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# Fully mechanized latex immunoassay for serum lipoprotein(a)

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#### Abstract

We have developed a fully automated system to quantify lipoprotein(a) (Lp(a)) in human serum, based on the latex-enhanced turbidimetric immunoassay by application of the Immuno Chemistry Analyzer 501X. This assay was carried out with undiluted serum and was able to detect at Lp(a) levels higher than 4.0 mg/l. When judged to be out of range of the calibration (>600 mg/l), the sample was automatically re-tested after automatic 10-fold dilution. Within-run C.V.s ranged from 1.9 to 2.1% and between-run C.V.s from 2.7 to 3.9%. Results by the present method were in good agreement with those by the in-house ELISA (r = 0.978) and the commercial ELISA (r = 0.990). The distribution of Lp(a) levels in sera from 508 healthy donors was highly skewed; the mean and median were 158 mg/l and 105 mg/l, respectively.

Key words: Fully automated latex immunoassay; Lipoprotein(a); Enzyme-linked immunosorbent assay; lsoforms; lmmuno-Chemistry Analyzer 501X

#### 1. Introduction

Recently, a number of studies have suggested that lipoprotein(a) (Lp(a)) is one of the strong independent risk factors of premature coronary artery disease, stroke and restenosis following angioplasty and bypass surgery [1–4]. Lp(a) resembles low density lipoprotein (LDL) in being cholesterol rich but it is distinguished by its content

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#### 2.2. Latex particles

Polystyrene latex particles having hydrophilic domain localized on the surface, 0.16  $\mu$ m in diameter, were obtained as a 50 g/l suspension from Tokuyama Soda Co. Ltd., Tokyo. The latex particles had been prepared by a seeded polymerization technique for the emulsifier-free copolymerization of styrene and glycidyl methacrylate [16].

# 2.3. Latex immunoassay

After dialysis of the purified antibody (2 g/l) against 0.1 mol/l glycine-NaOH buffer (pH 8.6), 1-ml aliquots were incubated with 1 ml of 10 g/l latex suspension at room temperature for 2 h. One milliliter of 0.1 mol/l glycine-NaOH buffer (pH 8.5) containing 10 g/l bovine serum albumin (BSA) was added and the mixture was incubated overnight at 4°C. The latex particles were pelleted by centrifugation (10,000  $\times$  g, 15 min) washed with 0.1 mol/l glycine-buffer (pH 8.5) containing 1 g/l BSA, 0.1 mol/l NaCl and 1 g/l NaN<sub>3</sub>, and resuspended in 2 ml of this solution. The purified antibody-coated latex solution (R2) was stored at 4°C. Immunoglobulincoated latex particles are unstable and tend to agglutinate spontaneously. However, the antibody-coated latex particles, prepared as mentioned above, could be used for at least 12 months when stored at 4°C.

In the Lp(a) assay, additional buffer (R1) containing 0.1 mol/l glycine-NaOH (pH 8.5), 5 g/l BSA and 1 g/l NaN<sub>3</sub> was used in order to dilute R2 and to control the reaction rate of agglutination. In the present automated Lp(a) assay, 300  $\mu$ l of R1 and 3  $\mu$ l of serum sample or standard solution were mixed and pre-incubated for 3.75 min at 37°C in the reaction cells. After addition of 50  $\mu$ l of R2, changes in scattered light intensity were measured. The Lp(a) concentrations of samples were calculated by fitting the standard curve values to a three-dimensional curve mode on the 501X. When Lp(a) concentrations of samples were judged to be out of range of the calibration (>600 mg/l), samples were automatically re-tested after automatic 10-fold dilution by the 501X with 0.1 mol/l Tris-HCl buffer (pH 8.2) containing 0.15 mol/l NaCl and 1 g/l NaN<sub>3</sub>.

# 2.4. Standard and serum samples

As a standard material for the latex immunoassay had not been established, we employed the in-house standards. Concentrated lipoprotein fraction obtained from a pooled serum [17] was added to pooled Lp(a)-rich sera. The secondary standards were prepared by lyophilizing the Lp(a) enriched serum and were calibrated by the in-house ELISA with Immuno (Vienna, Austria) reference serum [9], after dissolving in distilled water.

Serum samples were obtained from the Red Cross Blood Center, Gifu, Japan. They were from healthy adult subjects, aged 16-64 years (197 males and 311 females).

#### 2.5. Lp(a) phenotyping

Electrophoretic procedures for separation of apo(a) isoforms were performed under reducing conditions as described previously [18], using sodium dodecyl sulfate-4% polyacrylamide gel electrophoresis, immunoblotting and gold-silver staining. Isoforms were designated according to Utermann's original nomenclature [19].

# 2.6. Apparatus

Automated measurement of serum Lp(a) was carried out with an Automated Immuno Chemistry Analyzer 501X (A & T Corporation, Tokyo, Japan). The 501X is a system that quantifies antigen or antibody in samples by determining the rate of forward scattered light energy obtained at 660 nm on the latex agglutination reaction.

The 501X improves the reliability of the data through the use of a washing function, a reagent refrigerating function, post- or pro-zone check functions and two calibration curves. The operation is very easy because the system has automatic dilution function and automatic re-testing function and is operated by a personal computer (NEC 9801-RX, NEC Co., Tokyo) having multi-window interactive modes.

# 3. Results

## 3.1. Standard curves and linearity

The assays were carried out in a range from 0 to 600 mg/l of Lp(a). As shown in Fig. 1, two types of calibration curves were prepared by measuring the same stan-



Fig. 1. Calibration curves for Lp(a) determination. Rate of agglutination was expressed by difference of light scattered energy (DLSE). Lines [1] and [2] are the first and second calibration curves obtained from the 6.75 min and 1.75 min reactions, respectively.

dard series (0, 18.8, 37.5, 75.0, 150. 300 and 600 mg/l) automatically diluted by the 501X with the secondary standard. The first calibration curve was obtained from long photometric point interval (6.75 min), and was used for low Lp(a) concentration ranges (0–60 mg/l) of samples. The second calibration curve based on short interval (1.75 min) was automatically selected for measuring higher than 60 mg/l of Lp(a) samples. An antigen-excess phenomenon, due to an excess of antigen and resulting in a decrease of agglutination, was observed at Lp(a) concentrations higher than 1,000 mg/l. However, with the Reagent Blank Check (RBC) option on the 501X, the scattered light intensity of the early reaction rate of the test sample was compared with that of the highest standard (600 mg/l) and the antigen-excess phenomenon was able to check at least up to 3,000 mg/l of Lp(a).

In order to study the linearity of the assay some serum samples in the 90-1,140 mg/l Lp(a) range were diluted at various ratios with the buffer and Lp(a) concentrations assayed. Linearity was confirmed at least up to 1,140 mg/l.

# 3.2. Sensitivity and precision

The sensitivity of the present method, defined as the minimum amount of Lp(a) that is different from zero at the 95% confidence level, was 4.0 mg/l. The precision of the technique was estimated by measuring three serum pools with various concentrations of Lp(a). The between-run reproducibility was evaluated by determining three samples in duplicate assay once per day for consecutive days over 1 week. As shown in Table 1, the coefficients of intra-assay variation at three concentrations were approximately 2% and those of inter-assay were less than 4%.

#### 3.3. Analytical recovery

Recovery, calculated from data on addition of one-tenth of Lp(a)-rich serum (1,018 mg/l) by volume to several concentrations of sera, was from 97.0 to 107.7%.

## 3.4. Interference study

Possible interfering materials were investigated by the usual technique, with use of pooled human serum. Five grams per liter of hemoglobin, 250 mg/l of bilirubin

Lp(a) (mg/l)		C.V. (%)	
Mean	S.D.		
Within-run $(N = 10)$			
39	0.8	2.1	
276	5.2	1.9	
431	8.6	2.0	
Between-run $(N = 7)$	)		
93	3.6	3.9	
353	13.4	3.8	
495	13.5	2.7	

Table 1 Precision data for measuring serum Lp(a)

and 2.5 g/l Intralipid had no effect on the present Lp(a) assay. To determine that hypertriglyceridemia did not interfere with the assay, 21 turbid serum samples with triglyceride concentrations from 1,120 to 5,920 mg/l were analyzed by the present method (y) and by the in-house ELISA method (x). A good agreement was obtained between these two methods; y = 1.05x + 0.96, r = 0.950. We also estimated cross-reactivity with apo B and plasminogen and demonstrated that the antibodies used in this assay were little affected by the addition of these compounds; 1 g/l of apo B and 1 g/l of plasminogen were estimated as 5 and 10 mg/l of Lp(a), respectively.

# 3.5. Comparison with ELISA methods

In order to evaluate the ability of the present method to estimate various kinds of apo(a) isoforms, 52 serum samples, classified phenotypes by our method [18],



Fig. 2. (A) Estimation of apo(a) isoforms by the LIA method and the commercial ELISA method. Phenotypic classified sera: ⊙, F (N = 2); □, B (N = 3); ■, S<sub>1</sub> (N = 4); (Δ), S<sub>2</sub> (N = 14); O, S<sub>3</sub> (N = 15) and ●, S<sub>4</sub> (N = 14) were assayed by the LIA method (y) and by the Biopool ELISA method (x). (B) Comparison of the LIA method and the commercial Biopool ELISA method.

were assayed in parallel by the present method (y) and by the commercially available Biopool ELISA kit (x). Although the ELISA method does not, in and of itself, prove that the assay is not affected by variation in size of apo(a), the Lp(a) assays are usually performed in many laboratories with use of this method because it is considered that it is able to measure Lp(a) regardless of apo(a) isoforms [20]. The results, shown in Fig. 2A, suggest that the present LIA method is independent of variation of sizes of apo(a) and Lp(a).

The correlation of the present method and two kinds of ELISA methods with the individual sera were investigated. In another set of experiments, 79 serum samples were assayed by the present latex immunoassay (y) and by the Biopool ELISA kit method (x). As shown in Fig. 2B, there was a good agreement between these methods: y = 1.12x - 2.0 mg/l ( $S_{y/x} = 17.1 \text{ mg/l}$ , r = 0.990). Similarly, comparison of the results obtained by the present method (y) and by the in-house ELISA method (x) [9] for 105 samples showed a good correlation: y = 0.91x + 0.7 mg/l ( $S_{y/x} = 26.6 \text{ mg/l}$ , r = 0.978). The slope values may reflect the differences in standard materials used in these assays.

#### 3.6. Reference values

The distribution of serum Lp(a) concentrations determined by the present latex method was highly skewed as previously observed [18]. Values of mean, median and standard deviations (S.D.) for 508 serum samples from healthy adult Japanese were 158, 105 and 159 mg/l, respectively. The 90th and 95th percentiles were estimated as 351 and 521 mg/l, respectively. Lp(a) concentrations did not differ significantly between males and females, males having a mean of 146 mg/l and a median of 88 mg/l (S.D. = 149 mg/l), females having a mean of 165 mg/l and a median of 112 mg/l, respectively (S.D. = 165 mg/l).

## 4. Discussion

The development of a latex immunoturbidimetric method for automatic determination of Lp(a) in human serum is described in the present paper. This method is easy to perform, sensitive, specific, accurate and requires only a small amount of serum sample for analysis. In the assay, more than 100 samples can be measured within 1 h, 15 samples within 20 min and one sample within 15 min. The within-run and between-run C.V.s were apparently smaller than those found by the ELISA methods [9,10] and by the semi-automated ITA method [13].

Increasing attention is being paid to the measurement of serum Lp(a) not only as an aid to the clinical management of patients, but as a predictor of coronary artery disease and other atherosclerotic diseases. The present automated LIA method is suitable for screening large populations with regard to sensitivity, operation, precision and sample volume.

A main difficulty of latex immuunoassay is to control the stability of the antibodycoated latex particles, since this influences the specificity of the assay. We concluded, from the results on changes of values measured, sensitivity and reproducibility with use of this reagent, that the antibody-coated particles could be used for at least 12 months when stored at 4°C. The surface of the latex particles used in this reagent was presumed to be dotted with hydrophilic domains, giving stability to the particles [16].

An optimal Lp(a) assay requires specific antibodies that do not cross-react with plasminogen or with apo B, that can estimate similarly all apo(a) isoforms and must not be influenced by the size of Lp(a) particles with different apo(a) isoforms present in the sample. Pfaffinger et al. [15] demonstrated that low molecular weight apo(a) isoforms were preferentially associated with low density Lp(a), whereas high molecular weight apo(a) isoforms were found in high density Lp(a) particles.

These findings were confirmed in our laboratory with the use of density gradient ultracentrifugation [21]. Although the nephelometric or turbidimetric assays are highly sensitive to differences in size of the analyte being measured, the present LIA method was considered to be able to measure Lp(a) regardless of size of Lp(a) particles and apo(a) isoforms. Since it is very difficult to prove it [10], we have utilized an assumption in which the ELISA methods usually used in many laboratories are able to measure Lp(a) isoforms.

Although we used the apo(a) isoforms designated by Utermann et al. [19] in the present study, many investigators [22–25] recently reported developments of extremely sensitive methods with high resolving powers for determining the size of apo(a) polymorphs in plasma samples. However, the relationships of higher numbers of apo(a) polymorphs to the originally described isoforms or to each other are unclear at present.

In the quantitation of Lp(a) by immunoassay, the major problem to be considered is the intra- and inter-individual heterogeneity of apo(a) and Lp(a) in size. Therefore, the basic requirement that the composition of reference material and assay calibrator should be similar to the composition of the analyte in the samples is impossible to achieve for Lp(a). The only solution to this problem to be considered is to express the results in mol/l rather than mg/l. However, we are not able to express them in mol/l at present, because the standard material, of which molecular weight is demonstrated, is not generally available.

Although the present paper describes the automated Lp(a) measurement with the specific immunochemical analyzer 501X, identical results were obtained with the usual automated biochemical analyzers, such as TOSHIBA TBA-30R and HITACHI 7050. In the case of the TOSHIBA TBA-30R, the minimum detectable concentration was 3.0 mg/l at the 95% confidence level and the C.V.s of within-run with use of two kinds of serum samples (means of 26 and 521 mg/l of Lp(a)) were 1.2 and 1.3%, respectively. However, the dilution series of standards for the calibration and over-range samples had to be performed by manual operations.

The lack of standardization of Lp(a) measurement and calibration may lead to systematic differences in the results obtained with different calibrators, as was the case in our experiment. Presently, there is no commonly accepted primary reference materials for Lp(a) determinations, nor is there agreement on the reference method for the determination of Lp(a). Since Lp(a) measurements are already performed in many clinical laboratories, the standardization issue urgently needs to be settled in order to warrant unequivocal interpretation of the results obtained with different methods.

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