A Radioimmunoassay for 18-Hydroxycortisol in Plasma and Urine

John E. T. Corrie,1 Christopher R. W. Edwards,2 and Peter S. Budd1

Increased excretion of 18-hydroxycortisol has been proposed as a specific biochemical marker for differential diagnosis of primary aldosteronism. We describe the development of a direct RIA with an 125I label that permits measurement of the steroid in ≤0.5 μL of urine or ≤25 μL of plasma. For control subjects, the mean concentrations of 18-hydroxycortisol in urine and plasma were 310 (SD 178) nmol/24 h (n = 32) and 2.27 (SD 0.80) nmol/L (n = 37), respectively; patients with Conn’s adenoma or glucocorticoid-remediable aldosteronism had values for urine in the range 1084 to 6534 nmol/24 h and concentrations in plasma ranging from 6.49 to 31.20 nmol/L. Patients with idiopathic zona glomerulosa hyperplasia had values for urine and plasma ranging from 353 to 734 nmol/24 h and from 0.26 to 6.60 nmol/L, respectively. Concentrations of 18-hydroxycortisol in urine clearly discriminate patients with idiopathic hyperplasia from those with other forms of primary aldosteronism, but further work is required to assess the diagnostic accuracy of determinations in plasma.

Additional Keyphrases: reference interval · differentiating idiopathic zona glomerulosa hyperplasia from other forms of primary aldosteronism · liquid chromatography compared · steroids

Measurement of urinary 18-hydroxycortisol (I, Figure 1) may be definitive for the differential diagnosis of primary aldosteronism, because patients with a proven Conn’s adenoma or glucocorticoid-remediable aldosteronism reportedly excrete more of the steroid than do normal subjects or patients with idiopathic zona glomerulosa hyperplasia (I, 2). The ability to make such a distinction would be useful because current diagnostic techniques are not entirely specific (3) and, although evaluation of 18-hydroxycortisone in plasma has been proposed as an improved discriminator (4), its measurement, usually involving chemical modification before RIA (5), is technically demanding.

The original measurements of 18-hydroxycortisol in urine were by gas chromatography–mass spectrometry, but such equipment is not widely available. In our attempts to establish an assay for studying the physiology and pathophysiology of this new steroid and assessing its diagnostic utility, we first tried a “high-pressure” liquid chromatographic (HPLC) method. However, its low sample throughput, the requirement for solvent extraction of samples, and its susceptibility to analytical interference from drugs made it unacceptable for routine use. Subsequently we have established a sensitive and specific RIA procedure for 18-hydroxycortisol suitable for direct assay of urine and plasma samples.

Materials and Methods

Materials

18-Hydroxycortisol, 18-hydroxycortisone, and 18-oxocortisol were generous gifts from Professor C. Gomez-Sanchez, VA Medical Center, University of South Florida, Tampa, FL. Other steroids for cross-reaction studies were from Sigma Ltd., London, U.K. Na125I (cat. no. IMS.30) was from Amersham International, Amersham, Bucks, U.K. Donkey anti-rabbit antiserum and normal rabbit serum were from the Scottish Antibody Production Unit, Carluke, Lanarkshire, U.K. New Zealand White rabbits were from Ranch Rabbits Ltd., Croydon, Surrey, U.K.

Assay diluent, pH 4, was prepared by dissolving 10.5 g of citric acid monohydrate, 8.4 g of anhydrous Na2HPO4, and 1 g of gelatin in 1 L of distilled water. Polystyrene assay tubes (cat. no. LP3) were from Luckham Ltd., Burgess Hill, U.K.

18-Hydroxycortisol standards were prepared in assay diluent in doubling concentrations over the range 0.0258–13.20 nmol/L (1.96–1000 pg per assay tube) and stored in aliquots (one set per assay) at −20 °C.

Development of the Radioimmunoassay

Synthesis of 3-O-(carboxymethyl)loximino-18-hydroxycortisol. We dissolved 7.24 mg of 18-hydroxycortisol and 5.15 mg of carboxymethylxylamine hemihydrachloride in a half-saturated manuelic solution of sodium hydrogen carbonate (1.07 mL) and left this at room temperature for 25 min (6). After evaporating the solvent under reduced pressure, we dissolved the residue in 3.6 mL of water and acidified this to pH 4 with 0.1 mol/L hydrochloric acid. We extracted the aqueous mixture with four 3.6-mL portions of ethyl acetate and washed the combined extracts once with saturated NaCl solution, dehydrated them over anhydrous sodium sulfate, and evaporated the solvent under reduced pressure. We dissolved the residual solid in 5 mL of methanol and diluted an aliquot of this 100-fold for quantification by ultraviolet spectroscopy at 251 nm. On the basis of ε = 23 100 for 3-O-(carboxymethyl)loximino-18-hydroxycortisol, the calculated yield of the oxime was 5.50 mg (63.8%). Analysis by thin-layer chromatography on silica gel with a solvent of benzene/ethanol/acetic acid, 75/25/0.5 by vol, showed only a single component, which was more polar than 18-hydroxycortisol or 3-O-(carboxymethyl)loximino-18-hydroxycortisol. The NMR spectrum (methanol-d4) showed δ 6.36 and 5.64 (total 1H,

![Fig. 1. Structure of 18-hydroxycortisol (I), its 3-carboxymethylxylamine derivative (II), and conjugates with bovine serum albumin (III) and radioliodinated histamine (IV)](image-url)
both s, H-4; Z,E 2:3), 4.53 and 4.49 (total 2H, both s, =NOCH₂-), 4.32 and 3.74 (2H, d/d, H-18, J = 10Hz), 4.34 (1H, s, H-11), 3.73 and 3.58 (2H, d/d, H-21, J = 12Hz), 1.32 (3H, s, H-19). Because there was no evidence of contamination by starting material or 3,20-dioxime, we used this material after evaporation of the methanol without further purification.

**Synthesis of immunogen.** We dissolved 5.5 mg of the oxime in 0.1 mL of dimethylformamide and coupled it to 20 mg of bovine serum albumin by the modified Ellman procedure previously described (7). Analysis of the conjugate showed a coupling ratio of 13.5 18-hydroxycortisol residues per protein molecule. The steroid–protein conjugate was lyophilized and stored at −20 °C.

**Antiserum to the conjugate were raised in New Zealand** White rabbits. Each animal was injected by the multiple intradermal technique (8) with 100 μg of immunogen in 1 mL of an equimolar emulsion of saline and Freund’s complete adjuvant. Subsequent booster injections were made every six weeks by combined intramuscular and subcutaneous injection of the same dose in saline/Freund’s incomplete adjuvant. The animals were bled 10 days after each booster injection.

**Iodinated radioligand** was prepared by activating the oxime in dimethylformamide solution and subsequent coupling to radiiodinated histamine as previously described (9). The radioligand was recovered by extracting the reaction mixture three times with 0.25-mL portions of ethyl acetate and the combined extract was chromatographed on a plastic-backed silica gel plate (20 × 20 cm × 0.1 mm) in toluene/ethanol/acetic acid (70/30/1, by vol). The major radioactive band, Rₜ 0.18–0.24, was located by autoradiography (Kodak X-omat film), cut from the plate, and eluted with 6 mL of ethanol containing 60 mg of triethylamine. The recovered tracer solution was stored at −20 °C. After determining what dilution of freshly prepared tracer in assay buffer would yield 2 × 10⁴ counts/min per milliliter, we used the same dilution factor throughout the life of the tracer.

**Assay procedure.** Dispense into assay tubes 200 μL of standard solutions, urine specimens diluted 400-fold in assay diluent, and plasma specimens diluted eightfold in assay diluent. (For all dilutions and dispensings we used Hamilton Microlab M apparatus.) To each tube add 50 μL of radioligand (10 000 counts/min with freshly prepared tracer) and 50 μL of 7500-fold diluted antiserum. Mix, then incubate at room temperature for 4 h. Next add 50 μL of 10-fold diluted donkey anti-rabbit serum and 50 μL of 150-fold diluted normal rabbit serum containing 1 mmol of EDTA per liter, incubate at 4 °C overnight, then dilute with 1.5 mL of assay buffer and centrifuge (4 °C, 2000 × g, 0.5 h). Decant the supernates and count the radioactivity of the remaining (antibody-bound) fraction. (We used a Wallac Autogamma counter.) Process the data, using a modified four-parameter logistic fit (10) to construct the dose–response curve. If results exceed the upper concentration range of the assay, re assay the samples at 10-fold (for urine) or fivefold (for plasma) greater dilutions.

**Parallelism studies.** Urine specimens at initial dilutions between 100- and 1000-fold (the appropriate initial dilution being chosen from the concentration of 18-hydroxycortisol determined by prior assay) and plasma specimens, initially undiluted, were assayed in consecutive twofold dilutions over a 16-fold range. For each set of results, the values falling outside the working range of the assay (see Results) were excluded and the remaining highest value was serially divided by two. We then compared the derived values with the measured results by linear regression analysis. Because of differences of initial dilutions among samples, all results are expressed as picograms of 18-hydroxycortisol per assay tube.

**Results**

**Assay Development**

**Antiserum.** Every animal immunized showed at least a weak antibody response, and three of the six had antibody titers exceeding 1:3500 after the first booster injection. Antiserum R 22C/3 had the highest titer after a second booster, and was used in further study. Figure 2 illustrates a typical standard curve.

**Radioligand.** The iodinated radioligand showed good stability on storage as judged by binding to antibody in the absence of added 18-hydroxycortisol: 48.1% (SD 2.2%) over 150 days (2.5 half-lives for 1²¹¹T). The nonspecific binding was <3% of total added counts throughout this time.

**Specificity.** Cross reactions at 50% displacement (11) for 18-hydroxycortisone, 18-hydroxy corticosterone, 18-hydroxydeoxycorticosterone, 18-oxocorticosterol, and cortisol were 1.04, 0.2, 0.017, 0.002, and 0.001%, respectively. Aldosterone, 11-deoxycorticoid, corticosterone, spironolactone, and dezamethasone all cross reacted <0.0003%.

**Analytical Variables**

**Sensitivity and working range.** The formal sensitivity of the assay system, expressed as the mass of analyte required to decrease the percentage binding of tracer by 2.5 SD of the percentage of tracer bound in the absence of unlabeled 18-hydroxycortisol, was 1.6 pg per tube, corresponding to 8.5 ngmml/L in urine or 0.17 ngmml/L in plasma. The precision of determinations at the formal detection limit is poor for all RIAs, and in practice we have used only those results falling between 20 to 85% depression of R₀. This gives a working range for the assay of 5 to 240 pg per tube, i.e., 26–1270 and 0.5–25 ngmml/L in urine and plasma, respectively.

**Precision and accuracy.** Figure 2 shows the within-assay component of the variation of duplicate estimates of concentration for 184 samples assayed in six routine assays. Illustrated is the averaged precision profile (10) from the six individual assays.

18-Hydroxycortisol was added to pooled urine and plasma from normal subjects to assess analytical recovery for each fluid. The pools were stored in aliquots at −20 °C and analyzed in consecutive assays. Results for analytical recovery and between-assay variation are shown in Table 1.

**Parallelism.** Seventeen urine specimens, diluted serially, gave 79 results within the working range. Linear regression analysis of these results gave the equation y = 1.03x + 0.23

---

**Fig. 2. Typical RIA standard curve (●●●) and precision profile (shaded area: mean ± SD), showing the within-assay variation of duplicate mean estimations NSB, nonspecific binding**
Table 1. Analytical Recovery of, and Between-Assay Variation for, 18-Hydroxycortisol Added to Urine and Plasma

<table>
<thead>
<tr>
<th>18-Hydroxycortisol, nmol/L</th>
<th>Added</th>
<th>Measured</th>
<th>Recovery*</th>
<th>Between-assay CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Urine (n = 11 each)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>95</td>
<td>—</td>
<td>—</td>
<td>8.05</td>
</tr>
<tr>
<td>284</td>
<td>371</td>
<td>104</td>
<td>108</td>
<td>6.1</td>
</tr>
<tr>
<td>794</td>
<td>956</td>
<td>110</td>
<td>109</td>
<td>8.2</td>
</tr>
<tr>
<td>2381</td>
<td>2516</td>
<td>102</td>
<td>8.2</td>
<td></td>
</tr>
<tr>
<td><strong>Plasma (n = 12 each)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>2.20</td>
<td>—</td>
<td>15.2</td>
<td></td>
</tr>
<tr>
<td>1.32</td>
<td>3.73</td>
<td>106</td>
<td>10.9</td>
<td></td>
</tr>
<tr>
<td>3.97</td>
<td>6.34</td>
<td>103</td>
<td>8.7</td>
<td></td>
</tr>
<tr>
<td>11.90</td>
<td>18.91</td>
<td>120</td>
<td>8.3</td>
<td></td>
</tr>
</tbody>
</table>

* (Measured – endogenous)/added 18-hydroxycortisol, × 100%.

(r = 0.983, p < 0.001), where y represents measured values and x the corresponding values calculated from the highest in-range result for each sample. For plasma, 39 specimens assayed as described gave 108 within-range results, and linear regression analysis gave the equation y = 1.14x – 0.48 (r = 0.967, p < 0.001).

Comparison of HPLC and RIA methods for urinary 18-hydroxycortisol. Linear regression analysis of the 18-hydroxycortisol concentrations (nmol/24 h) in 14 urine specimens measured by HPLC (single estimations; unpublished data) and RIA (duplicate estimations) gave the equation (18-hydroxycortisol by RIA) = 1.32 × (18-hydroxycortisol by HPLC) – 35.5, with r = 0.982 and p < 0.001.

Stability of 18-Hydroxycortisol in Urine and Plasma

Samples of urine and plasma from control subjects and patients with primary aldosteronism were stored for as long as 72 h at 4 °C and at room temperature. We also acidified urines with concentrated hydrochloric acid (0.2 mL per milliliter of urine) and kept these at room temperature for 16 h to simulate the conditions of urine collection for measurement of metanephric. There was no significant decrease (Student's paired t-test) in the measured concentration of 18-hydroxycortisol under any of these conditions.

Reference intervals

Plasma and 24-h urine samples were collected from normal volunteers and patients known not to suffer from primary aldosteronism, and from patients with primary aldosteronism resulting either from adrenal adenoma or from idiopathic or dexamethasone-suppressible zona glomerulosa hyperplasia. The differential diagnoses were established by a combination of the following techniques: aldosterone response to posture and time, adrenal vein catheterization, computed tomography scanning, adrenal scanning with 131I-hypothyroid and coadministration of dexamethasone. All cases of adrenal adenoma were confirmed at surgery. In addition, 12 normal volunteers underwent a short corticotropin stimulation test, supplying blood samples at 0900 h—immediately before administration of the drug (0.25 mg of Synacthen intravenously)—and two more samples 30 and 60 min later. Results are shown in Table 2.

Discussion

In our initial efforts to measure 18-hydroxycortisol in urine we used an HPLC method, but it was soon apparent that this would be of limited value for the reasons given in the Introduction. Additionally, the plasma 18-hydroxycortisol concentrations subsequently determined by RIA would not be measurable by HPLC.

By contrast, the development of the RIA was straightforward. 18-Hydroxycortisol was readily converted on a small scale to its 3-carboxymethylxoxime without derivatization of the C-20 carbonyl group, the relative resistance of which to oximation has been noted previously (2). High-titer antisera that yielded sensitive standard curves with the radioiodinated ligand were easily raised in three of the six rabbits immunized.

To simplify the assay methodology, we wished to establish a single direct assay for both urine and plasma, but a preliminary experiment at neutral pH showed a loss of reciprocity for dilutions of plasma samples. This was unexpected, because Ullick et al. (12) have shown that 18-hydroxycortisol has a very low affinity for cortisol binding globulin, but we abolished the effect by conducting the assay at low pH. This strategy has been previously applied to assays for cortisol (13) and progesterone (14), and in the present assay it yielded satisfactory parallelism for dilutions of urine and plasma. However, we found that a twofold increase over the routinely used (25 µL) volume of plasma per assay incubation mixture led to negatively biased results. For plasma the routine assay therefore operates near its limit of proportionality, whereas for urine it is at least fourfold within this limit.

Formal cross reaction studies with a range of corticosteroids and related drugs showed that the antiseraum was highly specific, but those few urine samples we analyzed both by HPLC and RIA showed a positive bias towards the RIA. This could in fact be a consequence of negative bias in the HPLC method, which for a number of specimens was operating near its detection limit, but we now are examining the use of thin-layer chromatography before RIA, to

Table 2. 18-Hydroxycortisol Concentrations as Measured by RIA in Urine and Plasma

<table>
<thead>
<tr>
<th></th>
<th>Urine</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Range</td>
<td>Mean (and SD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Controls</strong></td>
<td>32</td>
<td>77–806</td>
<td>310 (178)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls receiving Synacthen:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal*</td>
<td>12</td>
<td>0.87–3.92</td>
<td>2.27 (0.80)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+30 min</td>
<td>12</td>
<td>1.15–4.99</td>
<td>2.87 (1.16)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+60 min</td>
<td>12</td>
<td>1.44–5.60</td>
<td>3.15 (1.23)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients with primary aldosteronism:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conn’s adenoma</td>
<td>4</td>
<td>1084–2503</td>
<td>1560 (557)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucocorticoid-remediable zona</td>
<td>2</td>
<td>2606–6534</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>glucocorticoid hyperplasia</td>
<td>6</td>
<td>353–734</td>
<td>618 (148)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Idiopathic zona</td>
<td>2</td>
<td>6.89–31.20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>glomerulosa hyperplasia</td>
<td>9</td>
<td>0.26–6.60</td>
<td>3.94 (1.88)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Except in the corticotropin-stimulation test, the time of plasma sampling was not controlled. *0900 h. **Significantly different from basal values: p < 0.001 (paired t-test).
establish beyond doubt the validity of the RIA results.

The pattern of results for measurement of urinary 18-hydroxycortisol (Table 2) is broadly in agreement with that described by Ulick and Chu (1, 2), although many of our measurements of excretion of 18-hydroxycortisol by control subjects (mean 310, range 77-606 nmol/24 h) lie above the reported value (<265 nmol/24 h), as do the values for patients with idiopathic zona glomerulosa hyperplasia. The range of the latter values substantially overlaps that for control subjects, but the means differ significantly (p <0.001, Student's t-test), with the idiopathic hyperplasia patients showing a tendency towards higher values. However, we have as yet found no overlap between the values in idiopathic hyperplasia and those for patients with either adenomas or dexamethasone-suppressible aldosteronism.

Concentrations of 18-hydroxycortisol in plasma, shown in Table 2, are the first reported for this steroid, and the small increase (mean 1.4-fold) in concentration after stimulation with corticotropin is consistent with the reported response in urinary excretion (1). For all forms of primary aldosteronism the mean values significantly (p <0.001) exceeded those for control subjects, although values for patients with idiopathic zona glomerulosa hyperplasia overlapped with the normal range. More importantly, for one patient with hyperplasia the concentration of 18-hydroxycortisol in plasma was within the range seen in Conn's adenoma. However, we have seen considerable intra-individual variability in plasma (and urinary) 18-hydroxycortisol concentrations, and by sampling on more than one occasion the diagnostic specificity of the test probably can be improved.

In conclusion: we have developed an RIA for 18-hydroxycortisol that is suitable for direct measurement of this steroid in normal and pathological specimens of urine and plasma, and we have verified the stability of the analyte in either fluid during any likely interval for sample transmission at ambient temperature from patient to laboratory. Our preliminary results substantiate Ulick's hypothesis that this steroid could be a specific biochemical marker of primary aldosteronism associated with an adenoma or that due to the rare form of hyperplasia which is corticotropin-dependent. If further work with idiopathic hyperplasia shows that this can readily be distinguished from the much more easily treatable forms of primary aldosteronism, then the measurement of 18-hydroxycortisol may prove to be extremely valuable in the differential diagnosis of this condition.

We are grateful to Dr. C. Gomez-Sanchez, University of South Florida, for the gift of steroids, and to Professor D.N. Kirk, Westfield College, London, for the NMR spectrum. We thank Drs. R. M. Carey, P. L. Padfield, R. T. Jung, J. M. C. Connell, and P. E. Belchets for providing specimens from patients under their care.

References