The Measurement of Cholesterol in Serum: 
Reference Method*

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ABSTRACT
Measurement of cholesterol is sufficiently important in the diagnosis of cardiovascular disease so that a reference method should be available in the laboratory for evaluation of the rapid screening procedures in common use. With this in mind, a critical evaluation of the Abell procedure was carried out. The extraction procedure, the effect of interfering substances, the effect of time and temperature, the effect of light, the saponification procedure, reproducibility, and the effect of variations in the preparation of the Liebermann-Burchard reagent were critically examined. The accuracy and reproducibility of the method were confirmed, and it was considered to be a most suitable reference procedure.

Introduction
Current interest in the relationship of lipids and cardiovascular disease has emphasized the importance of serum cholesterol levels as a diagnostically useful measurement. Various investigators have devised new methods and modified older procedures for measuring serum cholesterol in order to make these methods practical for mass screening. Simplified techniques are now available that may perform cholesterol determinations automatically, and manual methods have been developed that require only a few reagents.

Many of the methods are based either on the Liebermann-Burchard reaction (L-B), or the more recent ferric chloride-sulfuric acid procedure. Modifications of these procedures use serum directly without the need of preparing a purified lipid extract. Although most of these methods are adequate when properly used, they are subject to unexpected aberrations. In addition, interfering substances may be present in the serums so that spuriously high results are produced. Therefore, it is most desirable that the laboratory have available a reference method that has been demonstrated to be accurate and reproducible through the years. Kabara, Tonks, Henry and Martinek have critically studied the cholesterol methods available and have emphasized their drawbacks. Martinek’s most recent review is highly recommended for those interested in cholesterol methodology.

After a comprehensive review of cholesterol methodology, the procedure described by Abell et al was selected as the reference method which most investigators consider reliable. The procedure
has been critically evaluated and the accuracy and reproducibility of the method statistically studied.

**Principle**

After saponification with alcoholic potassium hydroxide, cholesterol is extracted from serum with petroleum ether. An aliquot is evaporated to dryness and treated with L-B reagent, producing an emerald green color which is measured in the spectrophotometer at 630 nm.

**Reagents**

**Stock Reagents**

Ethyl alcohol, C.P.

Petroleum ether (boiling point 30° to 60°C), C.P.

Acetic acid, glacial, C.P.

Acetic anhydride, C.P.

Sulfuric acid, concentrated, A.C.S.

**Working Reagents**

Aqueous potassium hydroxide, 33 percent. This reagent is made by dissolving 10 g of potassium hydroxide pellets in 20 ml of distilled water.

Standard cholesterol. Precisely 250 milligrams of National Bureau of Standards cholesterol is dissolved in ethyl alcohol C.P. and the volume made to 100 ml in a volumetric flask. This working solution has a concentration of 250 mg per dl.

**Procedure**

A fresh solution of alcoholic potassium hydroxide is made by mixing 6 volumes of potassium hydroxide (33 percent) with 94 volumes of ethyl alcohol. Five ml of this solution are transferred to 25 or 50 ml glass stoppered centrifuge tubes, labeled “X” and “S.” Exactly 500 microliters of serum are added to “X.” The same volume of the 250 mg per dl standard is added to “S.” The tubes are stoppered, shaken and placed in a water bath at 37° to 40°C for 50 to 60 minutes. During this time, the tubes are mixed twice by inversion. The tubes are allowed to cool to room temperature and exactly 10 ml of petroleum ether are added to each, followed by 5 ml of distilled water. The tubes are stoppered and shaken vigorously for at least one minute. After about two minutes, the solvent and aqueous layers separate, and the lower layer is discarded using vacuum aspiration. The tubes are then centrifuged for about two minutes and exactly 4 ml of the supernatant are transferred from each centrifuge tube into properly labeled 19 mm cuvettes.

The petroleum ether is evaporated to dryness by placing the cuvettes in a hot water bath on a hot plate (caution, no flames allowed in the vicinity) and a jet of clean air is admitted to hasten evaporation.

While the tubes are being evaporated, the L-B reagent is prepared by transferring 20 volumes of acetic anhydride to a glass stoppered bottle. The bottle is placed in an ice bath until the temperature of the acetic anhydride is at least 10°C. One volume of concentrated sulfuric acid is added drop by drop while rotating the flask in the ice bath. After incubating the flask in an ice bath for 8 to 10 minutes, 10 volumes of glacial acetic acid are added and the contents mixed and allowed to reach 25°C. For each cuvette to be assayed, 6 ml of the L-B reagent are needed.

The cuvettes “X” and “S”, an empty cuvette labeled “B” and the bottle containing the L-B reagent are placed in a 25° water bath in a dark place. Six ml of the L-B reagent are added to each cuvette, at one half minute intervals. Timing is important when a large number of samples are run. Incubation is allowed to continue for 30 to 35 minutes. At the end of that time, the spectrophotometer is set at a wavelength of 630 nm and the galvanometer is adjusted to zero absorbance with the blank (“B”). The absorbance of the
unknown and standard samples is measured at one half minute intervals.

**Calculation**

\[
\frac{250 \times \text{Absorbance of unknown}}{\text{Absorbance of standard}} = \text{cholesterol, mg per dl of serum}
\]

If the reading for any sample is greater than 0.9 absorbance, 2 ml of final reaction mixture from the cuvette are transferred to another similar cuvette and 4 ml of the blank are added. The contents are mixed and the absorbance is measured. The results obtained from the above formula are multiplied by 3. If no blank solution is available, the L-B reagent may be prepared for this purpose and used as the diluent without cooling to 10°C.

**Evaluation of the Method**

**Extraction**

Results from our laboratory are in agreement with Abell *et al.* and confirm that over 98 percent of the cholesterol is extracted by the petroleum ether under the conditions described.

**Interfering Substances**

Several direct methods for measuring cholesterol are known to give higher results in the presence of bilirubin, hemoglobin (hemolysis) and bromide. This effect was studied by using the procedure to analyze solutions of bilirubin representing 60 mg per dl, sodium bromide of 200 mg per dl and recrystallized human hemoglobin of 1000 mg per dl. The absorbances of these solutions were the same as that of the blank. Abell *et al.* have performed counter current distribution analysis and found that cholesterol is responsible for over 99 percent of the color produced in the L-B reaction.

**Effect of Light**

It is well known that direct light inhibits the development of color. This was studied by preparing large volumes of cholesterol-L-B reagent mixtures in proportions to produce a concentration of 500 μg in the 6 ml of final mixture. This mixture was transferred to two sets of cuvettes. In one set the reaction was allowed to proceed at 25°C in the light while the other set of cuvettes was incubated at the same temperature in the dark. The latter set produced from 29 to 33 percent more color than the former. It is thus mandatory that the cuvettes remain in the dark during the development of color.

**Time Temperature Effects**

The color produced by the L-B reagent is time and temperature dependent. The intensity of the color increases progressively to a plateau, and then starts to fade (figure 1). Under the conditions described, the authors' studies show that at 20°C color intensity reaches a maximum between 40 and 55 minutes. At 25°C (the temperature recommended for analysis), maximum intensity is maintained from 25 to 40 minutes. At 28°C, maximum intensity is reached at 23 minutes, and fading starts at 32 minutes. At 32°C, maximum is at 15 minutes and fading starts at 22 minutes. For the procedure, incubation temperature of 25°C (±2°C) and time of 30 to 35 minutes have been selected, as recommended by Abell *et al.*

**Spectrophotometric Studies**

The absorption spectra of the L-B reaction was first studied by Sunderman and Razek. There is a peak between 620 and 660 nm. Then the absorbance diminishes at about 530 nm and increases again in the violet and ultraviolet region of the spectrum. The large majority of investigators favors the 620 to 660 nm band. Using the method with 19 mm cuvettes and the Coleman Jr. II spectrophotometer, a serum containing 200 mg per dl has an absorbance of 0.470, and follows Beer's
THE MEASUREMENT OF CHOLESTEROL IN SERUM: REFERENCE METHOD

LIEBERMANN-BURCHARD REACTION

TIME TEMPERATURE EFFECT

Figure 1. Lieberman-Burchard reaction time temperature effect.

law to at least 400 mg per dl. By diluting the final mixture as described in the calculation section, accurate measurement of cholesterol concentrations up to 1,000 mg per dl may be made.

SERUM VERSUS PLASMA

Schönheimer and Sperry found that values obtained using serum and heparinized plasma were essentially the same. Oxalated plasma gave lower results in part owing to dilution from erythrocyte water owing to shrinkage. Grossly hemolyzed serum should not be used, for although hemoglobin has no effect, the hemolysate from the erythrocyte contains less cholesterol, and results will be lower than in non-hemolyzed serum.

VARIATIONS IN THE PREPARATION OF THE L-B REAGENT

The ratio between acetic anhydride and sulfuric acid may be varied from 0.8 to 1.7 times that recommended in the method without any apparent change in the color produced.

Saponification procedure

This particular step was carefully studied in our laboratories. After the popularization of the L-B reaction by Bloor and Knudson in 1916, it was found that the colorimetric responses of equal amounts of cholesterol and cholesterol esters were non-stoichiometric: cholesterol esters producing higher absorbances than cholesterol. Henry reviewed the literature and found that the values obtained for cholesterol esters were from 10 to 30 percent higher. This varied according to the proportion of the components of the L-B reagent, the temperature and the solvent used. Using the L-B reagent described by Abell et al., the authors found that the ratios between the following esters and cholesterol were 1.09 for the oleate, 1.14 for...
the linoleate, 1.11 for the linolenate and 1.10 for the palmitate, respectively. Since in most serum samples over 60 percent of the total cholesterol is in the ester form, it is necessary, for accurate results, to saponify the esters previous to the colorimetric step. Many different procedures for saponification have been recommended. Schönheimer and Sperry \(^\text{18}\) prepared an alcohol-acetone extract from serum, and for each ml of the extract added one drop of "33 percent" aqueous potassium hydroxide and incubated for half an hour at 37° to 40°C. Sperry \(^\text{19}\) later increased saponification time to 40 minutes. Kelsey \(^\text{11}\) extracted the choelsterol in alcohol-ether and added for each 10 ml, 0.3 ml of the "33 percent" potassium hydroxide, and incubated at 37°C for half an hour.

Trinder \(^\text{23}\) treated serum with 0.5 N alcoholic potassium hydroxide and brought the contents to boiling. Finally, Abell et al used the procedure described above and in two of their publications \(^\text{5,12}\) insisted on 55 minutes incubation at temperatures from 37° to 40°C. Anderson and Keys \(^\text{3}\) adapted the Abell et al \(^\text{1}\) method to a smaller volume of serum but under the same conditions and recommended an incubation time of 90 minutes. They stated that the incubation mixture may be left at 37°C for at least a week and at room temperature for even longer periods.

The reagent used for saponification is potassium hydroxide. In most of the studies, this was an aqueous solution made by dissolving 10 g of potassium hydroxide in 20 ml of distilled water. It is interesting that it is referred to as "33 percent" potassium hydroxide or in some cases "50 percent." \(^\text{4}\) In actuality, it was observed that the final volume after cooling was 24 ml, so that the actual concentration is about 41 g per dl. By acidimetry it was 6.3 N, although small variations may be found with different batches of potassium hydroxide. By calculation, the normality of the final mixture under the conditions described in the present procedure varied from 0.33 to 0.5 N. In the original Abell et al procedure, it was about 0.4 N.

Schönheimer and Sperry \(^\text{18}\) have made the only critical study of saponification times, and reported that cholesterol esters produced the same color intensity as did cholesterol. Their study was made in 1934, and it seemed that re-evaluation with modern instrumentation was indicated.

In studying the saponification step, the procedure described was followed, but larger proportional volumes of serum and alcoholic potassium hydroxide were used. At stated intervals, aliquots were removed and extracted with petroleum ether. Aliquots of the extract were evaporated and the residue taken up in alcohol and treated with digitonin. After 24 hours at room temperature, the cholesterol digitonide was collected, washed with acetone previously saturated with cholesterol digitonide, dissolved in hot glacial acetic acid and treated with L-B reagent free of acetic acid. Measurements of color intensity were then made. The percent of saponification was then calculated with the following results:

<table>
<thead>
<tr>
<th>Time, Min</th>
<th>Percent Saponification</th>
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<tbody>
<tr>
<td>5</td>
<td>23</td>
</tr>
<tr>
<td>10</td>
<td>54</td>
</tr>
<tr>
<td>15</td>
<td>69</td>
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<tr>
<td>20</td>
<td>82</td>
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<td>25</td>
<td>100</td>
</tr>
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<td>30 to 120</td>
<td>100</td>
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Another aliquot of the petroleum ether extract was evaporated to dryness and the residue dissolved in alcohol. This extract was treated by the hydroxamic acid method of Morgan \(^\text{15}\) for the determination of esterified fatty acids with almost exactly the same results.

Cholesterol treated with the alcoholic potassium hydroxide showed little destruction up to a saponification time of 120 minutes at 37°C, as measured by the L-B
reaction or with digitonin as described above. These studies indicate that in the Abell saponification procedures, the cholesterol esters in serum are completely saponified in 25 minutes and that cholesterol is not destroyed by saponification times up to two hours.

Reproducibility of the Method

Twenty determinations were performed on the same sample of serum. The average of all determinations was 205 mg per dl, with a standard deviation of 2.92 mg per dl, and a coefficient of variation of 1.42 percent.

Twenty different serum samples were assayed in duplicate. The concentrations varied from 130 to 388 mg per dl. The average difference between duplicates was 5.32 mg per dl. These statistical studies demonstrated good reproducibility of the reference method.

Conclusion

Studies of the Abell et al procedure confirm the accuracy and reproducibility of this procedure under the conditions described. The procedure should be used in the clinical laboratory to evaluate the more simplified cholesterol techniques which have been adapted for rapid mass screening.

References


21. Sundermann, F. W. and Razer, J.: Spectrophotometric studies of the color development in the analysis of sugar by the Benedict method and of cholesterol by the Liebermann-


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"It is the great beauty of our science, that advancement in it, whether in a degree great and small, instead of exhausting the subject of research, opens doors to further and more abundant knowledge, overflowing with beauty and utility."

Michael Faraday

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