A Simple Method for Plasma Progesterone Measurement

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SUMMARY

A simple, rapid, non-chromatographic method for the assay of progesterone in human peripheral plasma is presented. A single petroleum ether extract of plasma is partitioned twice with 28% aqueous ethanol, and progesterone is then measured by a competitive protein-binding assay using plasma from women receiving high oestrogen oral contraception. Reliability criteria of the method have been evaluated, and have shown the assay to be satisfactory for the measurement of plasma progesterone in the luteal phase of the menstrual cycle.

Many techniques have been described for the measurement of plasma progesterone in human peripheral blood. These include techniques involving double isotope derivatives,[1,2] enzymic transformation of progesterone to 20β-hydroxy-pregn-4-ene-3-one, which exhibits sensitive fluorescent characteristics,[3] and gas liquid chromatography, using electron capture detection of chloro-acetate esters.[4] The introduction of competitive protein-binding (CPB) methods by Murphy[5] was soon followed by the quantitation of progesterone by this method. Initially, preliminary purification by at least one thin-layer or paper chromatographic step was necessary.[6-10] However, simpler non-chromatographic competitive protein-binding procedures have been developed by Johansson,[11] Lurie and Patterson,[12] Swain[13] and Horth and Palmer.[14] More recently, progesterone has been measured by radio-immunoassay.[15,16]

This article describes a simple rapid human plasma progesterone assay in which the number of steps have been reduced to a minimum, and which we feel has distinct advantages over other non-chromatographic CPB methods so far described, while at the same time retaining similar precision, sensitivity and specificity. The assay has been found suitable for estimation of plasma progesterone in the luteal phase of the menstrual cycle. During a single working day, 22 patient samples can be measured in duplicate by one technician.

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MATERIALS

1. Water for blanks and diluting ethanol used in extraction was distilled and de-ionised.

2. Petroleum ether (30° - 60°C AR, Mallinkrodt Chemical Co., St Louis, or 40° - 60° AR, M & B Ltd, Dagenham, England) was not further purified.

3. Ethanol AR (Hopkins and Williams (SA) (Pty) Ltd) was not further purified.

4. Tris buffer solution (pH 8,0) was made by dissolving 2,42 g (0,01M) of 2-amino-2-(hydroxymethyl)-propane 1:3-diol in 100 ml of freshly distilled and de-ionised water, adding 80 ml of 0,1N HCl, 170 g (0,25M) of sucrose and 0,584 g (0,001M) EDTA. This mixture was made up to 2 litres and the pH adjusted to exactly 8,0.

5. 7α-^{3}H (n) progesterone (SA 5 Ci/mM) and 1β, 2β-^{3}H (n) corticosterone (SA 30 Ci/mM) from Radiochemical Centre, Amersham, UK.

6. Dextran-coated charcoal suspension was prepared by dissolving 0,005 g Dextran 40 (Sigma) in 200 ml tris buffer, and adding 0,5 g Norit A Charcoal (Pfanstiehl Laboratories). This suspension is stable for several weeks when stored at 4°C.

7. For liquid scintillation the Beckman model 233 was used for all radioactive measurements. Efficiency for tritium was 35%. Insta-gel (Packard Instrument (Pty) Ltd) was used for counting aqueous samples.

METHODS

Cleaning of Glassware

All glassware was soaked in 2% v/v Decon 75 (Atomic Export Import Corp. (Pty) Ltd), rinsed well with tap water and distilled water and then dried before use.

Preparation of Binding Protein (CBG-B³H)

Plasma was obtained from women receiving oral contraceptives, who were given 0,5 mg dexamethasone 12 hours before blood was drawn. The plasma was pooled and small aliquots were dispensed and kept frozen at −20°C. To achieve optimal binding (50 - 60%) in the absence of progesterone, 0,1 ml ^{3}H-corticosterone (250 μCi/25 ml) was added to a glass beaker and blown to dryness under a stream of nitrogen. To this was added 0,1 ml of the above plasma and diluted with 50 ml tris buffer. The solution was mixed and incubated at 45°C for 5 minutes before use.

Plasma Extraction

One thousand cpm of 7α-³H progesterone was added in 50 μL ethanol to a series of 10-ml glass test tubes, fitted with ground glass stoppers. One millilitre or less of plasma was then added, depending on the expected levels of progesterone, and extracted with 6 ml of petroleum by shaking mechanically for 5 minutes. The tubes were then centrifuged for 5 minutes at about 1 000 g and the petroleum ether poured off into 19-ml ground-glass stoppered test tubes after freezing the plasma layer in a mixture of dry ice/acetone. After this, 5 ml of 28% aqueous ethanol was added to the petroleum ether extract, the mixture was shaken mechanically for 5 minutes and then centrifuged. The lower layer of aqueous ethanol was removed by a Pasteur pipette and discarded. After a second aqueous ethanol wash, 5 ml of the petroleum ether was removed with a graduated pipette: 2 × 2 ml aliquots were placed in 12 × 75 mm glass test tubes and blown to dryness under a stream of nitrogen. A 1 ml aliquot was counted to estimate recovery.

Protein Binding Radioassay

Standards containing 0; 0,2; 0,4; 1,0; 2,0; 3,0; 4,0 and 5,0 ng progesterone in ethanol were pipetted into 12 × 75 mm glass test tubes, 2 ml petroleum ether added and then blown to dryness together with the unknowns. To all tubes 0,5 ml of CBG - B³H solution, containing approximately 10 000 cpm, was added. The tubes were quickly shaken on a vortex mixer, incubated in a water bath at 45°C for 5 minutes, then mixed again and transferred to an ice bath for 10 minutes. Dextran-coated charcoal suspension (0,5 ml) was added, the tubes shaken and allowed to stand in the ice bath for a further 10 minutes. The tubes were centrifuged in a refrigerated centrifuge at 4°C for 10 minutes, then 0,5 ml of the supernatant removed and dissolved in 10 ml Insta-gel scintillator and counted for 10 minutes or until an error of less than 2% was reached.

A standard curve was plotted from the results of the standards. The unknowns were calculated from the curve, corrected for recovery and expressed as ng/ml of plasma. In order to determine reproducibility of the standard curve, the cpm found when no progesterone was added was defined as 100%, and other points on the curve calculated accordingly (Fig. 1).

EVALUATION OF METHOD

Efficiency of Extraction

The mean recovery (± SD) of 7α-³H progesterone from individual plasma samples during the assay was found to be 75,0% ± 6,9 (N = 80). Mean recovery varied inversely with the volume of plasma extracted, being 71% for male and female follicular phase plasma (1 ml extracted), and 78% for female luteal phase plasma (0,5 ml extracted). Hence, recovery was determined for each specimen assayed and taken into account in calculating its result.
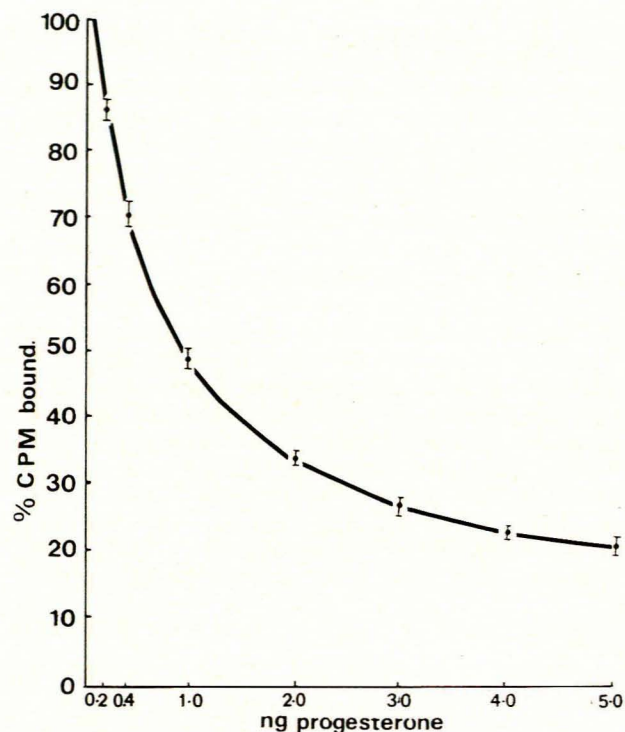

Fig. 1. Composite standard curve. Zero standard expressed at 100%. Each point on the curve represents mean ± SD.

Precision

In order to determine inter-assay precision, duplicate determinations of quality control pools of plasma at two levels of progesterone were performed. The coefficient of

variation (CV) was derived from the formula $S = \sqrt{\dfrac{d^2}{2N}}$,

where d = difference between duplicates and N = number of pairs of samples[17] (Table I). Intra-assay variability was determined by measuring replicate samples in a single assay (Table I). Coefficient of variation was low (7% or less) in both cases, showing a high degree of reproducibility both within and between assay.

TABLE I. INTRA- AND INTER-ASSAY REPRODUCIBILITY

Precision	Mean (ng/ml)	SD	CV (%)	N
Intra-assay				
Low pool	4,58	0,17	3,8	14
High pool	24,92	1,04	4,2	14
Inter-assay				
Low pool	4,54	0,38	7,0	7
High pool	24,59	1,98	5,3	7

Sensitivity

Blank values varied from 0,00 to 0,15 ng (mean 0,07 ng). On the standard curve (Fig. 1) 100 pg is significantly different from the zero points ($P<0,05$). With recovery ranging from 66% to 91%, and using 0,5 ml or 1,0 ml of plasma, approximately 400 pg/ml would be regarded as the lowest progesterone concentration detectable in plasma.

In an effort to evaluate the range between 0 and 400 pg, a second standard curve was constructed (Fig. 2). This
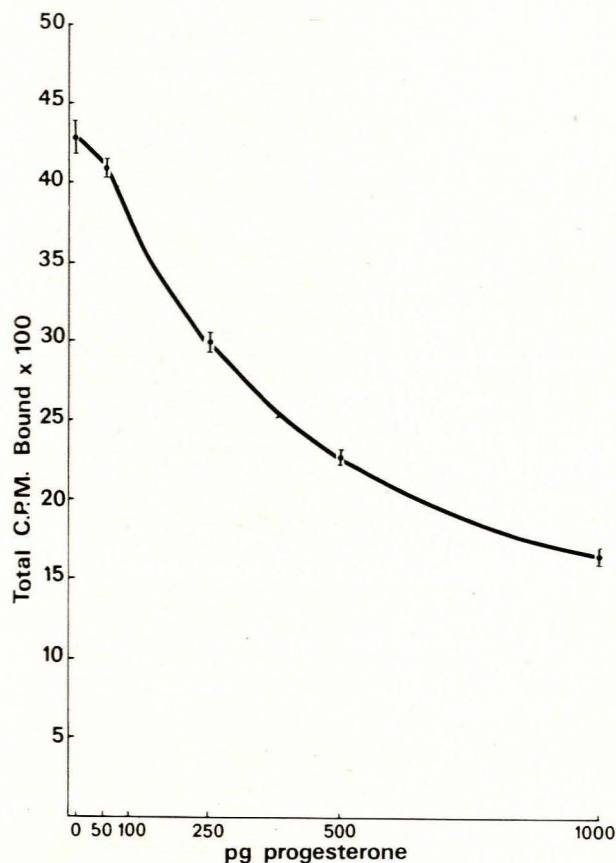

Fig. 2. A standard curve from 0 to 1 000 pg progesterone. The points are the means of duplicates.

shows that 50 pg is clearly distinguishable from zero, which is equivalent to approximately 200 pg/ml in plasma. Hence, we regard 200 pg/ml as the absolute sensitivity of this assay.

Accuracy

Accuracy of the method was determined by recovery and measurement of authentic progesterone added in different amounts to 1 ml of plasma in which progesterone could not be detected by previous assay. The regression time and correlation coefficient are shown in Fig. 3. The method is less accurate in the subnanogram per ml range, where CV tends to be rather high (Table II).
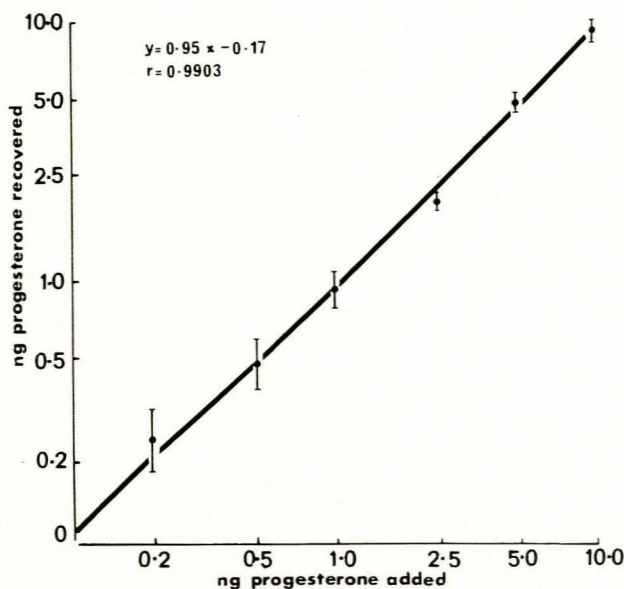

Fig. 3. Recovery of known amounts of authentic progesterone added to 'progesterone-free' plasma. Each point of the log curve represents mean ± SD.

TABLE II. ACCURACY OF METHODS AS DETERMINED BY RECOVERY OF AUTHENTIC PROGESTERONE ADDED TO 'PROGESTERONE-FREE' PLASMA

Progesterone added (ng/ml)	Progesterone recovered (ng/ml ± SD)	CV (%)	No. of determinations
0,2	0,25 ± 0,068	27,6	8
0,5	0,48 ± 0,10	20,9	13
1,0	0,93 ± 0,13	13,7	14
2,5	2,06 ± 0,21	10,2	12
5,0	4,81 ± 0,49	10,1	11
10,0	9,07 ± 0,62	6,9	10

Specificity

A selection of steroids, which could contribute to the assay by interfering with the competition of progesterone and tritiated corticosterone for binding protein, was added to pooled plasma with undetectable progesterone content, which was then assayed for progesterone. The specificity of the assay for progesterone is shown in Table III.

DISCUSSION

Numerous methods have been described for the measurement of progesterone in human plasma.[1-4,6-16] In developing the assay described above, an attempt was made to minimise the number of steps involved, and to simplify the method so that it can be easily applied in any clinical laboratory.

TABLE III. SPECIFICITY OF ASSAY AS DETERMINED BY % CONTRIBUTION MADE BY RELATED STEROIDS TO THE ASSAY

	Steroid (ng) added to 1 ml pooled plasma	Progesterone equivalent (ng/ml)	Contribution (%)	N
20α-hydroxyprogesterone	16	2,09	13,1	6
20β-hydroxyprogesterone	16	0,54	3,3	4
Testosterone	16	0,44	2,8	6
17α-hydroxyprogesterone	16	0,26	1,6	6
Androstenedione	16	0,21	1,3	4
Cortisol	20	0	0	2
11-desoxycortisol	20	0	0	2

TABLE IV. PLASMA PROGESTERONE CONCENTRATIONS FOUND BY DIFFERENT INVESTIGATORS

Investigator (method)	Follicular phase	Luteal phase	Males
Riondel et al.[2] (DI)	1,13 ± 0,49	10,4 ± 3,2	0,28 ± 0,13
Van der Molen and Groen[4] (GLC)	1,30	15,20	0,28
Johansson[11] (CPB)	0,37 ± 0,08	10 — 20	
Yoshimi and Lipsett[7] (CPB)	0,43 ± 0,37 (0,05 — 1,50)		0,33 ± 0,17
Swain[13] (CPB)	1,18 (0,76 — 1,77)	8,45 (3,42 — 17,13)	0,8 ± 0,39 (0,22 — 1,32)
Abraham et al.[15]	0,55 ± 0,1	8,56 ± 4,66	0,23 ± 0,06 (0,16 — 0,26)
Furuyama and Nugent[16] (RIA)	0,27 ± 0,19 (0,12 — 0,79)	8,28 ± 6,34 (1,41 — 17,47)	0,23 ± 0,10 (0,09 — 0,42)
Present method (CPB)	0,39 ± 0,32 (0,06 — 1,15)	7,05 ± 4,12 (0,67 ± 17,52)	0,29 ± 0,09 (0,13 — 0,40)

Water used for blanks and for diluting ethanol was distilled and de-ionised, but all other reagents were Analar grade and not further purified. Despite this, blank values were of the same order as those found in most other non-chromatographic procedures previously described.

To obviate the use of either paper or thin-layer chromatography, specificity of the assay is attained by the following:

1. A single petroleum ether extraction which excludes corticosterone, cortisol and 11-desoxycortisol. The last 2 steroids were tested and shown not to make any contribution to the assay (Table III).

2. Two aqueous ethanol washes. Advantage was taken of the different partition coefficient for progesterone and other interfering steroids such as testosterone, 17α-hydroxyprogesterone and 20α and β-hydroxyprogesterone.

3. A specific protein, corticosterone-binding globulin (CBG). Johansson[11] has shown that dehydro-epiandrosterone, oestradiol, pregnanediol and pregnenolone do not displace tritiated corticosterone from CBG, whereas 20α-hydroxyprogesterone, testosterone, progesterone and 17α-hydroxyprogesterone do so to varying degrees. Greatest displacement was achieved by 17α-hydroxyprogesterone, less by progesterone and least by 20α-hydroxyprogesterone and testosterone.

Table III shows that of all the steroids tested, only 20α-hydroxyprogesterone, which is secreted by the corpus luteum, makes any significant contribution to the assay (13,1%). Runnebaum et al.[18] reported a mean concentration

of 1,3 ng/ml of this steroid during the 16th to 25th day of the menstrual cycle. At this low level the contribution of 2α-hydroxyprogesterone to the final answer is insignificant.

Table IV shows a comparison of values obtained by our method and those described by other investigators using different methods. It can be seen that our results are in good agreement with those of Abraham et al.[15] and Furuyama and Nugent,[16] who assayed progesterone by radio-immunoassay. The low levels observed in female follicular phase and male plasma were obtained after subtracting blank values where these existed. Thus, we are able to detect plasma progesterone in these situations. However, in view of (a) variable blanks which are high in relation to the concentrations of progesterone normally found in follicular phase and male plasma, (b) the relatively high coefficient of variation at low concentrations found by us and others,[7,9] and (c) the fact that, although the contribution of testosterone to the assay was only 2,8%, it becomes significant at the plasma testosterone levels found in normal males, it is suggested that the assay should be reserved for luteal phase measurements only.

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