Human Lipoprotein(a) Quantified by ‘Capture’ ELISA*

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ABSTRACT

Plasma lipoprotein(a), Lp(a), is the most important known genetically controlled independent risk factor for the prediction of early atherosclerosis (AS) and coronary artery disease (CAD) in a significant subpopulation of Caucasians. A sensitive, specific ‘capture’ enzyme linked immunosorbent assay (ELISA) is reported for the assay of human plasma Lp(a). There is no interference from low density lipoprotein (LDL), plasminogen, or from endogenous lipids, hemoglobin, or bilirubin. An immobilized polyclonal rabbit antibody ‘captures’ the Lp(a) ligand, and then a monoclonal murine antibody ‘recognizes’ it. Alkaline phosphatase conjugated rabbit anti­mouse IgG and para-nitrophenyl phosphate substrate ‘detect’ and ‘indicate’ colorimetrically the amount of Lp(a) bound. Quantitation is relative to a commercially available secondary clinical standard.

The frequency distribution for a predominantly Caucasian reference population is highly skewed toward the higher concentrations. The median plasma Lp(a) concentration for healthy Caucasians is 80 mg per l. Relative risk for early myocardial infarction (MI) increases as plasma Lp(a) levels increase above 300 mg per l. Approximately 20 percent of Caucasians have plasma Lp(a) values above 300 mg per l. The frequency distributions of plasma Lp(a) in Blacks and Caucasian type II diabetics are different from the healthy Caucasian reference population. The percentiles of Lp(a) values greater than 300 mg per l in these latter groups is three times higher. Thorough epidemiologic and clinical studies where groups are segregated by race and ethnic origin are needed for accurate clinical interpretation of plasma Lp(a) results. Only neomycin and niacin are shown to lower plasma Lp(a) levels therapeutically, although anabolic steroid medication causes lower plasma Lp(a) concentrations. Endocrine malfunction also may influence plasma Lp(a) levels.

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Introduction

More accurate measurements of plasma lipoproteins than measurements of plasma lipids identify those individuals who are at greater risk in their younger years to develop atherosclerosis (AS) and coronary artery disease (CAD). Laboratory measurements of plasma apolipoprotein B-100 (apo B), apolipoprotein AI (apo AI), and lipoprotein(a) (Lp(a)) are replacing lipoprotein electrophoresis and plasma lipid measurements for diagnosis and management of cardiovascular disease. Lipoprotein(a) is gaining much attention as a quantitative, genetically controlled, independent risk factor for prediction of early myocardial infarction (MI). Lipoprotein(a) first was described in 1963 by Berg as the 'sinking' pre-beta band found by agarose lipoprotein electrophoresis in subjects with CAD. Now this unique lipoprotein is considered in Caucasians of northern European descent to be the most important genetically controlled independent risk factor for development of premature AS and CAD. However, information of its predictive value in other racial and ethnic groups still is inadequate.

It is certain, however, that the reference ranges determined for Caucasians may not be used to interpret plasma Lp(a) results for other population groups at risk. The shapes of the frequency polygons and the percentiles greater than 300 mg per l of plasma Lp(a) concentrations vary greatly among different racial and ethnic groups (table I). For example, 20 percent of Caucasians have plasma Lp(a) values above 300 mg per l, but Blacks may have a frequency of 60 percent as do Caucasian type II diabetics. Lipoprotein(a) levels in cord blood are barely detectable in 75 percent of samples from Caucasian or Black newborns. In a group of 126 Caucasian neonates, only two percent of cord blood Lp(a) values were above 100 mg per l. Moreover, early developmental changes in this lipoprotein are different from apo B and apo AI. Only neomycin and niacin therapy are reported to lower plasma Lp(a) concentrations. Diet and other environmental manipulations are ineffective. Interestingly, anabolic steroid medication and endogenous endocrine malfunction also may affect plasma Lp(a) levels.

Lipoprotein(a) is a low density lipoprotein (LDL)-like particle that contains an additional glycopolipoprotein, apolipoprotein(a) (apo(a)), that is attached by disulfide bonding to the apo B. The apo(a) moiety gives Lp(a) distinctly different biochemical characteristics from LDL. Lp(a) is found in a broad range of ultracentrifuge fractions up to a density of 1.21 g per ml. This great heterogeneity causes it to contaminate the other lipoprotein fractions that include LDL and high density lipoprotein (HDL).

The ultracentrifuge fraction richest in Lp(a) is 1.05 to 1.08 g per ml, where there is considerable overlap with HDL. Lipoprotein(a) carries approximately 12 percent of fasting plasma cholesterol compared to approximately 65 percent

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<th>Population Studied (Reference)</th>
<th>&gt; 300 mg per l Percent</th>
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<td>Caucasians 20</td>
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<tr>
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<tr>
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<td>8</td>
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<td>Easter Island Natives 5</td>
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for LDL and approximately 15 percent for HDL. Its specific biological and pathological roles are a mystery. It is considered a carrier of “bad” cholesterol as is LDL while HDL carries “good” cholesterol. Plasma Lp(a) is under strict genetic control, and concentrations are governed solely by the rate of synthesis in the liver. Its subsequent metabolism is influenced dramatically by the hydrophobic apo(a) constituent. Removal of Lp(a) from the plasma and subsequent catabolism by the “protective” specific LDL receptor mechanism probably is minor, while catabolism by the ‘atherogenic’ non-specific “scavenger” (or acetyl LDL) receptor pathway most likely is the major route. The increased virulence of atherosclerosis (AS) in unfortunate subjects who have concurrently increased plasma levels of Lp(a) and LDL is likely the result of competition for or blocking of the LDL receptor by Lp(a).

The finding that apo(a) has a great deal of structural homology with the plasma coagulation protein, plasminogen, has aroused much interest.7,26 It is speculated that Lp(a) may block conversion of plasminogen to plasmin by tissue plasminogen activator (TPA) and, thereby, prevent the normal lysis of fibrin clots. This may be the long sought link between atherogenesis and the plasma coagulation system. Researchers question whether or not this could be the initiating event of atheroma formation. Moreover, the association of Lp(a) plasma levels with specific phenotypes and their relative pathologic virulences presently are under active investigation.6,27,28

A sensitive, specific solid-phase microtiter plate ELISA was developed for the quantitation of human Lp(a) total concentrations in plasma or serum. The procedure is efficient and suitable for use in research or in clinical laboratories.

Materials and Methods

PRINCIPLE

This solid-phase ‘capture’ or ‘sandwich’ ELISA uses a high avidity, high specificity, polyclonal rabbit IgG preparation to coat the wells of a microtiter plate. After the Lp(a) ligand in standards and samples is ‘captured’, a highly specific monoclonal murine antibody11 reagent ‘recognizes’ the immobilized Lp(a). Rabbit anti-mouse IgG alkaline phosphatase conjugate (RAMAP) is bound stoichiometrically to the immobilized monoclonal antibody to ‘detect’ the sandwich complex. Bound alkaline phosphatase activity is determined by use of para-nitrophenyl phosphate substrate (PNPP) to ‘indicate’ colorimetrically the stoichiometric relationships between standards and samples.

REAGENTS

Polyclonal Rabbit Antiserum to Human Lp(a) was purchased* while the Monoclonal Mouse Antiserum to Human Lp(a)11 was provided from elsewhere.† Anti-mouse IgG Alkaline Phosphatase Conjugate (H&L, Rabbit) (RAMAP)‡ and Para-nitrophenyl Phosphate Tablets (PNPP)§ were both purchased.

STANDARDIZATION AND QUALITY CONTROL

A commercially available secondary clinical standard, Reference Standard Lp(a) Human,‖ was used as the calibrator. Identity of Lp(a) was confirmed by poly-

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‖ Immuno AG, Vienna, Austria.
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acrylamide gel electrophoresis (PAGE) combined with Western Blot analysis. Two lyophilized commercial control sera, Lp(a) Control¹ and Lipidophor Lipid Control Serum² and two native serum samples were run at two different dilutions each. The target absorbances covered the entire range of the calibration curve and thereby assured its analytical integrity. The calibration curve was slightly curvilinear.

SPECIAL APPARATUS

Titrtek Multiskan Plus¶ was the microtiter plate Absorbance measuring instrument.

SAMPLE REQUIREMENTS

Serum or plasma samples may be assayed. Incubation of a native serum sample for eight hours at 37°C resulted in only a 12 percent loss of the Lp(a) immunoreactivity.¹⁰ Therefore, serum samples may be kept at room temperature during analysis. Short-term storage (several days) at 4°C is permissible while longer storage (up to two years) at −20°C is possible. Fasting samples are preferred but not necessary.

PROCEDURE

Note: (1) Each protein-binding step is followed by washing the wells three times with phosphate buffered saline containing 0.5 percent Tween v/v (PBST)²⁹; and (2) Dilutions are in (PBST) unless otherwise stated.

1. The microtiter plate* wells are coated by placing 100 μl of working ‘capture’ antibody reagent into the individual wells and incubating at approximately 23°C for at least 15 minutes.

2. Eight dilutions of the Reference Standard Lp(a) Human are made to provide working calibrators that cover the range of 0.04 to 1.12 μg per ml. Four quality control samples are assayed at two different dilutions each. Patient samples are diluted 1000-fold for screening healthy groups. Samples containing markedly elevated Lp(a) concentrations are run at 5000-fold dilutions while samples containing very low Lp(a) concentrations are run at a 100-fold dilution. One hundred microliters of the dilutions are pipetted into individual wells. Calibrators and controls are run in duplicate, but unknown samples are run in triplicate. The covered plate is incubated at 37°C for at least six hours to ‘capture’ the Lp(a) ligand. Slow rotation may decrease the incubation time to less than six hours, but overnight incubation is convenient.

3. Working monoclonal murine anti-human Lp(a)¹¹ reagent (100-fold dilution), 100 μl, is added to each well except the nonspecific binding (NSB) control which receives PBST. The covered plate is incubated as before for 60 min to ‘recognize’ the immobilized Lp(a).

4. Working RAMAP (500-fold dilution), 100 μl, is added to each well except the substrate reagent blank control which receives PBST. The covered plate is incubated as before for 60 min to ‘detect’ the immobilized monoclonal antibody.

5. Alkaline phosphatase substrate, PNPP, (one mg per ml), 100 μl, is added to each well except the substrate reagent blank control which receives PBST. The covered plate is incubated as before until the absorbance of the highest working calibrator is approximately 1.2. Then the absorbances of all wells are measured by use of the microtiter plate reader.

CALCULATION OF RESULTS

Net absorbances of the calibrators are plotted on rectangular graph paper (or a computer generated plot may be used). Net absorbance values for controls and

¶ Eflaboy Laboratories, Helsinki, Finland.
* Immulon Microtiter Plate, Dynatech Laboratories, Alexandria, VA.
unknowns are converted to concentration units by use of the calibration curve. Multiplication by the appropriate dilution factors presents results in mg per l of sample Lp(a) total particle mass.

**Sources of Error**

If different antibodies or even different lots are used, they must be tested for avidity and specificity for the intact Lp(a) particle. There must be analytically insignificant crossreactivity with LDL or apo B, and with plasminogen. The LDL crossreactivity was tested at levels representing up to 10,000 mg per l. Plasminogen crossreactivity also was tested at levels representing up to 10,000 mg per l. Potential interferences by hemolysis, lipemia, and icterus were investigated. Sample stabilities were evaluated in the native state and after dilution with PBST.

**Reference Ranges and Clinical Interpretation**

The widely published reference value for plasma Lp(a) concentrations in Caucasians of northern European descent is 300 mg per l. Values above this arbitrarily chosen cut-off point are associated with a three-fold or greater relative risk for early development of clinically significant AS and myocardial infarction (MI). However, the relative risk not only increases concurrently with increases in plasma Lp(a) concentrations above 200 mg per l, but it also decreases concurrently with lower plasma levels. On the other hand, when both plasma Lp(a) and LDL concentrations are elevated, the relative risk increases dramatically.

It is important to note that the association of Lp(a) with coronary artery disease (CAD) has been studied extensively only in populations of Caucasians. The frequency distributions in other racial and ethnic groups vary greatly from the highly skewed pattern found in Caucasians (figure 1). The failure to use distribution-independent (nonparametric) statistical methods to summarize Lp(a) data further confuses comparisons of literature data. The 300 mg per l cut-off value should not be used indiscriminately for interpretation of laboratory results and treatment of subjects other than Caucasians until proper epidemiologic studies are reported. Moreover, the Bayes' Theorem model needs to be applied to the data so that clinical efficacy can be determined by use of sensitivity, specificity, and predictive value concepts.

**Results and Discussion**

**Preparation of Coating Antibody Reagent**

The IgG fraction of polyclonal rabbit anti-human Lp(a) serum was isolated at

![Figure 1. Frequency distribution of plasma Lp(a) for a predominantly Caucasian reference population (n = 118).](image-url)
23°C by protein-A sepharose column chromatography\(^\text{16}\) (figure 2). The eluate pH immediately was adjusted to 7.8 ± 0.1 by use of 1.5 M Tris buffer pH 8.7. This 3.1-fold enriched stock ‘coating’ antibody preparation is stable for at least one year when stored at 4°C. Recovery of total protein applied to the column was 91 percent, while recovery of anti-Lp(a) immunoreactivity (coinciding peaks) was 101 percent. The working ‘coating’ antibody reagent consists of a 200-fold dilution of the stock reagent in 0.1 M sodium carbonate buffer, pH 9.4. One hundred microliters of the working coating reagent contained 315 ng of IgG protein, of which 236 ng were bound to the microtiter plate.

**Verification of Reagent Specificities**

The lack of cross-reactivities of the ‘capture’ and ‘recognition’ antibodies with apo B and plasminogen were determined by polyacrylamide gel electrophoresis (PAGE) combined with Western Blot analysis. The polyclonal anti-human Lp(a) bound both the intact Lp(a) particle and the apo(a) fragments, but the monoclonal anti-human Lp(a) reacted with only the intact Lp(a) particle. Neither antibody cross-reacted with apo B (or LDL). Cross-reactivity with plasminogen was less than 10 percent for the monoclonal antibody and approximately one percent for the monoclonal antibody. The cross-reactivity with plasminogen in the ‘capture’ ELISA is less than one percent.

The importance of Lp(a) phenotypes is now reported.\(^\text{28}\) In this method development work, however, the specificity of the monoclonal ‘detector’ antibody for a defined kringle region was not yet determined.

**Dilutions of Antibody Reagents**

Analytical relationships between the ‘capture’ antibody and the Lp(a) ligand were determined by use of a standard ‘checkerboard’ titration. The analytically useful range is 0.05 to 0.76 μg per ml of Lp(a).

Serial dilution studies were performed sequentially for the protein-binding reagents in order to determine the optimum dilutions for analytical use. The monoclonal murine anti-human Lp(a), LHLP-1,\(^\text{11}\) dilution was 1:100, and the rabbit anti-mouse IgG alkaline phosphatase conjugate (RAMAP)* dilution was 1:500. The paranitrophenyl phosphate (PNPP) substrate tablets† were dissolved in 1 M diethanolamine buffer, pH 9.8\(^\text{29}\) to make one mg per ml.

**Optimization of Assay Conditions**

Bound/free relationships of all protein-binding reactants assured that adequate excess of reagents was present at each step. Non-specific binding by the

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* ICN, Immunobilogicals, Lisle, IL.
† Sigma Chemical Co., St. Louis, MO.
RAMAP was minimized by use of a one hour incubation period (figure 3). Kinetic studies were used to select the most appropriate incubation temperatures and intervals. Each protein-binding step rapidly approaches equilibrium except for the Lp(a) binding. This may be explained by the large molecular size (approximately 40 times larger than IgG) of this ligand and possibly steric factors that slow its diffusion and binding rates. A one hour incubation period at 37°C is adequate for each step except Lp(a) binding. A minimum of six hours or a convenient overnight incubation at 37°C is used. Constant slow rotation may decrease this incubation period.

VALIDATION OF METHOD PERFORMANCE

Method validation studies were performed according to a published recommendation. In figure 4 a typical dose/response curve is represented. As little as 0.05 µg per ml in each well of Lp(a) can be measured. This represents a 1000-fold dilution of a sample that contains 50 mg per l.

At a Lp(a) sample concentration of 160 mg per l, within-plate reproducibility was three percent, between-plate reproducibility was six percent, and day-to-day reproducibility was nine percent. In table II are presented additional day-to-day precision data. Recovery of Lp(a), added to serum pools at 25 percent to 200 percent of the starting concentrations, was 106 ± 11 percent. Parallelism data for two lyophilized controls and five native sera whose Lp(a) starting concentrations ranged from 80 to 1250 mg per l failed to demonstrate non-identity with the Lp(a) secondary standard. There was no analytical interference by lipemia (cholesterol = 3000 mg per l, triglycerides = 6000 mg per l), hemolysis (hemo-

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<th>Mean mg per l</th>
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globin = 164 g per l), or bilirubinemia (unconjugated bilirubin = 250 mg per l, conjugated bilirubin = 200 mg per l). Lipoprotein(a) data for 28 samples analyzed by the new ‘capture’ ELISA and a reference method\(^\text{11}\) gave a regression line \(y = 1.07 \pm 2.9\) and a correlation coefficient of 0.90. Six samples determined by independent methods to contain elevated levels of plasma Lp(a) all yielded abnormally elevated Lp(a) results, while samples that contained undetectable Lp(a) concentrations by other methods all yielded low Lp(a) results by the ‘capture’ ELISA. The frequency distribution of plasma Lp(a) in a predominantly Caucasian reference population (\(n = 118\)) closely resembled those previously reported in the literature.\(^\text{2}\)

**CLINICAL INTERPRETATION**

Plasma Lp(a) values can be interpreted most efficiently when combined with measurements of apo B and apo AI. Since the determined total apo B is comprised of the apo B from LDL (approximately 20 percent of LDL total mass) and the apo B from Lp(a) (approximately 10 percent of Lp(a) total mass), then subtracting the Lp(a) derived apo B from the total apo B determined gives a more accurate estimation of LDL. This becomes increasingly more important in subjects that have low plasma LDL and high Lp(a) levels. For example, when plasma LDL are approximately 2500 mg per l and Lp(a) is approximately 1000 mg per l, then the apo B from LDL is approximately 83 percent of the total and the total apo B value determined is still in the lower region of the normal limits. Whenever this occurs, a malginantly elevated relative risk for early MI, owing to elevated plasma Lp(a) concentration in a Caucasian subject, is artificially hidden by present interpretative procedures that fail to consider Lp(a). The normal apo B result in this case is an erroneous indicator that suggests low risk of developing early AS and MI. Therefore, whenever there exists a normal plasma LDL concentration and an elevated plasma Lp(a) concentration, there may be reported by the laboratory a normal plasma apo B value even though (in Caucasian subjects and perhaps other groups as well) there may indeed be a malignantly elevated relative risk for early MI. The authors propose that the clinical usefulness of lipoprotein/apolipoprotein assays should be improved by using combined data from the three analytical methods now available, apo B-100, apo AI, and Lp(a). Application of Galen’s Predictive Value Model will further refine the clinical use of these data.

**Acknowledgment**

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**References**


