AN IMMUNOASSAY FOR A PREGNANCY-ASSOCIATED α-MACROGLOBULIN USING ANTIBODY–ENZYME CONJUGATES

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1. Introduction

The existence of additional serum proteins in the blood of pregnant women is an established fact (for example [1–8]). One of these, a high molecular weight α-globulin [4], is the most commonly found and has been obtained in a pure form [7,9]. It is a glycoprotein containing 10% carbohydrate and having an iso-electric point of 4.6 [9]. This pregnancy-associated α-macroglobulin (PAM) has been quantitated in the blood of subjects with a variety of conditions and its level appears to be particularly dependent on the presence of oestrogens [6,8,10,11]. However the protein has been shown not to be involved in steroid transport [12–14]. A recent investigation has indicated that peripheral blood leucoctyes are responsible for the synthesis of PAM [15] but its biological role is as yet uncertain.

This study was carried out to produce a sensitive method for assaying PAM that could be employed the necessity for expensive equipment. The use of radioactive labels as markers for proteins is well known but they are often difficult to use because of the short half-life of the isotopes employed and because of the possibility of radiation damage to the molecule, which could effect its immunochemical reactivity. For these reasons an enzyme was used as marker [16–18] for the antibody to PAM in the 'sandwich' immunoassay developed.

2. Materials and methods

2.1. *Reagents*

Horse radish peroxidase (HRP), grade 1, RZ = 3.0, was obtained from Miles-Seravac; glutaraldehyde (25% aqueous solution) from B.D.H.; bovine serum albumin, Fraction V, from Sigma; 5-aminosalicylic acid from Emmanuel, Wembley, England, polystyrene test tubes (3 ml) from Sarstedt: Analar grade reagents were used wherever possible.

Pure PAM [9] and Freunds' complete adjuvant were used for immunising rabbits and the resultant antiserum was prepared by 50% ammonium sulphate precipitation and DEAE-cellulose fractionation [19] of the serum obtained. The anti-PAM was absorbed four times with 4B Sepharose coupled to male serum proteins by the cyanogen bromide method [20]. The specificity of the antiserum was checked by two dimensional immunoelectrophoresis [21].

Except, where indicated, 0.04 M phosphate buffer, pH 7.4, containing 0.5 M NaCl was used in the following procedures.

2.2. *Preparation of antibody–HRP conjugates*

1 mg antibodies to PAM and 5 mg HRP were dissolved in 2 ml 0.04 M phosphate buffer, pH 7.4, containing 2.5% glutaraldehyde and shaken at room temperature for 3 hr [18]. No precipitation occured during this procedure. The solution was then subjected to gel filtration on a column (1.6 × 40 cm) of Sephadex G-150 equilibrated with the buffer described above, at 4°C. The flow rate was 6 ml/hr and the effluent was continuously monitored for protein at 280 nm. Fractions of 2.4 ml were collected and the tubes containing the conjugate were pooled (fig. 1) and stored at −20°C.

2.3. *Determination of HRP activity*

A substrate solution was prepared by dissolving 40 mg 5-aminosalicylic in 100 ml of 0.02 M phosphate buffer, pH 6.0, containing 5 μl 30% H_2O_2 [17]. In the

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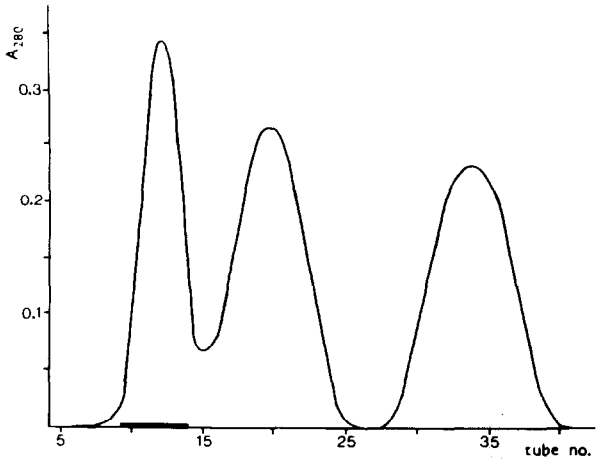

Fig. 1. Gel chromatography of antibody–HRP conjugate on a column of Sephadex G-150. The fraction indicated by the black bar was collected.

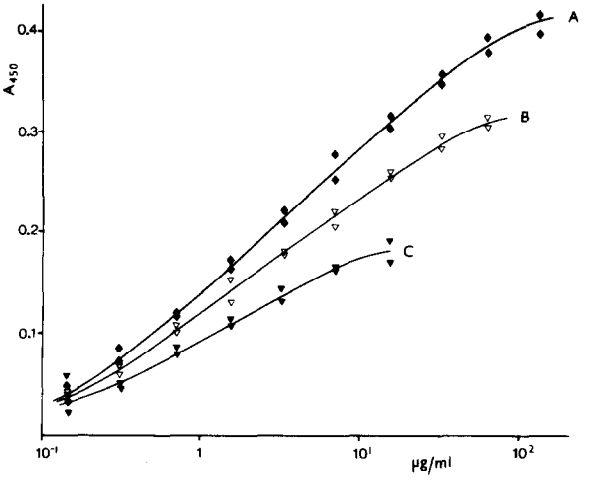

Fig. 2. Standard curves for PAM assay with various antibody dilutions. A (1:1000), B (1:3000), C (1:10 000).

assay 1 ml of freshly prepared substrate was added to the empty polystyrene tubes and the absorbance at 450 nm read after 1 hr at room temperature.

2.4. *Preparation of antibody coated tubes*

The anti-PAM was diluted with buffer and 0.6 ml of the solution incubated in each polystyrene tube [22] for 15 min at room temperature. The tubes were then washed three times with buffer, filled with 1% bovine serum albumin and re-incubated. The tubes could then be stored at 4°C until used.

2.5. *Enzyme-immunoassay procedure*

A 0.5 ml sample (standards 0–200 μg/ml PAM or pregnancy serum 1:10–1:100 diluted) was placed in each antibody-coated tube and incubated at 37°C for 45 min. The tubes were then washed three times with buffer containing 0.1% Triton X-100 and 0.6 ml aliquots of diluted antibody–HRP conjugate, containing approximately 20 mIU HRP activity, were added. After a further incubation of 2 hr at room temperature the test tubes were washed three times with buffer and the HRP activity determined as above.

3. Results and discussion

The conjugate was purified by gel chromatography and a typical elution profile is shown in fig. 1. The unreacted HRP (second peak) was adequately separated from that bound to antibody and the fractions indicated were pooled and normally used at 1:30–1:40 dilution. In fig. 2 three standard curves for the assay are indicated. The lower limit of sensitivity is approximately 0.2 μg/ml while the upper limit, with an antibody dilution of 1:1000, is 100 μg/ml. Thus a wide range of concentrations can be covered with a single standard curve is necessary. The method was compared with the Laurell 'rocket technique' of quantitative immunoelectrophoresis [23] by determining the PAM concentration in the serum of 30 pregnant women. The correlation between these test methods is shown in fig. 3.

The use of detergent in the post-antigen washing procedure was found to be of prime importance in reducing non-specific adsorption of serum proteins onto the bound antibody. Repeated assays with the purified glycoprotein were extremely reproduceable without the presence of Triton X-100, but very inconsistent results were obtained from serum samples unless the detergent was incorporated into the buffer. The incubation times employed were not necessarily optimum. Longer periods, of up to 24 hr, produced a more sensitive assay but the reproduceability was poor, perhaps in part due to HRP instability. However the time periods used provided an assay of sufficient sensitivity to cover the expected range of PAM concen-

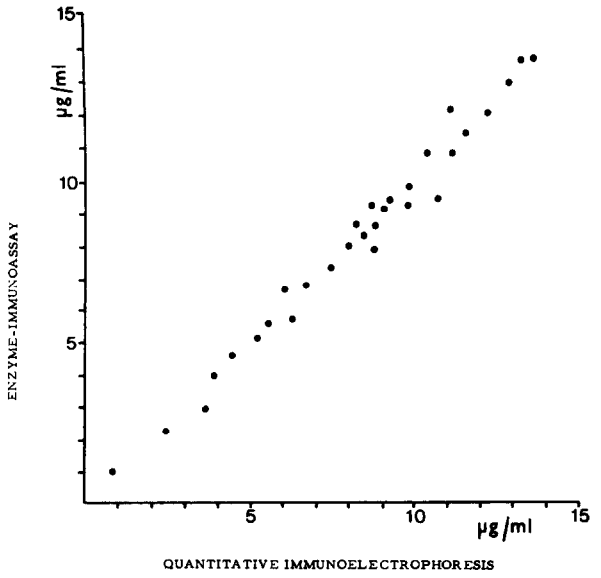

Fig. 3. Correlation between PAM concentration of 30 samples of pregnancy serum as measured by the enzyme-immunoassay and quantitative immunoelectrophoresis.

trations [11] and allowed the assay to be completed in a single working day.

By employing a 'sandwich-type' immunoassay with HRP as marker and the antibody bound to polystyrene tubes a simple, sensitive method has been developed for estimating PAM concentrations in serum. This should facilitate further studies into the role of the macromolecule.

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