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A RAPID AND PRECISE METHOD FOR MEASUREMENT OF PHYSIOLOGICAL VARIATIONS OF HUMAN PLASMA PROGESTERONE

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SUMMARY

A practical and precise radioimmunoassay for plasma progesterone (P) is described using a highly specific antiserum produced in the sheep immunized against progesterone-11α-succinyl-BSA. Purification of the extracts was achieved by a rapid efficient chromatography on small Sephadex LH-20 columns whereby a dye is included to monitor the P fraction. Recovery of ^{3}H-P after extraction and chromatography was 63 ± 7.9 (S.D.). The sensitivity of the assay was 10 pg. The intra and inter-assay coefficients of variation were 7.7 and 15.1% respectively. Mean values of plasma P were 9.5 ± 6.5 (S.D.) in males and 8.5 ± 6.6 ng/100 ml in females during follicular phase of the menstrual cycle. There was no significant difference between the two groups. Plasma samples obtained during luteal phase (day 17–26) varied from 119 to 1697 ng/100 ml. In all instances, non-chromatographed samples yielded consistently higher values, thus emphasizing the need for adequate purification if precise P values are needed for physiological studies.

INTRODUCTION

Among the various approaches applied to the measurements of the relatively low plasma progesterone (P) in male and female during the follicular phase, there are the competitive protein binding technique [1–3], the double isotope dilution technique [4], gas–liquid chromatography [5] and radioimmunoassay (RIA) [6–12]. The RIA is the most widely used and with the production of highly specific antiserum, direct assays were developed with satisfactory results for routine determinations [7, 10, 11]. However, few reports have documented higher values when the chromatographic step was omitted [13–14], thus cautioning the use of direct assays in physiological studies which require precise P determinations. The present communication describes a simple RIA for plasma P in which an efficient purification step is performed on small Sephadex LH-20 columns using a marker dye to locate the fraction.

MATERIALS AND METHODS

All solvents for plasma extraction and chromatography were of reagent grade (Fisher Scientific) and were purified and redistilled before used [15]. The second band of Sudan III (ChromaGesellschaft Schmid & Co., Stuttgart-Untertürkheim) used as the reference marker was purified as previously described [16]. Ethylene glycol (Baker Analyzed Reagent, Spectrophotometric), succinic anhydride (Eastman Kodak Company), pyridine (Baker analyzed Reagent), bovine serum albumin (Sigma) bovine γ-globulin (Cohn Fraction II, Sigma), tri-*n*-butylamine, iso-butyl chloroformate (K & K Laboratories Inc.) and Dextran T 70 (Pharmacia) were used as supplied. Norit A charcoal (Fisher Scientific) was washed several times with deionized water to remove fine particles and dried in an oven before use. Non radioactive steroids were supplied by Steraloids Inc., NY. P 1α, 2α-H^3 (n) (53 Ci/mmol), obtained from Amersham Searle, was purified prior to use by paper chromatography in a Bush A system isooctane–methanol–water (5:4:1). Sephadex columns (0.5 × 18 cm) were prepared in the mixture isooctane–benzene–methanol (85:10:5) as previously described [16]. The P fraction and the dye (a component of Sudan III) appears as a compact peak between 8–9 ml; interfering compounds such as 5α-pregnan-3,20 dione, pregnenolone, deoxycorticosterone and 17-hydroxy P are eliminated from the P fraction [16].

All assays were carried out in siliconized 12 × 75 mm disposable glass tubes. All samples were counted in low background plastic vials (NEN) using 10 ml of the scintillation fluid which was made up with 19 g PPO (2, 5 diphenyloxazole, Amersham Searle) and 76 ml of methanol in 1 gallon of toluene. Reusable glasswares were washed in acid, thoroughly rinsed with tap water, deionized water, dried in an oven and rinsed with purified methanol before use.

Preparation of antibody. 11α-hydroxy P was succinylated and conjugated to bovine serum albumin (BSA) by the mixed anhydride method as described by Erlanger *et al.* [17]. The reaction mixture was then dialysed for 48 h against 3 changes of 4 l. of deionized water. Differential U.V. absorption at 243 mμ indicated 25 steroid molecules were bound per mole BSA. Two adult sheep were immunized by multiple intradermal injections on the back (with 3 mg of the antigen dissolved in 1 ml of normal saline emulsified

with 2 ml of Freund's complete adjuvant). Subcutaneous booster injections were repeated every 4–6 weeks in four separate sites on the back. The antibody used in this study was obtained eight months after the initial immunization.

Plasma extraction, chromatography and recovery. Blood samples were obtained by venipuncture from normal healthy subjects (age 20–41) between 8 and 10 a.m. and the plasma was stored at −15°C until processed. The first day of the menstrual cycle was defined as the day on which bleeding occurred.

5,000 c.p.m. of [^{3}H]-P was added to plasma ranging from 0.1 to 2 ml (samples from male and female during the follicular phase of the menstrual cycle) to estimate recovery. When necessary, the volume of each sample was brought up to 2 ml with deionized water. The samples were then extracted with 10 volumes of petroleum ether and the organic phase was evaporated to dryness. The extracts were then dissolved in 0.1 of the column elution mixture containing the dye [16] and transferred onto the column. This procedure was repeated twice to ensure complete transfer of the sample. The columns were then eluted with the same mixture and the orange band (8–9 ml) containing P [16] was collected into assay tubes, evaporated to dryness in a vacuum oven at 37°C and redissolved in 1.2 ml of methanol. 0.1-ml aliquots were removed for liquid scintillation counting to correct for losses, and the appropriate aliquots (2 × 0.5 ml for samples from male and female during the follicular phase of the menstrual cycle) removed for assay. For the direct assay, appropriate aliquots of the organic extract were transferred into assay tubes, evaporated and processed in the same manner as the chromatographed samples.

Standard solutions of P were made up in methanol to contain 10–10,000 pg/0.1 ml and the tracer standard contained 5,000 c.p.m./0.1 ml. A standard curve of 0–10,000 pg was constructed in duplicate by transfer of 0.1 ml of labelled and unlabelled standards and methanolic ethylene glycol solution (1% solution, v/v) to assay tubes. Counts in unknown samples were estimated from the recovery aliquots and adjusted to the same as those used in the standard curve by transferring the appropriate volumes from the standard tracer solution using an adjustable automatic pipette "Pipetman P 200" (Mendel Scientific Co. Ltd., Montreal, Canada); 0.1 ml of the methanolic ethylene glycol solution was also added to the sample tubes. After evaporation of the methanol in a vacuum oven at 40°C, the contents were concentrated at the bottom of the assay tubes in a small droplet of ethylene glycol which is completely soluble in the assay medium. 0.5 ml of the antibody solution which was made up in 0.05 M borate buffer pH 7.8 and 0.1% gelatin was added to each tube, vortexed and incubated at 37°C for 30 min and at 4°C for 2–16 h. Free and bound hormone was separated by the addition of 0.5 ml of dextran-coated charcoal solution (0.25% charcoal and Dextran T70 in borate buffer and gelatin 0.1%), vortexed and after 10 min incubation centrifuged at 3000 rev./min for 10 min at 4°C. 0.5 of the supernatant was transferred into counting vials followed by 10 ml of the scintillation solution. The vials were tightly capped and shaken in a mechanical shaker for 3 min, equilibrated in a refrigerated scintillation counter (Nuclear Chicago, Mark II) for at least 1 h and then counted for 10 min per sample. The standard curve was constructed by plotting the percentage bound counts against pg per tube.

RESULTS

Serial dilution of the antiserum (sheep No. 103) indicated that at the dilution of 1:10,000, 0.5 ml of the solution binds 50–60% of 5,000 c.p.m. of [^{3}H]-P. This dilution was used for all subsequent assays.

Specificity. The cross-reaction studies of the antiserum calculated according to Abraham [18] are indicated in Table 1. The majority of the steroids tested showed little or no cross-reaction with the antiserum; the compounds (pregnenolone, 5αP, 5βP) which could interfere with the assay would be eliminated by the column chromatographic step [16].

Sensitivity. The standard curve ranges from 0–10,000 pg. At 95% confidence limit, 10 pg was significantly different from zero pg (n = 14) and the coefficient of variation at each point of the standard curve was less than 5%; the useful range is 10–1000 pg. The blank, assessed by extraction of 2-ml aliquots of deionized water and processed in the same manner as the plasma samples gave values that were consistently indistinguishable from the zero binding. Such values were only obtained with purified solvents in all steps. The dye used to monitor the P fraction as

Table 1. Cross-reaction of various steroids with anti-progesterone-11α-BSA serum (Sheep No. 103)

Steroids	% Cross-reaction
Progesterone P)	100.0
Cholesterol	0.00
Pregnenolone	1.63
17-OH Progesterone	0.21
20 β-OH Progesterone	0.48
20 α-OH Progesterone	0.35
5 β Pregnan 3,20 dione (5βP)	2.80
5 α Pregnan 3,20 dione (5αP)	1.00
11-deoxycorticosterone (DOC)	0.61
Corticosterone (B)	0.54
Aldosterone	0.00
11-deoxycortisol (S)	0.00
Cortisol (F)	0.00
Androstendione	0.00
Testosterone	0.00
Dehydroepiandrosterone	0.00
Estriol	0.00
Estrone	0.00
Estradiol	0.00
Spironolactone	0.00

% Cross-reaction 100 = x/y. x = pg of P required to displace 50% of [^{3}H]-P. y = pg of steroid required to displace 50% of [^{3}H]-P.

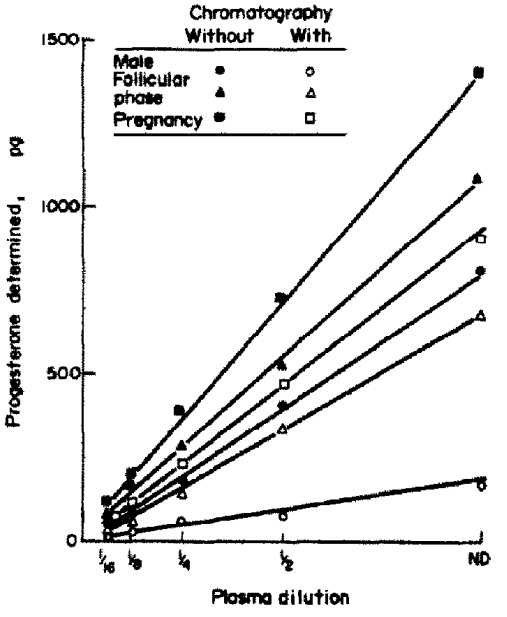

Fig. 1. Progesterone content of different plasma samples serially diluted. N.D.: non diluted.

previously verified in a protein binding assay [16], does not interfere with the standard curve nor the actual assay.

Precision. The intra and inter-assay coefficients of variation for a plasma pool of 8 ng/100 ml were 7.7% ($n = 8$) and 15.1% ($n = 18$) respectively.

Accuracy. 150 and 500 pg of P were added to 2-ml aliquots of charcoal-stripped plasma pool and processed through the entire procedure. After correction for losses, the amounts recovered were 148.5 ± 7.6 (S.D.) and 496 ± 17.9 ($n = 8$). Figure 1 illustrates the effect of serial dilutions of purified and non purified plasma samples from male and female (follicular phase and pregnancy). There is no evidence for deviation from linearity. This indicates that irrespective of dilution or purification, the unknown quantitated in the plasma sample reacts with the antibody in an identical manner as standard P.

Recovery. After solvent extraction and column purification, the mean recovery of tracer P from 93 samples was 63 ± 7.9% (S.D.).

Plasma P levels. The level of plasma P in 44 normal male subjects was 9.5 ± 6.5 ng/100 ml (mean ± S.D., range 1.8–29.5) (Fig. 2).

Thirty-four normally menstruating women in ther follicular phases had values of 8.5 ± 6.4 ng/100 ml (range 1.5–28) (Fig. 2). The plasma P levels in these two groups were not significantly different. During the luteal phase (days 17–26) of the menstrual cycle, plasma P levels ranged from 119 to 1697 ng/100 ml with a mean of 606 ng/100 ml ($n = 14$). Mean plasma P levels measured in 3 pregnant subjects during different stages of pregnancy were 3196 (9–12 weeks), 5251 (14–24 weeks) and 12,657 ng/100 ml (30–35 weeks) respectively.

Table 2 shows a comparison of plasma P values with and without column chromatography. In all instances, omission of the purification step resulted in higher values. The phenomenon is most evident in the plasma samples obtained from men and from women during the follicular phase of the menstrual cycle.

Reference method. 5 ml plasma samples from normal men and women in the follicular phase ($n = 14$) were analysed by protein-binding method [1] using a 3% solution of oestrogen treated dog plasma and the same Sephadex LH-20 chromatography previously described [16]. The results found (10.5 ± 6.1 ng/100 ml (S.D.)) were not significantly different from the present assay.

DISCUSSION

The present communication describes a practical, sensitive and reliable method for the determination of plasma P. A rapid and efficient chromatographic step (20 min) is included in the procedure to isolate the P fraction from steroids which could interfere with the binding assay. The use of a visual dye (a component of Sudan III) which has the same mobility as P [16], further simplifies the monitoring of the purification step.

Our normal values are somewhat lower than most of the figures documented in the literature, but are in agreement with the ranges reported by Cameron *et al.*[11] and Milewich *et al.*[19] for samples

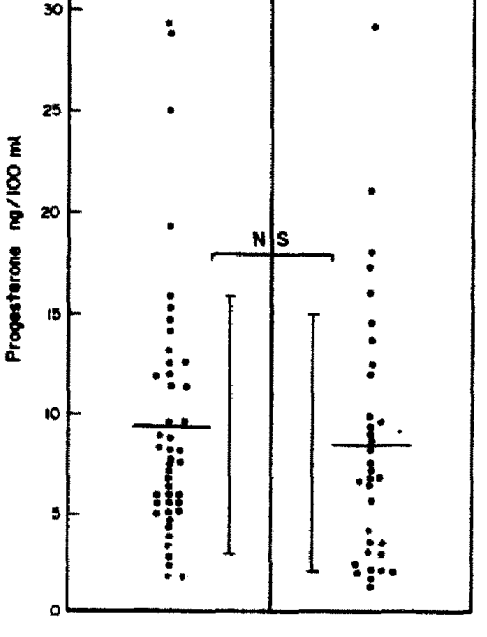

Fig. 2. Plasma progesterone values in male and female during the follicular phase of the menstrual cycle. Horizontal bars represent the means and vertical bars the standard deviation. N.S.: non significant.

Table 2. Comparison of plasma progesterone (ng/100 ml) with and without column chromatography

Subjects	n	Without chromatography		With chromatography	
		Mean	Range	Mean	Range
Men	10	13.2	9.3–15.2	8.1	3.8–13.4
Women (follicular phase)	8	28.7	20.8–47.6	9.6	7 –13.3
Women (luteal phase)	10	469	253–710	422	240–690
Pregnancy (2–12 weeks)	6	3201	2209–3875	2619	1689–3095

obtained from females in the follicular and luteal phases of the menstrual cycle. Very recently, Milewich *et al.*[19] also reported normal male values of 7 ± 1 ng/100 ml which are similar to ours. The observation that there was no significant difference between male and female during the follicular phase of the menstrual cycle has previously been reported [7, 10, 13, 20]. The lower plasma P values obtained by the present method could be due to (1) our use of the efficient small LH-20 column chromatography step which eliminates steroids with similar structures (such as 5α-pregnane-3,20 dione and possibly other steroids or metabolites which could cross-react with our antiserum) from the P fraction [16], (2) the highly specific antibody employed in the binding assay, and (3) the complete absence of blank values due to the use of purified solvents for the extraction and purification steps.

Despite the use of a highly specific antiserum, omission of the purification step resulted in two to threefold increase in plasma P in men and in preovulatory women (Table 2), a phenomenon also noted by others [13, 14] using less efficient purification steps. However, for unknown reasons, this difference is less evident with higher plasma values from women during the luteal phase and pregnancy; similar findings were reported by Youssefnejadian *et al.* during the luteal phase [14].

It is known that 5α-pregnane-3,20 dione, a naturally occurring metabolite of P in the human [21], cross-reacts with the most commonly used binding proteins for P assays. While this compound has nearly 100% displacement capacity for pregnant guinea pig plasma [21, 22], it cross-reacts in various degrees with almost all anti-P antisera [7, 8, 11, 13, 14, 20]. Hence depending upon the inherent characteristic of the binding protein employed for the final assay, and also on the purification methods used which could effectively eliminate 5α-pregnane-3,20 dione from the P fraction, lower values for P could be obtained which would correspond to the true value. This is particularly evident in pregnancy samples in which 5α-pregnane-3,20 dione could contribute to about 35% of the actual P concentration [21, 23].

Thus our observations, and also those documented in the literature indicated that the direct assay of plasma P can be a useful index for a functioning corpus luteum and in the monitoring of pregnancy for routine clinical purposes. However, if precise measurements of the hormone are required, the purification step should be included despite the use of a highly specific antiserum in the binding assay. Finally, we would like to point out that the column used in the present method could be a very useful adjunct in P radioimmunoassays in which a less specific antiserum is employed.

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