Effect of Serum Lyophilization on the Rate Constants of Enzymatic Methods for Measuring Cholesterol

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We determined the equilibrium absorbances and rate constants for two enzymatic methods, aca (DuPont) and RA-1000 (Technicon), used in determining cholesterol in reconstituted lyophilized serum. The lyophilized materials included two serum pools, three control materials, a College of American Pathologists’ survey material, and Standard Reference Material no. 909. We calibrated the reagents with aca standards for cholesterol (DuPont). The difference in the mean concentrations of cholesterol (aca — RA-1000) was -0.09 g/L overall and was not statistically significant by analysis of variance. The mean rate constant for all materials was 0.23 min⁻¹ for the aca and 1.42 min⁻¹ for the RA-1000, significantly different (P < 0.001). Lyophilization causes lower results for the aca method than for the RA-1000, because the reaction rate for the aca method is slower and has not reached equilibrium when the final absorbance reading is made.

Additional Keyphrases: standardization · variation, source of bias · discrete analysis · random-access analysis · absorbance at equilibrium

Accurate determination of cholesterol is an important part of the National Cholesterol Education Program’s plan to detect, evaluate, and treat high blood cholesterol concentrations (1–3). Maintaining a minimum bias for all cholesterol-measuring methods is essential, because the error in diagnosis is approximately twice the magnitude of the bias (4, 5). Biases do exist between several of the enzymatic methods and the Reference Method for determining cholesterol as performed at the Centers for Disease Control (6, 7). Understanding the cause of bias is crucial to resolving the inaccuracy problem.

Materials and Methods

Apparatus. We used the Lambda 4B High Performance Spectrophotometer (Perkin-Elmer Analytical Instruments, Norwalk, CT 06856).

Reagents. We used two sets of reagents to determine cholesterol: those for the RA-1000 (Technicon Instruments Corp., Tarrytown, NY 10591) and for the aca (DuPont Co., Clinical Systems Division, Wilmington, DE 19898). The aca reagents were cut out of their packs and consolidated on the day of each experiment. To calibrate the reagents at equilibrium, we used aca Standards, Levels 1 through 4 (DuPont). We tested seven lyophilized materials. Pools A and B were prepared for us and contained no preservatives (8). Three lyophilized materials were from Gilford (Ciba Corning Diagnostics Corp., Irvine, CA 92714): Elevated Lipid Control (lot no. 080603, expiration date August 1989) and two unassayed Normal and Abnormal Controls used in our laboratory. We used two reference materials, Standard Reference Serum (SRM 909) from the National Institute of Standards and Technology (NIST, Gaithersburg, MD 20899) and survey material c-18 (December, 1988) from the...
College of American Pathologists (CAP, Skokie, IL 60077).

Procedures. For the aca method, we pipetted 4 µL of sample into a cuvette sitting in the spectrophotometer at 37 °C and then added 996 µL of aca reagent incubated at 37 °C. Alternatively, for the RA-1000 method, we pipetted 7 µL of sample into the cuvette and then added 700 µL of RA-1000 reagent incubated at 37 °C. We recorded the absorbance as a function of time for each material.

For equilibrium measurements, we took the maximum absorbance from each scan and plotted absorbance vs the concentration of cholesterol in the aca standards. We analyzed the results by linear regression. After averaging the absorbance for each material, we calculated the concentration from the equilibrium absorbance, using the relationship that cholesterol concentration = (absorbance - y-intercept)/slope. Further, using analysis of variance, we tested the significance of the difference between the concentration of cholesterol determined by the aca and RA-1000 reagents.

To determine the rate constants, we plotted -ln(A_{max} - A) vs time, where ln is the natural logarithm, A_{max} is the maximum absorbance, and A is the absorbance at time t. We analyzed these plots by linear regression and averaged the slopes. The slope is equal to the rate constant for the reaction.

Results

Plots of the maximum absorbance vs the concentration of cholesterol in the aca standards gave a slope of 0.162 A per g/L and a y-intercept of 0.0162 for the aca reagents and a slope of 0.220 A per g/L and a y-intercept of -0.0205 for the RA-1000 reagents. Table 1 shows the concentration of cholesterol at equilibrium, the difference between the results with the aca and RA-1000 reagents, and the composite SD for the difference. The difference between the mean concentrations of cholesterol for the aca and RA-1000 reagents (all the materials) was -0.09 g/L, which was not statistically significant (P > 0.6) by analysis of variance.

Table 2 shows the rate constants for the aca and RA-1000 reagents for each material. The mean rate constant for all the materials with the RA-1000 reagents was 1.42 min⁻¹ (SEM 0.08 min⁻¹). The mean rate constant for all materials with the aca reagents was 0.23 min⁻¹ (SEM 0.04 min⁻¹). The difference between these mean rate constants is statistically significant (P < 0.001) by Student's t-test.

The reaction rate of the aca reagents with the aca standards was faster than that with the other materials: all levels reached equilibrium in <3 min. For the aca standard level 3, as a typical example, the rate constant was 1.40 ± 0.22 min⁻¹.

Discussion

We successfully transferred to a spectrophotometer the enzymatic methods for determining cholesterol in the aca and RA-1000. This transfer allowed us to monitor the reaction rate and to closely examine the reaction in progress. The aca and RA-1000 methods for determining cholesterol are both endpoint methods—i.e., it is assumed that the final absorbance reading is taken at equilibrium. We examined the difference between the two methods for the equilibrium reading and found that the difference was not statistically significant. Unfortunately, because the sample volumes were small, the errors in these equilibrium measurements were high. Thus, future studies may find a significant difference. The assigned value for the SRM 909 is 1.43 g/L, which neither reagent system attained in our studies. In a previous study, we found the mean concentration of cholesterol in the SRM 909 measured in the aca to be 1.32 g/L and in the RA-1000 to be 1.49 g/L (6). The results for the RA-1000 reagents are within experimental error, but those for the aca reagents are not. The equilibrium concentration for the aca reagents was measured at 20 min for the SRM 909, which gave results higher than those obtained by using the instrument that measures at 4.36 min (6). Therefore, even though all the cholesterol may react by equilibrium, a method involving a reaction rate that is too slow may underestimate the results (Figure 1).

Rate studies. In the rate studies we found that the aca method is slower than the RA-1000 method. In the aca method the reaction is not complete at the time of the final absorbance reading (4.36 min), whereas that of the RA-1000 (5 min) has finished. Figure 1 graphically shows this difference for Pool B, which happens to be typical for all the materials. For the aca, the absorbance at the final reading was only 60% of the equilibrium value. Further, we found that the progress of the reaction varies with the material, ranging from 40% to 80% completion. The progress of the reaction is easily calculated from the equation

\[ \text{Fraction completed} (F_c) = 1 - e^{-kt} \]

where \( k \) is the rate constant for the material and \( t_c \) is the time of the final reading. The reaction for the RA-1000 ranges between 99.5% and 99.97% completion, which
would not significantly affect the assay results. The difference between the lowest and highest values of progress of the reaction for the aca, however, is large enough to significantly affect the results.

What rate would be minimally acceptable for the reaction on the aca? The rate should be fast enough so that the reaction would be essentially complete by the time the absorbance is read. If the reaction is not complete when the absorbance is read, the absorbance at this assumed equilibrium will always be less than that at true equilibrium. For the aca we could calculate a minimum rate constant (\(k_c\)) for which the reaction is essentially complete. A rate constant greater than \(k_c\) would indicate that the reaction had reached equilibrium by the final absorbance reading, but a rate constant less than \(k_c\) would indicate that the reaction would be incomplete by the final absorbance reading and the calculated concentration would be undervalued. If we choose 3% as our maximum bias \((9)\), then we could consider the reaction to be essentially complete when it had progressed 97%. We solve for \(k_c\) by rearranging equation 1:

\[k_c = \frac{-\ln(1 - F_2)}{t_2}\]  

where \(F_2\) is in this case 0.03 and \(t_2\) is the time of the final reading, which is 4.36 min for the aca. The minimum rate constant then is 0.804 min\(^{-1}\). The differences in rate constants for different materials hampers the ability of these materials to act as standards. Further, any attempt to use these methods in a kinetic mode of measurement is doomed to error, because in all kinetic methods it is assumed that different samples will have identical rate constants \((10)\).

Reference materials and the effect of lyophilization. We conducted this study to examine the effects of using lyophilized samples on cholesterol determination. Some important materials are lyophilized: SRM 909, CAP survey materials, many quality-control materials, and some calibrators. The slowness of the reaction rate for the aca shows that lyophilized materials are not suitable for checking standardization for all methods. Lyophilized CAP survey material cannot be used as a measure of accuracy for any method for which lyophilization affects the reaction rate; the aca is just one such method. Lyophilized calibrators are a potential source of error in some methods for determining cholesterol, because absorbance at the time of reading is less than that at equilibrium when the reaction rate is diminished. A slope of absorbance of the lyophilized calibrator vs the concentration of cholesterol will be low compared with the slope for fresh samples, and calculations for unlyophilized samples will be too high. Further, not all lyophilized materials react at the same rate with the same method or in the same pattern between methods \((Table 2)\). Therefore, the lyophilization effect varies with the method used for the lyophilization and with what exactly is being lyophilized, and it would be difficult to establish a standard adjustment factor for lyophilization.

In light of the current recommendations for accuracy in cholesterol measurement \((9)\), better reference materials for standardization are needed. Instruments or reagents could be certified at the factory where they are manufactured and corrections made before the products are shipped. Reference materials are a necessity for individual laboratories because they are used to check the standardization periodically, to prove that a method is accurate, and to adjust a method so that results agree with those obtained by the Reference Method. Lyophilized materials have been used for this because they are easy to store and other analytes are most stable in them. Frozen materials may be adequate, but they present their own set of logistical problems.

Chemical reactions are characterized by their rate and equilibrium constants, and a study of reaction rates is useful in determining the reaction mechanisms \((11)\). The equilibrium constant is proportional to the absorbance. Other factors affecting the reaction—the path length of the cuvette, the molar absorptivities of the chromophores, and the relative activities of chromophore formation—are all constant. In this study, we have characterized the reaction for two methods chosen because they represent extremes in the lyophilization effect. The aca was maximally affected and the RA-1000 minimally affected \((8)\); other methods should fall somewhere between these two. Our results imply that the decreased reaction rate in lyophilized samples—in this case used with the aca—is the principal source of lyophilization-related bias. In future studies of the lyophilization effect, determination of the rate constant could be useful. If the newly determined rate constant is less than the minimum rate constant, \(k_c\), then the method is affected by lyophilization of samples; if the rate constant is \(\geq k_c\), then the method may be free of the lyophilization effect. In the latter case, the effect of the equilibrium absorbance must be determined. This test is a simpler way to check for the lyophilization effect with lyophilized materials than comparing bias before and after lyophilization; determining the rate constant is more sensitive than comparing means and is more closely related to the major cause of the lyophilization effect, the retardation of the reaction rate.

Sources of bias. Each step of the reaction could potentially be involved in the mechanism of the lyophilization effect. However, the aca and RA-1000 methods for determining cholesterol have essentially similar reaction steps. Fatty acids are cleaved from the cholesteryl esters by means of cholesterol esterase \((12, 13)\); then oxygen in the
The presence of cholesterol oxidase is converted to hydrogen peroxide (12, 13). The final step, reaction of the hydrogen peroxide with a chromogen to form a quinoneimine dye in the presence of horseradish peroxidase, differs slightly for the two methods. The chromogen used with the aca is N,N-diethylalanine - HCl/4-aminantipyrine, and the chromophore is measured at 540 nm (12). The chromogen used with the RA-1000 is 4-hydroxybenzoate plus 4-aminantipyrine, and the chromophore is measured at 500 nm (13). In neither method should this last reaction step be affected by lyophilization of the sample, because all the reactants are soluble in water, including the hydrogen peroxide. Further, the reagents used for the aca method should react at the same rate as those for the RA-1000, because the average reaction rate (1.40 min⁻¹) for the aca standards (calibrators) determined with the aca was the same as the average rate (1.42 min⁻¹) for the RA-1000 with all the materials.

The first two enzymatic steps are the most likely to be affected by the lyophilization. For example, considerable work has shown that the source (animal vs microbial) of the cholesterol esterase affects the activity displayed with different fatty acid esters (14–16), so the enzyme may have difficulty contacting the cholesteryl esters in lipoproteins that have been altered by lyophilization. The step involving the oxidation of cholesterol and production of hydrogen peroxide is also a potential candidate. The cholesterol oxidases used by the manufacturers may differ in their activities and ability to interact with lyophilized lipoproteins. Further, oxygen may have difficulty entering the complex of lyophilized lipoproteins and cholesterol oxidase. The mechanism of the lyophilization effect is not certain, but we believe that studying reaction rates will help elucidate it.

In conclusion, we have shown that a decrease in the reaction rate for the method used in one instrument, the aca, is the main cause of its lyophilization effect. The etiology of the lyophilization effect with other methods may be similar. Demonstration that a decreased reaction rate produces the lyophilization effect is important, because the lyophilization effect is one of the chief causes of bias between methods for determining cholesterol. Further study of the rate problem may lead to a solution of the bias problem in cholesterol determinations. In addition, study of the lyophilization effect in cholesterol detection can have important implications for determining other analytes. If all the lipoproteins are affected by lyophilization, the accuracy of measurement will be decreased even further. Moreover, the activity of enzymes may be affected by lyophilization. We will continue to study the reaction rate of cholesterol methods by varying the assay conditions, exchanging reagents, and isolating the steps of the reaction.

References