Separation of lipids by Silica Gel G column chromatography

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SUMMARY A column chromatographic procedure utilizing Silica Gel G is described for separating lipid components of serum and lipoproteins into individual fractions containing hydrocarbons (I), cholesterol esters (II), triglycerides (III), cholesterol (IV), free fatty acids (V), and phospholipids (VI). Silica Gel G required no pretreatment except adjustment of moisture content to 10%. The method affords a rapid, complete separation of all major lipid classes except diglycerides. Recoveries of serum and tissue phospholipids were approximately 60–80%, whereas those of the other major lipid classes were essentially quantitative.

RESEARCH STUDIES under way in our laboratory required a rapid procedure whereby the lipid classes in human serum and lipoprotein fractions could be separated completely and obtained in adequate amounts for subsequent quantitative chemical and radioactivity analyses. One or more characteristics of the available methods utilizing silicic acid (1, 2) or Florisil (3) tended to preclude their use for this purpose, particularly when numerous samples were to be separated. A column chromatographic procedure utilizing Silica Gel G as adsorbent was therefore developed.

Adsorbent. Silica Gel G[1] (E. Merck, Darmstadt, West Germany) has the following specifications, according to the manufacturer: $CaSO_4 \cdot {}^1/_2H_2O$, 13%; chloride (maximum), 0.02%; iron (maximum), 0.015%; pH, 6.7 ± 0.3%; density, approximately 30 g/100 ml; grain size, 5–20 μ; drying loss after 3 hr. at 150°, 3.5 to 6%; and surface loss at 900° for 1 hr, 7.8 (average) to 9%. The Silica Gel G in each container was analyzed using an Ohaus moisture balance and sufficient water was added to obtain a moisture content of 10%. The contents of each container were shaken thoroughly, stored in tightly stoppered bottles, and used without further treatment.

Solvents. The following were used: petroleum ether (bp 30–75°, redistilled before use), Baker Chemicals; normal hexane (bp 40–60°), Phillips Petroleum Co.; methanol (redistilled before use), chloroform (redistilled before use), ethyl acetate (reagent grade), and glacial acetic acid (reagent grade), Fisher Scientific Co.; diethyl ether (USP), Mallinckrodt; and absolute ethanol, U.S. Industrial Chemical Co.

Column Preparation and Elution. Silica Gel G, suspended in hexane, was packed to a height of 5 cm in a 1.8 × 45 cm column. Applying slight air pressure during packing resulted in a uniform distribution of adsorbent, which was supported and covered by small glass wool plugs.

About 10–15 mg of lipid mixture per g of Silica Gel G was generally an optimal column load; when a markedly disproportionate quantity of one lipid class was present only 7–10 mg of lipid per g of adsorbent was applied.

The lipid components of whole serum or serum lipoproteins were extracted with chloroform–methanol 2:1 (v/v) (4) and added in 5–10 ml of petroleum ether to the adsorbent column. Separation into individual fractions containing hydrocarbons (I), cholesterol esters (II), triglycerides (III), cholesterol (IV), free fatty acids (V), and phospholipids (VI) was effected by serial elution with 50 ml of petroleum ether (I), 50 ml of 6% diethyl ether in petroleum ether (II), 150 ml of 10% ethyl acetate in petroleum ether (III, IV), 50 ml of diethyl ether (V), and 100 ml of methanol–acetic acid–water 8:1:1 (VI). Elution was carried out at room temperature under slight nitrogen pressure and the eluate was collected in 5- and 10-ml fractions. Good lipid resolution was markedly dependent on flow rates; between 1 and 2 ml/min gave best results.

"Bleeding" from the Silica Gel G columns was negligible in Fractions I through IV, and less than 10 mg in Fraction V. The extraneous material thus eluted did not interfere with any of the lipid assay procedures described.

Analyses. A 25 μl aliquot of each fraction eluted from the column was analyzed by Silica Gel G thin-layer chromatography (5) to identify lipid(s) present and evaluate completeness of separation. The chromatoplates were developed with a mixture of either 5% diethyl ether in petroleum ether or, for separating phospholipids, chloroform–methanol–water 65:25:4 (6). The chromatoplates were sprayed with 50% sulfuric acid and charred on a hot plate. Eluate fractions containing the same lipid class were combined, solvents evaporated in vacuo under nitrogen and the following analyses applied: hydrocarbons by weighing; cholesterol esters and cholesterol colorimetrically (7); phospholipids by determination of lipid phosphorus (8, 9); triglycerides, diglycerides, and monoglycerides by the method of Van Handel and Zilversmit (10); free fatty acids by extraction and titration (11).

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[1] Silica Gel G is distributed in the United States by Brinkmann Instruments, Inc., Great Neck, L.I., N.Y. and by Terra Chemicals, Inc., New York, N.Y.

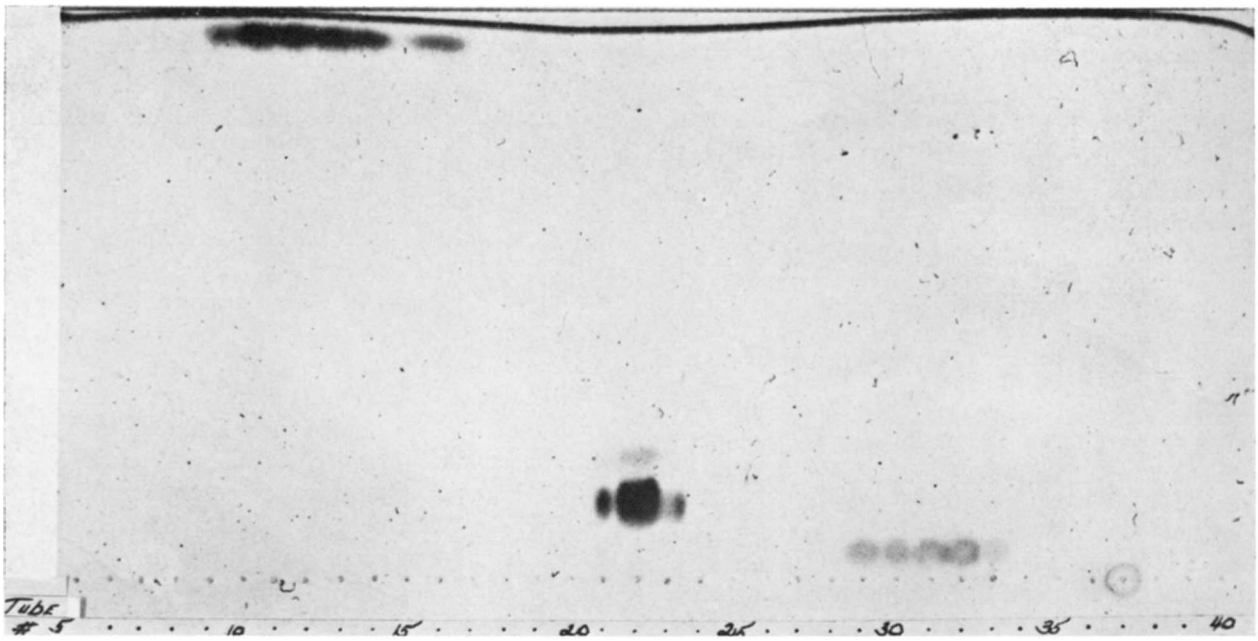

FIG. 1. Thin-layer chromatographic analysis of the eluates from a serum lipoprotein extract. A 25 μl aliquot of each eluate fraction was placed on the chromatoplate, developed with a mixture of 5% diethyl ether in petroleum ether, sprayed with 50% sulfuric acid, and charred on a hot plate to demonstrate any lipid material. Tubes 1–6 contained the 10-ml fractions eluted with petroleum ether; no hydrocarbons (I) were demonstrated. Tubes 7–17 contained the 5-ml fractions eluted with 6% diethyl ether in petroleum ether; cholesterol esters (II) were demonstrated in tubes 9–16. Tubes 18–34 contained the 10-ml fractions eluted with 10% ethyl acetate in petroleum ether; triglycerides (III) were demonstrated in tubes 21–23 and cholesterol (IV) in tubes 29–33. Tubes 35–40 contained the 10-ml fractions eluted with diethyl ether; free fatty acids (V) were present in tubes 36 and 37.

Separation of Serum and Tissue Lipids. The individual lipid classes were eluted from the column in distinct bands without trailing or overlapping (Fig. 1). Representative recoveries of non-phospholipids from serum and an artificial lipid mixture are presented in Table 1. Results obtained when a mixture containing cholesterol, monopalmitin, and 1,2-dipalmitin was eluted with 10% ethyl acetate in petroleum ether indicated that dipalmitin was eluted as a contaminant in the last several cholesterol fractions, whereas monopalmitin was obtained subsequently as a distinct fraction.

Column chromatographic separation with Silica Gel G has proved equally successful for separating tissue lipids, although good recoveries were obtained only for the "neutral" lipids. Representative recovery data for the lipids extracted from rat liver with an ethanol–petroleum ether mixture 10:8.5 (v/v) (12) are presented in Table 1.

TABLE 1 RECOVERY OF LIPIDS AFTER SILICA GEL G COLUMN CHROMATOGRAPHY

Sample	Cholesterol Esters (II)			Triglycerides (III)			Cholesterol (IV)			Free Fatty Acids (V)		
	Charge	Recovery		Charge	Recovery		Charge	Recovery		Charge	Recovery	
	mg	*mg*	*%*	*mg*	*mg*	*%*	*mg*	*mg*	*%*	*mg*	*mg*	*%*
Whole Serum "A"	13.7	13.00	94.9	5.8	5.3	91.4	5.3	4.9	92.5	1.74*	1.75	100.6
Whole Serum "B"	5.3	5.40	101.9	44.9	42.8	95.3	3.3	3.2	97.0	0.83*	0.79	95.2
Artificial Mixture†	6.0	5.80	96.7	7.9	8.1	102.5	18.7	17.3	92.5	—	—	
Palmitic Acid	0.0	0.00		0.0	0.0		0.0	0.0		7.57	7.75	102.4
Rat Liver	0.62	0.61	98.4	23.4	22.7	97.0	5.8	5.3	91.4	—	—	

— indicates no analyses were performed.
• Values are reported as equivalent milligrams of palmitic acid.
† Contained cholesterol stearate, tripalmitin and cholesterol.

No phospholipid contamination of the hydrocarbon fraction was observed when artificial lipid mixtures or samples of serum and liver were separated, in contradistinction to the reports of Lovern (13) and Riley and Nunn (14) that petroleum ether eluted phospholipids from the silica gel preparation they employed (British Drug Houses, Ltd.). An incomplete recovery of phospholipids extracted from serum and tissue was, however, repeatedly obtained. Even using the very polar mixture methanol–acetic acid–water 8:1:1, only 60–80% of the lipid phosphorus extracted from serum or tissue could be eluted. On the other hand, this mixture eluted cephalin and sphingomyelin, individually applied, almost quantitatively. Since in our studies the phospholipids are normally precipitated from the lipid extract by acetone, further investigation of this difficulty was not undertaken.

The Silica Gel G column chromatography procedure for separating serum and tissue lipids offers, in our hands, several advantages over established methods using silicic acid. The pretreatment procedure required with silicic acid is quite laborious and time consuming (1, 2). Silica Gel G, in contrast, requires no pretreatment except adjustment of moisture content to 10%. The degree of hydration is not as critical for Silica Gel G as for silicic acid; complete separation of the lipid classes was obtained when the moisture content was varied between 5 and 11%. Comparable results have been obtained using a number of different batches of Silica Gel G during a period of 18 months. Complete separation (as judged by thin-layer chromatography) of each major serum lipid class except diglycerides can be achieved, and the time required for column preparation and development is substantially less than for other adsorbents. The free fatty acid fraction obtained by this procedure is separated from other lipid fractions without the overlapping which has been observed with the silicic acid and Florisil methods and the eluting solvent does not interfere with subsequent titrimetric analyses (11), as obtained with the Florisil method (3).

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References

1. Hirsch, J., and E. H. Ahrens, Jr. *J. Biol. Chem.*, **233:** 311, 1958.
2. Horning, M. G., E. A. Williams, and E. C. Horning. *J. Lipid Res.*, **1:** 482, 1960.
3. Carroll, K. K. *J. Lipid Res.* **2:** 135, 1961.
4. Folch, J., I. Ascoli, M. Lees, J. A. Meath, and F. N. LeBaron. *J. Biol. Chem.* **191:** 833, 1951.
5. Mangold, H. K. *J. Am. Oil Chemists' Soc.* **38:** 708, 1961.
6. Wagner, H., L. Hörhammer, and P. Wolff. *Biochem. Z.* **334:** 175, 1961.
7. Sperry, W. M., and M. Webb. *J. Biol. Chem.* **187:** 97, 1950.
8. Fiske, C. H., and Y. Subbarow. *J. Biol. Chem.* **66:** 375, 1925.
9. Youngburg, G. E., and M. V. Youngburg. *J. Lab. Clin. Med.* **16:** 158, 1930.
10. Van Handel, E., and D. B. Zilversmit. *J. Lab. Clin. Med.* **50:** 152, 1957.
11. Dole, V. P. *J. Clin. Invest.* **35:** 150, 1956.
12. Alaupovic, P., B. C. Johnson, Q. Crider, H. N. Bhagavan, and B. J. Johnson. *Am. J. Clin. Nutr.* **9** (Part II): 76, 1961.
13. Lovern, J. A. *Biochem. J.* **63:** 373, 1956.
14. Riley, C., and R. F. Nunn. *Biochem. J.* **74:** 56, 1960.