One-Step Competitive Immunochromatographic Assay for Semiquantitative Determination of Lipoprotein(a) in Plasma

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Numerous studies have associated high concentrations of lipoprotein(a) [Lp(a)] with atherosclerosis. We developed a rapid, one-step competitive immunochromatographic assay to measure Lp(a) in plasma. The assay is performed on a nitrocellulose membrane strip and the result is determined by a visual readout of rust-colored colloidal selenium. The assay is based on the principle that Lp(a) in the sample will compete with Lp(a)-coated colloidal selenium for binding to the anti-Lp(a) monoclonal antibody immobilized on the assay strip in the format of four ladder bars. The number of capture bars that appear as a result of the formation of colloidal selenium color is proportional to the concentration of the Lp(a) protein in the samples. The strip assay semiquantitatively measures Lp(a) concentrations ranging from 0 to 180 mg/L of Lp(a) protein in serum, plasma, or fingerstick whole-blood samples. This assay appears very useful for quick identification of individuals with above-normal concentrations of plasma Lp(a) protein (>70 mg/L), and has potential for monitoring a patient's response to treatment with Lp(a)-lowering drugs.

Indexing Terms: lipoproteins · immunochromatography · nitrocellulose reagent strip · dipstick methods · colloidal selenium · monoclonal antibodies

In 1963, Berg (1) described lipoprotein(a) [Lp(a)] as a genetic variant of low-density lipoprotein (LDL). Later, the protein composition, electrophoretic mobility, particle size, and buoyant density of Lp(a) were found to differ from those of LDL (2). After a simple disulfide reduction, Lp(a) dissociates into LDL-like and apolipoprotein(a) [apo(a)] molecules, which suggests that the apo(a) molecule is covalently linked to apo B-100 by a disulfide bond (3–5). The apo(a) molecule on the Lp(a) particle was determined to have a cDNA sequence similar to that of human plasminogen (6, 7).

The Lp(a) particle is more atherogenic than LDL (8, 9), probably because, in part, Lp(a) disturbs the balance between thrombogenesis and fibrinolysis. High concentrations of Lp(a) may favor atherosclerotic plaque formation by inhibiting plasminogen activation by tissue plasminogen activator (7, 10, 11). Numerous studies have indicated that high concentrations of plasma Lp(a) are strongly associated with atherosclerosis (12–16). When Lp(a) is >300 mg/L, the relative risk of coronary atherosclerosis is doubled. When LDL and Lp(a) are both above normal, the relative risk is increased about fivefold (16). Given reports that patients with heterozygous familial hypercholesterolemia with high plasma Lp(a) may develop atherosclerosis earlier than those with low Lp(a) (17–19), the measurement of plasma Lp(a) is considered a very strong predictor of premature coronary artery disease.

Lp(a) has been measured quantitatively by radioimmunoassay (20), radial immunodiffusion (21), rocket immunoelectrophoresis (22), and more recently by ELISA (23–25). All of these methods require either multiple-step procedures or a long assay time. Here, we report on a rapid, one-step, noninstrumented, competitive immunochromatographic method that measures plasma Lp(a) semiquantitatively to identify individuals with higher risk for coronary artery disease.

Materials and Methods

Animal and Human Plasma

Female BALB/c mice 8–10 weeks old were purchased from Charles River Labs., Portage, MI. Human plasma used for Lp(a) isolation and testing was ordered from Interstate Blood Bank, Milwaukee, WI. Purified Lp(a), apo(a), and 29 clinical plasma samples were obtained from A. M. Scanu and G. M. Fless, University of Chicago, Chicago, IL.

Isolation of Lp(a) from Human Plasma

One unit of human plasma was collected from 1 L of whole blood. To minimize proteolysis, we added 30 mL of protease inhibitor stock solution (containing, per liter, 0.2 mol of sodium EDTA, 200 g of chloramphenicol, 25 g of sodium azide, 10 g of gentamicin sulfate, 20 million units of kallikrein inactivator, 1 mol of benzamidine, 0.2 mol of phenylmethylsulfonyl fluoride, and 0.3 mol of NaCl, pH 7.4) to the plasma. Total lipoproteins were isolated by adjusting the plasma to density 1.21 kg/L with solid NaBr and centrifuging in a Beckman 60Ti rotor (Beckman Instruments, Palo Alto, CA) at 477 000 × g (59 000 rpm) for 20 h at 15°C. Total lipoprotein fractions were then dialyzed against NaCl (0.15 mol/L) plus sodium EDTA (0.1 g/L, pH 7) for 24 h with three changes to remove the extra salt. Total lipoproteins were further passed through an apo(a)-specific monoclonal antibody-affinity column, prepared by coupling antibody 4F2 (see next sections) to Affi-Gel 10 (Bio-Rad, Richmond, CA). The bound Lp(a) was eluted from the 4F2-affinity column with glycine·HCl (0.1 mol/L, pH 2.8), and the eluted fractions were immediately adjusted to neutral pH by titration with Tris-base buffer (1 mol/L, pH 10.5). The concentration of Lp(a) protein was determined by using a modified Lowry method (26). Purity of

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1 Nonstandard abbreviations: Lp(a), lipoprotein(a); LDL, low-density lipoprotein; and apo, apolipoprotein.

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the isolated Lp(a) was evaluated by sodium dodecyl sulfate–polyacrylamide gel (66 g/L) electrophoresis under reducing conditions and by immunoblotting to see whether human plasminogen contaminated the Lp(a) purification (27, 28). We observed only apo B-100 and apo(a) bands on the stained gels. This isolated Lp(a) was used to prepare the Lp(a)-seelenium colloid conjugate for the Lp(a) strip assay.

The Lp(a) standard was obtained from A. M. Scanu’s laboratory at the University of Chicago. Isolation and characterization of Lp(a) were described by Fless et al. (5, 25). The Lp(a) standard had the following chemical composition based on weight fraction (g/kg): protein 236, phospholipid 218, free cholesterol 80, cholesteryl ester 376, and triglyceride 90. The factor for converting Lp(a) protein to lipoprotein is 4.2 (5). Therefore, a 70 mg/L Lp(a) protein standard is equivalent to 300 mg/L for total Lp(a) lipoprotein mass, the concentration recommended as the cutoff point for higher risk of atherosclerosis (16).

Anti-Lp(a) Monoclonal Antibody

Production. Female BALB/c mice were immunized four times at 2- to 3-week intervals with 50 μg of Lp(a) protein emulsified with Ribi adjuvant (Ribi Immuno-Chem Research, Inc., Hamilton, MT). Four days after the last boosting, the mice were killed and their immune spleen cells were fused with myeloma cells SP2/0, according to the procedure reported by Geffter et al. (29). After 2 to 3 weeks, spent tissue culture media were collected from hybrid-growing wells of microtiter plates and tested for Lp(a)-binding monoclonal antibodies. The screening procedure was carried out with an ELISA method, in which spent culture media were first incubated on an Lp(a) or apo(a)-coated microtiter plate and then with horseradish peroxidase–goat anti-mouse IgG (G + M) conjugate. An enzyme substrate solution, o-phenylenediamine, was added to each well for signal development, and absorbances were read at 492 nm with a CLS microtiter plate reader (Cambridge Life Sciences, Ltd., Cambridge, UK).

Characterization. Initially, 29 Lp(a)-binding monoclonal antibodies were selected from four cell fusions. Ten of the antibodies reacted with apo B-100 on the LDL particle but not with the isolated apo(a) molecule. Four monoclonal antibodies (8B4, 4D2, 1E1, and 4F2) with high affinity for Lp(a) and apo(a) were purified from mouse ascites fluid with a Protein A–agarose column, and antibodies were eluted from the column with 0.1 mol/L citric acid, pH 3.0. The purified monoclonal antibodies were further characterized in terms of cross-reactivity with human plasminogen and the ability to bind to different Lp(a) isoforms. As much as 1 g/L of human plasminogen did not significantly inhibit the binding of these four monoclonal antibodies to Lp(a)-coated plates in the ELISA. Also, each of these four Lp(a)-specific monoclonal antibodies recognized all of the different isoforms of Lp(a) that were previously separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions.

Preparation of Assay Strip Components

Lp(a)-coated colloidal selenium. Colloidal selenium was prepared essentially as described by Devereaux et al. (30). After adjusting 1 mL of colloidal selenium solution (A440 = 15) to pH 8.0 by adding 50 μL of borate buffer (100 mmol/L, pH 8.5), we added 10 μg of Lp(a) protein, gently vortex-mixed the colloidal mixture for 2 min, and added 10 μL of 100 g/L polyethylene glycol (M, 20 000) to block and stabilize the Lp(a)-coated colloidal selenium. This conjugate was stable in liquid form for 2 months, and in lyophilized form for ≥8 months at room temperature under low humidity.

Assay strip. As illustrated in Figure 1, the floppy assay strip consisted of a sample loading area, a conjugate pad, a measurement region, and an end-of-assay indicator.

Conjugate pad. The assay strip’s conjugate pad is a piece of glass-fiber material (0.8 × 0.3 cm) called Lydall (Lydall, Inc., Hamptonville, NC), which contains dried Lp(a)-coated colloidal selenium, sheep anti-human erythrocyte antiserum, and casein. The anti-erythrocyte antiserum traps erythrocytes in the pad when whole-blood samples are tested; thus, only plasma flows into the membrane strip. The casein helps the colloidal selenium migrate through the membrane strip. The conjugate pad was prepared by saturating a piece of Lydall (18 × 0.8 × 0.01 cm) with 1.5 mL of the conjugate mixture [Lp(a)-coated colloidal selenium at A440 = 10 and a 50-fold dilution of sheep anti-erythrocyte antiserum in (final concentration) 20 g/L casein, 20 mmol/L Tris buffer, pH 7.2]. The conjugate pad was frozen at −40 °C for 30 min and then dried at 10 °C for 16 h in a shelf lyophilizer (VirTis, Inc., Gardiner, NY).

Immobilization of antibody onto nitrocellulose membrane. The measurement region of the assay strip is a piece of nitrocellulose membrane (5 × 0.3 cm), to which was immobilized the Lp(a)-specific monoclonal antibody 8B4 in a four ladder-bar format. A computer-controlled reagent jet was used to dispense the anti-Lp(a) monoclonal antibody reagent, 4 g/L, in a straight line (18 ×...
0.1 cm) onto a piece of 5-μm pore size nitrocellulose membrane (20 × 15 cm) mounted on a mobile platform (31). The reagent jetting rate was 2 μL per inch. Two jetted lines without spacing formed the first capture bar (18 × 0.2 cm). The second, third, and fourth capture bars were printed in a single jetted line (18 × 0.1 cm) with 0.2-cm spacing between each bar.

End-of-assay indicator. The end-of-assay indicator bar was located at the end of the assay strip. A pH-sensitive dye, quinaldine red, was used as the end-of-assay indicator. At pH <1.4, the quinaldine red solution is colorless. When the pH is >3.2, the indicator turns red. The quinaldine red solution (0.2 g/L, pH 0.9) was dispensed with the reagent jet onto the nitrocellulose membrane in a 1-mm-thick line located 2.5 cm above the fourth capture bar. When a plasma sample (pH >5) flows through the end-of-assay indicator bar on the assay strip, the bar turns from colorless to red, indicating that the assay is completed.

Assembly. The schematic assembly of the assay strip is shown in the bottom panel of Figure 1. The conjugate pad (15 × 0.8 cm) is attached to the top side of the antibody-immobilized nitrocellulose membrane (15 × 5 cm) 0.5 cm below the first capture bar, with a 0.1-cm junctional area. The assay strip is then laminated on both sides of the conjugate pad and membrane with 8-cm-wide adhesive tape (Adhesive Research, Inc., Glenrock, PA). The assembled conjugate pad and membrane are then attached to the top of a clear, transparent plastic film (3M, St. Paul, MN) with double-sided adhesive tape. A 1-cm length of plastic film extends beyond the conjugate pad to be used as the sample loading area. This whole assembly is cut into 6 × 0.3 cm assay strips (Figure 1) with a slicing cutter (Schleicher & Schuell, Keene, NH).

Comparison Methods

The Lp(a) ELISA developed by A. M. Scanu’s laboratory was described in detail by Fless et al. (25). Briefly, microtiter plates were coated with 100 μL of affinity-purified rabbit anti-human apo(a) antibody for 2 h at 37 °C. After incubating the samples and then washing away the unbound fraction, we incubated each well of the microtiter plates with 100 μL of goat anti-human apo B antibody for 1 h at 37 °C. After washing the plates, we added 100 μL of rabbit anti-goat IgG–alkaline phosphatase conjugate per well and incubated the plates for 1 h at 37 °C. After washing again, we added 100 μL of p-nitrophenyl phosphate substrate solution per well and incubated the plates in a dark chamber for 30 min at room temperature to develop color. The absorbance of the plates was read at A(492).

The Terumo (Elkton, MD) Lp(a) sandwich assay was carried out according to the manufacturer’s instructions. We added 10 μL of the samples, calibrators, or controls to 2 mL of dilution buffer to make a 201-fold dilution, then pipetted 100 μL of the diluted samples into Lp(a) monoclonal antibody-precoated wells of the Terumo microtiter plate. The plate was incubated at room temperature for 1 h on a rotating platform. After washing away the unbound portion, we pipetted 100 μL of anti-Lp(a)–horseradish peroxidase conjugate into each well. The plate was incubated as above for 20 min and then washed. We then added 100 μL of o-phenylenediamine substrate solution and incubated the plate for 20 min to develop color. The enzyme reaction was stopped and the absorbance of the plate was read at A(405). The concentration of Lp(a) in the samples was determined from a standard calibration curve of total Lp(a) mass (sum of lipid and protein) ranging from 0 to 800 mg/L.

Results and Discussion

Assay Performance

Twenty-five microliters of serum or plasma, or 50 μL of whole blood was applied to the sample loading area. The liquid portion of the samples rehydrated the dried Lp(a)-coated colloidal selenium pad and flowed through the nitrocellulose membrane strip. Lp(a) in the sample competed with the Lp(a)-coated colloidal selenium for binding to the immobilized anti-Lp(a) monoclonal antibody on the capture bars. When the end-of-assay indicator developed, the assay was complete. The assay was completed in 8–10 min with serum or plasma samples and in 12–15 min with whole-blood samples. The results of the assay were determined by the number of bars seen. The number of capture bars that turned a rust color was a function of the concentration of Lp(a) protein in the sample.

Assay Dynamic Range

The dynamic range of the assay was determined by using the assay strips to assay a set of calibrators containing various amounts of purified Lp(a). Each successive capture bar that was stained by Lp(a)-coated colloidal selenium was defined by a range of Lp(a) protein concentrations. As shown in Figure 2, when Lp(a) in the samples

![Image](https://via.placeholder.com/150)

Fig. 2. Illustration of Lp(a) strip assay performance and assay dynamic ranges. The sample is applied to the sample loading area. When the end-of-assay (EOA) indicator has developed, the assay is complete. The number of capture bars that appear is a function of the concentration of the Lp(a) protein in the sample. Each successive capture bar that is stained by the Lp(a)-coated colloidal selenium color is defined by a range of Lp(a) protein concentrations as shown under each strip.

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was <40 mg/L of Lp(a) protein, all the Lp(a)-coated colloidal selenium color would be retained in the first capture bar by the immobilized anti-Lp(a) monoclonal antibody. When the samples contained 40 to <70 mg/L Lp(a) protein, the first two capture bars developed color. In individuals with above-normal plasma Lp(a) concentrations—>70 mg/L of Lp(a) protein has been suggested as the high-risk cutoff point for coronary atherosclerosis—the third capture bar developed color. The dynamic ranges of Lp(a) protein for the third and fourth capture bars were 70 to <120 mg/L and 120 to 180 mg/L, respectively. When all four capture bars had fully developed color on the assay strip, the Lp(a) protein in the samples was ≥180 mg/L. In such cases, the Lp(a) protein assay needed to be repeated after a twofold dilution of the sample. When only a partial area of the highest bar on each assay strip developed color, the Lp(a) protein concentration was further estimated proportionally within the assay range of that capture bar.

Accuracy and Reproducibility

Twenty-nine clinical samples with known lipid/lipoprotein profiles, including Lp(a) concentrations that had been previously determined by Fless et al. (25) with their ELISA, were assayed by the strip assay and the Terumo ELISA to determine Lp(a) concentrations. A set of strips run with these 29 clinical samples is shown in Figure 3. By comparing the number of capture bars developed on each strip with the Lp(a) protein standard calibrated assay range of each capture bar as illustrated in Figure 2, we determined the concentration of Lp(a) protein in these samples semiquantitatively. Sixteen of 29 strips developed color only within the first capture bar, indicating that the Lp(a) protein in these samples was <40 mg/L. Furthermore, when only a partial area of the first capture bar developed color, the Lp(a) protein concentration between 0 and 40 mg/L was estimated proportionally by the visual ratio of the partial area and the whole area of the first bar.

This strip assay was very reproducible between runs with the same sample. A 98% identical agreement was obtained from the same set of assay strips read by two researchers. The Lp(a) concentrations of these 29 samples measured by our strip assay, the ELISA from Fless et al.'s laboratory, and the Terumo ELISA are shown in Table 1. The linear correlation between Fless et al.'s ELISA and the Lp(a) strip assay was $y = -0.57(± 0.45) + 1.06(± 0.05)x$. In the Terumo ELISA, Lp(a) concentration was expressed as total Lp(a) lipoprotein mass. To obtain a comparable unit, we multiplied the Lp(a) protein concentrations measured by the strip assay and Fless et al.'s ELISA by a conversion factor of 4.2 to convert to total Lp(a) lipoprotein mass. The linear correlation of the Terumo ELISA with the strip assay was $y = 5.35(± 3.58) + 1.06(± 0.10)x$. The linear correlation of the Terumo ELISA with that of Fless et al.’s was $y = 8.01(± 3.10) + 0.96(± 0.08)x$. Eleven of the 29 clinical samples (41%) were detected by all three methods as having an above-normal concentration of Lp(a) protein (>70 mg/L) or Lp(a) lipoprotein (>300 mg/L).

Because the Lp(a) strip assay is quick, easy to use, and reliable, we have used it routinely to screen for high Lp(a) in blood donors from local blood banks. The Lp(a) concentrations in 25 plasma samples obtained from Interstate Blood Bank donors were determined by the strip assay (x) and the Terumo ELISA (y); the linear correlation of the results was $y = -2.14(± 2.54) + 1.39(± 0.09)x$. In contrast to the clinical samples from Fless et al.'s laboratory, 41% of which had increased Lp(a) protein concentrations, only 16% to 20% of samples from regular blood donors had increased Lp(a).

Overall, the results from these studies indicate that Lp(a) measured by the semiquantitative strip assay was comparable with that measured by the quantitative Lp(a) ELISA of Fless et al. However, we observed some discrepancy between the Terumo assay and the strip assay. This may be due to the different reagents used and the assay formats or to detection of different apo(a) isoforms in these samples.

Interfering Substances

Recently Eaton et al. (6) and McLean et al. (7) reported that the apo(a) molecule shares a cDNA sequence homology with human plasminogen. Therefore, it was very important to check whether the plasminogen present in the samples would interfere with the strip assay. We found that adding as much as 600 mg/L of plasminogen to 25 plasma samples with different concentrations of Lp(a) had no visual effect on the readings.
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* Determined in the laboratory of Fleiss et al.; see Materials and Methods section.

We thank Angelo Scanu and Günther Fleiss at the University of Chicago for their advice and for providing Lp(a) and clinical samples for this study. Without their help, this project could not have been initiated. We also thank Samer Kundu for his assistance in measuring Lp(a) in 25 samples with the Terumo ELISA.

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from the bar strips when compared with the readings for those strips run with the same samples but without added plasminogen. This also indicated that the anti-Lp(a) monoclonal antibody used as a capture antibody in this assay did not cross-react with human plasminogen at physiological concentrations (120-250 mg/L). As seen in Table 1, although some samples had total triglyceride concentrations >2500 mg/L, there was reasonable agreement between the ELISA of Fleiss et al. and the strip assay. We also found that plasma samples collected with different anticoagulants, such as EDTA and sodium citrate, resulted in identical assay performances.

**Stability of the Assay Strip**

The assembled floppy strip is stable for ≥5 months at room temperature and low humidity. The strips prepared from the same lot were tested every week to evaluate their stability. We saw no change in assay performance with these strips when compared with those originally run with the identical concentration of Lp(a).

In summary, this rapid, noninstrumented, one-step immunochromatographic assay measures Lp(a) concentra-
tein (a) and coronary heart disease in familial hypercholesterolemia [Letter]. Lancet 1988;ii:405.