A Solid-Phase Enzyme-Linked Immunosorbent Assay for the Quantitation of Human Plasma $\alpha_1$-Acid Glycoprotein

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We describe a solid-phase enzyme-linked immunosorbent assay for $\alpha_1$-acid glycoprotein in human plasma. Plasma samples are incubated with alkaline phosphatase-linked, purified $\alpha_1$-acid glycoprotein in $\alpha_1$-acid glycoprotein-specific antibody-coated polystyrene tubes. The alkaline phosphatase that becomes attached to the tube via an immunological reaction between the $\alpha_1$-acid glycoprotein and the specific antibody is measured spectrophotometrically. This assay is accurate, reproducible, simple, and economical. As little as 4 mg of $\alpha_1$-acid glycoprotein per liter can be detected. The normal range for $\alpha_1$-acid glycoprotein in the plasma of healthy adults, as measured by this method, is 0.48–1.27 g/L; the range is significantly different, 0.29–0.73 g/L, for women who are taking oral contraceptive pills.

Additional Keyphrases: alkaline phosphatase • enzyme-linked immunosorbent assay (ELISA) • oral contraceptives

Concentrations of protein-bound neutral sugars in the human blood were reported to be increased in patients with cancers or acute inflammatory diseases (1). Subsequent development of methods for preparation of the "seromucoid" fraction (2, 3) greatly facilitated the study of plasma glycoprotein in health and disease. The seromucoid fraction of human plasma was found to increase in diseases associated with inflammatory, neoplastic, degenerative, thrombotic, or traumatic processes (4–7). Serial determinations of seromucoid can help assess the adequacy of tumor surgery and monitor the recurrence after surgery (8).

Seromucoid, the phosphotungstic acid precipitate of the perchloric acid-soluble fraction of human plasma, contains several plasma glycoproteins (9), of which $\alpha_1$-acid glycoprotein ($\alpha_1$-AG) is the major component. This protein has been isolated in pure form by various methods (10–15), including a large-scale fractionation method introduced by Hao and Wickerhauser (16).

$\alpha_1$-AG is one of the most extensively studied plasma proteins. Its carbohydrate structure has been recently reviewed (17) and the complete amino acid sequence elucidated (18). Clinical studies on $\alpha_1$-AG by various assay methods reveal that concentrations of this plasma glycoprotein are significantly increased in patients with neoplastic disease, acute inflammation, or surgical trauma, and in recipients of anabolic hormones (19–25). It has been reported that serial determination of $\alpha_1$-AG and $\alpha_1$-antitrypsin, in addition to determination of carcinoembryonic antigen, can improve the prediction of the course in colon cancer over that based on the measurement of carcinoembryonic antigen alone (26).

Materials and Methods

Materials

Calf intestinal alkaline phosphatase (EC 3.1.3.1), Type VII suspended in ammonium sulfate, was purchased from Sigma Chemical Co., St. Louis, MO 63178. Horse antiserum against human whole serum was a product of Hyland Laboratories (Costa Mesa, CA 92626). Human plasma $\alpha_1$-AG was given us by Dr. M. Wickerhauser, American National Red Cross Blood Research Laboratory, Bethesda, MD; this product migrated as a single broad band in disc polyacrylamide-gel electrophoresis (Figure 1) and appeared as a single immunological component in immunoelectrophoresis (Figure 2).

Blood specimens were donated by healthy hospital employees aged 20 to 62 years old. The specimens were immediately placed into tubes containing EDTA. Plasma fractions were assayed immediately or stored at −20 °C until use.

Methods

Disc polyacrylamide gel electrophoresis: Disc polyacrylamide gel electrophoresis was performed in the presence of sodium dodecyl sulfate, according to the procedure described by Weber and Osborn (27). We used acrylamide separating gel (45 g/L, 0.5 × 11.5 cm) and detected protein by staining with Coomassie Blue R250.

Immunoelectrophoresis: Immunelectrophoresis was carried out on a microscopic slide (2.5 × 7.5 cm). After dissolving agarose (15 g/L) in sodium barbital buffer (40 mmol/L, pH 8.8), we poured enough onto the slide to obtain a 3-mm thick gel. The purified $\alpha_1$-AG was subjected to electrophoresis for 90 min at a constant current of 5 mA/slide. Horse antiserum against human whole serum was placed in the trough, and the slide was transferred to a humid chamber. We continued the immunodiffusion at room temperature for 48 h until diffusion ceased. The slide was then washed, dried, and stained with Amido Black (10 g/L).

Production and isolation of antibody: We injected young male New Zealand white rabbits intradermally in the flanks and foot pads with 450 µg of purified $\alpha_1$-AG in Freund's complete adjuvant twice weekly for four weeks. Blood was collected by cardiac punctures 10 days after the last immu-
Electrophoresis

Fig. 1. Polyacrylamide gel electrophoresis of \( \alpha_1 \)-acid glycoprotein in the presence of sodium dodecyl sulfate
Electrophoresis carried out as described under Methods with migration from top to bottom. Amount of \( \alpha_1 \)-acid glycoprotein was 56 \( \mu \)g of protein

Conjugation

Adsorption of antibodies onto polystyrene tubes: To make an antibody coating, we pipetted 4 \( \mu \)g antibody protein dissolved in 1 mL of sodium carbonate buffer (0.1 mol/L, pH 9.8) into a polystyrene tube (12 × 75 mm; Sherwood Medical Industries, Inc., St. Louis, MO 63103). The tube was then capped and incubated at 37 °C for 3 h with constant shaking. The antibody-containing tube was then stored at 4 °C overnight before it was used in the assay.

Conjugation of alkaline phosphatase and \( \alpha_1 \)-AG: The conjugation procedure basically followed that described by Avrameas (28). In brief, 1 mL of alkaline phosphatase (5 g/L) in an ammonium sulfate suspension was centrifuged at 10,000 \( \times \) g for 10 min at 4 °C. The resulting precipitate was dissolved in 0.33 mL of \( \alpha_1 \)-AG solution (1.65 g per liter of phosphate-buffered saline, 55 mmol/L, pH 7.4). The solution was then dialyzed against the same phosphate-buffered saline at 4 °C overnight. To the dialysand, glutaraldehyde was added to make a final concentration of 2 g of glutaraldehyde per liter. The resulting solution was allowed to stand at room temperature for 2 h and then applied on a Sephadex G-150 column (1.5 × 85 cm). The column was eluted downward with 50 mmol/L tris(hydroxymethyl)methylaminoHCl buffer, pH 8.0. Fractions in the void volume containing the alkaline phosphatase–\( \alpha_1 \)-AG conjugate were pooled and stored at -20 °C in the presence of bovine serum albumin (1 g/L) and NaCl (0.1 g/L). This enzyme–antigen complex was found to be stable for at least six months under these conditions.

Enzyme immunoassay of \( \alpha_1 \)-AG: The antibody solution in the plastic tube was discarded. The tube was then washed three times with 3 mL of a solution containing 0.15 mol/L NaCl and 0.5 g/L Tween 80. The \( \alpha_1 \)-AG standard solution was prepared by serially diluting the purified \( \alpha_1 \)-AG in phosphate-buffered saline. Plasma specimens were diluted 20,000-fold with phosphate-buffered saline. To each antibody-coated tube were added 0.5 mL of standard \( \alpha_1 \)-AG in phosphate-buffered saline or diluted plasma, and 0.1 mL of 200-fold diluted alkaline phosphatase–\( \alpha_1 \)-AG conjugate. The reaction mixtures were incubated at 25 °C overnight. The content of each tube was then discarded, and the tube was washed three times with 3 mL of 0.15 mol/L NaCl and 0.5 g/L Tween 80 solution to remove the unbound alkaline phosphatase–\( \alpha_1 \)-AG conjugate. To each tube was added 1 mL of the enzyme substrate solution containing, per liter, 2.7 mmol of \( p \)-nitrophenol phosphate and 1 mmol of Mgl2 in 50 mmol/L sodium carbonate buffer, pH 9.8. The reaction was carried out at 25 °C for 30 min and terminated by adding 0.1 mL of 1 mol/L NaOH.

Absorbance at 400 nm was recorded and a standard curve was constructed, based on the absorbances of the serially diluted \( \alpha_1 \)-AG solutions. The value of \( \alpha_1 \)-AG in plasma was obtained by reading the absorbance against the standard curve. All assays were carried out in duplicate. Assays with a coefficient of variation greater than 10% were rejected and the procedure was repeated.

Results

Enzyme immunoassay

We found a concentration of antibody IgG of 4 mg/L in the coating solution to be suitable, as was our 200-fold dilution of alkaline phosphatase–\( \alpha_1 \)-AG conjugate (Table 1). Alkaline phosphatase–\( \alpha_1 \)-AG conjugate was not adsorbed onto the uncoated tubes or tubes coated with rabbit non-specific IgG. This indicates that alkaline phosphatase–\( \alpha_1 \)-AG conjugate was adsorbed onto the tubes through an immunological reaction with the specific antibody. The alkaline phosphatase attached to the tubes through its conjugation with \( \alpha_1 \)-AG retained the enzymatic activity of its native form. Because the

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<th>Table 1. Adsorption of Alkaline Phosphatase–( \alpha_1 )-Acid Glycoprotein Conjugate to the Antibody-coated Plastic Tubes</th>
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<tr>
<td>Dilution of alkaline phosphatase–( \alpha_1 )-acid glycoprotein conjugate ( * )</td>
</tr>
<tr>
<td>Uncoated tubes</td>
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<tr>
<td>Rabbit non-specific</td>
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<td>IgG-coated tubes</td>
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* No free \( \alpha_1 \)-acid glycoprotein was included.
Fig. 3. Standard inhibition curve of $\alpha_1$-acid glycoprotein of the immunoassay

O, purified $\alpha_1$-acid glycoprotein; ●, human plasma with known amount of $\alpha_1$-acid glycoprotein. Lines and slopes were generated by least-squares analyses.

enzyme reaction was linear for at least 30 min at 25 °C, we chose a 30-min incubation time for this immunoassay. The standard curve obtained when assaying purified $\alpha_1$-AG was parallel to the one obtained by assaying a serially diluted plasma specimen (Figure 3), which indicated that the assay was specific for $\alpha_1$-AG in the human plasma. Coefficients of variation in the within-day assay of three plasma samples, 20 measurements each, were 6.9, 5.1, and 6.6%. Quantitation of $\alpha_1$-AG in Human Plasma

Plasma samples of 47 healthy individuals were assayed for $\alpha_1$-AG by the immunoassay method. These 47 individuals were divided into four groups: males, premenopausal females, premenopausal females on oral contraceptive pills, and postmenopausal females. The mean values of plasma $\alpha_1$-AG in these four groups were 0.77, 0.74, 0.54, and 0.66 g/L, respectively (Table 2). Analysis of variance and multiple-comparison tests revealed that only the third group was different from the other three groups. The three groups with no difference among them were combined into a single group to represent healthy adults. Estimating the plasma $\alpha_1$-AG level in this combined group by the non-parametric method gives a range of 0.48–1.27 g/L (90% probability). Likewise, the normal range in the premenopausal females who are on oral contraceptive pills should be 0.29–0.73 g/L.

Discussion

Enzyme-linked immunosorbent assay (ELISA) (29, 30) has gained popularity in recent years for assaying minute quantities of components in human blood. It has the advantage of being sensitive, simple, and economical; uses stable reagents and, unlike radioimmunoassay, totally avoids radiation; and is suitable for automation when a large number of assays are needed. The application of this assay in clinical medicine has been reviewed recently by Scharpé et al. (31) in this journal.

Immunological methods currently employed for the quantitation of plasma $\alpha_1$-AG are single radial immunodiffusion (32) or electrophoresis (33) on antibody-incorporated gels. A commercial kit based on the radial immunodiffusion is available (34), but our method is much more sensitive, having a detection limit of 4 μg/L vs. 100 mg/L (34). Because $\alpha_1$-AG is abundant in human plasma, a high grade of sensitivity may not be needed. However, in the biological fluids where concentrations of $\alpha_1$-AG are usually low (asctic fluid, pleural fluid, etc.), these amounts may not be detectable by Mancini's method without a prior concentration step. In these circumstances our assay will be superior to Mancini's.

In contrast to our assay, both Mancini's and Laurel's methods consume large amounts of specific antiserum. Besides being a sensitive and economical method, our assay can be potentially automated when the assay of a large number of specimens is needed.

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References


