Lipoprotein(a) Quantified by an Enzyme-Linked Immunosorbent Assay with Monoclonal Antibodies

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This new, sensitive, specific "sandwich"-type enzyme-linked immunosorbent assay (ELISA) for quantifying lipoprotein(a) (Lp(a)) in human serum and in ultracentrifugal lipoprotein fractions is based on use of a monoclonal antibody raised against apolipoprotein(a) as coating protein and a polyclonal antibody, raised against either apo B or against Lp(a) and conjugated with peroxidase, for detection of bound Lp(a). Mean intra- and interassay CVs for assay of 16 samples were 3.0% and 5.6%, respectively. Sample pretreatment with urea did not enhance Lp(a) immunoreactivity, and with treatment with nonionic detergents decreased binding to the monoclonal antibody. Results correlated well ($r = 0.99, n = 38$) with those by radial immunodiffusion (RID). The ELISA assay, however, detects amounts corresponding to Lp(a) contents of 10 to 1000 mg/L in plasma samples diluted 1000-fold, compared with 100–500 mg/L for RID. For 92 normolipidemic subjects, the mean Lp(a) concentration was 120 (SD 130) mg/L. In patients undergoing coronary angiography, Lp(a) concentrations increased with the severity of the disease but were not correlated with either HDL cholesterol, triglycerides, apo A-I, or apo B, and only weakly with plasma cholesterol and apo A-II. These two correlations were even weaker in normal subjects, and only the correlation with total cholesterol was valid. Lp(a), measured at birth and at seven days and six months, steadily increased with age. This assay is well suited for measuring Lp(a) in plasma and in lipoprotein fractions and also for screening programs evaluating this significant genetic risk factor for the development of atherosclerosis.

Additional Keyphrases: heart disease · screening · radial immunodiffusion compared · atherosclerosis · neonates

Measurement of lipoproteins and apoproteins in plasma has established the relation between their abnormal concentrations and risk for development of coronary heart disease (1–3). Among these lipoprotein fractions, Lp(a) has attracted interest, because several studies have shown a two- to threefold increase of the risk for coronary heart disease in subjects whose Lp(a) concentrations exceed 300 mg/L (4, 5). The Lp(a) concentration in serum is largely determined by heredity and seems insensitive to either diet, lifestyle, or therapy with hydroxymethylglutaryl-CoA reductase (EC 1.1.1.88) inhibitors (6, 7). The Lp(a) molecule consists of one molecule of apo B and one molecule of apo(a), linked by a disulfide bridge (8). Because of its high homology to plasminogen (9), quantification of Lp(a) requires the use of either affinity-purified polyclonal antisera or, preferably, monoclonal antibodies that do not react with plasminogen.

Immunological assays for Lp(a) for radial immunodiffusion (RID) (10), electroimmunoassay (11), radioimmunoassay (12), and ELISA (13), with polyclonal antisera, have been reported, as has one ELISA assay with monoclonal antibody (14).

We describe here a sensitive and rapid "sandwich"-type ELISA assay, in which a combination of a specific monoclonal antibody is used for coating and a stable polyclonal anti-apo B-peroxidase conjugate is used for detection. Although Lp(a) concentrations range from <5 to >1000 mg/L in the population, the ELISA assay provides both the sensitivity and the assay range required for measurements of Lp(a) in plasma samples and in lipoprotein fractions. After validating the assay by comparison with RID, we applied it to the characterization of Lp(a) concentrations and distribution in neonates and in adults.

Materials and Methods

Isolation of lipoproteins and production of antisera. The LDL were obtained by ultracentrifugal flotation of fresh plasma at densities between 1.03 and 1.05 kg/L (15). Lp(a) was purified by ultracentrifugation of plasma from donors with high Lp(a) concentrations (>500 mg/L) at densities between 1.06 and 1.10 kg/L. It was further purified by gel filtration on columns of either Sepharose 6B or Supersose FPLC ("Fast Protein Liquid Chromatography"; Pharmacia, Uppsala, Sweden) (13). Only the first-eluting fraction, the one that contains pure Lp(a), was retained. Its purity was checked by gradient gel electrophoresis and by a sensitive ELISA for apo A-I and B. No contamination with either LDL or HDL was observed.

Apo(a) was obtained after reduction of the Lp(a) lipoprotein fraction in the presence of dithiothreitol, 0.5 mmol/L, for 1 h at 23°C, followed by ultracentrifugation (16). Apolipoproteins A-I and B, and their respective antisera, were prepared as previously described (17).

Rabbits were immunized by subcutaneous injection of 0.4 mg of Lp(a), dissolved in 0.5 mL of 5 mmol/L NH₄HCO₃ buffer and emulsified with an equal volume of Freund's complete adjuvant (Difco, Detroit, MI). Booster injections of 0.25 mg of Lp(a), in Freund's incomplete adjuvant, were given at three-week intervals until reasonable titers were obtained. Antisera were obtained by bleeding the animals 10 days after the last injection.

For the generation of monoclonal antibodies, spleen cells of Balb/c mice immunized with Lp(a) were fused according to standard procedures (16). We obtained 14 stable clones, which we tested for specificity against LDL, plasminogen, and apo(a); they reacted only with apo(a) (16). In our assay...
we used the 1D1 clone, an IgG1 κ immunoglobulin fraction.

Preparation of the enzyme-antibody conjugates. Affinity columns for Lp(a) and for apo B were prepared by covalent linkage of 3 μg of Lp(a) or LDL to 1 g of CNBr-activated Sepharose 4B (Pharmacia). Antibodies to human lipoprotein Lp(a) and apo B immunoglobulins were isolated from rabbit antisera by immunosorbent affinity chromatography as described for apo A-I (17).

Horseradish peroxidase (EC 1.11.1.7) was covalently linked to the affinity-purified anti-Lp(a) and anti-apo B immunoglobulins by use of the periodate procedure as previously described (17) to generate the conjugates, which were stored at −70 °C in an equal volume of glycerol.

Quantification of Lp(a) by sandwich ELISA. We coated polystyrene microtiter plates ("Nunc Immuno" plates, "high-binding" quality; Intermed, Roskilde, Denmark) with purified monoclonal antibody 1D1 at 4 μg per milliliter of coating buffer (16). Plates were blocked with the assay buffer, a sodium phosphate buffer (0.05 mmol/L, pH 7.4, plus 0.15 mol of NaCl and 1 g of casein per liter) for 1 h at 23 °C. Between incubations, we washed the plates with assay buffer containing 0.5 mL of added Tween 20 (Bio-Rad Labs, Richmond, CA) per liter.

The sandwich ELISA for Lp(a) was performed as follows. Dilute plasma samples and conjugate 2000- and 6000-fold, respectively, with the same buffer as is used for blocking. Incubate the samples with either the immobilized antibody or the conjugate for 2 h at 37 °C. Determine the peroxidase activity, using o-phenylenediamine as substrate, in analogy with the assays previously developed (17), and measure the absorbance at 490 nm. Prepare calibration curves by making the appropriate dilutions of purified Lp(a).

We verified the concentration of the Lp(a) standard by the classical Lowry procedure for protein and by measuring phenylalanine by HPLC (17). We also compared the response of the primary standard with that of a calibrated serum from Immuno GmbH, Vienna, Austria.

Three pooled sera from normolipidemic donors with low, medium, or high Lp(a) concentrations, respectively, were apportioned and stored at −20 °C for use as secondary standards and controls (17). We prepared standard curves by plotting the absorbance at 490 nm as a function of the logarithm of the Lp(a) concentration and fitted them to an equation of Rodbard with three parameters (17). The specificity of the assay was controlled by checking its cross-reactivity with LDL, apo A-I, albumin, and lipoprotein-free serum.

Subjects. Blood, collected from normal and hyperlipidemic subjects after they had fasted overnight, was treated with disodium EDTA (final concentration 1 g/L), aprotinin ("Trasyllol"; Bayer, Leverkusen, F.R.G.; 104 kallikrein units/L), and sodium azide (0.2 g/L). The separated plasma obtained was stored at −20 °C for no longer than three months before analysis. The normal subjects had been recruited for the WHO-MONICA project, for monitoring coronary risk factors in the general population. Hyperlipidemic subjects were classified according to Fredrickson et al. (18). Plasma was sampled from patients who were undergoing coronaryography and who were classified as having one-, two-, or three-vessel disease on the basis of the angiogram. The familial history of coronary heart disease in these patients was also recorded. Plasma was also obtained from newborns at birth; seven, 30, and 90 days postpartum; and at age six to eight months.

Lp(a) distribution. Lipoproteins were separated from 0.5 mL of fresh plasma by isopycnic ultracentrifugation in a sodium chloride-sucrose gradient (19). Apo A-I and B, and Lp(a) were quantified in all fractions by immunonephelometry (18, 20) and by our sandwich ELISA, respectively. Lipids were determined enzymatically (19).

Sample treatment. To investigate the influence of sample pretreatment with detergents or denaturants on the Lp(a) concentrations, we treated three plasma samples [Lp(a) concentrations of 347, 550, or 830 mg/L] by diluting them 1000-fold with buffer containing urea, 4 mol/L, Tween 20, 0.5 g/L, or Triton X-100, 0.10 g/L (final concentrations). We compared the Lp(a) concentrations measured after these various treatments.

Lp(a) quantification by RID. For this we used immunodiffusion plates, reagents, and standards from Immuno GmbH.

Results

Assay conditions and standardization. The calibration curve obtained with fresh plasma samples paralleled that obtained when purified Lp(a) was used as a primary standard (Figure 1). We investigated the specificity of the ELISA assay for Lp(a) by constructing standard curves with purified apo A-I, human albumin, and lipoprotein-free serum as a source of plasminogen; LDL (d 1.03–1.05) as a source of apo B; and apo(a). As shown in Figure 1, the specificity of the monoclonal antibody used for coating sufficed to keep the cross-reactivity of these antigens with the monospecific anti-apo B conjugate at 0.20% for apo(a), 0.02% for LDL and for plasminogen in the lipoprotein-free serum, and <0.01% for apo A-I and albumin.

Standard curves obtained with the two types of conjugates—polyclonal anti-apo B and anti Lp(a), both labeled with peroxidase—are shown in Figure 2. The assay range is similar for both conjugates (2–50 ng) and the standard curves obtained with Immuno reference serum are quite comparable, as are the values determined for 25 samples. This suggests that both conjugates react with epitopes to apo(a) and apo B, respectively, both of which are equally accessible. We tested the influence of sample pretreatment on Lp(a) immunoreactivity either by exposing samples to denaturing agents (urea) before dilution or by adding detergents to the dilution buffer. Exposure to 4 mol/L urea had no significant effect on the immunoreactivity of Lp(a), whereas the nonionic detergents, Tween 20 and Triton X-100, decreased the Lp(a) immunoreactivity by 17% and 35%, respectively. The intraassay CV of the assay was greater (3% vs 9%) when the detergents were used. Deter-

![Figure 1](https://example.com/fig1.png)

**Fig. 1.** Standard curves, constructed with purified Lp(a) (×), a fresh serum (□), LDL (○), apo(a) (+), apo A-I (△), and lipoprotein-free serum (▼).
gent denaturation of epitopes might decrease binding of the monoclonal antibody to this epitope.

Analytical variables. The ELISA detects as little as 1.0 ng of Lp(a) per assay. Its working range is 2.5 to 50 ng (Figure 1). For samples diluted 2000-fold, this corresponds to Lp(a) concentrations in plasma between 50 and 1000 mg/L.

For samples with a lower concentration, a 500- to 1000-fold dilution is recommended. The standard curve was fitted to the three-parameter Rodbard equation by least-squares analysis, and unknown concentrations were calculated from the fitted curve.

The pooled-plasma samples with low, intermediate, and high concentrations of Lp(a) were analyzed 10 times on the same day and on 12 separate days to calculate the assay precision. Mean intra- and interassay CVs were 3.0% and 5.8%, respectively.

Lp(a) concentrations in plasma. We determined the Lp(a) concentrations in plasma from 92 normolipemic individuals and from 141 patients who were undergoing coronary angiography (Table 1). The mean Lp(a) concentration was 120 (SD 130) mg/L for the normolipemic individuals by the proposed sandwich ELISA. We observed no significant sex-related differences.

As summarized in Table 1, the Lp(a) values in the coronaryography patients increased with the severity of the lesions. We observed a significant difference between the patients with no vessel obstruction and the group with three-vessel disease, in agreement with previous reports (6). When we classified the same individuals according to their lipid values, we observed significantly increased Lp(a) concentrations in the group of Type IIA hyperlipoproteinemic subjects. However, 92% of this group consisted of patients with documented two- or three-vessel coronary artery disease, we could not clearly define the association of Lp(a) values with lipids within this group. Plasma Lp(a) concentrations ranged between 5 and 1600 mg/L in normolipemic and hyperlipidemic individuals. They were not correlated with either HDL cholesterol, apo A1, apo B, or triglyceride concentrations. We observed only a weak correlation with apo AII and with plasma cholesterol.

We also measured Lp(a) in newborns, to compare the evolution of the Lp(a) profiles with those of LDL apo B in the same infants. As shown in Table 2, the Lp(a) in plasma increased most between birth and seven days, as observed for apo B. However, Lp(a) increased up to six months, whereas the concentrations of the other apolipoproteins had stabilized after 30 days (19). With this assay we could detect infants with high Lp(a) concentrations at birth, for whom we confirmed a hereditary pattern (21).

Distribution of Lp(a) in plasma. Utermann et al. (6) suggested a significant association between the Lp(a) concentration, its maximal density, and the size of the apo(a) glycoprotein. We therefore monitored the distribution of Lp(a) in five subjects with Lp(a) concentrations of 200, 700, 780, 900, and 1200 mg/L by fractionating 0.5 mL of plasma by density-gradient ultracentrifugation (19). Figure 3 shows the Lp(a) profile in the individual with a Lp(a) concentration of 1200 mg/L, characterized by a maximal density of 1.075 kg/L. In the four other individuals studied, the density of the Lp(a) maximum ranged between 1.057 and 1.075 kg/L. The corresponding phenotypes of the glycoprotein were S3 for the subject with the lowest concentration and S2 for the others, in agreement with the predicted relationship between concentration and electrophoretic mobility (5).

Comparison of the ELISA and RID assays. We assayed 38 samples from normal and hyperlipidemic individuals both by ELISA and by radial immunodiffusion. The range of Lp(a) concentrations was quite broad, 5 to 1450 mg/L. The lower detection limit for the RID assay was 50 mg/L.

We found a high correlation between Lp(a) as measured by the two techniques (r = 0.99). The relation between the Lp(a) concentrations (mg/L) assayed by ELISA and by RID

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Table 1. Concentrations of Lipid, Apolipoprotein, and Lp(a) in Plasma from 141 Patients Undergoing Coronarography and in Normolipemic Controls

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol, g/L</td>
<td>2.2 (0.4)*</td>
<td>2.3 (0.6)</td>
<td>2.5 (0.5)</td>
<td>2.4 (0.5)</td>
<td>1.8 (0.3)</td>
</tr>
<tr>
<td>HDL-cholesterol, g/L</td>
<td>0.5 (0.2)</td>
<td>0.4 (0.1)</td>
<td>0.5 (0.1)</td>
<td>0.4 (0.08)</td>
<td>NT</td>
</tr>
<tr>
<td>Triglycerides, g/L</td>
<td>1.2 (0.5)</td>
<td>1.4 (0.7)</td>
<td>1.3 (0.7)</td>
<td>1.7 (2.0)</td>
<td>NT</td>
</tr>
<tr>
<td>Apo B, g/L</td>
<td>0.9 (0.2)</td>
<td>1.0 (0.3)</td>
<td>1.0 (0.7)</td>
<td>1.0 (0.1)</td>
<td>1.2 (0.3)</td>
</tr>
<tr>
<td>Apo A-I, g/L</td>
<td>1.1 (0.2)</td>
<td>1.1 (0.1)</td>
<td>1.1 (0.2)</td>
<td>1.1 (0.1)</td>
<td>1.4 (0.2)</td>
</tr>
<tr>
<td>Apo A-I/ apo B</td>
<td>1.28 (0.35)</td>
<td>1.10 (0.3)</td>
<td>1.14 (0.4)</td>
<td>1.11 (0.3)</td>
<td>1.24 (0.4)</td>
</tr>
<tr>
<td>Apo A-II, g/L</td>
<td>0.3 (0.05)</td>
<td>0.3 (0.04)</td>
<td>0.3 (0.06)</td>
<td>0.3 (0.05)</td>
<td>0.4 (0.07)</td>
</tr>
<tr>
<td>Lp(a), mg/L</td>
<td>150 (220)</td>
<td>270 (280)*</td>
<td>250 (300)*</td>
<td>320 (320)*</td>
<td>120 (130)</td>
</tr>
<tr>
<td>No. of subjects</td>
<td>36</td>
<td>30</td>
<td>43</td>
<td>32</td>
<td>92</td>
</tr>
</tbody>
</table>

* Values are mean and SD.

b. Significantly different from results from controls by * P < 0.01 or ** P < 0.001 (Wilcoxon test).

NT, not tested.

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Table 2. Cholesterol, Apolipoprotein, and Lp(a) Concentrations in Plasma of Newborns

<table>
<thead>
<tr>
<th>Days postpartum</th>
<th>n</th>
<th>Total cholesterol, g/L</th>
<th>Lp(a), mg/L</th>
<th>Apo B, g/L</th>
<th>Apo A-I, g/L</th>
<th>Apo A-I/apo B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>39</td>
<td>0.7 (0.2)*</td>
<td>17 (20)</td>
<td>0.3 (0.04)</td>
<td>0.8 (0.1)</td>
<td>2.9 (0.4)</td>
</tr>
<tr>
<td>7</td>
<td>136</td>
<td>NT</td>
<td>50 (60)</td>
<td>0.6 (0.2)</td>
<td>1.0 (0.3)</td>
<td>1.9 (0.7)</td>
</tr>
<tr>
<td>30</td>
<td>39</td>
<td>1.4 (0.3)</td>
<td>47 (60)</td>
<td>0.6 (0.1)</td>
<td>1.2 (0.2)</td>
<td>2.0 (0.7)</td>
</tr>
<tr>
<td>90</td>
<td>27</td>
<td>1.6 (0.4)</td>
<td>72 (115)</td>
<td>0.6 (0.1)</td>
<td>1.3 (0.2)</td>
<td>2.4 (0.7)</td>
</tr>
<tr>
<td>180</td>
<td>141</td>
<td>1.6 (0.3)</td>
<td>80 (100)</td>
<td>0.7 (0.2)</td>
<td>1.3 (0.2)</td>
<td>1.9 (0.4)</td>
</tr>
</tbody>
</table>

*Values are mean and SD.

NT, not tested.

![Graph](image)

Fig. 3. Lp(a) profile in a patient with a high concentrations of Lp(a), 1200 mg/L, in his plasma.

Fresh plasma (0.5 mL) was fractionated by isopycnic ultracentrifugation and the concentrations of apo A-I (A), B (B), Lp(a) (C), and cholesterol (X) were measured throughout the density range.

was: $C_{ELEISA} = 50.0 + 0.99 C_{END}$. The bias between the two techniques is especially pronounced at low concentrations, owing to the lower sensitivity of the RID technique. When the correlation was recalculated for 24 samples with concentrations $>50$ mg/L, the following applied: $C_{ELEISA} = 6.2 + 0.97 C_{END}$; the correlation coefficient ($r$) was 0.97 for the 24 samples.

Discussion

In this technique for quantification of human lipoprotein Lp(a), we used a combination of a monoclonal antibody against apo(a) to "capture" the antigen and a polyclonal antibody against apo B, conjugated with peroxidase, for detection. This experimental procedure combines both the specificity of the monoclonal antibody and the stability and easy coupling procedure for the polyclonal antibody. Moreover, it takes advantage of the nature of the Lp(a) particle, which is coupled to the antibody on the microtiter plate through its apo(a) moiety and is detected through its apo B content. This two-step ELISA assay is rapid, sensitive, specific, and simple to perform.

Previous studies (4, 6, 12) reported the plasma Lp(a) concentrations in normo- and hyperlipidemic subjects and in myocardial infarction survivors. Because of the high homology of Lp(a) with plasminogen, the values reported earlier with immunoassays involving polyclonal antisera may have been overestimates (12). For the Lp(a) assay, the use of a monoclonal antibody to apo(a) without cross-reactivity with plasminogen (16) should ensure specificity.

Moreover, owing to the large concentration range observed within a normal population (<5 to >1500 mg/L), the Lp(a) assay must span a broader range than for the other apolipoproteins. In this respect, less-sensitive assays such as RID and enzyme immunoassay do not fulfill the requirements for plasma Lp(a) measurements in a total population.

In patients undergoing coronary arteriography, we have confirmed the observations of previous authors who reported that Lp(a) increases with the severity of the disease. We monitored the Lp(a) concentrations in newborns, finding a significant increase in both Lp(a) and apo B between birth and the seventh postnatal day. The Lp(a) concentrations increased further up to eight months, whereas apo A-I and B concentrations remained constant. Our assay should also be suitable for use in screening newborns, because we could detect infants with above-normal Lp(a) concentrations at seven days (21).

This assay is therefore well suited for population studies as well as for the screening of individuals with high values for Lp(a), which may represent an additional risk factor for development of atherosclerosis and should therefore be detected at an early age.

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