Improved Stability of Apolipoproteins A-I and B in Filter-Paper Blood Spots Impregnated with Ascorbic Acid, Snezana Micic† and Bent Nørgaard-Pedersen (Dept. of Clin. Biochem., Statens Seruminstitut, Artillerivej 5, DK-2300 Copenhagen, Denmark; †author for correspondence: fax Int +45 32 68 38 78)

In population screening for familial hypercholesterolemia by assaying apolipoprotein A-I and B (apo A-I and apo B), stability of the samples has been a problem (1, 2). We have previously shown (3) that blood-spot specimens should be analyzed for apolipoproteins within 3 days when kept at 4°C or at −20°C. Furthermore, the blood-spot specimens must be transported to the laboratory within 6 h, and kept at 4°C until quantification. By our double rocket immunoelectrophoresis (RIE) assay (3), moreover, we observed a more rapid increase in apo B immunoreactivity from the blood on filter paper than in serum for samples stored at room temperature (data not shown). Others have reported the same findings by ELISA, for apo A-I (2). These findings suggest that dried blood-spot specimens are much more exposed than serum specimens to factors that lead to immunological alterations of apolipoproteins. Initiation of oxidative modification of LDL may be mediated via a free radical-induced peroxidation in the presence of a micromolar amount of transition metals (e.g., iron) in whole-blood spot specimens.

To prevent lipid peroxidation and therefore, also degradation and/or conformational changes of apo A-I and apo B (4–7), we investigated the use of antioxidants and chelating agents (e.g., EDTA) impregnated in filter paper to increase the stability of the apo B/A-I ratio in blood-spot samples.

The double RIE was performed as previously described (3). In brief, 3.2-mm-diameter disks were punched from blood spots dried on untreated filter paper (samples, calibrators, or controls) and subjected to elution in 30 µL of buffer containing Triton X-100 and sodium dodecyl sulfate. The eluates (5 µL) were immunoelectrophoresed on agarose gel containing antisera to both apo A-I and apo B, and the ratio apo B/A-I was determined. Blood-spot calibrators were prepared by mixing equal volumes of standard serum from Immuno AG (Vienna, Austria) with erythrocytes washed in isotonic saline; 50-µL samples were applied on filter paper, air-dried, and kept in sealed plastic bags containing silica gel at −20°C for 2 weeks. The control was prepared from pooled patients’ serum, lyophilized, and kept at −20°C; aliquots of this lyophilized serum were used to prepare control blood spots in the same way as the calibrators.

Stability of the control during the week after thawing (days 0, 1, 3, and 7) is shown by the corresponding apo B/A-I ratios—0.76 (n = 9), 0.74 (n = 8), 0.75 (n = 10), and 0.74 (n = 10)—and the respective CVs: 5.6%, 5.8%, 11.6%, and 5.7%. Average within-assay CVs for the blood control included in 61 assay series during a 3-month period were 4.9% for apo B, 3.9% for apo A-I, and 3.3% for the apo B/A-I ratio (n = 20). The between-assay CVs were 16.5% for apo B, 13.9% for apo A-I, and only 7.2% for the ratio B/A-I.

In a pilot study we tested several antioxidants—buthylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), α-tocopherol, and ascorbic acid—with or without EDTA, EDTA alone, and various antibacterial and antiproteolytic agents. Some of these agents, BHT and BHA at 0.1 g/L, were without effect, whereas at higher concentrations (up to 10 g/L) the apolipoproteins and the ratio apo B/A-I were stabilized in blood spots stored at room temperature. However, the apparent apolipoprotein concentrations were greater (by >30%) than in blood spots on untreated filter paper. Furthermore, the higher BHA concentrations changed the appearance of the “rockets” in RIE. Filter paper impregnated with a mixture of ascorbic acid and EDTA gave the best stability of the apo B/A-I ratio when the dried blood spots were stored at room temperature.

To prepare the filter paper, we sprayed the strips of filter paper (no. 2992; Schleicher & Schull, Dassel, Germany) with a freshly prepared solution of 15 g/L ascorbic acid (Merck, Schuchardt, Germany) and 0.5 g/L EDTA-K (Fluka, Buchs, Switzerland) in 950 mL/L ethanol (Uvasol, Merck). After drying in horizontal position at room temperature for at least 30 min, the dried impregnated filter paper and filter-paper blood samples were stored at room temperature in sealed plastic bags containing a silica gel capsule.

To evaluate the efficiency and stability of the ascorbic acid impregnation procedure, we exposed impregnated filter paper to various environmental conditions and lengths of storage (up to 9 weeks)—4°C, room temperature, direct sunlight, 35°C, higher humidity (close to the water bath), and with and without sealed plastic bags and silica gel—before application of blood spots.

Venous blood specimens from 13 nonfasting persons of the laboratory staff (collected in accordance with the Helsinki Declaration of 1975) were used to examine the stability of the apo B/A-I ratio in dried blood spots from ascorbic acid-impregnated filter paper. Blood samples from eight subjects were spotted onto filter paper with ascorbic acid, and blood from five subjects was spotted onto filter paper without ascorbic acid; all were then dried for 2 h at room temperature. The apolipoproteins from each subject were assayed the same day, i.e., day 0, with and without added ascorbic acid, and after storage on filter paper at room temperature in sealed plastic bags (with and without silica gel capsules) on days 1, 3, and 7. All blood-spot samples from the same person were assayed on the same plate in duplicate.

We used paired Student’s t-test to compare the ratio apo B/A-I values from blood spots from the same patients treated in various ways. In comparing the results from two different groups of patients, the apo B/A-I ratios were expressed as a percentage of the value on day 0; for this comparison we used an ordinary two-sample t-test.

The initial concentrations of apolipoproteins and the apo B/A-I ratio (day 0) were somewhat higher on ascorbic acid-treated paper but not significantly so (all P > 0.05). The apo B concentrations (mean ± SD, n = 13 each) were 0.99 ± 0.28 and 1.04 ± 0.26 g/L on nonimpregnated and impregnated filter paper, respectively; 1.83 ± 0.36 and
2.07 ± 0.43 g/L for apo A-I; and 0.53 ± 0.19 and 0.52 ± 0.18 for the ratio apo B/A-I. The apparently higher apolipoprotein concentrations in ascorbic acid paper might be due to the effect of impregnation on the porosity of the filter paper.

Figure 1 (top) shows the percentage changes from day 0 of the apo B/A-I ratio during a week with and without treated filter paper. In ascorbic acid-impregnated filter paper, the apo B/A-I ratio did not change in the first 3 days; at day 7, the ratio increased by −10.9% (P < 0.05). In filter paper without ascorbic acid, the apo B/A-I ratio increased −40% during the first 24 h; this increase was less marked when the samples were stored with silica gel in the plastic bags but was still significant (P < 0.001). The higher apo B/A-I ratios were caused by apparent increases in apo B concentrations. The presence of silica gel in the storage bags to keep the humidity low also significantly affected the ratio apo B/A-I between samples from ascorbic-impregnated paper and samples from nonimpregnated filter paper. The ascorbic acid-impregnated filter paper in this experiment had been stored for 4 weeks at room temperature before blood application. We repeated the same procedure with ascorbic acid impregnated paper stored for 7 and 9 weeks. The difference in results was not significant between 4 and 7 and 9 weeks of storage, but the apo B/A-I ratios tested after 7 days of storage were higher (18%) on the impregnated paper stored 9 weeks than on that stored for 4 weeks.

There was no significant difference in the apo B/A-I ratio in blood spots on ascorbic acid-impregnated paper that had been stored for 4 weeks under different environmental conditions before blood application (Fig. 1, bottom). However, at day 7 the ratio B/A-I increased significantly (11.3%), except for blood spots applied to ascorbic acid-impregnated paper that had been stored at 4 ºC.

We conclude that blood samples dried on ascorbic acid-impregnated filter paper could be useful in screening programs for familial hypercholesterolemia by quantification of apo B and A-I, expressed as the ratio apo B/A-I.

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References

Rapid and Reliable Identification of Human Apolipoprotein E1 (Gly127→Asp, Arg158→Cys) Variant, Giso Feussner, 1 Jens Lohmann, and Jürgen Dobmeyer [Abt. Innere Med. I, Endokrinol. und Stoffwechsel, Med. Universitätsklinik Heidelberg, Bergheimer Str. 58, D-69115 Heidelberg, Germany; 1 author for correspondence: fax Int + 0049-(0)6221-565226]

The apolipoprotein (apo) E1 (Gly127→Asp, Arg158→Cys) variant has been described in patients with hyperlipidemia, especially those with the genetic lipidoprotein disorder type III hyperlipoproteinemia (HLP) (I–4). Investigators speculate, therefore, that this variant predisposes the carriers dominantly with late or incomplete penetrance to the phenotypic expression of type III HLP (2). Two other apo E1 variants described, apo E1Harrisburg (Lys1146→Glu), (5, 6) and

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