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Note

Three-step one-dimensional thin-layer chromatographic separation of neutral lipids

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During radioisotopic investigations of receptor-mediated breakdown of triphosphoinositide to inosito triphosphate and diglyceride (DG) and conversion of the latter compound to monoglyceride (MG) and fatty acid (FA), a thin-layer chromatographic (TLC) system was needed that would ensure that FA, MG and DG would be sufficiently separated from each other and other neutral lipids to allow them to be scraped from the plate and counted. Of the existing chromatographic systems for the separation of neutral lipids[1–6], only the systems of Freeman and West[6] and Chabard *et al.*[3] seemed to provide this degree of separation. The latter system, however, requires specialized equipment[3] and the separation reported by Freeman and West[6] could not be reproduced as FA overlapped DG. Studies were therefore undertaken to design a system that would provide the required separation. The resulting system effects a separation that allows the following lipid fractions listed in order of increasing R_F values, to be scraped from the plate for determination: phospholipids (PL, at the origin), FA, MG, cholesterol (C), DG, triglyceride (TG) and cholesterol esters (CE). The system described here was compared with that of Freeman and West[6], which provided guidelines in designing the present chromatographic system.

EXPERIMENTAL

Materials

TLC plates were prepared with the following silica gels: without binder, silica gel 60 HR (Merck), silica gel 60 H (Merck), silica gel (Camag, D-O); and with binder (calcium sulphate), silica gel G (Merck). A Desaga spreader (Merck) was used to give an adsorbent layer 0.3 mm thick. Commercial TLC plates were silica gel 60 (Merck). Cholesterol palmitate, triolein, 1,2-diolein-*rac*-glycerol, 1-monooleoyl-*rac*-glycerol, linoleic acid and arachidonic acid were obtained from Sigma, linolenic acid from Fluka and cholesterol from Merck. Phospholipid was a mixture of phosphatidylcholine and phosphatidylethanolamine, both prepared from egg yolk[7]. All reference substances were more than 99% pure. Organic solvents were of analytical-reagent grade, except triethylamine, which was a Merck product for synthesis, purity 99%.

Thin-layer chromatography

TLC plates were activated for 1 h at 105°C prior to use and delineated into

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2–3-cm lanes by means of a needle. In addition, a front line was scored corresponding to the longest distance of development. After each development, including predevelopment when used, the plates were dried for 5 min with a hair-dryer set in the "no-heating" mode. Samples in hexane–diethyl ether (9:1, v/v) were applied 2 cm from the lower edge of the plates. Approximately 20 μg of each reference compound were applied.

RESULTS AND DISCUSSION

Fig. 1 shows the separations obtained at each stage of the three-step chromatographic analysis of a mixture of reference compounds. The first solvent resolves the mixture into DG, C, MG, a mixture of CE and TG and a mixture of FA and PL which remains at the origin (*cf.*, chromatogram 1). When development is done with the first solvent followed by the second solvent, an additional separation of CE and TG is obtained while the mixture of FA and PL still remains at the origin, as seen in chromatogram 2. Resolution of the mixture of FA and PL is obtained with the third solvent, as seen in chromatogram 3, which shows the complete separation of neutral lipids by the proposed chromatographic system with PL remaining at the origin.

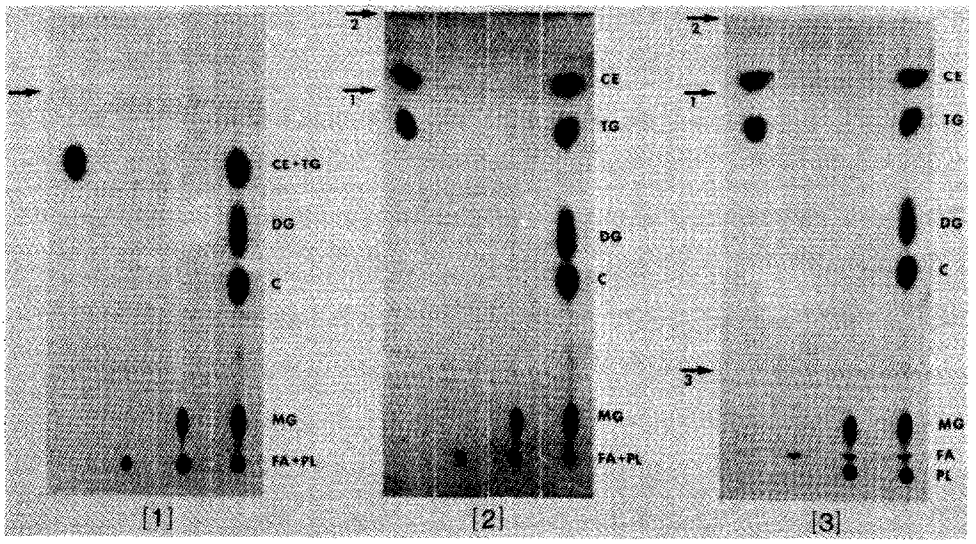

Fig. 1. Progression of lipid class separation by TLC using three-step development. Solvent systems (proportions by volume): 1, diethyl ether–benzene–ethanol–triethylamine (40:50:2:1); 2, diethyl ether–hexane–triethylamine (10:90:1); 3, diethyl ether–hexane–acetic acid (75:25:2). Chromatogram 1, developed with solvent 1 up to arrow 1, *ca.* 16 cm; chromatogram 2, developed with solvent 1 up to arrow 1, *ca.* 16 cm, and with solvent 2 up to arrow 2, 19 cm. Chromatogram 3, developed in succession with solvents 1, 2 and 3 up to arrows numbered correspondingly, *ca.* 16, 19 and 6 cm, respectively. Before application of samples, the plates were predeveloped with solvent 1, 19 cm. CE, cholesterol ester; TG, triglyceride; DG, diglyceride; C, cholesterol; FA, fatty acid; MG, monoglyceride; PL, phospholipid. The four samples applied across each plate, from left to right, were (i) cholesterol palmitate and triolein, (ii) linoleic acid, (iii) 1-monooleoyl-*rac*-glycerol, linoleic acid and phospholipid and (iv) cholesterol palmitate, triolein, 1,2-diolein-*rac*-glycerol, cholesterol, 1-monooleoyl-*rac*-glycerol, linoleic acid and phospholipid. The amount applied of each compound was *ca.* 20 μg. Detection by charring with 95% sulphuric acid.

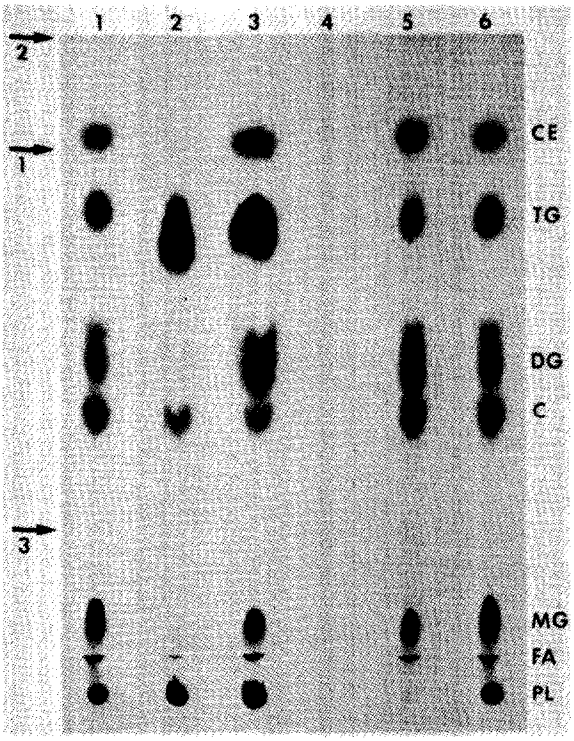

Fig. 2. Examination of neutral lipids of egg yolk and egg white by TLC using three-step development. Conditions as described for chromatogram 3 in Fig. 1. Applications: lanes 1 and 6, reference compounds mentioned in Fig. 1; lane 2, egg yolk lipid; lane 3, lipids extracted from egg yolk to which a mixture of neutral lipid reference compounds had been added; lane 4, lipid extract from egg white; and lane 5, lipids extracted from egg white to which a mixture of reference compounds had been added. The samples applied to lanes 2, 3, 4 and 5 were prepared by extracting 25 mg of egg yolk, 25 mg of egg yolk to which was added 0.5 mg of each reference compound, 250 mg of egg white and 250 mg of egg white to which was added 0.5 mg of each reference compound, respectively. The extracted lipid samples were dissolved in 0.5 ml of hexane–diethyl ether (9:1, v/v) and 20 μl were applied. Abbreviations and detection as in Fig. 1.

Fig. 2 shows the results obtained when the chromatographic system is used to study the neutral lipids of a biological material. It can be seen (lane 2) that TG and C are the only important neutral lipids in egg yolk. Trace amounts of the other neutral lipids were discernible on the original chromatogram, but only FA are visible on the photographic reproduction. Lane 3, which represents the separation of the lipids extracted from egg yolk enriched with standards, shows the usefulness of the system in separating neutral lipids likely to be encountered in a biological material. Lane 4 shows the absence of neutral lipids from egg white and lane 5 shows that standards added to egg white are recovered by the lipid extraction and adequately separated in the subsequent chromatography. The elongated spot of DG on the chromatograms in Figs. 1 and 2 contains the two isomers of diolein, owing to isomerization on the adsorbent. The 1,3-isomer is known to have a slightly higher R_F value than the 1,2-isomer[8].

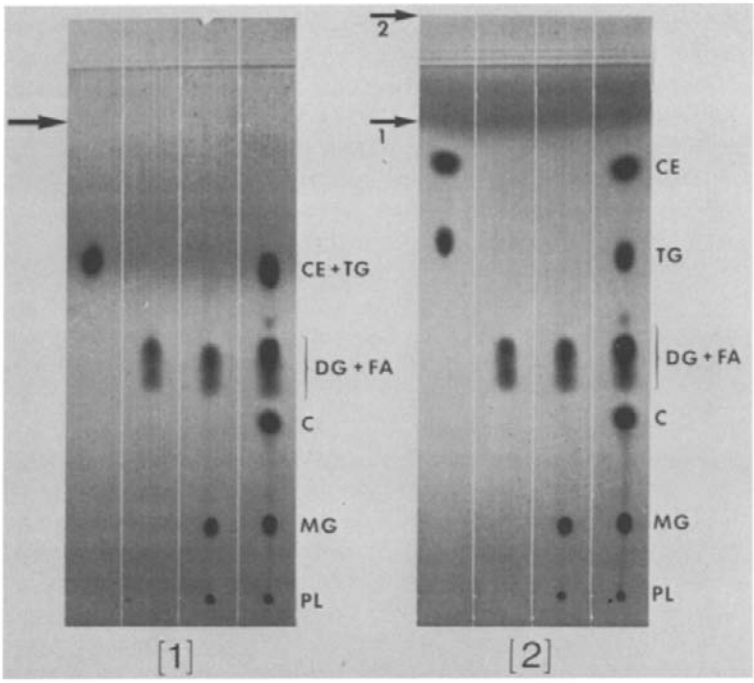

Fig. 3. Separation of neutral lipids by the double-development procedure of Freeman and West[6]. Chromatogram 1: solvent system, diethyl ether–benzene–ethanol–acetic acid (40:50:2:0.2), development up to arrow, *ca.* 27. Chromatogram 2: solvent system, diethyl ether–benzene–ethanol–acetic acid (40:50:2:0.2), development up to arrow 1, followed by development in diethyl ether–hexane (6:94) up to arrow 2, 33 cm. The plates (20 × 34 cm) were prepared with silica gel G, 0.3 mm thick. No predevelopment was performed before application of samples. The four samples applied across each plate, from left to right, were identical with those in Fig. 1. Abbreviations and detection as in Fig. 1.

Fig. 3 shows the results using the system of Freeman and West[6]. The first development (chromatogram 1) separates the mixture of standards into PL, which remains at the origin, MG, C, a mixture of DG and FA and a mixture of CE and TG. When the first development is followed by the second, CE and TG are separated whereas DG and FA remain unresolved, as seen in chromatogram 2. When the chromatograms in Fig. 3 were compared with those reported by Freeman and West[6], it was clear that the only disagreement concerned the chromatography of FA, which according to them migrates to a position between that of MG and C.

The natural strategy for constructing a modified system was therefore to preserve the successful separation of CE, TG, C and MG and direct the migration of FA to a vacant area of the chromatogram by using suitable basic and acidic solvents. Consequently, a basic component, triethylamine, was substituted for acetic acid in the first solvent of the system of Freeman and West[6] and was also included in the second solvent of that system while maintaining approximately the same proportions of the other organic solvents. When silica gel H plates predeveloped with the first solvent were used, FA remained at the origin during development with the first and second solvents, as might be expected because FA exist as their polar anions under these

conditions. At the same time, the good separation of the other neutral lipids reported by Freeman and West was preserved (*cf.*, Fig. 1, chromatogram 2). In the subsequent third development with the acidic solvent, FA are converted to their undissociated form of far less polarity allowing migration to the middle of the vacant area between the origin and the position of MG (*cf.*, Fig. 1; compare chromatograms 2 and 3).

In order to localize the positions of the various fractions in the analysis of biological samples, a mixture of reference compounds must be applied on the far left and right lanes of a plate, as done in Fig. 2. After chromatographic development the reference lanes are rendered visible by exposure to iodine vapour while covering the remainder of the chromatogram with a glass plate. The zones corresponding to various fractions are then delineated in the unexposed lanes with a needle guided by the positions of the reference compounds on the iodine-treated lanes.

The designed three-step TLC system works equally well with different brands of silica gel without binder. In contrast, it does not work with commercial plates and plates prepared with silica gel G. The system employs very short periods of drying without heating between chromatographic developments and consequently the risk of autoxidation of unsaturated lipids during the chromatographic analysis is small. No sign of autoxidation during the analysis was observed even when the very unsaturated fatty acid arachidonic acid was chromatographed.

It is of interest to compare the present system with that of Chabard *et al.*[3], because the chromatography of FA is controlled by similar principles. In that system, which also employs three-step one-dimensional development, FA are likewise kept at the origin during development with the first two solvents by maintaining FA in their anionic form. Chabard *et al.*[3] achieved that by the use of a 3-cm lower adsorbent layer made from silica gel G slurried in 0.4 *M* sodium hydroxide solution. The remainder of the plate was coated with silica gel G slurried in water. Development with an acidic third solvent was finally used to move FA from the origin to a vacant area of the plate. Approximately the same degree of separation is achieved in the two systems, but the method of controlling the chromatography of FA used in this work is more convenient and only standard equipment is required. It is believed that the present system, in addition to its usefulness in the investigations mentioned in the Introductory remarks, constitutes a valuable supplement to already existing systems which may be preferable in other quantitative studies of neutral lipids. Naturally, if quantitative data for FA are not required in a particular study, it is convenient to omit development with the third solvent.

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