ISOLATION OF LIPIDS FROM PLASMA BY AFFINITY CHROMATOGRAPHY

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SUMMARY: Dodecylamine-agarose was used for the isolation of lipids from plasma by affinity chromatography. All of the major neutral lipids and phospholipids, commonly found after extraction by classical procedures, are recovered from plasma with this procedure. It was also shown that treatment of serum with dodecylamine-agarose quantitatively removed the lipoproteins. These preliminary data suggest that dodecylamine-agarose, with its broad specificity, may become a very useful tool for the extraction and fractionation of lipids and lipoproteins.

Presently lipids are separated from other plasma components by extraction with cholorforform-methanol or other organic solvents (1,2) while lipoproteins are fractionated by density gradient ultracentrifugation (3). We report here preliminary data showing that affinity chromatography, using dodecylamine-agarose, can be utilized for the separation of lipids and lipoproteins from plasma.

EXPERIMENTAL

Dodecylamine and 2,4,6,-trinitrobenzenesulfonic acid were obtained from Eastman Kodak. Agarose (Sepharose 4B) and cyanogen bromide were from Pharmacia and Matheson, Colman and Bell, respectively. Standard mixtures of lipids and thin layer plates were from Supelco, Inc. All solvents used for thin-layer chromatography were reagent grade obtained from J. T. Baker Chemical Company. Plates for agarose electrophoresis of lipoproteins were from Bio-Rad Laboratories.

Preparation of Dodecylamine-Agarose. One hundred ml of settled agarose was activated with 10 grams of cyanogen bromide by the method of Cuatrecasas et al (4). Six grams of dodecylamine, dissolved in 160 ml of ethanol, was added to a 150 ml suspension (0.3 M Na_2CO_3-HCl buffer, pH 10.2) of the acti-

vated agarose and the slurry was stirred for 24 hours at 5°C. The reaction mixture was warmed to room temperature, to solubilize any dodecylamine which precipitated in the cold, and washed with 6 liters of ethanol and then with 10 liters of water. Covalent attachment of dodecylamine to agarose was qualitatively shown by the 2,4,6-trinitrobenzenesulfonate reaction (5) which gave a stable orange color with the beads.

Treatment of Serum with Dodecylamine-Agarose. This initial experiment was conducted to determine if the affinity adsorbent could specifically remove lipid components from serum. Seven ml of fresh serum was stirred with 20 ml of settled dodecylamine-agarose (equivalent to about 8 grams of moist packed dodecylamine-agarose) for 30 minutes at room temperature. The serum was then separated from the affinity adsorbent by vacuum filtration through a 10 μ nylon net and saved. As a control, serum was treated in the same manner with lysine-agarose, an adsorbent used by Deutsch and Mertz (6) for the purification of plasminogen by affinity chromatography. Serum treated with dodecylamine-agarose and lysine-agarose in this manner was analyzed for total cholesterol, triglycerides and lipoproteins.

Isolation of Lipids from Plasma by Affinity Chromatography. Batch affinity chromatography procedures were used for the adsorption, washing and elution steps. In a typical experiment, 20 ml of settled dodecylamine-agarose and 20 ml of fresh citrated plasma were mixed for 30 minutes at room temperature. The agarose derivative was washed under vacuum on a Buchner funnel with 1 liter of 0.15 M NaCl, pH 7.5, and with 200 ml of distilled water. The moist dodecylamine-agarose cake was then stirred with 40 ml of ethanol in a beaker at room temperature for 10 minutes. The suspension was filtered and washed with an additional 40 ml of ethanol and the resulting 80 ml of eluate concentrated to 2 ml by evaporation under nitrogen. The precipitate found in the concentrate was removed by centrifugation. The same procedure was performed using chloroform-methanol (2:1) for elution. A control, using lysine-agarose, was run in parallel with all ex-

periments. The plasma lipids obtained by affinity chromatography were analyzed by thin-layer chromatography (TLC).

Analysis of Lipids by Thin-Layer Chromatography. The major classes of neutral lipids were separated by one-dimensional TLC using a hexane/ether/acetic acid solvent system 70/30/1 (7). Components were detected by spraying with dichromate-sulfuric acid (7). Standards were run in parallel with unknowns.

The major classes of phospholipids were identified by two-dimensional TLC as described by Turner and Rouser (8). Standards were run on the same plate as the unknown in each solvent system for identification of the affinity chromatography products.

RESULTS AND DISCUSSION

After treatment of serum with dodecylamine-agarose, approximately 50% of the triglycerides and nearly all of the cholesterol and lipoproteins are removed (Table 1). In this initial experiment serum was employed, instead of plasma, for compatability with some of the lipid assays. As expected, in the lysine-agarose treated serum, the cholesterol, triglycerides and lipoproteins do not deviate appreciably from normal serum. The small differences are probably attributable to the non-specific adsorption which is known to occur with agarose derivatives under these conditions (5). Incomplete removal of the triglycerides from the serum may be due to saturation of the dodecylamine derivative. Alternatively, the affinity adsorbent may have specificity for only certain types of triglycerides.

All of the major neutral lipid and phospholipid classes are isolated from plasma by affinity chromatography using dodecylamine-agarose. TLC analysis of the neutral lipids eluted from dodecylamine-agarose with ethanol or with chloroform-methanol indicates that the cholesterol esters, cholesterol, triglycerides, diglycerides, monoglycerides and free fatty acids are recovered (Figure 1). The use of chloroform-methanol for elution was discontinued, however, since the yields were lower and the beads assumed a

TABLE 1

CHOLESTEROL, TRIGLYCERIDES, AND LIPOPROTEINS IN NORMAL, LYSINE-AGAROSE, AND DODECYLAMINE-AGAROSE TREATED SERUM

Sample	Cholesterol[a] (mg/100ml)	Triglycerides[b] (mg/100ml)	Lipoproteins[c] Present
Normal serum	125-250[d]	74-174[d]	β, preβ, α
Lysine-agarose treated serum	164	62	β α
Dodecylamine-agarose treated serum	2	32	No stainable lipoproteins

[a]Total serum cholesterol was determined by the method of Levine and Zak (9) as modified by Claude et al (10).

[b]Triglycerides were assayed by the procedure of Kessler and Lederer (11).

[c]Lipoprotein analysis was performed using agarose gel electrophoresis according to the procedure of Noble (12).

[d]University of Colorado Medical Center, Central Laboratory's normal range.

gelatinous consistency. Two-dimensional TLC analysis of the phospholipids eluted with ethanol from the affinity adsorbent indicates that phosphatidyl choline and sphingomyelin are the major components with lysophosphatidyl choline and phosphatidyl ethanolamine present in smaller amounts (Figure 2). These data are consistent with that obtained by classical extraction of plasma which shows that phosphatidyl choline is the most abundant phospholipid followed by sphingomyelin and lysophosphatidyl choline (8).

One of the most interesting features of affinity chromatography with

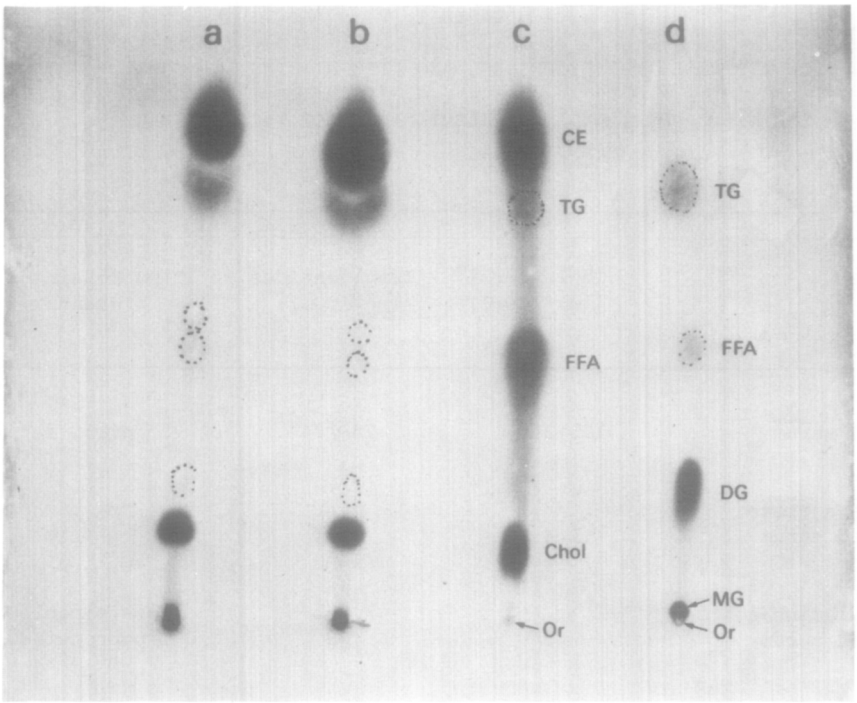

Fig. 1.

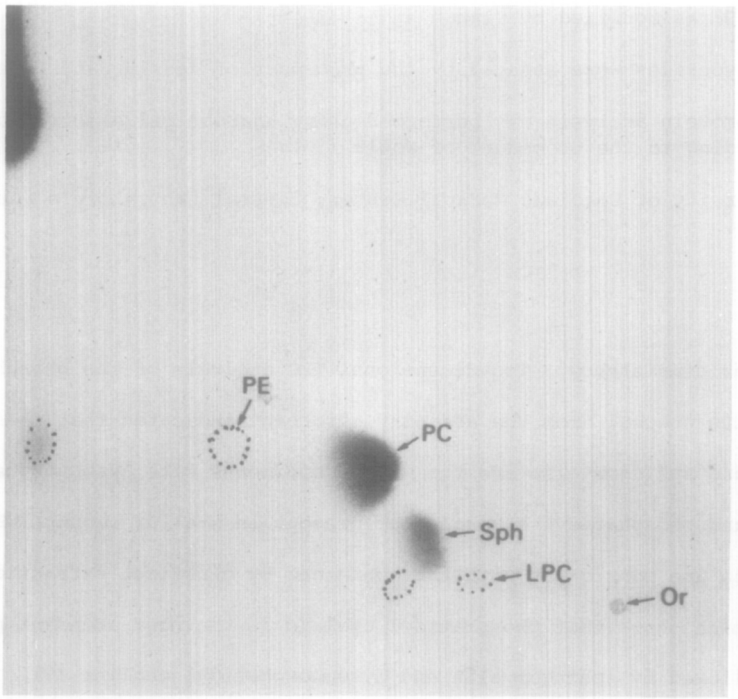

Fig. 2.

the dodecylamine-agarose adsorbent is the very broad specificity it exhibits. All of the major neutral lipids and major phospholipids as well as the main lipoprotein classes are sequestered from plasma by dodecylamine-agarose. Accordingly, this adsorbent should find immediate use for separation problems involving lipid and non-lipid materials, especially where organic extraction would be deleterious to enzymes of interest. Also, since lipoproteins are bound to dodecylamine-agarose, this adsorbent may find practical use in their fractionation from plasma as an alternative to density gradient centrifugation. This would be realized by using a gradient (with increasing concentrations of organic solvents or salts) for the elution of plasma lipoproteins from a dodecylamine-agarose column. In conclusion, these preliminary data suggest that dodecylamine-agarose may become a very useful method for the extraction and fractionation of lipids and lipoproteins.

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Figure 1. Thin-layer chromatography of neutral lipids prepared by affinity chromatography. TLC was performed as described in the "EXPERIMENTAL" section. a) Twenty microliters of the 2 ml ethanol eluate applied. b) Fifty microliters of the 2 ml chloroform-methanol eluate applied. c) Two hundred micrograms of each reference compound applied. d) Two hundred micrograms of total mixture applied. The eluate from the lysine-agarose control gave no detectable neutral lipids. Abbreviations: Or, origin; MG, monoglycerides; TG, triglycerides; DG, diglycerides; CE, cholesterol esters; Chol, cholesterol; FFA, free fatty acids.

Figure 2. Thin-layer chromatography of phospholipids prepared by affinity chromatography. Two-dimensional TLC was performed as described in the "EXPERIMENTAL" section. Twenty microliters of the 2 ml ethanol eluate was applied at the origin. The first solvent system was run from the bottom of the plate to the top and the second solvent system was run from right to left. The eluate from the lysine-agarose control gave no detectable phospholipids. Abbreviations: Or, origin; LPC, lysophosphatidyl choline; Sph, sphingomyelin; PC, phosphatidyl choline; PE, phosphatidyl ethanoamine.

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