Studies of Cholesterol Methodology and Application to Population Surveys

Nell F. Hollinger,* Elizabeth Austin,† Dorothy Chandler,* and Richard K. Lansing‡

The present interest in the relationship of cholesterol to atherosclerosis has increased the frequency with which the determination of serum cholesterol is utilized as a diagnostic aid. Two general lines of evidence support the conclusion that most of the currently available methods of cholesterol determination are not entirely satisfactory in all laboratories. First, there are a large number of methods which have been and are presently being proposed for this determination (1). Second, there is an apparent inability of laboratories to achieve as high a degree of accuracy in the determination of cholesterol as in the determination of some other substances (2). The variability of cholesterol values reported from different laboratories may arise from the use of different methods in each laboratory or from some more subtle difference in the conditions under which the reported values are obtained. In order to investigate the former in the absence of the latter, 7 different methods for the determination of cholesterol were evaluated.

Studies by Dawber et al. (3) in Framingham, Mass., have indicated that the probability of arteriosclerotic heart disease (ASHD), code 420 WHO (4) is highest among the segment of the population which may be characterized as having hypercholesteremia, hypertension, and obesity. A survey of 3992 longshoremen in the San Francisco Bay Area, previously reported by Buechley et al. (5) did not show obesity to be a factor associated with increased incidence of ASHD.

From the School of Public Health, University of California, Berkeley,* Sacramento State College, Sacramento,† and Newel Laboratories, Fresno,‡ California.

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Analysis of data on a 4.9% sample from this survey (193 individuals) is considered with reference to the factor of hypercholesteremia. The incidence of ASHD is estimated at 87 per 1000 among longshoremen having a total cholesterol of 230 mg./100 ml. or over; below that cholesterol level the incidence is 54 per 1000 as estimated from the 4.9% sample. Contrary to findings in the Framingham study hypertension and obesity were not found to be associated with ASHD (6).

METHODS

Repetitive total cholesterol determinations were carried out using the method of Schoenheimer and Sperry, SS (7), Bloor, B (8), Schmidt-Thomé, Schettler, and Goebel, ST (9), Michaels, Margen, and Kinsell, MT (10), Zlatkis, Zak, and Boyle, Z (11), Forbes and Irving, FI (12), Abell, Levy, Brodie, and Kendall, A (13), and Carr and Drekter, CD (14). The SS, B, FI, A, and CD methods utilize a variety of extraction procedures but all terminate in the production of the Liebermann-Burchard color reaction. In the MT method the cholesterol is precipitated as the digitonide, which is then resuspended in alcohol and stabilized with gum ghatti to produce a turbidity which is estimated photometrically at 420 m\(\mu\). In the ST method the cholesterol is precipitated with a known large amount of digitonin, and the amount of digitonin in excess of the cholesterol is determined by titration with standardized red blood cells. In the method of Zlatkis et al., the Liebermann-Burchard reaction is replaced with a color reaction with ferric chloride. All methods were performed as indicated in the original papers with 2 exceptions; the FI tests were read at 420 m\(\mu\) instead of 540 m\(\mu\); 95% ethyl was substituted for the absolute ethyl alcohol in the MT method.

The reproducibility of each method was determined by performing replicate determinations on cholesterol standards and on pooled sera. Various methods were compared by performing paired determinations on a series of different specimens.

As a test of the purity of commercially available cholesterol preparations, samples from 3 brands were subjected to infrared spectroscopy.\(^1\) The spectra of 2 brands\(^2\) were not found to be different. Neither was subjected to purification when used as a standard. One brand\(^3\) was found to yield an altered spectrum and was not used in this study. Various lots of digitonin were tested and were found to

\(^1\) Infrared spectra by courtesy of Donner and Radiation Laboratories, U.C.
\(^2\) Pfanstiehl and Paul Lewis.
\(^3\) Nopco.
be similar with respect to visual hemolytic criteria; other differential criteria were not employed.

The clinical data are from the longshoremen’s survey reported by Buechley et al. (5). Blood specimens were obtained from every third man among the first 95 examined on Monday of 7 consecutive weeks. Those specimens which were to be used for cholesterol determinations were kept under refrigeration. Paired determinations for total cholesterol by the Forbes-Irving method were completed by the second day following collection.

RESULTS

Some statistical estimates calculated from the results of replicate analyses for total cholesterol on several serum pools by each of 6 methods are shown in Table 1. The estimate of correlation is high as obtained on paired reports for determination by 2 of these different methods. For FI reports, paired with values for total cholesterol obtained by each method in the table, the correlation coefficient is 0.85 or better. The correlation of SS values on FI reports is equally high, and the premise is adopted that the FI reports are equivalent to those obtained by the accepted reference method of SS. As a result of the poor correlation between FI and ST, the ST method was dropped from consideration for inclusion in the table. The possibility exists that these 2 methods do not measure the same constituent; for the FI and ST 273 paired tests gave a very low correlation ($r=0.0$). An eighth method also was excluded from Table 1 when it became apparent that under the conditions specified for the determination of Zlatkis, ferric chloride reacted with so many agents in blood serum as to preclude specificity for cholesterol (15).

Most of the methods reported were found reproducible to a similar degree, approximately ±10%. In all cases for which data or estimates were obtained, the reproducibility was less than indicated by the original investigator. This lack of reproducibility cannot be construed as a deficiency in technical proficiency, interest, or academic background of those performing the determinations. Rather, it is clear that results in one laboratory cannot always be duplicated in rigorous trials in another laboratory, a tenet fully accepted in the fields of basic research. Findings by the C.A.C.L. (16) and by Benenson (17) support this view on variability of cholesterol results.

In the choice of a method to be used by a laboratory, accuracy, as well as reproducibility, is a primary consideration. If pure standards are available, the accuracy of a method frequently is related to
Table I. STATISTICAL ESTIMATES ON REPLICATE ANALYSIS OF CHOLESTEROL STANDARDS AND ON POOLED SERA

<table>
<thead>
<tr>
<th>Method</th>
<th>Serum pool</th>
<th>200 mg./100 ml. or less</th>
<th>Over 200 mg./100 ml.</th>
<th>Reproducibility data given by original investigator</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS Schoenheimer Sperry</td>
<td>65</td>
<td>11.5</td>
<td>42</td>
<td>9.2 49 5.3</td>
</tr>
<tr>
<td>B Bloor</td>
<td>80</td>
<td>10.2</td>
<td>32</td>
<td>8.5</td>
</tr>
<tr>
<td>FI Forbes</td>
<td>111</td>
<td>11.8</td>
<td>120</td>
<td>16.6 148 39</td>
</tr>
<tr>
<td>CD Carr</td>
<td>52</td>
<td>6.2</td>
<td>32</td>
<td>9.4 28 6.2</td>
</tr>
<tr>
<td>MT Michaels Turbidimetric</td>
<td>149</td>
<td>7.2</td>
<td>85</td>
<td>400 73 29 Unpublished</td>
</tr>
</tbody>
</table>

specificity. The specificity of the SS method has not been challenged seriously. It is not readily apparent whether such specificity may be attributed to the fat solvent extraction process or to precipitation by digitonin. Specificity due solely to the use of digitonin seems unlikely; digitonin itself can replace cholesterol in the Liebermann-Burchard colorization (1). One cholesterol determination in which digitonin is employed and colorization eliminated has a poor correlation with a method retaining this colorization and eliminating digitonin precipitation, that is, the ST and FI relationship presented earlier. No evidence obtained or reviewed here substantiates the suggestion that the Carr-Drekter method lacks specificity. The
method of choice on the basis of reproducibility, accuracy, and ease of performance would be the Carr-Drekter (see Table 1 and (14)). The time and number of steps involved in the CD is greatly reduced over the SS. Of the other methods evaluated none is as precise as the CD if results on both standards and pooled specimens are taken into consideration. For the CD, twice the coefficient of variation ($2CV_2$) has a value of 6.2, 9.4, and 6.2 in 3 series of determinations in which the number of tests was 52, 32, and 28, respectively (Table 1). Inspection of the table for estimates of $2CV_2$ does not reveal any equally low absolute values on 3 series of tests by 1 method. Professional personnel adopting the CD for laboratory use uniformly accredit this method with ease of performance. The effectiveness of control charts for use with CD standards and control sera has been demonstrated here and reported by others (6, 14, 18). The reporting investigators found that failure to employ such charts or equivalent comparisons resulted in loss of accuracy.

**CHOLESTEROL AND ARTERIOSCLEROTIC HEART DISEASE (WHO CODE 420), ASHD**

In the survey of 3992 longshoremen (5), a total cholesterol was obtained for 220 men, that is, for 5.5% of the larger closed population surveyed. For 193 of these men clinical data, laboratory reports, and a 5-year follow-up history were available in addition to the cholesterol value. These 193 cases, a 4.9% sample, are deemed representative; the incidence of age, weight, and ASHD is the same in the 4.9% sample as in the larger survey. For this reason additional estimates were made from data for the 193 cases.

Of the 193 cases, 120 were tabulated as normal; these cases had negative findings on clinical and laboratory data on 10 screening tests (vision, hearing, chest x-ray, EKG, blood pressure, serology, hemoglobin, blood and urine sugar, and urine protein). At the time the screening tests were reported, none of the 120 was referred to a physician, a practice which had been adopted for the larger survey population studied (5). A subsequent 5-year history for ASHD among the 120 and diagnosis for the remaining 73 cases was obtained as described by Buechley et al. (5).4

For the group of 120 normal individuals the mean and median value for total cholesterol was 210 mg./100 ml. blood, a value which is in conformity with that of Sunderman (19). For this sample with

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4Appreciation is expressed to the Bureau of Chronic Diseases, California State Department of Public Health, for access to screening data and for diagnoses.
Table 2. Incidence of ASHD According to Cholesterol Level

<table>
<thead>
<tr>
<th>Cholesterol level</th>
<th>Population at risk</th>
<th>Cases of ASHD (420)</th>
<th>Incidence (N/1000)</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥ 230 mg./100 ml.</td>
<td>46</td>
<td>4</td>
<td>87</td>
</tr>
<tr>
<td>&lt; 230 mg./100 ml.</td>
<td>147</td>
<td>8</td>
<td>54</td>
</tr>
</tbody>
</table>

10 negative screening tests the standard deviation for cholesterol values was 24.7 mg./100 ml. Tabulations of cholesterol by age and weight group have been made for all 193 cases (6). No significant difference in the cholesterol level tabulated by age or weight was found by inspection or by statistical estimate. The number of cases of ASHD (Code 420) was too small to warrant statistical treatment of many small categories according to the presence or absence of the 3 diagnostic criteria, obesity, hypertension, and hypercholesteremia. Of importance at this time and for resolution by additional longitudinal studies is the fact that for the sample of 193 men, the incidence of ASHD among the individuals with a cholesterol of 230 mg./100 ml. or over was 87 per 1000 and that among the individuals with lower cholesterol levels the ASHD incidence was 54 per 1000, as shown in Table 2.

SUMMARY

Comment is made on 7 methods with respect to replicate analysis and correlation with results of the Schoenheimer-Sperry determination of serum cholesterol. Estimates of variance are tabulated for 5 of the methods in terms of twice the coefficient of variation ($2CV_x$); values range from 6.2% to 11.8%, averaging approximately 10%. For cholesterol standards, reproducibility of color development is somewhat better for standards equivalent to more than 200 mg./100 ml.; the range of values for $2CV_x$ is 3.8% to 39% above 200 mg. per 100 ml.; below that level $2CV_x$ is 9.4% to 16.6% for photometric methods. On the basis of reproducibility and ease of performance the method of choice for the determination of total cholesterol is the Carr-Drekter.

As estimated from findings for 12 cases of atherosclerotic heart disease (ASHD) in a 4.9% sample representative of a survey of 3992 longshoremen, the incidence of ASHD is 87 per 1000 longshoremen having a total cholesterol of 230 mg./100 ml. or over; below that cholesterol level the incidence is 54 per 1000.
REFERENCES

15. Mt. Zion Hospital Biochemistry Department, San Francisco. Personal communication.