A Semiautomated Procedure for the Determination of Serum Cholesterol Using the Abell-Kendall Method

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Abstract

A semiautomated method for the measurement of serum total cholesterol, based on the Abell-Kendall procedure, is described by which 100 to 140 samples may be analyzed in duplicate per day. The method was made possible by the development of a Liebermann color reagent which is stable for 8 hr. Mechanized pipetting units are used to measure all reagents and the 0.1 ml samples of serum. One analyst can analyze 60 samples in duplicate per day. Determinations are carried out on an assembly line basis so that 2 analysts can analyze more than twice as many samples as can one analyst. Representative data collected over a period of 2 years for the analysis of routine samples showed that the standard deviation for duplicates, randomized over 6-day periods, ranged from 2.7 to 3.0 mg% after repeating the analysis of the 6 to 8% of samples whose duplicates differed by more than 9 mg%.

Introduction

An accurate method for the determination of serum cholesterol in large numbers of samples would be very desirable. In the present publication a semi-automated method is reported which has been used over a period of 2 years for the precise and accurate determination of serum total cholesterol in duplicate for 500 to 750 subjects per week.

This procedure is based on the Abell-Kendall method (1). The latter procedure has been used in many studies of the role of cholesterol in heart disease and has been shown to give results which parallel very closely those obtained with the more specific procedures involving precipitation of the steroid with digitonin (2–6) or separation of cholesterol by means of gas-liquid chromatography (7,8).

The development of the present method was made possible by the finding that the Liebermann reagent of the Abell-Kendall method could be used for at least 8 hours if kept in a dry atmosphere. Furthermore, the analysis of large numbers of samples was facilitated by the use of commercially available mechanized pipetting units of high precision and reproducibility. The method should be of interest, particularly when large populations are involved, in investigations of environmental and physiological factors which influence the level of serum cholesterol.

Experimental

Reagents

The reagents are made up as described by Abell et al. (1) with the following exceptions: It is unnecessary to redistill the absolute alcohol or the high boiling petroleum ether. Skellysolve B, bp, 65–80C, purchased in 15-gallon drums is a suitable type of petroleum ether. This solvent must, however, be filtered before use through Eaton-Dikeman filter paper 512 in order to remove a fine deposit. Cholesterol was purified through the dibromide according to Fieser (9). Four standard solutions of cholesterol in absolute alcohol containing 100, 200, 300, and 400 mg% of the sterol are prepared. These solutions are kept in the dark in tubes with Teflon-lined screw caps in desiccators over absolute alcohol at room temperature and under these conditions are stable for 1 or 2 months.

The Liebermann reagent for color development is prepared in the following way: Into a 3-necked standard taper 1000 ml round bottom flask equipped with a silica gel drying tube and a thermometer, weigh 442.3 g of acetic anhydride (97.0% purity). Immediately insert a 250 ml standard taper separatory funnel with stopcock which has been lubricated with sulfuric acid and closed. After weighing the set-up, weigh 38.1 g of sulfuric acid (98.0% purity) into the separatory funnel and then cool the contents of the flask to 7C in an ice bath. Add the sulfuric acid at the rate of 4 to 5 drops per second with continued swirling of the flask so that the temperature does not rise above 12C. Then remove the flask from the ice bath and add with swirling of the flask 200 ml of glacial acetic acid (99.9% purity) through the separatory funnel taking care to rinse the sulfuric acid from the sides of the funnel. Keep the reagent protected from moisture with the silica gel drying tube. Before use bring the reagent to 25C in a water bath. Under these conditions the reagent will be stable for about 8 hr. These quantities yield 620 ml of reagent, an amount sufficient for 300 determinations.

Equipment

Mechanized Pipetting Units. Four mechanized pipetting units are used to measure reagents and serum. The units are the Trimatic model #2642, 2 pipette units, which were supplied by the Research Specialties Company. This supplier is now the Instrument Division, Warner Chilcott Co., Richmond, California. These units are equipped with mechanically operated syringes which have polypropylene barrels and glass pistons. These syringes may be connected in a variety of ways to measure and deliver the required aliquots of samples and/or reagents. The syringes may be set up 1) to withdraw a sample from a tube into the glass pipette and then wash the sample out with a reagent or 2) to deliver one or two reagents. The reagent reservoir for each unit is kept in a 25C bath. All units except (d) have valve systems which contain nylon end elements. Glass delivery pipettes of 1 mm bore are used for all units except unit (a).

Unit (a): (Fig. 1) One syringe of this unit is of 0.25 ml capacity and is equipped with a pipette of 2 mm bore. This unit is set to remove 0.1 ml of serum from the sample tubes. The other syringe is 1 ml in capacity and is connected in series with the

FIG. 1. Mechanical pipetting unit (a) for pipetting serum and alcoholic potassium hydroxide.

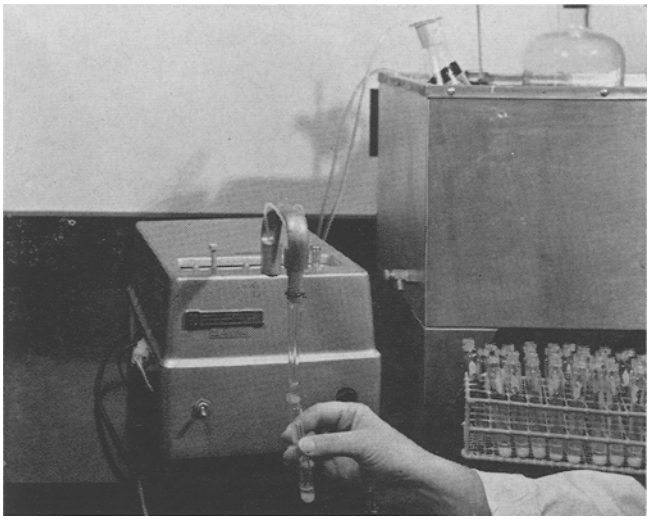

FIG. 2. Mechanical pipetting unit (b) for pipetting petroleum ether and water.

first. One milliliter of alcoholic potassium hydroxide is drawn into this syringe from the reagent reservoir while 0.1 ml of sample is drawn simultaneously into the 2 mm pipette on the first stroke of the unit. The sample and then the measured aliquot of alcoholic potassium hydroxide are expelled from the pipette on the second stroke. All tubing which comes in contact with potassium hydroxide is Teflon. At the end of a work day, the unit is rinsed with distilled water which is left in the unit. The glass pipette is treated with silicone once a week.

Unit (b): (Fig. 2) This unit is equipped with polypropylene tubing and each syringe has a needle valve to regulate air flow. One syringe is of 4 ml capacity and is set to deliver 2 ml of petroleum ether; the other is of 1 ml capacity and is set to deliver 1 ml of water. Each syringe is connected to a separate pipette and measured aliquots of the reagents are delivered simultaneously. The syringes must be freed of air bubbles before use. Reagents are left in this unit at all times.

Unit (c): This unit has syringes of the same size as those in unit (b), but both syringes are connected to the same delivery pipette. The mechanical pipetting unit is set up to measure a 1 ml aliquot of petroleum ether extract from a tube and a 2 ml aliquot of petroleum ether from the reagent reservoir on the first stroke and to deliver the extract followed by the solvent through the pipette on the second stroke. Other features of this unit are the same as unit (b). Precautions are the same as with unit (b) except that air flow must be regulated by the needle valve to avoid the formation of bubbles in the tip of the pipette.

Unit (d): Teflon tubing is used in this unit and the valve system has Teflon end elements. Only one syringe of 4 ml capacity set to deliver 2 ml is connected in this unit. The Liebermann color reagent is left in this unit at all times. Before use, fresh reagent is flushed through the system.

Vacuum Oven equipped with silica gel drying tube for air inlet, dry ice-alcohol trap for solvent and vacuum oil pump.

Kahn or Platform Shaker modified to hold tubes in horizontal position.

Beckman DB Spectrophotometer.

Water Baths. Two water baths at 25C.

Oven at 45C.

Procedure

Calibration of Mechanized Pipetting Units. The mechanized pipetting units are calibrated to the appropriate volume at the factory. This calibration should be checked initially and then every 3 or 4 months using water as a calibration fluid. The units should be checked each day for absence of leaks and presence of bubbles.

Analytical Procedure.

1. Each day's analyses include duplicate standard cholesterol solutions of 100, 200, 300 and 400 mg%, respectively, and 5 control samples. Also one reagent blank is run for each 45 determinations. The reagent blank includes all steps of the analyses. Serum samples are analyzed in duplicate. The samples are divided at the time of collection and each is stored in 2 vials. Subsequently all sample vials are randomized over a 6-day period without regard to sample number so that a given pair of duplicate samples may be analyzed on the same day or as many as 6 days apart. The serum samples, standards, and control samples are placed in random order in each day's analytical run. Serum samples are kept in tightly capped screw cap vials at −20C except when thawed for analyses. The caps are either sealed with tape or the vials are kept in vial boxes in order to prevent the caps from loosening during storage.

2. Withdraw 0.1 ml of standard, serum sample, or control serum, each at 25C (unit a), and rinse the pipette with 1 ml of alcoholic potassium hydroxide (unit a), into a 13×100 mm borosilicate glass screw cap tube. Cap tubes tightly with Teflon-lined caps.

3. Incubate the saponification mixture in an oven at 45C for 60 min. The saponification mixture may be kept at room temperature in the dark for as long as three days without change in results. Usually incubate in groups of 45.

4. Add 1 ml of distilled water at 25 C and 2 ml of petroleum ether at 25C with unit (b) to each tube at room temperature. Cap tightly.

5. Clamp each tube in the horizontal position in a Kahn-type shaker and shake for 5 min at the rate of 280 strokes per minute. Allow the tubes to stand for 15 min. Usually they are shaken in groups of 45.

6. Pipette 1 ml aliquots from the petroleum ether phase and rinse with 2 ml of petroleum ether into 15 × 85 mm borosilicate glass tubes using unit (c). Be careful to avoid the lower aqueous phase. When pipetting, unstopper only one tube at a time in order to prevent evaporation of the petroleum ether phase. At this stage the extracts will keep at least a week in the dark at room temperature.

7. Evaporate the samples to dryness at 40–50C in a vacuum oven equipped with a silica gel drying tube over the air inlet, dry ice-alcohol trap and vacuum pump. If the tubes are cold when taken from the vacuum oven, immediately warm them in the drying oven at 45C for 10 min to avoid condensation of moisture. All tubes should be allowed to come to room temperature before going on to the next step.

8. The color is developed in groups of 45 tubes containing the dry residue from the petroleum ether extraction. Since samples, standards, and control sera have been randomized for the daily run, at least one standard and/or control usually are included in each group of 45 tubes. At 40-sec intervals add 2 ml of Liebermann reagent from unit (d) to each tube being careful to wash down the entire inner surface of the tube. Place the tube in a water bath equipped with agitator so that uniform bath temperature of 25C is obtained. Read the absorbance of each sample in the Beckman DB spectrophotometer at 620 mμ against the Liebermann reagent blank at exactly 30 min after the color reagent is added to that sample. One blank is used for each group of 45 tubes. A set of 3 matched cuvettes, each of approximately 3 ml capacity and 10 mm light path, is used; one cuvette contains the blank, the other 2 are used for the samples. The latter two cuvettes are allowed to drain on paper towels between readings. The cuvettes chosen for the readings are alternated with each successive tube. It is essential for proper maintenance of the spectrophotometer that the cuvettes placed in the instrument always be capped.

Results and Discussion

Precision of Mechanized Pipetting Units

The precision of these pipettes, expressed as coefficient of variation and measured for 20 aliquots of water, was 0.4 to 1.0% for the 0.1 ml unit and 0.1% or less for the mechanized pipetting units which delivered larger volumes. Over a period of 2 years during which time the precision was checked in this manner every 4 months, the means of the volume of water measured did not differ by more than 1.5% for any of the pipettes.

TABLE I

Stability of Liebermann Reagent in Dry Atmosphere at 25C

Experiment	Age of reagent (hours)	Maximal absorbance at 620 mμ	Time of maximal color development (min)
1	0	550	29.0–32.5
	1	539	30.0–32.5
	2	545	29.5–32.5
	4	542	29.0–32.0
	5	555	31.0–33.0
	8	558	30.5–33.0
	24	540	37.5–40.0
2	2	576	28.0–30.0
	2[a]	556	35.0–37.0

[a] Usual reagent to which 0.1% (v/v) water has been added after reagent has been prepared 2 hr.

The rate of color development of the Liebermann reagent was investigated at various time intervals at 25C using a recording Beckman DB spectrophotometer. The colored solution contained 0.125 mg of cholesterol per milliliter.

Stability of the Color Reagent

Experiments illustrating the stability of the dry Liebermann reagent are given in Table I. The dry reagent appeared to be stable for 8 hr at 25C. The time of color development was increased when the reagent was kept for up to 24 hr although the maximal absorbance showed no change. If small amounts of water were present, the time of color development also was increased.

The addition of 2% sodium sulfate to the Liebermann reagent used in this study as recommended by Huang et al. (10) and Vanzetti and Gatti (11) did not increase the stability of this reagent.

Precision of the Method

Reproducibility of the Cholesterol Standard Curve. The values of the absorbances obtained from the cholesterol standards and the slopes of the standard curves relating cholesterol concentration to absorbances are quite reproducible. As can be seen in Table II, the coefficient of variation for the absorbances of the standards varied from 1.2 to 2.5%, whereas that of the reciprocal of the slope of the standard curve was 1.1%. In addition the total variation and within-day variation in the absorbances of the cholesterol standards were similar. Because of this low variability, the color reaction thus appears suitable for use in data processing systems which are based on calculations of an average slope of a standard curve.

In this procedure cuvettes were not rinsed but were allowed to drain for short periods between readings of absorbance. Rinsing the cuvettes between readings with the solution whose absorbance was to be measured did not increase the precision of the measurement of the absorbances beyond values given in Table II or change the value of the slope of the line relating absorbance and cholesterol concentration.

Analyses of Quality Control Samples. A measure of the reproducibility of the semi-automated method is given in Table III in which are presented representative daily analyses over a period of 6 months of a frozen pooled serum used as a measure of quality control. It can be seen that there was very little change in mean value for these specimens from day to day and month to month during this period. In fact, analysis of variance technique applied to these data revealed that the day-to-day variation was no greater than the usual within day variation indicating a high degree of reproducibility of the method. The standard deviation, calculated for each of the 6 weeks in Table III, ranged from 1.7 to 4.1 mg%.

Analyses of Routine Samples. The reproducibility

TABLE II

Precision of Measurement of Absorbances of Standards and of Slope of Standard Cholesterol Curve over a 20-Day Period

	Concentration of cholesterol in mg%				Reciprocal of slope
	100	200	300	400	
Mean absorbance at 620 mμ	0.121	0.243	0.364	0.484	824
Range	0.114 to 0.126	0.232 to 0.254	0.357 to 0.373	0.471 to 0.498	805 to 838
Coefficient of variation	2.5%	1.9%	1.2%	1.3%	1.1%

Duplicate standards were run over a period of 4 weeks for 5 days a week. These data were taken from the routine analyses of 100 to 125 samples per day. Analysis of variance technique (12) showed the day to day variation in absorbance to be significantly higher than the within day variation at the 0.05 level except with the most concentrated solution. Standard deviation was calculated from the formula:

$$\text{S.D.} = \sqrt{\frac{\Sigma(X - \bar{X})^2}{(n-1)}}. \qquad \text{Coefficient of variation} = \frac{\text{S.D.}}{\text{mean}}.$$

TABLE III
Reproducibilty of Analyses of Quality Control Samples Over a 6-Month Period

Month	Units	Mon. mg%	Tues. mg%	Wed. mg%	Thur. mg%	Fri. mg%	Weekly average mg%	Weekly precision, S.D. mg%
Oct.	Mean	184.8	184.2	184.9	183.3	183.4	184.1	2.61
	Range	6.6	3.3	10.6	3.2	4.9	5.7	
Nov.	Mean	184.6	184.7	183.0	186.5	182.8	184.3	2.28
	Range	8.2	5.7	3.3	3.4	4.0	4.9	
Dec.	Mean	185.2	182.9	182.7	183.9	184.1	183.8	3.27
	Range	5.2	11.9	10.4	6.1	6.9	8.1	
Jan.	Mean	183.2	182.5	182.2	182.9	183.7	182.9	2.22
	Range	7.5	2.5	1.4	6.8	5.9	4.8	
Feb.	Mean	184.5	183.4	183.8	184.4	184.5	184.1	1.66
	Range	7.3	4.2	3.5	3.4	2.5	4.2	
Mar.	Mean	182.9	182.1	183.0	184.5	184.3	183.4	4.13
	Range	4.0	20.7	3.2	4.9	2.5	7.1	

The analyses of one week per month were chosen at random for presentation in the table. Five aliquots of frozen serum were analyzed per day. Precision is expressed in the last column as standard deviation for analyses carried out in a given week. Standard deviation was calculated as in Table II. Analysis of variance technique (12) showed that the within day variation was not significantly different than the day to day variation.

of the method also is illustrated in Table IV with representative data taken from the duplicate analyses of routine samples during a period of 2 years. The 2 analyses were performed at randomly selected times within the 6-day period as outlined in the *Procedure*; hence, the day-to-day variation is included in the measurement of precision. The standard deviations, based on differences between duplicates, for analyses performed in a given week, ranged from 5.5 to 10.1 mg%. In this laboratory analyses were repeated in duplicate on those samples whose duplicates differed by more than 9 mg%.

In the representative group of analyses reported in Table IV from 6 to 8% of the samples were reanalyzed. When the original results in Table IV are corrected for the repeated analyses, the corrected weekly standard deviations, which are a measure of the precision of the values actually reported, ranged from 2.7 to 3.0 mg%.

If the less rigid criterion of rejecting those analyses whose duplicates differed by more than 16 mg% had been used, only 3.5% of the samples would have had to be reanalyzed. The "corrected standard deviations" for the analyses of each of the weeks given in Table IV would have increased by approximately 20% to values ranging between 3.2 and 3.6 mg%.

TABLE IV
Precision of Routine Analyses for Serum Cholesterol Before and After Correction of Analyses Which Were Repeated

Week	Number of samples analyzed per 5-day week	Precision of analyses, standard deviation, mg%			% Analyses repeated	
		Original	Corrected for repeat analyses above 9 mg%	Corrected for repeat analyses above 16 mg%	At 9 mg% level	At 16 mg% level
1	395	10.1	2.9	3.6	8.2	4.1
2	605	7.9	2.8	3.3	5.9	3.2
3	725	8.7	3.0	3.4	7.0	3.6
4	535	6.4	3.0	3.3	7.5	3.6
5	711	5.5	3.0	3.3	6.0	3.4
6	722	6.6	2.7	3.2	6.4	3.6
Weighted average					7.1	3.6

These data are taken from routine analyses from serum cholesterol carried out over a period of 2 years during which from 400 to 700 samples were analyzed in duplicate per week. The weeks included in the table were chosen by a process of random selection. The standard deviation was calculated on the basis of the difference between duplicates according to the equation $S.D. = \sqrt{\Sigma R^2/2N}$ where R equals difference between duplicate determinations and N equals the number of samples. Duplicate samples were analyzed from 0 to 6 days apart. The "corrected standard deviation" was calculated after substitution of the values obtained after repeating duplicate analyses of samples in which duplicates differed by more than 9 mg% for the original values on these samples. For purposes of illustration, another "corrected standard deviation" also was calculated by substitution of the second analysis of specimens whose duplicates differed by more than 16 mg% to show what the effect would have been on the overall precision if 17 mg% had been chosen as the difference between duplicates which required repeat analyses.

In the 2 years in which this semi-automated method has been used in this laboratory for the analyses of approximately 40,000 samples, the analyses of about 7% of these samples have been repeated because duplicates differed by more than 9 mg%. This percentage of repeated samples is in excellent agreement with the precentage of repeated samples in the representative data in Table IV. Therefore, it is highly probable that if values above 16 mg% had been chosen as the unacceptable difference between duplicates, only about 3% of the analyses would have been repeated and the reported precision still would have been approximately 3 mg%. This degree of precision would be satisfactory for most epidemiologic or physiologic investigations of cholesterol levels.

Comparison of Results of the Semi-Automated Procedure with Those of the Usual Abell-Kendall Method. Experiments set up to compare the results obtained with the semi-automated Abell-Kendall procedure and those obtained with the usual method employing volumetric pipettes are given in Table V. It was found that the mean value was 3.4 mg% higher with the semi-automated procedure than with the usual manual method. This 1.5% difference was significant at the 0.001 probability level. Investigation of the various points of difference between the semi-automated and manual Abell-Kendall methods failed to reveal a reason for this difference in results. However, since both procedures are based on the same method of purification of cholesterol and on the same color reaction, there is no reason to anticipate that discrepant results due to the presence of interfering materials would affect the semi-automated method to a different extent than it would affect the manual procedure. In this connection it is of interest that Antonis, Platt, and Thorpe (13) also have reported an approximately 1% difference between mean values for the acyl ester level of serum as determined by an automated method and those determined by a manual procedure.

TABLE V
Comparison of the Results of the Semiautomated with Those of the Manual Abell-Kendall Method

Method	Number of samples	Range of values mg%	Mean value mg%
Manual	102	126–334	219.0
Semi-automated	102	127–345	222.4

The samples were analyzed in duplicate using both methods. Duplicates were randomized over a period of 6 days and analyses were carried out at the same time by both methods. The difference between means is significant at the level $p = 0.001$ when calculated by the t-test for paired observations. The standard error of differences between means was 0.44 mg%.

Number of Samples Analyzed. With the usual equipment an analyst is able to analyze 14 samples in duplicate in one day by the manual Abell-Kendall procedure. With the equipment and modifications described in this communication, the same analyst can determine 50 to 60 samples in duplicate per day, an increase in rate of analyses of 3- to 4-fold. This estimate of the number of samples is based on the assumption that 8 standard solutions and 5 aliquots of control serum also are analyzed. Because of the high reproducibility of the method, more than one analyst may analyze the same group of samples without noticeable change in results. When two analysts work together, it is possible to analyze somewhat more samples, i.e., a total of 120 to 140 samples per day, than with the two analysts working separately. The analyses presented in Table IV were carried out by two analysts working together. However, as a practical consideration, it is not possible for more than two analysts to work simultaneously in order to increase the number of samples analyzed per day unless a second spectrophotometer is available or parts of the procedure are delayed overnight.

A further saving in manpower is brought about by this modified procedure, since it is unnecessary to wash large numbers of pipettes. All of the glassware in this procedure with the exception of the mechanized pipetting units, the standard taper glassware, and the spectrophotometer cuvettes, may be cleaned by mechanical dishwashers.

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