A Specific ^{125}I Radioimmunoassay Method for Progesterone

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ABSTRACT

A specific and sensitive radioimmunoassay method for progesterone and its application to clinical practice are described. Antiserum of progesterone was prepared in rabbits by repeated subcutaneous injections of 11 α-hydroxyprogesterone-bovine serum albumin conjugate emulsified in complete Freund's adjuvant. The antiserum was so specific that the chromatographic purification could be omitted for sample preparation. A conjugate of 11 α-hydroxyprogesterone with tyrosine methyl ester was also prepared, and radioiodinated by the chloramine-T method. Immunoreactivity of the labeled progesterone was stable at 4°C for up to 40 days. Sensitivity of the assay was 0.05 ng/tube. The intra- and inter-assay coefficient variations were 7.0% and 14.4%, respectively. There was a linear correlation ($r=0.95$) between the amounts of progesterone added and the values determined by the radioimmunoassay method. The mean recovery rate was 86.4%. Serum progesterone levels in volunteers and infertile patients with histological diagnosis of normal luteal function are statistically higher in mid-luteal phase than any progesterone levels in patients with histological diagnosis of luteal insufficiency. This indicates that any single determination of serum progesterone levels in mid-luteal phase might be useful in evaluating corpus luteum function.

INTRODUCTION

Radioimmunoassay for progesterone, first described by Abraham *et al*[1], has been reported by many investigators[2,3]. However, most of these methods need chromatographic purification for sample preparation. Recently, several investigators[4-6] have demonstrated a highly specific antiserum which made it possible to omit the chromatographic step for sample preparation. On the other hand, ^{125}I-labeled tracer has been used in radioimmunoassay[7,8] with potential advantages over the ^{3}H-tracer;

i.e., the higher radioactivity of iodine and operative simplicity associated with the use of γ-counting techniques instead of β-liquid scintillation. In this paper, ^{125}I-labeled progesterone and a specific antiserum were prepared to use for a radioimmunoassay. The clinical application of this method is also described.

MATERIALS and METHODS

Preparation of antigens

11 α-Hydroxyprogesterone (Sigma Chemical, St. Louis, USA) was used without further purification. 11 α-Hydroxyprogesterone hemisuccinate was prepared according to the method described by Massagria *et al*[9]. Conjugation of 11 α-hydroxyprogesterone hemisuccinate to bovine serum albumin (BSA, fraction V, Katayama Chemical, Osaka) was carried out according to the method of Abraham *et al*[1]. Preparation of the conjugate of 11 α-hydroxyprogesterone with tyrosine methyl ester (TME, Nakarai Chemical, Kyoto) was achieved by the method of Oliver *et al*[10].

Preparation of antiserum

One mg of 11 α-hydroxyprogesterone-BSA conjugate in 0.5 ml of 0.15 M NaCl was mixed with an equal volume of complete Freund's adjuvant (Difco Lab., Detroit, USA) and injected subcutaneously into a rabbit. The injections were repeated at 3-week intervals and bleedings were performed 10-14 days after each injection. Antiserum was stored in small aliqouts at -20°C.

Radioimmunoassay method

Radioiodination: Progesterone-TME conjugate was iodinated by the method of Greenwood and Hunter[11]. The ^{125}I-progesterone-TME conjugate was separated from free iodine by passing through a 1×20 cm column of Sephadex G 25 (medium), which was equilibrated with 0.01 M sodium phosphate buffer, pH 7.4 containing 0.9% NaCl. The labeled progesterone was diluted by 0.05 M sodium phosphate buffer, pH 7.4 containing 0.1% BSA and kept at -20°C.

Radioimmunoassay procedure: Serum samples (0.05-0.1 ml) were adjusted to a final volume of 0.1 ml by the addition of distilled water. One ml of hexane was added to a 0.1 ml serum sample. The mixture was shaken on a mixer and centrifuged for 15 min at $1,500 \times g$. An aliqout (0.8 ml) of organic phase was placed into an assay tube and evaporated under an N_2 gas stream at 37°C.

To each tube containing standard progesterone or serum extract, 0.3 ml of 12.5 mM EDTA-buffer, pH 7.4 containing 0.1% BSA, and 0.1

ml of antiserum (1:1,750) were added. The solution was shaken thoroughly and left at room temperature for 30 min. Then, 0.1 ml of ^{125}I-labeled progesterone was added and incubation was continued overnight at 4°C. The antibody-bound progesterone was precipitated by addition of 0.1 ml of bovine γ-globulin (15 mg/ml, fraction II, Sigma) and 0.7 ml of a 25% solution of polyethylene glycol (PEG 6,000, Katayama)[12]. The tubes were centrifuged at 1,500×g for 30 min at 4°C. The supernatant was aspirated and the radioactivity of the precipitate was determined in a well-type scintillation counter (ALOKA TDC-501).

All standards and samples were assayed in duplicate.

Clinical subjects

Four volunteers and 30 infertile patients were selected for this study. All subjects have shown biphasic basal body temperature for at least 2 months. Serial serum samples were obtained in the preovulatory and luteal phases of the cycle. Serum progesterone levels were determined by a radioimmunoassay. In 4 volunteers, serum luteinizing hormone (LH) and follicle stimmulating hormone (FSH) levels were also determined using a radioimmunoassay kit (Daiichi Radioisotope Inc., Tokyo). An endometrial biopsy was performed in all infertile patients. According to the criteria of Jones *et al*[13], the cases were divided into two groups: (group A) histological patterns showed good mature endometrium which was adapted to the day of menstrual cycle, (group B) histological patterns were 2 days or more behind the expected date. Each group consisted of 15 patients. Values have been related to the first day of menstruation (M) following the studied cycle.

RESULTS

Labeled progesterone

Separation of the ^{125}I-labeled progesterone from free iodine by a Sephadex G 25 column is illustrated in Fig. 1. An iodination mixture showed 2 radioactive peaks. Most of the immunoreactive labeled progesterone was eluted at the second peak, which was pooled and used for radioimmunoassay. Immunoreactivity of labeled progesterone was stable at 4°C for up to 40 days.

Effect of incubation time

The binding percent of ^{125}I-labeled progesterone with excess amounts of antibody reached plateau within 1 h either at 4°C or at room temperature (Fig. 2).

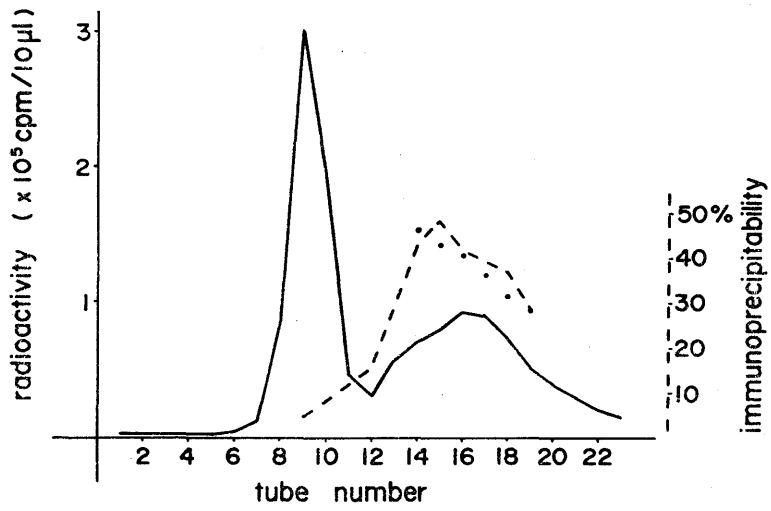

Fig. 1 Separation of labeled progesterone and free iodine on a 1×20 cm column of Sephadex G 25 (medium). Solid line represents the radioactivity of each fraction. Broken line and dots represent the immunoprecipitability of ^{125}I-progesterone on the day of separation (broken line) and after 40 days storage at 4°C (dots), respectively.

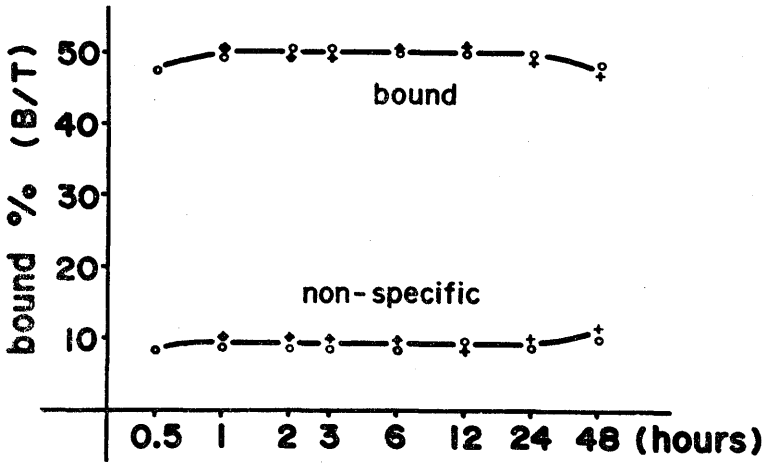

Fig. 2 Effect of incubation time on the binding percent of ^{125}I-labeled progesterone with excess amounts of antibody at room temperature (○), and at 4°C (+).

Specificity

The specificity of the progesterone antiserum was investigated by cross-reaction studies with various steroids (Table 1). 5 α-Pregnanedione, 11 β-hydroxyprogesterone and 11 α-hydroxyprogesterone showed cross-

Table 1 Cross reaction of various steroids with the antiserum to 11 α-hydroxyprogesterone BSA

Steroids	%Cross reaction
progesterone	100
5 α-3, 20 pregnanedione	37.5
pregnenolone	1.6
pregnanediol	<0.1
17 α-hydroxyprogesterone	1.6
11 α-hydroxyprogesterone	15.5
11 β-hydroxyprogesterone	21.4
deoxycorticosterone	2.0
corticosterone	0.9
deoxycortisone	<0.1
cortisone	<0.1
cortisol	<0.1
testosterone	<0.1
androsterone	<0.1
androstendione	<0.1
estrone	<0.1
17 β-estradiol	<0.1
estriol	<0.1

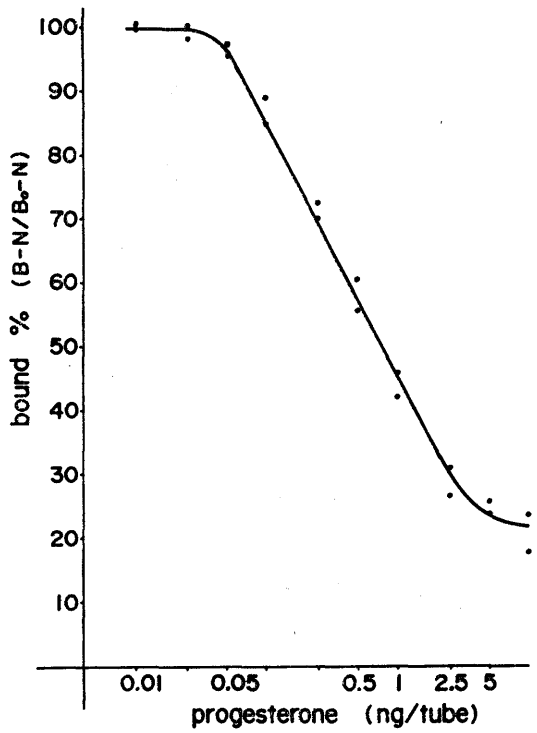

Fig. 3 A typical standard curve for progesterone

Table 2 Accuracy of progesterone radioimmunoassay

progesterone added to male serum (pg/ml)	progestrone determined			recovery rate (%)	number of assays
	mean (pg/ml)	standard deviation (pg/ml)	coefficient of variation (%)		
0	180	24	13.3		6
50	216	12	5.7	71.6	6
100	272	15	5.6	91.7	6
250	404	22	5.3	89.7	6
500	618	45	7.3	87.7	6
1000	1092	124	11.4	91.2	6

regression equation

$Y = 0.92X + 175$

X : progesterone added (pg/ml)

Y : progesterone determined (pg/ml)

corelation coefficient

$r = 0.95$

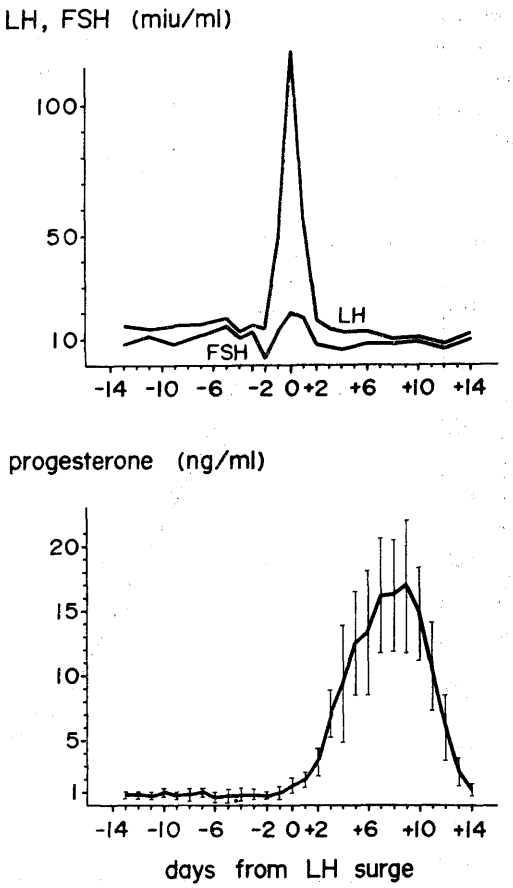

Fig. 4 Serum LH, FSH and progesterone levels of 4 regularly menstruating volunteers. The day of LH surge is designated as day O. Serum progesterone levels started to rise on the day O and reached plateau on the day +7. The vertical bar represents one standard deviation.

reactivities of 37.5%, 21.4% and 15.5%, respectively. Other steroids tested showed little or no cross-reaction.

Sensitivity

Fig. 3 shows a typical standard curve displaying a linear relationship between 0.05 and 2.5 ng/tube. The smallest amount of progesterone that can be assayed was 0.05 ng/tube.

Accuracy and precision

Known amounts of progesterone ranging from 0.05 to 1 ng were added to male serum. There was a linear correlation between the amount of progesterone added and that determined by the radioimmunoassay

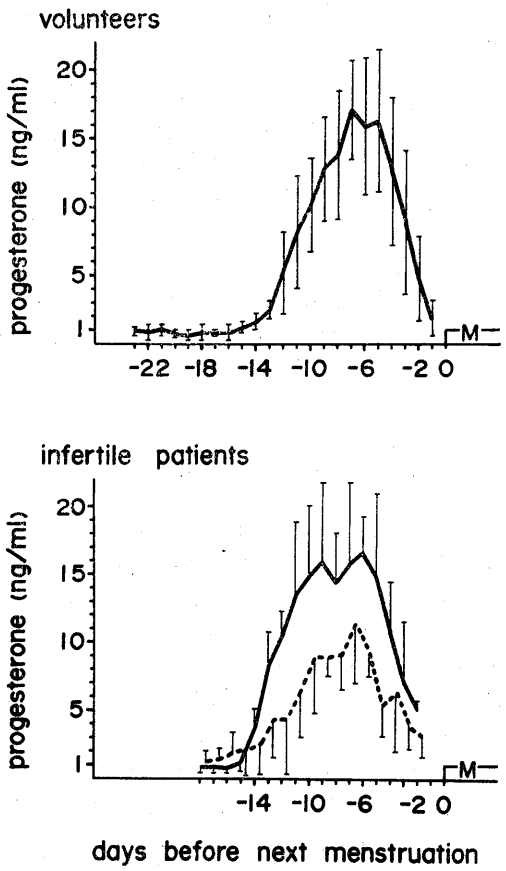

Fig. 5 Mean (±SD) serum progesterone levels of volunteers, infertile patients with histological diagnosis of normal luteal function (group A,——) and infertile patients with histological diagnosis of luteal insufficiency (group B,……).

method (Table 2). The mean recovery rate was 86.4%. One patient's serum, run in 7 different assays, gave a coefficient of variation of 14.4 %, i.e. 9.0±1.3 ng/ml (mean±SD). Five duplicate determinations, run in the same assay, resulted in a coefficient of variation of 7.0%, i.e. 8.6±0.6 ng/ml (mean±SD).

Clinical studies

Serum progesterone, LH and FSH levels in 4 volunteers throughout a menstrual cycle are presented in Fig. 4. Serum progesterone levels remained low during follicular phase. A significant ($p<0.05$) rise of progesterone was observed on the day of LH surge. During the next 6 days progesterone levels increased and reached plateau 7 days after ovulation with a mean level of 16.4±4.2 ng/ml (mean±SD). Progesterone levels gradually decreased from 10 days after ovulation and fell to 1.2 ng/ml 4 days thereafter.

Infertile patients were divided into 2 groups consisting of 15 patients each. Fig. 5 represents the serum progesterone levels of each group. Serum progesterone patterns in group A patients were similar to those of volunteers. In group B patients, serum progesterone levels started to rise on day M -14 and reached a maximum level of 10.4±4.4 ng/ml on day M -7 followed by rapid decline to day M -1. On day M -13, it was first observed that serum progesterone levels in group B patients were significantly lower ($p<0.05$) than those in group A patients. Significant differences in progesterone levels were observed on each menstrual day from M -13 to M -4.

DISCUSSION

A number of purification techniques[7,9] for the ^{125}I-labeled substances have been reported. In the present study, column chromatography was selected because of its ease of sample preparation[8]. The elution profile showed two radioactive peaks, which agreed with the results of Niswender[7]. Massaglia and co-workers reported[9] that the iodination mixture contained 3 radioactive components which corresponded to unreacted iodine (10-20%), monoiodinated progesterone (75-85%) and diiodinated progesterone (5-10%), and that the diiodinated form underwent a rapid degradation within 10 days. They emphasized that the labeling always had to be followed by a purification of the monoiodinated progesterone by preparative thin layer chromatography, and that monoiodinated progesterone showed an excellent stability when stored in ethanol solution at 4°C. In the present study, immunoreactivity of the second peak was found to be stable for more than 40 days without further purification.

As for the specificity of anti-progesterone serum, 5 α-pregnanedione, 11 β-hydroxyprogesterone and 11 α-hydroxyprogesterone showed cross-reactivities on the radioimmunoassay system. This result had been expected since BSA was attached at the 11 position of progesterone[7]. Serum pregnanedione levels are approximately one-seventh of that of progesterone through the luteal phase[14], and in physiological condition, 11 α- and 11 β-hydroxyprogesterone can not be detected in the blood[5,6]. Therefore, the cross-reactivity of these three steroids might be negligible. The other steroids showed little or no cross-reaction. These results indicate that the antiserum is specific enough to omit a chromatographic step for sample preparation. In fact, the blank with water was always determined to be zero, when progesterone was prepared by hexane extraction without chromatographic step. Furthermore, a linear correlation was obtained between the quantity of progesterone determined by the present method and the volumn of sera ranging 0.005 to 0.1 ml, which was examined in sera from 3 ovulatory women and from 3 pregnant women.

In this study, all volunteers had a mid-cycle gonadotropin peak, and the length of time from the mid-cycle LH surge to the onset of the next menstruation was at least 13 days in duration. These results would provide reasonable evidence of a normal menstrual cycle[15-17]. Serum progesterone levels of volunteers concur with the findings of many investigators[18-22] who utilized various assay methods.

Many investigators[23-25] reported that endometrial histological patterns serve as a bioassay for corpus luteum function. In this study, progesterone levels of group B were significantly lower than those in group A on each menstrual day from M -13 to M -4. Serum progesterone determination would be an appropriate diagnostic method to evaluate corpus luteum function in clinical practice. In addition, serum progesterone levels in volunteers or group A patients between M -10 and M -5 are statistically higher than any progesterone levels in group B patients. Johansson[18] and Radwanska *et al*[26] also reported that serum progesterone levels of 10 ng/ml in mid-luteal phase (from day M -10 to M -5) represented the demarcation between adequate and inadequate luteal function. If the time of sampling would be carefully taken into consideration, the single serum progesterone determination would be a practical method for evaluation of corpus luteum function.

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