Rapid method for determining free and esterified cholesterol in plasma extracts

BENJAMIN B. ZEITMAN

Physiology Branch, Environmental Biology Division, NASA, Ames Research Center, Moffett Field, California

SUMMARY A method has been developed for the quantitative measurement of free and esterified cholesterol in extracts

of rat plasma after separation by thin-layer chromatography. As little as 0.2 ml of plasma may be used in the determinations.

KEY WORDS cholesterol · free · esterified · determination · plasma · rat · thin-layer chromatography · fluorometry

FREE AND ESTERIFIED CHOLESTEROL have been determined after separation by column chromatography (1) or digitonin precipitation (2) utilizing the Liebermann-Burchard color reaction (3, 4). Albers and Lowry (5) developed a fluorometric method sensitive to 0.1 μg for determining cholesterol. The following method utilizes the speed and high resolution of thin-layer chromatography (TLC) for separation and the sensitivity of fluorometry for quantification. It has been applied to rat plasma only.

Materials. All chemicals were reagent grade. Diethyl ether, *n*-hexane, and chloroform were redistilled over $FeSO_4$ and stored under nitrogen. Anhydrous methanol (J. T. Baker Chemical Co., Phillipsburg, N. J.) was used without further purification. Cholesterol (California Corporation for Biochemical Research, Los Angeles, Calif.) was twice recrystallized with absolute ethanol and dried to constant weight under vacuum. Cholesteryl palmitate, >99% pure (Applied Science Laboratories, State College, Pa.), was used without further purification. The 2,7-dichlorofluorescein reagent consisted of 400 mg of 2,7-dichlorofluorescein (Eastman Organic Chemicals, Rochester, N. Y.) per liter of 50% ethanol.

Extraction. Albrink (6) has described the quantitative extraction of plasma samples with chloroform–methanol 2:1 (v/v). In contrast to her method, a minimum of 0.2 ml of plasma extracted with 4.0 ml of chloroform–methanol was sufficient for determining total cholesterol and the ratio of free to esterified cholesterol. Other lipid constituents may be determined on the same extract if the volume of plasma and solvent is increased. The results presented in this paper were obtained on 1.0 ml plasma samples extracted with 20.0 ml of chloroform–methanol.

The samples were extracted for 1 hr in 50-ml round bottom centrifuge tubes, which were agitated with a gentle horizontal motion on a shaking machine. Distilled water (20 ml) was then added down the side of the tubes. The tubes were capped and allowed to stand overnight in the refrigerator.

The extraction mixture separated into a lower chloroform phase and an upper aqueous phase. The precipitated proteins concentrated as a disk between the two clear phases. A pipette attached to a syringe was carefully passed down the side of the tube through the aqueous phase and protein disk to remove the chloroform phase. A 0.2 ml aliquot of the extract was taken for total cholesterol assay; 1.0 ml was used for determining free and esterified cholesterol. A reagent blank containing 21.0 ml of distilled water and 20.0 ml of chloroform–methanol was carried along with the plasma samples.

A standard mixture of free and esterified cholesterol was prepared which contained amounts similar to those in extracts of rat plasma. Aliquots containing 30–45 μg of cholesterol were used for both direct total cholesterol determinations and for TLC of free and esterified cholesterol.

Thin-Layer Chromatography. Thin-layer plates (20 × 20 cm) were coated with a layer of Silica Gel G (Merck AG, Darmstadt, Germany) containing 2,7-dichlorofluorescein. Silica Gel G (25 g) was added to a mixture of 30 ml of dichlorofluorescein reagent and 20 ml of distilled water. Thin-layer plates of about 250 μ in thickness were prepared and dried for at least 2 hr at 110°. The plates were stored at this temperature until needed.

The 1.0 ml aliquot containing free and esterified cholesterol was evaporated to about 0.1 ml in a vacuum desiccator equipped with a 40° sand bath maintained by a heating mantle. The desiccator was evacuated by a water aspirator. The concentrated samples were quantitatively transferred to thin-layer plates with three 50-μl portions of chloroform. Care was taken to keep the spots less than 6 mm in diameter. One reagent blank, one standard, and four plasma samples were spotted on each plate.

A solvent system of petroleum ether–diethyl ether–acetic acid 90:10:1 (v/v/v) was used. Development to 150 mm was achieved in 50 min. The plates were removed from the solvent tank and air-dried for 5 min. Lipid spots were detected with an ultraviolet lamp and outlined with a pencil. Regions of appropriate R_F and with areas corresponding to those of the free and esterified cholesterol spots were employed as reagent blanks. A microscope slide was used to scrape the silica gel into 15-ml glass-stoppered conical centrifuge tubes. Four milliliters of diethylether–*n*-hexane 5:95 (v/v) was added to each tube. The samples were extracted for 2 min and centrifuged for 5 min at 1500 rpm. The clear solvent was carefully decanted into a 15 ml centrifuge tube. The extraction was repeated twice with 3- and 2-ml portions of solvent. The combined extracts were evaporated just to dryness with a gentle stream of nitrogen. The aliquots for total cholesterol were also evaporated to dryness at this point.

Fluorometry. A Turner Model 110 fluorometer (G. K. Turner Associates, Palo Alto, Calif.) was equipped with an ultraviolet lamp (General Electric No. F4T4/BL) and a Model 110-655 door. Primary filters numbers 1–60 and 58 (546 mμ excitation) and secondary filter number 23A (570 mμ) were used. The fluorescence was

TABLE 1 MEAN RECOVERY OF FREE AND ESTERIFIED CHOLESTEROL STANDARDS AFTER TLC

Sample	Number of Determinations	Known*	Found ± SEM	Recovery
		μg	μg	%
Mixture	11			
Cholesterol and cholesteryl palmitate		8.97†	8.69 ± 0.1	96.9
		22.6‡	21.4 ± 0.4	94.7
Cholesterol	6	38.4	37.0 ± 0.2	96.2
Cholesteryl palmitate	6	40.0	39.0 ± 0.1	97.5

* The known values for cholesterol were based on the weights of standards on a microbalance.
† Free cholesterol.
‡ Esterified cholesterol.

measured at 10× sensitivity in 12 × 75 mm Pyrex tubes.

The cholesterol fluorescence was developed as described in the Turner Manual of Fluorometric Clinical Procedures (7). A blank tube and three aliquots of the standard mixture served as instrument blank and reference standards, respectively. All blanks, standards, and unknowns were treated in the following manner:

(*a*) Pipette 0.2 ml of acetic acid–chloroform 3:2 (v/v) and mix.

(*b*) Add 5.0 ml of chloroform–acetic anhydride 10:3 (v/v) and mix.

(*c*) Add 0.2 ml of concentrated sulfuric acid and mix immediately.

The fluorescence was read between 35 and 50 min after the addition of sulfuric acid. The fluorometer was zeroed with the instrument reagent blank tube. "Repipets" (automatic dispensing pipettes, Labindustries, Berkeley, Calif.) were used in steps (*b*) and (*c*). A Vortex Junior mixer (Scientific Industries, Inc., Queens Village, New York) was used for rapid mixing.

The dichlorofluorescein dye was not extracted by the ether–hexane mixture and did not interfere with the fluorescence measurement. Typical readings for the reagent blanks for the free and esterified cholesterol areas were 1.0 and 2.5% of full scale, respectively. Forty micrograms of cholesterol gave a reading of approximately 79% of full scale under the operating conditions described.

Results and Discussion. Recovery experiments were performed on replicates of free, esterified, and mixed standards. The recovery of standards from thin-layer plates was found to be >95% (Table 1).

Extracts of six different rat plasma samples were analyzed directly for total cholesterol and, after thin-layer separation, for free and esterified cholesterol. The sum of the free and esterified cholesterol values was compared with that of the total cholesterol to determine the thin-layer chromatographic recovery; the mean recovery was found to be 96.0%. The method was also found to be satisfactory for determining free and esterified cholesterol from chloroform extracts of liver and adrenal glands.

The mean percentage of plasma cholesterol that was unesterified was determined on extracts from six female Sprague-Dawley rats which had been fasted 24 hr. It was found to be 36.6 ± 0.4% of the total cholesterol.

The results presented indicate that free and esterified cholesterol separated by TLC can be quantitiatively determined by fluorometric means. The speed of separation and the relative simplicity of the operations employed are distinct advantages over tedious column chromatographic or precipitation methods previously described.

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