating the plate at room temperature in a tank saturated with iodine vapor (Fig. 1), or by spraying the plate with a 20% ethanol solution of phosphomolybdic acid, then heating it in an oven at 120° for at least 15 min [16] (Fig. 2).

RESULTS AND DISCUSSION

The advantages of two- or three-step migrations with two or three solvent systems are illustrated in Figs. 1 and 2. Fig. 1 shows the separation obtained with solvent systems I and II. Compared with migration in system I alone [10] we not only obtain the four classical phospholipid fractions (PE, PC, SP, LL), but also the separation of neutral lipids in six spots: MG, NEFA, FC, 1,2DG,

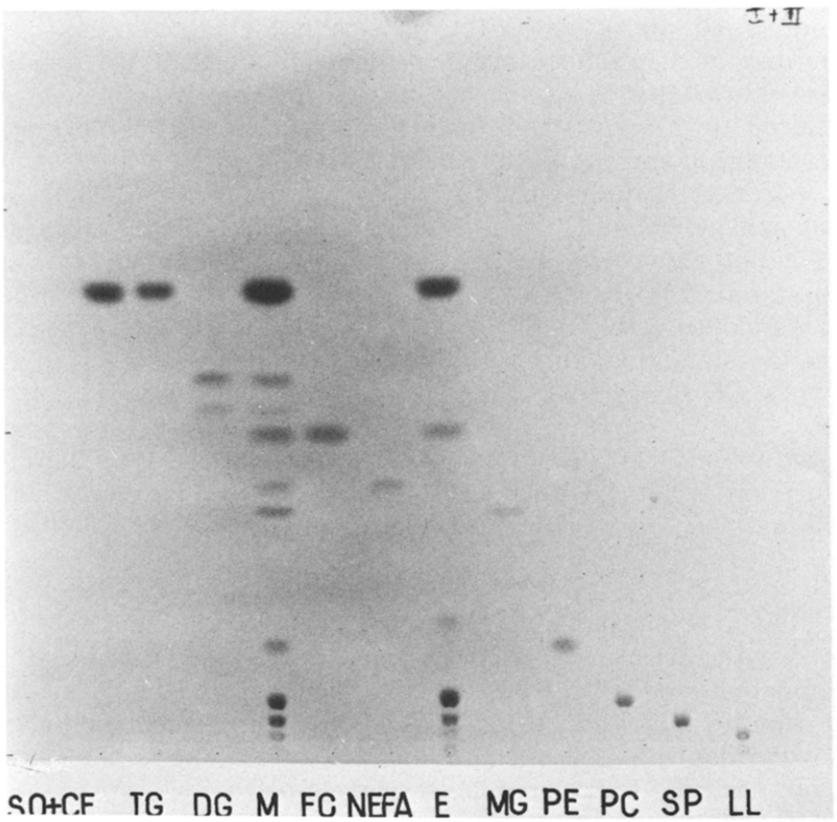

Fig. 1. Thin-layer chromatogram of lipids developed in solvent systems I + II. SQ = Squalene; CE = cholesterol ester; TG = triglycerides; DG = diglycerides (diglyceride 1,3 = 1,3DG and diglyceride 1,2 = 1,2DG); M = reference mixture of the twelve standard lipids; FC = free cholesterol; NEFA = non-esterified fatty acid; E = human serum lipid extract; MG = monogiyceride; PE = phosphatidyl ethanolamine; PC = phosphatidyl choline; SP = sphingomyelin; LL = lysolecithin.

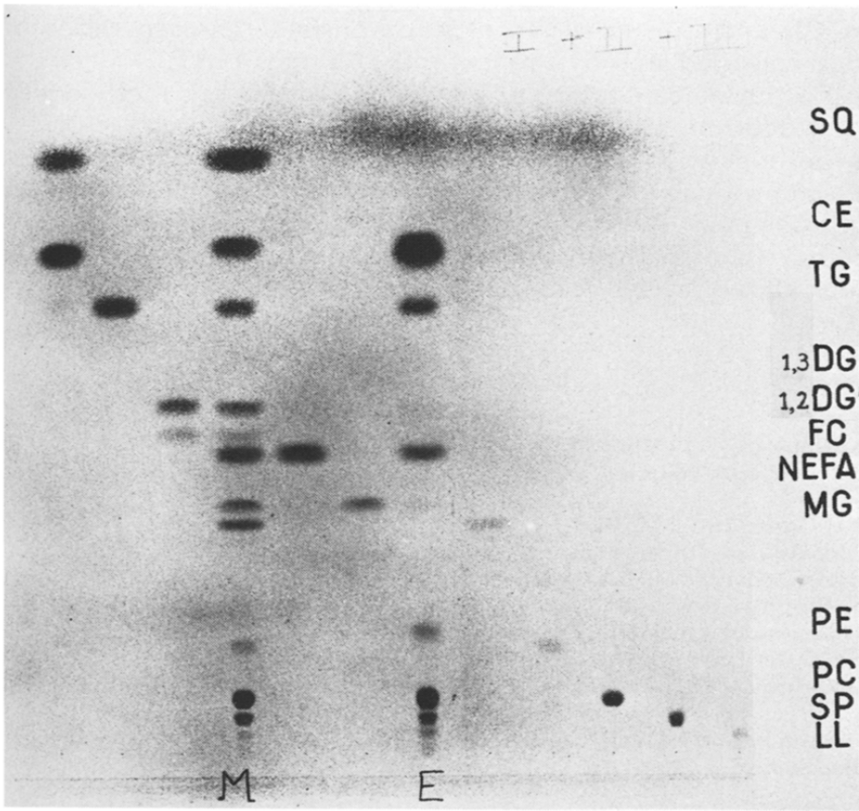

Fig. 2. Thin-layer chromatogram of lipids after subsequent development in solvent III. For abbreviations see legend to Fig. 1.

1,3DG, and a spot containing SQ, CE and TG together. The separations of MG and NEFA, which are in high concentration, and of 1,2DG and FC are particularly good.

When the third solvent system is used, TG, CE and SQ are now separated while the other compounds are unaffected (Fig. 2).

As an example of the practical use of this method we ran a sample of human serum. A blood sample was taken from a normal healthy male volunteer after 12 h of fasting. Lipids were extracted from 1 ml of serum using the method of Folch et al. [17]. The dried extract was dissolved in 1 ml of chloroform—methanol (1:1, v/v) and 10 μl of this solution were spotted on the plate at the place marked E in the figures. As in the case of the standard solution M, one observes in Fig. 2 the separation between TG and CE. One can note not only the normal absence of SQ but also the absence of DG and MG because their concentrations in serum are too low to be detected under the conditions used in this experiment. In spite of the very low NEFA concentrations in normal blood, their presence can be observed on both plates.

With the serum sample, the CE spot is larger than the one obtained with the standard mixture. This is due to the fact that blood serum contains several CE whereas in the standard mixture only cholesteryl linoleic ester was present. The

separation between CE and TG, however, is accomplished. The separation of CE can be obtained in a second step by the method of Morris [18].

The complete and accurate separation of each lipid renders this method well suited for quantitative determinations.

Of the different methods proposed up until the present study, for lipid separation in biological samples using TLC in one direction, the best results appear to be those of Wildgrube et al. [14] who obtained nine different spots on their chromatograms. We have shown that the use of three different solvent systems in a convenient order allows twelve fractions to be obtained without many experimental difficulties.

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