Fractionation of cholesterol esters by thin-layer chromatography

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» Cholesterol esters, as a class, are readily separated from other lipids in biological extracts, or from cholesterol or cholesteryl acetate in synthetic preparations by silicic acid adsorption chromatography, either on columns or on thin-layer plates (e.g., 1–3). More detailed analysis of a given cholesterol ester fraction is also readily achieved by gas-liquid chromatography of the component fatty acids, as their methyl esters. It is not so easy, however, to separate individual cholesterol esters from each other. Some resolution has been achieved on an analytical scale by thin-layer chromatography (e.g., 3–5), and on a preparative scale by the column chromatographic method of Klein and Janssen (6); the latter, however, is somewhat tedious.

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It has recently been shown that silicic acid impregnated with silver nitrate allows ready chromatographic separation of methyl esters and triglycerides differing in number and type of double bonds (7–9). Separation of cholesterol esters according to degree of unsaturation is now shown to occur rapidly and quantitatively on thin layers of silicic acid impregnated with silver nitrate.

Thin-layer plates were prepared with Silica Gel G (E. Merck, A.G., Darmstadt, Germany) using the Desaga equipment (Desaga, GmbH, Heidelberg, Germany). Impregnation was accomplished either by spraying silicic acid plates with a 10% aqueous solution of silver nitrate or, for better reproducibility, by using silver nitrate solution instead of water in preparing the slurry of Silica Gel G for coating the plates. Less silver nitrate is necessary than was originally supposed (8, 9); 2% impregnation of Silica Gel G with silver nitrate (w/w) gave as good separation as 20–30% impregnation, but 5% impregnation was taken as the standard (i.e., the slurry was prepared by mixing 23.75 g Silica Gel G with 50 ml water containing 1.25 g silver nitrate). Plates were dried and activated by heating at 110° for 30 min. These plates were not particularly sensitive to light but should be protected from laboratory fumes and strong direct light to prevent discoloration.

Human serum cholesterol esters were isolated by preparative thin-layer chromatography on Silica Gel G. The separated components were located under ultraviolet light after spraying with 2′,7′-dichlorofluorescein, the cholesterol ester zone scraped from the plate, and the cholesterol esters eluted from the adsorbent with purified diethyl ether and recovered. Chromatography of the cholesterol ester mixture on silver nitrate-impregnated silicic acid, with diethyl ether–hexane 1:4 and with pure diethyl ether as eluting solvents in separate runs, resulted in separation of a total of seven components as shown by Fig. 1A and 1B, sample S. Preparative thin-layer chromatography on silver nitrate-impregnated plates was carried out, using, first, pure ether to fractionate components 4, 5, and 6, and, subsequently, ether–hexane 1:4 to fractionate components 0–3 on a second chromatogram. The bands of adsorbent containing the individual zones were scraped from the plate into microchromatography columns and immediately eluted with 5-ml amounts of suitable solvents to isolate the cholesterol ester fractions. Components 0, 1, 2, and 3 were each isolated in this way by elution with pure diethyl ether while the more unsaturated components 4, 5, and 6 were eluted with chloroform–methanol 2:1. Recovery of all components by this procedure was virtually quantitative (>95%). Any entrained silver nitrate was readily removed by a water wash. The

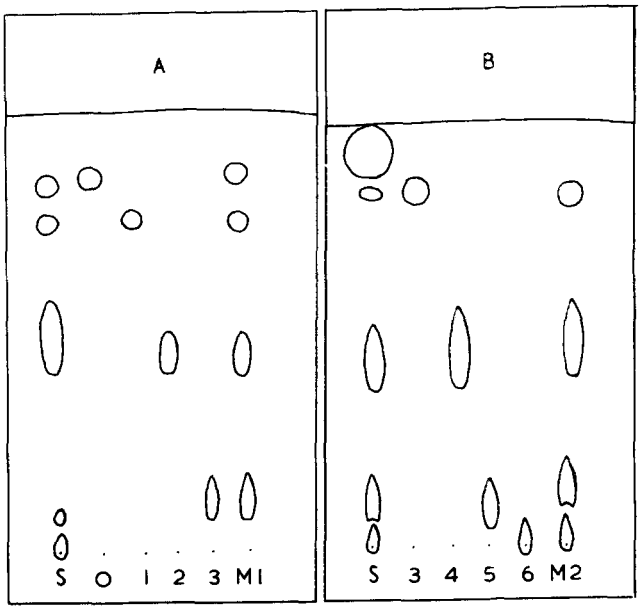

FIG. 1. Thin-layer chromatograms of cholesterol esters on Silica Gel G impregnated with silver nitrate (5% w/w). Solvent systems were (A) diethyl ether–hexane 1:4, and (B) diethyl ether. Components were visualized under ultraviolet light after spraying with a 0.2% solution of 2′,7′-dichlorofluorescein in ethanol and were reproduced by tracing. Samples were of cholesterol esters of the following types of fatty acids: S, mixed, from normal human serum; 0, saturated; 1, monoenoic; 2, dienoic; 3, trienoic; 4, tetraenoic; 5, pentaenoic; 6, hexaenoic; M1, mixture of 0, 1, 2, and 3; M2, mixture of 3, 4, 5, and 6.

suggested order of isolation of compounds (4–6 before 0–3) is preferable, to minimize chances of degradation of the more highly unsaturated components. The chance of autoxidation is reduced by carrying out all operations, including application of samples to the plate, under an atmosphere of nitrogen.

The isolated components were shown to be free from each other and from degradation products by rechromatography on silver nitrate-impregnated plates as illustrated in Fig. 1. An aliquot of each fraction was converted to methyl esters by transmethylation and was analyzed by gas-liquid chromatography on an ethyleneglycol-adipate polyester stationary phase. Each fraction contained only fatty acids of the same degree of unsaturation, as assigned in Fig. 1. Some contained homologous acids (e.g., component O contained myristic, palmitic, and stearic acids), whereas some had only one acid (e.g., component 4 contained only arachidonic acid).

Between 25 and 50 mg of mixed cholesterol esters were fractionated on single thin-layer plates as described. Even in preparative work on this scale, no advantage was found in increasing the level of impregnation from 5% to 20 or 30% of silver nitrate (w/w).

Thin-layer chromatography on silicic acid, impregnated with silver nitrate, has been shown to have considerable value in qualitative studies of cholesterol ester mixtures. The proportions of the individual unsaturation classes of cholesterol esters are readily determined by one of the standard colorimetric determinations (cf. 4), after recovery of the separated components from the plate.

Further resolution of these unsaturation classes, according to chain length, may be achieved by a reversed phase partition procedure (e.g., 5). However, such partition systems are not so suitable for preparative work, which is likely to be the most important application of the procedure here described. Quantitative resolution of 25 mg of mixed cholesterol esters in single samples was obtained in the course of this work using the two solvent systems described; by using thicker layers or the spread-layer technique, it may be possible to resolve even larger amounts. For amounts in excess of 100 mg, however, column chromatography on silicic acid impregnated with silver nitrate, according to de Vries (8), may be preferable to multiple thin-layer separations.

One obvious and potentially valuable application of this procedure would be in the preparation of radioactively labeled cholesterol esters. For example, instead of rigorously purifying individual methyl esters of fatty acids prior to the synthesis of the corresponding cholesterol esters, a mixture of methyl ester vinylogues of one chain-length could be used and the separation of individual compounds deferred until after the conversion to cholesterol esters. Randomly labeled fatty acids are conveniently produced by *Chlorella pyrenoidosa*, and their methyl esters are readily separated into single chain-length fractions by distillation in a whirling band column (10). Cholesterol esters could then be made with each chain-length fraction by transacylation with the acetate of either normal or radioactively labeled cholesterol (3), and the separation and purification of the individual cholesterol esters achieved simultaneously by thin-layer or column chromatography on silicic acid impregnated with silver nitrate.

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