Enzyme-Linked Immunosorbent Assay of Apolipoprotein B in Blood Spotted onto Filter Paper, Suitable for Neonatal Screening

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This assay was developed to measure apolipoprotein (apo) B in blood samples spotted onto filter paper. The long-term aim is to detect young families with the dominantly inherited familial hypercholesterolemia, during current neonatal screening programs. The interassay CV was 5.6%, and apo B concentrations correlated closely with values measured by radial immunodiffusion. In the present assay, the primary apo B standard selected and the serum samples behaved similarly. Use of Triton X-100 in the extraction of apo B from dried blood prevented the decrease in apo B immunoreactivity that otherwise occurred during storage for 20 days at 4 °C. In 57 neonates two to six days postpartum, the mean (and SD) apo B concentration in whole blood was 186 (78) mg/L, and the apo A-I/apo B ratio was higher in female than in male neonates (P <0.001), as is also true for adults. The assay is suitable for use in screening programs for newborns, and the observations add to our understanding of lipid metabolism in neonates.

Additional Keyphrases: apo A-I/apo B ratio · radial immunodiffusion compared · sex-related differences

Apolipoprotein B-100 (apo B) is the principal protein moiety of the atherogenic low-density lipoprotein (LDL), the main cholesterol-carrying protein in the blood. Apo B is the ligand mediating uptake of cholesterol into cells by the LDL-receptor pathway (1), and apo B and LDL cholesterol concentrations in serum increase when there is a decrease in the number of functioning LDL receptors. This occurs in the dominantly inherited familial hypercholesterolemia (FH), and to a lesser extent with obesity and a diet high in saturated fat (2, 3). Concentration of apo B in the circulation may also be increased because of increased biosynthesis (4). Values for apo B and LDL cholesterol in serum are closely correlated. Because both are taken up by the LDL receptors, very high concentrations of either of these analytes should be useful in identifying FH. However, several studies (5–8) suggest that circulating apo B more sensitively predicts premature coronary artery disease than does LDL cholesterol.

In developed countries, blood is sampled by heel prick from all neonates during the first postnatal week and is spotted onto filter paper to test for phenylketonuria, hypothyroidism, and sometimes other disorders, in newborn screening programs. We have explored the feasibility of using measurements of apo B in such blood spots to detect families with FH, and possibly other inherited disorders resulting in increased circulating apo B (9–13). Our aim is to implement family-based coronary-disease prevention, because such families constitute a high-risk group for premature vascular disease (13). Our results so far suggest that high-risk families can be identified by this neonatal screening approach, and that their incidence is about 1 in 500 of the screened population (12), as compared with 1 in 12 500 and 1 in 4000, respectively, for phenylketonuria and hypothyroidism, and 1 in 2500 and 1 in 30000 for cystic fibrosis and galactosemia (14). Recent primary prevention studies indicate that dietary and risk-factor intervention and, where necessary, drug therapy are effective in reducing cardiovascular risk (2, 15, 16).

Earlier we developed a radial immunodiffusion (RID) method for measuring apo B directly in dried blood-spot samples (9). This method gave satisfactory results, but was somewhat cumbersome and not easily automatable. Furthermore, the immunoreactivity of apo B declined with time of storage at 4 °C (10, 17). In contrast, an enzyme-linked immunosorbent assay (ELISA) technique for apo B measurement can provide results within 24 h, is easily automatable, and has been reported to minimize storage problems (18). Here we report on the development of an ELISA assay to measure apo B both in dried blood spots and serum. We have also explored the effects of temperature and storage on apo B immunoreactivity in dried blood spots. We also describe the results of a pilot study of apo B values in 57 neonates during the first postnatal week and their relation to apo A-I concentrations.

Materials and Methods

Subjects

Twenty hypercholesterolemic adults (mean total serum cholesterol 8.18, SD 1.73, mmol/L) and six healthy normocholesterolemic volunteers provided venous blood samples after an overnight fast. Serum was separated from each, and blood from all six normal subjects and from six of the hypercholesterolemic patients was also spotted onto filter paper for the storage study described below.

Venous serum and blood spot samples were also obtained from five children, ages two months to 10 years; and from 57 neonates (21 boys and 36 girls), we obtained a blood sample by heel prick two to six days postpartum and spotted it onto filter paper. The latter samples were taken in the course of routine neonatal screening. All heel-prick blood samples were obtained by a standard newborn screening technique in which blood is allowed to flow freely onto filter paper and to spread by capillary action to cover a defined circle (diameter 14 mm). The filter paper used was specially manufactured for newborn screening and is of known absorbency and thickness (cat. no. SS 2992, grade 908; Schleicher & Schuell, Keene, NH).
Standards and Controls

Calibration apolipoprotein serum (lot no. 15537715-01; Boehringer Mannheim, Mannheim, F.R.G.) was used as our primary standard. Pooled serum, calibrated against the primary standard, was used to set up secondary dried blood-spot standards. We mixed equal volumes of washed fresh erythrocytes and a solution of standard serum or control serum diluted with phosphate-buffered saline (PBS; 10 mmol of sodium phosphate and 150 mmol of sodium chloride per liter, pH 7.4) containing 50 g of bovine serum albumin (BSA) per liter (19). A 50-μL aliquot of this mixture was spotted onto filter paper and air-dried as described below. In seeking a primary standard, we also used Behring standard apo B serum (lot no. A042237A; Behringwerke AG, Marburg, F.R.G.). A reference serum (no. 1883) obtained from the Centers for Disease Control (CDC), Atlanta, GA, was used as an external quality control on each ELISA plate. Behring control serum (lot no. 063604A; Behringwerke AG) and another pooled serum were used as internal quality controls. Dried blood spots were prepared by spotting 50 μL of fresh blood, or standard, or control blood mixtures (9) onto the filter paper, which was allowed to air-dry for 2 to 3 h at room temperature before being stored in plastic bags at −70°C.

Storage Conditions

All standard and control sera were stored in aliquots at −70°C, and all serum samples were stored at −70°C for no longer than 15 days before apo B assay.

Dried blood-spot samples from neonates were stored at −20°C for less than 24 h after collection, and then transferred to −70°C. They were assayed within 15 days of −70°C storage. Dried blood-spot samples for the Triton X-100 storage study (reported below), obtained from five pediatric patients, six healthy laboratory staff, and six hypercholesterolemic patients, were stored at −70°C immediately after the spots were air-dried. Then blood spots from each subject were brought to 4°C on different days so that samples from each subject had been stored at −70°C for 20, 15, 10, 8, 6, 4, 2, and 0 days, by the time of assay. All blood spots from the same patient, stored at 4°C for different periods, were assayed on the same ELISA plate.

Procedures

Preparation of anti-apo B–horseradish peroxidase conjugate. Immunopurified specific sheep anti-apo B antisera (lot no. 10799020-01; Boehringer Mannheim) and Sigma horseradish peroxidase (EC 1.11.1.7; lot no. 125F-9640, HRPO Type II; Sigma Chemical Co., St. Louis, MO) were used to prepare the conjugate by the method of Goding (20). We then mixed the conjugate with an equal volume of PBS containing BSA, 20 g/L, and stored this in 300-μL aliquots at −20°C in sterilized vials. Each thawed aliquot was stored at 4°C until used up. The working dilution of the stored conjugate was 1:200.

ELISA assay of apo B in serum. Assays were carried out with quality-certified microtiter plates (lot no. 1887, Nunc-Immuno Plate II; Nunc, Roskilde, Denmark) at room temperature, with gentle shaking except during the color reaction, when shaking was vigorous. Wells were coated with capture antibody by exposure to 100 μL of the sheep anti-apo B antiseraum 10 mg/L in PBS for 6 h. Unbound antisera was drained off and wells were “blocked” by treatment with 150 μL of 5 g/L BSA solution in PBS for 1 h to reduce nonspecific binding interactions. After washing the wells four times with washing buffer (PBS containing Tween-20, 5 g/L) and shaking them dry, we added to each well 100 μL of the apo B standard, control, or unknown (diluted in PBS containing BSA, 5 g/L) and incubated for 15 h. We then washed the plates five times with washing buffer, and added to each well 100 μL of anti-apo B HRPO conjugate (diluted 200-fold in PBS containing 5 g of BSA and 1 g of Tween-20 per liter), incubated for 2 h, and then washed them again five times with washing buffer and dried by shaking. To start the color reaction, we added to each well 100 μL of 2.2 mmol/L o-phenylenediamine solution in pH 5.0 buffer (per liter, 51.4 mmol of disodium hydrogen phosphate and 24.3 mmol of citric acid) containing hydrogen peroxide, 3.5 mmol/L, and let the reaction proceed for 30 min in the dark, after which we added 100 μL of 2.5 mol/L sulfuric acid reagent. The absorbance of the product was measured at 490 nm within 1 h in a Dynatech Model MR700 microplate reader (Dynatech Laboratories Ltd., West Sussex, U.K.) with integral data processing module and printer. The instrument presents the results as a plot of the absorbance (linear scale, ordinate) vs the logarithm of the apo B concentration (abscissa); the data processor of the reader plots standard curves by using a linear least squares program.

ELISA assay of apo B in dried blood spots. We punched 3-mm (diameter) discs from dried blood spots, and incubated the discs for 1 h at room temperature with occasional shaking in the test tubes containing 250 μL of buffer. We assessed several eluting buffers, including PBS alone and PBS containing BSA (5 g/L) or various detergents: Triton X-100 (1, 10, or 50 g/L), Tween-20 (5 g/L), and Teric G.12A12 D212-21 (10 g/L; I.C.I. Pty Ltd., Sydney, Australia). The eluents were then diluted 10-fold with PBS/BSA (5 g/L), and 100-μL aliquots were assayed in duplicate according to the ELISA method described above.

In the dried blood-spot storage study, the 3-mm discs were placed directly into previously coated and blocked wells, in 200 μL of one of the eluting buffers described above. After 3-h incubation, we removed the eluted discs and washed the wells five times with washing buffer, before resuming the normal assay procedure, i.e., the addition of anti-apo B HRPO conjugate.

Apo A-I. The concentrations of apo A-I in dried blood spots were measured by an ELISA assay previously described (21).

Testing eluted blood spot discs for residual apo B. Dried blood-spot discs stored at 4°C for 0–20 days were eluted in test tubes as described above, with either PBS or PBS/Triton X-100 (10 g/L). After the eluent was removed, discs were washed further with PBS (five times, 3 mL each), transferred to new microplate wells, and suspended in 100 μL of anti-apo B HRPO conjugate solution (diluted 200-fold in PBS containing BSA and Tween-20, as in the serum assay) for 15 h at room temperature. The discs were then washed again six times with 300 μL of PBS, transferred to new microplate wells, and suspended in 100 μL of the o-phenylenediamine substrate solution for 10 min at room temperature. We then stopped the color reaction with 100 μL of 2.5 mol/L sulfuric acid, as described above, and transferred 100-μL aliquots of the reaction solutions to new microplate wells and measured the absorbance at 490 nm.
Statistics

We measured the coefficient of variation (CV) of our assays at various concentrations of apo B, adopting the "precision profile" procedure of Ekins (22). The intersample intra-assay CV was assessed by calculating the CV between the duplicates of all the assayed samples. Then the mean CV was obtained from all the samples within the concentration range, which would include most of the population apo B values (0.4–2.5 g/L). The mean intra-sample intra-assay CV was obtained by assaying 20 replicates of Behring control serum and also of CDC reference serum in three plates. The intra-sample inter-assay CV was assessed by using values for Behring control serum apo B, assayed in duplicate in 11 plates.

Results

A Primary Apo B Standard Serum

In seeking a reliable primary standard apo B serum we investigated standard sera, supplied by Boehringer Mannheim and Behring. In six ELISA plates we measured standard curves of Boehringer Mannheim standard, Behring standard, and pooled serum dilutions. There was consistent agreement between curves of Boehringer Mannheim standards and pooled serum dilutions, and an example is shown in Figure 1. By contrast, the slope of the Behring standard curve was consistently greater than the slopes of the other two curves. The mean ratio of the slopes of the Boehringer Mannheim standard and pooled serum curves (± SD) was 1.018 ± 0.099 (n = 6), whereas the mean ratio of the slopes of Behring standard and pooled serum curves (± SD) was 1.253 ± 0.078 (n = 6).

Serum from a single healthy adult was found to behave essentially the same as Behring standard serum at concentrations in our working range, but at higher concentrations it gave higher A₄₅₀ values than Behring standard serum.

However, when assayed by RID, the slope of the Boehringer Mannheim standard curve was substantially steeper than that of the Behring standard curve (Figure 2). When the pooled serum sample was assayed by RID and compared as before with either the Boehringer Mannheim or the Behring standard curves, the pooled serum had (ring diameter)² values that fit precisely on each of the appropriate standard curves. We found it interesting that the standard curves prepared with Behring control serum and CDC reference serum were essentially coincident with the curve of Behring standard serum measured by RID. Nevertheless, because Boehringer Mannheim standard serum behaved in the same way as the natural sera in both ELISA and RID assays, we chose it to be the primary calibration standard serum. CDC reference serum was used as a quality-control serum in each assay, as were Behring control and another pooled serum, which were all used diluted 8000-fold. The calibrated apo B concentration in pooled serum was 1.44 (SD 0.07) g/L by RID and 1.5 (SD 0.1) g/L by ELISA. The use of Boehringer Mannheim serum as the primary standard for the ELISA assay in our laboratory underestimated the stated apo B concentration of the CDC serum by 27%.

Analytical Considerations

Correlation between RID and ELISA results for serum apo B. The mean (± SD) apo B values for 29 serum samples assayed by both ELISA and RID were respectively 1.48 ± 0.81 and 1.48 ± 0.75 g/L. The differences were not significant by both t- and F-test; by linear regression, the correlation coefficient between the two methods was 0.849, P <0.001 (Figure 3). Samples with apo B concentrations >2.0 g/L correlated less well by the two methods. In particular, three samples had apo B >3 g/L measured by ELISA but ≤2.5 g/L by RID.

Precision. The precision profile technique was used to assess the reproducibility of the ELISA assay (22). For serum samples the average intra-sample intra-assay CV was 3.9% at 1.15 g/L, and 5.4% at 0.7 g/L concentrations of apo B. The mean between-sample intra-assay CV was 4.5 (SD 1.8%) for apo B concentrations between 0.4 and 2.5 g/L. Between-assay intra-sample CV was 8.6% at 1.15 g/L for 11 plates. In dried blood-spot assays, intra-assay intra-sample and between-sample CV was 3.4 (SD 1.4%) within the apo B working concentration range (0.1 to 0.7 g/L); between-
assay intra-sample CV from blood spots supplemented with the CDC serum on eight plates was 5.8%.

Effects of differing elution procedures on blood-spot apo B measurements. Blood-spot apo B values did not alter when the eluting time was varied from 1 to 15 h. Elution with different buffers—PBS, PBS/BSA, PBS/Tween-20, and PBS/Triton X-100—similarly did not change apo B measurements in fresh blood-spot samples.

Effects of storage on apo B concentrations in dried blood spots. The immunoreactivity of apo B in dried blood spots stored at 4 °C decreased with time when PBS was the eluting buffer. By day 20 the assayed apo B immunoreactivity had decreased by approximately 28% (Figure 4). Inclusion of Triton X-100, 10 g/L, in the eluting buffer prevented the decrease in immunoreactivity in all 12 post-fasting blood samples, but two nonfasted blood samples showed no response to Triton X-100. Increasing Triton X-100 concentration to 50 g/L had no further effect, whereas Triton X-100 at 1 g/L was effective in only two samples with apo B <1.8 g/L. Elution of dried blood spot apo B with PBS containing Tween-20 or Teric was no better than elution with PBS alone.

Lack of residual apo B on discs after elution. We tested previously eluted dried blood-spot discs for traces of residual apo B as described in Materials and Methods. No apo B immunoreactivity was detected in these washed discs; the absorbance of all wells was the same as that produced by blank discs. There was no difference between PBS- and PBS/Triton-eluted discs, and samples stored for as long as 20 days showed no difference from fresh samples in this assay.

Clinical Studies

Pilot study of dried blood spots for measuring concentrations of apo B and apo A-I in neonates. For 57 healthy neonates two to six days postpartum, the mean (± SD) blood-spot apo B value was 0.186 (0.078) g per liter of whole blood. The mean apo A-I concentration in the same samples was 0.373 (SD 0.112) g/L, giving an apo A-I/apo B ratio of 2.00. Apo A-I concentrations were significantly (P <0.001) higher in female babies (0.399 ± 0.115 g/L, n = 36) than in males (0.348 ± 0.080 g/L, n = 21). However, apo B values were similar for female and male neonates, 0.189 ± 0.084 and 0.183 ± 0.065 g/L, respectively. The results for individual neonates (including birth weight and date of sampling) are available from the authors.

Correlation between venous serum and blood-spot concentrations of apo B. The concentrations of apo B measured by ELISA in venous serum and blood-spot samples from 11 healthy subjects ranged from 0.35 to 2.1 g/L and correlated closely (r = 0.87).

Discussion

The primary aim of this study was to develop and validate an assay for apo B that could be incorporated into current neonatal screening programs for inexpensive and efficient identification of young families having enhanced cardiovascular risk because of their genetically determined increased concentrations of circulating apo B. Our results indicate that the present assay is suitable.

Crucial to the establishment of any screening assay is the choice of a primary apo B standard (29). The long-term ideal may be to use pure dry apo B as the weighed source of that standard. However, faced with difficulties in maintaining this hydrophobic protein in aqueous solution, we investigated the use of a commercially prepared solution of apo B as an alternative standard. We found that the curve for A_{405} vs apo B concentration in dilutions of Boehringer Mannheim standard serum was similar to the curve obtained with the same dilutions of pooled serum, as measured with either ELISA (Figure 1) or RID (Figure 2) methods. Behring standard and control sera, on the other hand, although behaving similarly to pooled serum and also to an individual serum in dilution in the RID method (Figure 2), had higher immunoreactivity than the pooled serum by ELISA (Figure 1). Thus we chose to use the Boehringer Mannheim serum as the primary standard for calibration. Pooled serum was considered suitable for calibration and use as a secondary standard. An appropriate external quality-control standard is also essential, to obviate the possibility of a systematic error because of differing values obtained for the sample when different assay methods are used (24). CDC apolipoprotein serum may be a valuable candidate for this, and its widespread adoption could facilitate international standardization. However, investigators should be aware of the difference in its behavior in the ELISA assay at high concentrations.

We used RID to assess the accuracy of ELISA for serum and dried blood-spot apo B estimations. We found signifi-
cant discrepancies between the results obtained by the two methods at high apo B concentrations: when apo B exceeded 3 g/L by ELISA, RID values were lower (Figure 3). There are several reasons why this might be so. First, because RID is a gel method, its results may be affected by the size and shape of antigens. Second, the gel reaction requires six to seven days for completion at 20–23 °C, which could be long enough for there to be some denaturation of apo B, particularly at higher concentrations. Third, the concentration of very-low-density lipoproteins in serum may affect serum apo B measurements by RID but not by ELISA (25).

Our earlier study identified decreasing apo B immunoreactivity in stored samples assayed by RID as a potentially significant problem for large-scale neonatal screening (10). The present study shows that Triton X-100 in the eluting buffer attenuates the decrease in dried blood-spot apo B immunoreactivity, an observation also reported by Ohta et al. (18). The reasons for the loss of immunoreactivity and the mechanism of Triton X-100-induced attenuation of it are not clear. Our study nevertheless indicates that neither retention of more apo B particles on the filter paper with time nor altered elution rates of blood-spot apo B are factors. Perhaps structural and (or) chemical changes in apo B-containing particles during storage make epitopes less available to the polyclonal antiserum than when the dried blood spots are fresh and unaltered. Interestingly, the protective effects obtained with Triton X-100 in the eluting buffer were not obtained with the other detergents (Tween-20 and Teric).

When we applied the ELISA assay to the estimation of dried blood-spot apo B concentrations in the 57 neonates, the mean apo B concentration in whole blood was 25% lower than that obtained in our previous study of more than 4500 neonates (10); and the 9% higher concentrations of apo B in female neonates noted in that study were not seen in the smaller population of the present study, despite a trend in that direction. However, when apo B concentrations were related to apo A-I concentrations measured in the same sample by ELISA, the apo A-I concentrations in the female neonates were significantly higher than in the males, yielding correspondingly increased apo A-I/apo B ratios. In premenopausal women this ratio is higher than in age-matched men and is associated with a reduced risk of vascular disease (26). Therefore, the protective higher ratio of apo A-I/apo B found in premenopausal women is already present during the first postnatal week. This occurs despite the slightly higher concentrations of apo B in the female neonates and may be relevant to an understanding of lipid-transport mechanisms in the neonatal period.

In summary, we have investigated methodological problems relevant to the establishment of an ELISA assay that could be used to measure apo B in dried blood spots obtained during current neonatal screening programs, and that could also be used for adult screening. Our results suggest that an ELISA assay with polyclonal antibodies is suitable.

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References