Radioimmunoassay of 17α-Hydroxyprogesterone in Saliva, Parotid Fluid, and Plasma of Congenital Adrenal Hyperplasia Patients

Richard F. Walker, Graham F. Read, Ieuan A. Hughes, and Diana Riad-Fahmy

We report a radioimmunoassay sensitive enough to determine 17α-hydroxyprogesterone concentrations in 200 µL of parotid fluid or mixed whole saliva. Because the correlation of concentrations in matched samples of parotid fluid and saliva was excellent (r = 0.98), we exclusively used saliva, which is easier to collect, in later studies. The assay is specific; saliva samples assayed with and without thin-layer chromatographic purification showed no significant difference. The assay is also precise, and has a lower limit of sensitivity of 4 pg per assay tube. In 14 patients having congenital adrenal hyperplasia from a C21-hydroxylase enzyme deficiency, all of whom were receiving cortisol replacement therapy, the range in 17α-hydroxyprogesterone concentrations observed in saliva (67–26 300 pmol/L) was about 20-fold that seen in 32 healthy children (90–1520 pmol/L). The close correlation (r = 0.91) between 17α-hydroxyprogesterone concentrations in matched samples of saliva and plasma from these patients indicates that determination of steroids in saliva could well replace determination in plasma. This concept is supported by 17α-hydroxyprogesterone concentrations monitored throughout 24 h from one patient and following stimulation with synthetic corticotropin in another patient.

Additional Keyphrases: diagnosis of congenital adrenal hyperplasia • steroids • monitoring therapy • pediatric chemistry • normal values

Plasma concentrations of 17α-hydroxyprogesterone are valuable (1, 2) in the initial diagnosis of congenital adrenal hyperplasia (CAH) in newborn infants. This common inborn error of metabolism is usually characterized by deficiency in the C21-hydroxylase enzyme system, and necessitates steroid replacement therapy. Adequacy of treatment has been monitored by determining circulating 17α-hydroxyprogesterone concentrations (3, 4). Because of the notorious difficulty of venipuncture in children, and because serial samples are often required, determination of 17α-hydroxyprogesterone concentrations in saliva could be of great value.

Recent studies at the Tenovus Institute indicate that salivary cortisol concentrations can be used not only to assess adrenal function but also to determine potency of the hypothalamic–pituitary–adrenal axis after stimulation and suppression tests (5, 6). Concentrations of steroid hormones in saliva reflect the free hormone rather than the protein-bound fraction in plasma (7) and are therefore low. Radioimmunoassay (RIA), which combines high sample throughput with the required specificity and sensitivity, is a technique well suited to salivary steroid determinations.

This paper describes an RIA for 17α-hydroxyprogesterone in 200 µL of whole saliva. In the assay a tritiated ligand is used and dextran-coated charcoal is used to separate antibody-bound and free steroid. Using this method, we determined concentrations of 17α-hydroxyprogesterone in matched samples of whole saliva, parotid fluid, and plasma collected from a group of treated CAH patients attending a pediatric outpatient clinic. A provisional “normal range” for 17α-hydroxyprogesterone in mixed whole saliva was determined by assaying samples collected from 32 apparently healthy infants and children.

Materials and Methods

Patients

Fourteen patients, one to 16 years old and having a C21-hydroxylase enzyme deficiency, were investigated. For replacement therapy all patients received cortisol (15–20 mg per day per square meter of body surface), taken in divided doses three times a day. The eight salt-losers in this group also received 0.1–0.15 mg of fludrocortisone (Florinef) daily. All samples from these patients were collected between 0900 and 1000 h on their visits to an outpatient clinic. Additional samples were also provided by patients SA and JH. Patient SA, a 15-year-old female salt-loser with persistently supra-normal concentrations of circulating 17α-hydroxyprogesterone, provided matched whole saliva, parotid fluid, and plasma at approximately 2-h intervals over a 24-h period. Patient JH, a 14-year-old male non-salt-loser, provided matched samples of whole saliva and plasma just before receiving synthetic corticotropin (Synacthen), 0.25 mg intramuscularly; matched samples were then collected at 30-min intervals for 4 h.

Sample Collection

The collection of parotid fluid by fitting a Curby cup over the duct of the parotid gland and stimulating parotid secretion by administering a citric acid syrup has been described in detail elsewhere (5). Mixed whole-saliva samples were usually collected by spitting directly into glass tubes (75 × 12 mm). In infants, however, this procedure was impractical; samples were therefore obtained by placing a wide-bore plastic tube under the tongue and gently aspirating saliva into the tube by applying slight negative pressure with an attached syringe. Collection of saliva always preceded that of parotid fluid, to avoid contamination with citric acid syrup. Blood samples from superficial veins were collected in lithium heparin-containing tubes and centrifuged. Because painful venipuncture may increase steroid concentrations in salivary secretions, collection of parotid fluid and saliva always preceded venipuncture.

Reagents

Antiserum to 17α-hydroxyprogesterone, raised in rabbits to a 17α-hydroxyprogesterone-3-(O-carboxymethyl)oxime/
bovine serum albumin conjugate, was kindly donated by Dr. D. L. Loriaux, National Institutes of Health, Bethesda, MD. The antiserum was diluted to 100-fold in assay buffer and stored in 0.5-mL aliquots at -20 °C.

Radioligand [1,2,3H]-17α-hydroxyprogesterone (specific activity, 55 kCi/mol) obtained from NEN Chemicals GmbH, Dreirich, G.F.R., was diluted in benzene/methanol (9/1 by vol) and stored at 4 °C for not longer than four months.

Common solvents and reagents used in this assay procedure have been listed in an earlier publication (5).

Petroleum ether (bp, 40–60 °C) and hexane ("Analar" grade) were purified by washing first with concentrated sulfuric acid, then with water; after drying over calcium chloride, they were re fractionated. Diethyl ether (laboratory grade) was purified just before use by washing twice with sodium hydroxide (40 g/L), then with de-ionized water, and re fractionating.

Ethyl acetate ("Analar" grade) had to be redistilled at monthly intervals.

The following reagents, previously described in detail for use in a testosterone RIA procedure (9), were used without modification.

*Phosphate-buffered saline, pH 7.4.*

Solutions contained 1 g of gelatin per liter.

**Dextran-coated charcoal suspension.** Assay buffer containing dextran (0.25 g/L) and charcoal (2.59 g/L).

17α-Hydroxyprogesterone stock standard solution, 100 mg/L, was prepared by dissolving 1 mg of 17α-hydroxyprogesterone in 10 mL of absolute ethanol and stored at 4 °C for not longer than four months. Ethanolic standard solutions for use in the dose–response curve contained 25, 50, 100, 150, 200, and 250 pg/10 μL and were prepared by appropriate dilution of the stock standard solution just before use.

Tritiated 17α-hydroxyprogesterone solution for use in the assay was prepared when required by drying a 100-μL aliquot of the stock tritiated-ligand solution under nitrogen and redissolving in 25 mL of assay buffer.

Antiserum, diluted in assay buffer to a final concentration of 1 mL/24 L, was freshly prepared for each assay.

**Assay Procedure**

Assay of 17α-hydroxyprogesterone in whole saliva, parotid fluid, and plasma differed only in the initial stages, because extraction of this steroid from plasma required a slightly more polar solvent (petroleum ether/diethyl ether, 50/50 by vol) than that used for saliva and parotid fluid (petroleum ether/ether 65/35 by vol). To avoid unnecessary delay in reporting results, we diluted all plasma samples from CAH patients 10-fold and 20-fold before extraction.

To extract 17α-hydroxyprogesterone, transfer 200-μL aliquots of saliva, parotid fluid, and plasma samples to disposable glass tubes (100 × 12 mm). Add 3 mL of the appropriate solvent and place stoppered tubes in a multiple vortex-type shaker for 10 min. After shaking, centrifuge briefly to break the slight emulsion that may form in some tubes. Freeze the aqueous layer by placing tubes in acetone/ solid CO2 and decant the organic phase into clean, labeled glass assay tubes (75 × 12 mm). Transfer 10-μL aliquots of the ethanolic standards used in the dose–response curve to similar tubes, and add 3 mL of the appropriate solvent. Evaporate solvents under nitrogen at 30 °C.

Add 100 μL of antiserum, vortex-mix briefly, and incubate at 30 °C for 30 min. Add 100 μL of tritiated 17α-hydroxyprogesterone containing 16 667 dpm to all tubes and equilibrate at 30 °C for 1 h. Transfer the tubes to an ice bath and cool for 10 min. Add 500 μL of well-stirred, ice-cold, dextran-coated charcoal suspension to all tubes to separate antibody-bound and free steroid, stand the tubes in iced water for a further 15 min, and centrifuge at 2500 rpm for 10 min at 4 °C. Transfer 500-μL aliquots of the supernates to scintillation vials, add 6 mL of scintillant, and count the radioactivity associated with the antibody-bound steroid.

The dose–response curve was obtained and the concentration of 17α-hydroxyprogesterone in the samples was calculated by using the four-parameter fit model of Rodbard and Hutt (9). Hemolysis per se and high lipid concentrations in the samples caused no significant interference in the assay of plasma samples. Freshly collected parotid fluid was a clear solution, in marked contrast to the turbid whole-saliva samples. After the samples were frozen and thawed, a white precipitate, possibly protein, was seen in all tubes. This precipitate neither adsorbed 17α-hydroxyprogesterone nor interfered in the assay.

**Results**

**Analytical Variables**

The standard curve is shown in Figure 1. Six replicate assays established that the assay was satisfactorily precise at all fixed points.

Specificity of the antiserum was assessed by the criteria of Abraham (10); the cross reactivity of steroids structurally related to 17α-hydroxyprogesterone and those given as replacement therapy is presented in Table 1. We examined the specificity of the assay by assaying 12 samples of parotid fluid, 12 of saliva, and 12 of plasma, with and without pre-assay thin-layer chromatographic purification on silica gel plates that were developed in ethyl acetate/hexane (60/40 by vol). In this system, 17α-hydroxyprogesterone had an Rf of 0.65, and androstenedione (Rf = 0.65) was used as a marker to locate 17α-hydroxyprogesterone following chromatography. 17α-Hydroxyprogesterone concentrations in parotid fluid, whole saliva, and plasma showed no significant difference when assayed with and without purification, so the assay is specific (r > 0.9).

*Sensitivity*, defined here according to Kaiser and Specker (11) as the least amount distinguishable from zero at the 95% confidence level, was 4 pg per assay tube, corresponding to
17α-hydroxyprogesterone concentrations in parotid fluid, saliva, and undiluted plasma of 61 pmol/L.

**Precision.** Three pooled groups of saliva samples having high, medium, and low 17α-hydroxyprogesterone concentrations were established. Sixteen aliquots of each pool were determined in one assay, data from which were used to assess the intra-assay variance (Table 2). These pools were then split into aliquots, stored at −20 °C, and used as quality controls for subsequent routine assays giving the inter-assay variance (Table 2).

**Analytical recovery.** Tritated 17α-hydroxyprogesterone was added to parotid fluid, saliva, and plasma samples having 17α-hydroxyprogesterone concentrations approximating the lowest and highest standards used in the dose–response curve, and the samples were incubated overnight at 4 °C. Because recovery on extraction exceeded 95% in all samples, we deemed it unnecessary to monitor recovery in routine practice.

**Clinical Data**

At the beginning of this study we were uncertain which buccal fluid to use because mixed whole saliva, although easily collected from children of all ages, contains considerably more “debris” than parotid fluid. From the older children among the CAH patients, therefore, we collected and assayed 18 matched samples of whole saliva and parotid fluid. Because there was an excellent correlation ($r = 0.98$) between 17α-hydroxyprogesterone concentrations in saliva and parotid fluid (Figure 2), only whole saliva was collected in the latter part of this study.

To establish a provisional “normal range” for salivary 17α-hydroxyprogesterone concentrations, we collected samples from 32 apparently healthy children. The mean 17α-hydroxyprogesterone concentration in these samples was 527 ± 353 pmol/L (±SD), ranging from 90 to 1520 pmol/L. In the group of treated CAH patients, the concentrations measured

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**Table 1. Specificity of the 17α-Hydroxyprogesterone Antiserum**

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Cross reactivity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>17α-Hydroxyprogesterone</td>
<td>100</td>
</tr>
<tr>
<td>5α-Pregnanedione</td>
<td>3.4</td>
</tr>
<tr>
<td>11-Deoxycorticisol</td>
<td>3.3</td>
</tr>
<tr>
<td>5β-Pregnanedione</td>
<td>1.8</td>
</tr>
<tr>
<td>21-Deoxycorticisol</td>
<td>1.6</td>
</tr>
<tr>
<td>17α-Pregnanedione</td>
<td>0.5</td>
</tr>
<tr>
<td>Progesterone</td>
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</tr>
<tr>
<td>Cortisol</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Cortisone</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Testosterone</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Androstenedione</td>
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</tr>
<tr>
<td>11-Deoxycorticosterone</td>
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</tr>
<tr>
<td>Pregnanetriol</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>9α-Fludrocortisone (Florinef)</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>&lt;0.1</td>
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**Table 2. Assay Variance of Pooled 17α-Hydroxyprogesterone Samples**

<table>
<thead>
<tr>
<th>Plasma</th>
<th>Intra-assay variance</th>
<th>Inter-assay variance</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>High</td>
<td>Medium</td>
</tr>
<tr>
<td><strong>Saliva</strong></td>
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<td></td>
</tr>
<tr>
<td><strong>Plasma</strong></td>
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<tr>
<td><strong>Plasma</strong></td>
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</tbody>
</table>

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**Fig. 2.** Correlation between 17α-hydroxyprogesterone concentrations in 18 matched samples of parotid fluid and mixed whole saliva.

**Fig. 3.** Concentrations of 17α-hydroxyprogesterone in mixed saliva from normal subjects and patients with congenital adrenal hyperplasia.
Fig. 4. Correlation between 17α-hydroxyprogesterone concentrations in matched plasma and mixed whole saliva from patients with congenital adrenal hyperplasia showed a 20-fold greater variation, ranging from 67 pmol/L in patients receiving excessive glucocorticoid dosage to 26,300 pmol/L in inadequately treated patients (Figure 3).

17α-Hydroxyprogesterone in matched samples of saliva and plasma collected from CAH patients correlated well (r = 0.91) over a wide range of concentrations, indicating that concentrations of 17α-hydroxyprogesterone in saliva reflect those of plasma (Figure 4).

Data obtained on assay of matched samples of mixed saliva, parotid fluid, and plasma collected at frequent intervals over a 24-h period from an inadequately controlled female salt-losing patient (SA) are presented in Figure 5. Despite the markedly increased 17α-hydroxyprogesterone concentrations in plasma and the effect of oral replacement therapy given at the times indicated in the figure, the data suggest the presence of a diurnal rhythm in this patient. Matched saliva and parotid fluid show a pattern identical to that described for plasma; peak values for saliva are about 38-fold those observed in healthy children. Matched samples were also collected from a non-salt-losing male patient (JH) during the course of a Synacthen stimulation test (Figure 6). Plasma and salivary 17α-hydroxyprogesterone concentrations both reached peak values 30 min after Synacthen administration and declined to baseline values after 4 h. These results further support the concept that data on salivary 17α-hydroxyprogesterone concentrations are of value. They also indicate that because serial saliva samples are easily collected by comparatively stress-free techniques, salivary steroid concentrations may well replace those in plasma for determining endocrine status in CAH patients.

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References