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Radioimmunoassay of Progesterone and Estriol in Plasma Using Antibodies Immobilized onto Protein A–Sepharose CL-4B

MARIA ANGELA BACIGALUPO AND GIACOMO CARREA*

Istituto di Chimica degli Ormoni, CNR, Via Mario Bianco 9, 20131 Milano, Italy

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ABSTRACT

Protein A-Sepharose CL-4B was used as a solid phase for antibodies in the radioimmunoassay of progesterone and estriol. The method was fast and easily standardizable. Immobilized antibodies had the same binding capacity as free antibodies and gave good correlation curves (r = 0.996 for progesterone and r = 0.989 for estriol). Sensitivity was 12.5 pg/tube for progesterone and 8.0 pg/tube for estriol. Comparison of progesterone radioimmunoassay with chemically immobilized antibody onto Sepharose CL-4B was also carried out.

Index Entries: Solid-phase radioimmunoassay, of progesterone and estriol; protein A–Sepharose, antibodies as immunomatrix for; CNBr method, of antibody immobilization; radioimmunoassay of progesterone and estriol by immobilized antibodies; progesterone, immobilized antibody radioimmunoassay for; estriol, immobilized antibody radioimmunoassay for; plasma, radioimmunoassay of progesterone and estriol in; antibodies, radioimmunoassay of progesterone and estriol by immobilized.

*Author to whom all correspondence and reprint requests should be addressed.

INTRODUCTION

Immunological assays based on immobilized antibodies have advantages such as simple separation of free and antibody-bound antigen and shortened assay time. Nevertheless, the immobilization of antibodies onto various solid supports is not easily standardizable and immunoglobulins are often incorrectly oriented, which reduces their binding capacity and results in a waste of material.

To overcome these limitations, protein A-containing *Staphylococcus aureus*, which binds the Fc region of immunoglobulins without affecting specific Fab sites (*1,2*), has been proposed as a separating agent in immunoassays (3–7). Several antigens (*8–10*) and haptens (*10,11*), including steroid hormones (*12,13*), have been assayed using a suspension of formaldehyde- and heat-treated *S. aureus* cells.

In the present article, a radioimmunoassay (RIA) of plasma progesterone (P) and estriol (E_3) utilizing, as the adsorbent for antibodies, purified *S. aureus* covalently linked to Sepharose CL-4B is described. The assay requires no technical skill for standardization, and furthermore, immobilized antibodies possess the same binding capacity for steroids of the free ones.

MATERIALS

Estriol (E_3) and progesterone (P) standards were obtained from Vister (Milan, Italy); 2,4,6,7-^{3}H-estriol and 1,2,6,7-^{3}H-progesterone (specific activity 2.7×10^9 kBq/mmol) from NEN (Boston, USA). Dextran-coated charcoal (10% dextran T70), bovine serum albumin (BSA), and antisera raised in rabbits were obtained from Sorin (Saluggia, Italy) (immunogens, estriol-3-*O*-carboximethyl oxime-BSA and progesterone 11α-hemisuccinate-BSA; working dilution titer 1:20,000 for antiserum anti-estriol and 1:13,000 for antiprogesterone). Sepharose CL–4B and protein A–Sepharose CL-4B were purchased from Sigma (St. Louis, USA) and scintillation liquid Picofluor 30 from Packard (Milan, Italy). The other chemicals were obtained from Merck (Darmstadt, FRG).

METHODS

Immobilization of Antibodies onto Protein A–Sepharose CL-4B

Protein A–Sepharose CL-4B (6 mg) was swelled in 0.05*M* sodium phosphate buffer, pH 7.4, and 0.01*M* EDTA, and diluted with Sepharose CL-4B (200 μL of settled gel). Antiserum, enough for 200 RIA tubes, was added, and the suspension (final volume 1 mL) was gently shaken for 30 min at room temperature. Then, the suspension was diluted 10-fold with

the buffer, centrifuged at 1000*g* for 5 min, and the pellet resuspended in the buffer containing 0.15% BSA (final volume 20 mL).

The binding capacity of immobilized antibodies in comparison with free antibodies was measured by RIA after addition of a known amount (about 10,000 cpm) of ^{3}H-progesterone or ^{3}H-estriol, and centrifugation. The supernatant was also tested for residual binding capacity. The same measurements were carried out in the presence of non-immune bovine immunoglobulins (0.1%, w/v). Nonspecific absorption of steroids was evaluated in glass and polytene tubes using protein A–Sepharose CL-4B bearing nonspecific bovine immunoglobulins.

Immobilization of Antibodies onto CNBr-Activated Sepharose CL-4B

Sepharose CL-4B was activated at pH 10.5 according to Axen et al. *(14)* using 20 or 100 mg CNBr/mL of settled gel. The coupling of antiserum (enough for 200 RIA tubes) to CNBr-activated matrix (20 μL of settled gel) was performed in 0.1*M* sodium phosphate buffer, pH 7.5, under gentle stirring at 4°C overnight (final volume 0.5 mL). After addition of Sepharose CL-4B (200 μL of settled gel) the suspension was diluted 10-fold with the pH 7.4 buffer and centrifuged at 1000*g* for 5 min. The pellet was then resuspended in the buffer containing 0.15% BSA (final volume, 20 mL).

Sample Preparation

Plasma samples (100 μL) were extracted by 2-min vortex mixing with 10 vol of *n*-hexane in the case of P, or diethylether in the case of E_3. After freezing of the aqueous phase, 300 μL of the organic phase, corresponding to 30 μL of plasma, was pipeted, in duplicate, into assay tubes and dried under nitrogen. Residues were dissolved in 100 μL of the pH 7.4 buffer, containing 0.15% BSA, and tested by RIA. Recovery was determined using 2500 cpm of ^{3}H-hormone as internal standard.

Assays

One hundred μL of sample or a dilution series of standard hormone (12.5–1600 pg/tube for P and 8–400 pg/tube for E_3) was incubated in glass or polytene tubes (11 × 45 mm) for 2 h at 2°C with 100 μL of tritiated hormone (10,000 cpm) and 100 μL of a homogeneous suspension of immobilized antibody. After centrifugation at 1000*g* for 10 min the supernatant was decanted, and the pellets were mixed with 2 mL of scintillation liquid. The radioactivity was then counted in a Tricarb 2425 Packard counter. A glass vial with a central support was employed to keep the tube vertical.

Comparisons with free antibodies were carried out using the same working dilution titer and adsorbing free steroids with dextran-coated charcoal (1 mg/tube).

RESULTS

Properties of Antibodies Immobilized onto Protein A–Sepharose CL-4B

The steroid-binding capacity of antibodies immobilized onto protein A–Sepharose CL-4B was not significantly different from that of free antibodies. The residual binding capacity present in the supernatant was smaller than 0.5% of the total. The addition of nonimmune bovine immunoglobulins, severely decreased the amount of labeled hormone present in the pellet after incubation and centrifugation. This finding indicates that the interaction between the solid matrix and antibodies takes place through protein A, since antibodies are displaced by other immunoglobulins that bind to protein A.

Immobilized antibodies were stable for about 6 mo at 4°C in buffer solutions with a pH between 4.5 and 9.

Radioimmunoassay

Standard curves for P and E_3 are shown in Figs. 1 and 2, respectively. It can be seen that curves obtained with immobilized antibodies are almost superimposable to those obtained with free antibodies. This

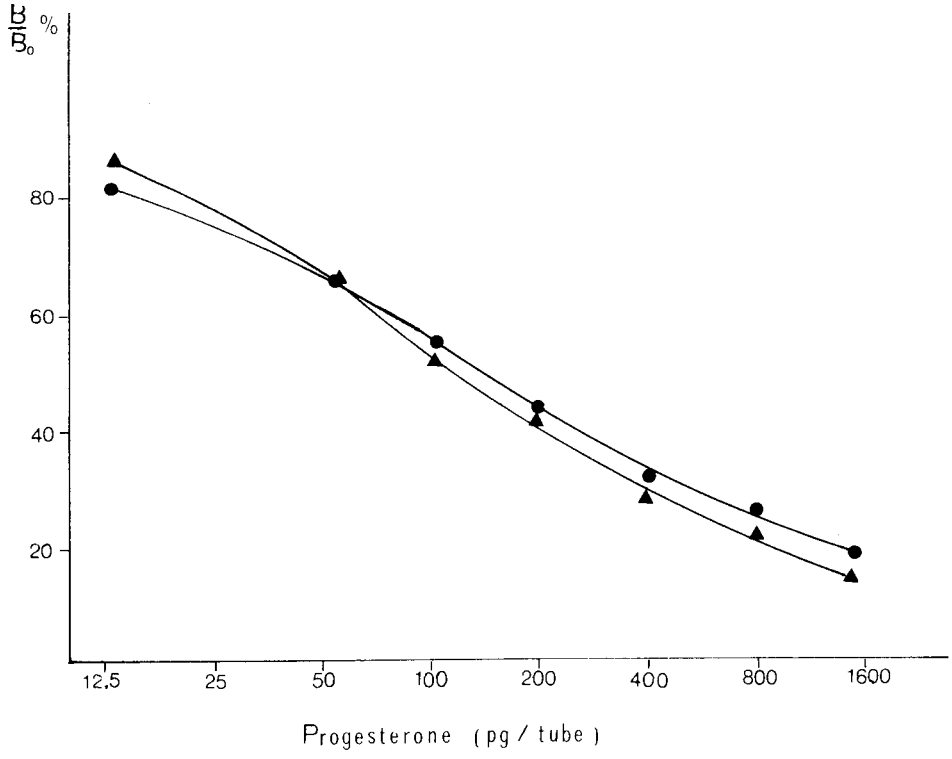

Fig. 1. Comparative standard curves for P, obtained with antibody immobilized onto protein A–Sepharose CL-4B (●) and free antibody (▲).

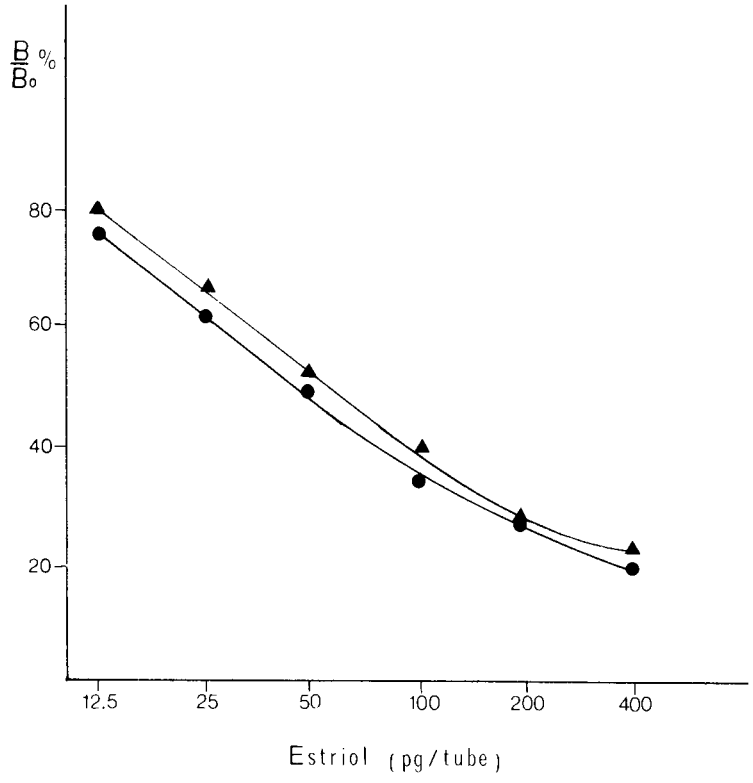

Fig. 2. Comparative standard curves for E_3 obtained with antibody immobilized onto protein A–Sepharose CL-4B (●) and free antibody (▲).

indicates that free and immobilized antibodies have the same binding capacity and sensitivity since the same dilution titer was used in both cases.

Nonspecific adsorption was negligible in glass tubes, whereas it was 8.3 ± 2.3% in polytene tubes. Hormone recovery after extraction with organic solvents was determined with the internal standard method. It was 82 ± 3.2% for P and 80 ± 2.1% for E_3. In calculation, values were corrected for both nonspecific adsorption and recovery.

Assay sensitivity, defined as the smallest amount of hormone giving a response significantly different from zero at twice the standard deviation, was 12.5 pg for P and 8 pg for E_3.

Coefficients of within-assay and between-assay variation, for plasma samples with low and high steroid concentration, were between 3.5 and 10% (Table 1). Coefficients of correlation between the values obtained with immobilized antibodies and those obtained with free antibodies were $r = 0.996$ for P and $r = 0.989$ for E_3.

Comparison with Antibodies Immobilized onto CNBr-Activated Sepharose CL-4B

Progesterone was also assayed using chemically immobilized antibodies. The yield of immobilization of antibodies onto Sepharose CL-4B

TABLE 1
Coefficients of Variation of P and E_3 Determination

Hormone	Mean concentration, ng/mL	Coefficient of variation, % (n = 8)	
		Within assay	Between assay
P	0.8	6.5	9.6
	9.56	3.5	8.2
E_3	1.55	7.8	10.2
	11.0	4.3	9.8

was 95 or 99% when 20 mg CNBr/mL of matrix or 100 mg CNBr/mL of matrix were used, respectively. Antibodies immobilized with low CNBr activation showed the same sensitivity, but a binding capacity 27% lower than that of antibodies immobilized through protein A, whereas those immobilized with high CNBr activation showed the same binding capacity but a lower sensitivity (20 pg progesterone/tube instead of 12.5 pg/tube). Therefore, chemically immobilized antibodies were less efficient and, furthermore, required more skill to be prepared than antibodies simply adsorbed onto protein A–Sepharose CL-4B.

DISCUSSION

The separation of free from antibody-bound antigens is the most laborious stage in competitive immunoassays. Solid-phase coupled antibodies greatly simplify the process, but often standardization is difficult for the variability of materials and conditions of activation, and for nonspecific adsorptions. Instead, the method here described, which utilizes protein A–Sepharose CL-4B as adsorbent for antibodies to P and E_3, is easily standardizable, simple, and fast. Immobilized antibodies have optimal spatial orientation, as demonstrated by the fact that they maintain the same binding capacity and sensitivity as the free ones. The stability of bound antibodies is very high in a wide pH range.

Potentially, the use of protein A–Sepharose CL-4B could be easily extended for the assay of a variety of haptens and antigens since no immobilization specific for each kind of antibody is necessary. Also, work is in progress aimed to verify whether other matrices, such as polystyrene and nylon, are suitable for protein A immobilization.

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