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Notes on Methodology

Separation of tissue cholesterol esters and triglycerides by silicic acid chromatography

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► Unequivocal class separations of tissue lipids are important particularly when high temperature gas chromatography is used at the same time for the determination of the long-chain acid composition of major lipid classes. This problem was studied most recently by Hirsch and Ahrens (1); their procedure involves silicic acid column chromatography with elution by ether-petroleum ether mixtures for "neutral" lipids, and methanol for phospholipids. The separation of cholesterol esters from triglycerides is difficult to achieve; the Hirsch-Ahrens separation procedure is the most detailed and most effective one that has been described.

Benzene-hexane (and benzene-petroleum ether) mixtures applied to a silicic acid column can usually be made to give the same kind of results as ether-petroleum ether mixtures, and Borgström's method (2) for class separations was based on the use of the former solvent pair. In these procedures and that of Lipsky *et al.* (3), as in the ether-petroleum ether methods of Fillerup and Mead (4) and Luddy *et al.* (5), the key to successful duplication of separations lies in the preparation of the silicic acid. Since benzene-hexane mixtures are more convenient to use than ether-petroleum ether mixtures, the following method was developed for this solvent pair; the relatively large changes in benzene concentration make the neutral lipid separations particularly easy to accomplish.

MATERIALS AND METHODS

Preparation of Silicic Acid. Mallinckrodt silicic acid (catalogue number 2847), labeled "suitable for chromatographic analysis by the method of Ramsey and Patterson," is prepared for use by the following procedure.

A preliminary screening is carried out by dry sieving, and all material passing a 325-mesh screen is discarded. Approximately 100 g of silicic acid is treated with 250 ml of 3 N hydrochloric acid in a glass vessel and stirred for 30 minutes with a nonmetallic paddle. The silicic acid is allowed to settle, the aqueous acid decanted, and the residue washed repeatedly with deionized water for two days until the supernatant liquid reaches a pH of 4.5 to 5.5. Usually 20 to 40 changes of water are required. The silicic acid is then washed with acetone, filtered, and resuspended several times in fresh acetone and dried in glass trays under an infrared lamp for 16 to 20 hours. A final screening provides material of 100- to 200-mesh size, which is dried in an oven with 20 to 25 inches vacuum for 2 hours at 50°. It may be kept in closed containers for several months without apparent change. Used silicic acid is washed with methanol, followed by deionized water and acetone, and dried as in the original preparation.

The moisture content of this material as determined by drying to constant weight for 6 hours *in vacuo* at 150° is 9% to 10%. The behavior of the column is dependent on rigid adherence to details in the washing and drying procedure.

Solvents. Hexane (technical grade, from Phillips Petroleum Co. or Matheson, Coleman and Bell, Inc.) is distilled through a 20-inch Vigreux column before use. A 500 ml sample is concentrated to 2 ml in a Roto-Vap apparatus and the residue examined for high boiling components with the same gas chromatographic columns as those employed for the analytical work.

The benzene should be thiophene-free, and other solvents should be reagent grade.

Apparatus. The preferred analytical column is the Hirsch-Ahrens glass column (1). The jacket protects the column against air drafts, but circulating water is not needed for the solvent systems used here.

Preparation of the Column. An 18 g quantity of silicic acid is slurried with hexane and placed in the column after covering the glass filter plate with a filter paper disk. The column, which is about 5.5 inches high, is covered by a second filter paper disk, and is compacted by 30 psi air pressure applied intermittently until the top of the column is firm. About 300 to 350 mg of mixed lipids may be used as a column charge.

Elution. The major classes of neutral lipids are separated by stepwise elution with the following benzene-hexane mixtures: 6% (v/v) benzene-hexane (I), 18% (v/v) benzene-hexane (II), 60% (v/v) benzene-hexane (III), and benzene (IV). Phospholipids may

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be eluted by varying concentrations of methanol in chloroform (6) or methanol (1). If recovery of a total phospholipid fraction is desired, a suitable procedure is to follow the benzene with 100 ml of chloroform, followed by 100 ml of 50% (v/v) chloroform-methanol and 150 ml of methanol.

The most satisfactory operating conditions with 100 to 350 mg of total lipids is to collect 10 ml fractions at a flow rate of 3 to 5 ml per minute, which is obtained without pressure. The entire separation through fraction IV requires 90 to 115 tubes and 3.5 to 4 hours for human plasma lipid samples.

RESULTS

The chief objective of the work was to determine conditions for the quantitative separation of cholesterol esters and triglycerides from other lipid components without the use of low boiling solvents (i.e., ether). It was considered desirable to use relatively large changes in solvent composition for class elutions, and the method described was found to be more rapid than those developed earlier. The behavior of the column was tested initially with a mixture containing cholesterol palmitate (50 mg), tristearin (100 mg), and cholesterol (25 mg). Quantitative recoveries were obtained in fractions II, III, and IV, respectively. The column behavior was tested extensively with lipid fractions from blood, liver, intestinal tissue, marrow,

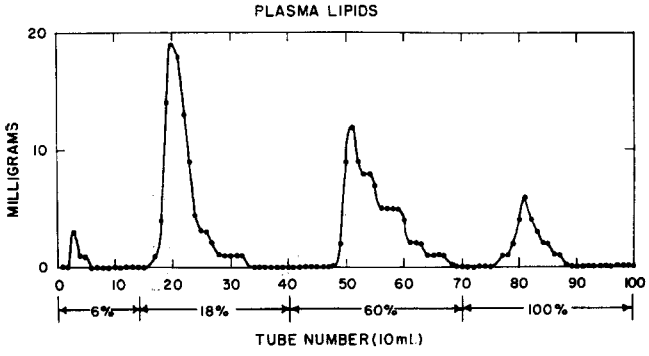

FIG. 1. Chromatographic separation of human plasma neutral lipids. The solvents are varying concentrations (v/v) of benzene in hexane.

and adipose tissue. Figure 1 shows a typical elution pattern for human plasma lipids from which the phospholipids had been removed with silicic acid. The cholesterol ester fraction was obtained as a relatively sharp cut, coming after a small "hydrocarbon" fraction. The triglyceride fraction showed a characteristic trailing; there was also some trailing in the cholesterol fraction.

The number of tubes required for each fraction varies with the amount of material in the fraction. With tissues containing small amounts of cholesterol esters, fraction II may be greatly reduced in volume. Also, fraction II sometimes was eluted more rapidly, but always as a relatively sharp fraction.

The uniformity of fraction II was established by gravimetric and colorimetric determination of sterol by the procedure of Hanel and Dam (7); the trailing tubes and the combined tubes for the entire fraction gave no triglyceride test, indicating that there was no overlap in the separation from fraction III. The analytical data for a human plasma lipid separation are given in Figure 2.

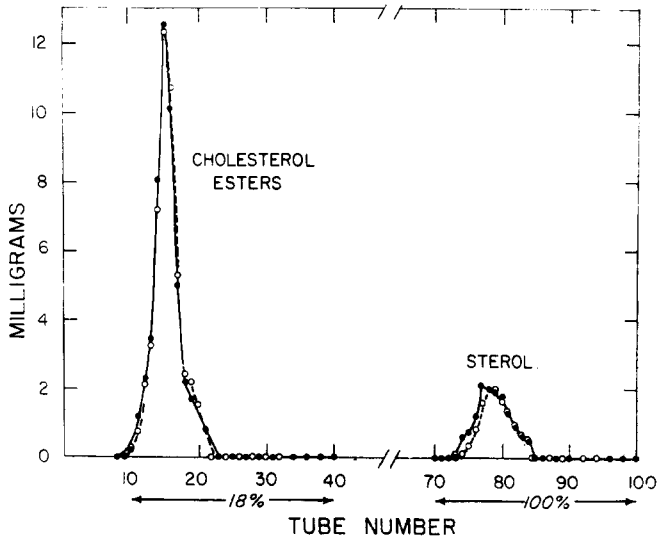

FIG. 2. Chromatographic separations of cholesterol esters and sterol fractions of human plasma; ● = gravimetric determination; o = colorimetric determination (7). The total values amount to 48.7 mg (gravimetric) and 48.4 mg (colorimetric) for cholesterol esters, and 13.6 mg (gravimetric) and 11.5 mg (colorimetric) for cholesterol. The occurrence of a band impurity is evident in the sterol peak.

The uniformity of fraction III as a triglyceride fraction free of contamination from either cholesterol esters (II) or sterol (IV) was established by use of the triglyceride procedure of Van Handel and Zilversmit (8) (using corn oil triglycerides as a standard), and the cholesterol procedure of Hanel and Dam (7). Figure 3 shows the data for gravimetric and colorimetric determinations in a human plasma lipid separation of fraction III. The leading and trailing tubes, and the combined tubes for the entire fraction, gave no test for cholesterol or cholesterol esters by the Dam or Liebermann-Burchard tests.

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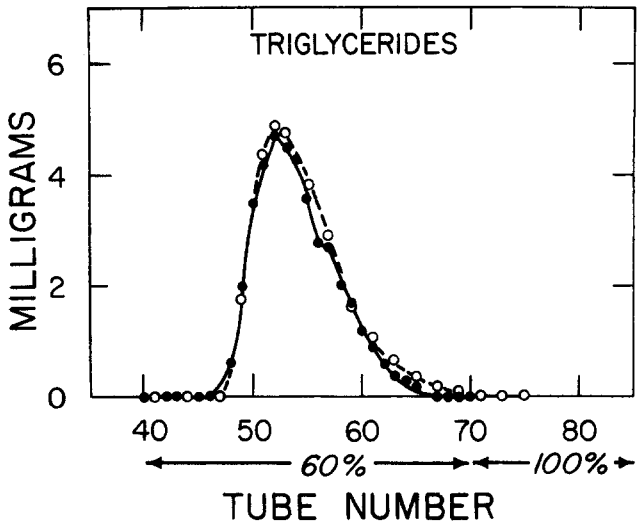

FIG. 3. Chromatographic separation of the triglyceride fraction of human plasma; • = gravimetric determination; o = colorimetric determination (8).

Figure 2 shows the examination of fraction IV by gravimetric and colorimetric (7) determinations of sterol. It was found that in the early tubes of the fraction the gravimetric values exceeded the colorimetric ones. Yet the leading tubes and the combined tubes for fraction IV gave no triglyceride test. Apparently the weight of fraction IV may not be used for the determination of sterol.

In order to characterize fractions III and IV in greater detail, a lipid sample obtained in the course of an animal experiment with acetate —1-C^{14} was employed. This material contained labeled fatty acids in the triglycerides, and some labeled free fatty acids were also present. The separation of fraction III, according to radioactivity levels, is shown in Figure 4. The trailing effect shown gravimetrically was duplicated by the radioactivity measurements; it is believed that this effect is due to fractionation within the class. The absence of free fatty acids was demonstrated by partition of the triglyceride fraction between hexane and 1 N potassium hydroxide solution; radioactivity was found only in hexane-soluble material.

An examination of fraction IV showed the presence of free fatty acids (1, 5). When the cholesterol fraction from the same column was partitioned between hexane and 1 N potassium hydroxide solution, about 60% of the radioactivity was associated with an alkali-soluble part of this fraction. When this free acid fraction was recombined with the cholesterol and the column separation repeated, a single peak again resulted. This does not indicate whether the free acids form molecular compounds with cholesterol, or whether they co-

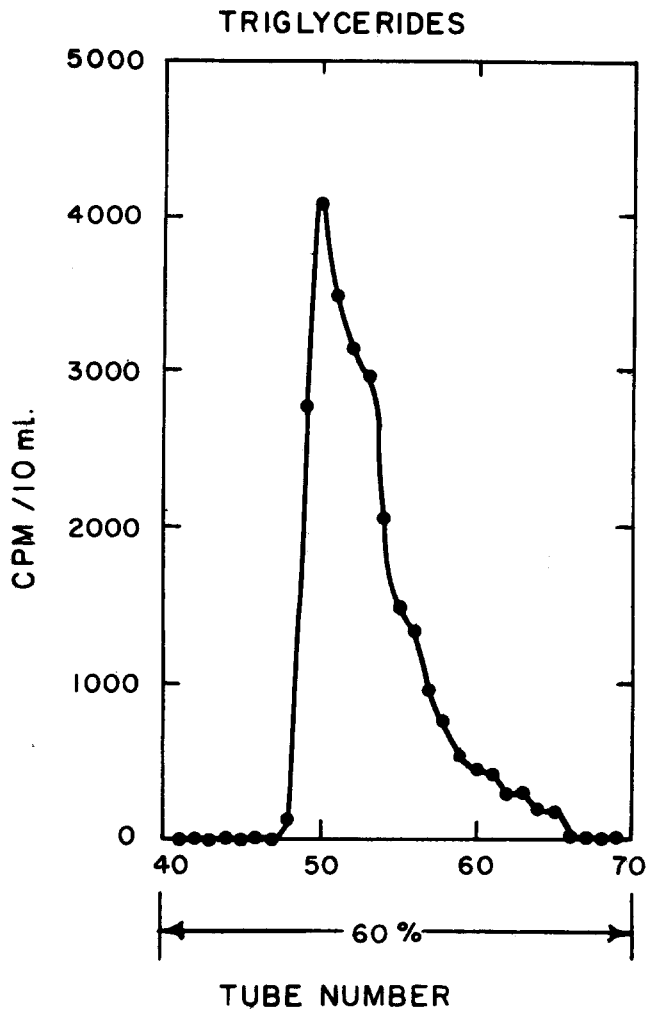

FIG. 4. Chromatographic separation of triglyceride fraction labeled with C^{14}. Leading and trailing tubes contained no radioactive material. No free acid was present.

chromatograph with the sterol. In confirmation of this behavior, a corresponding fraction from human plasma was examined by infrared spectroscopy (potassium bromide disk) and was found to contain strong carbonyl absorption characteristic of the carboxylic acid group. Since fraction IV was not of primary interest, no further characterization was attempted.

No attempt was made to determine the elution behavior of mono- and diglycerides, but they should follow cholesterol.

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