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Enzyme Immunoassay of Lipoprotein(a)

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Sheep polyclonal antibodies to human lipoprotein(a) were used for the development of sandwich enzyme immunoassay. These antibodies virtually do not interact with human plasminogen, apoB-100, or fibrinogen in this assay. The method permits measurements of lipoprotein(a) in a wide range of concentrations: from 2 to 500 mg/dl. The variability of analyses within the range of 5 to 180 mg/dl is approximately 4.5% in a plate and 10.5% in different tests. Correlation analysis of the results of the enzyme immunoassay modification developed in our laboratory and of its foreign analogs showed a high similarity of the methods, the correlation coefficient being 95%.

Key Words: *enzyme immunoassay; lipoprotein(a); atherosclerosis*

Lipoprotein(a), or Lp(a), is a lipoprotein particle containing a protein apo(a) unique to the lipoprotein family; it is bound to molecule apoB-100 with a covalent disulfide bond, and this molecule is the principal protein component of a corpuscle of low-density lipoprotein (LDL). Lp(a) was discovered by Berg in 1963 as a new antigen of human plasma and as a variant of LDL corpuscles [2]. Further studies showed that Lp(a) differs from LDL not only in composition, molecular weight, and electrophoretic mobility, but also in physiological role and metabolism [12].

Active studies of Lp(a) were started after it was shown to be associated with the risk of atherosclerotic involvement of the heart [4]. Further investigations clearly demonstrated that a high level of Lp(a) in the blood is closely related to the appearance and development of atherosclerotic lesions in various arteries [7,12].

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Some scientists believe that Lp(a) may act as a bond in the processes of thrombosis and atherogenesis [10] (due to the high homology of the primary structure of apoprotein(a) with the plasminogen molecule [5]) and that it is involved in fibrinolysis, competing with plasminogen for fibrin binding [6].

At present, various immunological methods for measuring Lp(a) have been described: radial immunodiffusion, electroimmunodiffusion, immunonephelometry, and immunoturbidimetry [1,9], and different types of enzyme immunoassay (EIA) exist [3,9, 13]. Commercial kits for measuring Lp(a), manufactured by Biopool, Terumo, etc., are available. Still, because of the specific structure of an Lp(a) particle, its heterogeneity, and its mobility, there is a need for new methods facilitating its study.

MATERIALS AND METHODS

Lp(a) and LDL were isolated from the blood plasma of patients with elevated concentrations of

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these lipoproteins by ultracentrifugation in a stepwise density gradient of neutral NaBr salt [11]. Polyclonal sheep antibodies to Lp(a) were obtained by fractionation of antiserum by ammonium sulfate, followed by ion-exchange chromatography [1]. Monoclonal antibodies to apoB-100 were isolated as described previously [14]. Immunoblotting was carried out after a method described elsewhere [8], with monospecific sheep polyclonal antibodies to human Lp(a) used as the first antibodies, and rabbit immunoglobulins to peroxidase-conjugated sheep immunoglobulins (Sigma) as the second antibodies.

Linbro plates for EIA were incubated overnight at 4°C with a solution of polyclonal antibodies to Lp(a) (1 μg protein per well) in phosphate buffer containing 0.01% NaN_3 as an antiseptic. The plates were then washed in water and incubated 1 h with phosphate buffer containing 0.2% albumin and 0.05% Tween-20. The solutions of the samples and reference were prepared using the same buffer. Test samples of human plasma or serum were diluted 100-fold and titered at step 3. The reference sample was diluted 200 times and titered at step 1.4. Thus, seven dilutions of the reference sample were prepared. The test and reference samples were placed in wells (100 μl), incubated for 1 h at room temperature, and then washed with water. Polyclonal antibodies to Lp(a) or monoclonal antibodies to apoB-100 conjugated with horseradish peroxidase were used for conjugate preparation. 150 μl of conjugate solution were placed in a well and incubated 1 h at room temperature, after which the plates were thoroughly washed, and incubated 10 min with ortho-phenylenediamine solution in citrate buffer. The reaction was stopped by adding 25 μl of a 50% aqueous solution of H_2SO_4. Optical density was measured at wavelength 492 nm with a Titertek Multiscan device (Flow). A curve reflecting the relationship between the optical density and concentration of Lp(a) in the standard sample was then plotted, and this relationship was used as the calibration curve (Fig. 1). The concentrations of the test samples were estimated from the points on the linear portion of the calibration curve. Lyophilized Lp(a) calibrated after the Immuno standard was used as the reference. With monoclonal antibodies to apoB-100, plasma precalibrated after the same curve and stored at -20°C for 6 months was used.

RESULTS

Antibodies adsorbed on the plastic and forming a monolayer were selected for the development of EIA. These antibodies were titered at step 2 starting from the concentrations of 20 and 15 μg protein per well. The second antibodies were rabbit antibodies to peroxidase-conjugated sheep antibodies. The optimal concentration of antibodies providing for a distribution approaching a monolayer was attained at a concentration of 1 μg/well.

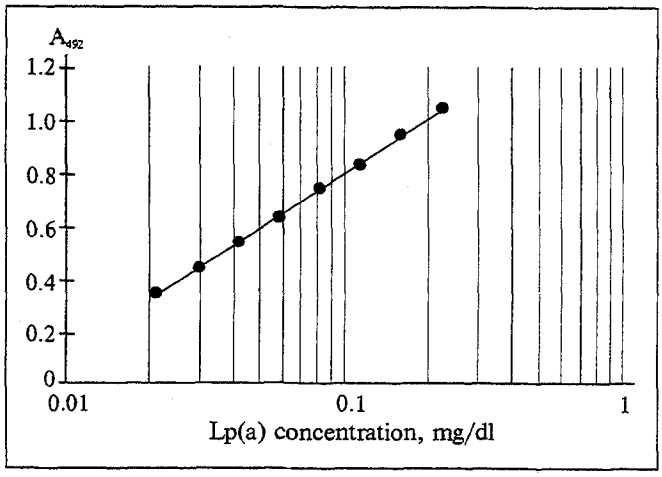

Fig. 1. Calibration curve for EIA. Abscissa: Lp(a) concentration in reference sample diluted 200 times.

Since the primary structure of apoprotein(a) is highly homologous to the plasminogen molecule [5] and Lp(a) is affine to fibrinogen as a protein rich

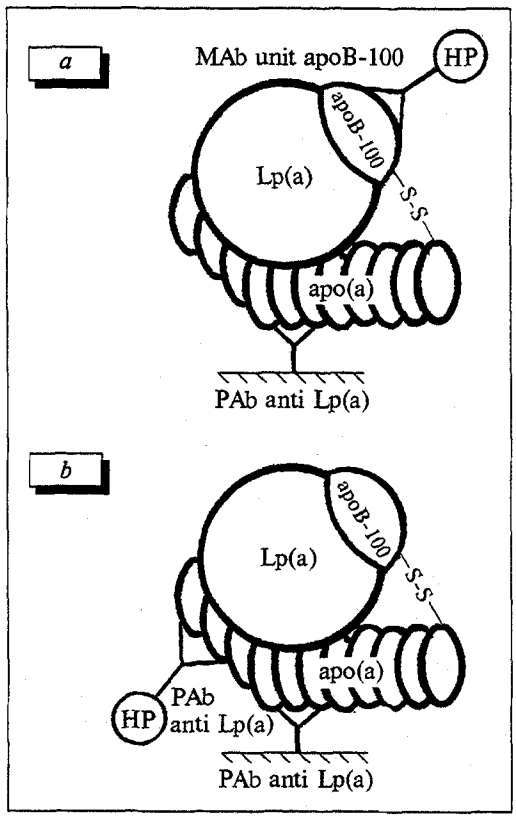

Fig. 2. Scheme of two variants of EIA. *a*) polyclonal antibodies (PAb) to Lp(a) used as adsorbing antibodies, horseradish peroxidase (HP) – conjugated monoclonal antibodies (MAb) to apoB – 100 as the developing antibodies; *b*) PAb to Lp(a) used as adsorbing and developing antibodies.

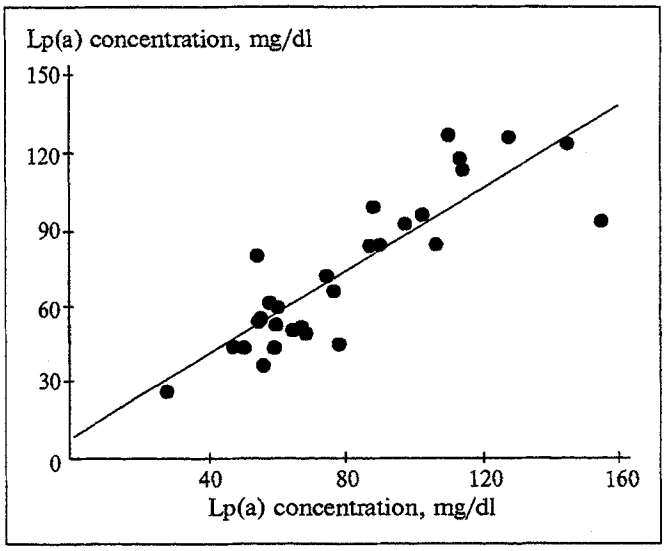

Fig. 3. Regression analysis of Lp(a) measurements by two EIA methods. Abscissa: Lp(a) values according to EIA scheme presented in Fig. 2, *b*; ordinate: Lp(a) values according to EIA scheme presented in Fig. 2, *a*.

in lysine [6], we tested the interaction between our polyclonal antibodies to Lp(a) and these components.

Preparations of human Lp(a), LDL, plasminogen, and fibrinogen were added to EIA plates with monospecific polyclonal antibodies to Lp(a). The preparations were titered at step 2 starting from the concentration corresponding to the higher value of the normal range for each of the above components. The rest of the experiment proceeded as described previously. The antibodies did not interact with fibrinogen, and the reactions with plasminogen and LDL did not surpass the background in the range of dilutions used to measure Lp(a).

Moreover, joint studies with the Institute of Human Genetics (Innsbruck, Austria) demonstrated that polyclonal antibodies to Lp(a) recognize all Lp(a) isoforms in accordance with the classification proposed by Prof. Utermann [15].

TABLE 1. Data on Reproduction of the Results of the Elaborated EIA ($M \pm m$)

No. of sample	Lp(a) concentration, mg/dl	Coefficient of variation, %
In a plate ($n=6$)		
1	5.7±0.18	3.2
2	52.6±2.20	4.3
3	101.3±3.40	3.3
4	189.5±13.2	7.0
Between experiments ($n=8$)		
1	5.4±0.79	14.7
2	50.3±4.70	9.3
3	110.2±8.59	7.8
4	177.5±18.20	10.3

Note. *n*: number of experiments.

A typical calibration EIA relationship is presented in Fig. 1. The linear portion of the calibration curve contains at least one point of any test sample diluted 100, 300, 900, and 2700 times. Despite the small number of samples that can be measured in one plate (no more than 18), a great advantage of such a scheme of EIA is the possibility of simultaneously measuring an Lp(a) concentration of anywhere from 2 to 500 mg/dl in different samples. This greatly simplifies measurements of Lp(a) concentrations, which vary very widely [10], and makes it easier to store Lp(a), whose corpuscles are rather labile and subject to change upon every repeated freezing and thawing.

Table 1 presents standard deviations and the coefficient of variation for a single measurement (upper part of the table) and for different experiments (lower part of the table) with plasma samples with very high, high, medium, and low concentrations of Lp(a).

The data indicate that the coefficient of variation is maximal for samples with an Lp(a) concentration approaching the lower limit of calibration. However, for EIA employed in clinical and even biochemical studies such an error in measurements is negligible.

Due to the presence of two proteins in Lp(a) - apo(a) and apoB-100 - there are two possible methods of measuring Lp(a) by EIA (Fig. 2).

The first of these was described by Dac [3]; it is based on Lp(a) measurement using two types of antibodies. Polyclonal antibodies to Lp(a) are adsorbed on the plastic, and peroxidase-conjugated monoclonal antibodies to apoB-100 are used as the developing antibodies. However, with this method Lp(a) can only be measured together with apoB-100 (Fig. 2, *a*).

The other EIA technique developed in our laboratory is based on Lp(a) measurements making use of only sheep polyclonal antibodies to human apo(a) (Fig. 2, *b*). Such an EIA modification makes it possible to assess the whole pool of apo(a), that is, free protein, the Lp(a) corpuscle proper, and apo(a) within the corpuscles rich in triglycerides.

Thirty plasma samples from cardiovascular patients with elevated Lp(a) were tested by the two EIA methods. Regression analysis of the results is presented in Fig. 3. The coefficient of correlation was 85%.

The results of our solid-phase EIA were also compared with those of the EIA modification developed at the Institute of Human Genetics (Innsbruck) [13]. The correlation coefficient was 98%. The concentrations of Lp(a) were measured in 20 serum samples from cardiovascular patients using two kits,

the one developed in our laboratory and the commercial Tint Elisa Lp(a) kit (Biopool); the correlation coefficient was 95%.

Hence, we have developed an EIA method permitting measurements of the Lp(a) concentration in human plasma and serum which is highly accurate and easily reproducible.

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