Simultaneous Quantification by Double Rocket Immunoelectrophoresis of Apolipoproteins A-I and B in Blood Spotted on Filter Paper

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We describe double rocket immunoelectrophoresis for simultaneous quantification of apolipoprotein A-I (apo A-I) and B (apo B) in blood on filter paper. The apolipoproteins from blood spots on filter paper were eluted with detergents (sodium dodecyl sulfate and Triton X-100). The eluates were subjected to electrophoresis on agarose gel containing antisera against both apolipoproteins. Within- and between-assay CVs for apo B/A-I ratios were <5.5% and 7.2%, respectively. The apo B/A-I ratio was influenced by length and temperature of storage. In results for 121 venous blood samples, the apo B/A-I ratios in dried blood spots correlated well with those in serum (r = 0.92) and correlated somewhat with the ratios for low-density lipoprotein/high-density lipoprotein cholesterol in serum (r = 0.87). Of these specimens, 68 were from patients with known familial hypercholesterolemia, all of whom had an apo B/A-I ratio > 0.90. We think this method will be of value for detecting familial hypercholesterolemia and possibly familial hyperapobeta- and hypoalphaproteinemia.

Additional Keyphrases: screening · heritable disorders · hypercholesterolemia · electrophoresis, agarose gel · heart disease

The concentrations of certain lipoproteins in blood are significantly correlated with the development of coronary artery disease (1, 2). However, several studies (e.g., 3-6), indicate that the concentrations of apolipoproteins identify individuals at high risk of coronary artery disease even better than do the values for lipids, the risk increasing as the concentrations of apolipoprotein (apo) B—the major apoprotein of LDL in serum—increases and as the concentrations of apo A-I—the major apoprotein of HDL—decrease.3

Certain familial lipoprotein disorders are familial in childhood, e.g., familial hypercholesterolemia and familial hyperapobetalipoproteinemia, characterized by an increase of apo B in serum (7-9), and familial hyperalphaproteinemia, characterized by a low concentration of apo A-I in serum (10). Moreover, atherosclerosis leading to coronary artery disease in later life starts in childhood (11, 12). Thus it is important to identify early the children with high-risk families (those with a high incidence of coronary artery disease) who have high concentrations of LDL (serum apo B) or low concentrations of HDL (serum apo A-I), or both, in their serum.

Concentrations of LDL and HDL cholesterol measured in serum from children whose cases were followed prospectively for as long as nine years seem to "track"; i.e., the LDL and HDL values remain within the same quintile(s) in an individual from childhood to adulthood (13). Such individuals must be identified if we are to reduce the increased risk of early coronary artery disease in these children through normalization of their serum lipoproteins and through modulation of other risk factors for coronary artery disease, such as tobacco smoking, hypertension, obesity, and a sedentary lifestyle.

For screening purposes, determination of the apo B/A-I ratio seems particularly appropriate because a result exceeding an age-, sex-, race-specific range would lead to further investigation as to the cause of the above-normal ratio. Here we describe a screening method based on determination of apo B and apo A-I in blood presented as samples dried on filter paper.

Materials and Methods

Materials

The 121 venous blood specimens were taken from 68 patients with familial hypercholesterolemia (24 males and 44 females, ages four to 42 years), and 53 from normal subjects (18 males and 35 females, ages five days to 49 years). Capillary blood obtained by ear prick and corresponding venous blood specimens were taken from 11 normal subjects. About 50 µL of blood was spotted onto filter paper (no. 2992; Schleicher & Schull, Dassel, F.R.G.), allowed to dry at room temperature for 2 h, and then stored at 4 °C in sealed plastic bags until quantification of apo B and A-I on the following day. Cholesterol and triglyceride concentrations in serum obtained from fasting patients, and also the individual lipoproteins, were analyzed enzymatically with an Ektachem Analyzer (Eastman Kodak, Rochester, NY), VLDL cholesterol was measured after ultracentrifugation at 40 000 x g for 20 h, HDL cholesterol after precipitation of VLDL and LDL from serum with dextran sulfate—MgCl₂ (14). The concentration of LDL cholesterol was calculated by subtracting the concentrations of HDL cholesterol and VLDL cholesterol from the concentration of total cholesterol. Between-assay CVs were less than 2% for cholesterol and 5.3% for HDL cholesterol.

Antiserum

The antigens used for immunization were a gift from Prof. T. Olofsson, Umeå, Sweden. LDL and HDL were isolated from EDTA-treated plasma collected from healthy donors after overnight fasting. The LDL was isolated by repeated ultracentrifugation at d = 1.063 for 18 h at 40 000 x g, and apo A-I was isolated from HDL by chromatography on diethylamineoethyl cellulose. HDL was isolated by ultracentrifugation at d = 1.210 for 48 h at 40 000 x g. The homogeneity of each fraction was confirmed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Antisera were raised in rabbits by intradermal immunization with 100 µg of protein, as described previously (15). Antisera from different animals were pooled.
Calibrators and Controls

A commercial standard serum for apo B and apo A-I was obtained from Behring, Marburg, F.R.G. Blood calibrators were prepared as described (15) by mixing equal volumes of the standard serum and plasma erythrocytes that had been washed three times. Fifty microliters of the blood calibrator was spotted onto the filter paper, dried for 2 h at room temperature, and kept at 4 °C in sealed plastic bags for four days. The control was prepared from pooled patients’ serum, lyophilized, and kept at -20 °C. Blood controls were prepared from this lyophilized serum in the same way as the blood calibrators were. A control was run in the beginning and in the end of the plate, and all samples were assayed in duplicate.

Assay

We used double rocket immunoelectrophoresis (RIE) (16) for simultaneous quantification of apo B and apo A-I in the dried blood spots (Figure 1). We moulded between two glass plates (200 × 110 mm) a 1.5-mm thickness of 10 g/L agarose gel (Type II, Medium, EEO; Sigma Chemical Co., St. Louis, MO) in Tris-barbital buffer (20 mmol/L, pH 8.6) containing rabbit antiserum to apo B and A-I. Discs, 3.2 mm in diameter, containing about 2.9 μL of dried blood were punched from filter paper and eluted with 30 μL of the Tris-barbital buffer containing 4 g of Triton X-100 (Fluka AG, Buch, Switzerland) and 0.4 g of sodium dodecyl sulfate (Calbiochem, La Jolla, CA) per liter, by gentle shaking for 1.5 h at 4 °C. We placed 5 μL of eluate in each of the 36 wells (2.5 mm diameter) punched out in the gel and carried out electrophoresis at 2.5 V/cm for 24 h at 12 °C. Afterwards we pressed and washed the gel three times ( 16), then air-dried it. We stained with Sudan Black (Flucka AG) in zinc acetate solution as described by Dudman et al. (17) and then with Ponceau-S solution (Serva, Heidelberg, F.R.G.). The apo A-I rocket was stained red, and the apo B rocket was stained blue. The concentrations of apo A-I and B from blood samples on filter paper were calculated from rocket heights (in mm) by comparison with calibrators run on the same plate. We quantified apo B and apo A-I in serum with the same technique after dilution of sera and standards in a phosphate buffer (10 mmol/L, pH 7.4) containing NaCl (0.15 mol/L) and gelatin (1 g/L).

Results

We tested the specificity of our antiserum by crossed immunoelectrophoresis, which showed a single precipitation peak against human serum. Furthermore, we compared the antisera prepared in our own laboratory with three commercially available preparations: those from Boehringer Mannheim, Mannheim, F.R.G.; Behring-LN; and Daplatex, Glostrup, Denmark. All investigated antisera reacted identically with human serum and calibrators. Several detergents were tested: Tween 20 (Merck, Schuchardt, F.R.G.), Apovax (Ortho Diagnostics, Beerse, Belgium), Thesit (Desitin, Werke Klink, Hamburg, F.R.G.) (17, 15), Triton, and sodium dodecyl sulfate. A combination of Triton (4 g/L) and sodium dodecyl sulfate (0.4 g/L) gave the optimal elution and the most distinct immunoprecipitation peaks of both apolipoproteins.

Errors related to the varying volume of blood in the spots on filter paper and hematocrit were minimized by simultaneously quantifying both apolipoproteins, expressed as the ratio between apo B and apo A-I. We punched 12.32-mm discs from one blood spot. Table 1 shows that the absolute amounts of apolipoproteins within one blood spot from the same patient may differ. The apo B/A-I ratios, however, remained almost constant.

The within-assay coefficient of variation was measured for three pools from blood spot eluates, with each pool analyzed on the same plate (n = 20 each). The mean concentrations were 1.67, 1.17, and 0.58 g/L for apo B pools, and 2.05, 1.40, and 0.51 g/L for apo A-I pools. The respective CVs were 2.5%, 4.4%, and 7.9% for apo B; 1.9%, 3.0%, and 6.9% for apo A-I; and 1.9%, 2.6%, and 5.5% for the apo B/A-I ratio. The between-assay CV was calculated from the values for the blood control, which was included in 61 measurements made during three months. The CV was 16.5% for apo B, 13.9% for apo A-I, and 7.2% for the ratio B/A-I. The mean concentration of apo B was 0.88 and of apo A-I, 1.16 g/L.

The influence of sample storage for seven days at room temperature, 4 °C, and -20 °C is shown in Figure 2. At 4 °C the apo B/A-I ratio did not change within the first three days of storage (P = 0.056). At day 7, however, the ratio increased slightly (P = 0.048). At -20 °C the apo B/A-I ratio did not change.

Table 1. Variation of Apo B, Apo A-I, and B/A-I Ratio within a Single Blood Spot

<table>
<thead>
<tr>
<th>Apo B</th>
<th>Apo A-I</th>
<th>B/A-I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (and SD)</td>
<td>1.41 (0.18)</td>
<td>1.54 (0.18)</td>
</tr>
<tr>
<td>Range</td>
<td>1.15–1.84</td>
<td>1.31–1.71</td>
</tr>
<tr>
<td>CV, %</td>
<td>12.9</td>
<td>11.7</td>
</tr>
</tbody>
</table>

n = 12.32-mm discs each.

![Fig. 1. Apo B/A-I double rockets, with apo A-I corresponding to the higher and apo B to the lower peaks of each pair](image-url)

Immunoelectrophoresis of 5-μL blood spot eluates on gel containing 125 and 375 μL of calibrators to apo B and apo A-I, respectively, per 30 mL of agarose. C, blood control; 1–8, samples in duplicate; S1, apo A-I calibrators (Behring): 1.487, 1.173, 0.89, 0.44, and 0.22 g/L; S2, apo B calibrators (Behring): 1.136, 0.568, 0.284, 0.142 g/L per liter of whole blood.

![Fig. 2. Effect of storage on dried blood spots on the apo B/A-I ratio](image-url)

Samples were stored at -20 °C (○), 4 °C (●), and room temperature (△). Results are the mean of n = 7 each.
change for up to 7 days (P = 0.47). Storage at room temperature always resulted in higher apo B/A-I ratios (P = 0.030), primarily owing to higher apo A concentrations, compared with storage at 4 °C and -20 °C.

Regression line calculation for log (ln) transformed filter-paper blood spot apo B/A-I ratio (y) and log transformed serum apo B/A-1 ratio (x) was performed for 121 specimens with the following concentration ranges: LDL cholesterol, 1.09–8.21 mmol/L; HDL cholesterol, 0.57–1.87 mmol/L; triglycerides, 0.41–4.69 mmol/L; and apo B/A-I ratio, 0.21–1.95. The regression equation was \( y = 0.912x + 0.242, r = 0.92, S_x = 0.191. \) A similar comparison between the log (ln) transformed filter-paper blood spot apo B/A-I ratio (y) and LDL/HDL cholesterol in serum (x) values also showed a linear correlation (y = 0.731x − 1.2319, r = 0.87, S_y = 0.193). The apo B/A-I ratio for capillary blood spots (y) and venous blood spots (x) correlated well (y = 1.06x − 0.07, r = 0.98, n = 11).

**Discussion**

Our aim here was to establish a simple, reliable method for quantifying apolipoproteins in blood spotted onto filter paper. Several methods, based on immunomuassays, for measurement of apolipoproteins from dried blood spots have been described, such as: RIE, radial immunodiffusion (RID), and immunonephelometric assay (INA). By RID (17) and RIE (18) only apo B was measured, whereas both apo A-I and B were measured by INA (15). Simultaneous measurement of apolipoproteins by RIE has been described elsewhere (19), but only for serum.

In comparison with the INA method, the present RIE method for simultaneously quantifying both apolipoproteins is carried out on less material from blood spots, and it requires less antibody. Furthermore, the apolipoproteins are determined in the sodium dodecyl sulfate and Triton eluate from the blood spot in one analytical step.

The error associated with the use of filter-paper blood spots is related to the actual volume of blood analyzed. Factors of importance are the hematocrit, the position of the punch within the spot, and the amount of blood spotted onto filter paper (20). By use of the apo B/A-I ratio, however, these errors can be minimized (15), as is confirmed by the present study.

Earlier studies have shown that apo B and apo A-I from blood dried on filter paper are stable at 4 °C for only a few days (17) or for one month (15, 18). Our investigation of the stability of the apolipoproteins on storage showed that the apo B/A-I ratio is time- and temperature-dependent. Blood samples exposed to prolonged storage or higher or variable temperatures show increases in apo B concentration, probably owing to its degradation. Apo A-I seems to be more stable under the conditions mentioned. For standardization of the storage conditions, blood-spot specimens should arrive in the laboratory on the day of sampling, be kept at 4 °C, and be analyzed within three days, or they should be kept at -20 °C and analyzed within a week. Our results concerning stability are in agreement with the Australian findings (21), where apo B was measured by RID.

The correlation experiments indicate that the apo B/A-I ratios obtained from the blood spots compare well with those obtained from serum. Furthermore, there was a significant correlation between the apolipoprotein ratios for blood spots and LDL/HDL cholesterol ratio in serum. The correlations were always stronger when apo B/A-I ratios were used than when the components were compared separately.

Evidently, the apo B/A-I ratio is the best variable to use when the apolipoproteins are measured in blood that has been spotted on filter paper.

Furthermore, preliminary results confirmed that the method described here can identify patients with known familial hypercholesterolemia, because all of them, in contrast to normal persons, had an apo B/A-I ratio >0.90, which is in agreement with the literature (22).

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**References**


