Radioimmunoassay for 17α-Hydroxyprogesterone in Plasma, for Diagnosing and Managing Congenital Adrenal Hyperplasia

K. H. James Yeo and Wendy J. Whittau

This rapid, inexpensive, and sensitive radioimmunoassay (RIA) for plasma 17α-hydroxyprogesterone involves radioiodination. A single extraction with toluene/hexane removes an average 93% of the hormone from 0.1 mL of plasma. The extract is evaporated and the hormone is estimated by a simple, precise, and accurate $^{125}$I RIA involving a specific rabbit antiserum. A suspension of dextran-coated charcoal is used to separate free and bound steroid. Inter- and intra-assay CVs were <15 and <10%, respectively, and the sensitivity was 3 pg per assay tube. The regression equation for data on 17α-hydroxyprogesterone added to steroid-free plasma was $y = 0.94x + 2.2$ ($r = 0.99$). However, the turnaround time is only one-half to one-tenth that for most $^{3}$H RIA (3 h vs 6 to 30 h). The ranges of values found for plasma from normal subjects, treated and untreated patients with congenital adrenal hyperplasia, and infants with recently detected congenital adrenal hyperplasia were, respectively, 1 to 11, 0 to 20, 30 to 620, and 270 to 4900 nmol/L.

Additional Keyphrases: steroids · $^{3}$H and $^{125}$I radioimmunoassays compared · reference interval

Plasma 17P is measured to aid the diagnosis of CAH with 21-hydroxylase deficiency (1–5). Most methods for such estimation require extraction with organic solvent or column chromatography followed by RIA, with tritiated 17P as tracer (5–10). Here we describe a rapid assay with $^{125}$I tracer, involving a single extraction with toluene/hexane, then proceeding directly to RIA in which specific rabbit antiserum raised against 17P-3-CMO-BSA is used. This assay is much faster (~3 h) than most $^{3}$H RIAs reported, and it costs significantly less than the corresponding $^{3}$H assay.

Materials and Methods

Materials

Chemicals. Bovine gamma-globulin (Cohn Fraction II), bovine serum albumin (Cohn Fraction V), 17P, and activated neutralized charcoal were purchased from Sigma Chemical Co., St. Louis, MO 63178; 17P-3-CMO-BSA from Steraloids Inc., Wilton, NH 03806; Dextran T-70 and Sephadex LH-20 were from Pharmacia Fine Chemicals AB,Upsala, Sweden; and silica gel 60 F$_{254}$ thin-layer chromatography plates (art. 5554) from E. Merck, Darmstadt, F.R.G. Other buffer chemicals, from BDH Chemicals Ltd., Poole, U.K., were of analytical grade. Toluene from J. T. Baker Chemical Co., Phillipsburg, NJ 08865, was used as supplied, but hexane and methanol were glass-distilled. [1,2,6,7-3H]17P (specific activity: 55 kCi/mol) and Na$^{125}$I (13.4 kCi/g) were from Amersham International, Bucks., U.K. We purified $^{3}$H-17P by thin-layer chromatography, using chloroform/ethyl acetate (4/1 by vol) as the developing solvent. Glass-distilled water was used throughout, and all reagents were stored at 4 °C.

Reagents

Assay buffer: Boric acid (0.1 mol/L, pH 7.4) containing, per liter, 1 g of bovine serum albumin, 0.5 g of bovine gamma-globulin, and 1 g of sodium azide.

Gelatin buffer: Boric acid (0.1 mol/L, pH 7.4) containing 1 g each of gelatin and sodium azide per liter.

Acetate buffer: 0.1 mol/L, pH 5.0, containing 1 g of sodium azide per liter.

Dextran-coated charcoal: Mix 1.0 g of Dextran T-70 and 5.0 g of activated neutralized charcoal in 1 L of gelatin buffer.

17P standards. Standard solutions, 5 to 400 pg per tube, were prepared in assay buffer from a 100 ng/mL stock solution of 17P.

Procedure

Rabbit antiserum to 17P. A single 250-μg dose of 17P-3-CMO-BSA, in complete Freund’s adjuvant, was given by multiple intradermal injection to four New Zealand White rabbits. Antisera were harvested 4.5 months later. The serum showing the highest titer was stored at −80 °C. Antiserum diluted 100-fold in assay buffer was stored at −20 °C.

Samples. Each of 114 blood specimens, anticoagulated with EDTA, was promptly centrifuged and the plasma stored at −20 °C. Steroid-free plasma was prepared by mixing 20 mL of plasma with 1 g of Amberlite XAD-2 polymeric beads (BDH Chemicals Ltd.) at 37 °C for 2 h. The slurry was filtered and this treatment was repeated three times.

Radioiodination of tracer. 17P-3-CMO, prepared by the method of Janoeki et al. (11), was purified by thin-layer chromatography on silica gel, with equal volumes of toluene, methanol, and acetone as the developer. The derivative was radioiodinated by the method of Nars and Hunter (12), and further purified by chromatography through a 48 × 1.2 (i.d.) cm column of Sephadex LH-20 at 4 °C, being eluted with methanol and the eluate collected in 2-min fractions at a flow rate of 0.8 mL/min. The major product appeared between fractions 50 to 60, while free iodide was between fractions 70 to 80 (see Figure 1). The tracer (specific activity 315 kCi/mol) was stored at 4 °C.

Proposed method. To 100-μL plasma samples in glass tubes, add 0.9 mL of the pH 5.0 acetate buffer and 4.0 mL of toluene/hexane (1/4 by vol). Mix by rotating the tubes horizontally at 30 rpm for 10 min. Transfer 0.4 mL of the upper layer into glass 75 × 12 mm assay tubes, evaporate under air in a 40 °C water bath, and reconstitute the residue with 100 μL of assay buffer.

Place standards, 100 μL each, into additional assay tubes.
Add 100 μL of [125I]17P tracer (40 000 dpm) and 100 μL of antiserum solution, diluted 28 000-fold, in assay buffer. Vortex-mix the contents of the tubes, and incubate at 37 °C for 15 min, then at 4 °C for 15 min. Add 1.0 mL of well-stirred, cold, dextran-coated charcoal suspension, allow to stand at 4 °C for 10 min, and centrifuge (1720 × g, 4 °C, 15 min). Discard the supernate and count the radioactivity of the charcoal-bound steroid.

Results

Figure 2 illustrates a typical standard curve. The sensitivity (i.e., the smallest amount of unlabeled 17P that can be distinguished from zero with 95% confidence) was 3.1 pg per assay tube. The specificity is summarized in Table 1, which shows the cross reactivities as calculated by comparing the standard curves for 17P or related steroids in assay buffer (13).

The interassay CVs, assessed by measuring 17P at 15 and 100 nmol/L in steroid-free plasma (used for internal quality control) in 16 assays, were 14.6% and 7.4%, respectively. The corresponding intra-assay CVs, from analysis of seven replicates in duplicates, were 6.7% and 3.5%, respectively.

To assess extraction recovery, we added tritiated 17P (2500 dpm) to plasma samples. For the extracts, recoveries were 93.6% (SD 10.6%, n = 190). The same tracer (5 × 10^6 dpm) was added into 4 mL of normal plasma, incubated at 37 °C for 30 min, and extracted; analytical recovery was 93.5% (SD 1.6%, n = 15).

For data on quadruplicate analyses of five steroid-free plasmas with added 17P ranging from 5 to 250 nmol/L, the regression equation was y = 0.94x + 2.2 (r = 0.99), where x is the exogenous 17P concentration (see Figure 3).

The range for 30 normal subjects, aged 10 to 65 years, was 1.1–11.3 nmol/L (mean 4.7, SD 2.4). Untreated infants with CAH had very high 17P concentrations (270 to 4900 nmol/L), while treated CAH patients had values ranging from 0 to 20 nmol/L. However, a group of untreated CAH patients had values ranging from 30 to 620 nmol/L (see Figure 4).

Discussion

We used a solvent mixture of toluene/hexane (1/4 by vol) for extraction rather than the polar solvent dichloromethane (6, 7) and obtained an extraction recovery of 93%, as measured by use of tritiated 17P.

Our radioligand has the carboxymethylxime bridge at the C-3 position of the steroid molecule to facilitate recognition of the tracer to the antibody (14). Further purification of this radioiodinated product through the Sephadex LH-20 column removed the free iodide, resulting in a standard curve with a steeper slope.

The proposed assay fulfills Abraham's criteria for assay practicability (15). The turnaround time is at least two- to 10-fold faster than for most 3H-based assays (1, 7, 8), and the cost of liquid scintillation cocktails and vials is eliminated.

Table 1. Cross Reactivity of Steroids in the [125I]17P Assay for 17α-Hydroxyprogesterone*

<table>
<thead>
<tr>
<th>Steroids</th>
<th>% cross reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>17P</td>
<td>100</td>
</tr>
<tr>
<td>17α-Hydroxyprogrenolone</td>
<td>7.47</td>
</tr>
<tr>
<td>Progesterone</td>
<td>3.33</td>
</tr>
<tr>
<td>5α-Pregnanedione</td>
<td>1.50</td>
</tr>
<tr>
<td>11-Deoxycortisol</td>
<td>1.05</td>
</tr>
<tr>
<td>21-Deoxycortisol</td>
<td>3.42</td>
</tr>
<tr>
<td>Prednisone</td>
<td>1.09</td>
</tr>
<tr>
<td>5β-Pregnanedione</td>
<td>0.52</td>
</tr>
<tr>
<td>Deoxycorticosterone</td>
<td>0.28</td>
</tr>
<tr>
<td>17α,20α-Dihydroxyprogesterone</td>
<td>0.095</td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>0.041</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>0.026</td>
</tr>
<tr>
<td>Cortisone</td>
<td>0.016</td>
</tr>
<tr>
<td>Cortisol</td>
<td>0.019</td>
</tr>
</tbody>
</table>

* Aldosterone, cholesterol, dehydroepiandrosterone, testosterone, 17β-estradiol, and prednisolone cross reacted by <0.01%.
range of concentrations of 17P, whereas treated CAH patients had low or near-normal 17P concentrations. We suggest that this simple assay is useful for a rapid diagnosis in patients with CAH and in infants having ambiguous genitalia or suspected of having CAH.

We thank Dr. M. S. Scandrett for his technical advice; Mr. W. A. Sedler for designing the computer system for the assay calculation; and Drs. M. G. Metzal and M. H. Abermethy for their helpful suggestions in the preparation of this manuscript.

References