AN ASSAY PROCEDURE FOR PLASMA PROGESTERONE BASED ON ANTIBODY-ENHANCED CHEMILUMINESCENCE

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1. Introduction

Existing radioimmunoassay (RIA) procedures have the advantage of extreme sensitivity, but depend on the availability of a suitable radiolabeled ligand and of expensive equipment. Furthermore, such RIAs require a time-consuming phase-separation step [1]. Several approaches have been suggested to overcome the drawbacks of RIA while retaining the specificity of an immunoassay [2–4], but the procedures proposed thus far suffer from inadequate sensitivity. We have explored the possibility of using chemiluminescent markers, since chemiluminescent compounds are commonly detectable at pM levels [5–7].

In the method described here, an isoluminol derivative is attached covalently to a carboxy derivative of a steroid. The resulting steroid–chemiluminescent marker conjugate emits light upon oxidation with hematin compounds and H_2O_2. When the steroid–chemiluminescent marker conjugate is bound to a specific binding protein, the total light production of the conjugate is enhanced. This binding and the consequent enhancement of light emission is prevented by the addition of unaltered steroid in a competitive manner. A 'homogeneous' immunoassay, viz. one requiring no separation of bound and free hormone, can thus be designed. To illustrate this approach, the results of an immunoassay for plasma progesterone based on the monitoring of chemiluminescence are reported in the following sections.

2. Materials and methods

2.1. *Reagents*

Progesterone-11α-hemisuccinate (4-pregnene-3,20-dione-11α-hemisuccinate) was purchased from Steraloids, Wilton, NH; Microperoxidase (MP-II), carbodiimide, *N*-hydroxysuccinimide, bovine serum albumin (fraction V) (BSA) from Sigma, St. Louis, MO; luminol [5-amino-2,3-dihydrophthalazine-1,4-dione] from Aldrich Chemicals, Milwaukee, WI; DEAE-cellulose from Whatman, England; and 30% hydrogen peroxide solution from Merck, FRG.

Antiserum to progesterone was raised in rabbits, using a progesterone-11α-hemisuccinate–bovine serum albumin conjugate as the immunogen [8].

Anti-progesterone IgG fraction was prepared by ammonium sulfate precipitation, followed by DEAE-52 cellulose column chromatography [9] and dialysis against phosphate buffered saline (PBS). The titer and specificity of anti-progesterone IgG fraction were determined by radioimmunoassay procedures [8], using 0.05 M Tris (pH 8) containing 0.1 M NaCl and 0.1% NaN_3, as the assay buffer. The amount of specific antibody in 1% IgG solution, determined according to [10], was 0.8 mg/ml.

The assay buffer in the chemiluminescent reactions was 0.02 M phosphate (pH 8.6) containing 0.01% BSA.

Stock solutions of steroids and chemiluminescent compounds were prepared in ethanol and diluted to the desired concentration in assay buffer when required.

Microperoxidase was dissolved at 1 mg/ml in 0.01 M Tris–HCl (pH 7.4); this stock solution was kept at 4°C. The working solution was obtained by diluting the stock solution in assay buffer to 1 μM enzyme.

The oxidant solution was prepared by adding

100 μl 30% H_2O_2 solution to 5 ml borate buffer (pH 8.6, 0.06 M).

2.2. *Synthesis of 6-{N-ethyl-N (6-(4-pregnene-3,20-dione-11α-hemisuccinylamido)-hexyl)amino } 2,3-dihydrophthalazine-1,4-dione (progesterone–AHEI conjugate)*

The progesterone–AHEI conjugate was synthesized in two steps.

1. The hemisuccinate derivate of 11α-hydroxy progesterone (compound I, fig.1) was converted to an activated N-succinimide ester (compound II, fig.1).
2. The activated ester was reacted with an equivalent amount of 6[*N*-(6-aminohexyl)-*N*-ethyl] -amino-2,3-dihydrophthalazine-1,4-dione (AHEI) [7], in the presence of two equivalents of sodium bicarbonate.

The resulting progesterone–AHEI conjugate (compound III, fig.1) was separated by chromatography on silica Gel 60 and crystallized: m.p. 155–157°C; mass spectrum, *m/e* 714 (M^+); ultraviolet absorption peaks at 241 nm (ε 16 000), 294 nm (ε 16 200) and 320 nm (ε 7200).

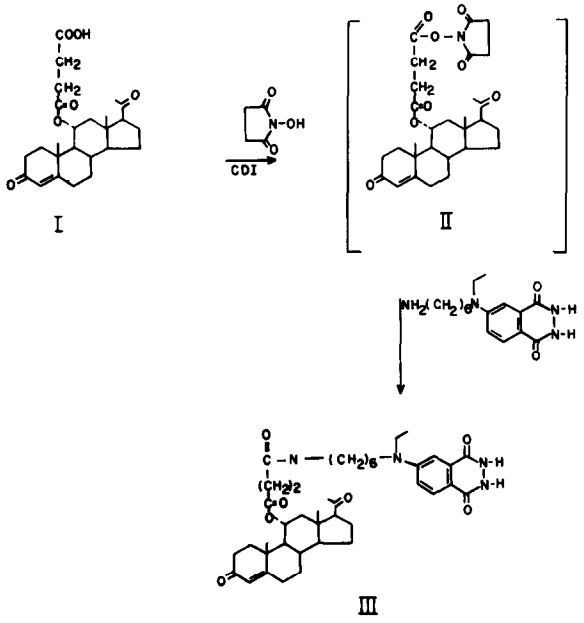

Fig.1. Reaction sequence for the synthesis of progesterone–aminohexylethyl isoluminol (AHEI) conjugate; CDI = carbodiimide.

2.3. *Light measurements*

Measurements of total light emission were made with a custom-built instrument. The reaction vessel (6 × 50 mm disposable test tubes, Kimble, Toledo, OH) was placed in a light-tight housing over the aperture of a photomultiplier (PM 270 C, International Light, Newburyport, MA). Each tube contained the chemiluminescent compound and the reagents specified in 40 μl. The light generating reaction was initiated by rapid injection of 100 μl oxidant with an Eppendorf pipette mounted on the cover of the light-tight compartment. The output of the photomultiplier was measured simultaneously with a research radiometer (International Light, Model 700 with power supply Model 760) reading in W/cm^2 and with a storage oscilloscope (Type 543, Tektronix, Beaverton, OR) connected to the radiometer, reading in mV. The area under the curve traced by the oscilloscope was integrated. All reactions were done in quadruplicate; the mean values and their standard errors are reported.

3. Results

3.1. *Determination of chemiluminescent markers*

To determine optimal conditions, the oxidation of luminol with H_2O_2 was studied in various buffers and with different heme catalysts (e.g., microperoxidase, horse radish peroxidase). The microperoxidase–H_2O_2 oxidation system at pH 8.6 afforded the most sensitive system for detection of luminol. The total light production measured within 0.1 s after addition of the oxidant increased linearly with luminol concentration and the lower limit of detection was ~0.1 fmol/tube.

When the isoluminol derivative 6[*N*-(6-aminohexyl)-*N*-ethyl] -amino-2,3-dihydrophthalazine-1,4-dione (AHEI) was oxidized with the microperoxidase–H_2O_2 system, the total light yield was 20% that of free luminol. The total light yield of AHEI relative to luminol was further reduced after covalent attachment to progesterone-11α-hemisuccinate, and the limit of detection of progesterone–AHEI conjugate (compound III, fig.1) was 0.14 pmol/tube.

3.2. *Effect of anti-progesterone IgG on the light yield during oxidation of progesterone–AHEI conjugate*

Varying levels of the γ-immunoglobulin fraction

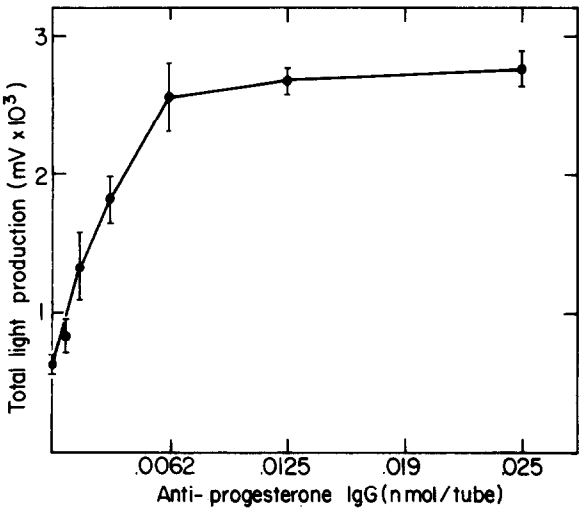

Fig.2. Effect of anti-progesterone IgG concentration on the total light yield from progesterone–AHEI conjugate. Varying levels of anti-progesterone IgG were incubated for 30 min at 4°C with 0.2 pmol progesterone–AHEI in a total volume of 30 μl assay buffer. Microperoxidase (10 μl of a 1 μM solution) was added, the reaction vessel was mounted in the light-tight compartment, and 100 μl of the oxidant solution was injected. Oscilloscope measurements of light emission was started within 0.1 s after addition of the oxidant and measured for 1 s.

(IgG) of an antiserum to progesterone-11α-hemisuccinate–bovine serum albumin were incubated for 30 min at 4°C with 0.2 pmol progesterone–AHEI. Figure 2 shows the total light produced by the conjugate upon oxidation as a function of the concentration of the antiserum. The total light yield increased with increasing anti-progesterone IgG concentrations up to 6.2 pmol IgG/tube (4-fold light emission), but remained constant at higher concentrations. Heterologous IgG, e.g., IgG from anti-cortisol sera, did not enhance light production (data not shown).

In additional experiments, varying levels of progesterone–AHEI conjugate were incubated with or without a fixed amount of anti-progesterone IgG (6.2 pmol/tube) for 30 min at 4°C (fig.3). In the absence of anti-progesterone IgG, the total light yield produced by oxidation of progesterone–AHEI increased linearly with the concentration of conjugate up to 1.4 ng/tube. When anti-progesterone IgG was added, the total light yield increased linearly with the concentration of progesterone–AHEI up to 0.5 ng/tube (fig.3). The antibody-induced enhancement over this

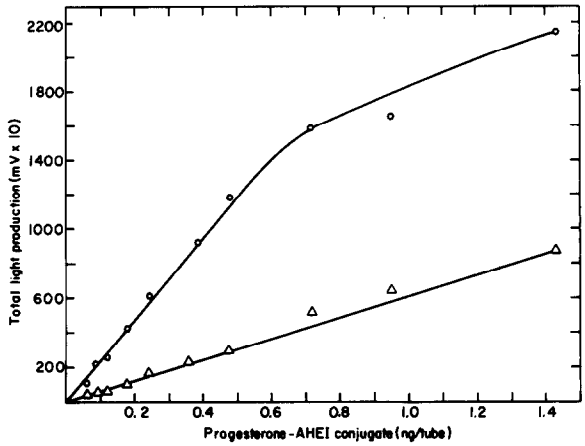

Fig.3. Effect of progesterone–AHEI conjugate concentration on IgG-enhanced light emission. Reaction mixtures containing various levels of progesterone–AHEI conjugate in 30 μl assay buffer were incubated at 4°C for 30 min with (○) or without (△) 6.2 pmol anti-progesterone IgG. Further treatment as described in legend to fig.2.

range was 4-fold. There was no further antibody-induced light increment, indicating saturation of the antibody.

3.3. *Validation of the progesterone immunoassay*

Competitive binding reactions were carried out by incubating varying levels of free progesterone with a constant amount of anti-progesterone IgG for 15 min at 37°C and 30 min at 4°C. The enzyme solution and the progesterone–AHEI conjugate were then added and after a brief incubation (30 s) at 4°C, the oxidant was injected. The light yield measured over 1 s decreased linearly with increasing progesterone concentration over 25–400 pg/assay tube. Figure 4 shows a composite standard curve obtained from four consecutive assays. The sensitivity of the assay was ~25 pg/assay tube, but is capable of further improvement (unpublished data).

The specificity of the immunoassay using anti-progesterone IgG and chemiluminescence was similar to that observed when using the same anti-serum in a radioimmunoassay. Light emission was unaffected by addition of cortisol or estradiol. Minor crossreaction was observed with 17α-hydroxyprogesterone (1.5%), testosterone (2%), 11α-hydroxyprogesterone (17%) and 11β-hydroxyprogesterone (23%).

A pool of human male plasma was serially diluted

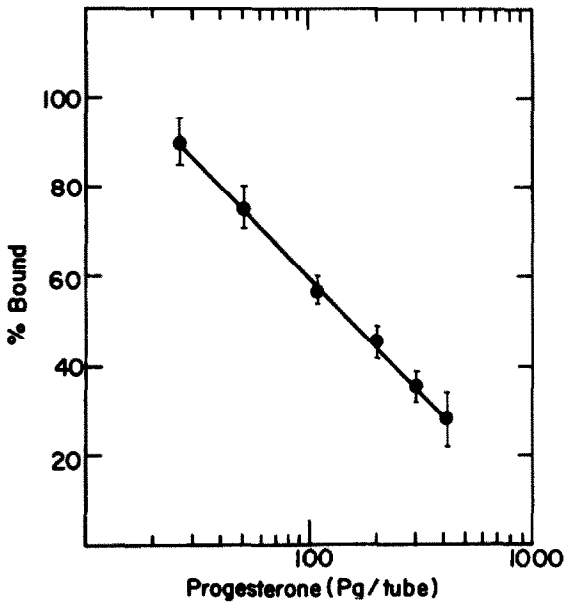

Fig.4. A standard curve for progesterone measured by chemiluminescence immunoassay. Varying levels of free progesterone were incubated with 6.2 pmol anti-progesterone IgG in a total volume of 20 μl assay buffer for 15 min at 37°C and 30 min at 4°C. The enzyme (10 μl of a 1 μM solution) and the progesterone–AHEI conjugate (140 pg in 10 μl assay buffer) were then added, and after brief incubation (30 s) at 4°C, the oxidant was injected. Shown are mean values ± SEM (n = 4).

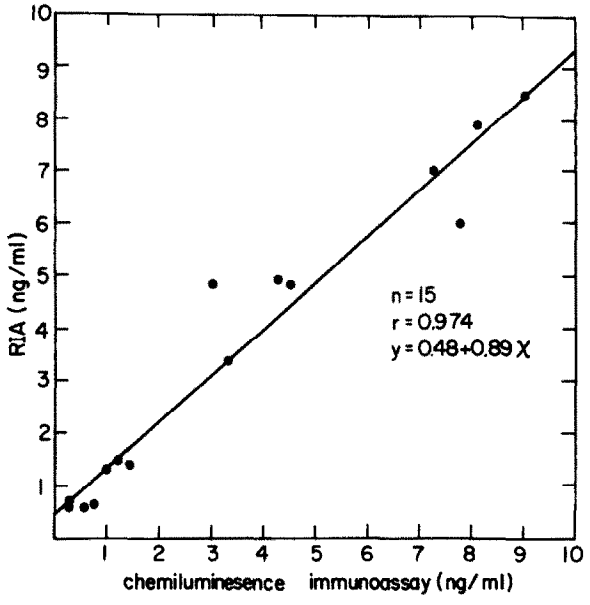

Fig.5. Comparison of plasma progesterone levels as determined by RIA or by chemiluminescence immunoassay.

with the assay buffer and extracted with petroleum ether. The extracts were then assayed for progesterone by chemiluminescence immunoassay. The regression of dilution factor on measured progesterone content was linear (y = 160.2 x + 7.3) and the correlation coefficient was r = 0.994.

Several plasma samples from non-pregnant women taken at different stages of the menstrual cycle (n = 15) were extracted with petroleum ether, and the extracts were assayed for progesterone by radioimmunoassay and by chemiluminescence immunoassay, using the same antiserum. The results of the two methods agreed well: r = 0.974; y = 0.48 + 0.89 x, where y corresponds to values determined by radioimmunoassay (fig.5).

4. Discussion

The progesterone assay described does not require physical separation of bound and free ligand, radioactivity counting or expensive equipment and can be accomplished in 50 min. The sensitivity achieved (25 pg/tube) is comparable to that obtained by radioimmunoassay, and is satisfactory for the measurement of progesterone in normal plasma. Furthermore, the steroid–chemiluminescent marker conjugate is stable at room temperature, can be characterized by physical methods and can be determined in < 1 s.

The chemiluminescence immunoassay developed for progesterone is simple: it consists of only four reagents, viz. the oxidant, the enzyme, the steroid–chemiluminescent marker conjugate and the specific binding protein, in addition to the sample to be determined.

Although at this stage recording of the light production is made manually, the assay is amenable to automation. The method eliminates the health hazards and isotope disposal problems of RIA. Chemiluminescence immunoassays are likely to be particularly useful in small clinical laboratories and in developing countries, where isotope counting equipment is not readily available, and in larger centers where rapid results are desired.

Acknowledgements

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