A Radio-immunoassay of 17-alpha-Hydroxyprogesterone

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SUMMARY

A method is described for the determination of 17α-hydroxyprogesterone (17-OHP) in plasma. Antisera were raised in rabbits against 11-deoxy cortisol-21-hemisuccinate-bovine serum albumin (11-DOC-21-HS-BSA) and 17α-hydroxyprogesterone-3-carboxymethyloxime-bovine serum albumin (17-OHP-3-CMO-BSA). An antiserum to 11-DOC-21-HS-BSA exhibited cross-reaction with progesterone (29%), 11-deoxycortisol (100%), cortisol (17%) and testosterone (10%) and was therefore not appropriate for quantitation of 17-OHP in plasma. An antiserum to 17-OHP-3-CMO-BSA cross-reacted with progesterone (9.7%), 11-deoxycortisol (50%) and less than 1% with all other major naturally occurring steroid hormones. A radio-immunoassay (RIA) was developed using the antiserum to 17-OHP-4-CMO-BSA. The intra-assay and interassay coefficients of variation were 7.2% and 10.5% respectively. The normal ranges (nmol/l plasma) of samples extracted with hexane:benzene (1:1) and purified by Sephadex LH-20 chromatography were 0.28 - 4.7 for men, 0.84 - 3.0 for women (follicular phase), 3.0 - 11.0 for pregnant women, 18.5 - 123.9 for cord blood, 0.12 - 5.0 for children and 56.3 - 1.032 for persons with congenital adrenal hyperplasia (CAH) due to a 21-hydroxylation enzyme defect. Sephadex LH-20 purification of plasma extracts could be omitted when using the RIA as a screening procedure for CAH due to a 21-hydroxylation enzyme defect.


Radio-immunoassay (RIA) is a widely used method for the measurement of steroids in biological fluids. Several methods have been reported for the measurement of 17α-hydroxyprogesterone (17-OHP) using antibodies raised against 17-OHP conjugated in different sites. Owing to substantial cross-reaction of the antibody to 17-OHP with other naturally occurring steroids, especially progesterone, purification of the sample by thin-layer chromatography or column chromatography on Celite or Sephadex LH-20 is necessary. In this article we describe the production of antiserum to 17α-hydroxyprogesterone-3-carboxymethylxime-bovine serum albumin (17-OHP-3-CMO-BSA) and 11-deoxycortisol-21-hemisuccinate-bovine serum albumin (11-DOC-21-HS-BSA) and the development of the RIA for plasma 17-OHP.

MATERIALS AND METHODS

Phosphate-buffered saline (PBS) (126 mM disodium hydrogen phosphate, 37 mM sodium dihydrogen phosphate, 154 mM sodium chloride, pH 7.0) was made up in de-ionized glass-distilled water. Gelatin PBS was prepared by dissolving gelatin (0.1% w/v) in PBS. Dextran-coated charcoal solution consisted of Dextran T-70 (Pharmacia, Uppsala) (0.25% w/v) and activated charcoal (Sigma Chemical Co., St Louis) (2.5% w/v) in PBS. Progesterone, 17-OHP, 17α-acetoxy-4-pregnen-3,20-dione, 11-deoxycortisol, 11-deoxycorticosterone, cortisol, testosterone, oestradiol and pregnenalone were obtained from Sigma Chemical Co. or Ikapharm, Tel Aviv. 1,2-H-17-OHP, specific activity 40.4 Ci/mmol, from New England Nuclear Corp., Boston, was diluted with ethanol, an aliquot was dried under nitrogen and the residue dissolved in 1% methanol in PBS (methanol PBS) to give approximately 20 000 dpm/100 μl. Carbodimide hydrochloride (1-ethyl-3-(3-dimethyl-amino-propyl)) and O-carboxymethyl hydroxylamine hemihydrochloride were from Sigma Chemical Co. All other reagents were analytical grade.

Preparation of the Steroid Derivatives

17-OHP-3-CMO: 17α-acetoxy-4-pregnen-3,20-dione (1.35 mmol) and O-carboxymethyl hydroxylamine hemihydrochloride (1 mmol) were dissolved in anhydrous pyridine (40 ml) and allowed to stand overnight at room temperature. The pyridine was evaporated under reduced pressure. The residue was dissolved in chloroform (50 ml) and washed with water (4 × 15 ml). The chloroform was evaporated and the extract dissolved in 5 ml 10% aqueous pyridine, to which potassium hydroxide (8.9 mmol) was added and the reaction mixture was allowed to stand at 40°C in a nitrogen atmosphere for 48 hours. The pyridine was evaporated, 20 ml water added, and the pH adjusted to 3.0 with concentrated hydrochloric acid, causing precipitation of 17-OHP-3-CMO, which was washed with water. Thin-layer chromatography on silica gel with methylene chloride:acetone (8:2) showed a single compound. Mass spectroscopy showed a pure product and confirmed the molecular weight to be 403.5 with the first degradation product being 17-OHP-3-methyl oxime (MW 360). The ultraviolet absorption had a λmax of 252 nm.

11-DOC-21-HS: 11-deoxy-cortisol (1.44 mmol) and succinic anhydride (5.0 mmol) were dissolved in 5 ml pyridine and kept at 75°C for 3 hours and then at room temperature overnight. The mixture was extracted with chloroform (20 ml × 3) and washed with 200 ml water. The chloroform was evaporated at 100°C and the residue...
dissolved in 3 ml acetone. On reduction of the volume of acetone, crystallization occurred. The crystals were filtered and recrystallized from acetone. Thin-layer chromatography on silica gel with methylene chloride : acetone (8 : 2) showed a single compound (R: 46,6).

Conjugation to Bovine Serum Albumin (BSA)
The derivatives (100 mg) were each dissolved in 5 ml 50% aqueous pyridine and mixed with 300 mg carbodiimide dissolved in 50% aqueous pyridine, by stirring at room temperature for 30 minutes. BSA (Miles Laboratories, Cape Town) (60 ng/ml water) was added dropwise, with stirring, over 10 minutes. The mixture was stirred at room temperature for 2 hours, dialysed against tap water for 24 hours and lyophilized.

Preparation of Antiserum
Three 3-6-month-old mixed-breed rabbits were injected with the freeze-dried antigen (0,6 mg) dissolved in saline (0,1 ml) and emulsified with 1 ml Freund's complete adjuvant in multiple intradermal sites. Booster injections with conjugate in incomplete adjuvant were administered when no further increase in titre was noted.

RIA Method
The procedure was similar to those described. Plasma samples were stored at -20°C until assay. For recovery estimates ~2000 dpm ³H-17-OHP was added to serum aliquots (0,5 ml), which were then extracted by shaking in stoppered glass tubes with hexane : benzene (1 : 1) for 20 minutes. The aqueous phase was frozen in acetone - dry ice, the organic phase was decanted and dried under a stream of nitrogen at 40°C. The residue was dissolved in 3 drops benzene : methanol (95 : 5), 2 drops of azobenzene 0,1% w/v in benzene : methanol (95 : 5) were added and the mixture applied to columns (0,5 x 28 cm) containing 4 ml Sephadex LH-20 previously equilibrated with benzene : methanol (95 : 5). The tubes were washed with a further 3 drops of the same solvent which was transferred to the columns, which were then eluted with benzene : methanol (95 : 5). The yellow azobenzene fraction containing progesterone and the following 0,6 ml were discarded. The next 2 ml were collected, evaporated to dryness under a stream of nitrogen at 40°C and dissolved in 1 ml acetone. An aliquot (0,5 ml) was dried in a counting vial, 10 ml Demilume (Packard Instrument Co., Downers Grove, Ill.) added and the radioactivity determined for recovery. Other aliquots of 0,1 or 0,2 ml (depending on 17-OHP concentration) were pipetted into RIA glass tubes in duplicate, evaporated to dryness and redissolved in 100 µl methanol-PBS. Duplicate standards in methanol-PBS were set up covering the range 0,009 - 5,0 pmol 17-OHP per tube. Antiserum 100 µl (1 in 200) was added, followed by ³H-17-OHP (~20 000 dpm) and then incubated at 37°C for 60 minutes. After cooling to 4°C, dextran-coated charcoal solution (0,75 ml) was added, the tubes were allowed to stand for 10 minutes at 4°C and centri-

fuged at 2 000 g for 10 minutes at 4°C. The supernatant was decanted into vials containing 10 ml Demilume and the radioactivity determined by liquid scintillation spectrometry. For each plasma sample, the value of bound ³H-17-OHP was calculated and the 17-OHP concentration read from the standard curve. Plasma 17-OHP was calculated as follows:

\[
\text{plasma 17-OHP} = \frac{C}{R \times A \times P} \text{ nmol/l,}
\]

where \( R \) = fractional recovery, \( A \) = acetone aliquot volume (ml), \( P \) = volume of plasma extracted (ml), and \( C \) = 17-OHP content of aliquot (pmol).

**RESULTS**

**Standard curve:** Under the conditions employed in our assay increasing quantities of unlabelled 17-OHP in the range 0,009 - 5,0 pmol per tube displaced ³H-17-OHP. A representative standard curve using an antiserum raised against 17-OHP-3-CMO-BSA is shown in Fig. 1.

![Fig. 1. Representative standard curve for 17-OHP using an antiserum to 17-OHP-3-CMO-BSA at a final dilution of 1 : 600 (v/v).](image)

**Sensitivity:** At the 95% confidence limits 0,09 pmol 17-OHP caused significant displacement of ³H-17-OHP when compared with the zero.

**Precision:** The intra- and inter-assay coefficients of variation over the range of the assay were 7,2% \((N = 6)\) and 10,5% \((N = 11)\) respectively.

**Accuracy:** The accuracy of the method was established by adding 17-OHP to plasma containing 0,27 pmol 17-OHP/100 µl. When 0,08, 0,64 and 2,5 pmol 17-OHP were added to 100 µl plasma, the mean volume values \((± SEM)\) obtained after subtraction of the endogenous 17-OHP were 0,10 ± 0,01, 0,68 ± 0,01 and 2,21 ± 0,18 pmol respectively.
Specificity: Cross-reaction of the two different antisera as calculated by the comparative mass of steroid required to displace 50% of the $^3$H-17-OHP bound to antibody is set out in Table I. The antiserum raised against 17-OHP-3-CMO-BSA exhibited less than 10% cross-reaction with progesterone and 11-deoxycortisol and less than 1% with other naturally occurring steroids. By contrast, the antiserum against 11-DOC-21-HS-BSA exhibited high cross-reactivity with most of the steroid hormones tested.

**TABLE I. SPECIFICITY OF THE ANTISERA**

<table>
<thead>
<tr>
<th>Cross-reaction (%) of antisera against</th>
<th>17-OHP-3-CMO-BSA</th>
<th>11-DOC-21-HS-BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-OHP</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Progesterone</td>
<td>9,7</td>
<td>29</td>
</tr>
<tr>
<td>11-deoxycortisol</td>
<td>5,0</td>
<td>100</td>
</tr>
<tr>
<td>11-deoxycorticosterone</td>
<td>&lt;1</td>
<td>25</td>
</tr>
<tr>
<td>Cortisol</td>
<td>&lt;1</td>
<td>17</td>
</tr>
<tr>
<td>Testosterone</td>
<td>&lt;1</td>
<td>10</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

Recovery of labelled internal standard: The amount of $^3$H-17-OHP recovered from plasma samples after extraction and chromatography ranged from 65% to 96% with a mean of 84,7% (N = 132).

**Plasma 17-OHP concentrations**: The mean and range of 17-OHP concentrations in plasma from normal men, women and children and from children with congenital adrenal hyperplasia (CAH) is shown in Table II. Since the plasma concentration of progesterone is approximately 7-fold greater than that of 17-OHP during the luteal phase of the menstrual cycle and approximately 20-fold greater during pregnancy, an antiserum having a cross-reactivity of 9,7% with progesterone would give rise to substantial overestimation of 17-OHP. Removal of progesterone by column chromatography is therefore essential. However, in 11 plasma samples from normal men, prepubertal children and children with CAH due to 21-hydroxylation enzyme deficiency, column chromatographic removal of progesterone was not essential. When comparing the values of 17-OHP obtained with and without chromatographic purification of these samples, a correlation coefficient ($r$) of 0,97 and a slope of 0,73 was obtained. The range of values was 0,8 - 1 032 nmol/l.

**DISCUSSION**

Antisera binding 17-OHP were produced by injecting rabbits with 17-OHP conjugated to BSA via carbon atom C$_6$ and 11-deoxycortisol conjugated via carbon atom C$_19$. Conjugation via C$_6$ tends to obscure functional sites which would distinguish 17-OHP from other naturally occurring steroids, differing in the region C$_{17}$ - C$_{19}$. Thus, as anticipated cross-reaction with progesterone (differs at C$_{11}$), 11-deoxycortisol (differs at C$_{19}$), 11-deoxycorticosterone (differs at C$_{11}$ and C$_{19}$), cortisol (differs at C$_{11}$ and C$_{19}$) and testosterone (differs at C$_{19}$) was high. Correspondingly, conjugation via C$_6$ gave rise to a specific antisera since the functional sites (C$_{17}$, C$_{19}$ and C$_{21}$) which distinguish the other steroids from 17-OHP are well exposed and unaltered. Thus, this antisera is potentially more able to specifically quantitate 17-OHP in plasma extracts.

The RIA developed using the antiserum raised against 17-OHP-3-CMO-BSA compares favourably with published methods in specificity, sensitivity, accuracy and precision.$^{5,8}$

The means and ranges of plasma 17-OHP concentrations for the different groups of people agree with those previously described.$^{5,8}$ The correlation of 17-OHP concentrations obtained with and without chromatography in samples containing relatively low concentrations of progesterone was good, indicating that 17-OHP may be determined without chromatography in the screening of infants for CAH. It is known that plasma progesterone is raised in CAH, but only to approximately 5% of the 17-OHP level.$^9$

In summary, we have produced a specific antiserum for 17-OHP by immunization of rabbits with 17-OHP-3-CMO-BSA, which can be used to assay 17-OHP in plasma extracts without chromatography in infants with CAH due to 21-hydroxylation enzyme deficiency. Thus the assay can be used as a simple screening procedure for CAH and for monitoring therapeutic response. However, measurement of 17-OHP in pregnancy or during the luteal phase of the menstrual cycle requires column chromatographic removal of progesterone.

**TABLE II. MEAN AND RANGE OF VALUES FOR 17-OHP IN PLASMA**

<table>
<thead>
<tr>
<th>Number of determinations</th>
<th>17-OHP (nmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
</tr>
<tr>
<td>Men</td>
<td>0,28 - 4,7</td>
</tr>
<tr>
<td>Women</td>
<td>0,84 - 3,0</td>
</tr>
<tr>
<td>Follicular phase</td>
<td>3,0 - 11</td>
</tr>
<tr>
<td>Luteal phase</td>
<td>4,6 - 22</td>
</tr>
<tr>
<td>Pregnancy 6 - 40 weeks</td>
<td>18 - 124</td>
</tr>
<tr>
<td>Cord blood</td>
<td>0,12 - 5,0</td>
</tr>
<tr>
<td>Children (2 days - 10 yrs)</td>
<td>56 - 1 030</td>
</tr>
</tbody>
</table>

* Due to 21-hydroxylase enzyme deficiency.
The excellent technical assistance of Di Searle, Ilse Wegener and John Morta is greatly appreciated.

REFERENCES

Maternal Post-delivery Weight in the Assessment of Obesity

SUMMARY
Maternal weight recorded on the day after delivery correlated significantly with maternal skinfold thickness measured at multiple sites. Skinfold thickness probably reflects total body fat stores. It is suggested that the relationship between maternal weight and fetal birth weight may be mediated by the amount of maternal fat available to meet the energy requirements of the growing fetus.


During pregnancy, most women accumulate subcutaneous fat which contributes to their overall weight gain. It is therefore frequently assumed that maternal weight at delivery, and especially immediately after delivery, reflects the total maternal fat store. This assumption that post-delivery weight is an index of relative obesity remains unproven. Other factors such as muscle bulk, skeletal weight and extracellular fluid volume may also influence maternal weight.

METHODS
The distribution of post-delivery weight for Coloured primigravidas in Cape Town was used to define light, intermediate and heavy women. Women below the 25th or above the 75th percentiles were regarded as light and heavy respectively, while women between these percentiles were regarded as being of intermediate weight. Consecutive primigravidas giving birth to liveborn, singleton infants at term were weighed on the day after delivery to obtain a quota of 15 light, 15 intermediate and 15 heavy mothers. The height of each woman was also measured, and the weight/height ratio calculated. Skinfold thickness was measured at multiple sites on the left side of the body using a Harpenden caliper. A standard method was used to determine skinfold thickness at triceps, subscapular, supra-iliac and mid-thigh sites. Abdominal skinfold thickness was measured 3 cm lateral to the umbilicus. In addition, a total skinfold thickness measurement was determined.

Student's unpaired t test was used in comparing the three study groups.

RESULTS
The size of the mothers in the heavy, intermediate and light groups is shown in Table I. By design, there was a significant difference between the weights of the women in the three study groups. The heavy women were significantly taller than either the intermediate or the light women, while the weight/height ratio was significantly different between groups. The individual and total skinfold thicknesses were significantly different between groups, but the difference was less marked at the triceps site.

DISCUSSION
Taggart et al. have documented the pattern of increasing skinfold thickness during pregnancy. They found a characteristic distribution of subcutaneous fat due to the accumulation of adipose tissue over the trunk, buttocks and upper thighs, with sparing of the arms and lower legs. They also showed a good correlation between skinfold thickness and body weight in early pregnancy. The correlation between skinfold thickness and weight at term was not, however, assessed.

The effect of oedema on skinfold thickness in pregnancy is unclear, but Taggart et al. found that oedema was not associated with a greater increase in skinfold thickness in late pregnancy or with a greater loss in skinfold thickness after delivery.