Simultaneous Measurement of Plasma Apolipoproteins A-I and B by Electroimmunoassay

Jean-Charles Fruchart, Ibrahim Kora, Claude Cachera, Véronique Clavey, Patrick Duthilleul, and Yves Moschetter

We describe a simplified electroimmunoassay for quantification of human apolipoproteins A-I and B on prepared plates. A solution of agarose at 55 °C, containing hydroxyethylcellulose and antibodies, is poured onto a plastic film and allowed to gel. Wells are punched in the gels and the plates are dried for storage. Before use, they are rehydrated and buffered. We use succinylated Sudan Black to prestain lipoprotein fractions of plasma. After electrophoresing samples of plasma or standards for 3 h at 4 °C at 12.5 V/cm, we measure the peak heights and read the results from a standard curve prepared by using calibrated sera of known apolipoprotein B and A-I content as secondary standard. The within- and between-assay coefficients of variation were <4% in all cases. Results correlated well with those obtained by classic electroimmunoassay. Subjects with confirmed atherosclerotic lesions had significantly (p < 10^-5) lower ratios of apolipoprotein A-I to apolipoprotein B, compared with ratios in controls.

Additional Keyphrases: electrophoresis, agarose gel, atherosclerosis, heart disease

Abnormal concentrations of plasma lipoproteins have been known for many years to be a major risk factor in the development of premature ischemic heart disease (1).

Although the major lipoprotein fractions are generally evaluated in terms of lipoprotein cholesterol, apolipoproteins may be better predictors than lipids in discriminating between atherosclerotic patients and controls (2).

The risk for vascular disease seems to be particularly associated with an increase in the concentration of apolipoprotein B (Apo-B), the major protein moiety of low-density lipoproteins, and a decrease in apolipoprotein A-I (Apo A-I), the major polypeptide of high-density lipoproteins.

Apolipoproteins have been measured by immunoassays such as radial immunodiffusion (3, 4), electroimmunoassay (5, 6), radioimmunoassay (4–8), immunonephelometry (9–11), and enzyme immunoassay (12).

In this report we describe a simple yet precise and accurate electroimmunoassay involving the use of commercially prepared plates for simultaneous determination of human Apo A-I and B.

Materials and Methods

Blood was drawn after an overnight fast, allowed to clot at room temperature for 60 min, and the serum was separated by low-speed centrifugation. Patients were men and women, ages 20 to 70 years, who were undergoing coronary angiography at the Cardiologic Hospital (Prof. Bertrand), Lille, France. Survivors of myocardial infarction were excluded from the study if the event had taken place less than six weeks before examination. We also investigated a population of apparently healthy volunteers chosen according the criteria set out by Siest and PetitClerc (13).

Lipid determination. Cholesterol and triglycerides were determined with enzymic test kits from Boehringer Mannheim, Mannheim, F.R.G. (14).

Isolation and purification of Apo B and A-I. Lipoprotein-B was obtained by preparative ultracentrifugation (12). Its purity was assessed by double-diffusion analysis and electrophoresis on polyacrylamide gel (15). High-density lipoproteins were isolated by ultracentrifugation of plasma between densities 1.063 and 1.21 kg/L. After centrifugation, the high-density lipoproteins were resuspended at density 1.21 kg/L and dialyzed overnight against a solution of, per liter, 10 mmol of Tris (pH 7.4), 0.1 mol of NaCl, 10 mmol of Na3PO4, and 100 mg of EDTA. The high-density lipoproteins were delipidated with diethylether/ethanol according to Scars and Edelestein (16), and Apo A-I was fractionated by ion-exchange chromatography (17, 18) or by preparative isoelectric focusing (10).

Its purity was assessed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (19) and by immunodiffusion techniques with specific antisera.

Antisera. Antisera to Apo B and to Apo A-I were prepared in adult female goats or rabbits. We emulsified 1 to 10 mg of antigens with an equal volume of Freund's complete adjuvant and injected the emulsion according to Vaitukaitis et al. (20). After six weeks, a second injection was administered. The animals were bled two weeks later. The specificity of the antisera was assessed by double immunodiffusion and two-dimensional immunoelectrophoresis.

Plates for electrophoresis (Apofilms A1 and B). An 8 g/L solution of agarose at 55 °C containing 12 g of hydroxyethylcellulose per liter, and antisera at a suitable dilution, was poured onto plastic films and allowed to gel at room temperature. Wells, 30 per film, were punched in the gel and the plates were dried. Before use, they were rehydrated for 30 min with distilled water and buffered with phosphate buffer (pH 9.2). They should be stored for no longer than 24 h at +4 °C before use.

Electroimmunoassay of apo B and apo A-I. For pretraining lipoproteins from sera or standards, we used succinylated Sudan Black (equal volumes of Sudan Black solution, 1 and 0.1 mol/L saline). We then placed 2 μL of this mixture into each well and electrophoresed for 3 h at 4 °C with a field strength of 12.5 V/cm. Immunoprecipitates ("rosettes") corresponding to Apo B (low peaks) and Apo A-I (high peaks) were then visible (Figure 1).

The height of each rocket was measured from the center of the antigen well to the apex of the peak. The plates were then soaked 1 h in distilled water and dried at room temperature. We included in each run three dilutions of a standard of

1 "Apofilm," "Apofilm Buffer," "Sudan Black Solution," and "Apoprotein Serum Control" were all obtained from Laboratoire Sebia, 92130 Issy les Moulineaux, France.
known concentration, for calibration and also for control of reproducibility.

Results

Standardization of the assays. The commercial control serum was calibrated by use of purified lipoprotein-B (for Apo B) and purified Apo A-I (for Apo A-I). The protein content of the lipoprotein-B and Apo A-I standards was determined by the method of Lowry et al. (21). Figure 2 shows the relationship between rocket heights and Apo B and A-I content of the standards.

Reproducibility. Within-run variation (CV) was measured in 30 aliquots of three specimens (with low, normal, and high values for Apo B and Apo A-I); CVs were, respectively, 4.3, 2.7, and 1.8% for Apo B; 2.9, 2.7, and 2.6% for Apo A-I; and 3.5, 2.3, and 2.1% for the ratio Apo A-I/Apo B. For a rapid assessment of day-to-day imprecision, we analyzed one specimen (with normal values for Apo A-I and B) once per day for 20 consecutive working days. The CVs were 2.9% for Apo B and 2.7% for Apo A-I.

Specificity. The antisera selected for use in the preparation

![Graph](image-url)

Fig. 1. Immunoprecipitates ("rockets") corresponding to Apo B (lower peak of each pair) and Apo A-I (higher peaks)

![Graph](image-url)

Fig. 2. Relation between rocket height and Apo B and A-I content of standards

![Table](image-url)

Table 1. Regression of Estimates of Apo A-I and B obtained by the Proposed Method (y) on the Values Given by Classic Electromiunodiffusion (x)

<table>
<thead>
<tr>
<th></th>
<th>Apo A-I</th>
<th>Apo B</th>
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<tbody>
<tr>
<td>Mean (x), g/L</td>
<td>1.10</td>
<td>1.14</td>
</tr>
<tr>
<td>Mean (y), g/L</td>
<td>1.11</td>
<td>1.08</td>
</tr>
<tr>
<td>Range, g/L</td>
<td>0.4–1.8</td>
<td>0.55–2.10</td>
</tr>
<tr>
<td>Data pairs (n)</td>
<td>82</td>
<td>83</td>
</tr>
<tr>
<td>r</td>
<td>0.91</td>
<td>0.94</td>
</tr>
<tr>
<td>Regression equation</td>
<td>$y = 0.91x + 0.11$</td>
<td>$y = 0.91x + 0.12$</td>
</tr>
<tr>
<td>$s_{yx}$</td>
<td>0.146</td>
<td>0.102</td>
</tr>
</tbody>
</table>

of plates gave a single peak on crossed immunoelectrophoresis against whole human, showing that it was directed against a single antigen.

Accuracy. We have compared results obtained by the proposed method with those given by classic electromiunodiffusion (22), generally accepted as a reference-quality method, for 100 different samples from patients, selected to include a wide range of apolipoprotein concentrations. Results by the two methods correlated well and the means were closely similar (Table 1).

Clinical value in coronary artery disease. Lipid and apoprotein concentrations were determined in normal controls (group I, n = 146) and in patients with (group II, n = 141) and without (group III, n = 102) coronary artery disease demonstrable by coronary angiography. All subjects were chosen without conscious bias. The results are given in Table 2. Patients with coronary artery disease had increased concentrations of lipids and apo B, decreased concentrations of apo A-I, and a decreased ratio of Apo A-I/Apo B. The diagnostic value of each of these variables was then studied for its ability to differentiate between group I and group II (Figure 3). Histograms of the distribution of cholesterol and triglyceride values indicated a great overlap, but the distribution of apoproteins between the two populations is clearly bimodal, with only a slight overlap.

![Table](image-url)

Table 2. Mean Concentrations of Lipids and Apoproteins in Serum of Apparently Healthy Persons (Group I) and in Patients with (Group II) and without (Group III) Coronary Disease

<table>
<thead>
<tr>
<th></th>
<th>Group</th>
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<tr>
<td></td>
<td>I</td>
<td>II</td>
<td>III</td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>146</td>
<td>141</td>
<td>102</td>
<td></td>
</tr>
<tr>
<td>Serum cholesterol, mmol/L</td>
<td>(1)</td>
<td>(1)</td>
<td>(1.4)</td>
<td></td>
</tr>
<tr>
<td>5.23</td>
<td>6.32</td>
<td>5.8</td>
<td></td>
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<tr>
<td>Serum triglycerides, mmol/L</td>
<td>(0.58)</td>
<td>(0.55)</td>
<td>(0.70)</td>
<td></td>
</tr>
<tr>
<td>0.93</td>
<td>1.27</td>
<td>1.28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum Apo B, g/L</td>
<td>(0.19)</td>
<td>(0.12)</td>
<td>(0.18)</td>
<td></td>
</tr>
<tr>
<td>0.98</td>
<td>1.24</td>
<td>1.01</td>
<td></td>
<td></td>
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<tr>
<td>Serum Apo A-I, g/L</td>
<td>(0.12)</td>
<td>(0.17)</td>
<td>(0.16)</td>
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<tr>
<td>1.38</td>
<td>1.15</td>
<td>1.36</td>
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<tr>
<td>Apo A-I/Apo B</td>
<td>(0.26)</td>
<td>(0.20)</td>
<td>(0.28)</td>
<td></td>
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<tr>
<td>1.33</td>
<td>0.85</td>
<td>1.23</td>
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*Values in parentheses are standard deviations. NS, not significant.*
Discussion

Although large-scale epidemiological studies (23, 24) have focused attention on the association between above-normal concentrations of plasma lipids and coronary heart disease, quantitative apolipoprotein determination may be a more nearly accurate predictor in the recognition of risk factors for atherosclerosis. Our own efforts in this field have been directed towards the development of simple and precise immunological methods for quantitation of the two major apoproteins, Apo A-I and Apo B, with the main objective of enabling reliable measurements under routine laboratory conditions.

This electroimmunoassay for Apo B and A-I with use of prepared plates is very reproducible, inexpensive, and easy to perform. Furthermore, it requires no prior extraction of lipids, no staining and destaining of the gels, and only 20 μL of plasma. It seems well adapted in clinical practice for population screening.

We applied this method to the analysis of plasma of patients with and without coronary artery disease as shown by coronary angiography. Our results suggest that determinations of Apo B or of the ratio of Apo A-I to Apo B are better indexes than lipid measurement for the evaluation of atherosclerosis. Although this study was not a prospective one, in our opinion these data show the usefulness of an accurate and precise method for quantification of plasma apolipoproteins for assessing the risk of coronary artery disease in a single patient. This study, which at present is being continued and extended, also supports the close connection between plasma lipoproteins and atherosclerosis.

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References


