Aqueous Primary Standard for Use in Measuring Cholesterol by the Cholesterol Oxidase Method

John Abele and Hassan Khayam-Bashi

An aqueous primary standard is needed for measuring cholesterol by enzymatic procedures. Sodium deoxycholate solubilizes cholesterol in water. Crystalline cholesterol (1.00, 3.00, 4.00, and 5.00 g/L), added to a solution containing 150 g of this compound per liter of a 9 g/L saline solution, was measured by a cholesterol oxidase procedure, with a centrifugal analyzer. The solubilizer did not interfere. When compared to an isopropanol-based commercial cholesterol standard, the cholesterol standards in solubilizer showed excellent correlation (r = 0.986; m = 0.989). Day-to-day variation for the mixture during nine days was small (CV, 2.9% at 1.00 g/L, 3.7% at 3.00 g/L, and 1.8% at 4.00 g/L). Linearity was maintained up to 5.00 g/L. The cholesterol concentration in four reference sera so analyzed maintained CV's of <4%. The viscosity of the mixture was similar to that of serum. The standard mixtures, stored at room temperature for 360 days, remained stable. The solubilized cholesterol standard is shown to be suitable for use in the enzymatic procedure.

Additional Keyphrases: centrifugal analyzer · cholesterol standards · coronary artery disease · hypercholesterolemia · serum cholesterol · serum lipids · sodium deoxycholate · bile salts

The association of an increased value for total cholesterol in serum and increased risk of heart disease is documented (1, 2). The effectiveness of therapeutic measures to lower blood cholesterol is being further clarified, but there remains a need to measure total serum cholesterol (1, 3) and the cholesterol content of high-density lipoprotein fractions (3). Accuracy is necessary for diagnosing hypercholesterolemia, and precision is essential for monitoring its treatment. Both precision and accuracy are a function of analytical method, available equipment, and appropriate standards. For cholesterol determinations, the first two considerations are well satisfied in the cholesterol oxidase method (4) as adapted to the centrifugal analyzer (5).

Availability of an aqueous primary cholesterol standard remains a problem. Kumar and Christian (6) recently reviewed several alternative means of solubilizing cholesterol and found none to be totally satisfactory for their enzymatic method (7, 8). Considering the solubility of cholesterol in bile salts (9), we developed a convenient, stable, inexpensive cholesterol standard, with aqueous sodium deoxycholate as a solubilizer (10). This primary deoxycholate cholesterol (DOCC) standard can be easily prepared from safe, commercially available materials and is a useful alternative to the standards mentioned in the previously cited review.

Materials and Methods

We dissolved 150 g of crystalline sodium deoxycholate (Matheson, Coleman and Bell, Norwood, OH 45212) per liter in isotonic saline solution (NaCl, 9 g/L), with the help of gentle continuous stirring and warming to 40 °C. Crystalline cholesterol ("chromatography-grade," >99% pure; Sigma Chemical Co., St. Louis, MO 63178) was dispensed into volumetric flasks, and the sodium deoxycholate solution was added to yield primary cholesterol standards containing 0.00, 1.00, 3.00, 4.00, and 5.00 g/L. Dissolution was again aided by gentle continuous stirring at 40 °C. These DOCC standards were assayed by the cholesterol oxidase (EC 1.1.3.6) procedure of Allain et al. (4), as adapted (5) to a centrifugal analyzer (Centrifichem, Model 400; Union Carbide Corp., Rye, NY 10580) with use of commercial reagents (Worthington Corp., Freehold, NJ 07728).

We assayed 12 multiple aliquots of the mixture during nine days. The changes in absorbance were recorded by the instrument's computer in a "Data Points" format. Also included in these 12 assays were aliquots of four protein-based lyophilized reference sera: "Monitrol 1," "Monitrol 2," "Choles-Trol," and "Choles-Trol D" (DADE Division of American Hospital Supply Corp., Miami, FL 33152). The concentrations of cholesterol in these reference sera were obtained by several nonenzymatic methods (11), as well as an enzymatic method (Technical Services Dept., Union Carbide Corp.). In this paper, Choles-Trol and Choles-Trol D are referred to as DADE I and DADE II.

We also investigated a commercial cholesterol standard (NERL; New England Reagent Laboratories, East Providence, RI 02914), in which isopropanol is used as the solvent and which is available in concentrations ranging from 0.50 to 4.00 g/L. We analyzed aliquots of 100 samples of serum, using first the DOCC standards and then the NERL standards.

To evaluate the stability of the DOCC standards, we stored the 0.00, 1.00, and 3.00 g/L solubilized standards at 4 °C for seven months, then re-analyzed them as before. Additionally, NERL standards, Monitrol 1, Monitrol 2, DADE I, and DADE II were reassayed with the use of the aged standards. Also, three additional samples of the DOCC standard (0.5, 1.00, and 3.00 g/L) were stored at room temperature for 360 days, then re-analyzed for cholesterol content.

We spectrophotometrically scanned the DOCC and NERL standards at 1.00, 2.00, and 3.00 g/L cholesterol with a re-

Clinical Biochemistry Division, Clinical Laboratory, Bldg. 100, San Francisco General Hospital Medical Center, 1001 Potrero and 22nd St., San Francisco, CA 94110.

1 Address correspondence to this author.

2 Ed. note: See also the paper by Proksch and Bonderman, extending their work on this problem, in our November issue, p 1924. Received Oct. 25, 1977; accepted Sept. 25, 1978.
Table 1. Change in Absorbance of Cholesterol Standards on Storage

<table>
<thead>
<tr>
<th>Chol. concn., g/L</th>
<th>0.00</th>
<th>1.00</th>
<th>3.00</th>
<th>4.00</th>
<th>5.00</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \Delta ) Absorbance, multiplied by 1000</td>
<td>2.04 (-4.22)(^a)</td>
<td>195.4 (179.6)</td>
<td>597.5 (577.7)</td>
<td>817.2</td>
<td>1000</td>
</tr>
<tr>
<td>Mean</td>
<td>2.26 (3.83)</td>
<td>5.58 (4.54)</td>
<td>21.9 (13.32)</td>
<td>14.5</td>
<td>39.6</td>
</tr>
<tr>
<td>SD</td>
<td>2.9 (2.53)</td>
<td>3.7 (2.31)</td>
<td>1.8</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>CV, %</td>
<td>No. detns.</td>
<td>11 (9)</td>
<td>10 (7)</td>
<td>13 (9)</td>
<td>6</td>
</tr>
</tbody>
</table>

\(^a\) Values in parentheses are those for the seven-month-old deoxycholate cholesterol standard, kept at 4 °C.

Table 2. Total Cholesterol Content of Control Sera as Measured by Use of Deoxycholate Cholesterol Standards

<table>
<thead>
<tr>
<th>Reference serum stated values</th>
<th>Monitrol 1</th>
<th>Monitrol 2</th>
<th>DADE I</th>
<th>DADE II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration, g/L</td>
<td>1.01 ± 0.098(^a)</td>
<td>2.70 ± 0.88(^a)</td>
<td>1.89(^b)</td>
<td>3.65(^b)</td>
</tr>
<tr>
<td>Mean</td>
<td>0.8265</td>
<td>2.408</td>
<td>2.034</td>
<td>3.617</td>
</tr>
<tr>
<td>SD</td>
<td>0.0298</td>
<td>0.104</td>
<td>0.0740</td>
<td>0.0870</td>
</tr>
<tr>
<td>Range</td>
<td>0.77-0.88</td>
<td>2.21-2.48</td>
<td>1.92-2.21</td>
<td>3.41-3.86</td>
</tr>
<tr>
<td>CV, %</td>
<td>3.61</td>
<td>4.33</td>
<td>3.84</td>
<td>2.40</td>
</tr>
<tr>
<td>No. detns.</td>
<td>22</td>
<td>21</td>
<td>29</td>
<td>24</td>
</tr>
</tbody>
</table>

\(^a\) Reported by DADE (all methods, all 93 labs.; West Coast, Nov.-Dec., 1976).
\(^b\) Reported by Union Carbide for enzymatic analysis (see Methods).

cording spectrophotometer (Model 25; Beckman Instruments, Inc., Fullerton, CA 92634).

Results

With the commercial enzymatic reagents, no cholesterol was detected in the solubilizing medium alone, and it was used as a 0.00 g/L standard, which had a mean change in absorbance of 0.002 ± 0.004 (2 SD).

The relationship between cholesterol content and absorbance was used to develop a standard curve (Table 1). In 49 determinations of cholesterol concentrations between 0.00 and 5.00 g/L, the slope (m) was 4.938 g/L per unit of absorbance change, and the correlation coefficient (r) was 0.998. The coefficients of variation (CV's) for each concentration of standard were less than 4%. The cholesterol oxidase reaction was linear to 5.00 g/L for the solubilized DOCC standards. The relationship between the solubilized and NERL standards (n = 100) was linear (m, 0.986; r, 0.989). Table 2 summarizes the results of assays of the four reference sera with use of the solubilized DOCC standard. The CV's ranged from 2 to 4%.

Figure 1 shows spectrophotometric scans of the DOCC standards in the presence of all the reagents. The absorption curves at different cholesterol concentrations showed patterns for this standard similar to those for the NERL standard or lyophilized control sera. The molar absorptivity value, measured by use of the 3.00 g/L DOCC standard was 7.04 × 10\(^3\); this compares with 6.96 × 10\(^3\) for a similar NERL standard. The expected molar absorptivity was 6.89 × 10\(^3\) (Worthington/Gilford cholesterol reagent insert, September 1976).

The DOCC standards developed a slight turbidity during refrigeration, which disappeared after warming the standards at 37 °C for 30 min. In stability studies, a repeat of the DOCC standard curve data with use of seven-month-old standards is presented in Table 1 (values in parentheses). The slope was 5.17 g/L/\( \Delta \) absorbance and r was 0.998. Re-analysis of the three specimens of 360-day-old DOCC standards (0.5, 1.00, and 3.00 g/L) measured 0.50, 1.04, and 3.02 g/L, a mean of 102% for all three.

The NERL standards, when analyzed for cholesterol with

---

**Fig. 1. Absorption spectrum of cholesterol deoxycholate standard**

A, deoxycholate, 150 mg/liter; B, C, D: 0.50, 1.00, and 3.00 g/L cholesterol in 150 mg/L deoxycholate, respectively. Solid line: DOCC standard; broken line: New England Research Laboratories standard.

**Fig. 2. Cholesterol content of sera, as measured with use of New England Research Laboratories and DOCC standards**
Table 3. Total Cholesterol Content of Control Materials as Measured by Use of Seven-Month Old Deoxycholate Cholesterol Standard

<table>
<thead>
<tr>
<th>Material Stated values (A)</th>
<th>NERL standards 0.500</th>
<th>1.00</th>
<th>3.00</th>
<th>Montrol 1 1.77 ± 28 a</th>
<th>Montrol 2 3.28 ± 54 a</th>
<th>DADE I 1.89 b</th>
<th>DADE II 3.85 b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (B)</td>
<td>0.518</td>
<td>1.043</td>
<td>3.104</td>
<td>1.663</td>
<td>2.958</td>
<td>2.067</td>
<td>3.695</td>
</tr>
<tr>
<td>SD</td>
<td>0.0197</td>
<td>0.0520</td>
<td>0.0945</td>
<td>0.0566</td>
<td>0.0852</td>
<td>0.0854</td>
<td>0.1043</td>
</tr>
<tr>
<td>CV, %</td>
<td>3.8</td>
<td>5.0</td>
<td>3.0</td>
<td>3.4</td>
<td>2.9</td>
<td>4.1</td>
<td>2.8</td>
</tr>
<tr>
<td>No. detns.</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>9</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>[(B - A)/A] × 100</td>
<td>3.6</td>
<td>4.3</td>
<td>3.5</td>
<td>-7</td>
<td>-10</td>
<td>9.4</td>
<td>1.2</td>
</tr>
</tbody>
</table>

* Reported by DADE X ± 2 SD (all methods, all 144 labs., West Coast, May–June 1977).
* Reported by Union Carbide for enzymatic analysis (see Methods).
* Values for assays with use of fresh deoxycholate cholesterol standards.

the seven-month-old DOCC standard, had concentrations within 4% of the nominal value (Table 3). Assay values for the four control sera are also included.

Discussion

Simple determination of total serum cholesterol, with emphasis on precision and accuracy, has been shown to be as reliable an index of coronary artery disease as are the more sophisticated analyses for lipoproteins (3).

Kumar and Christian (6) discuss the problems in preparing aqueous cholesterol standards applicable to enzymatic methods. Cholesterol has a low solubility in water (2.6 g/L at 20 °C) and dissolves very slowly (12). Common organic solvents such as isopropanol, benzene, and ether readily dissolve cholesterol, but these solvents are toxic, flammable, do not resemble an aqueous-physiologic system, and are unsuitable for use in enzymatic methods. Alternative solvents for primary cholesterol standards have been proposed (7, 8), but the enzymatic studies of Kumar and Christian show these solvents to be unsatisfactory. Recently, a water-soluble cholesterol derivative for use in lyophilized sera was reported (13).²

The disparity between values stated (by the supplier) and the values found in Table 2 for two of the reference sera was expected. As noted, the values for Montrol 1 and 2 were those supplied by the manufacturer and represent a compilation from a variety of nonenzymatic methods. The assay values reported here were obtained by the enzymatic cholesterol oxidase method, which gives as much as 22% lower values than do nonenzymatic methods (14). Union Carbide Corp. specifically supplied our laboratory with their enzymatically determined values for the particular lots of DADE I and II used in this study. The concentration of cholesterol in DADE II, as determined with use of the DOCC standard, agreed with Union Carbide's enzymatic determinations of this material. The reason for the 9% difference between our cholesterol determination of DADE I and that of Union Carbide Corp. remains unclear.

The DOCC standard was stable for seven months (Table 3). Stability of standards at room temperature for 360 days was also excellent. Accordingly, we recommend that the DOCC standards be stored at room temperature. Storage at 4 °C causes the standards to develop a slight turbidity, which disappears on incubating the mixture at 37 °C for 30 min without any change in the cholesterol value. We routinely store the standard at room temperature for three months before making a fresh one.

Several lyophilized, protein-based control sera that might serve as secondary standards have been available for some time. These products have several disadvantages: incomplete dissolution on reconstitution, a relatively short shelf life after reconstitution, and availability only in supplier-determined concentrations. More importantly, the manufacturer's labeled cholesterol concentrations may be derived by use of comparison studies with another standard, not a primary standard. If the manufacturer's assay method is not enzymatic, the stated value is usually not applicable to values obtained by enzymatic assay.

The aqueous primary DOCC standard, with deoxycholate (150 g/L) as a solubilizer, provides a stable, reliable standard of known cholesterol concentration ranging up to 5.00 g/L. Deoxycholate does not interfere with the cholesterol oxidase method and remains stable at room temperature.

The advantages of the DOCC standard over commercial isopropanol-based standards include: use of a nonvolatile solvent that resembles a physiologic system, lower expense, and availability in any desired range of concentrations. Users of enzymatic cholesterol methods should consider aqueous primary standards rather than lyophilized commercial sera. The DOCC standard has now been evaluated in our laboratory for more than one year and is currently in routine use.

The editorial assistance of Hilda Griscom, Ph.D., is gratefully appreciated. We are also thankful to Vern Walter for technical assistance, to Ms. Susan Estvold for her assistance in completing these studies, and to Ms. Lori Lewis for graphics and secretarial assistance.

References

A Further Comparison of Radioassay Results for Serum and Plasma

Norman P. Kubasik and Harrison E. Sine

We evaluated results for serum vs. plasma for 12 selected radioassay procedures. Blood samples were collected with heparin, oxalate-fluoride, or ethylenediaminetetraacetate as anticoagulant and compared with the corresponding serum samples. In combination with a previous report [Kubasik, N.P., and Sine, H.E., *Clin. Chem.* 24 137 (1978)] we have now tested a total of 25 different analytes, using 31 manufacturers’ kits. Differences were again demonstrated between serum and plasma that may be of sufficient magnitude to alter clinical interpretation of the results. Assays also demonstrated differences based on the procedure used.

Additional Keyphrases: variation, source of “kit” methods

We have previously compared results for serum and plasma in 15 selected radioassays, using 19 manufacturers’ kits (1). In this report we compare the differences between serum and heparinized plasma, oxalate–fluoride-treated plasma, and ethylenediaminetetraacetate(EDTA)-treated plasma for 12 more selected radioassay procedures.

**Methods and Materials**

We used the following radioassay kits:
- *Aldosterone* ("125I Aldosterone Assay Kit"; Diagnostic Products Corp., Los Angeles, CA 90064).
- *Choriosomatotrophin* ("Phadebas hCS(hP) Test"; Pharmacia Diagnostics).
- *Digoxin* ("Quantitope 125I-Digoxin"; Kallestad Laboratories, Inc., Chaska, MN 55318).

**Folate** ("Dualcount, Vitamin B-12 (57Co)-Folic Acid (125I)"); Diagnostic Products Corp.

**Gentamicin** ("125I Gentamicin RIA Kit"; Diagnostic Products Corp.).

**Hepatitis B Surface Antigen** ("Austria 11-125"; Abbott Laboratories, North Chicago, IL 60064).

**Immunoglobulin E** ("Phadebas IgE Test"; Pharmacia Diagnostics).

**Lutropin** ("Immuo-LH"; Pantex, Malibu, CA 90265).

**Testosterone** ("Immuno-Testosterone-125I"; Pantex).

**Vitamin B12** ("Dualcount, Vitamin B12 (57Co)-Folic Acid (125I)"; Diagnostic Products Corp.).

Blood was sampled from 12 volunteers. Four specimens for serum and plasma samples were drawn at the same time from each volunteer. Serum was collected in an evacuated blood-collection tube (Vacutainer Tube no. 4670, red-stoppered; Becton, Dickinson and Co., Rutherford, NJ 07070); plasma samples were collected in three different Vacutainer Tubes: ammonium heparin, no. 3200-XF183, green-stoppered; potassium oxalate–sodium fluoride, no. 4500, gray-stoppered; and tripotassium EDTA, no. 4675, lavender-stoppered. Serum or plasma samples not immediately assayed after collection were divided and frozen.

Triplicate samples were assayed for the metabolite of interest within the same assay run, to eliminate run-to-run variability. All tritium-labeled assay supernates were decanted into 10 mL of “Aquasol” liquid scintillation solvent (New England Nuclear, Boston, MA 02118) and counted with an LS-230 liquid scintillation system with use of an automatic external standardization for quench corrections (Beckman Instruments, Inc., Irvine, CA 92664). Iodine-labeled samples were counted in a Model 1185 Gamma System (Searle Analytic, Des Plaines, IL 60018).

Radioassay data were statistically examined by use of a 9830 Hewlett Packard calculator, with a Rodbard (2) statistical approach in which variance-weighted least-square regression analysis was performed on the logit transformation of the percent bound (B/B0).

Statistical analysis of the data (analysis of variance and F-test) was done with the aid of the 9830 Hewlett Packard.