Assay Instrument-Dependent Matrix Effects in Standardization of Cholesterol Measurements

Parvin P. Waymack, W. Greg Miller, and Gary L. Myers

Human serum-based frozen reference materials have been used by the Centers for Disease Control and Prevention (CDC)—National Heart, Lung and Blood Institute Lipid Standardization Program to improve the precision and accuracy of blood cholesterol measurements. Occasionally, laboratories in the program have had problems obtaining results for patients' fresh serum samples equivalent to those obtained with frozen CDC standardization pools. This incompatibility of sample, reagent, instrument, and assay characteristics has been labeled broadly as a "matrix effect," which usually is attributed to unknown characteristics of the processed pool material. In this study we showed that a large negative bias obtained with CDC pools was attributable to use of the sample blank mode on the Cobas-Bio analyzer. However, under the same conditions, fresh patients' serum samples were analyzed accurately. The use of a blank absorbance immediately after mixing sample and reagents (the "autoblank" mode) allowed the instrument to accurately analyze both fresh serum samples and CDC standardization pools and thus allowed the documentation of traceability of the cholesterol measurements to the National Reference System for Cholesterol.

Indexing Terms: Reference Method • variation, source of • centrifugal analyzer • National Reference System

The Laboratory Standardization Panel of the National Cholesterol Education Program has made recommendations for improving the reliability of cholesterol measurements (1). The Panel recommends that cholesterol measurements made in all US clinical laboratories be standardized so that cholesterol values are traceable to the National Reference System for cholesterol (NBS/CHOL) (2), which consists of the Centers for Disease Control and Prevention (CDC) Reference Method (3, 4) and the National Institute of Standards and Technology Definitive Method (4, 5).4

The CDC—National Heart, Lung and Blood Institute Lipid Standardization Program has successfully used commercial sources of serum to formulate pools (sometimes supplemented with lipid concentrates) to establish the traceability of cholesterol measurements to NRS/CHOL. To evaluate analytical bias, the program uses calibrators and reference materials that have been assigned values by the Reference Method. This approach assumes that the reference materials and fresh human serum samples perform similarly in the cholesterol assay.

One problem that the CDC standardization program has encountered with some instrument systems is apparent differences between fresh serum and processed frozen CDC pool material. These differences, referred to as "matrix effects," cause disagreement between the results obtained from some chemistry analyzers and the Reference Method values, which limits the universal use of these materials for standardization (6–8). The nature and causes of matrix effects are poorly understood, but they can adversely affect accuracy if they are not recognized. For example, several recent studies indicate significant discrepancies between the cholesterol concentrations of lyophilized and frozen processed serum materials measured by some routine enzymatic methods and those measured by the Abell–Kendall method (8–13). In these cases, if one adjusts the calibration of the instrument system to obtain accurate values for the reference materials, the system produces inaccurate cholesterol measurements of patients' fresh serum samples.

We describe here the resolution of an apparent matrix problem encountered at the Medical College of Virginia (MCV) lipid laboratory, which was unable to meet CDC standardization program criteria for accuracy when a Cobas-Bio analyzer was used. We verified the overall accuracy of the MCV Cobas-Bio analyzer in comparison with the CDC Reference Method by assaying split samples of fresh human serum. Using a Cobas-Bio analyzer, the CDC laboratory confirmed the matrix problem we encountered in analyzing CDC pools. To solve the problem, we chose an alternative blanking mode that allowed accurate analysis both of patients' fresh serum samples and of CDC standardization pools in the same run.

Materials and Methods

Materials

Fresh serum samples were collected and analyzed at MCV; aliquots were then frozen and shipped to CDC on solid CO2. Aliquots were stored at -60 °C until analysis by the Abell–Kendall assay or further enzymatic analysis. The CDC standardization pools were prepared from human serum as previously described (14) and stored frozen at -18 or -60 °C. Single-use 0.22- or 0.45-μm (pore size) filter units (Millex-HV, SLHV025NS) from Millipore (Bedford, MA) were used for the filtration experiments.

1 Center for Environmental Health, Centers for Disease Control and Prevention, Public Health Service, US Department of Health and Human Services, Atlanta, GA 30333.
2 Medical College of Virginia, Richmond, VA 23286.
3 Author for correspondence. Fax 404-488-4609; SMTP: PPWI@CHEHBLLEM.CDC.GOV.
4 Nonstandard abbreviations: NBS/CHOL, National Reference System for Cholesterol; CDC, Centers for Disease Control and Prevention; and MCV, Medical College of Virginia.

Received November 2, 1992; accepted April 22, 1993.
Enzymatic analysis. In the MCV lipid laboratory, researchers using a Cobas-Bio analyzer (Roche Diagnostics, Nutley, NJ) with Boehringer Mannheim Diagnostics (Indianapolis, IN) Cholesterol High-Performance-K reagents and Preciset cholesterol calibrators analyzed for total cholesterol fresh serum samples in duplicate in two runs. They used the Cobas-Bio analyzer in either the type 1 or type 5 analysis mode (see below) with the optional settings listed in Table 1. At CDC, researchers analyzed the fresh serum samples and a set of CDC pools for total cholesterol, using either the type 1 or type 5 analysis mode with the same settings as listed in Table 1, Boehringer Mannheim Diagnostics Cholesterol High-Performance-K reagents, and Preciset cholesterol calibrators. Analysts also determined total cholesterol concentrations by using the same reagents and cholesterol calibrators with an Abbott (Abbott Park, IL) VP automated analyzer, set as recommended by the manufacturer.

Reference Method analysis. Analysts in the CDC Lipid Reference Laboratory used the Abell–Kendall Reference Method (15, 16) in 12 runs (n = 48) to determine reference values for total cholesterol in the CDC reference pools. They also measured fresh serum total cholesterol values in duplicate by the Abell–Kendall Reference Method.

Instrument Settings and Calculations

Type 1 analysis mode

\[
\left( A_n - A_{\text{AUX}} \right)_\text{sample} - \left( A_n - A_{\text{AUX}} \right)_\text{blank}
\times \text{calibration factor} = \text{concentration}
\]  

(1)

The type 1 analysis is a programmed protocol that dilutes the serum sample with water (4 μL of serum and 25 μL of water in this case) and measures the absorbance of the mixture. This estimate of the sample’s contribution to the final absorbance \( A_{\text{AUX}} \) is subtracted from the final reaction absorbance \( A_n \), which is measured after the reagents are added and the reaction incubation time elapses.

In the type 1 mode, the initial sample blank absorbance is accurate after reagent is added because the unique variable-pathlength optical system of the Cobas-Bio analyzer makes the absorbance measurement independent of reagent volume (17).

Type 5 analysis mode

\[
\left( A_n - A_0 \right)_\text{sample} - \left( A_n - A_0 \right)_\text{blank}
\times \text{calibration factor} = \text{concentration}
\]  

(2)

In the type 5 analysis protocol, the sample, diluent, and reagent are mixed, and \( A_0 \), the absorbance at \( t = 0 \) (5 s, in this case), is subtracted from the final absorbance \( A_n \), measured after the reaction incubation time. The difference between the type 1 and type 5 analysis modes is that the type 1 mode measures sample blank absorbance before the addition of reagent, and type 5 measures the absorbance blank of the sample plus reagent immediately after mixing but before significant reaction has taken place. The calibration factor (concentration divided by absorbance) for each of the type 1 and type 5 analyses is calculated automatically by the instrument program, which uses the average absorbance of three within-run standards.

Results and Discussion

Accurate Analysis of Fresh Samples Established

The results of the MCV lipid laboratory analysis of the CDC–National Heart, Lung and Blood Institute Lipid Standardization Program's quarterly survey pools with a Cobas-Bio analyzer revealed a consistent negative bias compared with the CDC target value. The negative bias exceeded 3%, the maximum acceptable bias in the CDC standardization program. To determine the basis for this large negative bias, we initiated a series of studies, including a split-sample comparison of 20 fresh serum samples that were analyzed along with the CDC pools. The results of the analysis of the split fresh samples (Table 2) indicated a good agreement of accuracy between the MCV Cobas-Bio enzymatic method and the CDC Reference Method, with similar results being obtained with CDC’s Cobas-Bio analyzer and Abbott VP analyzer. The Cobas-Bio intercepts and slopes were obtained by using the spreadsheet program of the Cholesterol Reference Method Laboratory Network. With this approach, one can establish and certify accuracy (i.e., the National Cholesterol Education Program criterion of 3%) of results for total cholesterol throughout the comparison range (from about 3.23 mmol/L (125 mg/dL) to 9.05 mmol/L (350 mg/dL) for these studies). The comparison focuses on accuracy at
the critical decision points for cholesterol, 5.17 mmol/L (200 mg/dL) and 6.21 mmol/L (240 mg/dL). The MCV laboratory previously carried out split-sample comparisons with both the CDC Lipid Reference Laboratory and a Cholesterol Reference Method Laboratory Network laboratory, establishing the setpoint in Table 1. An instrument calibrated (via set point adjustments) may lose accuracy above or below the comparison range this way. However, it is a common practice to calibrate instruments differently for accurate cholesterol measurements in the low range (for high-density lipoprotein cholesterol), an action that would be necessary for the Cobas-Bio instruments. For the comparison summarized in Table 2, the CDC instrument used the same setpoint (Table 1), not an optimized setpoint. Nevertheless, both the MCV and the CDC Cobas-Bio instruments were accurate, well within the National Cholesterol Education Program guidelines.

The similar relationship between fresh samples and CDC pools when analyzed with the MCV and CDC Cobas-Bio analyzers is shown in Figure 1. The results in Table 2 and Figure 1 indicate that, because the type 1 assay settings produced accurate results for fresh serum samples, adjusting the calibrator setpoint to give higher values for CDC pools (the results of which were negatively biased in the surveys) would result in an unacceptably high bias in analysis of the fresh serum samples.

The Reagent as a Possible Cause of the Negative Bias

Having established the accurate performance of the MCV and CDC Cobas-Bio analyzers and the Abbott VP analyzer for fresh serum samples (Table 2), we conducted further studies to clarify the effect of reagent, matrix properties, and instrument settings. Because the Abbott VP analyzer was (a) apparently free of matrix bias in one study (b) and (b) able to analyze CDC pools accurately for in-house studies, we thought this analyzer would be useful in evaluating the possible sample-dependent involvement of reagent.

The results of the analysis of three frozen fresh pools (FHS 1–3) and CDC standardization pools, which were performed with the CDC Cobas-Bio analyzer and type 1 settings, and the results from the analysis performed with the Abbott VP analyzer are included in Table 3. The accurate analysis of CDC pools with the VP analyzer is consistent with competent performance by the Boehringer Mannheim Diagnostics reagent in recovering cholesterol (i.e., it solubilizes and allows complete reaction of the apparent aggregates found in some CDC pools). This finding contrasts with the large negative bias found with the Cobas-Bio type 1 assay for CDC pools E, F, G, and H when the same reagent was used. Further studies with the CDC and MCV laboratory Cobas-Bio analyzers showed that incubation time, temperature, sample and reagent volume, and dilution factors did not explain the large negative bias in the analysis of CDC pools E–H. This finding indicates that factors other than the reagent are involved in this apparent matrix effect.

Type 1 vs Type 5 Analyses

Type 1 mode accounts for negative bias in the cholesterol assay. Table 3 also contains a qualitative description of the pool appearance and a typical value for the $A_{Aux}$ obtained for the standardization pools by type 1 analysis. As noted in Table 3, some of the pools contained a uniform suspension of particles visible to the
eye. However, pool G also contained larger sedimenting particles. $A_{\text{AUX}}$ increased as a function of total cholesterol concentration in both the affected and the unaffected pools. In general, however, those pools that showed a large bias by the type 1 analysis showed a much larger $A_{\text{AUX}}$. In the type 1 analysis, the difference between the fresh serum samples and the CDC pools seems to result from a higher $A_{\text{AUX}}$ in the affected pools, which causes a corresponding negative bias when subtracted from the final absorbance, $A_0$ (see equation 1). The light scattering due to turbidity of the pools makes the $A_{\text{AUX}}$ measurement poorly reproducible and invalidates the assumption of ideal solution behavior for a chromophore for the total solution volume-dependent, variable-pathlength, absorbance measurement in the Cobas-Bio analyzer. Also, during the reaction, the solubilizing effect of the reagent probably removes some of the initial turbidity that contributed to $A_{\text{AUX}}$.

**Type 5 mode improves negative bias in the cholesterol assay.** The use of the type 5 endpoint analysis instead of the type 1 analysis mode gave more accurate results for most CDC pools. As shown in Table 3, the large negative bias (vs. the Reference Method results for CDC pools D, E, F, and H) of results obtained with the type 1 analysis is not observed when the type 5 analysis is used. Only the results from pool G still showed large negative bias. Type 5 analysis tended to give slightly higher values in fresh pools (FHS 1–3).

In the type 5 analysis protocol, the sample, diluent, and reagent are mixed, and the approximate absorbance at $t = 0$, $A_0$ (actual minimum $t = 5$), is subtracted from the final absorbance after the programmed reaction time, $n$. Because the Abbott VP analyzer accurately analyzes pool G when the same reagent is used, the failure to recover the reference value probably is not due to slower kinetics or the lack of accessibility of this somewhat particulate sample to the reagents. Instead, the type 5 sample-plus-reagent blanking mode results in a more reliable sample blank absorbance value, because the microparticulate aggregates (presumably containing lipoproteins) are dispersed by the reagent formulation. Pool G is also the only pool that was not accurately analyzed by type 5 analysis (i.e., bias >3%). We surmise that during analysis, this pool, the only one that contained visibly larger and sedimenting aggregates, lost the larger aggregates to the cuvette walls by the centrifugation during analysis.

The condition of pool G was not typical of the CDC standardization pools in service at that time or of those currently in use. When we discovered these studies that pool G contained sedimenting particles, we immediately removed it from further use as a standardization pool.

**Correlation of type 1 and type 5 analyses in fresh samples.** Using 90 fresh serum samples, the MCV laboratory conducted an extensive comparison of the type 1 and type 5 analyses (Figure 2). Deming regression analysis showed: slope = 1.006, $y$-intercept = 0.032, $S_{xy} = 0.116$, and $r = 0.990$; type 5 mean = 5.84, and type 1 mean = 5.77. Thus, both analysis modes produce accurate results for fresh serum samples. However, the type 5 sample-blanking mode is more chemically robust to processed materials such as the frozen pools used in the CDC lipid standardization program.

<table>
<thead>
<tr>
<th>CDC-pool</th>
<th>Abell–Kendall cholesterol, mg/dL</th>
<th>% bias</th>
<th>$A_{\text{AUX}}$</th>
<th>Cobas-Bio type 5</th>
<th>Abbott VP</th>
<th>Appearance of serum pool</th>
</tr>
</thead>
<tbody>
<tr>
<td>FHS 1</td>
<td>185</td>
<td>-1.6</td>
<td>0.0054</td>
<td>+0.5</td>
<td>-1.0</td>
<td>Clear</td>
</tr>
<tr>
<td>FHS 2</td>
<td>200</td>
<td>+0.5</td>
<td>0.0072</td>
<td>+0.7</td>
<td>-0.7</td>
<td>Clear</td>
</tr>
<tr>
<td>FHS 3</td>
<td>224</td>
<td>+0.6</td>
<td>0.0093</td>
<td>+2.0</td>
<td>0</td>
<td>Clear</td>
</tr>
<tr>
<td>A</td>
<td>180</td>
<td>+1.2</td>
<td>0.0067</td>
<td>+0.8</td>
<td>-1.1</td>
<td>Clear</td>
</tr>
<tr>
<td>B</td>
<td>300</td>
<td>+1.3</td>
<td>0.0146</td>
<td>+0.7</td>
<td>+1.3</td>
<td>Clear</td>
</tr>
<tr>
<td>C</td>
<td>126</td>
<td>-0.2</td>
<td>0.0045</td>
<td>-0.3</td>
<td>-2.1</td>
<td>Slightly turbid</td>
</tr>
<tr>
<td>D</td>
<td>276</td>
<td>-2.5</td>
<td>0.0188</td>
<td>+0.4</td>
<td>-0.3</td>
<td>Slightly turbid</td>
</tr>
<tr>
<td>E</td>
<td>214</td>
<td>-7.6</td>
<td>0.0280</td>
<td>-1.4</td>
<td>-0.6</td>
<td>Very fine granular suspension</td>
</tr>
<tr>
<td>F</td>
<td>286</td>
<td>-8.7</td>
<td>0.0558</td>
<td>-2.2</td>
<td>+0.3</td>
<td>Very fine granular suspension</td>
</tr>
<tr>
<td>G</td>
<td>201</td>
<td>-5.8</td>
<td>0.0184</td>
<td>-5.0</td>
<td>-0.9</td>
<td>Coarse granular suspension with a few sedimenting particles</td>
</tr>
<tr>
<td>H</td>
<td>323</td>
<td>-5.6</td>
<td>0.0477</td>
<td>-0.7</td>
<td>+1.7</td>
<td>Milky turbidity</td>
</tr>
</tbody>
</table>

* FHS, frozen pools of fresh serum that were not filtered or supplemented.

![Fig. 2. Cholesterol concentrations measured in patients' fresh serum samples during 8 days with the Cobas-Bio analyzer in type 1 and type 5 sample blanking modes](image-url)

Conditions as listed in Table 1: setpoint = 283 mg/dL for type 1, and 280 mg/dL for type 5
Origin of the Turbidity in the Frozen Pools

The use of a Coulter-type particle counter-sizer (Particle Data Laboratories, Elmhurst, IL) confirmed the presence of aggregates (2–5 μm diameter) in pools E, F, G, and H. Our efforts at filtering these pools gave results consistent with their filtration behavior during preparation. The preparation of standardization pools requires extensive successive filtration in steps starting at 6 μm down to 0.22 μm. This is necessary because turbidity appears when pools are formulated by mixing the component serum units. The filters become rapidly clogged unless successively smaller filtration pore sizes are used.

A small loss in total cholesterol is typically found after each filtration step. In our experience, a cholesterol/triglyceride pool that has been filtered down to 0.22-μm-diameter particles may become slightly turbid and require refiltration if not dispensed within 24 h. Therefore, the filtration data are complex and hard to interpret quantitatively; i.e., clogging results in incomplete filtration or particles are partially removed, corresponding to the filter-pore diameter being used. Our one-step filtration attempt to filter pool G through a 0.22-μm filter resulted in rapidly clogged filters. Filtration through a 0.45-μm filter resulted in slower clogging and allowed us to collect enough filtrate to estimate lipoprotein losses (~8% of the total cholesterol).

We believe the significance of the larger aggregates in pool G is that the particle formation process may have advanced further in this much older pool (stored and used for almost 9 years) compared with other pools (next oldest stored, 3 years). Therefore, we speculate that, with time, storage leads to a progressive aggregation process, with particles becoming so large that they do not stay in suspension (i.e., they settle out, as in pool G). Another possible mechanism for particle formation besides the spontaneous mode described above is based on a common but not universal characteristic of pools stored frozen for longer than a year: the presence of clear ice on the vial walls and above the body of the frozen serum. This transfer of ice from the body of the serum probably leaves behind a partially dehydrated serum on the surface of the frozen serum. When the serum vial is thawed, the dehydrated serum may not redissolve completely, creating a suspension.

In summary, we conclude that the lipoprotein aggregates present in some CDC pools are the likely source of the matrix effect seen when the Cobas-Bio analyzer is used in the type 1 analysis mode. The type 5 analysis produced accurate cholesterol values in fresh serum samples and CDC pools (i.e., <3% bias from the CDC Reference Method). The presence of the visible aggregates described here may also be a source of matrix effects in other systems in which sample blanks are similarly subtracted. Furthermore, when less robust reagents are used, the potential exists for a matrix effect of a kinetic origin to influence measurements of aggregate-containing pools, because full recovery of cholesterol requires that reagents solubilize the aggregates during the reaction time allowed by the analyzer being used.

We thank Charlene Griffith for her capable analysis of samples for cholesterol by the Abell–Kendall method. Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or the US Department of Health and Human Services.

References

2082 CLINICAL CHEMISTRY, Vol. 39, No. 10, 1993