Measurement of Apolipoprotein B in Plasma from Hyperlipidemic Subjects Treated with Cholestyramine or Placebo: Differential Effects of Storage at \(-70^\circ\)C

Stephanie Kafonok, Paul S. Bachorik, and Peter O. Kwiterovich, Jr.

Using radial immunodiffusion, we measured apolipoprotein B (apoB) concentrations in fresh plasma from 216 hyperlipidemic male subjects and in aliquots of the same plasma stored at \(-70^\circ\)C for 22 months. At the time of sampling, 112 were being treated with cholestyramine, the rest with a placebo. ApoB concentrations in the stored samples were significantly and positively correlated with apoB concentrations in the fresh samples (\(r = 0.84, P < 0.001\)), the mean (and SD) being 1680 (320) mg/L and 1570 (320) mg/L, respectively. This 6.5% decrease in the stored samples was statistically significant (\(P = 0.0005\)), but there was no significant correlation between the decrease in apoB values in the stored samples and plasma triglyceride concentrations measured in the fresh samples. The correlation between the two apoB analyses was greater in the cholestyramine-treated group (\(r = 0.90\)) than in subjects treated with the placebo (\(r = 0.75\)).

Additional Keyphrases: lipoproteins \cdot radial immunodiffusion \cdot hyperlipidemia \cdot cholestyramine \cdot sample handling

Studies of patients undergoing coronary arteriography suggest that increased concentrations of apolipoprotein B (apoB) may discriminate better between patients with and without coronary artery disease than will TC and LDL cholesterol (1,2).\(^1\) Increases in apoB-containing particles are found in dyslipidemias associated with premature heart disease such as familial hypercholesterolemia, familial combined hyperlipidemia, and hyperapolipoproteinemia.

Although plasma lipoprotein components are usually measured in fresh plasma samples, practical considerations occasionally require that blood samples be stored for various periods before analysis. The analytical suitability of using stored samples depends upon the components to be measured and the methods used. In this study, we examined the stability of apoB measurements in stored plasma samples, using RID.

Materials and Methods

Patient population. We studied 216 men, participants in the Johns Hopkins component of the LRC Coronary Primary Prevention Trial (3), a double-blind study to assess the effect of lowering cholesterol on coronary risk. All subjects had increased concentrations of LDL-cholesterol but normal plasma TG concentrations (type IIa hyperapolipoproteinemia) on entry into the study. At the time of sampling, half of the subjects were being treated with a moderate cholesterol-lowering diet and a placebo, and the other half with the same diet and a bile acid sequestrant, cholestyramine (24 g/day) (3). All of the subjects were sampled on the occasion of their last formal visit (Closure Visit 1) before the trial ended and before the treatment group to which they belonged was identified. Adherence to cholestyramine for six months before this sampling was determined by self-report and packet counts.

Blood samples. All subjects had fasted for at least 12 h before venipuncture. Blood was collected into evacuated tubes containing disodium EDTA (final concentration 15 mg/L) as the anticoagulant. Plasma was separated from the cells within 3 h of venipuncture. One aliquot of each plasma was promptly analyzed; separate aliquots were transferred to sterile vials, sealed, and stored at \(-70^\circ\)C. Lipid, lipoprotein, and apolipoprotein concentrations were measured in the fresh samples within one week of collection. The fresh samples were stored at 4° C before they were analyzed.

Lipid and lipoprotein measurements. We measured TC and TG in isopropanol extracts of fresh plasma, according to the procedures of the LRC Program (4). In brief, we mixed 0.5 mL of plasma with 9.5 mL of isopropanol, then treated the extract with a zeolite mixture to remove interfering substances. The solids were sedimented and the isopropanol extract was used for analysis. We analyzed the lipids with an AutoAnalyzer II (Technicon Instruments, Tarrytown, NY); cholesterol with the Liebermann–Burchard reaction, using a serum calibration procedure (5) to adjust the observed values in accord with reference values; triglycerides, in the same extracts, with the LRC modification (4) of the method of Kessler and Lederer (6). HDL-cholesterol was measured in similar zeolite-treated isopropanol extracts after treating the plasma with heparin and MnCl\(_2\) (final concentrations 1.3 g/L and 46 mmol/L, respectively) to remove apoB-containing lipoproteins. We calculated the concentrations of LDL cholesterol, using the empirical relationship of Friedewald et al. (7).

Measurement of apoB. We measured apoB by RID, using commercially available immunodiffusion plates (Behring Diagnostics, Freehold, NJ). These plates contain a monospecific antibody to apoB, not reactive with apolipoproteins E or A-I. The antibody does not discriminate between apoB-48 and apoB-100. We diluted the plasma threefold with 0.15 mol/L NaCl, to place the measured value within the range of the standard curve, applied 5 \(\mu\)L of the diluted sample to the well, and corrected the measured value for the dilution. We confirmed that the measured apoB concentration in serially diluted samples was a linear function of the extent of dilution.

The apoB concentration of the serum standard supplied with the plates was 750 mg/L. This was determined by radioimmunoassay performed at the Northwest Lipid Research Clinic, Seattle, WA. We diluted the standard serially to provide two additional standards with apoB concentrations of 250 and 500 mg/L. We constructed a three-point standard curve from which we calculated the apoB concentrations of the samples.

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Nonstandard abbreviations: TC, total cholesterol; TG, triglyceride; apoB, apolipoprotein B; LDL, VLDL, HDL, low-, very-low-, and high-density lipoproteins; RID, radial immunodiffusion; and LRC, Lipid Research Clinics.

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We analyzed a lyophilized control pool (Omega; Cooper Biomedical Corp., Malvern, PA) in duplicate in each run. In most cases, we analyzed the same lot of the control pool with both the fresh and the stored samples. After we applied the samples, we covered the plates, placed them into a draft-free container, and allowed diffusion to proceed for 72 h at room temperature before we measured the diameters of the precipitin rings. We calculated the apoB concentrations of the samples from the regression equation that related the square of the ring diameters of the standards to their apoB concentrations. The standard curves were linear ($r > 0.999$) under the conditions of the assay.

**Study design.** We divided each plasma sample into two aliquots and analyzed the first aliquot within one week of collection. We transferred the second aliquot to a sterile 2-mL serum bottle, sealed the bottle with a rubber stopper and aluminum cap, and stored the sample at $-70 \, ^\circ C$ for 22 months. We analyzed the fresh samples in 10 runs over a seven-week period, and analyzed the stored samples in the same groups in 10 runs over a seven-week period 22 months later. Therefore, all of the samples were stored for the same amount of time, and both the fresh and the stored samples were presumably subject to the same amount of within-run and between-run variability during the two analysis periods.

We calculated the inter- and intra-assay variability, using the measurements of the control pool. For these calculations, we used the measurements from seven of the 10 analytical runs, because in these runs, the same lot of control pool was analyzed with both the fresh and the stored samples (Table 1). In the remaining three runs, the lot of control pool analyzed with the fresh and the stored samples differed, and these data were not included in the calculations. The intra-assay CV was 3.06% for the fresh samples and 2.34% for the frozen samples. The inter-assay CV was 1.11% for the fresh samples and 2.95% for the frozen samples. Therefore, the overall CV (4.17%) for the fresh samples was similar to that for the frozen samples (5.29%).

We used the CLINIP system for the statistical analyses. We used $t$-tests for paired samples to compare means, and linear regression analysis to assess the relationship between two variables. We set the type I error rate at 0.05.

**Table 1. Inter- and Intra-Assay Variation for Measurement of ApoB in the Control Pool When It Was Analyzed with the Fresh and Stored Plasma Samples from Study Subjects**

<table>
<thead>
<tr>
<th>Control pool values, mg/L</th>
<th>CV, %</th>
<th>Control pool values, mg/L</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh samples</td>
<td></td>
<td>Frozen samples</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>700 (0)*</td>
<td>0</td>
<td>700 (25)*</td>
</tr>
<tr>
<td>5</td>
<td>700 (20)</td>
<td>2.8</td>
<td>700 (18)</td>
</tr>
<tr>
<td>3</td>
<td>710 (12)</td>
<td>1.6</td>
<td>680 (12)</td>
</tr>
<tr>
<td>3</td>
<td>710 (82)</td>
<td>11.5</td>
<td>650 (12)</td>
</tr>
<tr>
<td>4</td>
<td>730 (25)</td>
<td>3.5</td>
<td>690 (16)</td>
</tr>
<tr>
<td>2</td>
<td>710 (14)</td>
<td>2.0</td>
<td>680 (28)</td>
</tr>
<tr>
<td>3</td>
<td>710 (0)</td>
<td>0</td>
<td>660 (0)</td>
</tr>
<tr>
<td>Overall 23</td>
<td>710 (6)*</td>
<td>3.1</td>
<td>678 (6)</td>
</tr>
</tbody>
</table>

$^*$CV within run
CV, between run
CV overall

*Mean (SEM).

**Results**

We measured the lipid, lipoprotein, and apoB concentrations in fresh plasma samples from 216 subjects. The values are shown in Table 2. The group mean (and SEM) apoB concentration in fresh samples was 1680 (20) mg/L, and was 1570 (20) mg/L after storage for 22 months at $-70 \, ^\circ C$ (Table 2). This was a decrease of 6.5% ($P = 0.0005$). There was no difference between the group standard deviations for the fresh and stored samples (320 mg/L), however, indicating that the distribution of apoB concentrations was unchanged.

Linear regression analysis was used to determine the relationship between the concentrations of apoB in the fresh and stored samples. ApoB concentrations in the stored samples were significantly and positively correlated with apoB concentrations in the fresh samples ($r = 0.84$, $P < 0.001$) (Figure 1). The regression equation relating the two measurements was:

$$[\text{apoB}_{\text{stored}}] = 0.88 [\text{apoB}_{\text{fresh}}] + 175 \, \text{mg/L}$$

With use of this equation, an apoB concentration of 1000 mg/L in fresh samples would be measured as 1005 mg/L in the same samples after storage, representing an increase of 0.5%. At a concentration of 2000 mg/L, the value predicted in stored samples would be 1840 mg/L, a decrease of 8.3%. In this study, most of the samples had apoB concentrations in the range of 1000 to 2500 mg/L.

Because treatment with cholestyramine is known to affect both the composition and the concentration of LDL, we examined the correlations between the measurements for fresh and stored samples separately after grouping the subjects according to treatment. All of the subjects were asked to take six packets per day of cholestyramine or the placebo. We divided the subjects into three subgroups: those

**Table 2. Concentrations of Lipids, Lipoproteins, and ApoB in Plasma Samples from 216 Subjects**

<table>
<thead>
<tr>
<th>Concentration, mg/L</th>
<th>Mean</th>
<th>SEM</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>2710</td>
<td>30</td>
<td>430</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>1720</td>
<td>45</td>
<td>660</td>
</tr>
<tr>
<td>HDL-C</td>
<td>470</td>
<td>8</td>
<td>110</td>
</tr>
<tr>
<td>LDL-C</td>
<td>1890</td>
<td>31</td>
<td>320</td>
</tr>
<tr>
<td>ApoB, fresh</td>
<td>1680</td>
<td>20</td>
<td>320</td>
</tr>
<tr>
<td>ApoB, stored*</td>
<td>1570</td>
<td>20</td>
<td>320</td>
</tr>
</tbody>
</table>

*22 months at $-70 \, ^\circ C$ as described in text.

Fig. 1. ApoB concentrations in stored vs fresh plasma

The linear regression equation of this line is: $[\text{apoB}_{\text{stored}}] = 175.0 \, \text{mg/L} + 0.88 [\text{apoB}_{\text{fresh}}] (r = 0.84)$
who complied with the prescribed cholestryramine treatment (subgroup I); those who did not (subgroup II); and those who were treated with the placebo (subgroup III). Subjects in subgroup I (n = 84) had maintained at least 10% adherence to the drug regimen during the Coronary Primary Prevention Trial. Subgroup II (n = 28) consisted of subjects who took less than 10% of the prescribed dose of cholestryramine, and subgroup III (n = 104) consisted of subjects who were assigned to take the placebo. The correlation between the apoB concentrations in the fresh and stored samples from subgroups I and II were the same (r = 0.90) (Figure 2, a and b), and were higher than for the entire group (r = 0.84) (Figure 1). The correlation in subgroup III was significantly lower (r = 0.75, P = 0.001) than in subgroups I and II (Figure 2c).

Subjects in subgroup I had significantly lower total cholesterol, LDL-cholesterol, and apoB concentrations than did the other two subgroups (Table 3). The lipid, lipoprotein, and apoB concentrations in subjects in subgroup II (noncompliers) were similar to those in subgroup III (placebo) (Table 3). There were no significant differences between the TG and HDL-C concentrations of the three subgroups, and in all three subgroups, the mean apoB concentrations in the stored samples were 100 to 110 mg/L lower than in the fresh samples.

We examined the paired differences between apoB concentrations in the fresh and stored samples for each subgroup. The mean (and SD) differences were (mg/L) 109 (16), 97 (13), and 108 (21) for subgroups I, II, and III, respectively.

### Table 3. Mean (and SD) Concentrations of Lipids, Lipoproteins, and ApoB in Subjects in Various Treatment Groups

<table>
<thead>
<tr>
<th></th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(compliers, n = 84)</td>
<td>(noncompliers, n = 28)</td>
<td>(placebo, n = 104)</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>2560* (510)</td>
<td>2730 (390)</td>
<td>2820* (330) 0.001</td>
</tr>
<tr>
<td>Triglyceride b</td>
<td>1820 (750)</td>
<td>1680 (690)</td>
<td>1640 (550)</td>
</tr>
<tr>
<td>HDL-C b</td>
<td>450 (100)</td>
<td>470 (140)</td>
<td>480 (110)</td>
</tr>
<tr>
<td>LDL-C b</td>
<td>1740 (520)</td>
<td>1920 (440)</td>
<td>2000* (370) 0.001</td>
</tr>
<tr>
<td>ApoB fresh b</td>
<td>1590* (350)</td>
<td>1700 (290)</td>
<td>1750* (290) 0.003</td>
</tr>
<tr>
<td>ApoB stored c</td>
<td>1480* (350)</td>
<td>1600 (310)</td>
<td>1640* (290) 0.002</td>
</tr>
</tbody>
</table>

- *Dunnett's multiple comparison of treatments against a single control group.
- Analyzed in fresh plasma samples.
- * Stored for 22 months at -70 °C before analysis, as described in text.
- *Identifies pairs for which the P values apply.

These means were not significantly different, but the standard deviation of the paired differences was larger for the placebo subgroup, and indicates that, in this subgroup, the analyses of stored samples were more variable than those of fresh samples.

We measured the apoB concentration of the same lot of the lyophilized control pool in duplicate in seven of the analytical runs in which fresh samples were analyzed, and again 22 months later in seven of the runs in which stored samples were analyzed (Table 1). The mean (and SEM) apoB concentration was 710 (6) mg/L when the pool was analyzed with the fresh plasma samples, and 678 (5) mg/L when the pool was analyzed with the stored samples. The decrease was 4.3% and was statistically significant (P <0.001) and could reflect differences in the lots of immunodiffusion plates, variability in the values assigned to the calibration sera, or deterioration of the control pool during storage. Presumably, these factors also affected the analysis of the subject samples, and probably contributed to the 6.5% decrease in the apoB concentrations of these samples after they had been stored. The precision of the measurements was the same during both analysis periods (Table 1).

We examined the relation between the change in apoB concentration during storage and the plasma triglyceride concentration to determine if triglyceride-enriched, apoB-containing lipoproteins were selectively sensitive to freezing. There was no significant correlation between the change in apoB concentration during storage and the triglyceride concentrations of the samples measured when the samples were fresh.

### Discussion

In this study we examined the apoB concentrations in plasma stored at -70 °C for two years. We collected the samples from 216 patients who had high LDL concentrations, and in most, the apoB concentrations were in the range of 1000 to 2300 mg/L. Although our study subjects did not represent the general population, the findings provide insight into the long-term stability of apoB concentrations measured by RID. We are unaware of any previous studies in which the reproducibility of apoB measurements was evaluated in plasma samples from a large number of subjects after storage at -70 °C for almost two years, although others (6-11) have examined the stability of apoB concentrations after storage for six months or less. The coefficient of correlation was 0.843 between apoB concentra-
tions measured in fresh and stored samples, and there was a mean decrease of 6.5% in the apoB concentrations of the stored samples. Our findings agree with those of Havekes et al. (8), who observed a 6% decrease in the apoB concentrations of 34 plasma samples from normal subjects after storage for one week at -20°C.

The lower apoB concentrations we observed in stored samples may be due in part to analytical variability, because the apoB concentration of the control pool also decreased by 4.3% during the storage period. Differences in the precision of the assays did not account for the decrease, because the overall coefficients of variation for the control pool were the same for both analysis periods. The decrease could have resulted from variations in the reagents, plates, or serum calibration pools we used during the two analysis periods, deterioration of the control pool, or a combination of such factors.

Triglyceride-rich lipoproteins are prone to self-association and aggregation after freezing and thawing. Havekes et al. (8), using a RID method, reported that isolated VLDL added to plasma samples increased the apoB concentration of fresh samples, but did not affect the measurements if the samples that contained the added VLDL were frozen and thawed before apoB was measured. This suggested that VLDL was preferentially sensitive to freezing, which diminished its immunoreactivity or ability to diffuse in the RID plates. We did not measure VLDL apoB separately, but we used plasma triglyceride concentrations as an indirect measure of VLDL concentration. The difference between apoB measurements in the fresh and frozen samples was independent of triglyceride concentrations, and suggests that the sensitivity of VLDL to freezing did not account for lower apoB values in the stored samples. The amount of VLDL added to the samples by Havekes et al. (8), however, would correspond to a plasma triglyceride concentration exceeding 10 000 mg/L. The triglyceride concentrations of the samples we used were much lower.

The mean apoB concentrations in the samples decreased by 110 mg/L, or one-third of one standard deviation for this population, after storage. Snideman et al. (12) found an association between high apoB concentrations (>1200 mg/L) in plasma and the presence of angiographically documented coronary artery disease in adults. Using that concentration as the cutoff, few of the subjects in our study would have been misclassified as having normal apoB concentrations on the basis of the values we measured for the stored samples. The apoB concentrations were high in the subjects we studied. If the observed 6.5% decrease in mean apoB concentrations in the stored samples from our study group represents a relative rather than absolute decrease, the mean apoB concentration in a normal epidemic population would be expected to change very little during storage under the same conditions.

Because cholestyramine is known to affect LDL composition, as well as lower LDL concentrations in the blood, we compared the correlations between the apoB measurements in fresh and stored samples from subgroups of subjects who were asked to take cholestyramine or the placebo. The correlation between apoB measurements in the fresh and frozen samples from subjects who had been assigned to the placebo subgroup was significantly lower than from subjects who were asked to take cholestyramine, regardless of how well they complied with the treatment regimen. The lipid, lipoprotein, and apoB concentrations of the noncompliers were similar to those of the placebo subgroup, but the correlation between apoB concentrations in the fresh and frozen samples was much lower in the placebo subgroup. Although the mean differences in the apoB concentrations were similar in all three subgroups, the standard deviation of the paired differences was highest in the subjects who took the placebo. The reason for the greater variability of the measurements in the placebo subgroup is not clear, but may be due to some influence of the placebo itself on the stability of the apoB-containing lipoproteins during long-term storage. The composition of the placebo was considered by the manufacturer to be proprietary information.

Because of the nature of the study population, the apoB concentrations were higher than those found in a normal population (<1200 mg/L) (12), but our findings would probably apply to stored samples from normal subjects also.

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References