Effect of Blood Collection and Processing on Radioimmunoassay Results for Apolipoprotein B in Plasma

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We studied the effects of different blood collection and processing procedures on quantification of apolipoprotein (apo) B by radioimmunoassay. High-density lipoprotein subfractions HDL3 and HDL2 and isolated apoA-I did not cross-react in the assay. Analytical recovery of apoB at different doses of very-low- and low-density lipoproteins were complete. Inter- and intra-assay coefficients of variation (CVs) averaged 7.4% and 6.0%, respectively. Blood from 20 subjects was collected into tubes containing EDTA alone or EDTA with antiproteolytic and antioxidant agents; one half of each plasma was separated immediately, half after 3 h at 4 °C. Regardless of the addition of protective agents or the time difference in separating plasma from other blood elements, freezing plasma at −70 °C decreased apoB content a similar amount, an average of 6.8%. This loss of apoB immunoreactivity was not related to apoB content in fresh plasma. Analysis of variance showed no differential effect on apoB content by the various additions to whole blood or plasma. No additional apoB content was lost in once-frozen aliquots of three human plasma pools during storage at −70 °C for up to 18 months. We conclude that concentrations of apoB in human plasma can be measured reliably after long-term storage, although the absolute value may decrease slightly as a result of freezing.

Additional Keyphrases: sample handling · lipoproteins

A direct association between high-concentrations of low-density lipoprotein cholesterol (LDL-cholesterol) and an increased risk of coronary heart disease (CHD) has been established (1–3). Apolipoprotein (apo) B, the sole structural protein component of LDL and a major apolipoprotein of very-low-density lipoprotein (VLDL), does not exchange between plasma lipoproteins. Numerous studies have shown an association between concentrations of apoB in plasma and risk of CHD (4–10). These results are not surprising because of the structural relationship of apoB with LDL-cholesterol. Among subjects undergoing coronary angiography, however, measurements of LDL-apoB may be superior to LDL-cholesterol in separating cases from controls (11). Furthermore, the progression of atherosclerotic disease and the development of new lesions in bypass grafts are more strongly associated with LDL-apoB than with LDL-cholesterol (12). These findings demonstrate the importance of LDL particle structure as a predictor of CHD and suggest that measurement of LDL-apoB or even plasma apoB may improve the prediction of CHD risk.

A large prospective study, the Atherosclerosis Risk in Communities Study (ARIC), was initiated by the National Heart, Lung, and Blood Institute, National Institutes of Health, to examine the association of various factors with the occurrence of atherosclerosis. Among these factors, apolipoproteins and lipids are considered very important. This study, which is investigating the etiology of atherosclerosis, involves monitoring many characteristics in 16 000 representative residents of four U.S. communities over six years (13). Plasma specimens collected in the four field centers are transported to the Central Lipid Laboratory for determination of lipid, lipoprotein lipid, and apolipoprotein concentrations.

A precise, accurate, and reliable assay for quantifying concentrations of apoB is essential for the accurate estimation of the association of apoB content with CHD. Among other considerations, the results must not be distorted by the methods used for sample preparation and storage, i.e., during the time between sample collection in the field centers and analysis in the Central Lipid Laboratory. After the establishment of the radioimmunoassay (RIA) for apoB, a pilot study was designed to determine the effect of sample processing and storage on apparent apoB content as measured by RIA. The effects of antibacterial (14) and antioxidant agents (15), which prevent structural damage from free radical formation, were assessed. In addition, antiproteolytic agents were added, either to the blood or to separated plasma, to prevent proteolysis of apolipoproteins (16, 17). We also evaluated the effect of the time between blood collection and plasma separation, to determine whether this would be critical for epidemiologic studies, for which home visits may be necessary.

Our studies show that freezing causes a slight decrease in apoB concentrations in plasma. However, the percentage decrease is not related to apoB concentrations in fresh plasma. Thus, concentrations of apoB can validly be determined in frozen plasma specimens to ascertain the relationship of apoB to atherosclerotic risk.

Materials and Methods

Study Design

To examine the effects of different procedures of blood collection, processing, and storage on the measurement of apoB, we obtained blood from 20 volunteers (14 women and six men). The mean (SD) concentrations of cholesterol and triglyceride in plasma were 4.91 (0.63) and 1.50 (1.20) mmol/L (189.8 (245) and 1332 (1059) mg/L), respectively. On each of four days, blood was drawn from five fasting subjects into four 10-mL evacuated collection tubes (A–D) per subject. Tubes A and C contained 15 mg of dry EDTA; tubes B and D contained 15 mg of EDTA, 500 kalilkrein inhibitory units of aprotinin, and 0.6 mg of ε-aminocaproic acid in a volume of 50 μL. These reagents had been filter-sterilized.
and were injected into the collection tubes by sterile techniques. The order of blood sampling into tubes A-D was randomized among subjects. Tubes were completely filled, to keep the dilution error to <1% (assuming a hematocrit of <50%). Tubes A and B were centrifuged immediately after blood collection (1800 x g, 20 min, 4°C). Tubes C and D were placed on crushed ice for 3 h before we separated the plasma. Plasma obtained from each tube (A-D) was divided into six 200-μL aliquots. To aliquots 1 and 2 from all tubes, we added 20 μL of isotonic saline (NaCl, 8.5 g/L), and to aliquots 3 and 4, 20 μL of "cocktail A," a solution containing chloramphenicol (0.5 g/L), gentamicin sulfate (1 g/L), sodium azide (2 g/L), aprotinin (500 kallikrein inhibitory units), and ε-aminocaproic acid (12 g/L). Aliquots 5 and 6 received 20 μL of "cocktail B," a solution containing glutathione (5 g/L) and butylated hydroxytoluene (0.2 g/L) in addition to the protease inhibitors and antibiotics described above for aliquots 3 and 4. Aliquots 1, 3, and 5 from all tubes were stored at 4°C and analyzed within 24 h. Aliquots 2, 4, and 6 from all tubes were stored at −70°C for six weeks, thawed with shaking in a water bath at 20-22°C for 60 min, and analyzed without delay.

Quantification of ApoB

The apoB RIA procedure of Schonfeld et al. (18) was used with minor modifications. LDL was used as standard and for tracer was prepared by zonal ultracentrifugation (19). Fresh human plasma was obtained from healthy volunteers. LDL from the pooled plasma was isolated by ultracentrifugation in a Ti 14 zonal rotor (Beckman Instruments, Brea, CA) with use of a density gradient of sodium bromide of 1.0–1.3 kg/L. The LDL peak was pooled and dialyzed for 24 h against 9 g/L sodium chloride solution containing 1.0 mmol of EDTA per liter, pH 8.0. LDL was then centrifuged at 1.8 x 10^6 x g min at 10°C in a fixed-angle-head rotor. The pellet LDL was dissolved in 4.0 mL of the saline EDTA solution, then filter-sterilized through 0.22-μm (pore-size) filters (Millipore Corp., Bedford, MA) and stored at 4°C under sterile conditions.

The purity of LDL preparations was ascertained by electrophoresis in 0.5% agarose and 5% polyacrylamide gels in the presence of sodium dodecyl sulfate, 1 g/L (20). Protein content was determined by the method of Lowry et al. (21), with bovine serum albumin as standard. Antibodies directed against apoB were produced by injecting zonally isolated LDL into rabbits. LDL was iodinated by the Chloramine-T method (22).

Before each assay, iodinated LDL was repurified on a column of Sephadex G-50 (Pharmacia, Piscataway, NJ). Antiserum was diluted so as to bind 50% to 60% of the tracer. The total volume of standards or diluted plasma specimens, iodinated apoB, 50 mmol/L barbital buffer (pH 8.6) containing 1.0 mmol of EDTA and 20 g of bovine serum albumin per liter, and anti-apoB antiserum was 0.5 mL.

After samples incubated for 30–60 h at 4°C, we added 0.1 mL of Immunoprecipitin* (Bethesda Research Laboratories, Gaithersburg, MD), diluted fivefold in barbital buffer, to each. Samples were incubated for 15 min at 25°C, then 2.0 mL of barbital buffer was added to all tubes. The samples were centrifuged for 30 min (2500 x g, at 4°C) and the supernates were removed. Radioactivity remaining in the tubes was counted with a gamma counter (Micromedic, Atlanta, GA) equipped with data reduction capabilities for calculating final concentrations of apoB by the LOGIT procedure (23).

Non-specific binding was <2% of the total counts/min. The apoB assay range was defined as 5–40 ng/dose, which corresponds to plasma contents of 150–1200 mg/L when dilutions are taken into account. VLDL (19) and HDL subfractions (24) were prepared by zonal ultracentrifugation in a Beckman Ti-14 zonal rotor in an L2-65B ultracentrifuge (Beckman). The apoB content of VLDL was measured by the method of Kane et al. (25), with use of tetramethylurea.

Statistical Analysis

To assess the effect of specimen handling and storage on apoB content, we used analysis of variance (ANOVA) (26), performed by using the ANOVA routine of the SPSS-PC package (27) in a four-factor factorial design (protective agents during blood drawing, separation time, storage time and temperature, and plasma additives) with repetition (i.e., plasma from 20 persons) on all four factors. Because proteolytic agents during blood drawing and separation time were nested within tube, these effects and their interaction were tested with the subject x tube interaction term used as the error term. All other effects were tested by using the aliquot x tube interaction term as the error term. For rejection of the null hypothesis, a probability of ≤5% was required. In linear-regression analysis, the influence of individual data points was estimated by using Cook's D test (28).

The pattern of missing data was studied by use of the missing-data program of BMDP (29). From the possible 480 data points, 12 were missing, such that only 14 of the 20 subjects had complete data. Missing data were not related to specimen handling or storage. To have a balanced design for the very complex ANOVA, we estimated these values by regression (29). Overall results were similar whether based on 14 subjects with complete data sets or 20 subjects with imputed data.

Results

Radialimmunoassay of ApoB

First we evaluated the specificity of apoB measurements. Isolated human HDL2 and HDL3, respectively 800 and 1200 ng of protein per assay, had less than 2.2 ng of apparent apoB content. Also, purified human apoA-1 (1000 ng per assay) had <1 ng of apparent apoB content. Thus, other relevant apolipoproteins did not cross-react in our assay.

To determine the accuracy of apoB measurements in plasma, we added to various plasma specimens different amounts of LDL in which apoB-100 was the sole protein and measured the analytical recovery of apoB (Table 1). In these experiments, the mean analytical recovery of apoB was 101% of the apoB added. To determine whether our RIA procedure gave apoB results similar to reported values, we undertook analysis of apoB concentrations in 381 adults (including both patients and participants of research studies). The mean (SD) of apoB concentrations was 819 (246) mg/L, which is comparable with results for other nonselected groups (11, 30). Because differences in the expression of apoB epitopes between VLDL and LDL may affect quantification of apoB in plasma by immunoassays

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* ApoB concentrations are expressed as mg/L, not nmol/L. The presence of various proportions of two apoB species, of different molecular masses, confounds the establishment of a conversion factor.
Table 1. Analytical Recovery of LDL Standard Added to Human Plasma

<table>
<thead>
<tr>
<th>Added*</th>
<th>Measured</th>
<th>Recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10.91 ± 0.93b</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>13.48 ± 1.08</td>
<td>107</td>
</tr>
<tr>
<td>5</td>
<td>15.09 ± 0.30</td>
<td>101</td>
</tr>
<tr>
<td>10</td>
<td>20.73 ± 1.41</td>
<td>105</td>
</tr>
<tr>
<td>15</td>
<td>25.06 ± 0.26</td>
<td>97</td>
</tr>
<tr>
<td>20</td>
<td>31.70 ± 1.04</td>
<td>103</td>
</tr>
<tr>
<td>25</td>
<td>35.37 ± 1.97</td>
<td>98</td>
</tr>
<tr>
<td>30</td>
<td>42.24 ± 2.39</td>
<td>103</td>
</tr>
<tr>
<td>40</td>
<td>48.11 ± 1.73</td>
<td>95</td>
</tr>
</tbody>
</table>

Mean (SD) 101 (4.2)

* Added to 0.0167 μL of a human plasma specimen of unknown content.

b Mean ± SD of triplicate determinations.

(31), we compared the apoB content of VLDL as determined by RIA and by the tetramethylurea procedure. In a typical experiment, the mean (SD) apoB concentration of a VLDL preparation was 1483 (88) mg/L for RIA and 1400 (154) mg/L for the tetramethylurea procedure.

To determine the precision of the assay, we analyzed 10 replicates of apoB standard for each dose (2.5–100 ng). The mean intra-assay CV was 6.0% for the 5–40 ng dose range (Table 2A). Furthermore, the intra-assay CV was examined in human plasma specimens from an unselected population (Table 2B). Triplicate determinations were performed in each specimen, and specimens were divided into three groups according to apoB concentration. The intra-assay CV was similar in all three groups.

Table 2. Intra-Assay Variation of ApoB Measurements

A. In Standards

<table>
<thead>
<tr>
<th>Dose, ng per assay</th>
<th>Measured</th>
<th>Apparent apoB, ng</th>
</tr>
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<tbody>
<tr>
<td>2.5</td>
<td>2.49</td>
<td>38.7</td>
</tr>
<tr>
<td>5.0</td>
<td>4.73</td>
<td>7.2</td>
</tr>
<tr>
<td>10.0</td>
<td>9.51</td>
<td>7.8</td>
</tr>
<tr>
<td>15.0</td>
<td>15.41</td>
<td>4.7</td>
</tr>
<tr>
<td>20.0</td>
<td>20.47</td>
<td>5.2</td>
</tr>
<tr>
<td>25.0</td>
<td>24.58</td>
<td>8.9</td>
</tr>
<tr>
<td>30.0</td>
<td>31.00</td>
<td>3.0</td>
</tr>
<tr>
<td>40.0</td>
<td>40.02</td>
<td>5.3</td>
</tr>
<tr>
<td>50.0</td>
<td>48.95</td>
<td>6.0</td>
</tr>
<tr>
<td>75.0</td>
<td>73.71</td>
<td>8.6</td>
</tr>
<tr>
<td>100.0</td>
<td>94.58</td>
<td>6.2</td>
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</table>

B. In Plasma

<table>
<thead>
<tr>
<th>ApoB concn, mg/L</th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;600</td>
<td>505.1</td>
<td>59.2</td>
<td>3.86</td>
</tr>
<tr>
<td>600–850</td>
<td>719.1</td>
<td>75.9</td>
<td>4.18</td>
</tr>
<tr>
<td>&gt;850</td>
<td>1020.2</td>
<td>130.1</td>
<td>4.56</td>
</tr>
</tbody>
</table>

* Intra-assay coefficient of variation was determined in 10 replicates of LDL standard for each dose, except for doses 15 and 40 ng where n was 9 after removal of outliers.

b Plasma specimens were analyzed in triplicate.

Table 3. Interassay Variation of ApoB Measurements

<table>
<thead>
<tr>
<th>Pool</th>
<th>Overall daily mean, mg/L</th>
<th>SD, mg/L</th>
<th>Interassay CV, %</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>636</td>
<td>989</td>
<td>1264</td>
<td>40.02</td>
</tr>
<tr>
<td>P</td>
<td>51.6</td>
<td>67.8</td>
<td>91.7</td>
<td>95</td>
</tr>
<tr>
<td>W</td>
<td>56</td>
<td>104</td>
<td>7.3</td>
<td>91</td>
</tr>
</tbody>
</table>

The overall daily mean is the mean of daily means, where n is the number of days. SD, is the SD of daily means from the overall daily mean. Interassay CV = (SD/overall daily mean) × 100.

To determine the reliability of the assay, we used three plasma pools containing low, normal, or high concentrations of apoB. In 56 or more independent analyses of each plasma pool, the interassay CV was ≤8% (Table 3). In these pools, which were stored in aliquots at −70 °C and only thawed once before analysis, the concentrations of apoB were very stable over 18 months and showed no decreasing trend with storage time.

Effects of Blood Collection and Processing

A complete four-factor model analysis was performed to examine the possible effects of specimen processing, as described above; results are shown in Table 4. Freezing lowered the apoB concentrations (P < 0.0001). Storage at −70 °C decreased the mean apoB concentrations in this experiment by 6.8% from a mean (SD) of 768 (202) to 716 (207) mg/L. There were no significant interactions between freezing at −70 °C and the omission or addition of antiproteolytic agents or different reagents contained in the cocktails.

To determine whether the decrease in apoB concentrations due to freezing was a constant percentage of the apoB value in untreated fresh plasma, we compared by linear-regression analysis the percentage changes in frozen, untreated samples with the apoB concentrations in fresh, untreated samples. The slope was −0.28, which was significantly different from 0 (P = 0.033). However, Cook's D test (28) indicated that data from one subject influenced the
results more than fivefold as much as did data from any other subject. Inspection of these data indicated that the apoB concentration in one fresh sample was 41% lower than the mean apoB obtained for all other fresh aliquots from the same subject. Omission of this point, identified as an outlier, gave a slope that did not differ from 0 \( (P = 0.207) \). A slope that did not differ from 0 was also found when percentage changes of the means of all six frozen aliquots per subject were regressed against the means of all aliquots in fresh plasma \( (P = 0.91) \). Finally, the same nonsignificant slope was found when the “fresh-frozen” subset of plasma separated immediately (as identified in Table 4) was so analyzed \( (P = 0.69) \).

Discussion

Our purpose in this study was to determine the effect of different conditions of specimen-processing on the apoB concentrations determined by RIA. With the establishment of an accurate and precise assay and defined specimen-processing conditions, one can examine the predictive value of apoB concentrations for CHD by using a central laboratory in multicenter prospective studies. Our initial efforts were directed towards establishing a reliable radioimmunoassay for apoB. Specificity of the RIA was demonstrated by the absence of displacement of iodinated apoB by large amounts of other apolipoproteins or lipoproteins within the range of concentrations of these apolipoproteins in plasma. The ability of the RIA procedure to accurately determine apoB concentrations was established in recovery experiments. Precision was demonstrated with apoB standards and human plasma.

In subsequent studies we addressed effects of blood collection and specimen processing on apoB values measured in plasma, by examining four major variables:

- the interval between blood sampling and separation of plasma, which may be of relevance in epidemiological studies where it may be necessary to sample participants at remote sites away from a facility with centrifuges;
- the use of antiproteolytic agents during blood collection, which have been shown to be important in maintaining the structural integrity of apoB \( (17) \);
- the storage at \(-70^\circ C\) for six weeks, which reflects conditions applicable for multicenter studies; and
- the addition of various additives to plasma before freezing to perhaps protect the integrity of apoB during freezing and (or) thawing of samples subsequent to storage.

We found no significant difference in measured concentrations of apoB among plasma specimens that were collected as outlined above and never frozen. Adding reagents to blood during collection and (or) to the isolated plasma to protect apoB antigenic determinants had no effect. Bacterial contamination as a source of structural damage to apoB played no role in apoB quantification in this study. However, addition of antibacterial agents may be necessary in conditions where standard laboratory procedures cannot be maintained. There also was no detectable destruction of the relevant apoB epitopes that might have occurred as a result of free radical production. A major concern was the proteolytic action of plasma proteases; however, we observed no significant difference in apoB concentrations in the absence or presence of these structurally protective reagents. Finally, time of separation of plasma from other blood elements did not appear to be critical in apoB quantification. These data suggest that the relevant antigenic determinants of apoB either are inaccessible or are not sensitive to the action of these enzymes or free radicals at the level of sensitivity studied. Thus, blood collected in EDTA-containing tubes does not require other protective reagents for quantifying apoB.

Our results demonstrate that the mean apparent apoB content in human plasma, whether isolated immediately or later, decreased by 6.8% after one cycle of freezing and thawing. Importantly, the percentage loss of apoB immunoreactivity was independent of apoB concentrations in fresh plasma. This loss of apoB immunoreactivity was not prevented by the addition of antiproteolytic, antioxidant, or antibacterial agents. Analyses of the apoB content of the three frozen human plasma pools showed that once-frozen apoB, after the initial 6.8% loss, retains the rest of the measurable apoB epitopes. The stability of apoB epitopes after one freezing thus allows for the establishment of quality-control pools that can be monitored over time. Our study shows that apoB values in frozen specimens may be normalized for comparison of apoB values measured in fresh samples. This initial loss of immunoreactivity for apoB with freezing was not observed for apoA-I under the same conditions \( (32) \).

Our RIA-based observations on the effect of storage of plasma are similar to the 6.5–6.8% decrease in apoB concentrations reported with use of radial immunodiffusion techniques \( (33, 34) \). Other techniques of apoB quantification may necessitate special conditions to maintain apoB concentrations during blood collection or storage. Some apoB epitopes, perhaps of quantitative importance in other procedures, may be sensitive to the processes that were discussed. These considerations of blood collection and specimen processing should be taken into account when designing clinical studies.

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References