CHROM. 6055

The separation of small amounts of free fatty acids from large amounts of neutral fats on Sephadex LH-20

Thin-layer chromatography is an excellent method for the separation of lipid classes. In adipose tissue, however, difficulties arise if it is required to isolate the minor fractions from the large bulk of the triglycerides. In particular, the free fatty acids (FFA) comprise such a minor fraction, representing a fraction of only $5 \cdot 10^{-2}$ to 10^{-3} of the total amount of triglycerides. Therefore, it would be necessary to overload the thin-layer plates with respect to triglycerides, in order to obtain a sufficient amount of the smallest fractions for further investigation. Separation of lipids by means of column chromatography on silicic acid is time-consuming and, moreover, gives an incomplete resolution of FFA and triglyceride peaks. Otherwise, the separation of total phospholipids from the other fractions by using a small silicic acid column is a common and simple method. Therefore, the critical factor is the quantitative isolation of the FFA from the bulk of neutral fats. ADDISON AND ACKMAN[1] showed that chromatography on Sephadex LH-20 is a suitable technique for the separation of FFA from other lipids. We adapted the method to analytical problems in the study of adipose tissue lipids, and proved the efficiency of the method by using radioactive test mixtures. The result is a standardized technique for an excellent separation of small amounts of FFA from large amounts of neutral fat (up to 300 mg) within 3 h. The recoveries of FFA and neutral fat are quantitative.

Methods

Preparation of the Sephadex-column. Sephadex LH-20 (35 g for 140 ml of gel bed) was allowed to swell overnight in chloroform containing 0.2 % (v/v) of glacial acetic acid (elution solvent). The slurry was poured in several steps into a glass

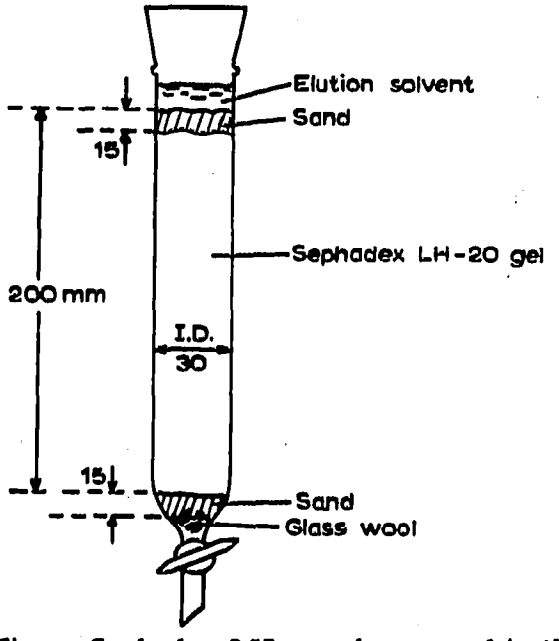

Fig. 1. Sephadex LH-20 column used in the experiments described. (All dimensions are in mm.)

column (I.D. 3 cm) fitted with a plug of glass-wool. This plug was covered with a 1.5-cm thick layer of washed sand (Fig. 1). Because the gel floats in chloroform, the lower chloroform layer was allowed to run off the column until a suitable gel bed had formed. Care was taken to ensure that the column did not run dry. A second layer of sand was placed on top of the gel bed in order to stabilize it. The column was pre-washed with 300–400 ml of elution solvent. The column was then ready for use.

We separated up to 32 mg of FFA from up to 300 mg of neutral fat on such a column. The capacity of the column was not exhausted by these loadings.

Chromatographic procedure. The sample was applied in a small volume of elution solvent. After the sample had moved into the gel at a suitable flow-rate, the surface was rinsed twice with a 4-ml portion of elution solvent.

The elution of neutral fats (fraction I) was carried out with 100–150 ml of solvent. After this, the FFA were eluted with a further 300 ml of the same solvent (fraction II).

The flow-rate, caused by gravity only, was maintained at 4 ml/3 min. After the elution of fraction II, the column can be re-used and up to 20 runs without any re-packing of the gel are possible. To exclude any contamination, the column was washed with a 300-ml portion of solvent before every run.

Although the elution pattern was reproducible, the end of peak I can be made visible by spotting small amounts of the effluent onto a thin-layer plate. In this instance we used the following elution scheme:

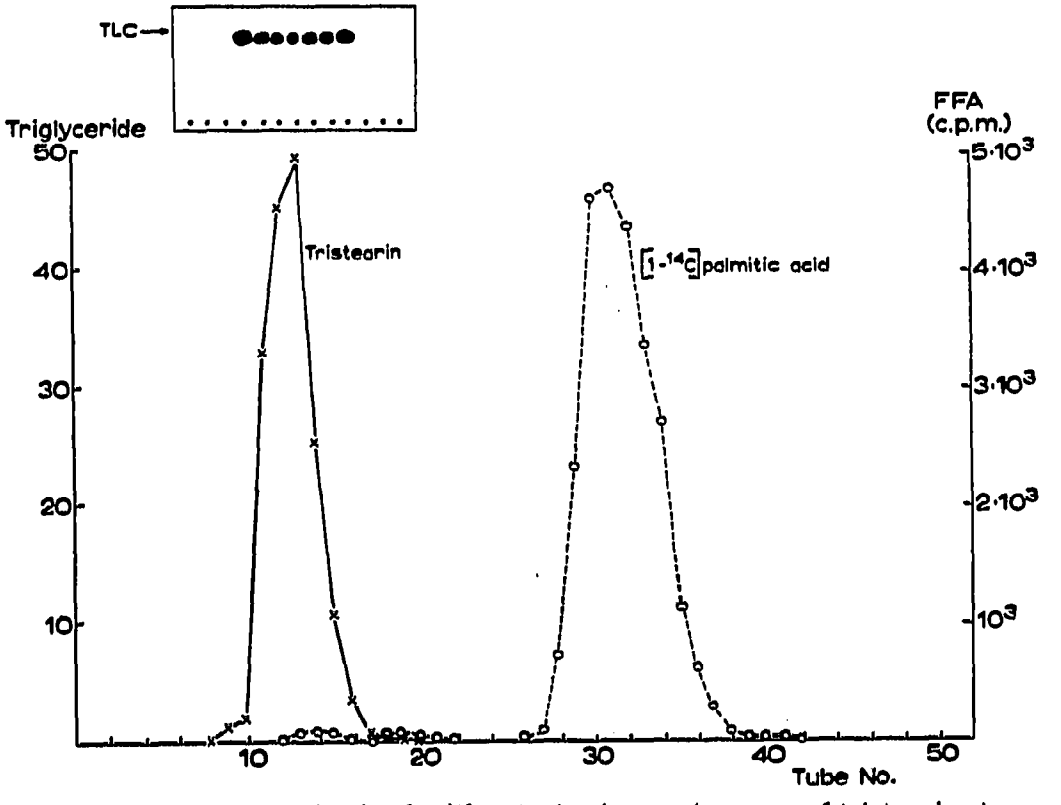

Fig. 2. Elution curve obtained with a test mixture (174 mg of tristearin + 20 mg of palmitic acid, containing $2.3 \cdot 10^6$ c.p.m. of [1-^{14}C]palmitic acid); 4-ml fractions; 35 g of Sephadex LH-20. Elution solvent: chloroform containing 0.2% (v/v) of glacial acetic acid. I.D. of column 30 mm; flow-rate 4 ml per 3 min. ×—×, tristearin; o---o, ^{14}C-activity.

(*a*) 60 ml of eluate (main fraction I);
(*b*) 10 × 4 ml of eluate in ten tubes;
(*c*) 300 ml of eluate (main fraction II).

About 0.02 ml (3 drops) from each of the tubes was spotted onto a thin-layer plate loaded with Kieselgel G (Merck). After development in dichloroethane (the solvent front moved 8 cm), the plate was allowed to dry in air. The lipids were made visible by spraying with phosphomolybdic acid (5 % solution in ethanol) and heating the plate at 180° for 15 min. The end of peak I is clearly demonstrated by the decreasing intensity of the triglyceride spots (Fig. 2). Three tubes, following the main fraction I, were added to the latter; all further tubes were pooled with the main fraction II.

Analytical methods. Triglycerides were determined by the chromotropic acid method[2]. Free fatty acids were measured by the method of NOVÁK[3].

Radioactivity was measured in a liquid scintillation counter (Packard Tri-Carb, Model 2100).

Results

Fig. 2 shows a typical elution curve. The column was run with a test mixture of 174 mg of tristearin and 20 mg of palmitic acid containing [1-^{14}C]palmitic acid ($23 \cdot 10^5$ c.p.m.).

Table I shows the recoveries in experiments with various test mixtures. Quantitative recoveries were found for both mass and radioactivity. The two peaks, identical with fractions I and II, were separated completely, and no tailing occurred.

Discussion

The method described has been used successfully in our laboratory for investigations on brown and white adipose tissue.

A further separation of the compounds of fraction I, derived from natural mixtures (mono-, di- and triglycerides, free and esterified cholesterol), by means of thin-layer chromatography is limited, but is not impossible. A suitable combination of Sephadex and thin-layer chromatography, therefore, was useful in the isola-

TABLE I

RECOVERIES OF TRIGLYCERIDE AND FFA IN TEST MIXTURES

Substance	*Amount applied*		*Recovery* (%)	
	mg	*c.p.m.*	*Mass*	*Activity*
Triglyceride	172		98.6	
	207		99.6	
	20		92.0	
FFA		$23 \cdot 10^5$		109
		$7.7 \cdot 10^5$		96
	29		99	
	32		98	
	1		103	
	1		101	

tion and determination of most of the lipid fractions of adipose tissue. The phospholipids can be separated first on a small silicic acid column.

The reproduction of the elution curve in Sephadex chromatography was excellent. The elution volumes are nearly independent of the amounts to be separated. Care was taken to maintain standard operating conditions. The distance between the end of peak I and the beginning of peak II is adequate with at least 30 ml elution solvent. Therefore, complete resolution of the fractions is guaranteed in all instances.

In our test experiments, we always found 2–4 % of applied radioactivity, due to FFA, within the neutral fats (peak I). We are not able to explain this phenomenon. It is possible that physicochemical effects, such as the formation of micelles or aggregation, play a role, and may contribute to the elution of traces of FFA together with neutral fats. This effect, however, is without any consequence and did not interfere with the results in any way.

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Department of Pathological Physiology,
Friedrich Schiller University,
Jena (G.D.R.)

L. WINKLER
T. HEIM*
H. SCHENK
B. SCHLAG
E. GOETZE

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* Stipendiate of the Ministry of Health of Hungary, Department of Pediatrics, University of Pecs, Hungary.