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# RADIOIMMUNOASSAY OF PLASMA PROGESTERONE

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## SUMMARY

A simple method is described for the determination of progesterone in peripheral venous plasma (0·1–2·0 ml) from men and women. Tritiated progesterone (2,000 d.p.m., 3·0 pg) is added as an internal standard and the plasma extracted with diethyl ether. Standards (0–150 pg) and aliquots of the unknowns (1–10%) are equilibrated with antiserum to progesterone-11α-hemisuccinate-bovine serum albumin and tritiated progesterone (20,000 dpm, 30 pg). The unbound steroid is removed with dextran-coated charcoal.

The mean total random theoretical percentage error is calculated to be less than 10% and the coefficients of variation on replicate analyses of male and female plasma ranged from 9–14%. The lowest level of progesterone which may be accurately determined is 3 ng/100 ml plasma. The concentration (mean ± S.D.; ng/100 ml plasma) of progesterone in plasma samples from a group of healthy men (aged 20–40 yr) was 49·5 ± 13·2. From women, during days 1–14 of the menstrual cycle, the value was 55·5 ± 26·5, during days 14–32 of the cycle 770 ± 249 and during pregnancy (12th week to term) 14,400 ± 6,600. After chromatography on Sephadex LH 20, the corresponding values were 23·0 ± 6·8 (percentage mean difference (PMD) 115%), 42·4 ± 18·7 (PMD 31%), 770 ± 190 (PMD 0), and 10,400 ± 5,500 (PMD 38%). The values are discussed.

## INTRODUCTION

RECENTLY, methods based upon the principles of radioimmunoassay have been applied to the determination of progesterone (4-pregnene-3,20-dione) in peripheral venous plasma from men and women. One procedure has been evaluated[1] which involves extraction with diethyl ether, chromatography on microcolumns of celite and equilibration with an antiserum to 11-desoxycortisol-21-hemisuccinate-human serum albumin. A similar method has been reported[2] using hexane for extraction, alumina for chromatography and an antiserum to progesterone-3-carboxymethyl oxime-bovine serum albumin (BSA).

Other antigens have been prepared by attachment of progesterone-20-carboxymethyl oxime[3], progesterone-6-carboxymethyl[4], and progesterone-6-carboxymethyl thioether[5] to BSA. The characteristics of antisera to these complexes and possible limitations in their use as reagents for the determination of progesterone were discussed. The most specific antisera, in terms of cross reaction with those steroids likely to be present in peripheral venous plasma, have been obtained after linkage of progesterone to BSA at carbon 11, using either the chlorocarbonate derivative[6] or the 11α-hemisuccinate[5].

This paper describes and evaluates a procedure for the determination of plasma progesterone using an antiserum to progesterone-11α-hemisuccinate-BSA. The mean and range of values in groups of healthy men and women are compared before and after chromatography on Sephadex LH 20.

## EXPERIMENTAL

### *Solvents and reagents*

Diethyl ether (peroxide free), benzene, methanol, and acetone were all Analar grade and redistilled before use. Water was glass distilled and deionized. Azobenzene was obtained from B.D.H. Chemicals Ltd., Poole, Dorset, England; and Sephadex LH 20 from Pharmacia, Uppsala, Sweden. Succinic anhydride and tributylamine were obtained from Eastman Organic Chemicals, Rochester, New York, U.S.A.; isobutylchloroformate from Pfaltz and Bauer Inc. Flushing, New York, U.S.A.; and bovine serum albumin from Sigma Chemical Co., St. Louis, Mo., U.S.A.

### *Buffer*

Phosphate buffered saline containing 0·1% gelatine (PBS) was prepared by dissolving 9 g sodium chloride, 1 g sodium azide and 1 g gelatine in 305 ml of 0·2 M disodium hydrogen phosphate and 195 ml of 0·2 M sodium dihydrogen phosphate. The pH was adjusted to 7·0, and the solution made up to 1 l with distilled deionized water.

### *Standards*

Progesterone, 11$\alpha$-hydroxyprogesterone (11$\alpha$-hydroxy-4-pregnene-3,20-dione), 17$\alpha$-hydroxyprogesterone (17$\alpha$-hydroxy-4-pregnene-3,20-dione), androstenedione (4-androstene-3,17-dione), and pregnenolone (3$\beta$-hydroxy-5-pregnen-20-one) were obtained from Sigma Chemical Co.; 11$\beta$-hydroxyprogesterone (11$\beta$-hydroxy-4-pregnene-3,20-dione), 16$\alpha$-hydroxyprogesterone (16$\alpha$-hydroxy-4-pregnene-3,20-dione), 20$\alpha$-dihydroprogesterone (20$\alpha$-hydroxy-4-pregnene-3, 20-dione), 5$\alpha$-dihydroprogesterone (5$\alpha$-pregnan-3,20-dione), and 6$\beta$-hydroxyprogesterone (6$\beta$-hydroxy-4-pregnene-3,20-dione) were donated by the MRC Steroid Reference Collection, Westfield College, London, N.W.3. All steroids were serially diluted in acetone to a concentration of 1 pg/$\mu$l.

[1,2,6,7,-$^3$H]-progesterone (S.A. 81·1 Ci/mmol) was supplied by New England Nuclear Corporation, Boston, Mass., U.S.A. A solution was prepared containing 10 Ci/ml in benzene/ethanol 95:5 (v/v). Subsequently, 100 $\mu$l were removed, dried and redissolved in 10 ml of PBS. One hundred $\mu$l containing approximately 20,000 dpm were used in the assay system.

### *Antiserum*

Progesterone-11$\alpha$-hemisuccinate was prepared from 11$\alpha$-hydroxyprogesterone by refluxing with succinic anhydride in pyridine. The crystalline hapten was joined to BSA using a mixed anhydride reaction according to the method of Erlanger *et al.*[7]. Hydrolysis of the antigen, followed by extraction and g.l.c., indicated that approximately 26 moles of hapten were originally joined to 1 mole of protein.

For immunization, 2 mg of antigen were dissolved in 0·5 ml of sterile isotonic saline and emulsified with an equal volume of Freund's complete adjuvant. This emulsion was injected subcutaneously at multiple sites into a rabbit. The procedure was repeated once a week for a further three weeks, then once a fortnight for the following month, and subsequently once a month. Of two rabbits so treated, one developed antisera which could be used at a dilution of 1:1,000 (v/v), 10

weeks after the first injection. The antiserum was diluted 1:5 (v/v) with PBS and divided into 2 ml aliquots, which were stored at −15°C, and diluted prior to use.

*Dextran-coated charcoal*

Norit A charcoal (Sigma Chemical Co.) was repeatedly washed with distilled deionized water to remove fine particles, then thoroughly dried. A solution was prepared containing 100 mg/ml of Dextran T70 (Pharmacia) and 1·0 mg/ml sodium azide. Charcoal (1·25 g) and 1·25 ml of the dextran solution were added to 500 ml of PBS. The solution was stored and used at 4°C. During use, the charcoal was maintained in suspension by a magnetic stirrer.

*Radioactivity measurement*

The samples were added to disposable glass vials containing 10 ml scintillant, prepared by dissolving 6·0 g of 2,5-diphenyloxazole (PPO) in 1 l of toluene and adding 500 ml of Triton X-100 (Koch-Light Laboratories Ltd., Colnbrook, Bucks., England). All samples were stabilised for a minimum of 90 min at 2°C in the dark, and were then counted for a total of 4000 counts (± 1·6%) in an automatic liquid scintillation counter. The counting efficiencies were determined by an external standard channels ratio method using a $^{133}$Ba source.

*Method*

Peripheral venous blood is withdrawn by a disposable syringe, transferred to a tube containing lithium heparin and centrifuged. The plasma is removed and processed immediately or stored at −15°C. The maximum volumes of plasma required for extraction and the aliquots subsequently removed for assay are shown in Table 1.

The procedure for the assay of progesterone is essentially similar to that described in detail for the determination of oestradiol [8] and testosterone [9]. Briefly, [$^3$H]-progesterone corresponding to 2,000 d.p.m. and 3·0 pg is added as an internal standard to correct for experimental losses. The plasma is extracted twice with 10 ml of diethyl ether using a vortex mixer. At this stage, the extracts may be dried in a counting vial or a pointed tube if a chromatographic step is to be included. For chromatography, the extracts are dissolved in a few drops of benzene: methanol 95:5 (v/v) containing azobenzene. Sephadex LH 20 is equilibrated with benzene: methanol 95:5 (v/v) and columns (7 cm) prepared in disposable Pasteur pipettes. After the extracts have been transferred, elution is performed with the same solvent mixture and the yellow fractions containing progesterone are collected in counting vials. 17$\alpha$-hydroxyprogesterone is eluted in a 2 ml fraction

Table 1. Volumes of plasma and aliquots of extracts taken for assay. Day 1 of the menstrual cycle is defined as the first day on which bleeding occurred

| Source | Volume of plasma (ml) | Aliquot for assay (%) |
|---|---|---|
| Men (aged 20–40 yr) | 2·0 | 10 |
| Women (days 1–14 of cycle and post-menopausal) | 2·0 | 10 |
| Women (days 14–end of cycle) | 0·2 | 10 |
| Pregnant women (12 week–term) | 0·1 | 1 |

immediately following the dye. After extraction or chromatography, the samples are dried in counting vials and redissolved in 1 ml of acetone. The appropriate aliquots (Table 1) are removed for assay, and the remainder subjected to liquid scintillation counting, in order to correct for losses. Standard curves and unknowns are prepared in triplicate, dried, and equilibrated with antiserum (100 $\mu$l) for 30 min at room temperature. After addition of labelled progesterone (20,000 d.p.m. corresponding to 30 pg) the solutions are mixed and equilibrated for a suitable period of time – either 5 min at 37°C followed by 10 min at 4°C or, if more convenient, at 4°C overnight. The unbound steroids are removed by addition of 1·0 ml of dextran-coated charcoal. After mixing, the tubes are allowed to stand for 15 min and are then centrifuged (15 min, 8°C, 2,000 r.p.m.). The dextran-coated charcoal adheres to the bottom of the tube, and the supernatant containing the antibody bound steroid is decanted into a counting vial containing scintillation fluid.

*Calculation of results*

A standard curve, where the amount of tritiated progesterone bound to antibody is expressed as d.p.m., is shown in Fig. 1. For each plasma sample, the mean number of d.p.m. in the aliquots removed for assay is calculated. The corresponding number of pg are read from the standard curve and corrected for the aliquot taken, losses incurred during the method and volume of plasma taken for assay.

A desk-top computer is used to calculate the result $S$, expressed as ng/100 ml plasma according to the following formula:

$$S = \left[\frac{T}{\alpha} \cdot \frac{D\beta}{d} - M\right] \frac{1}{10V}$$

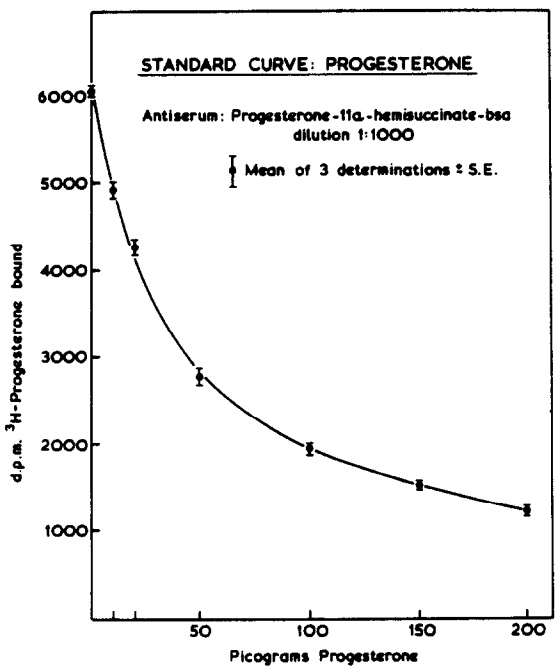


Fig. 1. Standard curve for progesterone, using an antiserum to progesterone-11α-hemisuccinate-BSA at a dilution of 1 : 1000 (v/v).

where $\alpha$ is the aliquot taken for radioimmunoassay and $T$ the reading from the standard curve in pg; $D$ is the d.p.m. added, and $d$ the d.p.m. in an aliquot $\beta$ of the unknown. $M$ is the mass of internal standard and $V$ the volume of plasma in ml.

## EVALUATION OF METHOD AND RESULTS

### *Theoretical assessment of error*

The random error on each term in Eq. (1) was assessed by replicate analyses and combined in order to determine the random error on the measurement of progesterone in an individual plasma sample [8, 9]. Using semi-automatic constant volume pipettes for all measurements, the total random theoretical percentage error was calculated to be less than 10%.

### *Practical, systematic errors*

The practical errors were determined from the recovery of known amounts of progesterone added to distilled deionized water and from replicate analyses of male and female plasma.

### *Accuracy*

The accuracy was assessed by replicate analyses of progesterone (in amounts similar to those found during the follicular and luteal phases of the menstrual cycle) added to distilled, deionized water. The mean values with and without chromatography on Sephadex LH 20 are shown in Table 2.

### *Precision*

The precision of the method was assessed from serial determinations of plasma from women during the follicular and luteal phases of the cycle. The coefficients of variation before and after chromatography on Sephadex LH 20 are shown in Table 3.

### *Sensitivity*

The lower limit of progesterone which may be determined depends upon the volume of plasma extracted, the aliquot taken for assay and the error associated with the measurement. As the standard deviation on 10 pg on the standard curve never overlaps the standard deviation of the corresponding 0, a reading of 10 pg on the standard curve may be safely taken as the lower limit of sensitivity. Referring to Table 1, this means that the lower limit of sensitivity is approximately 5 ng/100 ml plasma, when the method is applied to plasma from men, and women during the follicular phase of the cycle. However, in practice, the blank values

Table 2. Replicate analyses of authentic progesterone added to deionized, distilled water

| Amount added ng/100 ml | No. of deter. | Amount calculated ng/100 ml | Coefficient of variation (%) |
|---|---|---|---|
| 40 | 8 | 40 ± 3·9 | 9·8 |
| 40* | 8 | 41 ± 4·7 | 11·4 |
| 750 | 10 | 770 ± 63 | 8·1 |
| 750* | 10 | 710 ± 64 | 9·0 |

*Chromatographic step included.

Table 3. Replicate analyses [8] of progesterone in peripheral venous plasma from healthy women

| | ng/100 ml plasma mean ± S.D. | Coefficient of variation (%) |
|---|---|---|
| Female plasma pool (days 1–10) | 66 ± 6·1 | 9·2 |
| Female plasma pool* (days 1–10) | 69 ± 7·5 | 10·9 |
| Female plasma (day 24) | 980 ± 110 | 11·2 |
| Female plasma (day 24)* | 960 ± 130 | 13·5 |

*Chromatographic step included.

are sometimes higher especially if the chromatographic step is included. The corresponding values for sensitivity when applied to plasma from the luteal phase of the cycle and from pregnancy are 50 ng and 1·0 μg/100 ml plasma respectively. Under these conditions, the blank values are negligible.

The ability of the method to distinguish between two values may be assessed by taking readings from the mean of standard deviations of replicate determinations at two points on the extremities of the standard curve. These give a mean value of 2 pg and so the resolution of the three variations on the method are 1 pg, 10 ng and 0·2 μg/100 ml of plasma respectively. Accordingly, all unknown values are recorded to this degree of accuracy.

*Specificity*

The specificity of the method as applied to plasma samples from the luteal phase of the menstrual cycle and from pregnant women depends upon the characteristics of the antiserum. Increased specificity may be obtained by chromatography on Sephadex LH 20. The ability of steroids closely related to progesterone to compete for binding sites on the antibody has been investigated. Standard curves covering the same mass range are shown in Fig. 2. The relative amounts required to reduce the initial binding of [$H^3$]-progesterone by half, and where the mass of non-labelled progesterone required is defined as 100%, are as follows: 5α-dihydroprogesterone 80%; 11α- and 11β-hydroxyprogesterone 48%; 16α-hydroxyprogesterone 31%. The corresponding values for 17α-hydroxyprogesterone, 11-deoxycorticosterone and androstenedione are between 5–10%, and pregnenolone, 6β-hydroxyprogesterone and testosterone less than 5%.

The chromatographic step on Sephadex LH 20 completely removes all hydroxylated derivatives from progesterone, but approximately 90% of any 5α-dihydroprogesterone or androstenedione in the sample would be collected in the same fraction of eluate.

Evidence for the lack of non-specific interference from solvents and reagents during the procedure, may be deduced from the values obtained for varying quantities of distilled deionized water taken through the method. The mean and range of values, with and without chromatography, and corresponding to the same volumes and aliquots as taken for plasma samples, are shown in Table 4.

The specificity of the method when applied to peripheral venous plasma has been investigated by analysing the same samples before and after chromatography.

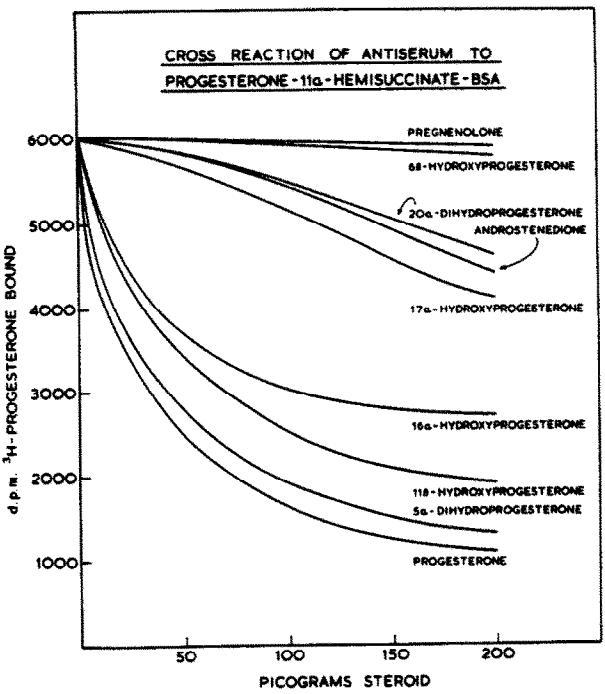


Fig. 2. The ability of various steroids related to progesterone to compete with [$^3$H]-progesterone for binding sites on an antiserum to progesterone-11$\alpha$-hemisuccinate-BSA.

Table 4. Method blanks. Replicate analyses of deionized distilled water (progesterone equivalents ng/100 ml) using the same volumes as for plasma samples

| Volume (ml) | No. of determinations | Without chromatography mean ± S.D. | range | With chromatography mean ± S.D. | range |
|---|---|---|---|---|---|
| 2·0 | 12 | 3·7 ± 2·7 | 0·1–7·3 | 9·0 ± 3·7 | 1·3–16·8 |
| 0·2 | 8 | 6·6 ± 2·6 | 2·9–9·8 | 13·2 ± 4·6 | 6·4–21·0 |
| 0·1 | 14 | 50·0 ± 70·0 | 1·0–200·0 | 40·0 ± 70·0 | 1·0–200·0 |

The mean and range of values from groups of healthy men and women are shown in Table 5.

*Recovery of labelled internal standard*

The amount of [H$^3$]-progesterone recovered (mean ± S.D.) is shown in Table 6. The values refer to the different volumes of plasma extracted, and after chromatography on columns of Sephadex LH 20.

## DISCUSSION

This method is similar in principle to those previously reported using different antisera and has similar merits in terms of sensitivity, accuracy, and precision. However, the use of antisera to progesterone-11$\alpha$-hemisuccinate-BSA may enable the chromatographic step to be omitted when analysing plasma samples from women. Thus, during the follicular phase of the menstrual cycle, the percentage mean difference between the determinations with and without chroma-

Table 5. Mean and range of values for plasma progesterone (ng/100 ml). Day 1 of the menstrual cycle is defined as the first day on which bleeding occurred

| Source | No. of determinations | Without chromatography mean ± S.D. | range | With chromatography mean ± S.D. | range |
|---|---|---|---|---|---|
| Men (aged 20–40 yr) | 72 | 49·5 ± 13·2 | 31–83 | 23·0 ± 6·8 | 8–37 |
| Women (days 1–14 of cycle) | 13 | 55·5 ± 26·5 | 29–106 | 42·4 ± 18·7 | 24–79 |
| Women (days 14–end of cycle) | 12 | 770 ± 249 | 100–1070 | 770 ± 190 | 90–980 |
| Pregnancy (12th week-term) | 42 | 14·4 ± 6·6* | 4–27 | 10·4 ± 5·5* | 4–23 |

*$\mu$g/100 ml plasma.

Table 6. The recovery (mean ± S.D.) of labelled internal standard

| Volume of plasma (ml) | Without chromatography | With chromatography |
|---|---|---|
| 2·0 | 91 ± 3 | 78 ± 6 |
| 0·2 | 94 ± 3 | 77 ± 3 |
| 0·1 | 93 ± 8 | 78 ± 5 |

tography was 31%. During the luteal phase there was no difference between the mean values, while during pregnancy the mean difference was 38%. On the basis of known characteristics of the antiserum and reported values for 17$\alpha$-hydroxyprogesterone[10], it may be deduced that any overestimation of progesterone in plasma from men and non-pregnant women is probably due to the presence of 17$\alpha$-hydroxyprogesterone. However, during pregnancy, the levels of various 16$\alpha$-hydroxylated steroids markedly increase and the overestimation of progesterone in plasma removed during this period may be due to relatively high levels of 16$\alpha$-hydroxyprogesterone.

The level of 5$\alpha$-dihydroprogesterone in peripheral venous plasma has not been determined by direct measurement. However, progesterone has been measured by techniques which either measure both steroids or progesterone alone. A comparison of values by both approaches suggests that the level of 5$\alpha$-dihydroprogesterone can only be relatively low (< 15% of progesterone)[2].

If the chromatographic step is omitted, useful information may be obtained as to whether or not a functioning corpus luteum is present and whether the subject is pregnant. These facts enable a rapid method with no internal standard and short equilibration times to be used, so that the results from up to twelve plasma samples may be obtained in 4 h.

If accurate measurements are required on plasma samples from men, then the chromatographic step and the internal standard must be included. In addition, increased sensitivity may be obtained by overnight equilibration with the antiserum at 4°C. Using these procedures the mean and range of values are similar to those reported[1, 2].

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