Automated Direct Method for Measurement of Serum Cholesterol, with Use of Primary Standards and a Stable Reagent

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A manual direct determination of serum cholesterol [CLIN. CHEM. **16**, 980 (1970)] has been adapted for automation. Optimal reaction temperature and reaction time were determined. The aqueous standard used is equivalent to NBS Standard Reference Material No. 911. Commercial control sera and 60 randomly chosen specimens were used to check accuracy and precision against the method of Abell et al. [*J. Biol. Chem.* **195**, 357 (1952)]. The correlation coefficient was 0.980. When these results were compared with those obtained with the SMA 12/60, the latter method had significant differences when there was interference from hemoglobin, bilirubin, gamma globulin, or gross lipemia. For a pooled serum, the inter-run relative standard deviation (coefficient of variation) was ±2.7%. Advantages over other automated direct methods for serum cholesterol include: use of primary standards, the stable and less noxious reagent, simplicity of the manifold, longer life of the reagent manifold tubing, and the decrease in photometric interferences.

Additional Keyphrases *AutoAnalyzer* • *ferric perchlorate–ethyl acetate reagent*

Several automated cholesterol methods are currently available to the clinical laboratory (*1*, *2*). Ferric chloride–sulfuric acid reagent (*1*) is either reacted with manually prepared isopropanol extracts or with extracts derived by use of a continuous filter. The direct method (*2*), which is based on a modification (*3*) of the Liebermann–Burchard reaction, is the method used with the SMA 12/60 (Technicon Instruments Corp. Tarrytown, N. Y. 10591).

Methods in which manually prepared isopropanol extracts are used can be used with primary standards, but are only semiautomated, the useful life of manifold tubing is short, and there are problems of sample evaporation. The more involved method, in which a continuous filter is used, requires secondary standards and the sampling rate is slow. The direct cholesterol method makes use of secondary standards, large amounts of reagent are consumed, and there is substantial photometric interference from bilirubin and hemoglobin.

In seeking an automated direct method for serum cholesterol that would obviate some of these problems, we considered the recently described ferric perchlorate–ethyl acetate reaction (*4*). A method reported to give results that correlate well with those of the reference method of Abell et al. (*5*) and to be essentially free of interference from bilirubin, hemoglobin, and gamma globulin in abnormal concentrations. We report here the automation of this direct cholesterol method.

Materials and Methods

Reagents

Ethyl acetate ("Nanograde," cat. No. 3427; Mallinckrodt Chemical Co., P.O. Box 5439, St. Louis, Mo. 63160). AR grade was found to be unsuitable.

Sulfuric acid (AR, concentrated).

Ferric perchlorate (cat. No. 40; G. Frederick Smith Chemical Co., Columbus, Ohio 43223).

Working reagent. In preparing this reagent, keep the temperature of the solution below 50°C. Dissolve 1.0 g of ferric perchlorate in 1200 ml of ethyl acetate that has been chilled to about 5°C. Add, gradually, while mixing, 400 ml of sulfuric acid (also chilled to about 5°C). Return the flask to a freezer or refrigerator until the temperature returns to about 25°C. Then gradually add, while mixing, an additional 400 ml of sulfuric acid. Return the flask to the freezer until the temperature again returns to about 25°C. Store at room temperature in an amber-colored bottle. This reagent is stable at least one year at 25°C (*4*).

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Cholesterol standards. "FermcoTest Aqueous Cholesterol Standard No. 291," 300 mg of cholesterol per 100 ml, equivalent to Standard Reference Material No. 911 of the National Bureau of Standards (Fermco Laboratories, P.O. Box 5110, Chicago, Ill. 60680). Dilute with "FermcoTest Special Diluent, No. 292." Add 10 mg of pure cholesterol (Schwarz-Mann Research Labs., Orangeburg, N. Y. 10006) to 10 ml of this standard, to make a standard having a cholesterol concentration of 400 mg/100 ml.

Human hemoglobin, gamma globulin, and bilirubin (Schwarz-Mann).

Instrumentation

The following AutoAnalyzer modules (Technicon Corp.) were used: Sampler II (50/h, 1:2 sampling cam), and Colorimeter with a 15-mm flow cell and 560-nm filters. The sealed aluminum heating-block module from the SMA 12/60 was disassembled and a modified phasing coil (cat. No. 157-0202-04) was substituted for the coil it contained. An 8-pin socket, similar to those on the thermoregulator strip of the SMA 12/60, was wired according to Figure 1. An adjustable temperature-control thermoregulator (Type TC 391K, 115 V, 60 cycle; Harrel, Inc., Norwalk, Conn.), was inserted into the socket. The entire unit–wiring, fuse holder, indicator bulb, socket, thermoregulator and insulated heating block—was installed in a metal housing. This enabled it to be operated as a independent unit, merely by plugging into any 120 V line. This type of heating assembly was used merely as a convenience. The same coil immersed in any type heater (oil or water) with temperature control, would suffice.

Procedure

The manual procedure described by Wybenga et al. (*4*) called for a sample-to-reagent ratio of 1:100, with the color reaction being developed in 1.5 min at 100°C. Cholesterol standards made up in acetic acid were unusable in this automated system because the sample line so quickly deteriorated. It was apparent that an aqueous solution of cholesterol (e.g., in solutions of bile salts) was required for this automated direct cholesterol. The aqueous solution marketed by Fermco Labs. proved to be a good working standard.

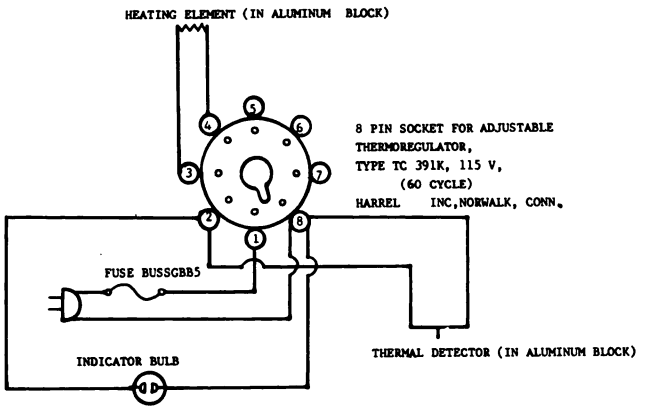

Fig. 1. Wiring description of the thermoregulator socket, enabling the SMA 12/60 adjustable heating module (95°C) to operate as an independent unit

In initial trials we examined the effects of temperature and time on the color reaction. Sera containing 100 or 200 mg of cholesterol per 100 ml and aqueous standards diluted to contain 100 or 200 mg/100 ml were used to study the optimum reaction time. Reaction time was varied by changing the length of the coil in the heating block and then comparing the length of time required for the reaction mixture to traverse the coil with the resulting absorbance value for serum and for aqueous samples; entry-to-exit time for the heating block was measured by timing a reagent segment containing ink. We selected manifold tubing sizes that would deliver volumes approximating the ratio used in the manual method and that would give absorbance readings within the range of the recorder.

The arrangement that we finally selected (Figure 2) was used to study the precision, accuracy, and recovery of the method. In a recovery study similar to that done for the manual method (*4*), the effect of hemoglobin, gamma globulin, and bilirubin on the cholesterol assay value was examined for our automated system. Sixty random specimens were assayed by this automated system and by the reference method of Abell et al. (*5*). Inter-run precision was checked by using three concentrations of cholesterol in commercial control sera and frozen pooled serum. Samples were run in random order, with 20 samples at each concentration. Results for 60 random specimens assayed with the SMA 12/60 were also compared to results obtained by using this method. A pooled serum sample was assayed on 50 days selected at random during $2^1/_2$ months. Percent carryover was assessed at different levels and the $W^1/_2$ (half-wash time) and L (lag phase) (*6*) were also assessed.

Results

Temperature. Absorbance of the reaction mixture increased with temperature of heating, and reached a maximum when the temperature exceeded 95°C (Figure 3), as determined with samples taken as the bath temperature increased from room temperature. We chose a thermoregulator setting of 95°C as a routine condition. The reaction mixture does not actually attain a temperature of 95°C during the 10-s heating, but the resulting absorbance falls within the proper absorbance range. In the paper by Wybenga et al. (*4*), it is mentioned

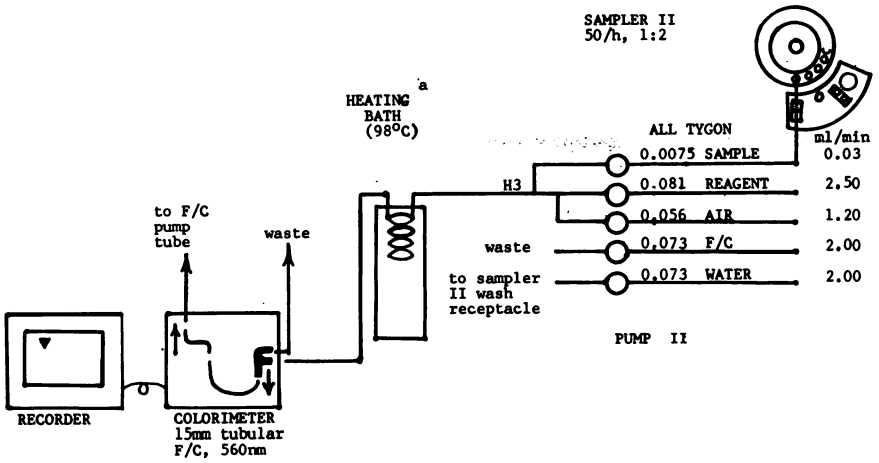

Fig. 2. Flow diagram for the direct cholesterol determination utilizing the ferric perchlorate–ethyl acetate reagent

(a), Heating Bath, a modified heating module from the SMA 12/60, contains a 4-turn coil (a phasing coil, Technicon No. 157-0202 = 04). See text and Figure 1 for details

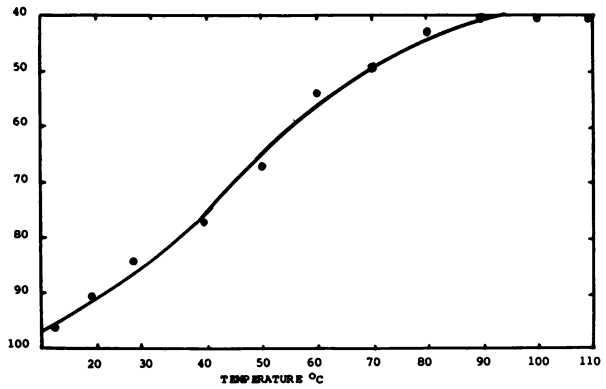

Fig. 3. Effect of reaction temperature upon the transmittance of a serum with a cholesterol concentration of 200 mg/100 ml

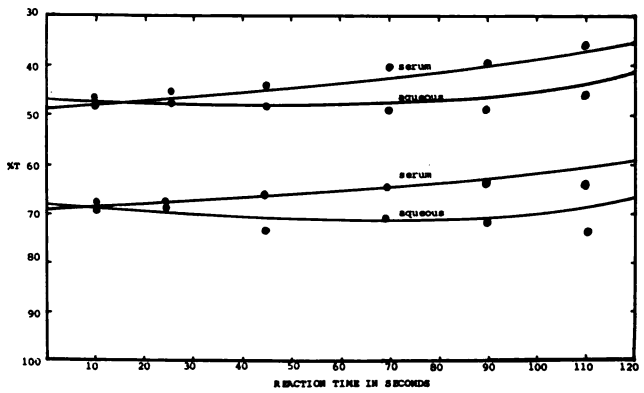

Fig. 4. Effect of reaction time upon the transmittance of a 100 mg/100 ml aqueous cholesterol standard and serum control (*lower curves*) and a 200 mg/100 ml aqueous standard and serum (*upper curves*)

that even though the reported reaction temperature is 100 °C, the 5-ml reaction volume attains a temperature of about 70 °C during the 1.5-min heating. We made no attempt to actually measure the temperature of reaction mixture itself, as we considered it unnecessary.

Reaction rate. We found that the aqueous primary standard and serum sample had slightly different reaction rates. At the recommended heating time of 1.5 min (*4*) values for serum and aqueous samples differed (Figure 4). Consequently we used a heating time of 10 s (as measured by entry-to-exit time of an ink-and-reagent segment), a time selected on the basis of the intersection points in Figure 4.

The sample and manifold tubing sizes selected provided results of sufficient precision and accuracy in subsequent trials. A calibration curve for dilutions of the Fermco standard shows linearity through 400 mg/100 ml (Figure 5).

Interferences. In Table 1, a hemoglobin concentration of 10 g/liter was found to significantly alter (15.7% positive error) the values for cholesterol, a result very similar to those previously reported for this interferant (*4*). Table 1 indicates one significant disadvantage of continuous-flow

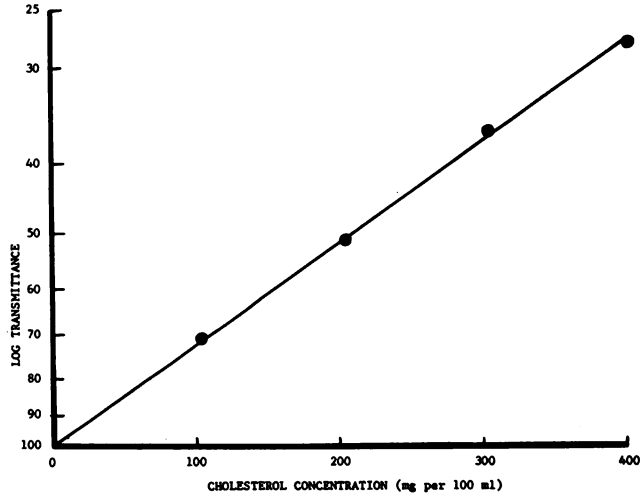

Fig. 5. Calibration curve for diluted Fermco cholesterol standards

analysis. The marked decrease in apparent cholesterol (25.4%) caused by 90 g of added gamma globulin per liter is most likely due to a hydraulic problem. The resulting increase in serum viscosity causes less sample to be aspirated. However, a

Table 1. Effect of Hemoglobin, Gamma Globulin, and Bilirubin on Total Cholesterol Values Obtained by the Proposed Automated Method

Added compound	Cholesterol, g/liter	Apparent change, %
Hemoglobin,[a] g/liter		
0	2.30	...
2	2.42	+5.2
4	2.48	+7.8
6	2.48	+7.8
8	2.56	+11.3
10	2.66	+15.7
Gamma globulin,[a] g/liter		
0[b]	2.28	...
15	2.30	+0.8
30	2.28	0.0
60	2.12	−7.0
90	1.70	−25.4
Bilirubin,[a] mg/liter		
0	2.32	...
25	2.36	+1.7
50	2.36	+1.7
100	2.44	+5.1
200	2.52	+8.6

[a] Human hemoglobin, gamma globulin, or bilirubin was added to a pooled serum.
[b] Gamma globulin initially was 27 g/liter.

specimen with 90 g of gamma globulin per liter will rarely be encountered, and so this method is faulty only in those specific cases. As much as 200 mg of bilirubin per liter caused only a 8.6% positive error in this cholesterol assay (Table 1).

In Figure 6, a comparison of this proposed automated method and the procedure of Abell et al. provided a correlation coefficient (r) of 0.980 with a paired t-test value of 0.995 (critical t = 2.000, 95% confidence limits). The regression line had a slope of 0.981 g per liter and a Y-intercept of 0.019 g per liter. The standard deviation of Y's around the regression line (Sy,x) was 0.131 g/liter.

Table 2 summarizes the values ascertained for

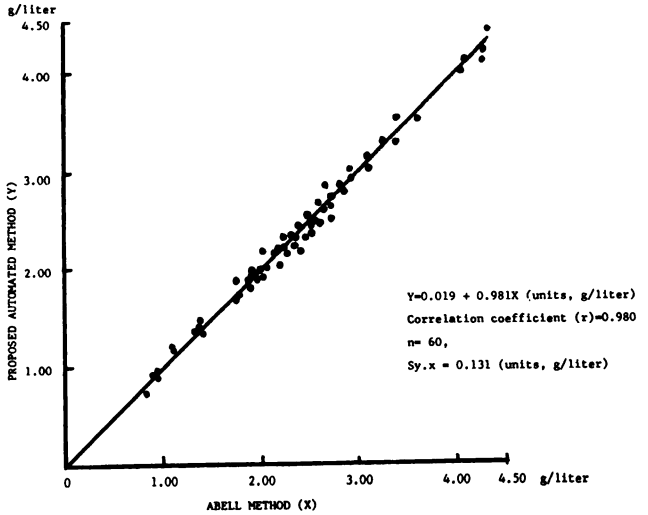

Fig. 6. Proposed automated method compared with the Abell method for the determination of total serum cholesterol

various commercial control sera. For pooled serum, day-to-day precision was ±3.3% (n = 50) and inter-run precision was ±2.7% (n = 20). The comparison of values by this method with those of the SMA 12/60 cholesterol values, gave a t = 1.889 (n = 60) with a critical t = 2.00 (95% confidence limits). Significant differences were observed in specific cases of interference (Table 3).

Percent interaction was 2.5%, as determined by running a specimen with a cholesterol value of 98 mg/100 ml before and after a specimen with a cholesterol concentration of 455 mg/100 ml. This degree of interaction is acceptable; a 357 mg/100 ml difference between succeeding samples will rarely be encountered, and if it does a repeat analysis will correct any substantial error. Repeat analysis of low values is common AutoAnalyzer practice. A difference of 150 mg/100 ml between the concentration of succeeding samples produces an interaction of about 0.5%.

The half-wash ($W^1/_2$) was 10 s with a lag phase (L) of 14 s, as calculated from the fall curve from a steady state (*6*). The percent of steady state was about 85%, using a sampling rate of 50/h. Tracings of the AutoAnalyzer recordings can be seen in Figure 7.

Table 2. Assay Values for Various Commercial Control Sera

Type	Supplier	Labeled value, mg/100 ml	n	Mean assay value, mg/100 ml	SD	CV, %
"Versatol A Alternate"	Warner-Chilcott	94	20	98.6	±3.9	±4.0
"Special Clinical Chemistry Control"	Hyland	180	20	187.0	±5.0	±2.7
"Serachol"	Warner-Chilcott	309	20	304.0	±10.9	±3.6

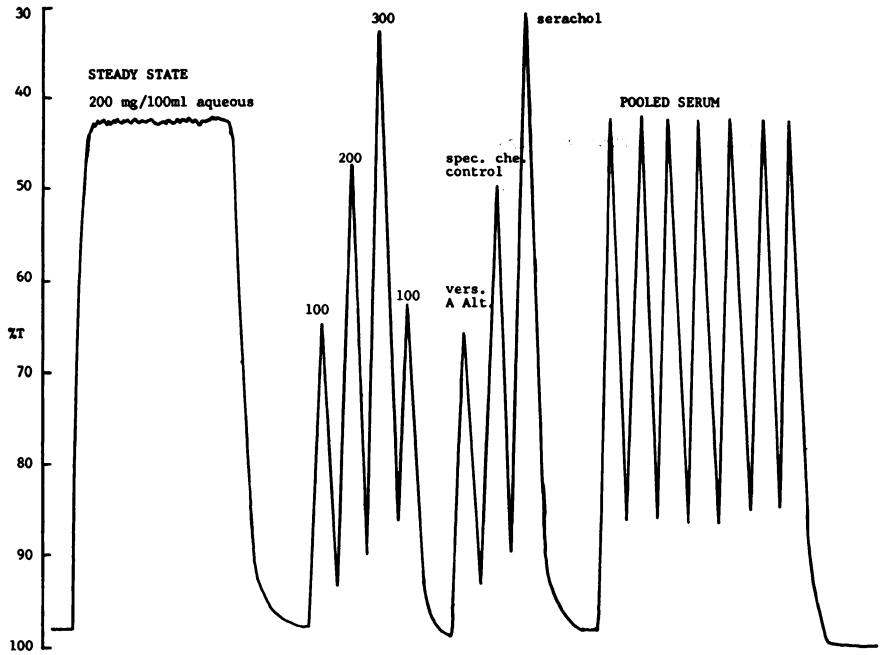

Fig. 7. Typical Auto-Analyzer chart for the automated ferric perchlorate–ethyl acetate method

Table 3. Comparative Effect of Photometric Interference on the Cholesterol Assay of 10 Sera by SMA 12/60 and by the Ferric Perchlorate–Ethyl Acetate Method Proposed

Cholesterol, mg/100 ml		
SMA 12/60	This method	Interference
240	190	bilirubin, 14.5 mg/100 ml
275	250	hemolysis
245	210	gamma globulin, 4.3 g/100 ml
225	200	bilirubin, 2.4 mg/100 ml
350	315	gross hemolysis
330	280	gross hemolysis
385	310	gross lipemia
400	355	gross lipemia
195	165	bilirubin, 5.2 mg/100 ml
225	185	bilirubin, 12.2 mg/100 ml

Discussion

Determination of the optimum reaction temperature and time enabled the design of an automated system for the direct determination of cholesterol with the ferric-perchlorate–ethyl acetate reaction. The FermcoTest cholesterol standard is described as being 93.5% pure, as compared to the NBS Standard No. 911, which is 93.2% pure (by differential scanning calorimetry).

The demonstrated accuracy and precision is likely to be adequate for the routine use of the method in the clinical chemistry laboratory. The kinetic parameters of half-wash time and lag phase offer a favorable comparison to values obtained by Thiers et al. (*6*) for other automated methods. Although only about 85% of steady-state absorbance is attained, this is necessary if reaction rates of aqueous standards and sera are to correspond. Operating at 85% of steady state apparently had no deleterious effect on the precision of the method. This method would probably be well adaptable to the AutoAnalyzer II.

Use of the SMA 12/60 heating module as an independent unit was found to be convenient in the modification of existing AutoAnalyzer I methodology to scaled down (reagent and sample consumption) versions and we are currently working on replacing the direct SMA 12/60 method with a method similar to the one used here.

The most noteworthy advantages of this automated procedure are: reagent stability (at least one year at room temperature), the ability to use primary standardization, longer life of reagent manifold tubing (we change them after about 40 to 50 h of use), and the markedly decreased photometric interferences as compared to other automated direct cholesterol methods.

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