# A SIMPLE PROGESTERONE RADIOIMMUNOASSAY WITHOUT COLUMN CHROMATOGRAPHY

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## ABSTRACT

A radioimmunoassay for plasma progesterone without chromatographic purification was developed. The 11-hemi-succinate of 11 -hydroxy-progesterone conjugated to bovine serum albumin was injected into rabbits to stimulate antibody production. The resulting antisera was used at a final dilution of 1:3500. The mean recovery of labeled progesterone added to 100 samples after ether extraction (88.9 ± 9.1%) was higher than the recovery obtained when column chromatography followed ether extraction (84.8 ± 7.5%). For comparison, plasma pools were assayed for progesterone with and without the use of columns. A female plasma pool (luteal phase) gave a mean of 546.3 ± 26.5 (SD) ng/100 mls (n = 5) without column chromatography and 557.2 ± 20.8 (SD)

ng/100 mls (n = 5) with column chromatography. Another female plasma pool (follicular phase) gave a mean of 87.9 ± 9.6 (SD) ng/100 mls (n = 24) with column chromatography and 93.3 ± 8.6 (SD) ng/100 mls ( n = 7) without column chromatography. A male plasma pool gave a mean of 22.8 ± 4.4 (SD) ng/100 mls (n = 13) with column chromatography and 21.8 ± 7.7 (SD) ng/100 mls (n = 3) without column chromatography. The intra assay and inter assay precision gave a coefficient of variation of 3.7 for six samples and 10.9 for 24 samples, respectively. The specificity of the antibody was determined by checking cross reactivity with 26 steroids. The sensitivity (25 pg) and accuracy were proven to be highly satisfactory.

## INTRODUCTION

A radioimmunoassay for progesterone has been developed which does not require the use of microcolumn chromatography for purification of the extract.

Other methods such as double isotope dilution (1) and gas liquid chromatography (2) require elaborate purification before measurement. Recently, competitive protein binding methods have been developed (3-9) but they are not as specific or as sensitive, and they require the use of some kind of chromatography for purification of the extract. Johansson (4) developed a non-specific competitive protein binding method which does not require further purification after extraction. This method, however, is only satisfactory in measuring progesterone levels in women during the luteal phase and during pregnancy. Its sensitivity is not satisfactory for measuring low progesterone concentrations in men, or in women during the follicular phase, or after menopause.

Other progesterone radioimmunoassay methods have been published (10,11) which possess high sensitivity, but their lack of specificity make the use of chromatography essential. A direct specific radioimmunoassay method for progesterone determinations was published recently (12) in which the investigators were able to measure the plasma progesterone levels of female rhesus monkeys. The sensitivity of the method, however, was probably unsatisfactory for the measurement of progesterone levels in humans. There was no attempt made in the paper to measure human progesterone.

Numerous papers have been published on the preparation of antisera for different steroids conjugated to protein at different positions (13,14, 15,16). The specificity of antisera in relation to the site of attachment of the steroid hapten to the peptide carrier has been studied for several ovarian hormones including progesterone (14). The need for antisera of high specificity has been emphasized so that time consuming separations can be reduced or omitted without loss of sensitivity and specificity. The site of conjugation determines the specificity of the antiserum. Those sites farthest away from the attachment to protein are the strongest determinants of specificity.

A direct, simple radioimmunoassay method is described below in which ether extraction is the only purification step. The use of microcolumns can be omitted since the results with and without chromatography are comparable.

## MATERIALS AND METHOD

Steroids:

1,2-$^3$H-progesterone (New England Nuclear Corp. 50.3 Curies/mM) was purified by paper chromatography using a system of hexane:methanol: water (100:70:30). The purity of the eluted material (in absolute ethanol) was checked by paper chromatography using two other systems:hexane/ formamide and isooctane:t-butanol:methanol:water (500:100:350:50). Non-radioactive steroids were obtained from Steraloids, Inc., Pawling,N.Y.

Solvents:

Analytical grade anhydrous ether (Baker) was used without distilling, methanol spectroquality (Matheson, Coleman and Bell), methylene chloride spectroquality (Mallinckrodt), hexane nanograde (Mallinckrodt), 95% ethanol reagent grade (Matheson, Coleman and Bell), absolute ethanol (Commercial Solvent Co.) toluene scintillation grade (Mallinckrodt) were used.

Other Materials:

Isobutyl chloroformate (K & K, Plainview, N.Y.), tri-n-butylamine (K & K), bovine albumin, fraction V (Sigma Chemical Co.), succinic anhydride (Matheson, Coleman and Bell) ammonium sulfate (Mallinckrodt) were used.

The scintillation fluid was prepared by adding 4 gm omnifluor (New England Nuclear) to 1 liter toluene.

All glassware required was disposable. Before use it was rinsed with a solution of methanol:methylene chloride (1:1).

The microcolumns used had the same dimensions as those of Mayes and Nugent (17). They were made out of borosilicate glass, with a teflon gasket, a nylon screen of 10 microns and a teflon stopcock with a bore size of 2 mm. The $Al_2O_3$ was purified prior to use (17).

Solutions:

Borate buffer, pH 8, containing 0.16% bovine serum albumin and 0.065% gamma globulin was stored at 4°C. Saturated ammonium sulfate was made with distilled water and kept cold at 4°C. Half-saturated ammonium sulfate solution was made by diluting the saturated solution 1:1 and kept at room temperature. Standard I solution was prepared by diluting the purified 1,2-$^3$H-progesterone to 12,500 DPM/0.05 ml with absolute ethanol. Standard II solution was made by diluting pure 1,2-$^3$H-progesterone to 2500 DPM/0.05 ml with absolute ethanol. Both

standard solutions were stored at 4°C.

Preparation of 11α-Hydroxyprogesterone Hemisuccinate:

A solution containing 2 gm of 11-alpha-hydroxyprogesterone (0.6 millimoles) and 6 gm (60 millimoles) of succinic anhydride in 20 ml of dry pyridine was refluxed for 15 hours. The solution was then poured into 115 ml of cold 1M $H_2SO_4$. The gummy dark brown product was isolated by filtration and washed with cold water. It was then dissolved in 150 ml of chloroform:methanol (2:1) solution. The organic layer was washed once with 25 ml of 1N $H_2SO_4$ and three times with water (25 ml methanol was added after each washing). The organic layer was dried over anhydrous sodium sulfate and taken to dryness with a rotary evaporator. Thin layer chromatography indicated that the reaction went to completion. Recrystallization from acetone-hexane yielded a yellowish solid weighing 1.4112 g (54.6% yield), m.p. 151.5-155°C. Second recrystallization from acetone and hexane yielded light yellow crystals m.p. 155-156°C. IR showed the disappearance of OH band at ca. 3450 $cm^{-1}$ and two new bands at ca. 1728 $cm^{-1}$ and 1747 $cm^{-1}$. Elemental analysis:Calculated for $C_{25}H_{34}O_6$:C, 69.72%; H, 7.96%; Found: C, 68.22%; H, 7.7%.

Preparation of 11α-Hydroxyprogesterone 11-Albumin Conjugate:

The preparation of 11α-hydroxyprogesterone 11-albumin conjugate used was similar to that of Erlanger (18). Assuming the molecular weight was 77,240, 20 of the 60 amino groups were substituted. Calculated: Amino-N = 0.74; Total N = 14.3. $\frac{\text{Amino N}}{\text{Total N}} = 0.052$. Found: Amino-N = .77; Total N = 14.4 (based on dried albumin conjugate) $\frac{\text{Amino N}}{\text{Total N}} = 0.0534$.

Production of the Antisera:

Three rabbits were immunized with periodical injections of 1 mg of 11α-hydroxyprogesterone 11-BSA conjugate per rabbit, emulsified in 1.0 ml of 50% complete Freund's adjuvant in saline. The first week the immunization solution was injected into three front and three back toe pads. The second week the rabbits were injected intraperitoneal, and the third week intramuscular in four different places along the back. Subsequent boosters were injected subcutaneous every four weeks.

All three rabbits made satisfactory antisera after eight months. The antiserum used for the assays was taken from the blood drawn 14 days after the ninth injection from the rabbit which gave the best titer. One

ml of this antiserum was diluted 1:10 with the assay buffer and stored in small quantities at -10°C. A final dilution of 1:3500 of the antisera was made before each assay run was performed.

Column Chromatography:

The progesterone elution method was as described by Furuyama and Nugent (11). This step was used only for comparisons. Each sample of the dry residue of extract dissolved in 1.0 ml of hexane was transferred to the $Al_2O_3$ microcolumns using individual pasteur pipettes. The columns were washed three times with 1.7 ml of 0.28% absolute ethanol in hexane. These washes were discarded. Progesterone was eluted with 3.4 ml of 0.45% absolute ethanol in hexane. The columns were calibrated so that they could hold a volume of 1.7 ml at a time. The progesterone eluates were collected in previously rinsed counting vials. After use, each column was washed 12 times (15): 2X with 95% ethanol, 4X with methanol, 3X with methanol:methylene chloride (1:1) and 3X with methylene chloride.

Radioimmunoassay:

The eluates in the counting vials were swirled to secure mixing. The 3.4 ml volume was divided into three 1 ml portions each containing about 2500 DPM. Duplicate aliquots were pipetted into 2 ml disposable glass tubes using a 1000 lambda Eppendorf pipette. The third 1 ml aliquot was pipetted into a counting vial and dried. This aliquot was employed in the determination of total radioactivity in the aliquots of eluates used for radioimmunoassay to check the recovery through the column and extraction. For the standard curve, eight standard solutions containing 0,10,25,50,75,100,200, and 500 pg of progesterone per 0.1 ml of absolute ethanol and 2500 DPM of 1,2-$^3$H-progesterone per .05 ml (Standard II) were pipetted in triplicate into 2 ml tubes. The same amount of 1,2-$^3$H-progesterone was added to three counting vials and dried in a 40°C vacuum oven for the determination of total radioactivity in the standards. The assay tubes containing the 1 ml aliquots were dried down to half their volume in a 40°C water bath using a semiautomatic air blowing device (19). They were then completely dried down in a vacuum oven at 40°C. The dried assay tubes were placed in an ice-water bath and 0.25 ml of diluted antiserum (1:3500) was added to each tube. A vortex mixer was used to mix the antiserum and the dried eluates in the tubes. All tubes were covered with parafilm and incubated overnight at 4°C. To separate free from bound progesterone, 0.25 ml of saturated ammonium sulfate was added to each tube and they were mixed thoroughly on a vortex mixer. After mixing, the tubes were then centrifuged at 3500 rpm for 20 minutes at 4°C. An automatic dilutor (Labindustries) was

used to transfer 0.2 ml of the supernatant into a counting vial followed by 10 ml of scintillation solution; 0.2 ml of half-saturated ammonium sulfate was transferred into the counting vials containing dried extracts for recovery determinations and the dry standards, followed by 10 ml of scintillation solution. All counting vials were shaken on a mechanical shaker for 10 minutes, and then counted for five minutes in a scintillation counter (Nuclear Chicago).

Calculations:

The standard curve was plotted on a semilog paper. Percent free of 1,2-$^{3}$H-progesterone was plotted in the linear y-axis and picograms of progesterone were plotted in the logarithmic x-axis. The percent free was calculated according to Furuyama and Nugent (11).

## RESULTS

In our standard curves, percent free 1,2-$^{3}$H-progesterone increased from 15% to 75% as the picograms of progesterone increased from 0 to 500.

Recovery:

The recovery of 1,2-$^{3}$H-progesterone varied between 69 and 100% when 200 samples were assayed. Following ether extraction and chromatography the mean recovery of 100 samples was 84.8 ± 8.8 (SD)%. When column chromatography was omitted, the mean recovery of 100 samples was 88.9 ± 10.3 (SD)%.

Precision:

The intra and inter assay precision was evaluated by multiple measurements of the same plasma pool sample in the same assay and in several different assays. On six duplicate determinations assayed on

the same day, the coefficient of variation was 4.8%. On 24 determinations assayed on different days, the coefficient of variation was 10.9%.

Accuracy:

Recovery experiments were made by adding 0,100,200, and 300 pg of progesterone in triplicate to 0.10 ml volumes of distilled water. The results are shown on Fig. 1. The analysis of variance gave the equation: pg of progesterone measured = 1.05 times pg of progesterone expected- 7.96. The correlation coefficient was $0.997 \pm 0.056$, $p < 0.001$.

Another experiment was performed to check the accuracy of our method. Increasing aliquots of pooled male plasma were assayed in triplicate at the same time without the use of chromatography. Fig. 2 shows the values of progesterone expected and those obtained when the aliquots increased from 0.25 ml to 1.00 ml. The analysis of variance gave the following equation: pg of progesterone measured = 1.04 times pg of progesterone expected - 2.63. The coefficient of correlation was $0.990 \pm 0.098$, $p < 0.005$.

Sensitivity:

The standard curves were constructed by plotting percent cpm free versus the logarithm of pg of progesterone added. At the 95% confidence limit, 25 pg was significantly different from 0 pg in every standard curve plotted. Fig. 3 shows a typical standard curve. The coefficient of variation at each point of the standard curve assayed in triplicate was always less then 6%.

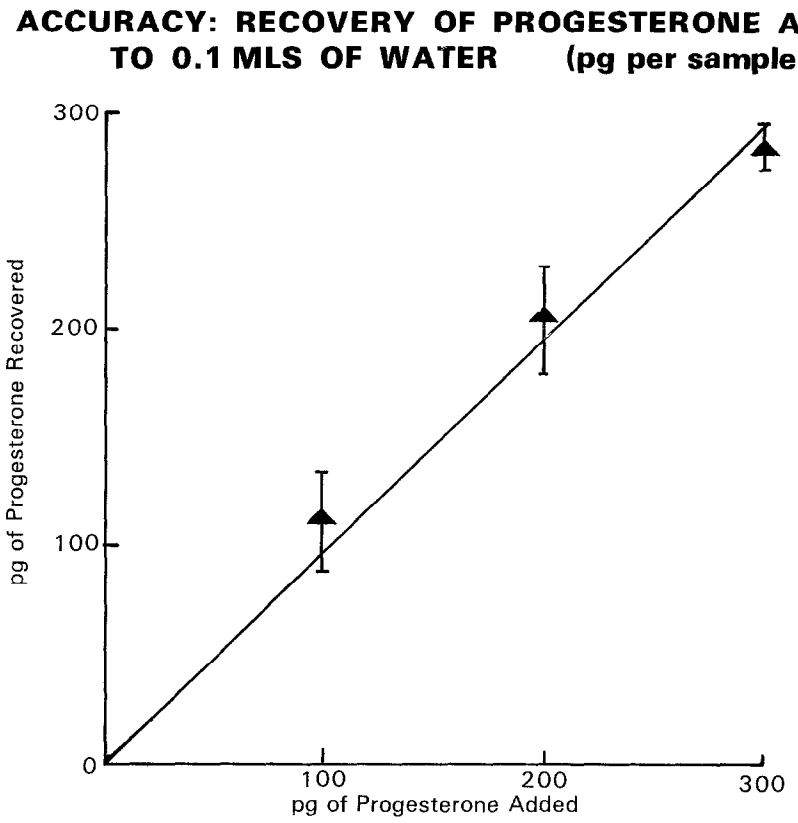


Fig 1. Recovery of progesterone added to 0.1 ml of water. Correlation coefficient = 0.997 ± 0.056 (SE), Y = 1.05X - 7.96, $p < 0.001$.

Blanks:

Double distilled water was used for blanks and one was run with each set of samples. When micro-columns were used, positive blank values were always obtained. The mean value for 10 blank samples run

using microcolumns as part of the purification step was 2.8 ± 1.1 (SD) pg per assay tube. When columns were omitted in the procedure, the blank value per assay tube was always zero.

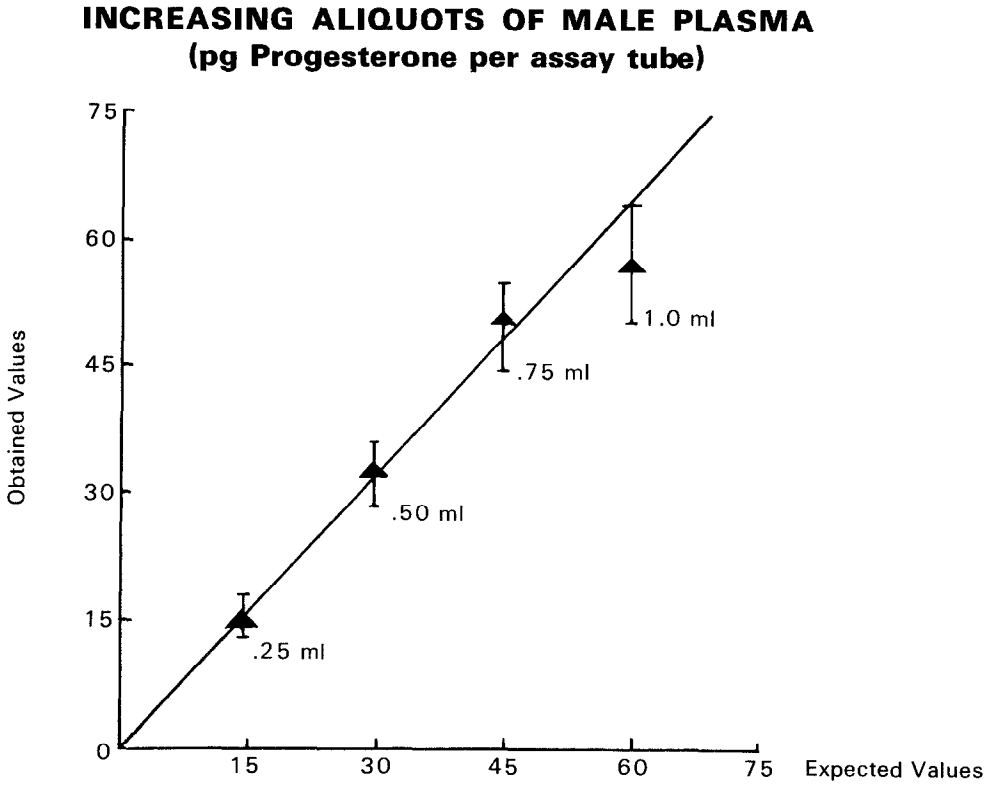


Fig 2. Accuracy Study. Values of progesterone expected and found when increasing aliquots of male plasma were assayed. Correlation coefficient = 0.990 ± 0.098 (SE), $y = 1.04x-2.63$, $p < 0.005$.

Specificity:

The specificity of a radioimmunoassay method is the uniqueness with which the antibody binds to the hormone. An antibody is specific

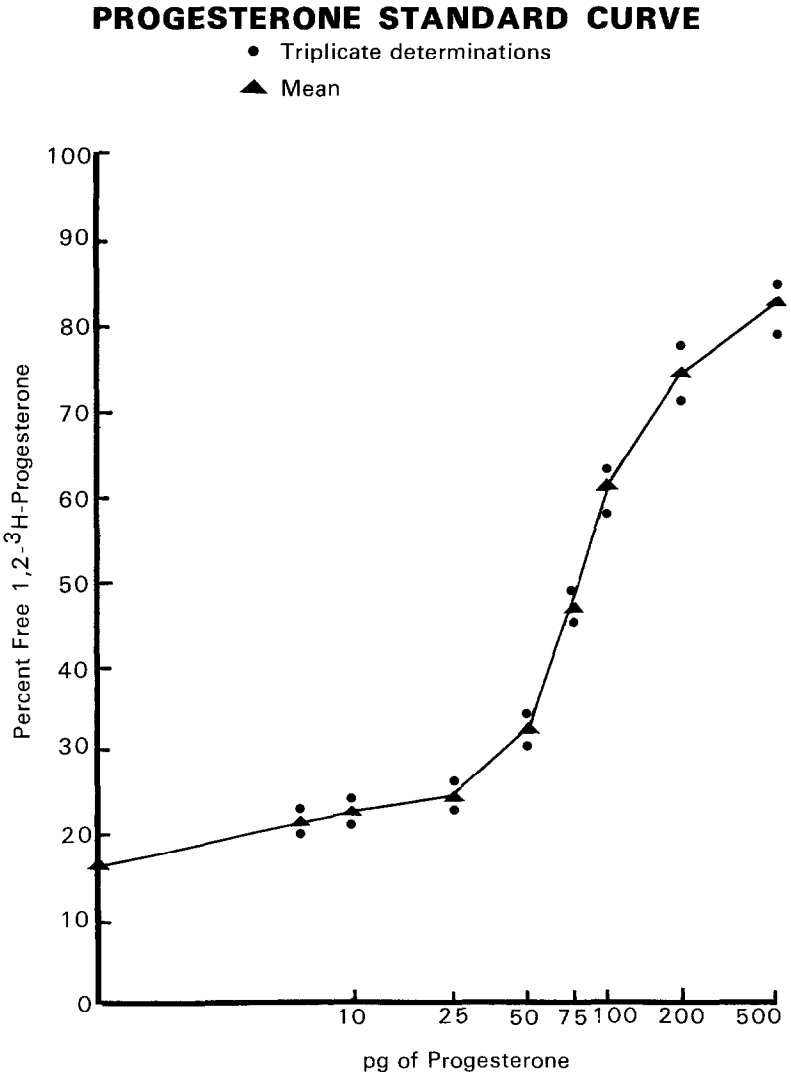


Fig 3. A typical standard curve for progesterone. The percent free of 1,2-$^{3}$H-progesterone is plotted as a function of the amount of unlabelled progesterone. The points represent the means of 3 replicates done in the same run.

only to plasma progesterone if it does not cross react with other steroids present in the plasma in significant concentrations.

The specificity of our antiserum was tested by its direct incubation with 26 different steroids. Percent cross reaction was calculated by the method of Abraham (20), which measures the displacement of 1,2-$^{3}$H-progesterone at the 50% bound line. Since our standard curve was plotted in terms of percent free, appropriate calculations were made to make our results comparable to those of Abraham et al. (10), Furuyama and Nugent (11) and others (12,14). Table I shows the percent cross reaction for the 26 steroids assayed and values previously obtained by other authors. The steroids tested in this study were chosen because they were present in the blood in significant concentrations, or because of their structural similarity to progesterone.

Plasma Progesterone Levels:

The levels of plasma progesterone in two normal male subjects varied from 17.5 ng/100 mls to 21.8 ng/100 mls. These values were obtained following our routine procedure without the use of column chromatography. Furuyama and Nugent (11) report a mean value of 22.6 ng/100 mls for male plasma progesterone, with values ranging from 9.2 to 42 ng/100 mls.

## DISCUSSION

The recovery of 1,2-$^{3}$H-progesterone in our assay improved from 84.8% to 88.9% when the use of the microcolumns was omitted.

TABLE I

% CROSS REACTION OF VARIOUS STEROIDS

| C18 Steroids | Our Paper | Nugent (11) | Abraham (10) | Spieler (12) | Lindner (14) |
|---|---|---|---|---|---|
| Estriol | <0.01 | <1 | <0.1 | | <0.3 |
| 17β-Estradiol | <0.01 | <1 | <0.1 | <0.2 | <0.3 |
| Estrone | <0.01 | <1 | <0.1 | <0.2 | <0.3 |
| **C19 Steroids** | | | | | |
| 4-Androstene-3,17-dione | <0.1 | | <0.1 | <0.2 | |
| 17β-Hydroxy-5α-androstan-3-one | <0.1 | | 2.3 | <0.2 | |
| Testosterone | <0.1 | <1 | 1.2 | <0.2 | |
| **C21 Steroids** | | | | | |
| 11α-Hydroxyprogesterone | 55 | | | 29 | 4.1 |
| Corticosterone | 0.8 | <1 | 2.6 | 0.8 | 0.4 |
| 11-Deoxycorticosterone | 2.3 | 4.7 | 35 | 3.0 | 8.5 |
| Aldosterone | <0.01 | <1 | <1 | <0.2 | |
| 3β-Hydroxy-5-pregnen-20-one | <0.1 | 7.3 | <0.1 | <0.2 | |
| Cortisol | <0.01 | <1 | 6.4 | <0.2 | |
| 17-Hydroxyprogesterone | 0.2 | 2.7 | 90 | 0.9 | 2 |
| 5-Pregnene-3β,20β-diol | <0.01 | | | | |
| 3β,17-Dihydroxy-5-pregnen-20-one | <0.01 | <1 | | | |
| 4-Pregnene-3,11,20-trione | 52 | | | | |
| Cortisone | <0.01 | | | <0.2 | |
| 5-Pregnene-3β,20α-diol | <0.01 | | <0.1 | | |
| 5-Pregnene-3β,17-20α-triol | <0.01 | | <0.1 | | |
| 20α-Hydroxy-4-pregnen-3-one | 0.1 | 1.5 | 1.2 | 1.8 | 8 |
| 17,21-Dihydroxy-4-pregnene-3,20-dione | <0.01 | <1 | | | |
| 3α,17,21-Trihydroxy-5β-pregnan-20-one | <0.01 | | <0.1 | | |
| 5β-Pregnane-3α,17,20α-triol | <0.01 | <1 | | | |
| 5α-Pregnane-3,20-dione | 6.5 | 51 | | 5.4 | 3 |
| 5β-Pregnane-3,20-dione | 4.8 | 8.5 | | | |
| Cholesterol | <0.01 | | <0.01 | | |

TABLE II

PROGESTERONE VALUES OF POOLED PLASMA AFTER RADIOIMMUNOASSAY WITH AND WITHOUT COLUMN CHROMATOGRAPHY (ng/100 mls)

| | Columns used | Columns omitted |
|---|---|---|
| Female plasma (Luteal Phase) | 546.3 ± 26.5 n=5 | 557.2 ± 20.8 n=5 |
| Female plasma (Follicular Phase) | 87.9 ± 9.6 n=24 | 93.3 ± 8.6 n=7 |
| Male plasma | 22.8 ± 4.4 n=13 | 21.8 ± 7.7 n=3 |

Furuyama and Nugent (11) reported a mean recovery of 66.1 ± 4.3 (SD)% for 66 samples assayed with the use of $Al_2O_3$ microcolumns. Abraham et al. (10) reported a mean recovery of 84.2 ± 4.8 (SD)% for 25 samples assayed using a Celite partition column for purification of the extracts. It could be concluded that recovery increases as the number os steps in an assay decreases.

The intra and inter assay precision of our method was very satisfactory. The mean obtained when the female pool (luteal phase) was assayed on the same day was 546.3 ± 26.4 ng/100 mls (N=24, coefficient of variation 10.9%). Furuyama and Nugent (11) examined intra assay precision by measuring progesterone concentration in pooled female plasma (follicular phase). The mean obtained was 18.1 ± 3.0 ng/100 mls (N=11, coefficient of variation 16.7%). Abraham et al. (10) obtained a coefficient of variation of 8.6% on 20 duplicate determinations

with values ranging from 3 to 45 ng/100 mls. In ten duplicate determinations in two different assays with values ranging from 9 to 43 ng/100 mls the coefficient of variation was 18%.

The sensitivity of our method (25 pg per sample) allows us to measure progesterone concentrations down to 5 ng/100 mls in 0.50 ml aliquots of plasma. This sensitivity is comparable to that of the authors mentioned above.

The experiments performed to check the accuracy of the method show very good correlation between the values expected and those obtained when known amounts of progesterone were added to blanks, and when increasing aliquots of pooled men plasma were assayed. Fig. 1 and 2 show the results. It should be noted that the amount of ether used for the extractions was increased as the volume of plasma increased. Since only two-thirds of the ether layer was separated each time, more than 10 volumes of ether had to be used to obtain a quantitative recovery. When less than 10 volumes of ether were used, lower values were obtained.

The double-distilled water blank values when columns were used were less than 3 pg per assay tube. When columns were not used, the blank value was always zero. Only once a positive blank value was obtained when columns were omitted. In this instance, the complete ether layer was separated and used (some of the interphase which contains ether and water was separated and used too). When the interphase

was avoided by using only two-thirds of the ether layer for the assay, blank values were zero.

A recent paper by Leyendecker et al. (21) reported repeated difficulties with blank values due to solvent residues and materials used for extraction and chromatography of serum samples. In spite of distillation and purification of solvents and materials it was impossible to eliminate blank values. Solvent residues in the incubation mixture seemed to exert some kind of effect on binding protein which could not by corrected for by subtracting a blank value for all samples. To eliminate the blank value in their assay, they had to protect binding proteins by diluting the small amount of binding protein in each assay tube with a large amount of gamma globulin.

In our method, omission of microcolumns and care in separating the ether from the lower layer (water or plasma) was the answer to the problem of eliminating positive water blank values.

Affinity is defined as the avidity with which an antibody binds to the antigen. Our antibody binds progesterone with an affinity about 1,000 times stronger than those steroids present in the blood in significant concentrations such as cholesterol, cortisol, 17$\beta$-estradiol, testosterone, 17-hydroxyprogesterone, 4-androstene-3,17-dione, etc. (22). The only two steroids that displayed considerable cross reaction were 11$\alpha$-hydroxyprogesterone (55%) and 4-pregnene, 3,11,20-trione (52%). This was expected since the protein molecule is attached at the

11 position of the hapten. However, these steroids are not secreted into the bloodstream. Therefore, the high degree of cross reactivity is irrelevant. The two other steroids that cross react to some extent, 5α and 5β-pregnane,3,20-dione (6.5% and 4.8%) are not present in the blood either (11). Furuyama and Nugent (11) obtained 51% cross reaction with 5α-pregnane,3,20-dione and 8.5% cross reaction with 5β-pregnane,3,20-dione. Of the remaining naturally occurring steroids, deoxycorticosterone (2.3%), corticosterone (0.8%) and 17-hydroxyprogesterone cross reacted to a small extent. The rest of the 26 steroids tested showed no cross reaction ($< 0.1\%$). The results show the high specificity of our antibody, which allows us to assay our samples without separating the other steroids that are extracted in the ether along with progesterone.

In the recent progesterone radioimmunoassay papers of Abraham et al. (10) and Furuyama and Nugent (11) the lack of specificity of the antibody necessitated sample purification prior to measurement. Abraham et al. (10) used a conjugate of the 21-hemisuccinate of 11-deoxycortisol coupled to human serum albumin. The specificity at the 21 position and neighboring 17 position was therefore destroyed. This can be seen from their cross reaction results. Two steroids showed 90% cross reaction: 17-hydroxyprogesterone and 11-deoxycortisol; 11-deoxycorticosterone (DOC) cross reacted to a significant extent (35%). These three steroids are present in the blood in significant concentrations;

therefore, chromatography is required for separation before radioimmunoassay can be performed.

In Furuyama and Nugent's case, the 3-oxime of progesterone was conjugated to BSA at the C-3 position of the progesterone molecule (11), thus, destroying the specificity at this position. They mentioned that omission of microcolumns resulted in values of progesterone double to those found when their routine method was used. This suggests that there are one or more steroids present in the blood that need to be separated by chromatography before assay since they seem to cross react with their antisera.

The specificity of the antisera used in the progesterone radioimmunoassay method of Spieler, et al. (12) is comparable to the specificity of our antisera, since their preparation of the antigen molecule is identical to ours. Their method was designed to measure progesterone levels in female rhesus monkeys which contain higher progesterone levels when compared to human values. The sensitivity of their method appears unsatisfactory for measuring human progesterone concentrations, except during pregnancy.

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