IMMUNOASSAY OF THE HUMAN CHORIONIC GONADOTROPHIN USING FLUORESCENCE POLARIZATION

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Received 22 May 1978
Revised version received 31 July 1978

1. Introduction

Classical immunoassay methods are based on the displacement of a labelled antigen by the antigen to be measured from sites on an antibody and necessitate the separation of the free and bound antigen fractions.

The fluorescence polarization method detects variations in the hydrodynamic volumes of fluorescent molecules; the larger the molecule, the greater the increase in the degree of polarized light emitted. This permits interactions between a fluorescent antigen and an antibody to be detected directly, without recourse to a separation step.

In the presence of a specific antiserum the antigen, labelled with a fluorochrome, is bound, resulting in an increase in the degree of fluorescence polarization. Upon addition of unlabelled antigen, bound labelled antigen will be displaced from the antibody and the fluorescence polarization will decrease accordingly.

This article describes the application of such a method to the measurement of human chorionic gonadotrophin.

2. Materials and methods

'Pregnyl' HCG, Organon (Oss, Holland) was used and measured using the 'RIA HCG K_1', double antibody kit from the 'Centre d'Energie Atomique' (Gif-sur-Yvette, France) by the method in [1]. Control clinical assays of urinary HCG were performed using the haemagglutination inhibition method with the 'Organon' kit (Oss, Holland).

Complete Freund's adjuvant (Perrin's modification) came from Calbiochem (San Diego, Calif.) and Biogel P2, P60 from Biorad Laboratories (Richmond, Calif.).

Fluorescein isothiocyanate from Aldrich-Europe (Beerse, Belgium) was measured in solution, at pH 8, from its $A_{493\ nm}$ value, Σ_M 1 cm = 8.52×10^4 [2]. Other chemicals were 'pro-analysis' grade from Merck (Darmstadt).

Optical densities were determined using a Perkin-Elmer Model 402 spectrophotometer, and fluorescence intensities with a FICA Model 55 MK II absolute differential spectrofluorimeter (Le Mesnil Saint-Denis).

For the determination of the degree of fluorescence polarization:

$$P = \frac{F_{\parallel} - F_{\perp}}{F_{\parallel} + F_{\perp}} \qquad (1)$$

the fluorescence intensity of the sample was measured when the polarizers for exciting and emitted light were parallel ($F_{\parallel}$) and perpendicular ($F_{\perp}$). A model 400 polarization fluorimeter (SLM Instruments, Urbana, Ill.) was used. The principle of operation of this type of instrument has been described [3,4]. This instrument allowed one to obtain the ratio $F_{\parallel}/F_{\perp}$ directly and averaged from

Abbreviations: HCG, human chorionic gonadotrophin; RIA, radio immuno assay; FITC, fluorescein isothiocyanate

10–100 values. Each determination was carried out with two samples and each reading (which corresponds to a mean of 10 values) was taken 3 times. The cuvette was held at 4°C by circulating alcohol Haake model F_2 K circulator; the contents being first passed through a millipore filter, 0.22 μm Millipore Filter Corp. (Bedford, Mass.).

2.1. *Purification of HCG*

'Pregnyl' HCG, at ~20 mg in 4.5 ml was first filtered on a 1.5 X 85 cm Biogel P2 (100–200 mesh) at 4°C using 0.1 M sodium phosphate buffer, pH 7.0. The excluded peak was submitted to analytical polyacrylamide gel electrophoresis (8% acrylamide, 0.4% bis-acrylamide) with 0.02 M Tris–glycine buffer, pH 8.6, and one major band was found with two minor rapidly migrating bands.

After a 10-fold concentration of the excluded peak and dialysis against 1 l 0.05 M sodium phosphate buffer, pH 7.0, at 4°C, an 0.8 ml sample of HCG (4.8 mg/ml) was filtered on a 1.1 X 30 cm Biogel P60 (100–200 mesh) column as in [5].

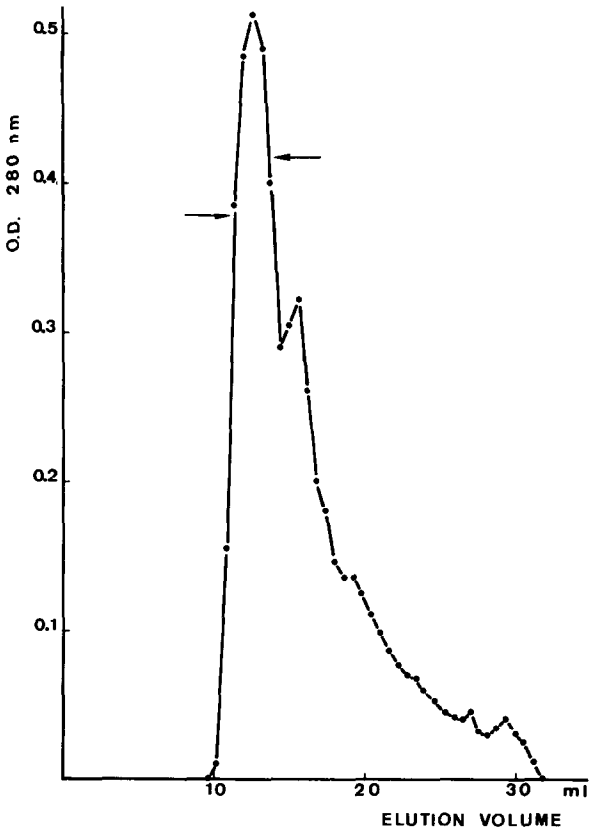

Fig.1. Gel filtration of partially purified HCG (3.8 mg in 0.8 ml) on a 1.1 X 30 cm column of Biogel P60 (100–200 mesh) with 0.05 M sodium phosphate buffer, pH 7.0; the fraction eluted between 11 ml and 14 ml contained electrophoretically pure HCG.

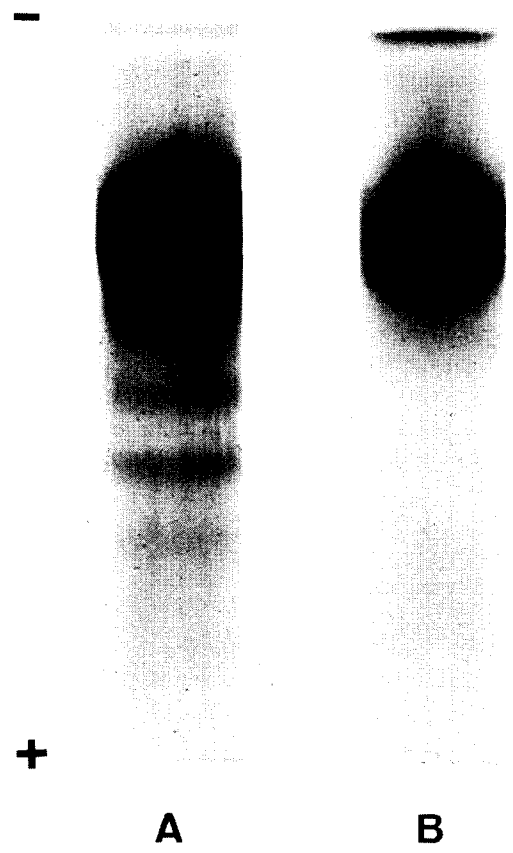

Fig.2. Polyacrylamide gel electrophoresis of HCG before (A) and after (B) filtration on Biogel P60.

Figure 1 shows the elution profile from this column; the protein fraction corresponding to the 11–14 ml elution volume, was submitted to gel electrophoresis as above (fig.2) and showed the removal of the 2 minor rapidly migrating bands by the gel filtration; the remaining band was comparable to that given by highly-purified HCG preparations [5–7].

2.2. *Raising and titer determination of antisera*

Anti HCG antisera were raised in 'Fauve de Bourgogne' rabbits after intradermal injection, at about 20 sites in the dorsal region, according to [8], of an emulsion containing 1 mg HCG in physiological saline and 0.6 ml complete Freund's adjuvant; after 4 booster injections, at 4–5 week intervals, the immunosera were collected and stored in small fractions at −15°C.

The titers of 3 antisera (AL_1, AL_2, AL_3) were determined by RIA in the presence of 2 ng HCG, 20 nCi radioactive tracer HCG (from the CEA HCG K_1 kit) and 0.05 M phosphate buffer, pH 7.5, with final vol. 0.4 ml. The dilutions of these antisera binding 50% of the antigen (2 ng) in this volume are shown in table 1.

3. Results and discussion

The unionised lysine Σ amino groups of the HCG were carbamylated with fluorescein isothiocyanate (FITC) by the modified method in [9]. The HCG was dialysed for 24 h against 2% sodium hydrogen carbonate, pH 8.5 at 4°C. To label protein in a 2 : 1 (FITC/HCG) ratio, 10 μl freshly prepared solution of FITC in acetone (19 mg/ml) was added slowly, to 1 ml HCG solution (7 mg/ml) at 4°C with gentle agitation. After 18–20 h at 4°C, the unreacted fluorochrome was removed by filtration of the sample on a 1.5 × 60 cm Biogel P2 column, using 0.1 M sodium phosphate buffer, pH 7.0; the excluded peak being collected.

The degree of labelling was estimated by measurement of protein concentration using the Lowry method [10]; the molecular weight of HCG being taken as 37 900 [11] and by absorbance measurement of FITC, taking into account an ~15% decrease in absorbance by protein-reacted FITC [12,13]. Under these conditions it was found that an average of 1.2 molecules of FITC had reacted with 1 molecule of HCG, and the complex retained 70% initial immunology activity (RIA determination).

Table 1
The titer (50% binding) of 3 rabbit antisera with 20 nCi HCG (2 ng)

Antisera	Final dilution
AL_1	1/12 000
AL_2	1/36 000
AL_3	1/14 000

Reaction vol. 0.4 ml

3.1. *Dilution curve for the antiserum using fluorescence polarization*

Samples of HCG–FITC, at final conc. 1 μg/ml were incubated for 3 h at 4°C with various dilutions of antiserum AL_2 in 0.1 M sodium phosphate buffer, pH 7.0, final vol. 2.0 ml. Figure 3 shows the increase in the amount of fluorescence polarization as a function of the concentration of the antiserum; 50% binding is found for the antiserum AL_2 at 0.25 mg/ml (final dilution 1/200). This result is in agreement with that observed in the determination titer by RIA (given that the HCG concentration in this experiment is 200 times greater than that used for the titer determination by RIA).

3.2. *Immunoassay*

Figure 4 shows the competition between increasing amounts of non fluorescent HCG (0.27–64 μg/ml) and a constant amount of HCG–FITC (1 μg/ml) with a constant amount of antiserum 0.65 mg/ml (final dilution 1/75) after incubation for 2 h at 4°C in 0.1 M sodium phosphate buffer, pH 7.0, final vol.

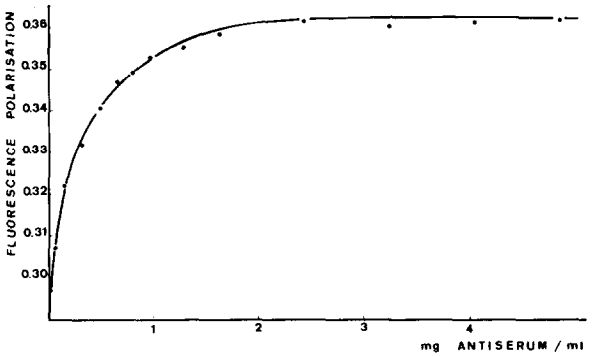

Fig.3. Dilution curve for the binding of HCG–FITC by antisera AL_2 showing the increase in fluorescence polarization as a function of the antiserum added. HCG–FITC (1 μg/ml and antiserum were equilibrated 2 h at 4°C in 0.1 M phosphate buffer, pH 7.0, with final vol. 2.0 ml before measurement of fluorescence polarization.

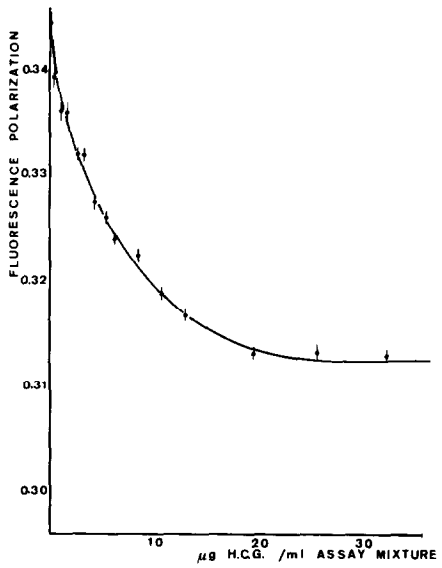

Fig.4. Displacement curve showing the decrease in fluorescence polarization of a solution of HCG–FITC (1 μg/ml assay mixture) and antiserum AL_2 (final dilution 1/75) caused by increasing amounts of standard HCG (0.27–64 μg/ml assay mixture). Tubes were equilibrated for 2 h at 4°C in phosphate buffer pH 7.0 with final vol. 2.0 ml, before measurement of fluorescence polarization.

2.0 ml. The competition was followed directly by reading the polarization of fluorescence. If we take the value of polarization with HCG to be 0.297 corresponding to 0% binding and the value of 0.345 corresponding to 100% binding, a maximum displacement of 70% is found under these conditions.

It can be seen that the displacement curve has a slope such that it can be used for the measurement of between 0.50 μg and 10 μg/ml HCG (3.3–66 IU/ml) and thus for the estimation of urinary HCG levels.

3.3. *Clinical applications*

Urine from pregnant women (6–10 weeks) and from women presenting a chorio-epithelioma or a molar pregnancy, were dialysed against bi-distilled water at 4°C and filtered through a 0.22 μm millipore filter. Quantities of 0.1, 0.2 and 0.6 ml were introduced into reaction media containing HCG–FITC and antibody such that final concentrations in 2 ml, were as described above.

The values obtained for fluorescence polarization were compared with a standard curve for 0.50–10 μg (3.3–66 IU) HCG.

Table 2 compares the levels of HCG (in IU)

Table 2
Urinary levels of HCG (IU/24 h) as measured by fluorescence polarization immunoassay and by haemagglutination inhibition assay for 7 clinical cases

	Urine sample (ml)	Fluorescence polarization immunoassay	Haemagglutination inhibition assay
Pregnancy (6–7 weeks)	0.1 0.25	31 000 15 000	20 000–100 000
Pregnancy (6–7 weeks)	0.5 1.0	18 000 10 000	10 000– 50 000
Pregnancy (10 weeks)	0.2 0.4	131 000 112 000	100 000–200 000
Pregnancy (11 weeks)	0.2 0.4	125 000 85 000	100 000–200 000
Molar pregnancy	0.5 1.0	53 000 45 000	20 000– 50 000
Epithelioma	0.1 0.2	327 500 201 000	> 200 000
Pregnancy?	1 1.5	640 760	< 1000

obtained by fluorescence polarization and those obtained by haemagglutination inhibition. It may be noted that the values obtained for small urine samples are higher than for larger volumes. In cases of commencement of pregnancy it is possible to rapidly concentrate urine samples to 1/2–1/3 volume by vacuum dialysis at 4°C.

4. Conclusion

The reference hormone used for the polarization of fluorescence assay was electrophoretically pure and stored in small fractions at −20°C, remains stable for at least 1 year.

The linkage of FITC to HCG diminishes the latter's immunological reactivity by 30%, but analytical electrophoresis of the protein before and after labelling showed it retained its native character. The excitation and emission maxima of the fluorochrome are not modified and linkage with the protein is remarkably stable [14]. The HCG–FITC has been found to be stable for at least 6 months at −20°C.

Analogous difficulties have been found in obtaining antisera with both a high titer and a high specificity for a glycoprotein such HCG, presenting similarities with HLH and FSH, to those in [15]. However, since the levels of HCG to be measured by this method were some 700-times higher than those of the latter hormones, the titer obtained appears sufficient for urinary HCG measurement and the specificity seemed to be of secondary importance [16].

Direct agreement was found between results obtained by polarization of fluorescence and by haemagglutination inhibition, and moreover the polarization of fluorescence method is reliable and rapid. After millipore filtration, an incubation period of 2 h at 4°C is followed immediately by direct reading of the percentages of free and bound HCG without recourse to a separation step. For highly coloured urines, found particularly when the urinary volume is small, a dialysis against distilled water before millipore filtration is recommended.

The sensitivity of the method is only 2 IU/ml, but its rapidity shows it to be useful not only for HCG determination during pregnancy, but also for urgently required determinations in certain pathological cases, such as the hydatiform mole or choriocarcinoma; such determinations being of increasing clinical interest [17,18].

Acknowledgements

This work was supported by grant from CNRS.

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