Immunonephelometry of Apolipoprotein A-II in Plasma

M. Rosseneu,¹ N. Vainaimont,¹ T. A. Musliner,²,4 D. Bernier,² P. N. Herbert,² and F. Belpaire³

A quantitative assay based on endpoint immunonephelometry was developed for human apolipoprotein A-II (apoA-II) in plasma or serum. Dilution of plasma samples with a 0.1 mol/L solution of sodium cholate enhanced the quantification. We used either purified apoA-II as the primary standard or plasma as a secondary standard. Results correlated well (r = 0.90) with those by a double-antibody radioimmunoassay for 63 serum samples from both normal and hyperlipemic individuals. The interassay coefficient of variation for the immunonephelometric assay was 7% within a working range between 0.05 and 0.7 µg of apoA-II per sample (corresponding to a 1500-fold final dilution of serum). No extraction of samples with organic solvent is necessary if the triglyceride concentration is <4 g/L.

Additional Keyphrases: hyperlipidemia · coronary heart disease · high-density lipoprotein · reference interval

Concentrations of high-density lipoprotein (HDL) cholesterol in plasma are widely estimated as an index to the risk of coronary heart disease (1,8). In general, HDL-cholesterol is considered to be the cholesterol remaining in plasma after polyanionic precipitation of low- and very-low-density lipoprotein cholesterol (2). The mean difference in HDL-cholesterol between age- and sex-matched controls and patients with heart disease is small (30–40 mg/L), often less than the coefficient of variation for the measurement itself (3). For this reason and because cholesterol accounts for only 20% of HDL mass there has been considerable interest in the utility of instead (or in addition) quantifying HDL apolipoproteins in assessing the HDL content of plasma. Although such quantification is still only a research technique, not a routine procedure, measures of apoprotein concentration evidently segregate coronary patients and controls at least as well as quantification of lipids (4). The major HDL apolipoprotein, apoA-I, has been assayed in several surveys (5–7) and shown to be useful in distinguishing patients with coronary heart disease. apoA-II, which comprises about 30% of the protein of HDL, is less widely measured. In two case-control studies no difference was found between apoA-II concentrations in subjects with heart disease and controls (8,9), whereas four similar studies from three laboratories demonstrated that low concentrations of apoA-II are associated with coronary heart disease (7, 10–12). Indeed, multivariate analysis in one of these (12) suggested that data on apoA-II concentrations were the most sensitive and powerful discriminator of all the parameters assessed.

ApO-A-II has been quantified by radioimmunoassay (13–15), radial immunodiffusion (16), electroimmunoassay (17), and enzyme immunoassay (18). We developed an immunonephelometric assay of apoA-II because the method is technically simple, easily automated, and readily applied to screening programs and population surveys. Here we report the details of this assay and its validation.

Materials and Methods

Isolation of apoA-II and preparation of anti-apoA-II serum. HDL were isolated from the plasma of normal volunteers by ultracentrifugal flotation between densities 1.080 and 1.21 kg/L in a Model L5-65 ultracentrifuge (Beckman Instruments, Fullerton, CA 92634). The HDL were delipidated with ether/ethanol (3:1 by vol) at −4 °C, and the apoA-II was purified by ion-exchange chromatography (19).

The purity of the apoA-II preparation was established by electrophoresis in polyacrylamide gel containing 8 mol of urea per liter and sodium dodecyl sulfate, and by immunodiffusion with antisera against apoproteins A-I, A-II, B, C-II, and C-III. The composition of the apoA-II used as a standard for immunonephelometry was determined with a Beckman 119CL amino acid analyzer after hydrolysis under reduced pressure in 6 mol/L HCl for 20 h at 110 °C (20).

The antisera to apoA-II was raised in rabbits as previously described for apoA-I and apoB (21,22). We checked the specificity of the antisera by immunodiffusion against apoproteins A-I, A-II, B, C-I, C-II, and C-III and noted a reaction only against apoA-II.

Immunonephelometric assay. We used a Hyland PDQ manual laser nephelometer (Hyland Div. Travenol Labs., Costa Mesa, CA 92628) and samples of plasma or serum diluted 75-fold with 0.15 mol/L NaCl solution containing 0.1 mol of sodium cholate per liter. The antisera to apoA-II was diluted 60-fold with 10 mmol/L phosphate buffer, pH 7.4, containing 0.15 mol of NaCl and 40 g of polyethylene glycol (M, 6000) per liter, then filtered through a 0.45-µm pore-size filter (Millipore Corp., Bedford, MA 01730). The reaction mixture, 1 mL of diluted antisera and 50 µL of the standard or test sample, was incubated at 23 °C for 4 h. The reagent blank contained 50 µL of diluted plasma and 1 mL of pH 7.4 phosphate buffer; diluted antisera was used as an antibody blank. To construct the calibration curve, we serially diluted the purified apoA-II protein solubilized in pH 7.4 phosphate buffer containing sodium cholate, 0.1 mol/ L. We followed the relative light-scattering (RLS) as a function of time for a given concentration of apoA-II, to determine the kinetics of antibody–antigen association. Standard curves were obtained by plotting RLS as a function of the apoA-II concentration.

Preparation of the test samples. Sera or plasma anticoagulated with EDTA (100 mg/L final conc) were collected after the subject had fasted overnight and apoprotein A-II was measured either in fresh samples or after storage at −20 °C. In agreement with the observations of Albers et al. (23), sera could be stored at −20 °C for as long as a year without any significant change in the apparent concentration. Total (24) and HDL cholesterol (25) and triglycerides (26) were determined by enzymatic methods.

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1 Algemeen Ziekenhuis Sint-Jan, Ruddershove 10, 8000 Brugge, Belgium.
2 Brown University Program-in-Medicine, Department of Medicine, The Miriam Hospital, Providence, RI 02906.
3 Heymans Instituut, Akademisch Ziekenhuis, R.U.G., Gent, Belgium.
4 Present address: National Regional Medical Center-Oakland, Oakland, CA.
5 Nonstandard abbreviations: apo, apolipoprotein; HDL, high-density lipoprotein; and RLS, relative light scatter.
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We tested the following in an effort to maximize exposure of the antigenic sites in purified and plasma apoA-II:

• dissolving the purified apoA-II in phosphate-buffered saline (phosphate 10 mmol/L, NaCl 150 mmol/L, pH 7.4) and diluting the test samples in the same buffer.

• dissolving the purified apoA-II, diluting the plasma samples in 6 mol/L guanidine chloride to a final concentration of 0.3 mol of guanidine chloride per liter in the nephelometric assay.

• dissolving the purified apoA-II or diluting the test samples with phosphate buffer containing one of several detergents: "Tween 20" (polyoxyethylene (20) sorbitan monolaurate, 0.2 and 0.4 g/L), sodium cholate (0.01 and 0.1 mol/L), and "Apovax" (Ortho Diagnostics, Beere, Belgium) (0.1 g/L).

• delipidation of the test samples with ether/ethanol (3/1 by vol) at 4 °C. Lipid-free plasma was diluted with phosphate buffer (10 mmol/L, pH 7.4) containing 150 mmol of NaCl and 8 mol of urea per liter.

**Double-antibody radioimmunoassay.** The double-antibody radioimmunoassay for apoA-II that we used has been described previously (20). Routine assays were designed for a working range of 3 to 15 ng of apoA-II. The intra-assay coefficient of variation (with the same tracer preparation throughout) was 3.5% (n = 20). For aliquots of a serum sample stored at -20 °C and assayed over a six-month period with multiple preparations of 125I-labeled apoA-II, the CV was 7.2%.

**Results**

**Kinetics of immune complex formation.** We studied the kinetics of immune complex formation in buffers containing either 0.3 mol of guanidine chloride or 0.1 mol of sodium cholate per liter (Figure 1). In both plasma and purified apoA-II, immune complexes formed more slowly in the presence of guanidine chloride than with sodium cholate (4 h vs 3 h), and was slower with plasma apoA-II than with the purified protein in both buffers. These data suggest that antibodies have less access to apoA-II in plasma HDL. A conformational change in HDL-proteins probably is required for easier access, which is induced by guanidine chloride and by sodium cholate. Temperature did not significantly affect the reaction (Figure 1) and we routinely performed incubations at 23 °C for 4 h.

**Maximizing reactivity of apoA-II in standards and test samples.** We compared the immunoreactivity of purified apoA-II in phosphate buffer with that of apoA-II in guanidine chloride or detergents. We incubated 50-μL aliquots of the purified apoA-II, in concentrations ranging between 0.05 and 0.8 g/L, at 23 °C for 4 h with 1 mL of diluted antisera and measured the RLS with the nephelometer. The greatest RLS was observed with 0.01 mol/L sodium cholate (Figure 2), followed by Apovax, 0.01 g/L, and Tween 20, 0.2 g/L (data not shown). RLS was less with 0.3 mol/L guanidine chloride and least in phosphate buffer alone. These findings agree with those of Musliner et al. (20), who observed an increased immunological response after either reduction of apoA-II or addition of a detergent. The light-scattering curves obtained for purified apoA-II and plasma apoA-II show a parallel response in 0.1 mol/L sodium cholate (Figure 2).

The reactivity of plasma apoA-II with antibody was also compared after extraction with organic solvent and after treatment with various dissociating agents (Table 1). Immunoreactive apoA-II increased by about 20% after extraction with organic solvent. Only pretreatment of the test samples with sodium cholate yielded comparable results. Treatment with Tween 20, Apovax, or guanidine chloride did not significantly increase the immunoreactive apoA-II, as also was reported for radioimmunoassay (20).

To ascertain analytical recovery with this method, we added 0.1 to 0.5 μg of purified apoA-II to constant volumes of normal serum. After the samples were incubated at 4 °C for 3 h, we measured the results by immunonephelometry. Using the phosphate buffer plus cholate, we detected all of the apoA-II added to serum, without including extraction with organic solvent (Table 1).
Table 1. Analytical Recovery of Immunoreactive ApoA-II in Plasma after Various Treatments

<table>
<thead>
<tr>
<th>ApoA-II added, µg</th>
<th>Untreated</th>
<th>Extraction with organic solvent</th>
<th>Guanidine HCl</th>
<th>Apovex or Tween 20</th>
<th>Sodium cholate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>81 ± 7</td>
<td>97 ± 8</td>
<td>77 ± 9</td>
<td>77 ± 8</td>
<td>97 ± 9</td>
</tr>
<tr>
<td>0.1</td>
<td>77 ± 8</td>
<td>93 ± 9</td>
<td>79 ± 12</td>
<td>78 ± 13</td>
<td>100 ± 11</td>
</tr>
<tr>
<td>0.3</td>
<td>79 ± 7</td>
<td>97 ± 11</td>
<td>78 ± 10</td>
<td>76 ± 10</td>
<td>96 ± 10</td>
</tr>
<tr>
<td>0.5</td>
<td>77 ± 10</td>
<td>96 ± 9</td>
<td>76 ± 10</td>
<td>79 ± 12</td>
<td>93 ± 9</td>
</tr>
</tbody>
</table>

*Means (± SD) of five determinations each. *Means for each were identical and results for both detergents were pooled. *None of the recovery differences in the presence of cholate were significant.

Assay variability and interfering substances. The intra-assay CV for 20 samples, tested in triplicate, was 5%. The interassay CV, determined by testing 10 samples twice a week for four weeks, was 7%. This precision is similar to that for apoA-I (21) and apoB (22) in our immunonephelometric assays.

Hemoglobin and bilirubin do not interfere with either immune complex formation or the nephelometric reading.

Plasma apoA-II concentrations in normal and hyperlipemic subjects. Table 2 summarizes concentrations of apoA-II in plasma of normal and hyperlipemic subjects as determined by immunonephelometry. These values agree with those of Cheung and Albers (16), who measured apoA-II by immunodiffusion. We observed no significant difference between the concentrations of apoA-II in normal persons and dyslipemic subjects, in agreement with previous reports (27).

Correlation between immunonephelometry and radioimmunoassay. We assayed 63 samples from normal and hyperlipemic individuals both by immunonephelometry and by radioimmunoassay. The cholesterol concentrations ranged from 1.3 to 3.9 g/L and the triglycerides from 0.3 to 7.5 g/L. In the presence of sodium cholate, hypertriglyceridemic samples containing as much as 4 g of triglycerides per liter could be assayed by immunonephelometry without prior organic solvent extraction. At higher concentrations, the sample blank values were too high to be read accurately with our nephelometer, i.e., they exceeded 25% of the sample RLS value. After delipidating these samples with ether/ethanol (3:1 by vol) at 4 °C and re-assaying with the buffer containing 0.1 mol of sodium cholate per liter, we found (Figure 3) that the two techniques correlated well (r = 0.90). In the range encompassing most normal subjects (0.1-0.4 g/L), the relation (n = 63) between the apoA-II concentrations assayed by radioimmunoassay (C_{RIA}) and by immunonephelometry (C_{INA}) is as follows: C_{RIA} = 0.026 + 0.92 C_{INA}.

Discussion

As with apolipoproteins A-I and B, the immunonephelometric technique is quite suitable for the quantification of apoA-II in plasma. The precision of the assay compares favorably with both radioimmunoassay and electroimmunoassay (28) and its sensitivity is similar to that of electroimmunoassay.

Table 2. Concentrations of ApoA-II (Mean ± SD) in Plasma from Normal and Dyslipemic Patients

<table>
<thead>
<tr>
<th>Subjects</th>
<th>n</th>
<th>ApoA-II, g/L (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal lipemic men</td>
<td>20</td>
<td>0.35 (0.07)</td>
</tr>
<tr>
<td>Normal lipemic women</td>
<td>30</td>
<td>0.37 (0.06)</td>
</tr>
<tr>
<td>Fredrickson type 2A</td>
<td>20</td>
<td>0.34 (0.06)</td>
</tr>
<tr>
<td>Fredrickson type 2B</td>
<td>25</td>
<td>0.36 (0.07)</td>
</tr>
<tr>
<td>Fredrickson type 4</td>
<td>14</td>
<td>0.35 (0.08)</td>
</tr>
</tbody>
</table>

Fig. 3. Correlation between immunonephelometric assay (INA) and radioimmunoassay (RIA) for the quantification of plasma apoA-II.

The apoA-II protein has several antigenic sites, some of which are masked by the dimeric structure of the native protein and its association with HDL lipids in plasma (20). The addition of guanidine chloride partly exposes these antigenic sites in both purified apoA-II and in HDL. This finding is consistent with observations of Nichols et al. (29), who reported a progressive dissociation of apoA-II from HDL after incubation with guanidine chloride. This effect was persistent after the dilution and incubation with the antiserum and was not observed when the plasma was placed directly into 0.3 mol/L guanidine chloride.

Sodium cholate in a concentration of 0.1 mol/L most effectively exposes the antigenic sites of purified apoA-II, doubling the RLS of the purified protein relative to the response in phosphate buffer. This treatment also increased the apoA-II reactivity in plasma HDL by about 20%, an effect comparable to that obtained by organic solvent extraction of plasma. These results agree well with those of Musliner et al. (20), as do the concentrations of apoA-II we found in normal and hyperlipemic sera.

This immunonephelometric assay of apoA-II, together with those for apoA-I and B, has been applied to apolipoprotein measurement in normal and dyslipemic individuals, in myocardial infarction survivors (30), and in newborns (31). Establishing its clinical value, however, will require much more experience. Quantification of both HDL apolipoproteins and HDL cholesterol should facilitate estimates of HDL subfraction distribution (7) and identify circumstances wherein both HDL composition and concentration are abnormal.

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References