A Method for Measurement of Cholesterol in Blood Serum

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A method for measuring cholesterol in 0.1 ml. of blood serum is described. This uses the saponification and extraction procedure of Abell et al. (4) and the color reaction of Zlatkis et al. (7). The procedure is shown to be accurate and reproducible. It is compared with several commonly used procedures and shown to be equal or superior to these. An adaptation of the method to filter-paper stored samples is described. The method is recommended for use in clinical laboratories.

THE METHODS for measuring serum cholesterol often perform badly in practical application (1). In part, this is related to the technical requirements for obtaining reproducible performance with the Liebermann-Burchard reaction, the basis of many of the methods. Because of its sensitivity, this reaction has made the use of small serum samples practical. Schoenheimer, and later Sperry and Webb (2), improved the specificity of this method with serum by using preliminary digitonin precipitation. This innovation also made possible the separate determination of total and unesterified cholesterol. There was then much clinical interest in the ratio of free to total cholesterol (3). There are two difficulties with the method of Schoenheimer-Sperry and Webb. It is tedious and, the Liebermann-Burchard reaction being very sensitive to temperature, the control of conditions during color development is of the utmost importance.

An advance was made when Abell *et al.* (4) introduced a modification of the procedure, which used either 0.2 or 0.5 ml. of serum and

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appreciably shortened the procedure. This was done by use of an alcoholic alkaline digestion of the serum followed by extraction of the mixture with a measured volume of hexane. This method would not allow partitioning of free and esterified cholesterol, but it was rapid, precise, and reproducible if careful attention was paid to the conditions of the Liebermann-Burchard reaction. Meanwhile, Trinder (5) had published a micromethod for serum based on the reaction of the solvent-extracted cholesterol with a mixture of acetyl chloride and sulfuric acid, and Carpenter *et al.* (6) had described a micromethod based on the fluorescence of cholesterol in trichlorethane and dry sulfuric acid.

We thought that the Abell procedure was sound and useful and might be improved by substituting the reaction of cholesterol with the reagent developed by Zlatkis *et al.* (7), namely a solution of iron in sulfuric and acetic acid. We have devised an eclectic method for serum cholesterol based on the Abell extraction of 0.1 ml. of serum and quantitated by application of Zlatkis' reaction with iron, which yields a stable violet color much less sensitive to time and conditions than the Liebermann-Burchard reaction. Furthermore, it is somewhat more sensitive and so permits use of a smaller serum sample.

The method has been used routinely in this laboratory for three years for measuring great extremes of cholesterol levels in both human and experimental animals. It has been compared with three other published methods (described here). The procedure has been informally circulated to a dozen or more interested laboratories, which have indicated successful application. This method has also been applied to the use of filter-paper storage of serum intended for cholesterol measurement as dscribed by Anderson and Keys (8). This has been an especially useful technic for field studies that involve transporting material back to the base laboratory.

Herrmann (9) has described a similar idea with his development of a method using 0.1 ml. of serum with essentially Abell's conditions of extraction and Zlatkis' reagent. Herrmann used glass stirring rods in the extraction mixture and removed the entire hexane extraction by repeated and meticulous aspiration. In his preliminary report, Herrmann found that the direct procedure of Zlatkis, as applied by McIntyre (10), gave high values when compared with either his own modification or the original Abell method. Herrmann also found the iron chromogen to be stable and exceptionally sensitive.

Morris (11) has compared several methods in an excellent study. He concluded that the acetyl chloride procedure of Trinder was the most reproducible but the adaptation of Abell's method by Anderson and Keys and the Sperry-Webb modification of Schoenheimer's procedure were very nearly as good. The adaptation of Anderson and Keys is different from Abell's procedure in only three aspects: (1) The incubation of the serum in alkaline alcohol was increased to 90 min. (Abell advised 55 min.); (2) The entire cholesterol in the digest was removed by repeated hexane extractions; and (3) The chromogen mixture in the Liebermann-Burchard reaction was measured repeatedly at 5-min. intervals, beginning at 20 min., in order to interpolate to the color maxima. In our experience, the first modification is necessary, but we prefer to heat 60 min. at 65°. The second and third modifications are not necessary with the more sensitive and more stable iron reagent described here.

Methods

Reagents

Alcohol-KOH mixture. Prepare each day, as needed, a mixture of 6 parts of a stock solution of 33% potassium hydroxide with 94 parts of absolute ethyl alcohol.

Petroleum ether. Redistill reagent grade solvent in a glass still, collecting the fraction that boils between 30° and 60°. Store in a brown bottle.

Iron stock solution. Dissolve 2.50 gm. $FeCl_3 \cdot 6H_2O$ in 25 ml. of glacial acetic acid. Store this in the cold room. When using, avoid the precipitate. This material will store many months.

Working iron-sulfuric acid reagent (FeSac). Measure exactly 1 ml. of the iron stock solution into a dry 100-ml. volumetric flask. Add reagent grade conc. H_2SO_4 cautiously and make to volume. Discard if and when a precipitate appears.

Cholesterol standard. Dissolve 100 mg. of recrystallized cholesterol (M.P. 148°–149°) in glacial acetic acid in a 100-ml. volumetric flask. Make to volume with glacial acetic acid. This stock standard contains 1000 μg./ml. The working standard is made by transferring 10 ml. of the stock standard to a 100-ml. volumetric flask. Make to volume with glacial acetic acid. This solution contains 100 μg./ml. The standards are stored at 23° no more than 3 months, and less if much used.

Equipment

Beckman Model B spectrophotometer
Water bath set for 65°
Pyrex test tubes No. 99650, 125 × 200 mm., with glass stoppers
Either Beckman square cuvets or round absorption tubes (The latter, with a light path of about 1 cm., are more convenient but slightly less accurate.)

Procedure

1. Transfer 0.1 ml. of well-mixed serum or plasma to the *bottom* of a digestion tube. The amount is critical; a Levy-Lang constriction pipet is used. Between samples rinse the pipet with water and blow out.
2. Add 5 ml. of freshly prepared alcoholic KOH; mix by swirling.
3. Place the tubes in a 65° bath for 60 min. with stoppers loose.
4. Remove the tubes and cool to room temperature. Add *exactly* 10 ml. of petroleum ether with a pipet, replace the stopper, and mix vigorously by repeatedly inverting for 1 min. Add 5 ml. of distilled water to each tube, stopper, and mix vigorously (by inverting) for 1 min.
5. When the petroleum ether layer separates, and if it is not centrifuged 10 min. at 1500 rpm, remove exactly 2 ml. of the ether layer with a pipet. Transfer this to the bottom of a test tube, 22 × 175 mm. The tube must be wide for subsequent mixing. Place the tube in the 65° bath until all the solvent evaporates. This requires about a half hour. If aeration is used it must be with nitrogen. Smell tube for the last traces of solvent. Cool the tubes to room temperature.
6. Add 4 ml. of glacial acetic acid to each tube. Prepare 4 additional tubes, one for a blank and 3 for standard solutions, containing 20 μg., 50 μg. and 100 μg. of cholesterol, respectively. Add 3.8, 3.5, and 3.0 ml. of glacial acetic acid to each standard as appropriate.
7. Add exactly 2 ml. of FeSac reagent to each graduated tube with a 5-ml. pipet. Do two tubes at a time and mix the contents by swirling. The tubes will heat and a transitory brown color will change to violet. Gas bubbles will form. If a petroleum ether residue is present, an emulsion will result. Discard such a tube.
8. After 20–30 min. at room temperature the tubes are cool. Read at 560 mμ. with the instrument set by the reagent blank.
9. Plot the standards on rectilinear paper. The optical density versus concentration is typically a straight line and this repeats well.

The conversion is simplified by calculating a multiplication factor for converting optical density to concentration.

The average of the ratios of cholesterol-in-standard to optical density multiplied by the appropriate dilution factor yields the multiplication factor.

If the optical density of the sample is more than 0.600, a smaller amount of petroleum ether should be used. If the optical density is under 0.10 a larger amount of solvent should be used.

The glassware used for color development must be dry.

If the quantity of serum is other than 0.1, adjust the petroleum ether proportionately.

Screw-cap culture tubes with Teflon cap liners occasionally leak when used for the saponification. We prefer test tubes with glass stoppers. When transferring the chromogen solution to the photometer tube avoid excessive agitation, which will produce troublesome bubbles.

Procedure with Filter Paper

When the samples are stored and transported on filter paper the procedure is almost the same. Either 0.1 or 0.2 ml. of serum is distributed on one half a Whatman No. 1 7-cm. filter paper. It is convenient to apply this directly from a Levy-Lang constriction pipet. Two samples can be easily placed on a paper, one on each half. The paper is dried for an hour at room temperature with care taken to handle only the edge bearing the penciled identity number. The papers are then stored and transported in envelopes.

The portion of the paper stained with serum is cut with scissors into 5 × 10-mm. sections that are then dropped through a funnel into the tube. The saponification fluid is added. Care is taken that all the paper fragments are submerged. The procedure continues as above except that *6 ml.* of water is added instead of 5 during the hexane extraction stage. This accounts for the little water absorbed by the paper and prevents an otherwise slightly low recovery, apparently due to a disturbance of the solvent distribution equilibrium during extraction.

Results

The stability of the chromogen produced at convenient intervals and room temperatures is shown in Fig. 1. Since the color reagent produces heating, it is desirable to wait 15 min. before measuring the

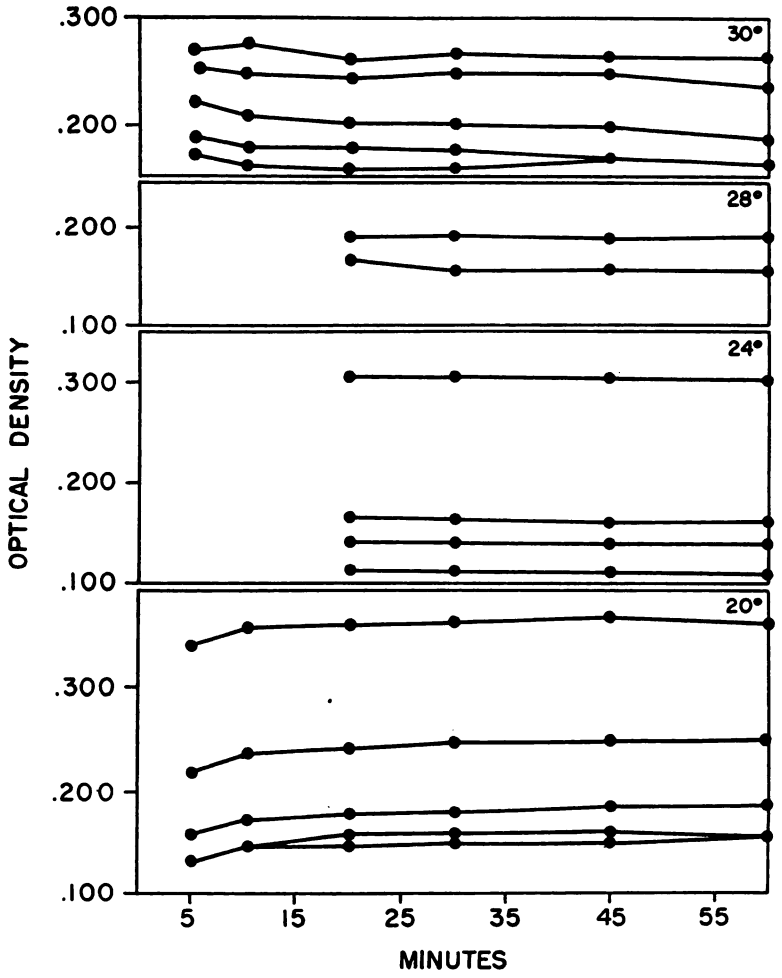

Fig. 1. Stability, with time at 4 temperatures, of the chromogen produced by the reaction of iron with cholesterol in sulfuric and acetic acids. Sera were selected to give a range of concentrations. Conditions are given in the text.

optical density. In practice the tubes may be measured between 15 and 60 min.

There are two useful dimensions for evaluating the performance of a quantitative method: The first of these is the precision—that is, the extent that replicates agree, which can be calculated as the Standard Error of a single observation:

$$S_x = \sqrt{\frac{\Sigma \text{ diff}^2}{2 \text{ pr.}}}$$

There are, however, two distinct kinds of such an estimate: that obtained when the operator knows he is checking; and that obtained without operator knowledge, by the use of blind replicates. The second dimension for evaluation is the accuracy—that is, the extent that a procedure conforms with other established procedures or the extent that it can be shown to measure the quantity intended. This is usually evaluated by recovery experiments done with a pure compound. This comparison is unnecessary in the present work in view of the demonstration of Abell *et al.*, by counter-current distribution methods, that the method they described did in fact measure cholesterol. The method of Abell *et al.* is our principal reference method but we have also used those of Sackett (12) and Pearson *et al.* (13) in the belief that the comparison would prove useful in determining the most appropriate method.

The comparison of methods was done by establishing these three other commonly used procedures and submitting to each sera selected to represent a range of values. This kind of evaluation always suffers from a bias; the local method will be better performed than the unfamiliar method, which we may wish to supplant. However this may be, the data in Table 1 show comparison of three of these four methods, with the trial sera arranged by level. A fourth method, that of Zlatkis *et al.* was abandoned after we were unable to obtain comparable results either in terms of precision or equivalence of means on single samples of sera.

An inspection of Table 1 indicates that the FeSac method agrees well with the Abell method both in terms of the equivalence of means and in terms of the precision, measured here by the technical error. The method of Pearson *et al.* agreed well with respect to means below 275 mg/100 ml. (at the two lower levels) but agreed poorly above

Table 1. PRECISION OF FOUR METHODS FOR MEASUREMENT OF CHOLESTEREMIA*

		Abell (4)		*FeSac*†		*Pearson (13)*		*Sackett (12)*	
Level	*N*	$\bar{x}$	S_x	$\bar{x}$	S_x	$\bar{x}$	S_x	$\bar{x}$	S_x
<210	15	187.8	6.40	179.9	5.55	179.1	7.68	237.3	3.16
211-274	15	231.8	4.08	228.3	5.67	225.2	8.80	288.0	3.18
275-499	14	368.0	5.22	383.5	7.55	340.0	15.07	451.6	12.85
>499	15	671.7	7.73	667.1	10.39	643.0	26.86	837.0	23.02
All levels	59	364.8	6.01	364.7	7.55	346.8	17.05	453.5	14.19

N represents number of determinations; $\bar{x}$, mean level; S_x, technical error (see text).
*Values in milligrams per 100 ml.
†Present method.

this. Additionally, this method showed significantly larger technical error estimates. The Sackett modification of the Bloor method, so widely used in clinical laboratories, showed consistently higher means at each of the four serum levels. This was an important difference at every level. An extension of this comparison with the Sackett method and with additional sera over a period of months is shown in Table 2. The technical error of the Sackett method was

Table 2. COMPARISON OF THE SACKETT AND FESAC METHODS FOR DETERMINATION OF SERUM CHOLESTEROL*

		Sackett		*FeSac*	
Month	*N*	$\bar{x}$	*S.D.*†	$\bar{x}$	*S.D.*†
11	58	280.2	±145	268.0	±162
12	45	250.2	±141	232.9	±142
1	10	142.5	± 67.5	132.7	± 63.48
2	15	252.3	±109	238.7	±110
3	16	315.3	±129	290.3	±124

*Values in milligrams per 100 ml.
†Standard deviation of distribution.

very small below 275 mg./100 ml. but at the high levels of serum concentration the technical error became excessively large. It must be borne in mind that these technical errors all represent estimates of the first kind; i.e., when the operator knew that he and the method were being checked.

In the course of a clinical study it was possible to separate a portion of the sera at the clinic when the blood was drawn and introduce that portion at the laboratory as though it were a sample from a different individual. This procedure, which provides an unbiased measurement of technical error, was introduced in the Framingham laboratory (14) in 1955, and has yielded a fine estimate of unbiased laboratory error for the several kinds of measurements done there. The summary of this kind of estimate of precision for the FeSac method is shown in Table 3. It is of some interest that the technical error here is approximately twice that obtained when the operator knew that the procedure was being checked. This has been our usual experience.

Also shown in Table 3 are estimates of S_B for the solvent duplicates. Routinely, sera in this laboratory are measured in duplicate only from the petroleum ether stage onward. This estimate of error,

Table 3. PERFORMANCE OF THE FESAC METHOD WITH BLIND DUPLICATES UNDER NORMAL WORKING CONDITIONS

	Serum duplicates	*Solvent duplicates**
Number of pairs	18	36
Mean level (mg./100 ml.)	207.2	206.9
S_d	11.0	5.45

*Duplicate hexane extracts of the same samples (not blind).

which is about half that of the blind replicates for total sera, is also influenced by the routine practice of repeating the run with a new sample of sera whenever the solvent duplicates disagree by 15 mg./100 ml. cholesterol or more.

A comparison of sera measured in the fresh state and after filter paper storage is shown in Table 4. This is a reliable and convenient variation of the method proposed.

Table 4. COMPARISON OF MEASRUEMENT OF SERUM CHOLESTEROL MADE WITH FRESH SERA AND WITH SERA DRIED ON FILTER PAPER

	Fresh	*Dried*
$\bar{x}$ (mg./100 ml.)	236.4	236.2
S.D.	91.42	93.40

Twenty determinations each were made with fresh and dried sera.

Discussion

Because we find the FeSac method faster, easier and cheaper, it has been adopted for routine use even though the Abell method may be slightly more precise. We believe the small advantage in precision of the Abell method is offset by these advantages. It is especially important that the FeSac chromogen is stable, thus avoiding the familiar difficulties with the Liebermann-Burchard reaction. Our experience with the widely used Zlatkis procedure confirms the finding of Morris, suggesting that the method may be unreliable.

Since there is no evidence that distinction between free and esterified cholesterol is relevant to studies of atherogenesis this limitation of the method is unimportant.

Experience with the FeSac method indicates that a complete batch analysis, which may conveniently include 18 samples and end with 40 measured optical densities, including a set of standards, will require about 4 hours and some waiting time.

The FeSac method has retained a distinct practical advantage of the original Abell procedure; that is, if the usual 2 or 4 ml. petroleum ether proves inappropriate, yielding optical densities under 0.100 or over 0.600, the operator can turn to the remaining petroleum ether. In practice, this is time saving.

A standard curve is run with the blank and three standards with every set of samples. The standards are run singly. The optical density—concentration relationship is invariably linear and has lead to the use of a multiplication factor for conversion of optical density to milligrams per 100 ml. This is more accurate than reading mg. per 100 ml. from a graph. However, this factor must be calculated with each set of samples because there are important variations.

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