The Standardization of Measurements of Serum Lipids for an Epidemiologic Study of Stroke

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INTRODUCTION

In any epidemiologic study of cardiovascular disease, it is necessary to standardize the methods used to measure total serum cholesterol (CH) and triglyceride (TG). Standardization not only allows for comparisons between studies, but also aids in the evaluation of secular changes within a single study group and permits the results from different surveys to be pooled1).

Since 1968, the Center for Adult Diseases (CAD) in Osaka has been a participant in the Cooperative Lipid Standardization Program2) of the Center for Disease Control (CDC) in Atlanta, Georgia, and after a four-year series of studies CH and TG standardization has been achieved3). Based on instructions from the CDC, the present authors (OCU) standardized the measurements under CAD controls until they were admitted to the program itself. In the two-year period beginning in 1979, standardization has been possible, although the following problems were encountered. 1) As opposed to the hospital laboratory situation requiring daily measurements, the field survey took such measurements only two or three times a year. 2) Staff members who do not habitually make analyses had to analyze 1,000 to 2,000 serum samples per 10 days. 3) Since the results were supposed to be reported to the subjects as soon as possible, multiple serum samples had to be analyzed quickly. To conclude, it was extremely difficult for the plural staff members to analyze the required samples within the range allowed for precision and accuracy. The present report discusses the method of standardization developed.

METHODS

Total serum cholesterol (CH) was measured by the Lieberman-Buchard (LB) method by using Technichon auto analyzer II (AA II)4).

Serum triglyceride (TG) was measured according to the acetyl-aceton method5) in which TG is extracted with isopropyl alcohol, and interfering substances (saccharides and phospholipids) are removed by adsorbents and the TG is saponified by potassium hydroxide to release glycerol. The glycerol is oxidized by metaperiodate to formaldehyde which reacts with acetyl-aceton and ammonia to form a yellow dihydrolutidine derivative, absorbing at 410 nm.

The CAD lipid standardization program, which conforms to that of CDC, consists of four parts.

Part 1 (Self-evaluation phase, using samples of known concentration): The samples were control sera
of known low, medium, and high concentrations. In a single run, each concentration was measured four times, and a total of six runs were performed. The results were subjected to self-evaluations based on the $X-R$ control chart. In the case of TG, in addition to the control sera, 60 serum samples were prepared to permit double checking by the CAD. Ten samples were measured in each run, and a total of six runs were made. Self-evaluation was on the basis of correlation chart. This part was repeated twice for TG.

Part 2 (The CAD precision control phase, using samples of unknown concentration): The samples included unknown but generally low, medium, and high concentrations control sera, and 60 others to double-check random combinations of various unknown concentrations.

In a single run, each concentration was analyzed four times, with a total of six runs performed. Ten double serum samples were analyzed in each of six runs. The control sera results were plotted on $X-R$ control chart, while the double sample results were reported to the CAD.

Part 3 (Standardization phase): Samples consisted of 30 control samples where concentration was completely unknown. In one run, three types were each analyzed twice, that is, two measurements were obtained for one. A total of 60 measurements were reported to the CAD. This was performed semiannually.

Part 4 (Long-term external quality control survey phase): The samples included 30 control preparations of three completely unknown concentrations. Ten runs were performed as in Part 3 above. Where field samples were available, they were measured. Accuracy was checked by the CAD. This part was performed twice annually.

If satisfactory results had not been obtained in any part, it was repeated 2 or 3 times. In principle, no more than two runs were performed during one week.

RESULTS

1. Standardization of total serum cholesterol measurements

   (1) Part 1 (April to May, 1980): The individual averages of three sera concentrations were 3 to 7% higher than those obtained by the CAD, according to whose instructions adjustments were made in the standard calibrations of Technicon reference sera. The findings from three control concentrations fell within standard CAD ranges.

   (2) Part 2 (June, 1980): The analytical values of control sera are shown in Table 1. Figure 1 shows the correlation chart prepared by the CAD based on the OCU report. From this and the $X-R$ control chart (Fig. 2), the CAD judged Part 2 to be complete.

   (3) Part 3 (Series 1: August, 1980; series 2: September, 1980; series 3: February, 1981; series 4: June, 1981): All control sera analyses are listed in Table 2. In the series 1 of Part 3 (3-1), the coefficient of determination for CAD and OCU measurements was good ($r^2=0.998$), but the CAD stated that control values should be under 100% of the CAD values. Therefore, the standard calibration was readjusted (Part 3-2). Control sera analytical values were thus generally reduced, to 96 to 99% of the CAD values (Table 2). Since only the high control sera fell to any extent, the CAD advised that Part 3-3 should be performed using the standard calibrations of Part 3-1. As shown in Table 2, the ratios of three concentrations to those by the CAD varied between 102, 98 and 95% and high concentration C still remained a low 95%. The linearity of the calibration curve was suspected by CAD, and investigations disclosed the main cause to be the colorimeter. The colorimeter required two months to be repaired and adjusted, after which calibration curve linearity was restored, and Part 3-4 was conducted using a stan-

<table>
<thead>
<tr>
<th>Table 1 Cholesterol control values for Part 2.</th>
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</thead>
<tbody>
<tr>
<td>Controls</td>
</tr>
<tr>
<td>Mean (a)</td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>L</td>
</tr>
<tr>
<td>M</td>
</tr>
<tr>
<td>H</td>
</tr>
</tbody>
</table>

L: Low pool, M: Medium pool, H: High pool.

CAD: Department of Epidemiology and Mass Examination for Cardiovascular Diseases, Center for Adult Diseases, Osaka.

OCU: Department of Public Health, Osaka City University Medical School.
standard calibration intermediate between Part 3-1 and 3-2 values. The findings from three kinds of control sera were 101, 100 and 98% of the values obtained by the CAD, and were similar to the results from Part 3-1. The CAD accepted this level of reproducibility.

(4) Part 4 (August, 1981): The results are shown in Table 3. The control serum findings, compared with those from the CAD, were 100% for low (D) and medium (E) concentration sera, and 98% for high concentration sera (F), which were better than those of Part 3-4. Thus reproducibility had been maintained.

Table 3  Cholesterol control values for Part 4.

<table>
<thead>
<tr>
<th>Control serum</th>
<th>CAD (mg/dl)</th>
<th>OCU (mg/dl)</th>
<th>b/a(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (a)</td>
<td>Mean (b)</td>
<td>S.D.</td>
</tr>
<tr>
<td>D</td>
<td>121</td>
<td>120</td>
<td>2.89</td>
</tr>
<tr>
<td>E</td>
<td>189</td>
<td>188</td>
<td>4.19</td>
</tr>
<tr>
<td>F</td>
<td>245</td>
<td>241</td>
<td>4.70</td>
</tr>
</tbody>
</table>

\[ r = 0.999 \ (p < 0.01) \]
\[ r^2 = 0.998 \]
\[ y = 1.023x - 2.327 \]

Table 2  Cholesterol control values for Part 3.

<table>
<thead>
<tr>
<th>Controls</th>
<th>CAD</th>
<th>OCU</th>
<th>(mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (a)</td>
<td>Mean (b)</td>
<td>S.D.</td>
</tr>
<tr>
<td></td>
<td>Part 3-1</td>
<td>Part 3-2</td>
<td>Part 3-3</td>
</tr>
<tr>
<td>A</td>
<td>132</td>
<td>134</td>
<td>2.60</td>
</tr>
<tr>
<td>B</td>
<td>186</td>
<td>195</td>
<td>5.08</td>
</tr>
<tr>
<td>C</td>
<td>243</td>
<td>239</td>
<td>2.33</td>
</tr>
</tbody>
</table>

\[ r = 0.999 \ (p < 0.01) \]
\[ r^2 = 0.998 \]
\[ y = 1.055x - 10.803 \]
\[ y = 1.109x - 14.469 \]
\[ y = 1.440x - 22.004 \]
\[ y = 1.056x - 9.524 \]

\[ r : \text{Correlation coefficient.} \]
\[ r^2 : \text{Coefficient of determination.} \]
Agreement with CAD values was fair. The coefficient of determination, \( r^2 \), was 0.982, and the regression line expressed as \( y = 0.792x + 7.72 \) gave excellent results. The series 2 was intended as a check of the reproducibility of Part 1-1. The coefficient of determination was high, \( r^2 = 0.988 \) and \( y = 1.061x - 3.021 \), but the low concentration was only 94% of that of the CAD. Therefore, when the day-to-day variations of double measurements were compared with those of all three control concentrations, the variations were found to be parallel.

(2) Part 2 (Series 1: September, 1979; series 2: June, 1980): The results from control sera are shown in Table 4. In Part 2-1 the low concentration was 94% of that of the CAD. Although there was a high coefficient of determination, \( r^2 = 0.978 \), and the regression line, \( y = 1.118x - 10.592 \), most OCU values were unstable falling in a range of 91 to 101% (mean = 96%), with the coefficient of variation (CV) ranging from 0.4 to 7.1% (mean = 3.5%). The standard deviation was larger than that of the CAD, and each mean OCU concentration was 4% lower. Accordingly, the present methodology was carefully reviewed, and the main problems were found in supernatant sampling of the deproteinized serum and serum sampling.

In Part 2-2, serum sampling and reagent injection were automated with an automatic dilutor, and the standard deviation was reduced for each concentration (CVs from 0.3 to 3.4%, mean CV = 1.9%) (Table 5). Precision had therefore been satisfactorily improved. The analytical values for the concentrations ranged between 96 and 98% (mean = 98%) which raised the level of accuracy. Correlation with the CAD values in Part 2-2 was acceptable, \( r^2 = 0.996 \) and \( y = 1.016x + 0.436 \).
DISCUSSIONS

1. Standardization of total serum cholesterol measurements

When the attempt was begun to standardize serum lipid measurements in 1979, CH was measured by a modified version of the Zak-Henly method (Za-H)\(^6-8\). On the basis of this, our laboratory was certified after successful completion of Part 1 to 4 of the program over the course of approximately a year\(^9\). But, with the addition of the three problems discussed under introduction above, the Za-H method itself made it technically difficult to mix the samples with sulfuric acid, and color presentations of the standard solution were unstable. In fact, the same samples had to be remeasured because control sera were not within the range permitted by internal quality control. In 1980, the installation of automatic analyzers made it possible to begin to standardize CH using the same method (LB) employed by the CAD. In Part 1 of LB standardization, self-evaluation was done only once because Za-H standardization had been complete by that time.

For Part 1 to 3, the CAD instructions were that all three concentrations should be under 100%. In fact, although the former were within the permissible CDC range, all three concentrations were 5 mg/dl higher than CDC reference values. For this reason, standard calibrations were repeated, and in Part 4, the control sera were 97 to 100% of the CAD values, so that good reproducibility had been obtained. Accuracy under the CAD external quality control will be checked twice annually in the future.

Now let us suppose that the CAD cumulative average is the CDC reference value, then do the present CH measurements satisfy the CDC reference value?

According to CDC calculations the mean (\(M\)) and standard deviation (S. D.) are obtained from double measurements of the three concentrations following Part 3. For example, assuming that the measurements for one run of a certain concentration are \(x\) and \(y\), that their difference is \(D\) and that \(N\) is the
number of runs, $M$ and S. D. may be obtained by:

\[
S_x^2 = \frac{\sum x^2 - (\sum x)^2}{N-1} \quad S_y^2 = \frac{\sum y^2 - (\sum y)^2}{N-1}
\]  

$S_{\text{overall}}^2 = \frac{S_x^2 + S_y^2}{2}$ (overall variance)  

$S_{\text{overall}} \sqrt{S_{\text{overall}}^2}$ (overall S. D.)  

\[
M = \frac{\sum x + \sum y}{2N} \quad \text{(overall mean)}
\]

Equation (1)

Equation (2)

Equation (3)

Equation (4)

Table 8 shows $M$s and S. D.’s from Parts 3 and 4.

According to the CDC criteria for acceptable accuracy, the $M$ of any concentration must fall within a range of $\pm 5\%$ of the reference value (R. V.). In Table 8, $a$’s are $5\%$ R. V. values for each concentration, and R. V. $\pm a$ or $b$ is the permissible range. The $M$s of CH measurements were within $b$, except for the high concentration (C) in Part 3-3, where, despite calibration readjustments, the analytical value was low, and a faulty colorimeter was the cause. Based on acceptable precision criteria, the upper limit of S. D. must be $7\,\text{mg/dl}$ for low and medium concentrations (100 to 199 mg/dl), and $8\,\text{mg/dl}$ for high concentration (200 to 299 mg/dl). All the S. D.’s of the CH measurements were less than $7\,\text{mg/dl}$ for $c$ of Table 8, or less than $8\,\text{mg/dl}$ for $d$. Therefore with the exception of Part 3-3, where there was autoanalyzer trouble, the criteria for precision and accuracy were met.

2. Standardization of serum triglycerides measurements

| Table 8 Cholesterol compared to CDC criteria for acceptable performance. |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Controls | CAD(mg/dl) | OCU(mg/dl) | Part 3-1 | Part 3-2 | Part 3-3 | Part 3-4 | Controls | CAD(mg/dl) | OCU(mg/dl) |
| R. V. | $a$ | $b$ | Mean $^*$ | S. D. $^*$ | Mean $^*$ | S. D. | Mean $^*$ | S. D. | Mean $^*$ | S. D. | Part 4 |
| A | 132 | 7 (125-139) | 134 3.20$^{**}$ | 130 1.73$^c$ | 134 2.16$^c$ | 133 1.68$^c$ | D | 121 | 7 (114-128) | 120 2.99$^c$ |
| B | 187 | 9 (178-196) | 189 5.16$^c$ | 183 4.97$^c$ | 183 3.60$^c$ | 187 2.38$^c$ | E | 187 | 9 (178-196) | 188 4.28$^c$ |
| C | 243 | 12 (231-255) | 239 2.39$^{**}$ | 231 3.82$^d$ | 231 4.99$^d$ | 238 2.42$^d$ | F | 245 | 12 (233-257) | 233 4.86$^d$ |

$^*$1: R. V. = Reference value (CAD is the reference value for the CDC).

$^*$2: $a$ = $5\%$ of R. V. (Maximum deviation from the Mean).

$^*$3: $b$ = R. V. + $a$ (Acceptable deviation).

$^*$4: Arithmetical mean of individual results for each control sample.

$^*$5: Overall standard deviation for square roots of the sum of the within day and day to day variance components.

$^*$6: $c$ = maximum overall standard deviation = $7\,\text{mg/dl}$.

$^*$7: $d$ = maximum overall standard deviation = $8\,\text{mg/dl}$.

| Table 9 Triglyceride compared to CDC criteria for acceptable performance. |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Controls | CAD(mM) | OCU(mM) | Part 3-1 | Part 3-2 | Controls | CAD(mM) | OCU(mM) | Part 4 |
| R. V. | $a$ | $b$ | Mean $^*$ | S. D. $^*$ | Mean $^*$ | S. D. | Part 4 |
| A | 0.79 | 0.10 (0.69-0.89) | 0.76 0.035$^{**}$ | 0.78 0.041$^c$ | D | 0.80 | 0.10 (0.70-0.90) | 0.80 0.025$^c$ |
| B | 1.15 | 0.11 (1.04-1.26) | 1.14 0.062$^{**}$ | 1.17 0.053$^d$ | E | 1.41 | 0.11 (0.30-0.52) | 1.42 0.034$^d$ |
| C | 1.65 | 0.11 (1.54-1.76) | 1.60 0.036$^d$ | 1.64 0.064$^d$ | F | 1.71 | 0.11 (1.60-1.82) | 1.68 0.040$^d$ |

$^*$1-5: See in Table 8.

$^*$6: $c$ = maximum overall standard deviation = 0.080mM.

$^*$7: $d$ = maximum overall standard deviation = 0.090mM.
According to CAD evaluations, \( \bar{X} - R \) control chart and correlation coefficients were satisfactory for Part 1-1. However, as experiments continued (Parts 1-2 and 2-1), only low concentration sera were lower than CAD values. The operating methods were completely reviewed, and the serum sampling and reagent injecting procedures were automated, whereupon, both accuracy and precision were improved significantly (Part 2-2 in Table 5). Comprehensive reviews by the CAD of Part 1 to 4 shared stable levels for the present TG measurements although these were lower than all CAD values.

As with CH, Ms and S. D.'s are obtained from the double measurements. However, in the case of TG, as standard substances vary in molecular weight, it is necessary to convert them into the mM units when comparisons are to be made with other data. This unit is obtained by multiplying the mg/dl (mg%) value by a certain factor, which differs for each standard substance. In the present case, since the standard substance was triolein, the factor was 0.0113. The Ms and S. D.'s from Parts 3 and 4 are summarized in Table 9.

According to CDC accuracy requirements, \( M \) must be within \( R \). \( V \geq 9 \) mg/dl (0.10 mM) for low concentrations (0 to 88 mg/dl), or within \( R \). \( V \geq 10 \) mg/dl (0.11 mM) for medium or high concentrations (89 to 176 mg/dl). In Table 9, \( a \) is the limit of \( M \) and \( R \). \( V \geq a \), or \( b \), is the permissible range.

For TG measurements, most \( M \) values were within \( b \). On the other hand, according to CDC precision requirements, the upper limit of \( S. D. \) must be 7 mg/dl (0.080 mM) for low concentrations, and 8 mg/dl (0.090 mM) for medium or high concentrations. Here \( c \) was found to be less than 0.080 mM and \( d \) less than 0.090 mM (Table 9). The criteria for precision and accuracy have thus been met.

**SUMMARY**

In any epidemiologic study of cardiovascular disease, standardized total serum cholesterol (CH) and triglyceride (TG) measurements are indispensable if different studies are to be compared and secular changes within a group evaluated. In the present study, a lipid standardization program was begun in 1979 under the directions of the Center for Adult Diseases (CAD) in Osaka which had already standardized CH and TG as part of a joint program of the U. S. Center for Disease Control (CDC). After two years, this laboratory was found to meet both precision and accuracy criteria when measuring serum cholesterol and triglyceride using CDC reference values.

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循環器疾患の疫学的研究における
血清脂質測定の標準化について

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循環器疾患の疫学的研究において血清総コレステロール（CH）と、中性脂肪（TG）の測定方法が標準化されると、種々の研究の相互比較が可能になるばかりでなく、集団内の経年推移を評価することなどができるようになる。著者らは、先に Center for Disease Control (CDC) のプログラムに参加して、CH と TG の標準化を達成した大阪府立成人病センター (CAD) に指導を受け、1979年より、Lipid Standardization Program に取り組んだ。その後2年を費やして、著者らの CH、TG の測定値が、正確度と精密度の両面において、CAD の基準値を通して、CDC の許容基準を満足していることを示した。

Key words: Standardization, Serum cholesterol, Serum triglyceride, Cardiovascular diseases, Epidemiologic study

標準化、血清コレステロール、血清トリグリセライド、循環器疾患、疫学調査

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