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Notes on Methodology

Rapid ultramicro estimation of serum total cholesterol*

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SUMMARY

An ultramicro cholesterol technique based upon a new color reaction and requiring only 10 μl. of serum is proposed. The need for extraction with lipid solvent is eliminated by drying serum on filter paper segments and eluting directly in the ferrous sulfate color reagent. This method has been successfully applied to sera obtained from 200 blood donors.

► In 1934 Schoenheimer and Sperry (1) described a microcholesterol method using the Liebermann-Burchard reaction which required 0.2 ml. of serum. Subsequently there has been a tendency toward the use of smaller blood specimens for cholesterol analysis. Recently Galloway *et al.* (2) modified the macro method of Sperry and Webb (3) to measure cholesterol in as little as 40 μl. of serum. Zlatkis *et al.* (4) introduced a new ferric chloride-cholesterol color reaction which is also sufficiently sensitive for estimation of this lipid fraction in small quantities of serum. A simple micro technique based on this color reaction which employs only 0.1 ml. of serum has been described by Zak (5). Rosenthal and Jud (6) have further modified this procedure for use with 50 μl. of serum. It is the purpose of this report to describe a method for cholesterol quantitation which utilizes even smaller amounts of blood. The ferrous sulfate color reaction previously described by Searcy and Bergquist (7) has been applied to 10 μl. of serum. This makes possible accurate quantitation of cholesterol on ultramicro quantities of serum, thereby conserving blood for additional laboratory determinations.

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METHODS

Measurement of Serum Total Cholesterol. An aliquot of 10 μl. of serum is quantitatively applied to a 2 cm. square of lipid-free filter paper (Whatman #1). To prevent serum loss during the drying process, the filter paper segments are supported by two glass stirring rods. Depending on room temperature and humidity, a 10-to-15-minute period is usually sufficient to dry serum spots. The filter paper segments are folded and placed in the bottom of small test tubes containing exactly 2.0 ml. of a saturated solution of ferrous sulfate in glacial acetic acid. The total cholesterol is eluted by allowing the serum-spotted strips to remain in the color reagent for at least 1 hour. Exactly 1.5 ml. of the cholesterol-ferrous sulfate reagent mixture, which represents 7.5 μl. of serum, is then transferred to a Coleman microcuvette (10 × 75 mm.) and mixed with 0.5 ml. of concentrated sulfuric acid. A small stirring rod may be used to effect uniformity. The salmon-pink color is allowed to develop for at least 10 minutes. The tubes are placed in a microcuvette adapter and the optical densities are measured at 490 mμ. against a reagent blank with a Coleman Junior spectrophotometer. The reagent blank is prepared by eluting unspotted filter paper with the color reagent. The standard cholesterol is prepared in the ferrous sulfate color reagent.

RESULTS AND DISCUSSION

Color Production with Increasing Cholesterol Concentrations. Amounts of cholesterol ranging from 10 to 60 μg. in 1.5 ml. of ferrous sulfate color reagent were mixed with 0.5 ml. of concentrated sulfuric acid. After 10 minutes the color intensity was measured against a reagent blank. Figure 1 demonstrates that the chromogenic response followed Beer's law with the cholesterol concentrations employed. The sensitivity of the color reaction makes practical the quantitation of total cholesterol in as little as 10 μl. of serum.

Serum Cholesterol Extraction Efficiency. Aliquots of 10 μl. of a pooled serum were applied to filter paper and placed in the ferrous sulfate color reagent or in chloroform-methanol (2:1, v/v) for 1 to 60 minutes. The amount of cholesterol was quantitated directly in the ferrous sulfate extracts but the chloroform-methanol eluates were taken to dryness prior to treatment with the color reagent. Figure 2 demonstrates that in 6 minutes each mixture contained some 60 per cent of the total cholesterol. However, with the glacial acetic acid reagent, extraction proceeds more rapidly after

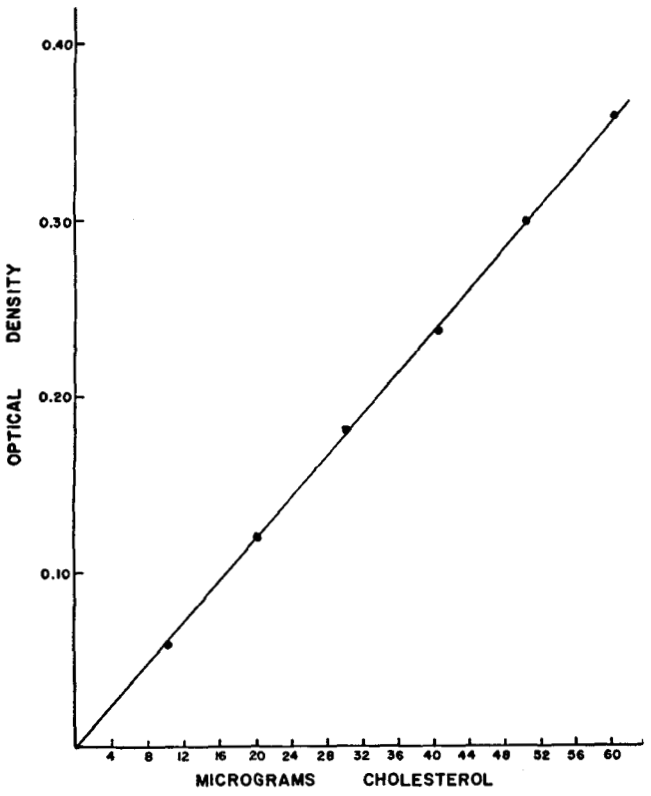

FIG. 1. Color production with increasing amounts of cholesterol treated with the $FeSO_4$ color reagent.

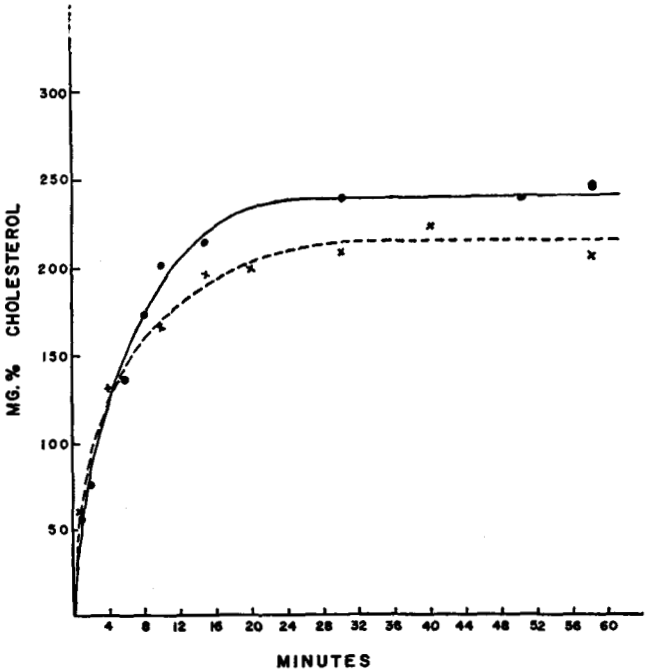

FIG. 2. Effect of time upon the extraction of cholesterol from serum applied to filter paper using $FeSO_4$ color reagent (————) and chloroform-methanol (- - - - - -).

this time and appears complete in 40 minutes. No appreciable change in cholesterol values was noted up to 16 hours of elution. In contrast, chloroform-methanol (2:1, v/v) failed to extract the total cholesterol during the time intervals studied.

Recoveries of Steroid Chromogen. Aliquots of a pooled serum were placed on filter paper and allowed to dry in air at room temperature from 15 minutes to 13 days. They were then extracted with the ferrous sulfate color reagent. During the first 24 hours after serum application, no significant reduction in steroid chromogen could be observed (Table 1). However,

TABLE 1. RECOVERY OF STEROID CHROMOGEN FROM SERUM APPLIED TO FILTER PAPER

Storage Time	Total Cholesterol	Storage Time	Total Cholesterol
	mg./100 ml.		*mg./100 ml.*
15 minutes	249*	6 hours	255
1 hour	251	24 hours	250
2 hours	253	6 days	221
4 hours	243	13 days	229

* Each figure represents the average of at least three total cholesterol determinations.

exposure for 6 to 13 days resulted in approximately a 10 per cent loss of chromogen as compared to the first 24-hour measurement.

A 10 μl.-aliquot of pooled serum having an average total cholesterol of 240 mg. per 100 ml. (7, 8) was applied to filter paper segments. Increasing amounts of a standard solution containing 200 mg. per 100 ml. cholesterol were placed on serum spots so that total cholesterols ranged from 320 to 440 mg. per 100 ml. The filter paper segments were dried and then eluted for 60 minutes in the ferrous sulfate reagent. The cholesterol recoveries shown in Table 2 range from 92 to 101 per cent of the theoretical values. The mean cholesterol recovery at the four concentrations was 95 per cent or above. These figures agree well with those reported by other workers (9) utilizing microcholesterol methods.

Reproducibility of the Method. Total cholesterol determinations were carried out in duplicate on 24 randomly selected sera from normal individuals (Table 3). The mean total cholesterol of these sera ranged

from 112 to 231 mg. per 100 ml. The differences between duplicates varied from 0 to 13 mg. per 100 ml. Expressed as a per cent of the mean, the variation averaged a little more than 3 per cent. These results compare favorably with those obtained by Clayton *et al.* (9) who report measurements of six aliquots of the same serum to be within 4.3 per cent of the highest cholesterol value.

Comparison of Ultramicro Technique with the Zak Cholesterol Method. Single cholesterol determinations were carried out on 42 randomly selected normal sera utilizing the proposed technique. Two-tenths ml. of the same sera was extracted with acetone-ethanol and quantitated, using the ferric chloride color reaction (8). Results of this study are illustrated in Figure 3. The solid line represents the theoretical relationship of cholesterol concentrations by the two methods. The actual cholesterol measurements by the macro and ultramicro techniques demonstrate a variation within acceptable limits.

Normal Values Obtained with the Method. Blood was collected from 200 male and female hospital blood donors. The serum was isolated and the total cholesterol was analyzed in 10 μl. aliquots. The normal values ranged from 122 to 350 mg. per 100 ml., with an average of 223 mg. per 100 ml.

TABLE 2. RECOVERY OF STEROID CHROMOGEN FROM CHOLESTEROL AND SERUM APPLIED TO FILTER PAPER

Cholesterol Present	Cholesterol Added	Total Cholesterol Found	Recovery
mg./100 ml.	*mg./100 ml.*	*mg./100 ml.*	*per cent*
240	80	322, 314, 303	101, 98, 96
240	120	357, 354, 344	99, 98, 96
240	160	385, 395, 374	96, 99, 93
240	200	437, 416, 406	99, 95, 92

TABLE 3. REPRODUCIBILITY OF ULTRAMICROMETHOD FOR SERUM TOTAL CHOLESTEROL

Duplicates		Difference	Duplicates		Difference
mg./100 ml.	*mg./100 ml.*	*per cent of mean*	*mg./100 ml.*	*mg./100 ml.*	*per cent of mean*
198	208	4.9	146	150	2.7
188	198	5.2	160	163	1.9
208	208	0	187	184	1.6
167	172	2.9	208	205	1.5
110	114	3.6	188	200	3.1
229	232	1.3	175	178	1.7
208	218	2.1	140	146	4.2
168	166	1.2	163	156	4.4
160	167	4.3	184	188	2.2
176	180	2.3	161	168	4.2
146	146	0	192	205	6.6
177	180	1.7	111	123	10.3

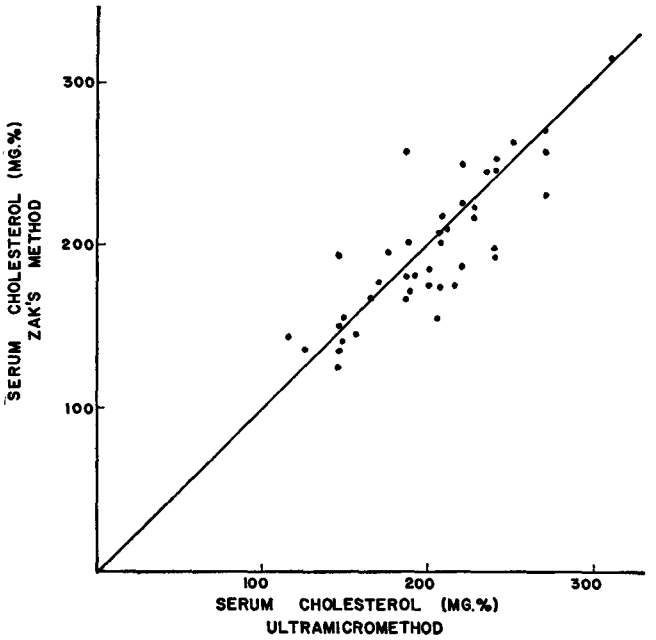

FIG. 3. Comparison of serum total cholesterol values obtained by the proposed ultramicromethod and the Zak technique.

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