Quantification of adipocyte free and esterified cholesterol using liquid gel chromatography

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Summary A reliable method for the separation of free and esterified cholesterol in adipocyte extracts is described. The procedure uses Sephadex LH-20, a lipophilic dextran gel, with a solvent system of chloroform–hexane 55:45 (v/v). Interference by excess triglyceride, such as that encountered in adipocyte total lipid extracts, was not observed, and overall recovery of both sterols exceeded 98%.

Supplementary key words cholesteryl stearate · adipocyte cholesterol storage · cell size

It is now well recognized that adipose tissue contains a significant portion of total body cholesterol in most animal species including man. In the last several years, however, attempts to quantitate the amounts of free and esterified cholesterol in isolated adipocytes have been contradictory. In this regard, values of 96% (1), 90% (2), and 76% (3) have been reported for the percentage of free cellular cholesterol. During the course of studies designed to elucidate the possible regulatory determinants of adipocyte cholesterol storage (4), we found that several conventional methods, although successfully employed for plasma samples, were not well suited for the purpose of separating the free and esterified fractions contained within isolated fat cells. The inadequacies of these methods were due to interference by the large quantities of triglyceride present in adipocyte extracts, quantities which exceed that of cholesterol by about 3 orders of magnitude (1000:1). For example, incomplete precipitation of free sterol was encountered in the digitonin procedure (5), confirming the work of others (3). Separation of the free and esterified cholesterol was also attempted using thin-layer chromatography (TLC) on silica plates of several thicknesses with a variety of solvent systems. Both sterol fractions, however, tended to cochromatograph to variable extents with triglyceride. This was due to the large excess of triglyceride in the cell extracts, which had to be spotted in order to obtain enough cholesterol for colorimetric analysis. Such "smearing" invariably interfered with the separation of free and esterified cholesterol. An enzymatic approach to the problem was also unsuccessful. In this procedure, solubilization of adipocyte extracts in aqueous solution containing various surfactants resulted in turbidity which interfered with the cholesterol oxidase procedure (6). In additional attempts to remove the excess lipid, triglyceride lipase was employed (7) prior to TLC. This procedure, however, proved to be tedious, variable, and expensive. We now report the successful use of column chromatography using the lipophilic dextran gel Sephadex LH-20 (8, 9) which provides excellent and dependable separation of free and esterified cholesterol in extracts derived from rat adipocytes. A preliminary report has appeared (10).

Methods and materials

All solvents and chemicals were analytical reagent grade. Sephadex LH-20, crystalline cholesterol, and cholesteryl stearate were purchased from Sigma Chemical Company, St. Louis, MO. Collagenase was the product of Worthington Biochemical Corporation, Freehold, NJ. The glass chromatography column and Teflon fittings were purchased from Lab-Crest Scientific Division, Warminster, PA. Corn oil was obtained through local food suppliers.

Abbreviation: TLC, thin-layer chromatography.

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In the present procedure 20 g of Sephadex beads were allowed to swell overnight in a mixture of chloroform–hexane 55:45 (v/v). After removing fine particles by elutriation (11), a slurry was gently added to the column (15 × 600 mm) and allowed to settle until a bed height of 38 cm was obtained. The solvent contained in the reservoir was connected to the top of the column in a constant stream by means of Teflon tubing and fittings. The height of the column was adjusted such that an eluant flow rate of 2.1–2.4 ml/min was achieved. These conditions provided consistent and reproducible separations.

In order to initially test and calibrate the column, a solution containing 25 g of corn oil and 12 mg each of cholesterol and cholesteryl stearate per 100 ml of chloroform–hexane was prepared. Such concentrations simulate the proportions of these lipids in adipocyte extracts (3, 4). Preliminary elution order and volume were estimated by TLC using appropriate standards, but quantitatively these parameters were determined by placing 0.5 ml of the sandard solution on the column and collecting 20 fractions of 6.0 ml each. Fractions were then dried under nitrogen and either were analyzed for triglyceride by the method of Levy (13) or were saponified at 60°C for 2 hr in 2% ethanolic KOH (12). After saponification, samples were extracted twice with light petroleum ether, washed with water, and taken to dryness. Cholesterol content was then determined by a micro-modification of the original colorimetric procedure of Zak et al. (14). Hydrolysis was necessary since it is known that fat cell triglyceride interfers with direct cholesterol measurements (3, 4).

Results and discussion

The elution curve derived from analyzing the corn oil standard is shown in **Fig. 1.** Esterified cholesterol is eluted in a single zone near the beginning of the profile, whereas the free sterol is retarded in its elution. The bulk of triglyceride eluted with cholesteryl ester, but significant amounts also appeared in the free cholesterol fractions. Triglyceride of adipocyte extracts eluted in a manner similar to that of the corn oil standard. In practice the column is not subjected to the large amounts of lipid depicted here. More triglyceride and therefore more sterol was used in the calibration, first to test the capacity of this system to fractionate large amounts of lipid without overlap or interference, and second, to compare the elution characteristics of this simulated adipocyte extract with that of an actual cell extract (see below). Recovery of the combined free and esterified cholesterol in corn oil solution averaged 92.7 ± 3.1% (mean ± SE, n = 10) when compared to an independent total cholesterol determination.

Two groups of male Sprague-Dawley rats were used in the present report to check recoveries from fat cell extracts and to determine if age affected the levels of cellular free and esterified cholesterol. The first group consisted of six fasted animals weighing an average of 503.1 ± 15.3 g (25 wks of age) (mean ± SE). Isolated fat cells were prepared from epididymal and

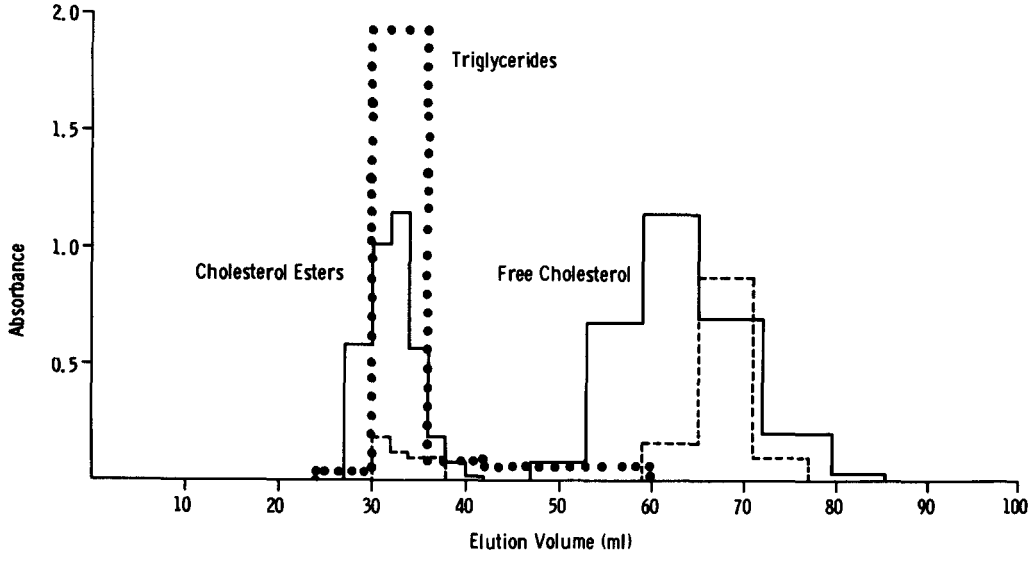

Fig. 1. Separation of cholesterol, cholesteryl ester, and triglyceride by Sephadex LH-20 gel chromatography. The elution profile of corn oil triglycerides (· · · · · ·) was determined by absorbance at 410 nm by the method of Levy (13). After saponification, free cholesterol and cholesteryl ester were measured at 560 nm (14) in both the corn oil–sterol standard solution (———) and in a representative adipocyte total lipid extract (– – – – – –). The eluting solvent was chloroform–hexane 55:45 (v/v) with a flow rate of 2.1–2.4 ml/min.

TABLE 1. Recovery of free and esterified cholesterol in adipocyte extracts

Adipocyte Cholesterol		Standard Added	% Recovery	
Free	Esterified		Free	Ester
μg				
203.6 (86.9–556.4)	133.8 (38.7–471.6)	Free	99.8 ± 3.8 (5)	
231.1 (116.2–561.6)	150.9 (40.9–470.4)	Ester		98.4 ± 3.7 (4)
96.0 (44.5–127.4)	39.6 (26.5–46.7)	Free + Ester	97.4 ± 3.5 (3)	97.9 ± 6.8 (3)
		Overall Recovery	98.9 ± 2.4 (8)	98.2 ± 3.2 (7)

Isolated adipocytes were prepared from the epididymal and perirenal depots of rats that weighed about 500 g. Total lipid extracts were prepared in isopropanol from aliquots of the cell suspensions such that a wide range of cell numbers was obtained. On the left is shown the average amount of free or esterified cholesterol chromatographed, with the range of values given in parentheses. To each lipid extract was added either 100 μg of crystalline cholesterol or 200 μg of cholesterol stearate or both. The percent recovery was determined by dividing the amount of sterol recovered after separation by the amount of sterol (cellular plus standard) placed on the column (×100). Numbers in parentheses indicate the number of lipid extracts tested for recovery. Data are reported as mean ± SE.

perirenal tissues by the method of Rodbell (15). From the washed cell suspensions numerous isopropanol–Zeolite adipocyte extracts (4, 16) were prepared with various triglyceride contents for recovery studies. Each fat cell extract was split into two equal portions and to one was added either 100 μg of crystalline cholesterol, 200 μg of cholesteryl stearate, or a combination of both. Aliquots of both portions were chromatographed. Recovery of added standard was determined as the difference in cholesterol in each fraction between these extracts with added standard and those without. A second group of 12 rats averaging 175 ± 2.0 g (6–8 wks of age), was killed and extracts were prepared from only isolated epididymal adipocytes as above to determine whether age affected the distribution of cellular free and esterified cholesterol. A previous study from this laboratory (4) has shown that total cellular cholesterol increases about 4-fold between these ages. In the small animals it was necessary to pool tissue from several animals in order to obtain sufficient lipid for cholesterol analyses.

TABLE 2. Effect of age on percent of total adipocyte cholesterol as ester

Age	Depot	Percent Ester
6–8 wk. (175 g)	Epi	29.6 ± 1.5 (5)
	PR	
25 wk. (503 g)	Epi	29.5 ± 2.4 (6)
	PR	30.0 ± 1.9 (6)

Adipocytes were prepared from either the epididymal or the perirenal depot. Lipid extracts and cellular free and esterified cholesterol were determined as described in the previous table. Numbers in parentheses indicate the number of animals in each group. Data are reported as mean ± SE. Data from the 25-wk-old rats is derived from data also shown in Table 1.

The elution curve derived from a typical fat cell extract is compared to the corn oil curve in **Fig. 1.** It can be seen that its behavior is similar to the corn oil–sterol standard profile. When known amounts of both cholesterol and cholesteryl stearate were added to adipocyte extracts from the first group of rats, it was found that recovery exceeded 97% for both fractions (**Table 1**).

Table 2 shows that in these older rats the percentage of total cellular cholesterol in the esterified form averaged 29.5 ± 2.4 for epididymal cells and 30.0 ± 1.9 for perirenal (mean ± SE). Thus, anatomical location did not affect the free–ester distribution in these large animals. In the younger group of rats it was found that the percentage of total cholesterol as cholesteryl ester was 29.6 ± 1.5 (Table 2). The data from both groups of rats agree most closely with those reported by Farkas, Angel, and Avigan (3) who used an indirect method for the determination of free and esterified adipocyte cholesterol content. Discrepancies with other investigators are probably due in large part to the utilization of methods that do not allow quantitative recovery of sterols in the presence of adipocyte triglyceride. Other factors cannot be disregarded, however, such as variation in the extent of collagenase treatment of tissues or variation among different strains of rats. Also, from the data reported above, there does not appear to be any difference in the ratio between epididymal and perirenal depots in adult animals. It is possible, however, that other conditions or factors may change or regulate the free–ester balance in adipocytes. In this regard, recent studies from our laboratory (16) have shown that Sprague-Dawley rats of comparable body weight fed semipurified diets from weaning until adulthood have an increased percentage of esters

(36%) compared to the chow-fed animals reported in Table 2. In addition, increasing the dietary load of cholesterol in at least one strain of rat (Fisher 344) increases both the total adipocyte cholesterol (6-fold) and the percentage that exists in the ester form (32–48%) (18). Such findings suggest possible physiological roles for the cholesteryl esters in adipose tissue which warrant further study.

In our hands this method has proved to be more reliable and reproducible than other methods attempted. Because of the problems encountered in trying to quantitate adipocyte free and esterified cholesterol levels, this method may prove to be the method of choice for studies regarding the function and turnover of these fractions in adipose tissue.

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