Method-Dependent Variations in the Stability of Apolipoprotein B in a Stabilized Liquid Reference Material

Joanne V. Mei,1 Mary K. Powell, L. Omar Henderson, S. Jay Smith, Gerald R. Cooper, Santica M. Marcovina,2 and W. Harry Hannon

Using accelerated Arrhenius-type short-term and long-term temporal studies, we evaluated the storage life of a stabilized, liquid-frozen reference material (SLRM) for human apolipoprotein B (apo B) developed by the International Federation of Clinical Chemistry. As measured by our candidate reference RIA, the concentrations of immunoreactive apo B in the SLRM showed pronounced degradation with exposure to increasing temperatures over time. The SLRM was stable for as long as 1 year when stored at −70°C, but its immunoreactive apo B declined by <10% when stored at 4°C for 10 months. Using radial immunodiffusion and an ELISA to assess the equivalency of measured mass for the accelerated thermal stability of the SLRM, we found a loss of immunoreactive apo B similar to that measured by RIA. Analyzing the same samples by liquid immunoprecipitation (nephelometry) resulted in the amount of apo B present being overestimated, especially in samples held for long periods. By using different immunological methods to evaluate this thermally aged SLRM, we demonstrated that its measured behavior varies depending on the method of quantitation.

Indexing Terms: immunoassays/intermethod comparison/variation, source of/Reference Material/sample handling

Clinical data suggest that concentrations of the protein components of high-density lipoprotein, apolipoprotein (apo) A-I, and low-density lipoprotein (LDL), apo B, may provide a more accurate indication of risk of coronary artery disease than cholesterol measurements alone (1, 2). Accordingly, interest in measuring these materials has grown. Efforts to standardize these measurements, however, have been complicated by the lack of an established accuracy base for these tests and by the heterogeneity of results produced by different analytical test systems. To alleviate some of the standardization problems, the Apolipoprotein Committee of the International Federation of Clinical Chemistry (IFCC) and several manufacturers of apolipoprotein products have worked to develop suitable accuracy-based reference materials to standardize values obtained with different immunoassays (3–5).

The IFCC developmental studies of secondary reference materials have culminated in the preparation of a lyophilized serum-based material for apo A-I known as SP1-01. Accelerated and temporal stability studies demonstrate that the immunoreactivity of apo A-I in SP1-01 remains constant during storage at low temperatures and for several months after removal from low temperatures and storage at higher temperatures (6). Measurements of this lyophilized serum preparation by several methods also showed linearity, parallelism, and lack of matrix effects. A program to transfer mass units to manufacturers' calibrators yielded results that were not significantly different among methods and that had low imprecision (CV) for three fresh-frozen serum pools (6).

Development of a lyophilized serum reference material for apo B, however, has been problematic. Unlike apo A-I, apo B tends to be unstable and can self-associate and form irreversible aggregates (7, 8). The values for apo B measured in lyophilized materials by different test systems were also inconsistent (9, 10).

Central to the standardization of apolipoprotein measurements by various analytical methods is the need for stable primary standards and reference methods. Purified apo A-I is stable in the lyophilized form and can be used as a primary standard with the appropriate analytical method (6, 11). Because of problems with the suitability of apo B in the lyophilized form, however, an ultracentrifugally isolated preparation of narrow-cut, human LDL (d = 1.030–1.050 kg/L) has been proposed as a surrogate primary standard (12, 13). Using an LDL preparation as a primary standard, we have developed a RIA to measure apo B. This method, which we used to quantify the amount of immunoreactive material present in a stabilized liquid reference material (SLRM) during stability studies, has been proposed by the Centers for Disease Control and Prevention as an interim candidate reference method for the apolipoproteins (14).

We monitored the IFCC secondary reference material for apo B, known as SP3-07, in both a short-term thermal stability study and a temporal stability study. We used the Arrhenius equation (15) to assess data generated by the accelerated stability study. The Arrhenius equation has been used to predict the chemical stability
and shelf life of pharmaceutical dosage forms of drugs and vitamins exposed to increased temperatures and to estimate the stability of serum-based reference materials (16).

For the accelerated stability study, SP3-07 was placed at increased temperatures and aliquots were sampled over 3 weeks. The long-term stability study was conducted for 10 months with samples stored at 4°C and 25°C; in addition, we measured samples stored for >1 year at −70°C. To minimize interassay variability, we removed thermally treated samples from frozen storage and analyzed them concurrently. This type of analytical design has been used to examine the biological variation for several protein analytes in serum samples from healthy subjects (16, 17), and was used as the basis for stability studies done to establish international reference methods for human serum proteins (18). Researchers found that variation in measurements of the analytes was significantly reduced when samples collected from individuals were frozen until the end of the study and then analyzed in one batch.

We used seven analytical methods in the accelerated stability study to analyze the apo B and apo A-I concentrations in SP3-07: RIA, ELISA, radial immunodiffusion (RID), and four liquid immunoprecipitin assays: fixed-time immunonephelometry (INA), end-point INA, rate INA, and end-point immunoturbidimetry (ITA). SP3-07 was measured in the long-term stability study by RIA and fixed-time INA only. Here we report the behaviors of the aged reference material with respect to method.

Materials and Methods

Materials

The stabilized, secondary serum reference material for apo B, SP3-07, was prepared by and obtained as a frozen liquid from Behringwerke AG (Marburg, Germany). Rabbit anti-apo B polyclonal antiserum, which was used in the RIA, was obtained as a gift from John Albers of the University of Washington (Seattle, WA). Goat anti-apo B antiserum was raised against narrow-cut LDL by immunization of normal goats as previously described (19).

The RIA for apo A-I was carried out with an optimized method of Henderson et al. (20). The RIA for apo B followed an optimized procedure (14). The ELISA was the monoclonal antibody-based candidate reference method for apo B (21, 22), the ELISA kits being provided as a gift from Richard Smith of Johnson and Johnson (San Diego, CA). The RID, the M-Partigen Apolipoprotein B kit (Behring Diagnostics, Somerville, NJ), was conducted in accordance with manufacturer’s instructions. Fixed-time and end-point INAs were conducted on a Nephelometric Analyzer (Behring Diagnostics) with the reagents and analysis protocol provided by the manufacturer and, in separate protocols, with the narrow-cut LDL as a calibrator. Fixed-time and end-point INAs were also conducted by replacing the manufacturer’s antiserum with a goat anti-apo B antiserum prepared in-house. All fixed-time and end-point INA tests were performed with the N Supplement Reagent containing detergent (Behring Diagnostics). Rate INA was performed with a Beckman Array nephelometer (Beckman Instruments, Palo Alto, CA), and end-point ITA was performed with a Cobas Fara II centrifugal analyzer (Roche Diagnostic Systems, Nutley, NJ). Both assays were conducted with reagents and protocols specified by the respective manufacturers.

Thermal Stability Studies

Accelerated thermal stability of SP3-07. Aliquots of the SLRM, SP3-07, were received in 1-mL glass containers from Behringwerke. They were thawed from storage (−70°C) by allowing the samples to come to room temperature. The samples were gently mixed for 30 min and then kept at five temperatures (4, 25, 37, 45, and 55°C) for 3 weeks. Experimental samples were removed at intervals on designated days throughout this period. Each sample was labeled, divided into aliquots, and stored in 4-mL glass vials (Wheaton, Millville, NJ) at −70°C. Control samples were thawed to room temperature and refrozen at −70°C. On the day of analysis, all experimental samples and controls, including a zero time sample that was stored frozen, were removed from storage and warmed to room temperature. The contents of each aliquot were gently mixed for 30 min. For RIA, all samples were assayed in triplicate, with the analysis repeated on 3 separate days. The concentration of apo B in the reference material was determined by RIA, ELISA, RID, fixed-time and end-point INA, rate INA, and ITA. To predict the stability of apo B in the material, we used the Arrhenius equation to evaluate data collected from the analysis of SP3-07 by RIA.

Accelerated thermal stability of apo B determined from fresh sera. Fresh serum samples were collected from four fasting adults. After removing the erythrocytes, we divided the serum from each patient into 0.25-mL aliquots and stored these at 4, 45, and 55°C for 3 weeks. The control samples were aliquots of the same serum samples stored at −70°C, without thermal treatment. The experimental samples were removed on designated days during the 3-week period and stored at −70°C until the day of analysis. All samples were analyzed for apo B concentration by RIA and fixed-time INA only.

Temporal thermal stability of SP3-07. To study the temporal thermal stability of SP3-07, we stored 1-mL aliquots of the reference material at 4 and 25°C for 10 months. Experimental samples were removed every month and stored at −70°C. Control samples were thawed one time and stored at −70°C until the day of analysis. Samples collected during the 10 months were analyzed for apo B concentration by RIA and fixed-time INA with a single analytical run for each assay. For RIA, all samples were assayed in triplicate, with the analysis repeated on three separate days.

The turbidity of samples was measured by the INA instrument with the manufacturer-defined settings for turbidity measurement. In this protocol, both a turbidity threshold and a turbidity factor must be established for a sample to be identified as turbid (Behring Nephelometer Instruction Manual).
Results

Stability studies with SP3-07. The apo B concentration in SP3-07 was immunologically stable when stored at -70°C. As shown in Fig. 1, the mean of 80 RIA determinations over a 400-day period was stable, with a low CV (2.61%). Linear least-squares regression of the data from Fig. 1 yields the equation $y = 0.961 + 3.08 \times 10^{-8}x$, where $y$ is measured apo B concentration and $x$ refers to days of exposure. The slope of the regression line was not significantly different from 0.0 ($P = 0.15$). The assigned apo B value for SP3-07 is 1.22 g/L, as determined by INA (23). The apo B value determined by RIA here should not be confused with the assigned value for SP3-07. Historically, RIA has given lower apo B values than nephelometry for various serum samples (24). With SP3-07 as a calibrator, harmonious values for apo B have been achieved with both RIA and fixed-time INA. These values reflect constant and consistent differences between the methods.

We measured by RIA the apo B concentration in SP3-07 samples exposed to increased temperatures over a 3-week period and subjected the resulting data to the classical Arrhenius analysis to determine stability (15). A series of plots was generated for the log relative concentration vs the age of the samples (in days) for the temperatures indicated. The data for samples stored at 55°C for 6 or more days were omitted for their nonlinearity, possibly because of protein degradation. The absolute value of the slope vs temperature was modeled with data for samples stored at 25–45°C. The estimated slope for samples stored at 4°C was not used because the experimental data did not show a significant change in negative slope (Table 1).

From the RIA data used to generate Arrhenius plots (secondary slope vs temperature), we predicted that, if the reference material were stored at 25°C, in 21 days 10% of the apo B in the reference material would degrade. This prediction agreed closely with the observed values measured by RIA (Table 1). The amounts of apo B in samples stored at 37°C and at 45°C also closely approximated predicted values. The degradation of apo B in 3 weeks could not be estimated in samples stored below 16°C, because the predicted slope for that temperature was zero, implying that little degradation occurs at or below that temperature. Fig. 2A shows a comparison of curves for apo B for samples stored at all temp-

| Table 1. Thermal degradation of apo B: predicted and observed values. |
|-----------------------------|-----------------------------|
| Temp., °C | Slope | Observed | Predicted |
| 4 | +0.0009 | +0.0803 | — | — |
| 25 | -0.0021 | -0.0022 | 21.6 | 21.2 |
| 37 | -0.0037 | -0.0035 | 12.4 | 13.1 |
| 45 | -0.0038 | -0.0040 | 12.0 | 11.6 |
| 55 | -0.0132 | -0.0044 | 3.5 | 10.5 |

*Time for 10% of apo B to degrade.

Fig. 1. Apo B content of liquid-frozen reference material, SP3-07, stored at -70°C.

Each point is the result of duplicate RIA determinations; dashed lines are mean ± 2 SD.

Fig. 2. Accelerated stability studies with SP3-07: (A) degradation curves for apo B stored at 5 temperatures (experimental sample vs control sample) as measured by RIA; (B) degradation curves of apo A-I in thermally treated SP3-07, measured by RIA specific for apo A-I.

(A), 4°C: (+), 25°C: (b), 37°C: (c), 45°C: (+), 55°C. Values plotted are the means of triplicate determinations, made on 3 days.
temperatures, analyzed by RIA; the y-axis shows the ratio of the result for an experimental sample divided by the result for a control sample that had been thawed and refrozen one time. RIA of the apo A-I concentration in the thermally aged SP3-07 showed degradation similar to that seen for apo B measured by RIA; however, the apo A-I in SP3-07 was stable at 4–45°C for 1 week before marked degradation was detected (Fig. 2B). Both RID and the monoclonal antibody-based ELISA showed similar patterns of apo B degradation for samples stored at 4, 45, and 55°C (data not shown).

We also used liquid immunoprecipitation assays to analyze the apo B content of the thermally aged SP3-07. Fixed-time INA of these samples showed apo B to be stable at 4°C over the duration of the experiment (Fig. 3A). At higher temperatures (25, 37, or 45°C), the apo B concentration apparently increased by 5–10% after 1 day (Fig. 3A). At 55°C, the apo B concentration decreased rapidly after 7 days, with the samples exposed >7 days identified as turbid by the INA instrument. End-point INA of these same samples showed patterns of apo B degradation similar to patterns found by fixed-time INA of thermally aged SP3-07. In a previous international investigation, the role of anti-apolipoprotein antisera was studied to evaluate the contribution of these antisera to the variance of among-laboratory measurements (19). We wished to test the role of different polyclonal antisera preparations as a possible explanation for the differences in results between RIA and the INA methods. When undiluted, polyclonal goat anti-apo B antisera (prepared in-house) was substituted for the polyclonal antisera provided by the manufacturer in both fixed-time and end-point INA, we observed patterns similar to fixed-time INA with commercial antisera, and only slight differences in slopes. Fixed-time INA analysis of apo A-I in the thermally aged SP3-07 showed degradation curves similar to those seen for RIA (Fig. 3B).

End-point INA analysis of SP3-07 for apo B showed trends similar to those found with fixed-time and end-point INA: The relative concentrations of experimental samples kept at 4°C were stable (with a slight positive slope), experimental samples kept at 25 and 45°C had measured apo B concentrations higher than those of the control, and the apo B concentration of experimental samples stored at 55°C began to decline after 5 days and dropped off considerably after 2 weeks (data not shown). Results of the analysis of apo A-I in SP3-07 by ITA were similar to those by fixed-time INA (Fig. 3B). Rate nephelometric analysis of SP3-07 showed that the apo B content in the samples stored at 4°C degraded by ~10% over the first week of exposure to increased temperature, and then stabilized (data not shown).

**Temporal thermal stability of SP3-07.** We investigated the temporal thermal stability of SP3-07, using both RIA and fixed-time INA. According to the RIA, SP3-07 lost >5% of the measurable apo B over a 3-month period when stored at 4°C, a pattern that continued for the next several measurement intervals (Fig. 4); at 25°C, the material was stable for 1 month, but it began to degrade after 2 months and lost ~45% of its
initial apo B concentration by the end of the study. However, when fixed-time INA was used to determine the apo B concentration of the same thermally aged samples, the apo B concentration appeared to increase; at 4°C, the material was stable for 2 months but showed a slight increase over the duration of the 10-month experiment; and at 25°C, the measurable apo B concentration increased by as much as 60% (Fig. 4). Before analysis, all samples were tested for turbidity by the INA. Unlike the accelerated stability study, none of the samples was detected as being too turbid to analyze. The samples were not checked for microbial growth.

Accelerated thermal stability of apo B determined from fresh serum. Serum samples from four adults were divided into aliquots and stored at 4°C for 3 weeks. The apo B concentration of the samples was determined by RIA and fixed-time INA. The samples from all four subjects were shown to be stable for 3 days at 4°C, but the apo B concentration as measured by RIA showed a decline over the 3-week period. Fixed-time INA of the same serum samples showed that, after 3 days, the apo B concentration in 3 of the 4 samples had an apparent increase of 10–20%. In general, both RIA and fixed-time INA showed that, for storage at 4°C, the serum samples demonstrated more variability in apo B results than did the SLRM (data not shown).

Discussion

SP3-07 has been accepted by the World Health Organization Expert Committee on Biological Standardization as the First International Reference Reagent for Apolipoprotein B. To evaluate the stability of SP3-07, we used an in-house reference RIA, fixed-time INA, and several other immunological methods to measure apo B. RIA of SP3-07 demonstrated that the concentration of apo B in the reference material remained constant for at least 1 year stored at −70°C, and that ~10% of the apo B immunoreactive material was lost when stored at 4°C for 10 months. We first used fixed-time INA to measure the concentrations of apo B in the thermally treated samples and found that results were greater than those we initially obtained for SP3-07 samples stored at −70°C. Upon analyzing the same samples by RIA, we saw the decrease in apo B content that we had predicted for a protein material exposed to denaturing conditions. Because SP3-07 has been proposed for use as an international primary standard for immunological tests, we tested the stability of the material with regard to other commonly used methods (RID, ELISA, ITA).

Using several analytical techniques to measure the degradation of apolipoproteins in SP3-07, we illustrated method-related differences in the measurements. We found a similar type of degradation with any of several immunochemical methods (RIA, ELISA, and RID). As determined by RIA measurements, the apo B in SP3-07 remained stable for as long as 3 weeks at 25°C, and lost ≤10% over 10 months at 4°C. Because the apo B concentrations in aged samples measured by fixed-time INA were higher than initial values, we also investigated apo A-I contents of the SLRM samples used for the accelerated stability study. Both RIA and fixed-time INA showed temperature-dependent degradation of apo A-I in the liquid material, in contrast to the stability shown by the lyophilized apo A-I reference material, SP1-01, even at increased temperatures (6). The stability of apo A-I in SP3-07 was studied only as a qualitative control (as another apolipoprotein component) for some of the analytical methods used in the apo B stability studies.

Fresh serum samples held for 3 weeks at temperatures ranging from 4 to 45°C also showed apo B degradation within this period when measured by RIA, but not by fixed-time INA: The contents of the samples remained stable for ≤3 days at 4°C. This difference between the reference material and fresh serum is probably attributable to the stabilizing agents added to SP3-07.

When we used ELISA to analyze aged SP3-07 samples, the apo B degradation exceeded that seen by RIA. This phenomenon was possibly due to the fact that the monoclonal antibody conjugate used in the assay was selected for a single epitope on the apo B molecule, which is less stable than the polyclonal antisemur used in RIA (22). A complex protein antigen such as apo B contains many epitopes, which give rise to a diverse population of polyclonal antibodies. Some of these epitopes are certainly more stable than others, and this difference would explain the differential response seen between RIA and ELISA.

Analysis of apo B in thermally aged SP3-07 by liquid immunoprecipitation methods did not show the same degradation patterns seen by RIA, ELISA, and RID. Fixed-time INA of patients' serum samples stored at 4°C also did not show the same degradation trends found by using RIA. These results are of concern, given the vital importance that the apolipoprotein concentration in reference materials be measured accurately, and that the material should mimic the characteristics of fresh serum. The RIA and ELISA procedures demonstrated an apparent degradation of SP3-07. The application of the Arrhenius equation to the RIA data confirmed a close agreement between the actual half-life and the predicted half-life of apo B in the reference material. However, we were unable to predict the half-life of apo B in SP3-07 on the basis of the fixed-time INA results because the latter showed no appreciable degradation of apo B in the SP3-07 samples subjected to accelerated thermal stability conditions, i.e., the INA data would have predicted high stability of the SLRM. If we had not used multiple immunoassay techniques to assess the stability of the SLRM, we would not have observed the differences in its immunoreactivity with different methods. For between-method harmonization, therefore, it is vital that SP3-07 be stored at −70°C, shipped on solid CO₂, and used without delay, to eliminate the possibility that the material may become compromised.

The deterioration of apo B in SP3-07 during stability studies, as measured by RIA, ELISA, and RID, followed normal and predictable degradation patterns. These assays rely on the interaction of epitopes with either
monoclonal or polyclonal antiserum reagents. The antibody/antigen reaction takes place either in solution (RIA), on a solid support (ELISA), or through lattice formation in a gel (RID). The analyte of interest is directly quantified either through a radioactive or enzymatic indicator or through complex formation in the gel. Because of the nature of the antibody/antigen reaction and the Law of Mass Action, RIA, ELISA, and RID all seemed to demonstrate similar apo B degradation trends, even though each assay involves a different detection method.

Large differences in the results obtained with the different methods are most probably not caused by the different antiserum reagents used with the various techniques. When the manufacturer's antiserum reagent for fixed-time INA determinations was replaced with an in-house polyclonal anti-apo B antiserum, we saw no difference in apo B values. The negligible effects of antiserum reagents of acceptable specificity and sensitivity have been documented by a study that tested the reactivity of an apolipoprotein reference material with a common antiserum and antiserum reagents obtained by test laboratories (19).

Fixed-time INA results indicated that none of the SLRM samples stored at 4°C or 25°C for the temporal stability study was turbid. Because we do not know the composition of the SLRM, additives in the material may have contributed to the difference in results obtained with immunochemical assays and those obtained with the several types of INAs. Samples with high triglyceride content can form large triglyceride-rich very-low-density lipoprotein particles (VLDL) and often cause nonspecific light scatter in INA and ITA systems (25). Although we do not know the cholesterol and triglyceride concentration of SP3-07, amounts of these analytes are not abnormally high and should not have contributed to large particle formation.

We obtained higher values of apo B in aged SP3-07 by the liquid immunoprecipitation assays than by the other methods. This overestimation of apo B concentration in both the accelerated and temporal stability studies could have resulted from the unmasking of epitopes on the apolipoproteins, such that exposed epitopes could react with the polyclonal antiserum, and thus create more immune complexes. More immune complexes would be translated into greater light scatter by the instrument, which would, in turn, give higher apo B values for samples that had decomposed. However, this hypothesis may not explain more complex interactions, such as the possible formation of larger lipoprotein particles, which may occur during the antibody/antigen reaction of liquid immunoprecipitation assays. All fixed-time and end-point INA tests were performed with a supplemental reagent that included detergent. The addition of detergent should have reduced any nonspecific effects from the formation of large VLDL particles (25).

In conclusion, we found that the liquid immunoprecipitation methods gave higher apo B values in aged samples than did other methods. Liquid immunoprecipitation methods, however, constitute the most frequently used methods of analyzing apolipoproteins in clinical chemistry laboratories. The IFCC and manufacturers of instruments and reagents have invested substantial resources in efforts to standardize apolipoprotein determinations made with different methods. Clearly, SP3-07, as a reference material for apo B, is a stable preparation at −70°C and at 4°C, as indicated by both accelerated and temporal stability studies. Our results emphasize the need to assess the stability of a serum-based, candidate reference material for apolipoproteins by using all methods in which the material is intended to be used. The issue of stability becomes a problem in certain analytical test systems, for which sample integrity can be measured accurately only during brief periods. In addition, not all methods are equally sensitive to sample degradation. Harmonization of measurement among methods can be achieved only if the reference material is stored and handled properly and if the analysis of the SLRM is acceptable for all analytical systems.

This is the first report to show the method-dependent stability of a protein analyte. A difference in the antigenic and functional stability of complement proteins (e.g., C3) has been found for several candidate reference materials (17, 18). Researchers found that, although the C3 protein was immunologically stable in the reference material despite thermal treatment, substantial enzymatic activity was lost during storage at room temperature; moreover, although the analyte could be deemed stable by immunological methods (INA and RID), its functional activity, as measured by a hemolytic assay, could not be sustained at high temperatures (18). Although our study did not measure functional activity, our results parallel these two reports, in that we also were able to demonstrate differential responses between analytical assays for antigens.

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References


