Direct Radioimmunoassay of Progesterone in Rat Serum

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Abstract

A direct method has been described which makes possible a specific assay of progesterone in rat serum without extraction.

Anti-progesterone serum was prepared in our laboratory by the immunization of three rabbits with 4-pregnen-3, 20-dione-3 CMO:BSA. This antiserum (Gunma OGP#1) displayed little or no cross reaction with 20α -dihydroprogesterone (0.38%), pregnenolone (0.44%), 17α hydroxypregnenolone ($<0.1\%$), 20β hydroxyprogesterone (2.4%), 17α hydroxyprogesterone (2.88%) or deoxycorticosterone (2.19%). The nonspecific inhibitory effect of serum was compensated for by adding progesterone-free serum to the standard curve tubes.

The sensitivity of this assay was 1.1 pg/tube and serum progesterone could be measured by using as little as 1 μ l of serum.

The working range of the standard curve was 1.25-2560 ng/ml.

Under the conditions of this assay (1 μ l of serum per tube), interference from steroid binding proteins did not affect the sensitivity, precision or reliability of the assay.

The intra-assay and inter-assay coefficients of variation were 5.5% and 8.7%, respectively, and the assay values correlated well with those obtained by the extraction method ($R=0.997$, $P<0.001$).

Analytical recovery indicates a close correlation between added and recovered progesterone concentrations ($R=0.992$, $P<0.001$), and the recovery rate averaged 96%.

Compared with the extraction method, the direct progesterone assay has the advantage of speed, precision and simplicity.

The method described is particularly suitable for routine assays of progesterone in rat serum.

Previously progesterone has been assayed only by bioassay, which was time- and money-consuming and had poor sensitivity and low accuracy.

Although development of the radioimmunoassay (RIA) of progesterone opened

a new era of reproductive endocrinology, initial RIA (van der Molen *et al.*, 1965; Johansson, 1969; Lurie *et al.*, 1970) had required complex extraction and purification steps, followed possibly by thin-layer or column chromatography. The availability of highly specific antisera has eliminated the purification step from many steroid RIAs

(Furuyama *et al.*, 1971; Dighe and Hunter, 1974; Riley *et al.*, 1972).

However, there remains the problem of endogenous steroid hormone binding proteins.

Measurement of serum progesterone requires an extraction step with organic solvents to separate the progesterone from serum.

A direct method omitting extraction would save labor, time and the cost of assays.

Such a method has been proposed for several studies in animal and human serum (Haynes *et al.*, 1980; Stupnicki, 1975; Stupnicki and Kula, 1982; Schanbacher, 1979; Mertens *et al.*, 1983; Jurjens *et al.*, 1975), but was found unsatisfactory for rat serum.

Progesterone concentrations in rat serum were higher (5 ng—50 ng/ml) than in any other animals and men.

We were therefore to use a 200-fold dilution of rat serum (1 μ l of serum per tube) in this direct assay.

Under these assay conditions, interference from steroid hormone binding proteins did not affect the sensitivity, precision or reliability.

In this report, we have described a direct RIA of progesterone in rat serum which was simple, precise, time-saving and was free from the interference of steroid hormone binding proteins.

Materials and Methods

Hormone Preparations

Progesterone-3-(0-carboxymethyl) oximino-(2-[¹²⁵I]) iodohistamine (IM 139, specific radioactivity 2000 Ci/mmol) and [1, 2, 6, 7-³H] progesterone (TRK 413 Batch 35, specific radioactivity 96 Ci/mmol) were obtained from Amersham International Plc., Buckinghamshire., England.

4-pregnen-3,20-dione 3-CMO: BSA (Batch 2677; 29 moles steroid: mole BSA., 25 mgmQ 2606) was purchased from Steraloids, INC.

WILTON, N. H.

Freund's complete adjuvant H Rv (Lot No SDR 9705) was obtained from Wako Pure Chemical Industries, Ltd.

Progesterone, pregnenolone, 20 α dihydroprogesterone, 20 β hydroxyprogesterone, 17 α hydroxyprogesterone, 17 α hydroxypregnenolone, deoxycorticosterone, cortisol, testosterone, dihydrotestosterone, androstenedione, dehydroepiandrosterone, estradiol, estrone and cholesterol, were purchased from Sigma Chemical Co., St. Louis, U. S. A.

Preparations of immunogen and antisera

4-pregnen-3,20-dione was used to immunize three male Japanese white rabbits which were purchased from the Imai Experimental Animal Farm, Saitama.

Immunization was performed according to the method described by Vaitukaitis *et al.* (1971).

Three mg of the antigen was dissolved in 3 ml of 0.15 M NaCl and emulsified with 3.3 ml complete Freund's adjuvant.

First, 1 mg/2 ml antigen was administered i. c. at about 30 sites in each rabbit. The injections were repeated weekly for 6 weeks at a dose of 0.5 mg/2 ml.

Thereafter, booster injections were given at 2 week intervals for 6 weeks and at 4 week intervals 3 times. The increases in titer were monitored by small blood samples taken at 6–11 days after each booster.

At the 7th month after the initial immunization, and 6 days after the last injection, the animals were bled through the carotid artery under light anesthesia. Collected sera were stored frozen at -80°C .

Then one 0.5 ml portion was dissolved in 99.5 ml of 0.05 M EDTA-50 mM PBS (pH 7.5) and frozen at -80°C in 0.4 ml aliquots (200-fold dilution).

Out of 3 animals immunized, 1 died after the 5th immunization and 2 responded well and produced antisera of sufficiently high titer and specificity to be used in RIA.

One of the antisera (Gunma OGP#1) was used throughout the study.

Progesterone-free serum

This was prepared by the method of Zarrow, Yochim and McCarthy (1964 a, b).

Adult male rats of the Wistar strain bred in our laboratory were used for these surgical

operations.

Adrenalectomy and orchidectomy were performed under ether anaesthesia and 0.9% saline was substituted for drinking water post-operatively in the case of these animals.

After one week of treatment, the rats were decapitated and examined for RIA of plasma progesterone.

These sera were suitable for progesterone-free serum when the concentration of progesterone was below 60–70 pg/ml. This can be checked by a reference method (by a RIA involving extraction).

Assay procedure

a) Antiserum

One 0.4 ml portion of 200-fold diluted antiserum was diluted with 199.6 ml of 0.5% NRS-EDTA-PBS (pH 7.5).

The dilution required to bind 40 to 50% of ca 4000–5000 cpm of ^{125}I -progesterone was found to be 1 : 100,000.

b) Standard curve

From the stock solution of progesterone in methanol (10 $\mu\text{g}/\text{ml}$), 100 μl was added to 78.125 ml of 1% BSA-PBS.

This contained 12.8 ng progesterone per ml and served as the working standard solution.

A series of consecutive two-fold dilutions was prepared so as to obtain the following concentrations: 2560, 1280, 640, 320, 160, 80, 40, 20, 10, 5, 2.5, 1.25 pg/0.2 ml.

c) Assay method

In the direct RIA method, 200 μl of assay buffer or standard solution (1.25 pg–2560 pg/tube), and 200 μl of serum samples or progesterone-free serum which was diluted 200-fold with assay buffer, were added to triplicate assay tubes and incubated with 200 μl of diluted antiserum in 0.5% NRS 0.05 M EDTA-PBS (1 : 100,000) for 24 hrs 4°C.

100 μl of ^{125}I -labelled progesterone tracer (ca 4000 cpm, 0.8 pg/tube) was then added to all tubes and incubated at 4°C for 24 hrs.

200 μl of second antibody, diluted further in 0.05 M EDTA-PBS, was finally added to all tubes except those for total count and incubated at 4°C for 48 hrs.

At the end of the third incubation, the tubes were centrifuged at 3000 rpm for 30 minutes (4°C) to separate the free from the bound tracer.

The supernatant was removed by decantation and the precipitates were counted in an Aloka

601 Automatic γ counter.

d) Sample extraction

0.5–0.8 ml aliquots of rat serum samples were pipetted into 18×110 mm glass test tubes and extracted by vortex mixing for 30 sec. with 5–6 vol of ether.

After clear separation of the two phases, the lower phase was quick-frozen by immersing in a cold methanol bath (–70°C).

The organic layer was decanted into 15×105 mm glass tubes and evaporated with a centrifuging evaporator (Yamato Scientific Co., Ltd. Tokyo).

The dried residues of the extracted progesterone were dissolved in 0.5–0.8 ml of assay buffer and then diluted 200-fold with assay buffer and transferred to assay tubes for RIA.

For calculation of the recovery after extraction, 0.5–0.8 ml aliquots of control serum samples which contained 0.1 ml of ^3H -progesterone (ca 1000 dpm, 1.5 pg/tube) were extracted with 5–6 vol of ether.

The dried residues were dissolved in 0.5–0.8 ml of assay buffer and added to counting vials containing 10 ml of scintillation fluid.

The radioactivity was counted for 5 min.

Rat estrous cycle

A total of 46 mature female Wistar rats, weighing 250–300 g, were housed under controlled temperature and lighting (lights on from 0500–1900 h) and supplied with water and food *ad libitum*.

Vaginal smears were taken daily, and only animals showing at least 2 regular 4-day cycles were used.

Rats were decapitated at 1800 hr of each day of the estrous cycle and also at 0700 hr, 1000 hr, 1600 hr and 2000 hr during proestrus, 1000 hr and 1400 hr during estrus.

Groups of four to eleven rats were used for each progesterone assay.

They were decapitated and blood was collected into 18×100 mm tubes, and the serum was separated by centrifugation and stored at –20°C until assayed.

Results

Specificity of antiserum

The specificity of the antiserum is documented in Table 1, which indicates the

Table 1. Specificity on rabbit antiserum (Gunma OGP #1) to progesterone 3-CMO-BSA conjugate.

Steroid	Percent cross reaction
Progesterone	100
20 α -Dihydroprogesterone	0.38
20 β -Hydroxyprogesterone	2.4
17 α -Hydroxyprogesterone	2.88
Pregnenolone	0.44
17 α -Hydroxypregnenolone	<0.1
Cortisol	<0.01
Deoxycorticosterone	2.19
Androstenedione	<0.1
Dehydroepiandrosterone	<0.1
Testosterone	<0.1
Dihydrotestosterone	<0.01
17 β -Estradiol	<0.01
Estrone	<0.1
Cholesterol	<0.01

percentage of cross-reactivity of other steroids with the antiserum, estimated from the values at 50% displacement of tracer binding.

The other steroids tested showed little or no detectable cross reaction.

Interference in hormone-antiserum binding by serum.

Fig. 1 and Table 2 demonstrate the inhibitory effect of serum on the binding of progesterone to antiserum.

It follows from the data that the greater the serum increase (from 1 μ l to 8 μ l of serum), the lower the zero-dose binding and assay sensitivity.

The slopes of the displacement curves generated by serial dilutions of progesterone-free serum were consistently parallel to that of the standard.

Sensitivity

Fig. 2 shows typical standard curves of the direct method and of the extraction method.

The means for the different progesterone concentrations obtained in 5 assays are on a straight line in both methods (log-logit

Fig. 1. Inhibition curves for a direct assay of progesterone in various amounts of rat serum. The direct method was carried out in the following system: 200 μ l of 1% BSA-PBS (A) or 200 μ l of progesterone-free serum diluted 200-fold (B: 1 μ l of serum/tube), 100-fold (C: 2 μ l of serum/tube), 50-fold (D: 4 μ l of serum/tube) and 25-fold (E: 8 μ l of serum/tube) with 1% BSA-PBS, 200 μ l of standard dissolved in 1% BSA-PBS (1.25 pg–2560 pg/tube), 200 μ l of diluted antiserum in 0.5% NRS 0.05 M EDTA-PBS and 100 μ l of 125 I-labelled progesterone.

Table 2. Parameters for the direct RIA of progesterone in various amounts of rat serum.

	Amount of serum per tube				
	A 0 μ l	B 1 μ l	C 2 μ l	D 4 μ l	E 8 μ l
Zero-dose binding (Bo/T)					
Mean (%)	48.9	46.8	44.7	43.0	39.6
S. D.	1.1	0.6	0.9	0.7	0.3
Sensitivity (pg/tube)					
Mean (pg/tube)	0.7	0.9	1.9	2.3	3.0
95% limit	0.5-0.9	0.8-1.0	1.6-2.2	1.9-2.7	2.6-3.5
Midrange concentration (X-intercept, "ED-50")					
Mean (pg/tube)	33.5	45.4	75.5	107.2	163.6
95% limit	29.3-38.5	41.9-49.3	69.1-82.6	95.9-119.7	142.2-181.9
Slope (decimal logit-log)	-1.743	-1.714	-1.824	-1.754	-1.691
S. E.	0.043	0.026	0.030	0.035	0.031

paper).

Some assay conditions and mean assay parameters for both methods are summarized in Table 3.

It follows from the data presented that 1 μ l of serum (serum standard) did not affect the sensitivity, as shown in Fig. 2.

Under these assay conditions, the standard curve for the direct method exhibited adequate sensitivity (a reasonably steep slope for the standard curve) throughout the range 1.25-2560 pg/tube.

As summarized in Table 3, the assay sensitivity, based on the 95% confidence limit of the mean of 5 replicate tests for the zero standard, was 1.1 pg/tube (1.1 ng/ml serum) and 0.8 pg/tube (0.8 ng/ml serum) for the direct and extraction method, respectively.

Fig. 2. Mean standard curves for the direct method and for the extraction method. The direct method was carried out in the following system: 200 μ l of assay buffer (1% BSA-PBS) or standard solution (1.25-2560 pg/tube), 200 μ l of serum samples or progesterone free serum which was diluted (1 μ l of serum/tube), 200 μ l of diluted antiserum (1:100,000) and 100 μ l of 125 I-labelled progesterone.

Table 3. Assay parameters for the direct and the extraction method. Note. The assay sensitivity is based on the 95% confidence limit of the mean of 5 replicate tests for the zero standard.

	Direct method	Extraction method
Amount of serum (μ l/tube)	1	0
Zero-dose binding (Bo/T)		
Mean (%)	54.6	57.7
S. D.	2.4	2.2
Sensitivity		
Mean (pg/tube)	1.1	0.8
95% limit	0.9–1.4	0.6–1.0
Midrange concentration (X-intercept, "ED-50")		
Mean (pg/tube)	51.6	35.4
95% limit	45.7–58.3	31.6–39.6
Range of applications (pg/tube)	1.25–2560	1.25–2560
Slope (decimal logit-log)	–1.756	–1.769
S. E.	0.043	0.039

The coefficient of variation at each point of the standard curve varied from 5 to 10% and 2 to 11% for the direct and extraction method, respectively.

Precision

The intra- and inter-assay variance was evaluated by triplicate measurements of the same assay and in five different assays.

Specimens were analyzed in triplicate by the direct RIA method, and single extracts of specimens were analyzed in triplicate by the extraction RIA.

In triplicate determinations of 12 samples in the same assay, which values ranged from 17.1 to 1177.5 ng/ml, the coefficient of variation was 5.5% for both methods (Table 4).

Interassay variance for both methods is shown in Table 5.

In five different assays, the coefficient of variation was 8.7% and 6.7% for the direct and extraction method, respectively.

Fig. 3. The recovery of various amounts of progesterone added to rat serum. These data were collected on five separate days with the extraction method on a single extract and on five separate days with the direct method.

Accuracy

To assess analytical recovery, we measured progesterone in 8 samples by both the direct and the extraction method.

Fig. 3 shows the linear regression line ($Y = AX + B$) for the correlation of added versus recovered progesterone quantities.

As summarized in Fig. 3, the recovery for the direct method averaged 96%, while the mean recovery for the extraction method was 103%.

Comparison of the direct method with the extraction method for progesterone in rat serum

We measured progesterone in 12 specimens from rats ranging from 10 ng/ml to

1280 ng/ml serum.

As shown in Fig. 4, results obtained by both methods agreed well with each other ($R=0.997$, $P<0.001$).

Peripheral serum levels of progesterone at various times during the estrous cycle.

Serum progesterone concentrations, which were assayed by direct and extraction RIA

Table 4. Intra-assay precision of progesterone determination. The intra-assay variance was evaluated by three determinations of the same samples in the same assay.

	Progesterone concentration (ng/ml)							
	Direct method				Extraction method			
	Mean	SD	CV (%)	No. of tubes	Mean	SD	CV (%)	No. of tubes
1	22.1	0.7	2.9	3	17.1	0.6	3.6	3
2	52.6	3.8	7.3	3	47.8	5.2	10.9	3
3	85.8	5.4	6.3	3	69.6	3.8	5.5	3
4	151.4	12.0	7.9	3	120.4	9.7	8.1	3
5	253.3	5.7	2.2	3	233.9	10.9	4.7	3
6	360.3	19.3	5.4	3	332.2	11.6	3.5	3
7	458.2	23.6	5.2	3	455.8	25.6	5.6	3
8	523.7	35.0	6.7	3	511.5	6.0	1.2	3
9	639.3	68.3	10.7	3	595.2	11.6	1.9	3
10	725.8	31.7	4.4	3	769.6	61.0	7.9	3
11	851.4	43.6	5.1	3	850.6	60.9	7.2	3
12	1098.0	24.4	2.2	3	1177.5	65.8	5.6	3

Table 5. Inter-assay precision of progesterone determination. The inter-assay coefficient of variation was evaluated by triplicate measurements in five different assays using the same samples.

	Progesterone concentration (ng/ml)							
	Direct method				Extraction method			
	Mean	SD	CV (%)	N	Mean	SD	CV (%)	N
1	23.4	1.5	0.6	5	17.8	1.5	8.2	5
2	53.9	2.7	4.9	5	43.9	3.8	8.6	5
3	82.5	6.2	7.5	5	67.9	3.5	5.2	5
4	143.6	6.3	4.4	5	129.6	7.5	5.8	5
5	245.4	8.1	3.3	5	240.2	9.6	4.0	5
6	337.8	24.4	7.2	5	343.7	22.3	6.5	5
7	429.1	53.2	12.4	5	453.1	25.6	5.7	5
8	507.6	19.8	3.9	5	531.1	30.3	5.7	5
9	582.6	81.2	13.9	5	632.4	46.9	7.4	5
10	762.7	107.8	14.1	5	779.8	57.4	7.4	5
11	829.4	65.3	7.9	5	861.4	64.3	7.5	5
12	1157.0	280.1	24.2	5	1315.1	108.3	8.2	5

Fig. 4. Comparison of the direct method and the extraction method for progesterone in serum from rat.

Fig. 5. Mean concentrations of serum progesterone during the 4-day estrous cycle of the rat, which was assayed by direct and extraction methods. Bars indicate standard deviation of mean, and numbers on top indicate number of rats from which blood samples were collected.

Fig. 6. Correlation between progesterone concentration in rat serum samples as estimated by the direct method and extraction method.

methods, throughout the estrous cycle in the rats are illustrated in Fig. 5.

The preovulatory rise in progesterone was observed at 1000 hr of proestrus, with a peak at 1800 hr (44.0 ± 15.9 ng/ml, and 44.7 ± 15.8 ng/ml in the extraction and direct method, respectively), and then returned to the base level at noon of estrus.

There was a rise in the progesterone concentration late on diestrus-I to 37.5 ± 3.6 ng/ml (extraction method) and 43.8 ± 6.0 ng/ml (direct method) at 1800 hr.

A further rise was found late in diestrus-II to 48.8 ± 11.7 ng/ml (extraction method) and 54.6 ± 24.8 ng/ml (direct method) at 1800 hr.

A comparison of the direct method and the extraction method for progesterone in serum from rats throughout the estrous cycle is shown in Fig. 6 ($R = 0.945$, $P < 0.001$).

Discussion

Ideally, it is desirable to perform progesterone RIA directly on serum; however, interference with serum constituents such as steroid binding proteins provide major obstacles.

These nonhormonal components in serum have been shown to exert nonspecific effects in a direct progesterone RIA.

In the direct RIA in this paper, steroid-free serum was previously added to the standard curve tubes in order to circumvent these nonspecific effects.

These methods had been reported by other workers (Haynes *et al.*, 1980; Stupnicki and Kula, 1982; Stupnicki, 1975; Ogihara *et al.*, 1977).

In the direct RIA method mentioned in our report, the components in serum inhibited ^{125}I -progesterone to antiserum in a

concentration-dependent manner.

The slope of the displacement curves generated by serial dilutions of progesterone-free serum were consistently parallel to that of the standard, indicating that the components causing the displacement of ^{125}I -labelled progesterone from antibody was immunologically identical to progesterone.

Since the progesterone concentrations in the estrous cycle of the rat vary from 5 ng to 50 ng/ml (Butcher *et al.*, 1974; Smith *et al.*, 1975; Nequin *et al.*, 1975; Kalra and Kalra, 1974), which is higher than for any other animals, e.g., ovine 0.2 ng–4.0 ng/ml (Pant *et al.*, 1977), bovine 0 ng–8.5 ng/ml (Stupnicki 1975), guinea-pig, 0.5 ng–3.2 ng/ml (Blatchley *et al.*, 1976), and also human, 0.4 ng–13.0 ng/ml (Moghissi *et al.*, 1972), we are able to use a 200-fold dilution of serum (only 1 μl of serum per tube) from rat.

Under these assay conditions, the standard curve for the direct method exhibits adequate sensitivity throughout the range 1.25 ng–2560 ng/ml serum.

The specificity of antiserum to steroids is dependent on the site through which the steroid molecule is conjugated to protein (Niswender, 1973).

Our results have confirmed the specificity to be expected of antiserum raised to progesterone 3-CMO: BSA conjugates.

The specificity of our antiserum with respect to pregnenolone (% cross reaction: 0.44%) was much better than reported by Scarisbrick (1975; % cross reaction 37%) and Niswender (1973; % cross reaction 13.3%).

For 20 β -hydroxyprogesterone antiserum (Niswender, 1973) at 0.3%, 17 α -hydroxyprogesterone antiserum (Scarisbrick, 1975) at 1.2% and deoxycorticosterone antiserum (Niswender, 1973) at 1.6% were somewhat better than ours with 2.40%, 2.88% and 2.19%, respectively.

Though the cross-reaction of 17 α -hydroxyprogesterone is relatively high (2.88%), the concentration of this steroid varies

between only 2 ng and 3 ng/ml (Shaikh and Shaikh, 1975). 20 α -dihydroprogesterone (20 α -DHP) is present in much higher concentrations than progesterone in rat serum (e.g., Nequin *et al.*, 1975, peak progesterone concentrations in peripheral plasma were 40 ng–60 ng/ml, whereas peak 20 α -DHP levels were about 230 ng/ml.), but its cross reactivity was only slight (0.38%).

Since our antibodies have high specificity for progesterone, other circulating steroids should not interfere with the direct assay technique.

The present direct assay meets all the requirements of specificity, reliability, sensitivity and practicability.

When compared with methods involving extraction, the direct assay gave very similar results ($R=0.997$, $P<0.001$; Fig. 4) and proved especially suitable for routine work.

The concentrations of serum progesterone obtained by direct RIA throughout the estrous cycle in the rats were in agreement with other reports (Smith *et al.*, 1975; Nequin *et al.*, 1975; Kalra and Kalra, 1974; Butcher *et al.*, 1974), except on the day of diestrus-II.

It is reported that the progesterone level rose dramatically on the afternoon and evening of proestrus, and slightly on diestrus-I.

Although the progesterone concentration on diestrus-II was reported in the range of 3 ng–15 ng/ml serum (Butcher *et al.*, 1974, Smith *et al.*, 1975), our data displayed high levels of progesterone on diestrus-II to 48.8 ± 11.7 ng/ml (extraction method) and 54.6 ± 24.8 ng/ml (direct method) at 1800 hr on diestrus-II.

The reasons for these discrepancies are unclear, but are probably related to the fact that the adrenal gland contributes to the pool of circulating progesterone, especially under conditions of stress (Barraclough *et al.*, 1971; Feder *et al.*, 1971; Feder *et al.*, 1968).

Because it does not require expensive and

dangerous extraction solvents, the assay described herein provides a convenient and economical procedure for assessing the physiological condition of the rat.

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