Quantification of Lipoprotein(a) in Plasma by Assaying Cholesterol in Lectin-Bound Plasma Fraction

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Lipoprotein(a) [Lp(a)] is a low-density lipoprotein (LDL)-like particle in which apolipoprotein(a) [apo(a)] is disulfide-linked to apolipoprotein B (apoB). High concentrations of Lp(a) in plasma are associated with an increased risk of coronary heart disease (CHD). Lp(a) has traditionally been measured by immunoassay and expressed as total mass of Lp(a). Measuring Lp(a) by its cholesterol content will provide a way to directly compare Lp(a) with other lipoproteins that are measured by cholesterol. We have developed an assay to quantify Lp(a) by its cholesterol content (Lp(a)-C), using lectin affinity to isolate Lp(a) from other lipoproteins, and then measuring the cholesterol within the isolated fraction. We compared the Lp(a)-C assay with an ELISA for Lp(a) mass in 47 plasma samples from normotriglyceridemic, fasting individuals with high Lp(a) contents (mean ± SD, 445 ± 350 mg/L). The mean Lp(a)-C concentration was 110 ± 89 mg/L and correlated very highly with Lp(a) mass (r = 0.9975). Lp(a)-C measurement is an alternative method to screen for this CHD risk factor.

Indexing Terms: coronary heart disease/apolipoproteins/standardization/wheat germ agglutinin/risk factors

The presence of lipoprotein(a) [Lp(a)] was first documented by Kåre Berg in 1963 to be associated with an increased risk of coronary heart disease (CHD) in men (1). Subsequently, Albers et al. (2) quantified Lp(a) by radioimmunoassay and demonstrated a higher mean Lp(a) concentration in men who had survived a myocardial infarction than in age-matched controls. Numerous case control studies have confirmed this association (3-10). Recently, Genest et al. (6) described an increased prevalence of Lp(a) excess [defined as an Lp(a) mass value exceeding the 90th percentile of normal] in patients with premature CHD and their family members. Despite numerous publications describing Lp(a) as a risk factor for CHD and cerebrovascular disease, screening for Lp(a) excess is not routinely performed in the US. Screening efforts for Lp(a) excess have been delayed for several reasons, one of which is lack of adequate standardization. Other methods to detect Lp(a) and to circumvent some of the difficulties posed in present Lp(a) assays should be explored. Widespread availability of standardized Lp(a) assays should increase the potential for therapeutic intervention for Lp(a) excess, e.g., with nicotinic acid (11).

Lp(a) has traditionally been measured by immunoassay directed against apo(a). However, other plasma lipoproteins generally are measured in clinical laboratories by their cholesterol content. Low-density lipoprotein (LDL) cholesterol is traditionally calculated by the Friedewald equation (12), in which LDL cholesterol is estimated as the difference between total cholesterol and the sum of high-density lipoprotein (HDL) cholesterol plus one fifth of fasting triglycerides. This LDL estimate includes intermediate-density lipoprotein, LDL, and Lp(a). Theoretically, there should be a difference in CHD risk in individuals with different amounts of Lp(a) in this LDL fraction. Devising a method to fractionate Lp(a) and LDL may be useful in estimating CHD risk. Measuring Lp(a) by its cholesterol content would allow for direct comparison of Lp(a) with other plasma lipoprotein fractions.

Lp(a) consists of an LDL particle with an attached molecule of apo(a), a large glycopeptide having a great deal of homology with plasminogen (13). Unlike plasminogen, apo(a) is heavily glycosylated, containing numerous O-glycosidic oligosaccharides rich in N-acetyl-
\[\text{D-neuraminic acid (NANA) and N-acetyl-D-glucosamine (GlcNAc) (14-17).} \]

apo(a) is reportedly 25-40% carbohydrate by weight, making it one of the most heavily glycosylated proteins in plasma. Apo(a) is covalently linked to apoB-100 within an LDL-like lipoprotein. Most Lp(a) has a flotation density of 1.050-1.110 kg/L, although some Lp(a) can be found throughout the density spectrum, with 2-9% of the immunoreactive Lp(a) being in the very-low-density lipoprotein (VLDL) density range and 1-5% of immunoreactive Lp(a) having a hydrated density of >1.21 kg/L (18-20). The differences in density are influenced by both apo(a) isoform size and lipid composition (17, 20). In general, the Lp(a) particle has the same degree of heterogeneity in lipid composition as does the LDL from the same individual (Seman, unpublished observation). Lipid composition and flotation densities of LDL and Lp(a) [after removal of apo(a)] are very similar (14, 17). In studies that clearly involved fasted samples, the quantity of immunoreactive Lp(a) in the VLDL fraction was generally <3% (18, 19). Furthermore, it is uncertain whether some or most of the Lp(a) with density >1.21 kg/L is an artifact of ultracentrifugation (18) or free apo(a). In the fasted state, apo(a) associated with triglyceride-rich lipoproteins ac-
counts for 0–4% of the total Lp(a) immunoreactivity (18, 19, 21). Lp(a) cholesterol may thus be a practical way to add screening for Lp(a) excess to existing clinical laboratory equipment. This approach would allow for a direct comparison with the concentrations of lipoproteins that are assessed by their cholesterol content.

We describe a method for rapidly separating Lp(a) from other plasma lipoproteins, so that the cholesterol content of Lp(a) can be measured with a routine automated chemistry analyzer. When measuring the cholesterol content in HDL or Lp(a), each of which is generally only a small fraction of the concentration of total plasma cholesterol values, the proportion of sample to reagent is generally increased to assure adequate sensitivity. Lower-concentration cholesterol standards are also used, ranging from 100 to 500 mg/L. In our studies, we took advantage of the high carbohydrate content of Lp(a), which is enriched in GlcNAc and NANA terminal residues. These oligosaccharides are excellent ligands for the lectin wheat germ agglutinin (WGA), which specifically binds GlcNAc and NANA terminal residues. Because of the large number of WGA binding sites per Lp(a) molecule, Lp(a) can compete strongly with other plasma glycoproteins that are present in plasma in much greater molar concentrations than Lp(a).

Materials and Methods

Plasma samples were obtained from patients seen in the Lipid Clinic at New England Medical Center, Boston, MA. Research procedures followed were in accordance with the ethical standards of the Tufts University institutional review board. Blood was collected by way puncture into glass tubes containing EDTA at a final concentration of 0.0037 mol/L (1.5 g/L) and centrifuged to isolate plasma. Plasmas were frozen at −20°C if not analyzed on the same day of collection.

All samples were initially analyzed for Lp(a) mass by Macro Lp(a)™ ELISA (Terumo, Elkonit, MD), which uses a monoclonal apo(a) antibody as the capture antibody and a polyclonal antibody to apo(a) as the detection antibody. The antibodies do not cross-react with plasminogen. The details of this commercially available assay have been previously described (6, 9). Intraassay coefficients of variation (CVs) for the ELISA were generated by repeated assay (n = 10) of control plasma samples of 150 and 400 mg/L. Interassay CVs were generated with the same controls run in duplicate on 62 different plates. The intra- and interassay CVs were 2.8% ± 0.4% and 4.3% ± 0.6%, respectively, for all concentrations of controls.

In the proposed assay, we incubated duplicate 1-mL samples of plasma with 0.5 mL of packed 4% agarose containing 7 g/L covalently bound WGA (Sigma Chemical Co., St. Louis, MO), equilibrated with 0.06 mol/L sodium phosphate-buffered isotonic saline, pH 7.35 (PBS). The plasma was allowed to incubate with the agarose-WGA for 1 h at room temperature, with intermittent agitation. We then washed the sample with agitation for 3 min with 2 mL of ice-cold sodium phosphate buffer (pH 7.35) containing 0.3 mol/L sodium chloride (wash buffer), decanted, and repeated for a total of three washes. After the last wash, the samples were centrifuged at 850g for 15 min, to remove as much of the wash as possible. We then incubated the samples with 0.5 mL of PBS containing 0.2 mol/L GlcNAc (Sigma) for 15 min before centrifugation to dissociate the plasma glycoproteins and the Lp(a) retained on the lectin.

We then applied 100-μL aliquots of this eluate to the Abbott Spectrum CCX™ cholesterol analyzer, with cholesterol reagents and standards (all from Abbott, Irving, TX) adjusted for cholesterol values between 20 and 250 mg/L. The calibration blank consisted of 0.1 mol/L GlcNAc in PBS. The final concentration of the elution buffer was 0.1 mol/L GlcNAc, given that the 0.2 mol/L GlcNAc elution buffer equilibrates with an equal volume of packed 4% agarose gel that does not contain GlcNAc but does contain 96% water, which contributes to the total liquid volume and freely exchanges with the equal volume of elution buffer; thus, the final concentration of GlcNAc is 50% of that of the starting elution buffer. All samples were tested in duplicate.

We then paired these cholesterol values with the ELISA values for Lp(a) mass and generated a linear regression plot. Our automated enzymatic cholesterol assays are standardized with the Centers for Disease Control and Prevention–National Heart, Lung, and Blood Institute Lipid Standardization Program.

The WGA-bound fraction of plasma was evaluated for lipid and lipoprotein content by subjecting this fraction to 4–30% acrylamide non-denaturing gels as previously described (22). Both unbound and bound plasma fractions were subjected to 3–12% polyacrylamide gel electrophoresis with sodium dodecyl sulfate and β-mercaptoethanol and silver stained as previously described (14). Lipid compositions were analyzed by gas chromatography, also as previously described (14).

The 4% agarose-WGA can be regenerated by adding three volumes of 2 mol/L Tris, pH 10. After a 5–10 min incubation, the Tris base is washed out of the agarose-WGA with PBS. We store the regenerated agarose-WGA in 2 mol/L NaCl in sodium phosphate buffer, pH 7.35, containing 0.1 g/L sodium azide, and refrigerate at 4°C.

Results

In the initial experiments with 4% agarose-WGA (n = 47 samples), Lp(a) could not be detected by ELISA in the unbound and wash fractions (detection limit 10 ng/L), confirming that all the Lp(a) was captured. When 6% agarose-WGA was substituted for the 4% agarose preparation, most of the Lp(a) remained unbound, unless very large amounts of lectin matrix were used. This difference was presumably due to either fewer WGA molecules or fewer pores with WGA molecules that would allow passage of Lp(a) into the 6% agarose, but the exact reason for the difference has not been determined. Therefore, we applied all samples to 4% agarose-WGA.

When fasting plasmas were applied, Lp(a) was the only lipoprotein retained by WGA: No other lipoproteins
were detected by nondenaturing polyacrylamide elec-
trophoresis (see Fig. 1). Under fasting conditions, <3% of
the total lipid mass retained on lectin was triglyceride.
This percentage of triglyceride corresponds to other pub-
ished reports of Lp(a) composition (14–16, 18), making
it unlikely that any significant amount of triglyceride-
rich lipoproteins was coisolating with the Lp(a).

In the sample population chosen, Lp(a) total mass
ranged from 60 to 1604 mg/L, with Lp(a) cholesterol
[Lp(a)-C] values ranging from 15 to 410 mg/L. The mean
(±SD) Lp(a) mass and Lp(a)-C were 446 ± 350 and 110.5
± 89 mg/L, respectively. The linear regression plot pair-
ing Lp(a)-C values with Lp(a) mass (Fig. 2) showed that
the two values were very closely correlated (r = 0.9975),
with a y-intercept of 3.58 and a slope of 3.98. The
Lp(a)-C content of plasma containing <60 mg/L Lp(a)
mass was undetectable by this method.

Intraassay CVs, generated by running 20 replicates of
plasma samples containing Lp(a) mass concentrations of
160, 400, and 800 mg/L, were 3.2% ± 1.2%. Interassay
CVs, calculated from duplicates of control plasmas at
160 and 400 mg/L, run on 14 consecutive assays, were
3.8% ± 1.3%.

**Discussion**

Our results indicate that the Lp(a)-C assay can be an
effective way to screen for patients with Lp(a) excess.
The sensitivity of the cholesterol assay, as modified for
these analyses, is greater than necessary for screening
Lp(a) excess, defined as that exceeding the 90th percent-
tile, or >380 mg/L Lp(a) mass, as determined by Genest
et al. (6). A more conservative definition of Lp(a) excess
would be the 80th percentile, which corresponds to ~300
mg/L Lp(a) mass, or 100 mg/L Lp(a)-C, according to data
from the Framingham Heart Study (23). Moreover, this
assay has proven to be sensitive enough to detect
Lp(a)-C concentrations as low as 15 mg/L, which is ap-
proximately equal to an Lp(a) mass value of 60 mg/L.
There is no evidence in the literature that Lp(a) values
in these low ranges are of any clinical relevance. How-
ever, potentially this cholesterol assay could be modified
to measure Lp(a) well below 15 mg/L. The obvious ben-
efits from using lipoproteins for standardizing Lp(a) are:
(a) the large body of accumulated experience of several
national and international bodies for standardizing cho-
lesterol determinations, including those associated with
the National Institutes of Health and Centers for Dis-
ease Control and Prevention; (b) the ability to compare
Lp(a)-C with other lipoproteins already measured by
their cholesterol content; and (c) widespread access to
laboratories that already measure HDL-cholesterol, us-
ing the same instrumentation and similar parameters
necessary for measuring Lp(a)-C.

The slope of 3.98, generated from the linear regres-
sion data (Fig. 2), suggests that the Lp(a) mass in this
population is ~25% cholesterol, which is close to esti-
mates of 26–34% calculated from the literature (14, 17,
24–26). Selection bias for increased concentrations of
Lp(a) mass (mean = 446 mg/L vs −145 mg/L in the
Framingham population (23)) was deliberate so that all
samples would be within the range of sensitivity of the
Lp(a)-C assay. Given the inverse correlation between
plasma concentrations of Lp(a) and apo(a) isofrom size,
the population tested overrepresents small apo(a) iso-
forms. It is unclear whether this overrepresentation of
small apo(a) isoforms biases either assay type or if this
bias is responsible for such high correlations between
Lp(a) mass and Lp(a)-C values. In the context of this

\[ y = 3.583 + 3.98x \quad r=0.997 \]

![Fig. 2. Linear regression plot generated from results for samples from 47 subjects with Lp(a)-C concentrations that were detectable by this assay (>15 mg/L).](image)

Lp(a) mass values were generated from a commercially available ELISA.

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3 The molecular mass of the fatty acids associated with cho-
lesterol esters was excluded in calculating the percentage of chole-
sterol.
limited pilot assay, Lp(a) associated with triglyceride-rich lipoproteins does not appear to have influenced the results in fasting plasma. None of the samples was known to be from overtly hypertriglyceridemic subjects (triglycerides >5.88 mmol/L).

In conclusion, WGA fractionation of plasma to separate Lp(a) from other lipoproteins, followed by cholesterol quantification of the WGA-bound fraction with assays designed to determine low concentrations of cholesterol, is a suitable alternative method to screen for Lp(a) excess. The method has the potential for increased sensitivity, which may increase the utility of this assay by accurately measuring concentrations of Lp(a) that are currently considered to be clinically insignificant but might prove relevant, given that the vast majority of Caucasians and Asians have low Lp(a) concentrations (27). Efforts are now being focused on comparing Lp(a)-C with immunoeasy-derived Lp(a) mass measurements for estimating risk of coronary artery disease.

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References