Double-Antibody Solid-Phase Radioimmunoassays for Gonadotropins in Serum*

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Synopsis

Double-antibody solid-phase (Dasp) method was applied to radioimmunoassays for follicle stimulating hormone (FSH) and luteinizing hormone (LH) in serum. The Dasp method uses second-antibody immunosorbent for the separation of free and antibody-bound antigen. Several parameters of the assays including concentrations of antiserum and second-antibody immunosorbent, reaction time and temperature were investigated. Reaction with second-antibody immunosorbent required only 2 hr at room temperature both in the FSH and LH assays. Nonspecific binding of labeled hormone to the immunosorbent was negligible. The FSH and LH assays were specific and sensitive enough to measure FSH and LH in serum. In these studies, the Dasp method proved to be useful for quantifying gonadotropins in large numbers of serum samples.

A good method of separating free from antibody-bound labeled antigen is essential to the development of radioimmunoassay. Recently, Den Hollander *et al.* (1972) developed a new separation method using second antiserum coupled to insoluble matrix. This Dasp method was applied to radioimmunoassays for human chorionic gonadotropin (HCG), urinary FSH and LH, and insulin. In these radioimmunoassays, the Dasp method was proved to be a rapid and versatile separation method. This paper describes the application of the Dasp method to radioimmunoassays for serum gonadotropins.

Materials and Methods

Hormones for iodination

Highly purified human pituitary FSH (LER 1366) having 3,014 IU FSH and 213 IU LH/mg and highly

purified human pituitary LH (LER 960) containing 923 IU LH and 1.9 IU FSH/mg were kindly supplied by the National Pituitary Agency (NPA), Endocrine Study Section and National Institute of Arthritis and Metabolic Diseases.

Reference preparations

Human pituitary standard for both FSH and LH (LER 907) having 20 IU FSH and 48 IU LH/mg by bioassay was supplied by NPA. Serum levels of FSH and LH were expressed in terms of ng LER 907/ml of serum. Urinary standard for both FSH and LH (2nd IRP-HMG) was supplied by the Medical Research Council, London. It has 40 IU of both FSH and LH per ampoule.

Antisera

Antiserum to human pituitary FSH (Batch #3), Anti-FSH, prepared in rabbits and pre-absorbed with HCG and antiserum to human pituitary LH, Anti-LH, prepared in rabbits were supplied by NPA.

Second-antibody immunosorbent

Gamma-globulin fraction of sheep antiserum to rabbit γ -globulin coupled to cellulose (Whatman CF 11), Anti-RGG immunosorbent, was kindly supplied by N. V. Organon, Oss, Holland.

HCG

Highly purified HCG containing 12,000 IU/mg

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was kindly donated by Dr. P. Donini, Istituto Farmacologico Serrone, Roma. Commercial HCG, Primogonyl (Schering), was also used.

Iodination

This was carried out by the chloramine-T method of Greenwood *et al.* (1963). Hormone (5 μ g) was radioiodinated with 125 I to specific activities of 100 to 150 μ Ci per μ g. Iodinated hormone was separated from inorganic 125 I by passing through a Sephadex G-75 column.

Diluent

The diluent for labeled hormone, antiserum, immunosorbent, standard and sample was composed of 0.01 M sodium phosphate, 0.15 M sodium chloride, 0.025 M EDTA and 0.5% BSA at pH 7.6.

Assay procedure

Labeled hormone (0.1 ml, 0.2–0.4 ng), antiserum (0.1 ml) and sample or standard (0.2 ml) were incubated (1st reaction). Then Anti-RGG immunosorbent (1 ml) was added and the reaction tubes were inverted continuously for a 2nd incubation time. After the completion of the 2nd reaction, the tubes were centrifuged, the supernatant was aspirated and the precipitate was counted for its radioactivity. The following parameters of the assays were investigated to obtain optimum conditions of the assays: concentrations of antiserum and Anti-RGG immunosorbent, reaction time and temperature.

Results

1. FSH radioimmunoassay

Concentrations of Anti-FSH and Anti-RGG immunosorbent

Anti-FSH was incubated with labeled FSH and 0.2 ml of diluent for 96 hr at 4°C. Second incubation was performed for 2 hr at room temperature. Effect of the concentrations of Anti-FSH and Anti-RGG immunosorbent on the recovery of the labeled FSH is shown in Figure 1. At a dilution of 1:5,000 of Anti-FSH and at dilutions of 1:5 through 1:20 of Anti-RGG immunosorbent, approximately 35% of the labeled FSH was recovered in the precipitate. The recovery of the labeled FSH was directly related to the concentration of Anti-FSH. At dilutions of 1:5 through 1:20 of Anti-RGG immunosorbent, the recovery of the labeled FSH was not significantly different,

Fig. 1. Effect of dilutions of Anti-FSH and Anti-RGG immunosorbent in the FSH assay.

but it decreased at a dilution of 1:40. When Anti-FSH was not added to the assay and Anti-RGG immunosorbent was used at a dilution of 1:20, the recovery of the labeled FSH was approximately 1%.

1st incubation time

When Anti-FSH and Anti-RGG immunosorbent were used at a dilution of 1:5,000 and 1:20, respectively, and the 2nd incubation was performed for 2 hr at room temperature, it required 96 hr for the 1st reaction to reach equilibrium both at room temperature and at 4°C (Fig. 2).

2nd incubation time

Anti-FSH and Anti-RGG immunosorbent were used at the same concentration as in the 1st incubation time study and the 1st incubation was performed for 96 hr at 4°C. The recovery of the labeled FSH reached maximum in 2 hr at room temperature and in 4 hr at 4°C (Fig. 3).

Fig. 2. Effect of 1st incubation time in the FSH assay. The solid line represents 1st incubation at 4°C and the interrupted line, 1st incubation at room temperature.

Based on the above observations, the final form of the FSH assay was determined as follows: concentrations of Anti-FSH and Anti-RGG immunosorbent 1: 5,000 and 1: 20, respectively, and 1st incubation, 96 hr at 4°C and 2nd incubation 2 hr at room temperature.

Characteristics of the FSH assay

In the above described condition, dose-response curves of LER 1366, LER 907, 2nd IRP-HMG and serum from a normal woman were parallel (Fig. 4). Purified HCG did not react in the assay, although commercial HCG did. LH cross-reacted in the FSH assay, but the degree of cross-reaction was less than 1% on a weight basis. Serum from a hypophysectomized patient did not react in the assay. Mean recovery of LER 907 added to serum from a normal man was 102%. Within- and between-assay coefficients of variation were 8 and 10%, respectively. The sensitivity of the assay was 10 ng/ml. In this FSH assay, 1 mg

Fig. 3. Effect of 2nd incubation time in the FSH assay. The solid line represents 2nd incubation at 4°C and the interrupted line, 2nd incubation at room temperature.

of LER 907 was equivalent to 78 IU of 2nd IRP-HMG.

2. LH radioimmunoassay

Concentrations of Anti-LH and Anti-RGG immunosorbent

Labeled LH and 0.2 ml of diluent were incubated with Anti-LH at various concentrations for 96 hr at 4°C and then 1 ml of Anti-RGG immunosorbent was added and the reaction tubes were incubated for further 2 hr at room temperature. At dilutions of 1: 10,000 of Anti-LH and 1: 20 of Anti-RGG immunosorbent, the recovery of the labeled LH was about 30% (Fig. 5). At dilutions of 1: 20,000 and 1: 40,000 of Anti-LH, it decreased markedly. The recovery of the labeled LH did not change significantly when Anti-

Fig. 4. Dose-response curves for LER 1366, LER 907, LER 960, 2nd IRP-HMG, HCG and serum in the FSH assay. Second IRP-HMG is plotted as mIU/ml, HCG as IU/ml and the other hormones are plotted as ng/ml.

Fig. 5. Effect of dilutions of Anti-LH and Anti-RGG immunosorbent in the LH assay.

Fig. 6. Effect of 1st incubation time in the LH assay. The solid line represents 1st incubation at 4°C and the interrupted line, 1st incubation at room temperature.

Fig. 7. Effect of 2nd incubation time in the LH assay. The solid line represents 2nd incubation at 4°C and the interrupted line, 2nd incubation at room temperature.

RGG immunosorbent was used at dilutions of 1:5 through 1:20, but decreased at a dilution of 1:40. Without Anti-LH added to the system, the recovery of the labeled LH was approximately 1% when Anti-RGG immunosorbent was used at a dilution of 1:20.

1st incubation time

Labeled LH, Anti-LH (1:10,000) and 0.2 ml of diluent were incubated for varying periods at 4°C and at room temperature. After the addition of Anti-RGG immunosorbent (1:20), the reaction was allowed to proceed for further 2 hr at room temperature. First reaction reached equilibrium in 96 hr at 4°C and at room temperature (Fig. 6).

2nd incubation time

After labeled LH, 0.2 ml of diluent and Anti-LH (1:10,000) were incubated for 96 hr at 4°C, Anti-RGG immunosorbent (1:20) was added and the tubes were incubated for varying periods at 4°C and at room temperature. The recovery of the labeled LH was

greatest after 2 hr at room temperature and 8 hr at 4°C (Fig. 7).

According to the preceding findings, the final form of the LH assay was set up as follows: concentrations of Anti-LH and Anti-RGG immunosorbent, 1:10,000 and 1:20, respectively, 1st reaction, 96 hr at 4°C and 2nd incubation, 2 hr at room temperature.

Characteristics of the LH assay

In the above conditions, the validity of the LH assay was investigated. LER 960, LER 907, 2nd IRP-HMG and serum from a normal woman gave dose-response curves similar in shape and slope (Fig. 8). FSH (LER 1366) cross-reacted and the degree of cross-reaction was 8% on a weight basis. LER 907 added to the serum from a normal adult man was recovered quantitatively (mean 104%). Serum from the hypophysectomized patient did not react in the assay. Within- and between-assay coefficients of variation were 7 and 14%, respectively. The sensitivity of the assay was 5 ng/ml. In this assay, 1 mg of LER 907 was equivalent to 250 IU of 2nd IRP-HMG.

Serum FSH and LH levels in women

Measurements on sera obtained from a normal cycling woman showed mid-cycle peaks of LH and FSH and in addition early follicular phase rise, preovulatory nadir, luteal phase depression and late luteal phase rise of FSH (Fig. 9). FSH and LH levels were also determined in sera obtained from an oligomenorrheic woman during two menstrual cycles, one control cycle and another cycle treated with clomiphene (Clomid). During the control cycle, FSH and LH levels were within normal range but acyclic. Administration of Clomid stimulated an elevation of both FSH and LH during treatment. After a dip, serum LH increased to a peak. In the period of early pregnancy, serum FSH slightly decreased whereas serum LH increased markedly, reflecting large amount of HCG (Fig. 10).

Discussion

Although double antibody method is appli-

Fig. 8. Dose-response curves for LER 960, LER 907, LER 1366, 2nd IRP-HMG and serum. Second IRP-HMG is plotted as mIU/ml and the other hormones as ng/ml.

Fig. 9. Serum FSH and LH levels during menstrual cycle.

Fig. 10. Serum levels of FSH and LH, their response to Clomid (lower) and basal body temperature (upper) in an oligomenorrheic patient. FSH (solid line) and LH (interrupted line).

cable to all radioimmunoassay systems, it requires considerable length of time for the immunoprecipitation reaction to reach equilibrium and conditions of the assay must be meticulously evaluated before establishing an assay system. The present Dasp radioimmunoassays for serum FSH and LH required only 2 hr for the 2nd reaction. Both in the FSH and LH assays, the recovery of the labeled hormone was similar when Anti-RGG immunosorbent was used at dilutions of 1:5 through 1:20. Thus, the range of suitable dilution of Anti-RGG immunosorbent is wide. Further, the Dasp method does not need carrier protein. The non-specific adsorption of the labeled antigen to the tube wall and to the immunosorbent was prevented by the addition of BSA to the diluent. In the absence of BSA, about 5% adsorption was found in the HCG radioimmunoassay (Den Hollander *et al.*, 1972). The present FSH and LH radioimmunoassays met most of the criteria required to validate the assay. Although FSH cross-reacted in the LH assay, this cross-reaction with FSH is considered to be due to the contamination of LH in the FSH preparation used. Serum FSH and LH levels in a normal cycling woman is similar to the other

reported studies including ours (Cargille *et al.*, 1969; Seki, 1972). Serum FSH depression in early pregnancy is in keeping with the result of Parlow *et al.* (1970) and provides further evidence for the specificity of the present FSH assay. In conclusion, the Dasp method was rapid, versatile and relatively easy to perform, and proved to be satisfactorily applicable to radioimmunoassays for serum FSH and LH, as previously shown in the radioimmunoassays for urinary FSH and LH (Den Hollander *et al.*, 1972).

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