New Assay of Apolipoproteins A-I and B by Rate Nephelometry Evaluated

James J. Maciejko,1,2 Stanley S. Levinson,2,3 Lois Markyvech,2 Michael P. Smith,1 and Roger D. Blevins1

We evaluated new, commercially available reagents for assaying apolipoproteins (apo) A-I and B by rate nephelometry (INA). Our initial linearity studies for apoA-I indicated that use of the commercial diluent resulted in incomplete immunoreactivity. Subsequent revision of the calibration line by the manufacturer compensated for this and improved the linearity for the apoA-I assay. We observed good linearity for the apoB assay. The within-run CVs were <4.0% and the between-run CVs were <5.5% for both assays. Results were 109% for apoA-I and 101% for apoB as compared with those measured for IUIS-WHO reference materials from the Centers for Disease Control. Recovery averaged 103% for apoA-I and 105% for apoB, for duplicate assays of three concentrations of purified apoA-I and low-density lipoprotein (LDL). Assaying sera from 45 patients, we demonstrated a good correlation between INA and radial immunodiffusion for both apoA-I (r = 0.92) and apoB (r = 0.95). Correlations between apoA-I and high-density lipoprotein cholesterol, and between apoB and LDL cholesterol compared favorably with previous reports. We conclude that these assays are accurate, precise, and easily automated for clinical application.

Additional Keyphrases: lipoproteins • cholesterol • immunoreactivity • antigenic sites • heart disease • radial immunodiffusion compared

Low-density lipoprotein (LDL) cholesterol is directly related to coronary atherosclerotic risk (1), whereas high-density lipoprotein (HDL) cholesterol is inversely related (2). However, recent evidence suggests that the protein components of these lipoproteins, apolipoproteins (apo), are better markers of coronary disease than are their corresponding cholesterol constituents (3, 4). The sera of patients with documented coronary disease contain higher concentrations of apoB (the major protein of LDL) and lower concentrations of apoA-I (the major protein of HDL) than sera obtained from control subjects. Therefore, measuring these apolipoproteins could be clinically useful in assessing the risk of coronary atherosclerotic disease.

In the past, measurement of apolipoproteins has not been routinely performed in most clinical laboratories because an accurate, cost-effective, and automated method has not been readily available. Apolipoproteins have been measured in research laboratories by radioimmunoassay (RIA), electroimmunoassay (EIA), and radial immunodiffusion (RID). The former two methods are technically difficult to perform and not easily automated. The latter method, although easier to perform and commercially available, is less precise and requires an incubation period of 48–96 h.

Recently, reagents for rate immunonephelometric assays (INA) of apoA-I and apoB have been developed (Beckman Instruments, Inc., Fullerton, CA 92634). The advantages of INA over other immunoassays are well known (5). The Beckman INA assays have been designed for automation on the “Array” analyzer (Beckman Instruments, Inc.), for use in clinical laboratories. Here we evaluate linearity, reproducibility, analytical recovery, and accuracy of the Beckman INA assays, and compare the results with those obtained by RID.

Materials and Methods

Preparation of Lipoproteins and Apolipoproteins

We isolated apoA-I from pooled plasma by procedures previously described (6). Briefly, chylomicrons, very-low-density lipoproteins (VLDL), and LDL were separated from HDL by ultracentrifugation of plasma at d = 1.063 for 16 h at 120 000 × g. The infranate, containing HDL (d > 1.063), was further centrifuged at d = 1.210 for 48 h at 120 000 × g. The supernate, containing HDL, was dialyzed overnight against de-ionized water containing EDTA (1 mmol/L, pH 7.4). After delipidating HDL with diethyl ether/ethanol (3:1 by vol), we isolated apoA-I by gel filtration on a column containing Sephadex G-150, with a guanidine HCl buffer (5 mol/L, pH 8.0). Protein in the purified sample was measured by the method of Lowry et al. (7).

We isolated LDL from pooled sera by ultracentrifugation at d = 1.019 for 16 h at 120 000 × g. The infranate, containing LDL, was further centrifuged at d = 1.063 for 16 h at 120 000 × g. The supernate, containing LDL, was dialyzed overnight against buffered saline (pH 7.4) containing 1 mmol of EDTA per liter. Again, protein in the final preparation was measured by the method of Lowry et al. (7).

Procedures

RID. For RID assays of apoA-I and apoB we used commercially available RID plates and reference sera (Diffu-Gen; TAGO Inc., Burlingame, CA 94011). Briefly, using the diluent provided, we diluted plasma twofold for apoB assays and sixfold for apoA-I, then added 5 μL of diluted sample to each well on the respective assay plates. Three calibrators were included for assay on each plate. Plates for apoB were

References

1 Lipid Research Laboratory, Center for Cardiovascular Research, and 2 The Department of Laboratory Medicine, Sinai Hospital of Detroit, 6767 West Outer Drive, Detroit, MI 48235.
2 Address correspondence to these authors.
3 Nonstandard abbreviations: LDL, low-density lipoproteins; HDL, high-density lipoproteins; VLDL, very-low-density lipoproteins; RID, radial immunodiffusion; EIA, electroimmunoassay; INA, immunonephelometric assay; PBS, phosphate-buffered isotonic saline; and apo, apolipoproteins.
4 Received May 11, 1987; accepted August 4, 1987.
incubated in a humidified incubator (Model 2220; Queue Systems, Parkersburg, WV 26101) to maintain a constant temperature of 37 °C for 96 h. Plates for apoA-I were incubated in a humidor at room temperature for 48 h. The reaction results on the plates were then read with a calibrating viewer (Model 2743; Transidyne General Corp., Ann Arbor, MI 48108).

Rate INA. To perform rate INA for apoA-I and apoB, we used an Immunochemistry System (ICS) Analyzer II (Model 6622; Beckman Instruments, Inc.) and Beckman reagents. Lyophilized apoA-I and apoB calibrators were provided in the same bottle. We reconstituted the calibrators and the lyophilized control sera samples with de-ionized water 30 min before assay, to ensure complete dissolution. The calibrators and controls are stable for a week after reconstitution when kept refrigerated at 4 °C. The commercial antisera for apoA-I and apoB were provided in separate bottles. The commercial diluent ("APO Diluent") used for diluting samples and the reconstituted calibrators and controls differed from that used for most tests performed with the ICS Analyzer II, in that it contained a detergent (formulation unspecified) in phosphate-buffered isotonic saline (PBS). We also tested a diluent containing 3.75 g of Tween 20 surfactant (J. T. Baker Chemical Co., Phillipsburg, NJ 08865) per liter of PBS (5). All assays were performed in a polymer-enhanced PBS that is routinely used with the ICS analyzer II.

The assays were performed according to the manufacturer's instructions: After reconstitution of the lyophilized calibrators and controls with 1 mL of de-ionized water, we further diluted threefold with de-ionized water the calibrator to be used for apoA-I. Using either the APO Diluent or the Tween 20 diluent, we prepared 36-fold dilutions of the calibrators, controls, and patients' samples. To calibrate the instrument, we used the calibration cards provided, and proceeded as follows: A cuvet containing 600 µL of polymer-enhanced PBS was placed in the measurement chamber of the ICS Analyzer II, and 42 µL of a diluted calibrator was added. Within 20 s, 42 µL of antisera was added. Results were recorded from the alphanumeric display. After the instrument was calibrated, we processed the controls and samples in a similar fashion. All samples were assayed in duplicate and the results averaged. The maximum concentrations that the instrument can read are 2.2 and 2.0 g/L for apoA-I and apoB, respectively; the respective minimum concentrations are 0.25 and 0.3 g/L.

Assay of lipids and lipoproteins in plasma. We assayed cholesterol in plasma and in the total HDL supernate by using an enzymatic method (Dri-STAT; Beckman Instruments, Inc.) and a Spectronic 2000 spectrophotometer (Bausch & Lomb, Rochester, NY 14625). Total HDL in plasma were separated by the dextran sulfate–Mg²⁺ precipitation technique (8, 9). We also used an enzymatic method to measure total triglycerides in plasma (Dri-STAT). All cholesterol and triglyceride assays were performed within 48 h of sample collection. To calculate LDL cholesterol, we used the modified Friedewald equation (10).

Samples for Apolipoprotein Studies

Beckman recommends using serum samples in apolipoprotein assays. However, in preliminary studies of 11 healthy volunteers, we found an excellent correlation between serum (x) and plasma (y) samples assayed for apoA-I (y = 1.03x – 8.81 g/L, r = 0.991) and apoB (y = 0.97x – 1.38 g/L, r = 0.994). The mean values were: apoA-I (plasma) = 1.29 g/L; apoA-I (serum) = 1.34 g/L; apoB (plasma) = 0.79 g/L; apoB (serum) = 0.82 g/L. The increased concentrations observed in serum relative to plasma is consistent with the known slight hemocencentration of proteins in serum.

Plasma samples used in our correlation studies were obtained from 45 adults, ages 25–60 years. Phlebotomy was performed according to guidelines established by the Lipid Research Clinics (11). Blood, collected with solid EDTA (1 mmol/L final concentration) as the anticoagulant, was promptly centrifuged at 4 °C to obtain the plasma. For linearity, reproducibility, and recovery studies, we used serum samples obtained in the same way as plasma, except with the anticoagulant omitted. All samples were stored frozen in aliquots at −20 °C until assayed.

Results

Linearity. We calculated the original calibration curves for apoA-I and apoB for the Beckman rate INA, using serum samples from patients with known high concentrations of either apoA-I or apoB, and samples of purified apoA-I and purified LDL. These samples were all diluted with goat sera to provide decreasing concentrations of apolipoproteins, then further diluted 36-fold with either the APO Diluent or the Tween 20 diluent. We used simple linear regression (unweighted, least-squares method) to establish correlations. Figure 1 (left) illustrates the original calibration curves for apoA-I; Table 1 lists the slopes and y-intercepts. The regression equation for patients' sera diluted with the Tween 20 diluent was very similar to the equation for purified apoA-I diluted with goat sera. However, the regression equations for patients' sera diluted with the APO Diluent had smaller slopes and greater y-intercepts as compared with the other curves. This nonparallelism and positive bias in the y-intercept suggested that the APO Diluent did not allow for complete immunoreactivity of apoA-I in sera, whereas the Tween 20 diluent allowed for virtually complete immunoreactivity.

Subsequently, the calibration cards for apoA-I were modified by the manufacturer, and we repeated the linearity studies. Figure 1 (right) illustrates the revised calibration curve for apoA-I. The revised regression equations for patients' sera diluted with the APO Diluent have slopes and y-intercepts very similar to the curve for pure apoA-I diluted with goat sera (Table 1). This indicates that the new calibration cards compensate for the apparent differences in immunoreactivity we originally observed.

The INA for measuring apoB also displayed good linearity (data not shown). There was parallelism of the various dilution curves, and the y-intercepts were nearly zero for purified LDL and for patients' sera containing high concentrations of apoB diluted with the APO Diluent. The regression equations were: (pure LDL) y = 1.41x + 0.033 g/L, r = 0.997; (serum 1) y = 1.45x – 0.042 g/L, r = 0.998; (serum 2) y = 1.76x – 0.059 g/L, r = 0.999.

Reproducibility. We determined precision by assaying serum pools along with the Beckman controls. The within-run CV was determined for the same sample pipetted 10 times. The within-run CV was 2.0%, 2.1%, and 4.0% for three pools with mean apoA-I concentrations of 1.72, 0.90, and 0.54 g/L, respectively. The within-run CV was 2.0%, 2.2%, and 2.3% for three pools with mean apoB concentrations of 1.71, 1.10, and 0.55 g/L, respectively. The means of duplicate determinations assayed on 10 different days were used to calculate the between-run CV, which was 5.5%, 4.7%, and 3.6% for three pools with apoA-I concentrations of
1.81, 1.34, and 0.63 g/L, respectively, and 5.0%, 3.5%, and 5.1% for three pools with mean apoB concentrations of 1.46, 1.04, and 0.54 g/L, respectively. The between-run CV for the Beckman control was 4.6% (apoA-I concentration 0.97 g/L), and 2.7% (apoB concentration 1.18 g/L). Although all samples in this study were prepared in duplicate, we never observed a substantial difference between the duplicates of any sample.

Analytical recovery. We added three known amounts of purified apoA-I or LDL to serum samples that contained known amounts of endogenous apolipoproteins, and then measured total amounts to assess analytical recovery. To calculate percentage recovery, we subtracted the amount of endogenous apolipoprotein from the measured total amount, divided by the added amount, and multiplied by 100. We calculated this in duplicate for apolipoproteins added to two different sera. Overall recovery averaged 103% for apoA-I and 105% for apoB (Table 2).

Accuracy. To determine accuracy, we assayed vials of the IUHIS-WHO apoA-I and apoB reference material. This material (CDC no. 1883; Centers for Disease Control, Atlanta, Table 1. Slopes and y-intercepts from ApoA-I Linearity Studies

<table>
<thead>
<tr>
<th>Slope</th>
<th>y-intercept</th>
</tr>
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<tbody>
<tr>
<td>Original calibration curves</td>
<td></td>
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<tr>
<td>Pure apoA-I in goat sera</td>
<td>2.324</td>
</tr>
<tr>
<td>Serum 1 in Tween 20 diluent</td>
<td>2.272</td>
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<tr>
<td>Serum 1 in APO Diluent</td>
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<tr>
<td>Serum 2 in APO Diluent</td>
<td>1.490</td>
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<tr>
<td>Revised calibration curves</td>
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<tr>
<td>Pure apoA-I in goat sera</td>
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<tr>
<td>Serum 1 in APO Diluent</td>
<td>1.740</td>
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<tr>
<td>Serum 2 in APO Diluent</td>
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</table>

Table 2. Analytical Recovery of ApoA-I and ApoB Added to Patients’ Sera

<table>
<thead>
<tr>
<th>Endogenous</th>
<th>Added</th>
<th>Measured</th>
<th>Recovery, %</th>
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</thead>
<tbody>
<tr>
<td>ApoA-I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum 1</td>
<td>0.88</td>
<td>0.34</td>
<td>1.03</td>
</tr>
<tr>
<td>Serum 2</td>
<td>0.57</td>
<td>1.13</td>
<td>1.64</td>
</tr>
<tr>
<td>ApoB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum 1</td>
<td>0.55</td>
<td>0.29</td>
<td>0.88</td>
</tr>
<tr>
<td>Serum 2</td>
<td>0.47</td>
<td>0.69</td>
<td>1.23</td>
</tr>
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</table>
GA 30333) is provided along with consensus values obtained from those reported by participating laboratories, which used various immunochemical methodologies. The results (mean ± SD) for two sets of four measurements of each apolipoprotein were: 1.23 ± 0.1 and 1.18 ± 0.1 for an apoA-I reference concentration of 1.10 g/L, and 0.59 ± 0.1 and 0.61 ± 0.0 for an apoB reference concentration of 0.59 g/L. This corresponds to 109% and 101% of the reference-concentration values for apoA-I and apoB, respectively, by the Beckman INA method.

Correlations between RID and Beckman INA. We calculated correlation coefficients by both linear regression and Spearman rank analysis. The relationship between apoA-I as assayed by RID and by INA is displayed in Figure 2 (left) and described by the equation \( y = 0.858x + 0.066 \) g/L \((r = 0.924, \rho = 0.941, n = 45)\). The relationship between apoB as assayed by RID and by INA is displayed in Figure 2 (right) and described by the equation \( y = 0.81x + 0.108 \) g/L \((r = 0.945, \rho = 0.951, n = 45)\).

Correlations between apolipoprotein and lipoprotein concentrations. We found a good correlation between apoA-I \(y\) and total HDI cholesterol \(x\), when the protein was assayed by either RID \(y = 1.689x + 0.658 \) g/L, \(r = 0.844, \rho = 0.860\) or INA \(y = 1.597x + 0.557 \) g/L, \(r = 0.859, \rho = 0.869\). Similarly, apoB \(y\) correlated well with LDL cholesterol \(x\), when assayed by either RID \(y = 0.661x + 0.023 \) g/L, \(r = 0.856, \rho = 0.851\) or INA \(y = 0.604x + 0.042, r = 0.913, \rho = 0.908\). The best correlations were observed by comparing the ratios for apoB/apoA-I vs LDL/HDL cholesterol, with the apolipoproteins assayed by INA \((r = 0.950)\). All correlations appeared to be slightly better for INA than for RID.

Discussion

Lipoprotein constituents are routinely measured in clinical laboratories, and the ratio of total cholesterol to HDL cholesterol is widely used to assess coronary risk \((12)\). Recent evidence suggests that apoA-I and apoB are superior markers for the presence or absence of coronary atherosclerosis disease, as compared with either total cholesterol or the cholesterol constituents of their corresponding lipoproteins \((3, 4)\). In addition, the synthesis of these proteins is genetically determined, and abnormalities may precede changes in the lipid composition of lipoproteins, thereby predisposing to coronary atherosclerosis \((13)\). Recent data from the Bogalusa Heart Study demonstrate that children whose fathers had suffered a myocardial infarction had higher apoB and lower apoA-I concentrations in plasma than did children whose fathers had no evidence of coronary atherosclerosis \((14)\). Despite the apparent clinical value of apoA-I and apoB measurements, however, the widespread measurement of these proteins has been limited, partially by the lack of reliable, reproducible, and automated assays.

Here we demonstrate good linearity, reproducibility, recovery, and accuracy of apoA-I and apoB assayed by a new rate INA method. For the apoA-I assay, our original linearity studies suggested that the APO Diluent resulted in incomplete immunoreactivity, as compared with use of the Tween 20 diluent (Figure 1). Rather than modifying the APO Diluent formulation, the manufacturer opted to revise the calibration cards, and our subsequent studies demonstrated improved linearity.

Our correlation experiments indicate that the results obtained by rate INA compare well with the results obtained by RID (Figure 2). However, apoA-I concentrations measured by INA are lower than those measured by RID, perhaps because of differences in the reference materials for apoA-I used by Beckman and TAGO, Inc., to establish their calibration curves. We also found good correlations between

![Fig. 2. Correlations between RID and nephelometry: (left) apoA-I; (right) apoB](image-url)
the apolipoproteins and the cholesterol constituents of their corresponding lipoproteins. The correlations compare favorably with previous reports involving INA procedures (15, 16), and are consistent with the known association between these lipoprotein constituents. The ratio of LDL/HDL cholesterol is generally considered to be a better indicator of coronary artery disease than either component alone, and is widely used to assess atherosclerotic risk. In our study, the ratio of apoB/apoA-I correlated with the ratio of LDL/HDL cholesterol, such that the results were equivalent 90% of the time ($r^2 = 0.903$). Furthermore, studies have demonstrated that apolipoprotein ratios, in and of themselves, may be superior to lipoprotein ratios as indicators of coronary disease risk (17, 18).

In our hands, the Beckman rate INA kits provided a simple, straightforward, reproducible, and accurate means of measuring apoA-I and apoB. Being easily automated, they should provide for cost-effective clinical applications.

We thank Beckman Instruments for supplying the reagents for this study, and J. Tim Jones for helpful advice.

References