Serum Cholesterol Methodology: 100 Years of Development*

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ABSTRACT

Serum cholesterol is the third nationally recommended health screening test. It is the first test which depends upon the chemical analysis of a serum compound. Over the past 100 years, the methods of measurement and methods of standardizing these measurements have improved owing to the combined efforts of clinical research, governmental and non-governmental actions, and commercial producers.

The future constancy of serum cholesterol measurement will be based on: (1) National Bureau of Standards (NBS) pure cholesterol standard,21 (2) NBS Definitive Isotope Dilution Mass Spectrography Method,5 (3) Centers for Disease Control (CDC) Lipid Laboratory Reference Method,10,11 and (4) CDC National Reference System for Serum Cholesterol.4

Introduction

In 1885, Lieberman16 reported the green-blue color produced by the reaction of cholesterol with concentrated sulfuric acid and acetic anhydride. Bur­chard3 followed in 1890 with a modification to increase the color developed by dissolving the cholesterol in chloroform; hence, the Lieberman-Burchard color reagent.

The modern measurement of serum cholesterol was influenced by the research of Bloor2 at Harvard in 1915 to 1922 and has involved illustrious members of the chemical pathology discipline over the past 70 years. The importance of identifying hypercholesterolemia in patients was recognized as a signal of out-of-control diabetes mellitus. In turn, this led to the postulation of a relationship between serum cholesterol and atherosclerosis as morphologic, and chemical analyses of atheromatous plaques in the aorta and coronary vessels revealed a high concentration of cholesterol.

Long-term studies dating back to the 1930s began to provide an epidemiologic background for the use of serum cholesterol as a predictor of coronary artery
thrombosis. The first conclusive study which had a sufficient number of subjects carefully followed over first a six- and then a 20-year period was the Framingham study (1951) conducted by the now renowned staff of the Harvard School of Public Health\textsuperscript{9,13,14} on the citizens of an affluent Boston suburb, Framingham, Massachusetts. In addition to a yearly physical examination, chest x-ray, blood pressure and serum cholesterol were monitored. Having a stable population with a high degree of follow-up from year to year, the prediction of coronary thrombosis in this population by elevated serum cholesterol was indisputable, and risk categories were established for clinical application. Since 1961, when the main Framingham data were reported by Kannel, Dawber, et al.,\textsuperscript{14} repeated trials have confirmed the original finding. Several international trials have confirmed this observation as well.

By 1984, there was sufficient evidence to establish the fact that modification of elevated serum cholesterol could influence the morbidity and mortality from coronary thrombosis. This led the National Institutes of Health Consensus Development Conference on Lowering Blood Cholesterol to Prevent Heart Disease\textsuperscript{20} to make a national screening recommendation with the anticipation that over a period of 20 to 30 years, the high incidence of death from coronary thrombosis could be significantly altered. The result was the publication of a document which established serum cholesterol as the third nationally recommended health screening tool for all citizens of the USA on a repeated five-year basis.

Therefore, in 1987 the NIH National Heart, Lung and Blood Institute established the National Cholesterol Education Program\textsuperscript{19} which convened a symposium on the current status of serum cholesterol as a public health screening tool. During the past two years, an entirely new literature has sprung into place regarding the implementation of this national recommendation.

The two previously nationally recommended national public health screening tools were (1) blood pressure and its predictive value in the prevention of the complications of hypertension and (2) cytologic screening of cells desquamated from the uterine cervix and its predictive value for cancer of the uterine cervix. Both of these screening measures have demonstrably influenced death rate figures for the respective areas of medical mortality.

Serum cholesterol is the first national screening tool which relies upon the chemical analysis of a substance in human serum. The experience over the past 100 years demonstrates the evolutionary pitfalls which have been encountered. It also is a good example of the maturation of a specific analytical methodology to include Reference Methodology\textsuperscript{11} and a Definitive Methodology.\textsuperscript{5} Through the combined efforts of clinical researchers, laboratory scientists and governmental and non-governmental leaders, the future decision-making backbone of the National Cholesterol Education Program\textsuperscript{19} of the National Institutes of Health is well established.

The Importance of Constancy of Serum Cholesterol Measurement

If reliable medical decisions are to be made, the serum cholesterol measurement must be held constant. The purpose of this presentation is to explore the background and progress in this regard. The present question is: “Do we now have the tools to keep cholesterol measurements comparable for the next 100 years?”

It is interesting to note that in 1950 Dr. Shields Warren,\textsuperscript{22} then Director of
the Division of Biology and Medicine of the Atomic Energy Commission, implemented a study of comparative cholesterol methods by four leading research groups in San Francisco, Cleveland, Pittsburgh, and Boston. This was necessary since the observed systematic bias between major research groups was excessive.

As with each of the methods for measurement of compounds in the human serum, serum cholesterol measurement evolved (1) with respect to photometric analysis (Sunderman); (2) with respect to pure standards (Fieser); (3) with respect to specific extraction isolation of cholesterol from the serum (Schoenheimer and Sperry, Kendall and Abell); and finally (4) through the efforts of CDC and NBS to control the explosion of automated methodology (1960-to-present) using non-extraction procedures and many uncontrolled secondary standards. This unexpected melange of methods of automated analysis affected both research and clinical efforts and almost prevented the final determination of cholesterol screening efficacy.

It is clear that the single institution which brought order out of chaos and made possible the confidence in the Lipid Research Clinics Cholesterol Programs upon which the NIH conferees acted was the Communicable Disease Center Lipid Laboratory led by Dr. Gerald Cooper. Important additions were (1) the National Bureau of Standards definitive method (1980), and (2) the NIH Manual of Laboratory Operation for the Lipid Research Clinics Program Coronary Diet and Coronary Drug studies requiring CDC cholesterol measurement supervision. The constancy of the cooperating Lipid Clinics cholesterol analyses has been all important.

The history of the development of cholesterol methodology has several important lessons which follow Sundermann's basic principles of clinical chemistry analysis that (1) pure chemical standards must be the basis for a method; (2) each step in the procedure must be analyzed for its effect on the final result; (3) reproducibility of results from day to day must be achieved; and (4) human reference risk values must be used for decision-making.

Cholesterol methodology will be discussed from the following viewpoints: (1) Instrumentation, (2) Sample preparation, color reaction, enzyme specific methods, calibrator decisions, (3) Pure standards, (4) Secondary reference materials, and (5) Present status.

Instrumentation

The first instrument used for quantitation was the human eye which could detect concentration differences on a (1) spot test; (2) a visual comparison with a set of calibrated standards in sealed tubes, and (3) the visual split field comparator. The split field comparator was the tool of choice from 1910 to 1945. In this device, a standard solution was viewed at a set depth, and the unknown sample was compared with the known standard solution on the split screen. The observer matched the color of the two half circles by changing the depth of the unknown sample. The molecular concentration of the unknown was inversely related to the linear depth of the standard. At the equality point, the same number of molecules were between the plunger face and the cup depth for both standard and unknown.

Sunderman and Razek in 1939 at the University of Pennsylvania, using a unique state of the art recording spectrophotometer, improved precision by introducing the concept of a time-related observation. The time-related observation was based on the recording spectroscopy data which showed that the
spectral transmittance color development curves of the cholesterol acetic anhydride sulfuric acid mixture coincided at 10 to 40 minutes of color development at 540 millimicrons.

By 1945 photoelectric cells were well known. Simple as well as sophisticated photometric-measuring instruments had become available. The two serious problems which had led to unreliable photocell instruments were the inconstant output of the photovoltaic cell itself and the minute-to-minute variability of the line voltage. Instability of the reading of the same tube could be the result of either input fluctuation or output fluctuation.

Klett stabilized his comparator photometer by balancing the working photocell output against a reference photocell output using a calibrated resistance. The reference photocell cancelled the effect of line voltage changes. An ingenious logarithmic unit scale on the resistance made possible an easy linear calculation of concentration.

Evelyn stabilized the power source by using a large six-volt battery with a low current draw, and this instrument achieved wide acceptance because of its stability.

The third instrument which appeared at this time was the Beckman quartz prism spectrophotometer which combined fine discrimination of the wavelength of incident light, a photomultiplier for detection of emergent light and a balanced reference circuit. This instrument, the Beckman DU, was renowned for 30 years and set a standard for photometric analysis. Later, Beckman and Carey introduced an advanced model which, until recently, was the United States National Bureau of Standards reference instrument.

In the 1960s and 1970s, two additional instruments were developed separately and then combined to make the present day ultimate measuring instrument, the mass spectrometer for component measurement with liquid chromatography for component isolation. Molecules are isolated by the gas chromatography and are analyzed at the molecular or submolecular level by the mass spectrophotograph. The introduction of stable isotope dilution in which a labelled isotope is clearly identified completes the cycle of improvement.

Against this background of steadily improving methods of analytical quantitation, there also developed a plethora of automated analyzers and reagent systems. The objective for these analyzers was (1) fast sample processing, (2) automated reagent additions, (3) automated sample reading, (4) simplified output, (5) small sample size, and (6) ability to process whole serum or whole blood. Many problems of systematic bias developed, usually giving a positive 10 to 30 mg per dl bias. The eventual successes with the automated instrument screening relate to improvements related to standardization and quality control programs to be considered.

Sample Preparation, Color Reaction and Enzyme Specific Reagent Methods and Calibration Decisions

Early work in the microchemical analysis of human blood and blood serum indicated that red cell proteins and serum proteins were major interferences in the analytical procedure for cholesterol color development. A protein-free filtrate of whole blood was developed as well as a protein-free filtrate of plasma. Between 1910 and 1940,$^{1,2,23}$ it was found convenient to precipitate the serum protein and extract the cholesterol and cholesterol esters using several solvent combinations—chloroform, ethyl ether, acetone and ethyl alcohol. After evaporation of the solvent, the choles-
terol and cholesterol esters were reacted with acetic anhydride and concentrated sulfuric acid to produce a green-blue color. This color was related directly to the cholesterol concentration. It was noted that the color for cholesterol esters developed faster than the color for unesterified cholesterol. Schoenheimer and Sperry in 1934 published a more specific method for total cholesterol in which the cholesterol esters were first hydrolyzed. The total cholesterol was precipitated as the digitonide. The precipitate was washed, redissolved, and reacted in the acetic anhydride-H₂SO₄ system. During the 1940s and early 1950s, this was considered the reference method by the clinical research community.

Copeland noted (1954) that when the digitonide cholesterol crystalline precipitate was washed, the crystalline material became a paste. A second wash of the pasty material was called for which seemed unnecessary. Several years later, Sperry affirmed this observation.

The elimination of the effect of the cholesterol ester from the color development and the general shift from visual colorimeter to stabilized photovoltaic cell detector took place in the 1940s. Although not carefully documented, a significant reduction in the average cholesterol levels took place, approximately minus 15 to 30 mg per dl.

The introduction in 1952 of the Abell-Kendall Total Cholesterol method substituted alkaline hydrolysis of the ester and extraction with petroleum ether for the digitonide precipitation and precipitate washing steps of the Schoenheimer-Sperry method. Abell-Kendall results were shown to be comparable to Schoenheimer-Sperry results, and the tedious digitonide precipitation and washing steps were avoided.

It was the Abell-Kendall method which was selected by the scientists at the Harvard School of Public Health as the basis for the Framingham Study in the 1950s and 1960s. It has continued to be the reference method used nationally in clinical research circles.

The CDC Lipid Laboratory, the National Bureau of Standards Lipid Laboratory, and the Food and Drug Administration (FDA) collaborated in the 1970s to develop a Definitive Method for Cholesterol in Serum—namely the Isotope Dilution-Mass Spectrography method. The definitive method then was used to confirm the validity of the Reference Method which was the Abell-Kendall Method as modified at the CDC Lipid Laboratory.

Several international comparative trials have been conducted using the Definitive Method with reference laboratories in Sweden and Norway. In this way, the international comparability of cholesterol standardization has been maintained.

In the mid 1970s, methods were introduced based on specific enzymes for splitting the ester linkage and oxidase specific reaction with cholesterol. These methods were shown to approximate the Abell-Kendall method. They received immediate acceptance owing to the avoidance of the caustic chemicals, acetic anhydride, and concentrated sulfuric acid.

The FDA with its authority in the medical appliance and instrument area undertook licensing of all commercial products for clinical testing in early 1970. Cholesterol was among the many kit tests licensed according to the basic principle that the test should measure cholesterol and be free of interfering compounds.

Many of the early automated reagent systems designed for automated use were found to be badly calibrated, and a significant number of unfortunate incidents occurred. This was due to two fac-
tors: (1) faulty calibration, and (2) normal reference ranges which were incorrectly defined. The usual systematic bias ranged from plus 15 to 30 mg per dl. At the present time, FDA requires that all acceptable kits must be traceable to CDC Lipid Laboratory or NBS standards. The most recent study of 41 current methods summarizes the 1988 College of Pathologists Chemistry Survey. This indicates that 30 percent of the methods have less than a five mg bias maximum error with respect to the CDC reference method value, and that 45 percent of the methods in use have less than a five to 10 mg bias with respect to the CDC value. Seventeen percent of the methods showed a bias outside 10 mg. These 17 percent would require parallel standardization.

Pure Cholesterol Standard Material

The liver has a specialized function for purification of cholesterol in the gallstone. Until 1969, analysts recrystallized cholesterol for standardization purposes. No pure, stable cholesterol was available from the early 1920s to 1969. Cholesterol was known to deteriorate and contain impurities and always required recrystallization. Fieser (1953) reported that oxidation of pure cholesterol occurred on standing and stated that a stable cholesterol compound was needed. Cholesterol dibromide was recommended by Fieser (1953) as a stable cholesterol material. In the 1950s and early 1960s, the cholesterol dibromide was the reference material of choice; however, it was not commercially available, and each laboratory had to purify the commercial product or prepare its own dibromide compound.

Lack of a readily obtainable stable cholesterol standard led the Standards Committee of the College of American Pathologists (founded in 1948 by Sunderland) to organize a two-day seminar in 1966 on the subject of serum cholesterol standardization. This meeting was held at the Harvard School of Public Health, June 16, 1966, and included Dr. Fieser and Dr. Stare from Harvard, and representatives from the National Bureau of Standards, the College of American Pathologists, the American Association of Clinical Chemists, and other interested scientists. The conclusion of the meeting was that a pure, stable cholesterol standard was needed at the national level. The second conclusion was that no commercial products met these specifications. A second meeting was held by the CAP Standards Committee September 1966 at which manufacturers and reagent companies were requested to consider the production of the recommended standard. No action was forthcoming. The Standards Committee of the College of American Pathologists at this point decided to produce the needed cholesterol standard itself unless the National Bureau of Standards decided to undertake this responsibility.

Fortunately, under the leadership of Dr. Thomas Mears and Dr. Wayne W. Meinke, the National Bureau of Standards Cholesterol Standard Reference Material SRM #91la was developed and marketed (1969). This was the first of a new NBS series of SRM's for laboratory medicine and clinical chemistry.

Development of Secondary Serum Reference Materials

In the 1970s, as part of the CDC Lipid Laboratory Standardization program, a series of secondary serum samples were produced for calibration of methodology in the long-term coronary Diet Trial and the long-term coronary Drug Trial and for the Lipid Research Clinics.
These samples were verified by the reference method (Abell-Kendall) and had the advantage of the traceability to the CDC Lipid Laboratory allowing all the data from approximately 12 to 15 research centers to be pooled. Without the central standardization, the data would have shown extensive methodology systematic bias which could have obscured the effect of these important studies. Fortunately, under the leadership of Cooper at the Communicable Disease Center Lipid Laboratory, a focal point of standardization was maintained.

These secondary reference materials from CDC were calibrated by the Isotope Dilution Mass Spectrography (IDMS) method as well as by the Reference Method (CDC). There was no question about the validity of the CDC secondary standard material.

**Fresh Parallel Secondary Calibration**

Several manufacturers whose instruments showed a negative bias with lyophilized secondary reference materials showed that when fresh serum samples are run by the CDC Reference Method (Abell-Kendall) and then are used as parallel secondary calibrators, the analyses based on the fresh parallel secondary calibrators, check very closely with CDC analyzed values.

This is the current status. Both the College of American Pathologists and the National Bureau of Standards have marketed lyophilized secondary reference material whose concentration has been determined by NBS, IDMS, and CDC. Extensive studies are now underway to extend the usefulness of the current crop of secondary calibrators. One approach has been to balance the cholesterol ester types in the lyophilized material. Another approach is to prepare the secondary standard as a liquid material. As shown in a recent analysis by Copeland, 32 of the 41 methods (80 percent) used by participants in the College of American Pathologists Chemistry Survey (1988) can use the present lyophilized material as an accuracy reference material. However, seven instruments show a distinct negative lyophilized serum bias and must be calibrated indirectly by parallel serum measurements using the Abell-Kendall CDC reference method. This is very inconvenient, and it prevents the user from having direct control over the accuracy status of her/his cholesterol analyses. Currently, the Food and Drug Administration has accepted this method of reference calibration. However, further research is in progress to eliminate this problematic bias.

**Present Status of Serum Cholesterol Standardization and Comparability Verification**

1. All analyses of cholesterol must be traceable to the Centers for Disease Control and National Bureau of Standards and Methods.
2. A Standard Reference Material of pure cholesterol is available (NBS). 21
3. Standard Reference Materials of cholesterol in serum lyophilized and frozen liquid are available (NBS and CAP). 21
4. A conjoint array of laboratories called the National Reference System for Cholesterol is available for parallel fresh serum analysis (by the CDC Reference Method) of split liquid serum samples allowing verification of individual laboratory calibration (CDC). 4
5. A nationwide program for comparative evaluation of cholesterol in lyophilized serum is available in which the national performance in 4,000 laboratories is monitored four times a year (CAP Survey). 25
<table>
<thead>
<tr>
<th>Method</th>
<th>Date Published</th>
<th>Standard</th>
<th>Measurement Instrument</th>
<th>Ester Hydrolysis</th>
<th>Extraction</th>
<th>Color Development</th>
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</thead>
<tbody>
<tr>
<td>Lieberman(^1)</td>
<td>1885</td>
<td>Developed the color reaction</td>
<td>cholesterol plus sulfuric acid plus acetic anhydride</td>
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<td>Burchard(^3)</td>
<td>1890</td>
<td>Improved color by chloroform solution of cholesterol</td>
<td></td>
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<tr>
<td>Bloor(^2)</td>
<td>1917</td>
<td>Laboratory purified cholesterol</td>
<td>Visual comparator</td>
<td>None</td>
<td>Chloroform</td>
<td>acetic anhydride- sulfuric acid- Lieberman-Burchard</td>
</tr>
<tr>
<td>Schoenheimer-Sperry(^{23})</td>
<td>1934</td>
<td>Laboratory purified cholesterol</td>
<td>Visual Comparator (Zeiss)</td>
<td>alkaline hydrolysis</td>
<td>Digitonide precipitate wash acetone ethanol</td>
<td>Lieberman Burchard</td>
</tr>
<tr>
<td>Sunderman(^{24})</td>
<td>1939</td>
<td>Laboratory purified cholesterol</td>
<td>Recording Spectrophotometer</td>
<td>Timed Color development</td>
<td>Chloroform</td>
<td>Lieberman Burchard</td>
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<td>1940 - 1950 Two shifts (1) Visual Comparator to photocell detector - (2) from no ester hydrolysis to ester hydrolysis before extraction, reduced total cholesterol by 10-30 mg/dl.</td>
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<td>1952 - 1956 Technical Group(^{22}) - Committee on Lipoprotein-Comparative Studies - First Major Comparability Study</td>
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<tr>
<td>Abell-Kendall(^1)</td>
<td>1952</td>
<td>Laboratory purified cholesterol</td>
<td>Photoelectric alcoholic photometer</td>
<td>petroleum ether hexane</td>
<td>Lieberman Burchard</td>
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<td>1952 Framingham Study selected Abell-Kendall method as the &quot;four clinics&quot; analytical method.</td>
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*COPELAND*
Cholesterol Standard Conference 1966 College of American Pathologists Standards Committee - meeting at Harvard School of Public Health: Recommended that a pure reference cholesterol material be marketed.


NIH-CDC-NBS 1974 Major cholesterol projects used CDC Lipid Laboratory as the Quality Control Monitor Coronary Drug Project Coronary Diet Project Lipid Research Clinics

CDC - Lipid Laboratory 1977 Selected Abell-Kendall Method as reference method for Lipid Standardization Laboratory.

1965 - 1985 Many automated, rapid methods introduced significant positive systematic bias 10-30 mg/dl.

Reference Method 1976 NBS-SRM High quality spectrophotometer alcoholic hexane Lieberman, Burchard (optimized)

Duncan Cooper 10,11

Definitive Method 1980 NBS SRM Isotope dilution hexane Particle size Mass spectrometry Cohen-Schaffer 5 Gas chromatography Mass spectrometer

National Reference Method 1989 NBS - SRM High quality spectrophotometer alcoholic hexane Lieberman, Burchard (optimized)

Laboratory Network Cooper (CDC) 1985 - 1989 Significant progress in controlling systematic bias in both large volume small sample screening procedures and in the other automated systems monitored by CAP Surveys.
6. The previous five programs are necessary to keep the proliferation of cholesterol measurement methods for human blood or serum within the comparability and transferability limits needed over the next 50 years for reliable medical decision-making.

Discussion

In retrospect of the past 100 years, the research and clinical evolution of cholesterol measurement has been the result of important contributions by individual scientists: Bloor, Lieberman, Burchard, Kendall, Schoenheimer, Sunderman, Sperry, Castelli, Dawber, Stare, Keys, Cooper, and others; by governmental agencies: Communicable Disease Center Lipid Laboratory, National Bureau of Standards Reference Material Section, Food and Drug Administration Section on Clinical Testing Devices, and NIH Committee on Cholesterol Education; by national organizations: including College of American Pathologists Standards Committee and the CAP Comparative Survey Program, and National Committee on Clinical Laboratory Standards, American Association of Clinical Chemists, and by many manufacturers who have developed and marketed cholesterol measuring systems.

There has been a constant striving for accuracy and precision. Comparability was preserved by the CDC and NBS. These first principles must be continually reviewed and redefined into the twenty-first century at least until 2,030 when the effect of cholesterol monitoring of the USA population can be definitively measured in terms of mortality statistics.

The NIH committee has been very proactive in its support of universal screening. Various subcommittees on quality control and accuracy have made major contributions to a disciplined approach to bringing this new health protection effort to fruition.

Conclusion

The recent implementation of a National Cholesterol Information Program has been built on 100 years of research. It appears that prospects for precise and accurate measurements for the present and future generations are guaranteed by the careful work of many investigators who have established basic principles which must be continued for the next 100 years.

References

7. COPELAND, B. E.: Personal communication with Dr. F. W. Sunderman, 1945.
8. COPELAND, B. E.: Personal communication with Dr. Warren Sperry, 1959.