IMMUNOASSAY USING ANTIBODY–ENZYME CONJUGATES

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1. Introduction

Most of the enzyme-immunoassays described in the literature employ enzyme-labelled antigen or hapten [1–8]. A few use enzyme-labelled anti-antibody (anti-IgG*) [8,9]. Enzyme-labelled antibodies against the substance to be determined, provide a third tool for enzyme-immunoassay.

In this report we describe two methods for the assay of human chorionic gonadotrophin (HCG), which employ anti-HCG labelled with horse-radish peroxidase (HRP). The methods are analogous to the 'immunoradiometric' [10] and the 'sandwich' [11] radioimmunoassays. Their sensitivities and the economy of reagents are compared with those of the Dasp enzyme-immunoassay for HCG, which employs HRP-labelled HCG. The latter method has been shown to be markedly more sensitive than the corresponding solid-phase assay [1].

2. Materials and methods

2.1. *Materials*

HCG was prepared by Van Hell et al. [12], according to previously described procedures. Antisera against highly purified HCG were raised in rabbits as described previously [13]. HRP, grade IV, was bought from Miles-Seravac, Maidenhead, Berks., England.

* *Abbreviations used:* IgG = immunoglobulin G; HCG: human chorionic gonadotrophin; HRP: horse-radish peroxidase; Dasp: double antibody solid phase.

Glutaraldehyde was bought from Schuchardt, Munich, W. Germany, as a 25% aqueous solution. Dasp® anti-rabbit (sheep antibodies against rabbit IgG, covalently linked to cellulose) is a product of Organon Teknika, Oss, The Netherlands.

2.2. *Preparation of immunosorbents*

HCG–Sepharose was prepared by coupling highly purified HCG (approx. 10 000 IU/mg by bioassay) to CNBr-activated Sepharose 4B according to Porath et al. [14]. (Anti-HCG)-cellulose was prepared by coupling the IgG fraction of anti-HCG serum (prepared by precipitation with 180 mg/ml of solid anhydrous Na_2SO_4 at room temperature) to cellulose according to the Gurvich method [15].

2.3. *Preparation of antibodies to HCG*

Anti-HCG serum (diluted with 9 volumes 0.05 M Na-citrate, pH 5.0) was passed through a column containing sufficient HCG–Sepharose to bind approximately half of the antibodies applied. After washing until $A^{1\,cm}_{280\,nm}$ was less than 0.025, the antibodies were eluted with 0.3 M glycine–HCl (pH 3.0). The eluate was immediately adjusted to pH 5–7 with 2 M NaOH, and concentrated by ultrafiltration to a protein content of approx. 5 mg/ml. All procedures were carried out at room temperature.

2.4. *Preparation of (anti-HCG)-HRP conjugates*

Antibodies to HCG were coupled to HRP using two different methods: (A) One-step method [17]. A solution containing 5 mg/ml of antibody, 5 mg/ml of HRP and 0.2% glutaraldehyde in phosphate–citrate buffer (pH 7.0) was shaken for 2 hr at room temper-

ature, centrifuged for 10 min at 1000 *g* to remove insolubilized protein and passed over a Sephadex G-50 column to remove unreacted glutaraldehyde; (B) Two-step method. This is a modification [17] of the procedure of Avrameas and Ternynck [16]. HRP was dissolved (30 mg/ml) in 0.05 M phosphate–citrate buffer (pH 7.0) containing 2% glutaraldehyde and incubated for 18 hr at room temperature. Unreacted glutaraldehyde was removed by gel filtration on Sephadex G-50. The HRP-containing eluate was diluted to a concentration of 2 mg/ml. 0.3 ml of this solution was mixed with 0.2 ml (anti-HCG) solution (5 mg/ml) and incubated for 2 hr at room temperature.

The reaction products of both coupling methods were purified by centrifugation on a linear 20–60% sucrose gradient at 281 000 *g* for 16 hr, as described previously [1].

2.5. *Preparation of HCG-HRP conjugates*

These conjugates were prepared either by a one-step or a two-step method. The one-step method has been described previously [1]. In the two-step procedure (a modification of the procedure of Avrameas and Ternynck [16]), 10 mg HRP were dissolved in 1 ml phosphate–citrate buffer (pH 7.0) containing 1% glutaraldehyde and incubated for 18 hr at room temperature.

Unreacted glutaraldehyde was removed by gel filtration on Sephadex G-50. The HRP-containing eluate was concentrated to 1 ml by ultrafiltration. 2.5 mg HCG were dissolved in the concentrate, which was then incubated for 2 hr at room temperature. The reaction products of both methods were purified by ultracentrifugation (see section 2.4).

2.6. *Enzyme-immunoassay procedure*

2.6.1. Immunoenzymometric method

This method is analogous to the immunoradiometric method of Miles of Hales [10]. 0.6 ml of sample (containing HCG) was incubated for 30 min with 0.1 ml of diluted* (anti-HCG)–HRP conjugate. Then 0.3 ml HCG–Sepharose suspension (in a concentration which allowed binding of all the added conjugate) was added, the mixture shaken for 2 hr and centrifuged for 5 min at 1000 *g*. The amounts of HRP-activity in the supernatant was measured as described previously [1].

2.6.2. Sandwich method

The analogous radioimmunoassay was described by Wide [11]. 0.6 ml of sample (containing HCG) was shaken for 2 hr with 0.3 ml (anti-HCG)-cellulose suspension (in a concentration related to the expected highest HCG-concentration in the test sample [17]). 0.1 ml of diluted* (anti-HCG)–HRP conjugate was added, the mixture shaken for 2 hr and centrifuged for 5 min at 1000 *g*. The amount of HRP-activity in the supernatant was measured as described previously [1].

2.6.3. Dasp method

The method was described in detail in a previous article [1]. 0.5 ml of sample (containing HCG) was incubated for 30 min with 0.1 ml of suitably diluted antiserum. 0.1 ml of diluted* HCG–HRP conjugate was added, and the mixture incubated for 30 min. After adding 0.3 ml of a Dasp anti-rabbit suspension, the mixture was shaken for 2 hr, and centrifuged for 5 min at 1000 *g*. The HRP-activity in the supernatant was measured as described previously [1].

3. Results and discussion

Labelled-antibody methods have one obvious advantage: every antibody–HRP conjugate can be prepared by the same method (provided that the antibodies are evoked in the same animal species). However, the need to prepare the antibodies by affinity chromatography forms a disadvantage.

In order to compare the sensitivities, we tested the effect of a dilution series of HCG in the three assays described in section 2.6. This experiment was performed with conjugates prepared by both the one-step and the two-step methods. The antibodies used – in conjugates, in immunosorbent and in solution – were all from the same antiserum. Different batches of HCG, of comparable purity, were used for the preparation of antiserum, immunosorbent and conjugates. The response curves are shown in fig. 1.

* The amount of conjugate in the incubation mixtures was so chosen that, in the absence of other immunoreagents, it would give an $A^{1\text{ cm}}_{450\text{ nm}}$ of approx. 0.4 in the final enzyme assay.

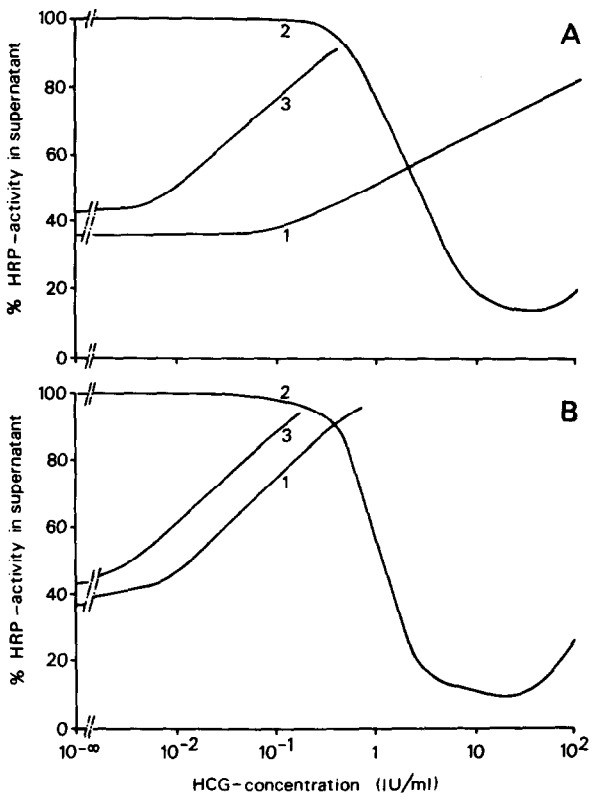

Fig. 1. Dose-response curves in different enzyme-immunoassays for HCG.
1. immunoenzymometric assay } employing (anti-HCG)-
2. sandwich assay } HRP conjugates
3. Dasp assay employing HCG–HRP conjugates
In part A. conjugates prepared by the one-step method were used. In part B. conjugates prepared by the two-step method were used.

Part A shows the curves obtained with the conjugates prepared by the one-step method, part B those obtained with conjugates prepared by the two-step method. The difference between one-step and two-step conjugates lies in their composition: the molar antibody (or HCG)/HRP ratio is less than one for one-step conjugates [18]. but at least equals and possibly exceeds one for two-step conjugates [16]. This difference should not affect the sensitivity of the Dasp and the sandwich assays, but the immunoenzymometric assay should be less sensitive when using one-step conjugates than when using two-step conjugates [17]. This was confirmed by the experimental findings: Curves A2 and B2 are close together, like curves A3 and B3, but curves A1 and B1 differ markedly. Using a two-step conjugate, it may be expected that the sandwich assay is about as sensitive as the Dasp assay and the immunoenzymometric assay [17]. It was found, however, that the andwich assay (curve B2) was 10–20 times less sensitive than the others (curves B1 and B3). This may be caused by steric hindrance, since the binding of the conjugate to the antigen occurs at the surface of the immunosorbent in the sandwich assay, but in solution in the other assays. Another reason may be that the affinity of the antibodies may be affected (possibly to different degrees) by coupling to HRP and to cellulose.

A rough calculation indicates that in the two-step procedure the amounts of HRP required to obtain reagents for the same number of HCG determinations, are approximately equal for all three assays. The sandwich assay, however, requires 3 times as much HCG as the other assays. In addition, considerably more antibodies are required for the labelled-antibody methods. In conclusion, in terms of sensitivity and economy, labelled-antibody methods are not preferable to the Dasp enzyme-immunoassay using HCG-HRP conjugates.

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