Apoprotein A-I Measured by Radial Immunodiffusion in Heparin–MnCl₂ Supernates

Xie-Rong Jiang¹,³ and Paul S. Bachorlk²,⁴

We compared measurements of apoprotein A-I (apoA-I) in plasma and in heparin–MnCl₂ supernates analyzed by radial immunodiffusion. The apoA-I values were similar when the samples were fresh (n = 41, mean (SD), mg/L: plasma, 1393.4(349); heparin–MnCl₂ supernates, 1364.9(332), p <0.01), but were >8% lower in heparin–MnCl₂ supernates after storage for seven days at 4 °C [plasma, 1348.0(351); heparin–MnCl₂ supernates, 1237.6(342), p <0.001]. Neither heparin nor MnCl₂ interfered directly with the immunodiffusion assay, and treating samples with tetramethylurea and urea to maximize the exposure of apoA-I did not prevent the decrease. MnCl₂ (46 mmol/L) added to isolated HDL (d 1.063–1.21) decreased apoA-I values by 5.6% when measured immediately and by 16.7% after storage at 4 °C for seven days. High-density lipoprotein cholesterol values were unchanged by MnCl₂. The results indicated that apoA-I was more stable in plasma than in heparin–MnCl₂ supernates, probably because of an interaction between apoA-I and MnCl₂.

Measurement of high-density lipoproteins (HDL) in plasma has shown an inverse relation between HDL concentration and cardiovascular risk (1–6). Cholesterol in HDL has generally been measured as the index to plasma HDL, and much of the epidemiological data that relate HDL with cardiovascular risk have been obtained (reviewed in 7). More recently, there has been a growing interest in measuring apoprotein A-I (apoA-I), the major apoprotein of HDL, in plasma (5, 8, 9). It is not yet entirely clear, however, which—apoA-I measurements alone or in conjunction with HDL cholesterol—is the better predictor of risk as compared with HDL-cholesterol alone (9–16).

Apoprotein A-I concentrations in plasma are usually measured by immunonephelometric methods. We measured apoA-I by radial immunodiffusion and evaluated the relationship between apoA-I in plasma and apoA-I in the HDL-containing fraction, which was isolated by precipitation with heparin–MnCl₂. We found close agreement between the two measurements when they were made in fresh plasma samples, but the values were about 8% lower for heparin–MnCl₂ supernates that had been stored at 4 °C for a week before analysis.

Materials and Methods

Plasma Samples

Blood was drawn from fasting subjects during routinely scheduled visits to the Johns Hopkins Lipid Referral Clinic. The samples, collected with disodium EDTA (final concentration 1.5 g/L) as anticoagulant, were cooled in an ice bath. Cells were removed within 2 to 3 h of venipuncture, and heparin–MnCl₂ supernates were prepared (17) within a few hours. We analyzed 74 samples, either immediately or after storage for seven days at 4 °C (see below). Of these, 41 had normal lipoprotein concentrations. The remaining 33 samples had the following lipoprotein patterns (18): Type IIa, 19; Type IIb, five; Type IV, nine. All samples had triglyceride concentrations <3.8 g/L. For data analysis, all the samples with above-normal triglyceride concentration (Types IIb and IV) were included in a single group.

Lipid and Lipoprotein Analyses

HDL-containing fractions were prepared by precipitating apoB-containing lipoproteins from 2.0 mL of plasma with heparin and MnCl₂ (final concentrations 1.3 g/L and 46 mmol/L, respectively) (17). This separation was performed within 3 to 4 h of venipuncture. We analyzed plasma cholesterol, triglycerides, and HDL-cholesterol enzymically (Cholesterol (enzymatic) Reagent Set and Triglycerides Reagent Set; Dow Diagnostics, Indianapolis, IN 46268). To measure HDL-cholesterol, we first precipitated MnCl₂ from the heparin–MnCl₂ supernates with NaHCO₃, as previously described (19). We measured apoA-I directly in heparin–MnCl₂ supernates.

ApoA-I Assay

ApoA-I was determined by radial immunodiffusion. We purchased agarose gel immunodiffusion plates (TAGO, Burlingame, CA 94010) that contained monospecific goat antiserum to apoA-I; except as indicated, we used them according to the manufacturer’s instructions. After first diluting the samples sixfold with isotonic saline (NaCl, 150 mmol/L), we applied 5-μL aliquots to the plates. The plates were kept at room temperature and the ring diameters were measured after 72 h.

Reference sera purchased from TAGO were used as the standards. The apoA-I content of this material was established by the manufacturer vs purified apoA-I as the primary standard. We used three concentrations of the standards to construct the standard curves. The area of the precipitin rings was a linear function of apoA-I concentration (r >0.999). To estimate precision, we analyzed a lyophilized serum pool (Omega Lipid Fraction Control; Cooper Biomedical, Malvern, PA 19355) once on each of two plates with each batch of apoA-I analyses. Based on 34 analyses of this pool in 17 analytical runs, the coefficient of variation of the ring diameter readings was 2.9% and that of the assayed apoA-I values (in g/L) was 5.9%. We expected the assayed

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⁵ Nonstandard abbreviations: HDL, high-density lipoproteins; apo, apoprotein; TMU, tetramethyl urea.

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values to be the more variable because they included the variation inherent in reading the ring diameters of both the standards and the pool.

In one set of experiments we measured the concentrations of apoA-I in heparin-MnCl₂ precipitates. We first washed the precipitates three times with isotonic saline containing heparin (1.3 g/L) and MnCl₂ (46 mmol/L), then dissolved them in 1 mL of NaHCO₃ (1 mol/L), and applied the samples to the plates without further dilution. The precipitates were therefore concentrated 12-fold with respect to plasma.

Results

We measured the apoA-I in each sample twice. The first time, the samples were applied to the plates within 4 to 5 h of venipuncture; we then stored aliquots of the samples for seven days at 4 °C and re-analyzed them. Results were similar for both the normal samples and those with abnormal lipoprotein patterns (Table 1). When the samples were fresh, the apoA-I values in heparin-MnCl₂ supernates were 1.8 to 2% lower than in plasma—which indicated that essentially all of the measurable apoA-I was in the heparin-MnCl₂ supernates. In contrast, the apoA-I values in the stored supernates were 7 to 8% lower than in the stored plasma samples.

The supernates were initially clear, but developed some turbidity during storage, presumably from the formation of manganese oxide (20). We also considered the possibility that residual heparin and MnCl₂ may have slowly precipitated some HDL during storage. Therefore, we collected the precipitates that developed in 20 samples, washed them three times with a heparin-MnCl₂ solution, suspended them in 100 mL of NaHCO₃ (1 mol/L), and assayed them. The precipitates contained less than 1 mg of apoA-I per liter. The decrease in apoA-I was therefore not due to the precipitation of HDL during storage.

We also considered that physical changes in HDL in the heparin-MnCl₂ supernates may have restricted the accessibility of apoA-I to the antibody. To maximize the exposure of apoA-I to the antibody, we treated samples with tetramethylurea (TMU) and urea (21). The samples were then analyzed when they were fresh and again after having been stored for seven days at 4 °C. The concentrations of apoA-I in TMU/urea-treated fresh plasma and heparin-MnCl₂ supernates were not significantly different from those in untreated samples (Table 2). After storage, however, the apoA-I concentrations in both untreated and TMU/urea-treated supernates were almost 11% less than in plasma (Table 2), suggesting that the lower concentrations of apoA-I in the supernates were not accounted for by diminished exposure of the apoprotein to the antibody.

We next examined the effect of heparin and MnCl₂ on the immunodiffusion assay itself. ApoA-I was measured in human HDL₃ (d 1.12–1.21) to which we had added either heparin (0 to 8 g/L) or MnCl₂ (0 to 46 mmol/L). Neither reagent changed the diameter of the precipitin rings (data not shown). In this experiment, the greatest heparin concentration we used exceeded by more than sixfold that routinely used to prepare supernates from plasma.

Finally, we examined the effect of the two reagents on isolated human total HDL (d 1.063–1.21). MnCl₂ (final concentration 0 to 91 mmol/L) was added to the lipoprotein and the mixture was assayed immediately and after storage at 4 °C for seven days. When analyzed immediately,

### Table 1. Effect of Storage on ApoA-I Measurements in Plasma and Heparin-MnCl₂ Supernates

<table>
<thead>
<tr>
<th>Lipoprotein pattern</th>
<th>No. of samples</th>
<th>Plasma</th>
<th>Heparin-MnCl₂ supernate</th>
<th>Diff. b</th>
<th>Plasma</th>
<th>Heparin-MnCl₂ supernate</th>
<th>Diff. b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>41</td>
<td>1393.4</td>
<td>1364.9</td>
<td>28.5</td>
<td>1348.0</td>
<td>1237.6</td>
<td>110.5</td>
</tr>
<tr>
<td>Ila</td>
<td>19</td>
<td>1347.4</td>
<td>1322.6</td>
<td>24.7</td>
<td>1367.4</td>
<td>1275.3</td>
<td>94.0</td>
</tr>
<tr>
<td>Hypertriglyceridemia</td>
<td>14</td>
<td>1307.8</td>
<td>1283.9</td>
<td>24.3</td>
<td>1265.7</td>
<td>1162.1</td>
<td>103.6</td>
</tr>
</tbody>
</table>

*The concentrations (mg/L) of lipids and lipoproteins in the samples were (mean and SD): Normals—cholesterol 2140 (392), triglycerides 1218 (553), HDL-cholesterol 539 (135), LDL-cholesterol 2953 (454), triglycerides 1287 (936), HDL-cholesterol 565 (181), LDL-cholesterol 2133 (382), and hypertriglyceridemia—cholesterol 2422 (510), triglycerides 2098 (1143), HDL-cholesterol 503 (196), LDL-cholesterol 1481 (481). Precipitation with heparin-MnCl₂ was performed in fresh plasma within several hours of venipuncture. Plasma and the heparin-MnCl₂ supernate were analyzed immediately and then after storage for seven days at 4 °C. a Difference between apoA-I in plasma and heparin-MnCl₂ supernates. b Significant (by paired t-test) at "p < 0.01 or "p < 0.001.

### Table 2. Assay of ApoA-I in Plasma and in Heparin-MnCl₂ Supernates from 12 Samples before and after Treatment with Tetramethylurea (TMU) and Urea

<table>
<thead>
<tr>
<th>Plasma</th>
<th>Heparin-MnCl₂ supernate</th>
<th>Diff. a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analyzed immediately</td>
<td>1465.0 (383)</td>
<td>1398.3</td>
</tr>
<tr>
<td>Analyzed after storage at 4 °C for seven days</td>
<td>1465.0 (383)</td>
<td>1398.3</td>
</tr>
</tbody>
</table>

a Difference between apoA-I in plasma and in heparin-MnCl₂ supernates. b Difference between results significant at p < 0.001 (paired t-test).
there was a slight linear decrease in the apoA-I values as the MnCl₂ concentration increased (Figure 1A). The regression line indicates a decrease of 5.6% at a MnCl₂ concentration of 46 mmol/L and about twice this much at 91 mmol/L. After storage for seven days there was a 14.4% decrease in apoA-I at the lowest MnCl₂ concentration, with a more gradual decrease at higher concentrations (Figure 1B). The decrease was 16.7% at a MnCl₂ concentration of 46 mmol/L and about 30% at 91 mmol/L. Heparin (up to 3 g/L) did not affect apoA-I values significantly (Figure 1A and B). Measurements of HDL-cholesterol in the stored samples remained constant regardless of the concentrations of MnCl₂ or heparin (Figure 1C).

**Discussion**

We previously reported that the measured values of HDL-cholesterol change when samples are stored (22, 23). We found that if the analyses were delayed, the changes were minimized when apoB-containing lipoproteins were precipitated and the heparin–MnCl₂ supernates were stored. In this study, we found that apoA-I values in plasma and in heparin–MnCl₂ supernates were similar when the samples were fresh, but after storage the values in the supernates were as much as 11% lower than in plasma. Valid comparisons could be made only between plasma–supernate pairs that were analyzed in the same analytical runs; however, we also noted that the slight difference between apoA-I values in fresh and stored plasma could be accounted for by analytical variability. Albers et al. (21) also found that apoA-I values were stable in plasma samples that were stored for four weeks at either 4 °C or −20 °C.

Because some of the immunocohemical recognition sites on apoA-I are masked in intact HDL, various denaturing techniques have been used to maximize the exposure of apoA-I to the antibody (24–26). The immunocohemical exposure of apoA-I in the plasma and supernates was apparently maximal, however, because treatment with TMUuras did not appreciably increase the apoA-I concentrations in either fresh or stored samples.

The findings with isolated HDL suggest that MnCl₂ may directly influence the apoA-I measurements, primarily in HDL₀. First, MnCl₂ decreased the apoA-I values in total HDL (d 1.063–1.21) but not in HDL₃ (d 1.12–1.21). Furthermore, after storage, the greatest decrease occurred at the lowest MnCl₂ concentration; a further four- to fivefold increase in MnCl₂ concentration had much less effect. This suggests that only a subfraction of HDL was susceptible to interference by MnCl₂. The decrease in apoA-I values could result from partial masking of immunoreactive sites on apoA-I by Mn²⁺, or by metal ion-catalyzed partial degradation of the apoprotein, as has been described for apoB, the major apoprotein in low-density lipoprotein (27). HDL itself was not lost from solution, however, because the HDL-cholesterol concentration was not decreased by MnCl₂.

These changes, although less pronounced in supernates prepared from plasma than in isolated HDL, were nonetheless detectable in fresh plasma. The effects may have been attenuated somewhat in the presence of other plasma proteins and by the removal of some of the MnCl₂ with the apoB-containing lipoproteins. Regardless of the mechanism, however, the apoA-I measurements were clearly more stable in plasma than in the supernates. This finding was unexpected in view of our earlier observations that HDL-cholesterol measurements were more stable in heparin–MnCl₂ supernates (23). The observations are relevant to the development of stable pools that can be used to standardize apoA-I assays and maintain their quality control.

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**References**


