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Effect of Lyophilization on Results of Five Enzymatic Methods for Cholesterol

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We studied the effect of lyophilization of serum pools on the determination of cholesterol with the enzymatic methods used in five automated analyzers: SMAC (Technicon), the RA-1000 (Technicon), the aca (Du Pont), the TDX (Abbott), and the Ektachem 700P (Kodak). We prepared two serum pools: pool A (2.2 g/L) and pool B (1.9 g/L). We separated each pool into three groups for treatment by lyophilizing or freezing at −20 and −70 °C. We determined cholesterol by the above methods at regular intervals during the next 270 days. For all methods, the measured concentration of cholesterol was less for the lyophilized serum than for frozen (P < 0.007 by the paired-sample t-test). After adjusting for the dilution effects of reconstitution, the decrease in original value was 0.7% for the SMAC, 1.7% for the RA-1000, 5.7% for the Ektachem, 9.4% for the TDX, and 14.3% for the aca. Lyophilization of cholesterol standards or serum samples may hamper the effective standardization of enzymatic methods and may be a source of apparent inaccuracy (bias) among enzymatic methods.

Additional Keyphrases: standardization - variation, source of

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whereas the assigned value was 1.43 g/L (5). Further, there was no evidence that the positive bias seen with serum samples or the negative bias observed with lyophilized material for the acc was caused by improperly assigned values for the calibrators (5). Apparently, lyophilization of serum caused a decrease in the apparent concentration of cholesterol. In this study, we evaluated this hypothesis.

**Materials and Methods**

**Instruments.** We determined cholesterol with the following instruments: SMAC and RA-1000 (Technicon Instruments Corp., Tarrytown, NY 10591), acc (Du Pont Co., Clinical Systems Division, Wilmington, DE 19898), TDx (Abbott Laboratories, Diagnostic Division, North Chicago, IL 60064), and Ektachem 700P (Eastman Kodak Co., Clinical Products Division, Rochester, NY 14650).

**Serum pools.** We prepared two large serum pools by combining serum from Clinical Center patients and treated these pools three ways. One group we lyophilized, one we froze and stored at −20 °C, and the third we froze and stored at −70 °C. One serum pool, designated "pool A," had a concentration of 2.1 g of cholesterol per liter, and the other, designated "pool B," had a concentration of 1.9 g/L, both as determined with the SMAC.

For lyophilized and frozen samples, we aliquoted exactly 3.00 mL of serum into glass lyophilization vials or plastic vials (Sarstedt 5000, cat. no. 55.535; Sarstedt Inc., Princeton, NJ 08540), respectively. The samples were lyophilized at the Center for Drugs and Biologics, Division of Product Quality Control, Food and Drug Administration, Bethesda, MD 20892. For lyophilization we used a Model 24FS40C Hull freeze-dryer. The samples were placed into the freeze-dryer, with a shelf temperature of −50 °C. During the next 2 h, the temperature was increased to −20 °C and the pressure was reduced to 3.325 Pa; these conditions were maintained for 17 h. The temperature was increased to 0 °C and held there for 24 h, then to 25 °C and held there for 36 h. The samples were capped while still under reduced pressure. (The moisture content of pool A was 0.74%; of pool B, 0.94%) This procedure follows the fundamental aspects of lyophilization (7).

We weighed the lyophilized cake in the pre-weighed vial before reconstitution and weighed 3 mL of the serum pool in the lyophilization bottle to determine the correct volume of doubly distilled, de-ionized water to add. The correct constitution volume of added water was 2.8 mL for both pools. We verified that the addition of water was correct by determining the concentration of Na⁺ and Cl⁻ with the SMAC each time a bottle of lyophilized pool was reconstituted.

**Procedure.** Instruments were calibrated according to the manufacturers' specifications. We had determined cholesterol with the SMAC, RA-1000, acc, and TDx for each pool before lyophilization or freezing. Then, at selected intervals, we reconstituted (with doubly distilled, de-ionized water) a lyophilized vial from each pool and thawed vials from frozen pools (−70 °C and −20 °C). We determined cholesterol in duplicate for each treatment by the above methods. About two-thirds of the way into the study, we also determined cholesterol with an Ektachem 700.

**Definitive method.** We determined cholesterol for all treatments, both pools, by isotope dilution/mass spectrometry, performed at the Center for Analytical Chemistry, National Institute of Standards and Technology, Gaithersburg, MD 20899 (6, 9).

**Statistical analysis.** We calculated means and used the paired-sample t-test to compare the lyophilization with the frozen treatments. We performed linear-regression analysis of each treatment over time to see if the effect of lyophilization was time-dependent. The final percentage change due to lyophilization was corrected for the error in volume used to reconstitute the lyophilized material (dilution error), based on the differences in the concentrations of Na⁺ and Cl⁻ determined between the frozen and lyophilized samples.

**Results**

The process of lyophilization resulted in a decrease in the value for cholesterol as determined by any of the four instruments (Table 1). The difference between the lyophilized and frozen treatments over time is statistically significant, with \( P < 0.007 \) for the SMAC and \( P < 0.00007 \) for the other instruments. The volume of water used to reconstitute the lyophilized cake exceeded what was required, which resulted in a decrease of 1.5% (SD = 0.1%, with \( n = 8 \)) for all the analytes. When the dilutional effect of reconstitution is taken into account, the decrease in the determined value of cholesterol because of lyophilization was 0.7% for the SMAC, 14.3% for the acc, 9.4% for the TDx, and 17.7% for the RA-1000.

An Ektachem 700 was not available to us until midway through the study. The mean values (±1 SD), obtained on three different days (\( n = 8 \)), were 1.70 ± 0.03 g/L (lyophilized), 1.78 ± 0.02 g/L (−20 °C), and 1.77 ± 0.02 g/L (−70 °C).

**Table 1. Mean Concentration of Cholesterol by Method and Treatment**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SMAC</th>
<th>acc</th>
<th>TDx</th>
<th>RA-1000</th>
<th>ID/MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pool A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None*</td>
<td>2.24 (0.007)</td>
<td>2.14 (0.014)</td>
<td>2.14 (0.01)</td>
<td>2.15 (0)</td>
<td>—</td>
</tr>
<tr>
<td>Lyophilizedb</td>
<td>2.18 (0.04)</td>
<td>1.77 (0.02)</td>
<td>1.92 (0.05)</td>
<td>2.12 (0.03)</td>
<td>2.05 (0.004)</td>
</tr>
<tr>
<td>−70 °Cb</td>
<td>2.22 (0.05)</td>
<td>2.10 (0.01)</td>
<td>2.18 (0.02)</td>
<td>2.18 (0.02)</td>
<td>2.07 (0.003)</td>
</tr>
<tr>
<td>−20 °Cb</td>
<td>2.23 (0.02)</td>
<td>2.11 (0.02)</td>
<td>2.15 (0.05)</td>
<td>2.19 (0.03)</td>
<td>2.07 (0.002)</td>
</tr>
<tr>
<td>Pool B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None*</td>
<td>1.87 (0.007)</td>
<td>1.81 (0.007)</td>
<td>1.78 (0.01)</td>
<td>1.84 (0.01)</td>
<td>—</td>
</tr>
<tr>
<td>Lyophilizedb</td>
<td>1.83 (0.03)</td>
<td>1.50 (0.04)</td>
<td>1.61 (0.04)</td>
<td>1.78 (0.04)</td>
<td>1.72 (0.009)</td>
</tr>
<tr>
<td>−70 °Cb</td>
<td>1.87 (0.04)</td>
<td>1.76 (0.02)</td>
<td>1.78 (0.08)</td>
<td>1.84 (0.02)</td>
<td>1.74 (0.003)</td>
</tr>
<tr>
<td>−20 °Cb</td>
<td>1.88 (0.02)</td>
<td>1.78 (0.02)</td>
<td>1.80 (0.05)</td>
<td>1.83 (0.03)</td>
<td>1.74 (0.004)</td>
</tr>
</tbody>
</table>

*\( n = 2 \); *\( n = 18 \) except for ID/MS, where \( n = 3 \). ID/MS, isotope dilution/mass spectrometry.

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(−70 °C) for pool B, and 2.02 ± 0.02 g/L (lyophilized), 2.11 ± 0.05 g/L (−20 °C), and 2.09 ± 0.02 g/L (−70 °C) for pool A. By the paired-sample t-test, the differences between the lyophilized and frozen treatments were always statistically significant (P < 0.001), but the difference between frozen treatments (−20 °C and −70 °C) was not significant (P > 0.2). The average effect of lyophilization of serum pools, as measured with the Ektachem, is to lower the determined value by 4.0%. The lyophilized material used for the Ektachem had been reconstituted with a smaller volume than the other samples in the study, with an apparent 1.7% increase in the concentrations of Na⁺ and Cl⁻. Adding the 1.7% dilutional effect to the effect of lyophilization gives an adjusted effect of 5.7% decrease in value. Thus, the Ektachem ranks third among the five analytical systems, the SMAC and RA-1000 having less and the acA and the TDx having more bias ascribable to lyophilization.

The isotope dilution/mass spectrometry method should be the best measure of the true value of cholesterol in the pools, and should not be affected by lyophilization. The difference between the lyophilized and frozen treatments was −1.1% for both pools, with no difference between the frozen treatments (Table 1). The difference between the lyophilized and frozen treatments is attributable to the dilutional effects associated with reconstitution as noted with Na⁺ and Cl⁻ (−1.5%, as mentioned above).

The effect of lyophilization on the enzymatic methods for cholesterol was studied over time. The data shown in Figure 1 are unadjusted for the dilutional effect. For each instrument and each treatment we analyzed the response by linear regression, starting with the ninth day. The slopes were not significantly different from zero (P > 0.05) for the entire time, except for the acA with pool A. After the first month, though, the slope for lyophilized pool A as measured with the acA was not significantly different from zero (P > 0.05). Thus, the effect of lyophilization is immediate and is not altered by storage.

Discussion

Lyophilization of serum pools is important in the standardization of enzymatic methods for cholesterol. Most calibrators for commercially available enzymatic methods are lyophilized, as are the controls. Two of the three Standard Reference Materials, SRMs 909 and 1952, are lyophilized. The survey materials from the College of American Pathologists are lyophilized. At present, the only viable alternative to lyophilization of these materials is distribution of frozen pools, a practice that has its own set of problems, such as thawing before use.

The current use of lyophilized material to calibrate enzymatic methods for cholesterol and to check standardization can be an important factor contributing to the bias observed among methods. Lyophilization of serum pools effects several changes to the determined values for cholesterol. For all the methods tested, the change in cholesterol concentration after lyophilization has always been a decrease. The percentage decrease varied widely among the methods, and the effect was immediate except for a slight decrease within the first month for the acA. After the first month, the effect of lyophilization appears to have stabilized for all the methods.

The cause of the effect of lyophilization on the determination of cholesterol is not clear. Lyophilization of serum does not affect results by isotope dilution/mass spectrometry (8, 9). The process of lyophilization may decrease the access of enzymes, either cholesterol esterase or cholesterol oxidase, to the cholesterol contained in the lipoproteins. The cholesterol itself is chemically unchanged, as shown with the isotope dilution method.

Lyophilization may affect some or all classes of lipoproteins. The lyophilized pools after reconstitution are more turbid than the untreated or frozen materials. The turbidity may represent simply a change in the size and shape of the lipoproteins or coalescence of the lipoproteins owing to a decrease in solubility caused by denaturation of the protein portion by lyophilization.

Barbarea (10) reported that the apparent activity of cholesterol in three different preparations of serum decreased by 4% to 5% when lyophilized by a process similar to ours. Unfortunately, this author did not specify the method used to determine cholesterol, and his results may have been due to dilution. It is important that lyophilization of serum affects methods differently. The varying response of methods to lyophilization complicates the standardization process for cholesterol, because different methods would produce different results for the same standardization material.

The crucial step in the lyophilization process may be the initial freezing of the serum. Here crystals are formed, sometimes of pure solvent. The interstitial fluids become more concentrated, supercooled, and eventually crystallize, often with crystals of differing chemical composition (7). The drying process after the freezing steps reportedly does not cause much physical or chemical change (7), but it may be the crucial step that causes the lyophilization effect.

Good materials are needed by manufacturers to standardize their methods, and by laboratories to calibrate and check standardization of the instruments in the field. We chose methods for cholesterol that would best demonstrate the varying effects of lyophilization. Our lyophilization study does not reflect the overall quality of these methods or their accuracy when used with fresh samples, but only the effect of lyophilization. The lyophilization of materials for cholesterol standardization can introduce bias among methods. Most manufacturers appear to be aware of the problem, and they attempt to adjust their instruments to
produce "accurate" results for fresh serum from patients; however, further studies of this effect will be needed if we are to understand the problem. In addition, our study has shown that for the CAP survey materials and the SRM 909 serum Reference Materials, lyophilization of the material plays a significant role in the bias observed on measurement with certain enzymatic methods for cholesterol. From our studies we cannot offer an immediate solution to this problem. It would be helpful to users if manufacturers would provide information on the effect of lyophilized materials for their instruments. Such information does not address the issue that the users of instruments should have adequate materials to verify that their own instruments are accurate. Confidence in our standardization procedures will be greatly enhanced when we better understand the effect of lyophilization.

We thank Don Hochstein and Mark Heintzelman for lyophilizing our pools; Polly Ellerbe, Lorna Sniegozski, and Edward White V for measuring cholesterol in our pools by isotope dilution/mass spectrometry; and Joan May for determining the moisture content of the lyophilized cakes.

References


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This is a multipoint kinetic method for simultaneously determining acetoacetate (AcAc) plus β-hydroxybutyrate and lactate plus pyruvate in a single cuvette of the Multistat III centrifugal analyzer. In the first step, AcAc and pyruvate are completely reduced, using 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) and lactate dehydrogenase (EC 1.1.1.27) in the presence of excess NADH at pH 7.5, to β-hydroxybutyrate and lactate, respectively. After dilution, the endogenous β-hydroxybutyrate and lactate and that resulting from reduction are simultaneously oxidized by their respective dehydrogenases in the presence of excess NAD+ at pH 9.0. Adjustment of the relative enzyme concentrations allows simultaneous estimation of AcAc plus β-hydroxybutyrate and lactate plus pyruvate by analyzing multipoint absorbance data, collected during the oxidation reaction, with use of a two-component linear-regression model. Total run-to-run CVs were 6.4% and 6.1% at 5 mmol/L β-hydroxybutyrate and 5 mmol/L lactate, respectively. The method was designed to be useful for identifying the cause of an increased anion gap in serum.

Additional Keyphrases: centrifugal analyzers • multiple analyte assay by enzymic methods • high anion-gap metabolic acidosis

High anion-gap metabolic acidosis results when various anions accumulate in the blood. Accumulation of lactate (LACT) leads to the development of lactic acidosis, whereas accumulation of β-hydroxybutyrate (BOH) and acetoacetate (AcAc) leads to the development of ketoacidosis (1). Uremic acidosis in patients with acute and chronic renal failure results from the accumulation of sulfate, phosphate, and various organic acid anions (2). High anion-gap metabolic acidosis is also caused by the ingestion of toxic substances such as salicylates, methanol, ethylene glycol, and paraaldehyde (1, 2). Diagnosis of these conditions is critical in emergency medicine and requires accurate and rapid measurement of the anions involved. Determination of LACT and pyruvate (PYRU) provides information on the state of tissue oxidation, whereas measurement of BOH and AcAc aids in the diagnosis of diabetic and alcoholic ketoacidosis (3–5). Routine measurement of these analytes

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