

A direct ³H-radioligand assay for serum progesterone compared with an assay involving extraction of serum

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Abstract

The direct assay of serum progesterone after denaturation of the binding proteins was investigated. 50ul of patients' serum was diluted with 750ul phosphate buffer (0.05M, pH 7.4) and heated to 65°C for 20 minutes. After cooling, 300ul of the treated serum was reacted with a rabbit antiserum to progesterone-11 α -hemisuccinyl-bovine serum albumin conjugate (Bioclin, U.K) and 1,2,6,7, tritium labelled progesterone. Separation of bound and free fractions was achieved with dextran coated charcoal. The method correlated well ($r = 0.98$) with an established method involving ether extraction of progesterone prior to assay. The mean sensitivity was 2.01nmol/L (range 1.90 - 2.23nmol/L). The proposed method considerably shortens assay time and removes a tedious and imprecise stage in the conventional method involving extraction of serum.

Key words: Heat treatment, serum progesterone, direct assay.

INTRODUCTION

Increasing clinical use of progesterone assays requires methods that are simple, rapid and robust and capable of higher sample throughput. Conventional methods involving extraction of progesterone from serum with organic solvents are technically complex, often imprecise and unsuited for heavy workload.^{1,2} Recently, methods have been described for the direct assay of progesterone in unextracted serum which employ danazol,^{3,4,5} cortisol,⁶ or 8-anilino-1-naphthalene sulphonic acid (ANS) and low pH⁵ to displace progesterone from binding proteins in serum. These allow progesterone to be assayed directly by radioimmunoassay.

In this study, heat treatment was used to release progesterone from serum binding proteins prior to direct radioimmunoassay of the hormone. The method is compared with the conventional extraction method.

MATERIALS AND METHODS

Steroid assay buffer²

The buffer used was 0.05M sodium phosphate containing 0.1g/L thiomersal and 1.0g/L gelatin. The pH was adjusted to 7.2 - 7.4 and diluted to 1L with distilled water.

Tritiated progesterone

1,2,6,7, tritium labelled progesterone (250 μ Ci/

250 μ l toluene) was purchased from Amersham International plc, diluted to 25 μ Ci/ml with 9:1 toluene:ethanol and stored in a dark airtight container at -20°C.

Progesterone antiserum

Rabbit antiserum against progesterone 11 α -hemisuccinyl-bovine serum albumin conjugate, Product No.1008 was purchased from BioClin Services, Cardiff, U.K, diluted to 1:100 in assay buffer and stored in aliquots of 125 μ l at -20°C.

Progesterone standard

Progesterone (4pregnene 3,20 dione anhydrous) was purchased from Sigma Chemical Co., St. Louis, USA. A stock solution containing 2.5 nmol/ml was prepared in 99.5% ethanol and stored at 4°C.

Charcoal suspension²

0.0625g dextran (T-70) was dissolved in 100ml assay buffer in a beaker. 0.625g charcoal was then added and stirred vigorously with a magnetic stirrer for 30 min. The charcoal reagent was stored at 4°C. This should be stable for up to one month.

Scintillation cocktail

The cocktail was prepared by dissolving 5g of 2,5-diphenyloxazole (PPO) in 1 litre of toluene.

The solution was mixed gently for 30min and transferred to a dark bottle with an airtight seal. This was stored at room temperature in the dark.

Optimizing release of progesterone from serum binding proteins

Fifty microliters of serum from each of 4 patients' samples, which were selected randomly, were equilibrated with 100 μ l ³H-progesterone (total count (TC) \approx 10,000 cpm) for 60 min at room temperature. To the mixture, 650 μ l steroid buffer was added, mixed vigorously for 30sec and heated at 65°C in a waterbath for 0, 3, 7, 10, 20, 30 and 40min. The samples were then cooled for 15min at room temperature. 200 μ l of charcoal suspension was added to the samples, mixed thoroughly for 30sec and incubated for 30min at 4°C. Separation of bound from free ³H-progesterone was achieved by centrifugation for 6min at 4°C and 800xg.

The supernatant containing the bound fraction was added to 5ml of scintillation cocktail in a 20ml glass vial and mixed on the Abbott clinical rotator at 180rpm for 1h at room temperature. After standing for a further 1h in the dark, the radioactivity was measured in a R-counter. The percentage of residual bound progesterone (Y) after heat treatment was calculated from the ratio of counts at different time points to counts at 0min. Therefore, the % of released progesterone (R) is given by $R = (100 - Y)\%$.

Sample, working tracer and antiserum preparation

Fifty microliters of each serum was added to 750 μ l steroid buffer, vortex mixed and heated for 20min at 65°C. After cooling, the samples were mixed and 300 μ l each of the denatured sera were pipetted into 12 x 75mm polystyrene assay tubes.

Working tracer was prepared by evaporating 130 μ l of ³H-progesterone stock solution to dryness with a jet of filtered air and redissolved in about 15ml assay buffer to give an activity of about 10,000cpm per 100 μ l. Working antiserum was prepared by diluting a 125 μ l aliquot of stock solution to 12.5ml with assay buffer.

Assay procedure

One hundred microliters of stock progesterone standard was diluted with 10ml assay buffer to a concentration of 2500fmol/100 μ l (top standard). The top standard was serially diluted with assay buffer to give concentrations of 2500,

1250, 625, 313, 156, 78 and 39fmol/100 μ l. 100 μ l of each standard was added into duplicate assay tubes and 100 μ l of assay buffer to 3 tubes for zero standard and 4 tubes for evaluating nonspecific binding. 300 μ l of denatured serum was added in duplicate to appropriately labelled assay tubes. Then, 100 μ l of working antiserum and working tracer were added to all tubes.

The contents of all tubes were diluted to 700 μ l with assay buffer, mixed thoroughly and incubated at room temperature for 2h. After cooling at 4°C for 10 min, 200 μ l of charcoal suspension was added to all tubes, mixed again and incubated for 30 min at 4°C. Bound and free W-progesterone was separated by centrifugation as described before.

The count rate of standards was plotted as a percentage of the zero standard count rate after subtracting non-specific counts against concentration of standards using Logit-log paper. Results were interpolated from the graph and corrected by the factor $1/18.8 \times 100/R$ to yield values in nmol/L.

Estimation of analytical recovery

Three series of samples for recovery experiments were prepared by adding three different amounts of progesterone to a male pool serum whose progesterone content was determined in the same assay. Recoveries were estimated by assaying the samples in replicates of six.

Method comparison

Progesterone as measured in direct assay of several serum samples was compared with results of an assay involving extraction in which the same ³H-progesterone, antiserum and charcoal suspension were used.

RESULTS

Fig. 1 shows the displacement of progesterone from serum binding proteins. The maximum binding of labelled progesterone varied from 41.2% to 62.1% with a mean of 53.1%. At 65°C, progesterone was released rapidly from 27.9% at 3min to 80.9% at 7min and more gradually thereafter. The optimum time of displacement was chosen as 20min and the average of displacement at this time was 84.070.

Table 1 shows the recovery of progesterone added to serum prior to direct assay. The mean progesterone level in the normal male serum pool used for spiking was 5.4nmol/L. Recovery of added hormone ranged from 85% to 97% and

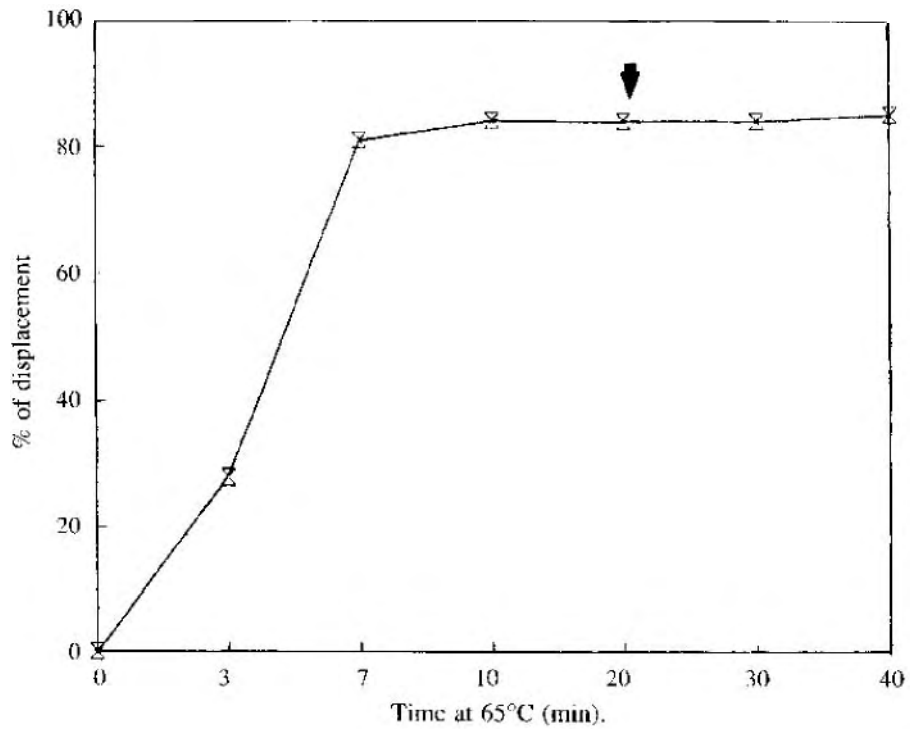


FIG. 1: Displacement of progesterone from serum binding proteins. The arrow indicates selected time for heat treatment.

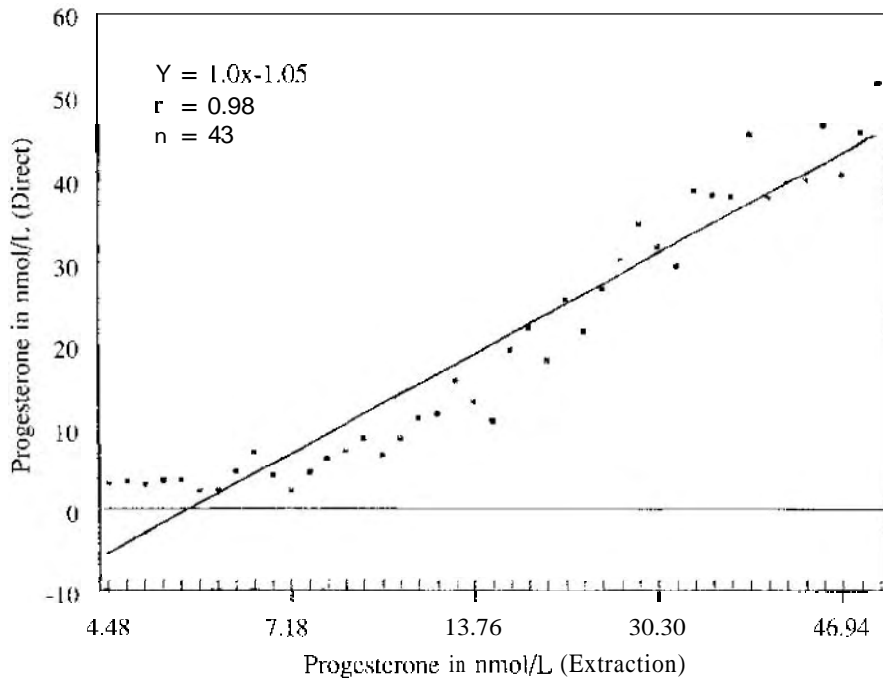


FIG. 2: Correlation of results for progesterone between the direct and conventional method.

TABLE 1: Recovery of added progesterone (Mean \pm S.D., n = 6)

Dose added (nmol/L)	Dose recovered (nmol/L)	% Recovery
10.2	9.4 \pm 0.6	91.3 \pm 5.5
41.0	39.7 \pm 1.7	96.8 \pm 4.1
82.1	70.0 \pm 5.0	85.3 \pm 6.2

it was better for low and **medium** dose than for high dose.

Fig. 2 shows the comparison of **results** for progesterone on 43 samples by the direct method and an extraction method using least squares analysis. There was good correlation between the heat treatment and the extraction method. The correlation coefficient was 0.98 and the correlation equation was $Y = x - 1.05$.

The sensitivity of the heat treatment method was determined by measuring the dose of triplicate samples of zero standard at 2SD of the mean count rate. Based on 3 assays, the sensitivity of the method was found to range from 1.90 to 2.23 nmol/l with a mean of 2.01 nmol/l.

DISCUSSION

The percentage displacement of progesterone from binding proteins by heat treatment at 65°C for 20 min ranged from 78-87% (mean 84%) for a total of 12 different serum specimens in 3 assays. As in extraction procedures, it is necessary for users of this method to demonstrate consistency in heat displacement of bound progesterone. If the differences are small, i.e. less than 10%, then the average displacement determined for 10-20% of samples in every assay batch may be used to correct for total serum progesterone in the calculation. Failing this, it would be necessary to determine percentage displacement of the bound hormone for each specimen.

It would appear that this displacement is largely of that fraction of the hormone that is bound to serum proteins other than cortisol binding globulin (CBG). Dunn *et al.*⁷ have reported that about 17.7% of serum progesterone

is bound to CBG which has the highest affinity for progesterone, while 79.3% is bound to albumin which has the lowest affinity for progesterone amongst the serum proteins.

The heat treatment technique gave good analytical recovery of progesterone from serum. The mean recovery was 91%, 97% and 85% for low, medium and high doses respectively. This compares favourably with recoveries of 92%-100% reported for other direct methods.^{4,5,6} The proposed method has the advantage that it does not require the addition of special chemicals for displacement of progesterone. Further, it considerably shortens assay time and removes a tedious and imprecise stage in the conventional method involving extraction of serum. As correlation with the reference method is good ($r = 0.98$) the direct method is an attractive alternative for laboratories.

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